Inhibin A is a follicle stimulating hormone-responsive marker of granulosa cell differentiation, which has both autocrine and paracrine actions in sheep

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Abstract

The aim of these studies was to examine the origin, control and local actions of inhibin A in monovular species, using the sheep as a model. Experiment 1 examined the pattern of mRNA expression for the inhibin subunits in relation to follicular size and pattern of expression to other differentiative markers in granulosa (P450 aromatase) and thecal cells (P450 17 α -hydroxylase). Experiment 2 examined the pattern of inhibin A production, in relation to oestradiol, by granulosa cells induced to differentiate *in vitro* with follicle-stimulating hormone (FSH). Experiment 3 examined possible paracrine and autocrine actions of inhibin A by determining the effect of addition of human recombinant inhibin A and/or antiserum to inhibin on gonadotrophin-stimulated cellular differentiation. The results of Experiment 1 showed that expression of mRNA encoding inhibin subunits α , β_A and β_B is greater ($P<0.05$) in large oestrogenic follicles than in small follicles but that only expression of the inhibin β_A subunit differs ($P<0.05$) between large oestrogenic and non-

oestrogenic follicles. Expression of 17 α -hydroxylase, but not of the luteinising hormone (LH) receptor, in thecal cells was related to both the size and the oestrogenicity of antral follicles, in a manner similar to that of the inhibin subunits. Experiment 2 demonstrated that the production of inhibin A by sheep granulosa cells is FSH-responsive after prolonged exposure ($P<0.001$) and precedes the production of oestradiol by around 48 h in the differentiative cascade induced in granulosa cells by FSH. The results of Experiment 3 showed that inhibin A can augment gonadotrophin-stimulated steroid production by both granulosa and theca cells ($P<0.01$), and that the addition of antiserum to inhibin can inhibit FSH-stimulated oestradiol production by granulosa cells from both small and large follicles ($P<0.001$). We conclude that inhibin A is an FSH-responsive marker of granulosa cell differentiation which has both autocrine and paracrine actions in sheep.

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Introduction

Inhibin consists of an α -subunit (18 kDa) linked by a disulfide bridge to one of two highly homologous β -subunits (14 kDa), designated β_A and β_B , to form either inhibin A or inhibin B. The name 'inhibin' is derived from its ability to inhibit the release of follicle-stimulating hormone (FSH) from the pituitary, and was initially isolated from ovarian follicular fluid as a classical endocrine hormone (Findlay 1994). However, since its isolation nearly 15 years ago, inhibin, along with its antagonist, activin, has been shown to be a member of the transforming growth factor- β (TGF β) superfamily, to be widely expressed, and to have local regulatory roles in a variety of tissues (in addition to the ovary), including the brain, the adrenal, bone marrow, the foetus and the placenta (reviewed by Knight 1996).

As an ovarian hormone, inhibin has both endocrine and local actions. Immunoneutralisation (Mann *et al.* 1990, Campbell & Scaramuzzi 1995, Campbell *et al.* 1995) and replacement (Mann *et al.* 1992a, Ramaswamy *et al.* 1998) studies have provided strong evidence to support an endocrine role for inhibin whereby it acts in concert with oestradiol to control FSH secretion by the pituitary; in addition, it appears likely that inhibin A is the principal isoform responsible for this activity in monovular species (Robertson *et al.* 1996). Both paracrine and autocrine local actions of inhibin have been reported. Inhibin A has consistently been shown to exert a potent stimulatory effect on luteinising hormone (LH)-induced androgen production by rat (Hsueh *et al.* 1987) and human (Hillier *et al.* 1991a) theca cells. Conversely, an inhibitory effect of inhibin on FSH-induced aromatase activity in rat granulosa cells has been described (Ying *et al.* 1986), but

this autocrine action has not been confirmed by others using rat (Sugino *et al.* 1988), bovine (Hutchinson *et al.* 1987, Shukovski & Findlay 1990) or primate (Hillier & Miro 1993) cells.

Despite the importance of inhibin as a major endocrine and local modulator of ovarian function, and in direct contrast to the situation in polyovular rodents, relatively little is known of the follicular origin, control of synthesis, and action of inhibin isoforms in monovular species. Studies examining the pattern of expression of mRNAs or protein for the inhibin α , β_A and β_B subunits have shown them to be widely expressed in the granulosa cells of follicles ranging from pre-antral to preovulatory follicles, there being a marked increase in the level of α -subunit expression in large antral follicles (primate: Jaatinen *et al.* 1994, Roberts *et al.* 1993, 1994; sheep: Englehardt *et al.* 1993, Braw-Tal 1994, Tisdall *et al.* 1994, Campbell *et al.* 1999). Initial studies examining the follicular origin of inhibin relied on bioassays that, although potentially confounded by the high steroid concentrations in antral fluid, showed that inhibin bioactivity was correlated with both the number of granulosa cells and their oestrogenicity (Tsonis *et al.* 1983). Until relatively recently, the available data on the synthesis of immunoactive inhibin was limited to assays that could not differentiate between inhibin A, inhibin B and non-bioactive isoforms of inhibin (McNeilly *et al.* 1989, Findlay *et al.* 1990). Early studies using these assays in sheep also indicated that the ovarian secretion of inhibin was positively correlated with oestradiol secretion (Campbell *et al.* 1990) and that, whilst immunoactive inhibin was secreted by both small and large antral follicles, the majority of the inhibin secreted was derived from large oestrogenic follicles (Campbell *et al.* 1991, Mann *et al.* 1992b). The more recent advent of specific, two-site, enzyme-linked immunosorbent assays for human inhibin A and B (Groome *et al.* 1994, 1996) revealed marked differences in the patterns of secretion in women between the inhibin isoforms: there was a peak of inhibin B secretion during the early follicular phase, followed by a marked increase in inhibin A secretion during the late follicular phase which is closely associated with the pre-ovulatory oestradiol surge. On the basis of this pattern of secretion, it has been suggested that there is a switch from inhibin B to inhibin A secretion during antral follicle development, but the evidence for this hypothesis is indirect. In sheep, the recent development of a specific inhibin A assay has shown that the pattern of inhibin A secretion closely parallels ovarian oestradiol secretion during periods of follicle growth (Souza *et al.* 1997a,b, Knight *et al.* 1998), but there are still no data on inhibin B secretion. Thus, it would appear that inhibin A is produced by granulosa cells that are also secreting oestrogen, but it is unclear if inhibin A secretion is constitutive or gonadotrophin-dependent. Furthermore, although these *in vivo* observations are consistent with the observed paracrine actions of inhibin in the upregulation of thecal

androgen synthesis, they are inconsistent with the autocrine action (the inhibition of aromatase activity; Ying *et al.* 1986).

Research in this area in monovular species has been hampered by the lack of suitable experimental tissue in the human, by the inadequate culture model and assay systems in ruminants, and by the relative scarcity of pure inhibins A and B. The development of specific assays for sheep/cattle inhibin A (Souza *et al.* 1997a,b) and serum-free culture systems for ruminant granulosa (Campbell *et al.* 1996, Gutierrez *et al.* 1997) and theca (Campbell *et al.* 1998) cells, along with an increased supply of human recombinant inhibin A, have overcome many of these problems. The aim of the present study, therefore, was to exploit these advances by examining, in more detail, the origin, control and local actions of inhibin A in monovular species, using the sheep as a model. The specific objectives were (i) to determine the pattern of mRNA expression for the inhibin subunits in relation to follicular size, and to relate this pattern of expression to other differentiative markers (P450 aromatase and 17 α -hydroxylase), (ii) to determine the pattern of inhibin A production by granulosa cells induced to differentiate *in vitro* by FSH, and to relate the pattern of production to oestradiol, and (iii) to examine possible paracrine and autocrine actions of inhibin A by using these culture model systems.

Materials and Methods

Experiment 1: mRNA expression of inhibin subunits, P450 aromatase, P450 17 α -hydroxylase and thecal LH receptor

Experimental design Experiments were conducted in accordance with the Animal (Scientific Procedures) Act of 1986. Oestrous cycles of eight mature Scottish Blackface ewes (ovulation rate 1.3 ± 0.1) were synchronised by the insertion of intravaginal progestagen sponges (Dunlop, Dumfries, UK) for 12–14 days, followed by the injection of 100 μ g of the prostaglandin F2a analogue cloprostenol (PG; Estrumate; Dunlop) 10 days after sponge removal. Animals were ovariectomised (Campbell *et al.* 1991) during the early follicular phase (24 h after a second PG injection administered on days 10–12 of the luteal phase). Both ovaries of each animal were fixed overnight in freshly prepared 4% paraformaldehyde in PBS, pH 7.4 (PFA), and, after fixation, ovaries were cut longitudinally into two or four slices, care being taken to cut through the largest follicles on each ovary (Engelhardt *et al.* 1993). The tissue was then dehydrated by passage through a graded series of alcohols and then embedded in paraffin wax. Serial sections of a 5 μ m thickness were mounted on slides coated with 3-aminopropyltriethoxy-silane (TESPA; Sigma, St Louis, MO, USA), incubated overnight at 50 °C, and then processed for *in situ* hybridisation as described previously (Harkness & Baird 1997).

Table 1 Details of ³⁵S-labelled riboprobe templates used for *in situ* hybridisation

	Vector	Size (bp)	Endonuclease		Polymerase		Reference
			Sense	Antisense	Sense	Antisense	
Probe							
hlnhibin α	pGEM3Zf(+)	360	EcoRI	HindIII	SP6	T7	Engelhardt <i>et al.</i> (1993)
hlnhibin β_A	pBluescript II SK +	618	BSSHII	BSSHII	T7	T3	Engelhardt <i>et al.</i> (1993)
hlnhibin β_B	pGEM3Zf(+)	623	EcoRI	BamHI	SP6	T7	Harkness & Baird (1997)
oLH _R	pBluescript II SK +/-		XbaI	EcoRV	T7	T3	Xu <i>et al.</i> (1995a)
bP450 _{17α}	pBluescript II SK +/-	400	EcoRV	XbaI	T3	T7	Xu <i>et al.</i> (1995b)
oP450 _{arom}	pGEM-T	429	Apal	NotI	SP6	T7	Campbell <i>et al.</i> (1999)

Abbreviations: h, human; o, ovine; b, bovine.

***In situ* hybridisation** *Template generation and cRNA probe synthesis* The details of the cDNA templates and cRNA probe synthesis are presented in Table 1. The inhibin subunit probes were based on human sequences but have previously been shown to hybridise in the sheep (Engelhardt *et al.* 1993, Campbell *et al.* 1999). All other probes were derived from ovine or bovine cDNA (see Table 1). ³⁵S-labelled sense and antisense riboprobes were generated from their cDNA templates using previously published methods (Harkness & Baird 1997).

In situ hybridisation All of the *in situ* hybridisation procedures were performed as previously described (our laboratory; Harkness & Baird 1997), all sections for a particular probe being processed at the same time to avoid variation between batches. Sections from an adult sheep ovary collected during the follicular phase and containing a large oestrogenic follicle were included in each run as a positive control.

Sections were deparaffinised twice in HistoClear (National Diagnostics, Atlanta, GA, USA), rehydrated through a graded series of ethanols, and post-fixed in 4% paraformaldehyde. The sections were washed and digested with proteinase K (20 μ g/ml; Sigma) in a 100 mM Tris/10 mM EDTA buffer. Sections were washed and refixed in 4% PFA, washed twice in 0.25% acetic anhydride in 0.1 M triethanolamine hydrochloride then washed again in PBS and saline, dehydrated through a graded series of ethanols (50, 70, 85, 95 and 100%) and air-dried.

The labelled probes were diluted to a concentration of 5×10^5 c.p.m./slide with a hybridisation buffer (62.5% deionised formamide; 25% of a 50% dextran sulfate solution; 7.5% 5 M NaCl; 1% 1 M Tris, pH 8.0; 0.2% 500 mM EDTA; 2.5% 50 $\times$ Denhardt's solution; 1% 1 M dithiothreitol (DTT) and 0.3% ultrapure water) heated to a temperature of 80 °C for 2 min and cooled on ice before application to sections (10 μ l per slide). Sections were overlaid with coverslips made from Gel Bond (FMC Bioproducts, Rockland, ME, USA) and the slides incubated overnight in a humidified chamber saturated with 2.5 $\times$ standard saline citrate (SSC) and 50% formamide at 55 °C.

Post hybridisation, the sections were washed in 5 $\times$ SSC with 0.01 M DTT at 55 °C, in 2 $\times$ SSC with 50% deionised formamide and 0.1 M DTT at 65 °C and then three times in NTE buffer (0.5 M NaCl, 10 mM Tris, 5 mM EDTA, pH 7.5) at 37 °C. Sections were then treated with ribonuclease type III A (Sigma; 40 μ g/ml in NTE) at 37 °C, then re-washed in NTE. The wash in 2 $\times$ SSC with 50% formamide and 0.1 M DTT at 65 °C was repeated. Sections were then washed at room temperature in buffers containing decreasing amounts of salt solutions (2 $\times$ and 0.1 $\times$ SSC) and then dehydrated through a graded series of ethanols (50% and 85% containing 0.3 M NH₄ acetate; 100% twice) and left to air-dry.

Autoradiographic dipping in liquid emulsion was performed in a dark room with a safety light. Sections were dipped in Kodak NBT-2 and exposed for between one and five weeks at 4 °C, developed in Kodak D-19 developer at 15 °C, and fixed in Kodafix at room temperature. Slides were counterstained with haematoxylin (BDH, Glasgow, UK), dehydrated and then mounted in Pertex mounting media (CellPath, Newtown, UK).

Image analysis For each animal, four randomly selected sections from different parts of the ovary were examined under dark field, using a microscope (Olympus Optical Company, Southall, UK) attached to a video camera and computer. The intensity of the *in situ* hybridisation signal was analysed using either Olympus PC-TAKS MANAGER Version 1.20A, CUE 2 image analysis morphometry (Olympus Optical Company) (for inhibin α , β_A and β_B , aromatase and the LH receptor) or IMAGE-PRO PLUS (Media Cybernetics, Leiden, The Netherlands) (for 17 α hydroxylase) (Campbell *et al.* 1999). The number of graphic pixels occupied by silver grains within a defined area of tissue section was counted and presented as the number of silver grains per unit of volume (e.g. 10⁶/cm³). Within each follicle, five separate fields were analysed for each probe, the coefficient of variation of these repeated measurements being <20% for each probe. Background hybridisation intensity, determined from sense probes run in parallel with antisense probes on alternate sections, was subtracted from the measurements obtained with the antisense probes

to give the final hybridisation signal. There was no significant difference ($P > 0.05$) in the hybridisation intensities obtained with antisense and sense RNA probes within a non-expressing region of a tissue section.

Follicle classification Follicles were divided into two size classes – small (1–3.5 mm) and large (≥ 3.5 mm) – based on the follicular diameters at which sheep follicles attain the ability to secrete oestradiol (Carson *et al.* 1979). The true maximum diameter of follicles close to this division point was determined using an eyepiece graticule in adjacent sections to ensure accurate classification. Follicles were morphologically classified as healthy or atretic on the basis of the presence or absence of degenerative changes (Turnbull *et al.* 1977), including the presence of pyknotic nuclei, thin or disrupted membrana granulosa, and destruction of the basement membrane. Follicles classified as atretic exhibited a low hybridisation intensity that did not differ significantly ($P > 0.05$) from background.

Experiments 2 and 3: production and local actions of inhibin A in vitro

Serum-free culture of granulosa and theca cells

The methodologies utilised for the collection and culture of sheep granulosa (Campbell *et al.* 1996) and theca (Campbell *et al.* 1998) cells have been described previously. All of the chemicals were obtained from Sigma-Aldrich (Poole, UK). Briefly, small (2–3.5 mm in diameter) and large (≥ 3.5 mm in diameter) ovarian follicles were dissected from ovaries collected, from the abattoir, in Medium 199 containing Hepes (20 mmol/l) penicillin (100 kIU/l), streptomycin (0.1 µg/l) and amphotericin (1 mg/l) at 37 °C, specific attention being given to the removal of all extraneous stromal tissue from the follicle wall. Small follicles were hemisected in DPBS[−] (Dulbecco's phosphate-buffered saline without calcium or magnesium) and the follicle halves were flushed repeatedly up and down the barrel of a 1 ml syringe. The thecal shells were allowed to settle, the granulosa-cell-rich supernatant was removed and the flushing procedure was repeated. The antral fluid of large follicles was removed using a 1 ml syringe and a 23 G needle before hemisection of the follicles and removal of the granulosa cells by gentle scraping with an inoculation loop. The granulosa cells were washed twice in culture medium (McCoy's 5a supplemented with penicillin (100 kIU/l), streptomycin (0.1 µg/l), L-glutamine (3 mmol/l), BSA (0.1%, w/v), transferrin (2.5 mg/l), selenium (4 µg/l), androstenedione (10^{-7} mol/l), bovine insulin (10 ng/ml) bovine insulin and Long R3 insulin-like growth factor-I (LR3-IGF-I; 10 ng/ml)) before being plated at a density of 75 000 viable cells per well into pre-prepared and equilibrated 96-well plates containing 200 µl culture medium.

The thecal shells were dispersed in an enzyme mix containing collagenase (5 g/l), hyaluronidase (1 g/l),

protease (1 g/l), deoxyribonuclease (2 g/l) and donor calf serum (0.002%, v/v) in 20 ml phosphate-buffered saline for 30–45 min at 37 °C with gentle agitation. The reaction was stopped by the addition of 2 ml donor calf serum, then the cells were washed twice in culture medium (DMEM:F12 with penicillin (100 kIU/l), streptomycin (0.1 µg/l), L-glutamine (3 mmol/l), BSA (0.1%, w/v), oestradiol (10^{-7} mol/l), transferrin (2.5 mg/l), selenium (4 µg/l), bovine insulin (10 ng/ml) and LR3-IGF-I (10 ng/ml)) before being plated at a density of 75 000 viable cells per well ($<1\%$ contamination by granulosa cells) into pre-prepared and equilibrated 96-well plates containing 200 µl culture medium.

Cells were cultured in a humidified atmosphere with 5% carbon dioxide in air at 37 °C. Granulosa and theca cells were cultured for a total of 8 and 6 days respectively, the medium being changed at 48 h intervals. In order to minimise disturbance of the cells, only 175 µl medium was gently removed and replaced at each change. The spent medium was stored at −20 °C before assay. At the end of culture, the number of viable cells per well was estimated using neutral red uptake; the results were expressed as ng hormone produced per 10 000 cells per 48 h.

Experimental design Experiment 2 was designed to examine the pattern of inhibin A production and responsiveness to gonadotrophic stimulation in granulosa cells from small follicles induced to differentiate *in vitro*. Granulosa cells from small follicles were cultured, in quadruplicate, with 0, 0.63, 1.25, 2.5, 5 or 10 ng ovine FSH/ml for the entire period of culture (10 days). Furthermore, the time and importance of time of addition of FSH in inducing cellular differentiation was tested by culturing cells with FSH (10 ng/ml) for the whole 10 days or for the last 8, 6, 4 or 2 days of culture. Within experiments, each treatment was replicated in quadruplicate and each experiment was repeated three times. The ovine FSH (oFSH) used was NIAMDD-oFSH-16, which has a bioactivity of 20 IU/mg and LH contamination of $0.04 \times \text{NIH-LH-S1}$ mg/ml. Normal circulating concentrations of FSH in sheep, using this standard, are 5–10 ng/ml, so the doses of FSH used covered the normal physiological range.

Experiment 3 was designed to examine the potential autocrine and paracrine actions of inhibin A through both exposure of granulosa and theca cells to exogenous inhibin and immunoneutralisation of endogenous inhibin. Granulosa (10 ng oFSH/ml) and theca (0.1 ng LH/ml) cells from small and large follicles were cultured with optimum gonadotrophin concentrations in the presence of human recombinant inhibin A (0, 0.01, 0.1, 1, 10, 100 ng/ml; NIH). In addition, FSH-stimulated (10 ng/ml) granulosa cells were cultured in the presence of polyclonal sheep antisera to inhibin (at dilutions of $1:10^2$ to $1:10^6$). The presence of sera in the cultures was controlled for by

parallel culture using normal sheep serum. The antiserum to inhibin was raised in sheep to the fusion protein of the entire human α -subunit (for the primary immunisation) and the human recombinant 32 kDa dimeric inhibin (for booster immunisation) and has been shown to lead to an increase in circulating FSH concentrations and to stimulation of follicle development following acute passive immunisation of sheep (Campbell & Scaramuzzi 1995, Campbell *et al.* 1995). Within experiments, each treatment was replicated in quadruplicate and each experiment was repeated three times.

Assays

Concentrations of oestradiol (Campbell *et al.* 1996), progesterone and androstenedione (Campbell *et al.* 1998) and inhibin A (Souza *et al.* 1997a) in unextracted culture media were determined using previously described radio-immunoassays. For inhibin A measurements, culture media had to be diluted to between 1:10 and 1:1000 in assay buffer. The sensitivities of the assays for oestradiol, androstenedione, progesterone and inhibin A were 50 pmol/l, 175 pmol/l, 380 pmol/l and 30 ng/l respectively. The intra- and inter-coefficients of variation for all assays were less than 15%.

Statistical analysis

Experiment 1 In large follicles, the presence of significant aromatase expression ($>10^6$ grains/cm³) was used to classify large follicles as oestrogenic or non-oestrogenic. Comparisons of levels of mRNA expression between small, large oestrogenic (LE) and large non-oestrogenic (LNE) healthy follicles were made using one-way analysis of variance on log-transformed data.

Experiments 2 and 3 With the exception of the time-course data, all hormone production data were expressed as amounts of hormone produced per 48 h per 10 000 cells (10 kcells). As accurate cell number data were not available at 96 h and 144 h of culture, the time-course data were expressed as concentrations. The significance of treatment effects was determined by analysis of variance, using replicate cultures as blocks. Individual comparisons between treatments were made using Bonferroni's test.

Results

Experiment 1: expression of mRNA encoding the inhibin subunits

Data for mRNA expression from 91 small, 10 large non-oestrogenic (LNE) and 8 large oestrogenic (LE) follicles respectively are presented in Fig. 1. As expected, aromatase expression was confined to the membrana

granulosa of LE follicles (by definition) and was absent in small and LNE (by definition) follicles. Expression of all the inhibin subunits was confined to the granulosa cells, as previously reported (Engelhardt *et al.* 1993, Tisdall *et al.* 1994, Campbell *et al.* 1999). Conversely, expression of 17 α -hydroxylase and the LH receptor was always observed in the theca cells, but LH receptor expression was not consistently observed in the granulosa cells of LE follicles. This unexpected finding was attributed to a low hybridisation signal due to an insufficient period of exposure to photographic emulsion; accordingly, LH receptor expression in granulosa cells has not been reported.

Expression for the inhibin α -subunit was detected in all follicle size classes and was found to be significantly higher ($P<0.05$) in LE follicles than in small follicles. Inhibin α -subunit expression in LNE follicles was intermediate between that in small and LE follicles, but was not significantly different from either (Fig. 1). Expression for the β_A -subunit varied markedly with size and oestrogenicity: the levels of expression in small follicles were low; levels were higher in LNE follicles ($P<0.001$); and the highest levels of expression were in LE follicles ($P<0.05$; Fig. 1). The level of expression for the inhibin β_B -subunit was markedly lower than that detected for either the α - or the β_A -subunit, but did differ between follicle classes with higher levels of expression ($P<0.05$) in LE than in small follicles (Fig. 1). LNE follicles were again intermediate, but not significantly different, from small and LE follicles. Of the thecal markers of follicular differentiation, LH receptor expression did not vary with follicle class; in contrast, 17 α -hydroxylase expression was detected in all follicle size classes and was found to be significantly higher ($P<0.05$) in LE follicles than in small follicles (Fig. 1). Expression of 17 α -hydroxylase in LNE follicles was intermediate between that in small and LE follicles, but was not significantly different from either.

Experiment 2: induction of inhibin A production by cultured granulosa cells

Granulosa cells from small follicles, in the presence or absence of FSH, produced negligible (0.5 ± 0.1 pg/10 kcells/48 h) amounts of oestradiol but appreciable (556 ± 69 pg/10 kcells/48 h) quantities of inhibin A during the first 48 h of culture (Fig. 2). Continued exposure to FSH resulted in the induction of oestradiol production after 144 h of culture; production continued to increase with increasing time of culture ($P<0.001$). In cells not exposed to FSH, however, oestradiol production remained low. Inhibin A production exhibited a pattern similar to that for oestradiol, but tended to precede oestradiol production by 48 h (Fig. 2). Thus, inhibin A production by granulosa cells from small follicles cultured in the presence of FSH increased ($P<0.01$) at between 48 h and 96 h, increased markedly ($P<0.001$) at between 96 h and 144 h

Figure 1 Expression of mRNA for P450 aromatase (a), inhibin subunits α (b), β_A (c) and β_B (d) in granulosa cells, and of the LH receptor (e) and cytochrome P450 17α -hydroxylase (f) in thecal cells of small (<3.5 mm; $n=91$), LNE (≥ 3.5 mm; $n=10$) and LE ($n=8$) ovarian follicles in sheep. Different letters indicate significant ($P < 0.05$) differences between follicle classes. The values are means $\pm$ S.E.M.

(a) Oestradiol **(b) Inhibin A**

Figure 2 Production of oestradiol (a) and inhibin A (b) by granulosa cells from small (<3.5 mm in diameter) ovarian follicles with increasing time in serum-free culture without (○) and with (●) oFSH (10 ng/ml). Values are the means $\pm$ S.E.M. of three replicate cultures with 4 duplicates per treatment dose within cultures. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with hormone production at 48 h within each dose of FSH (ANOVA).

(the time at which oestradiol was induced) and remained stable at between 144 h and 192 h of culture. In the absence of FSH, inhibin A production increased slightly ($P < 0.05$) from 48–96 h; then, for the remainder of the culture period, levels declined to those observed during the initial period. After 192 h of culture, both oestradiol

(a) Oestradiol **(b) Inhibin A**

Figure 3 Effect of FSH on oestradiol (a) and inhibin A (b) production by granulosa cells from small (<3.5 mm in diameter) ovarian follicles maintained in serum-free culture for 192 h. Note that hormone production has been expressed in terms of the number of granulosa cells per well at the end of culture. The values are means $\pm$ S.E.M. of three replicate cultures with 4 duplicates per treatment dose within cultures.

and inhibin A production exhibited a highly significant ($P < 0.001$) dose-responsive relationship with FSH (Fig. 3). The effective dose (50%) of FSH for inhibin A (0.7 ng/ml) production, however, was lower than that for oestradiol (1.8 ng/ml).

Experiments designed to examine the critical timing of exposure to FSH in relation to induction of inhibin A and oestradiol production showed that the absence of FSH from the culture wells for the first 48 or 96 h had no deleterious effect on the induction of either inhibin A or oestradiol production after 192 h of culture (when compared with cells exposed to FSH for the entire culture period) (Fig. 4). Surprisingly, the absence of FSH for the initial 48 h of culture resulted in an increase ($P<0.05$) in the induction of both oestradiol and inhibin A after 192 h of culture. Production of both inhibin A and oestradiol in cells exposed to FSH for just the final 48 h of culture (144–192 h) was lower ($P<0.05$) than that in cells stimulated with FSH for the entire culture period, but was higher ($P<0.05$) than that in cells not exposed to FSH (Fig. 4).

Experiment 3: paracrine and autocrine actions of inhibin A

Inhibin A enhanced FSH-stimulated oestradiol production by granulosa cells from both small ($P<0.01$) and large ($P<0.001$) follicles in a dose-responsive manner (Fig. 5a). Granulosa cells from large follicles appeared to be more sensitive to the stimulatory action of inhibin A than were cells from small follicles, as the magnitude of the effect was greater (100% vs 50%) and the dose-response curve began to plateau at a lower dose (10 ng/ml vs 100 ng/ml). Similarly, inhibin A enhanced LH-stimulated androgen production by theca cells from both small and large ($P<0.01$) follicles (Fig. 5b). Conversely, inhibin antiserum resulted in a marked inhibition ($P<0.001$) of FSH-stimulated oestradiol production by granulosa cells from both small and large follicles, dilutions as low as $1:10^4$ – $1:10^5$ inducing a significant suppression ($P<0.05$; Fig. 6). Furthermore, the slope of this inhibition curve differed markedly from that for non-immune serum (which depressed oestradiol production only at the lowest dilution, $1:10^2$), suggesting that the depression induced by inhibin antiserum was specifically due to blockage of endogenous inhibin activity (Fig. 6). As we have shown that the granulosa cells produce appreciable quantities of inhibin A in culture, no attempt was made to reverse the depressive effect of the antiserum with exogenous inhibin A. A $1:10^3$ dilution of inhibin antiserum, however, was able to prevent the stimulatory effect of human recombinant inhibin A (100 ng/ml) on thecal androstenedione production (data not shown).

Discussion

The results of these experiments are as follows. (i) The production of inhibin A by sheep granulosa cells is FSH-responsive after prolonged exposure and precedes the production of oestradiol in the differentiative cascade induced in granulosa cells exposed to optimum doses

(a) Oestradiol

(b) Inhibin A

Figure 4 Production of oestradiol (a) and inhibin A (b) by granulosa cells from small (<3.5 mm in diameter) ovarian follicles either deprived of oFSH (10 ng/ml) for the entire culture period (0–192 h), for the first 144 h of culture (0–144 h), for the first 96 h of culture (0–96 h) or for the first 48 h of culture (0–48 h), or stimulated with FSH throughout (0 h). Different letters indicate significant ($P<0.05$) differences between different times of exposure. The values are means $\pm$ S.E.M. of three replicate cultures with 4 duplicates per treatment dose within cultures.

of gonadotrophin and metabolic hormones *in vitro*. (ii) Inhibin A has both autocrine and paracrine actions in the augmentation of gonadotrophin-induced

(a) Granulosa cells

(b) Thecal cells

Figure 5 Effect of recombinant human inhibin A (rhInhibin A) on oestradiol production by granulosa cells (a) and on androstenedione production by thecal cells (b) isolated from either small (<3.5 mm) or large (≥ 3.5 mm) ovarian follicles and maintained in serum-free culture for either 192 h (granulosa) or 144 h (theca). The values are means $\pm$ S.E.M. of three replicate cultures with 4 duplicates per treatment dose within cultures. * $P < 0.05$; ** $P < 0.01$ compared with hormone production at 0 dose of inhibin (ANOVA).

differentiation of granulosa and theca cells respectively. (iii) Expression of mRNA encoding both the inhibin α - and the inhibin β_A -subunits is positively related to both the size and the oestrogenicity of antral follicles *in vivo*. Together, these observations provide strong evidence to show that inhibin A has, in addition to its endocrine role, a key local role in the augmentation of gonadotrophic stimuli in individual follicles, and hence in the mechanism of follicle selection and dominance.

The present study is the first to examine the expression of mRNA encoding the inhibin subunits in relation to the expression of other differentiative markers within the same ovarian antral follicles. This approach has allowed us to

classify follicles on the basis of both size and oestrogenicity, and the results show unequivocally that expression of all inhibin subunits is greatest in large oestrogenic follicles – a finding that supports our previous observation that follicles (assumed to be oestrogen-active on the basis of ovarian secretion *in vivo*) express mRNA for inhibin α , β_A and β_B at high levels (Engelhardt *et al.* 1993). In addition, while the present data show that expression of all the inhibin subunits increases markedly as small follicles grow into a large oestrogenic structure, expression only of mRNA encoding the inhibin β_A -subunit differed between LE and LNE follicles. Examination of these LNE follicles showed them all to be greater than 5 mm in diameter, and thus it

Figure 6 Effect of inhibin antisera (●) or normal sheep serum (○) on oestradiol production (expressed as a percentage of control cultures) by granulosa cells from either small (< 3.5 mm) or large (≥ 3.5 mm) ovarian follicles maintained in serum-free culture in the presence of FSH (10 ng/ml) for 192 h. Oestradiol production in the absence of serum (100%) was 485 ± 52 and 575 ± 75 pg/10 kcells/48 h for granulosa cells from small and large follicles respectively. The values are means $\pm$ S.E.M. of three replicate cultures with 4 duplicates per treatment dose within cultures.

is likely that these follicles, although not yet showing morphological signs of atresia, have begun to regress and lose their ability to produce oestradiol prior to inhibin A production.

An interesting feature of the inhibin expression data was that the level of expression for the inhibin β_B -subunit was several orders of magnitude lower than that for inhibin α or inhibin β_A . Although this difference could be related to probe specificity, this observation supports the fact that it has proved difficult to detect dimeric inhibin B in sheep follicular fluid (D T Baird & A S McNeilly, personal communication).

Of the thecal markers of cellular differentiation, expression of cytochrome P450 17α -hydroxylase was found to be related to follicle size and oestrogenicity in a manner similar to that for the inhibin α - or β_A -subunits – an observation in accord with the increase in the ovarian

secretion of androgens observed during pre-ovulatory follicle development (Campbell *et al.* 1990) and the increase in androgen concentrations in antral fluid during follicular development (Campbell 1988). The close relationship between the pattern of inhibin subunit and 17α -hydroxylase expression suggests that the ability of inhibin A to augment LH-stimulated androgen production by cultured thecal cells (see below) may be mediated, at least in part, via increased 17α -hydroxylase expression. In contrast, expression of the thecal LH receptor was not found to vary between follicle classes, and it is therefore unlikely that changes in the androgenicity of antral follicles are related to LH receptor expression.

The results for the sheep granulosa cell cultures support and extend our previous findings indicating that oestradiol production is FSH-responsive and that it increases over time in this serum-free culture system (Campbell *et al.* 1996) with oestradiol production continuing to increase for up to 8 days of culture. In contrast, our previous study showed that total immunoreactive inhibin production was not FSH-responsive on a per cell basis, although total inhibin production did increase over time in culture (Campbell *et al.* 1996). Similar findings have also been reported for the cow (Wrathall & Knight 1993). In the present study, the use of a specific assay for dimeric inhibin A has shown that inhibin A production is FSH-responsive on a per cell basis; these results therefore support those made using cultured rodent (Ying *et al.* 1986, Zhang *et al.* 1988) and primate (Hillier *et al.* 1991b) granulosa cells. The reason for the difference between these data and our previous results may be that other immunoreactive forms of inhibin masked the increase in inhibin A induced by FSH and/or that the secretion of other forms of immunoreactive inhibin decline in opposition to inhibin A with increasing FSH.

The results of the present study show not only that inhibin A production is responsive to FSH in a dose-dependent manner, but also that the ability of granulosa cells to synthesise inhibin A and oestradiol are closely related and that inhibin A secretion tends to precede oestradiol secretion. Thus the dose of FSH required to induce inhibin A secretion is around half that required for oestradiol production, and, accordingly, induction of inhibin A production by undifferentiated cells from small follicles precedes that of oestradiol by around 48 h. The fact that inhibin A production tends to precede oestradiol in this FSH-induced differentiative cascade suggests that significant production of inhibin A by granulosa cells of small follicles *in vivo* may occur at lower concentrations of FSH than are required to stimulate production of oestradiol.

The results of the studies in which the time of FSH-stimulation was delayed were somewhat unexpected. Surprisingly, this experiment showed that the absence of FSH for up to 4 days during the initial period of culture had no deleterious effect on oestradiol and inhibin A

production at the end of culture (compared with cells that had been exposed to FSH throughout). As induction of aromatase by FSH normally takes 4–6 days in these cultures, this observation suggests that non-FSH-dependent differentiative changes occur in the granulosa cells, during the initial periods of culture, that result in much more rapid FSH-dependent cellular differentiation if exposure to FSH occurs subsequently. The identity of these additional factor(s) in the differentiative cascade are unknown, but, as the presence of insulin and IGF-I in the culture media are essential for induction of aromatase *in vitro* using this culture system (Campbell *et al.* 1996), it is likely that these factors are induced by insulin and/or IGFs. The identification of these early factors in the differentiative cascade are a research priority in the elucidation of the mechanism of follicular recruitment and selection.

A paracrine action for inhibin A in the augmentation of LH-stimulated thecal androgen production has been suggested by a number of previous studies in rats (Hsueh *et al.* 1987) and in humans (Hillier *et al.* 1991a), but similar bovine studies have produced equivocal results (Shukovski *et al.* 1993, Wrathall & Knight 1995). The utilisation of a serum-free theca cell culture system that overcomes the problem of spontaneous luteinisation in the present study provides strong evidence that inhibin, as in other species, has a paracrine action in augmenting LH-stimulated androgen production by thecal cells in the ruminant. In contrast, the demonstration that inhibin A can augment FSH-stimulated oestradiol production by granulosa cells and that antiserum to inhibin can attenuate FSH-stimulated oestradiol production provides the first evidence that inhibin A has a positive autocrine effect during follicular differentiation. These results, therefore, contrast with the negative inhibitory effect of inhibin on FSH-induced aromatase activity in rat granulosa cells, described by Ying *et al.* (1986), or the lack of an autocrine effect reported by others using rat (Sugino *et al.* 1988), bovine (Hutchinson *et al.* 1987, Shukovski & Findlay 1990) and primate (Hillier & Miro 1993) tissue. A combined positive autocrine and paracrine action of inhibin A during follicular cell differentiation is consistent, however, with the observed relationship between FSH-induced oestradiol and inhibin A production by cultured granulosa cells (Experiment 2) and with the close relationship between mRNA expression for inhibin α - and β_A -subunits and aromatase and 17 α -hydroxylase (Experiment 1). It is notable, however, that the magnitude of the response to exogenous inhibin A supplementation in the current experiments was quite variable between replicate cultures. A possible explanation for this effect is differences between cultures in terms of the amount of endogenously produced inhibin A, which could also explain the equivocal nature of the data in the literature concerning this question. It is for this reason that immunoneutralisation proved to be a valuable means of confirming that inhibin has autocrine

actions – although it was necessary to control for the known deleterious effect of serum on oestradiol production by using this serum-free culture system (B K Campbell, unpublished observations).

The apparent positive relationship between oestradiol and inhibin A production by granulosa cells *in vitro* (Experiment 2) and expression of mRNA *in vivo* (Experiment 1) contrasts with a number of reports which have described a negative relationship between the concentration of oestradiol and inhibin A in the antral fluid of large dominant follicles in ruminants (Guilbault *et al.* 1993, Ireland *et al.* 1994, Sunderland *et al.* 1996, Mihm *et al.* 1999). There are a number of potential explanations for these differences, including species differences, assay differences in the detection of different inhibin isoforms, and differential dose effects. In our view, however, the most likely explanation for this difference is that antral fluid concentrations may not represent an accurate predictor of the synthetic capacity of the granulosa cells for both steroids and proteins. The concentration of oestradiol within antral fluid has been shown to be an accurate predictor of steroidogenic potential (Webb & England 1982) and this presumably reflects the fact that oestradiol is a small labile molecule that can move readily into the circulation and antral fluid. On the other hand, inhibin is a much larger molecule which has a range of molecular forms (Ireland *et al.* 1994), and, although it is unclear how its storage, processing and release from the follicle are controlled, studies in which antral fluid concentrations and *in vitro* release of immunoreactive (Campbell *et al.* 1991) and dimeric inhibin A (Mitchell *et al.* 2000) have been compared have shown that the level of inhibin in antral fluid may not be a reliable indicator of the synthetic capacity of the granulosa cells, particularly in LNE follicles.

In summary, the results of this work strongly support the hypothesis that inhibin A is a major local factor with paracrine and autocrine actions. The results of Experiment 2 indicate that production of inhibin A by granulosa cells is induced by prolonged exposure to FSH and precedes the induction of aromatase activity. The results of Experiment 3 indicate that inhibin A can augment gonadotrophin-induced granulosa- and theca-cell steroidogenesis and that immunoneutralisation of endogenous inhibin can block FSH-induced oestradiol production by granulosa cells. The results of Experiment 1 support the findings of these *in vitro* studies by showing that expression of mRNA encoding the inhibin A subunits is positively related to follicular size and steroidogenic activity *in vivo*. We conclude that inhibin A is an FSH-responsive marker of granulosa cell differentiation which has both autocrine and paracrine actions in sheep.

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