Inhibin production *in vitro* by granulosa cells from Booroola ewes which were either homozygous or non-carriers of a fecundity gene influencing their ovulation rate

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Summary. The production of inhibin by granulosa cells was studied *in vitro* using cells from follicles of various sizes and health. Follicles were recovered on Days 10–13 of the oestrous cycle, from Booroola × Romney ewes which were homozygous (*FF*) carriers or non-carriers (*++*) of the fecundity (*F*) gene. Inhibin was measured using a bioassay based on the suppression of follicle-stimulating hormone (FSH) output by cultured pituitary cells from ovariectomized Romney ewes and, in some instances, for comparative purposes, by radioimmunoassay also. Geometric mean inhibin production by granulosa cells from nonatretic follicles increased with increasing follicle diameter, during the first 24 h of culture, for both genotypes. The geometric mean production of inhibin by cells from nonatretic 3–4.5 mm diameter *FF* follicles (the largest follicles found in *FF* ewes), was significantly higher ($P < 0.05$) than that by cells from non-atretic 3–4.5 mm diameter *++* follicles, but similar to that of cells from non-atretic ≥ 5 mm diameter *++* follicles. The production of oestradiol-17 β by cells cultured in the presence of testosterone (1 $\mu\text{g/ml}$) followed a pattern similar to cellular inhibin production. There was a positive linear correlation between inhibin and oestradiol-17 β production during the first 24 h of culture, for both genotypes. In addition to acting as a substrate for oestradiol-17 β synthesis, testosterone generally had a slight, stimulatory effect on inhibin production. Irrespective of follicle size, or genotype, no detectable amounts of inhibin were produced by granulosa cells from atretic follicles during the first 24 h of culture, or by cells from nonatretic or atretic follicles during the second 24 h of culture.

These studies show that the highest mean amounts of inhibin produced by granulosa cells *in vitro* are similar for both genotypes. Moreover, they are achieved with cells from the largest nonatretic follicles in both *++* (i.e. ≥ 5 mm diameter) and *FF* (i.e. 3–4.5 mm diameter) ewes.

Keywords: Booroola ewes; inhibin; granulosa cells; oestradiol-17 β

Introduction

Studies of high fecundity Booroola ewes have shown that, in ewes bearing the fecundity (*F*) gene, high plasma concentrations of follicle-stimulating hormone (FSH) occur more frequently and follicles attain ovulatory maturity earlier, i.e. at a smaller diameter, relative to non-carriers of the gene (McNatty & Henderson, 1987). There is increasing evidence that inhibin may be important in regulating plasma FSH concentrations in sheep (Martin *et al.*, 1988; Findlay *et al.*, 1989; Mann *et al.*, 1989) and thereby follicular activity. Inhibin is therefore a possible candidate for mediating

the effects of the Booroola *F* gene. There may be differences between the Booroola genotypes in inhibin production by granulosa cells, these cells being the principal site of ovarian inhibin production (Henderson & Franchimont, 1983; Mann *et al.*, 1989; Rodgers *et al.*, 1989; Torney *et al.*, 1989). The purpose of this study was to investigate inhibin production by granulosa cells *in vitro* using cells from follicles of various sizes and health obtained from Booroola × Romney ewes which were homozygous (*FF*) or non-carriers (*++*) of the *F* gene. Inhibin was measured using a bioassay based on the suppression of FSH output by ovine pituitary cells *in vitro* (Henderson *et al.*, 1989) and by radioimmunoassay, for comparative purposes, in some instances.

Materials and Methods

Sheep. The Booroolas were 5–8 years old *FF* (*n* = 16) and *++* (*n* = 16) Booroola × Romney ewes which had been segregated on the basis of at least one ovulation-rate recording of ≥ 5 and 1 or 2, respectively (Davis *et al.*, 1982). The ewes were grazed on open pasture, and run with a vasectomized ram fitted with a marking harness to detect oestrous activity. Granulosa cells were recovered at Days 10–13 of the oestrous cycle. Pituitary cells for the inhibin bioassay were obtained from Romney ewes which had been ovariectomized for 4–8 weeks.

Reagents. Collagenase (type IV), deoxyribonuclease I, gentamicin, glutamine, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes), oestradiol-17β, sodium bicarbonate, sodium pyruvate and testosterone were obtained from Sigma Chemical Co., St Louis, MO, USA. Bovine serum albumin (BSA) was obtained from Immuno Chemical Products Ltd, Auckland, New Zealand; dispase from Boehringer Mannheim GmbH, Mannheim, W. Germany; amphotericin B from E.R. Squibb & Son Inc., Princeton, NJ, USA; penicillin from Glaxo Laboratories Ltd, Greenford, UK; Medium 199 with Earle's salts, Dulbecco's modified Eagle medium and non-essential amino acids from Gibco Laboratories, Grand Island, NY, USA. The sheep FSH radioimmunoassay kit and sheep LH preparations (NIDDK-oLH-I-3 and NIDDK-oLH-24) were gifts from the National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Pituitary Program, University of Maryland, USA. The ovine serum used in the granulosa and pituitary cell cultures was from a single batch, described previously (Henderson *et al.*, 1989), obtained from 4 ovariectomized Romney ewes.

A single pool of ovine follicular fluid was used as an inhibin reference standard in the inhibin bioassay. The fluid was collected from ovaries of sheep slaughtered at a local abattoir, and treated with dextran-coated charcoal, as described previously (Henderson *et al.*, 1986). The inhibin bioactivity of the pool of follicular fluid was calibrated in the inhibin bioassay against an inhibin standard (code no. 86/690), prepared from pig follicular fluid, and provided by the National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, UK. The dose–response curve of the sheep follicular fluid pool paralleled that of the inhibin standard. The potency of the ovine follicular fluid pool in the bioassay, in terms of the inhibin standard (86/690), was 10.4 Units/μl, with 95% confidence limits of 7.9 and 12.9 U/μl. The calibrated ovine follicular fluid pool was then routinely used as the inhibin standard in subsequent bioassays, and all inhibin bioactivity results were expressed in terms of the 86/690 standard.

Ovarian dissections. Pairs of ovaries were recovered from Booroola ewes at Days 10–13 of the oestrous cycle (Day 0 is day of oestrus). All the corpora lutea were dissected out and counted. All antral follicles ≥ 1.0 mm in diameter were individually dissected from each pair of ovaries, their diameters measured and then placed into sterile Medium 199 with Earle's salts (Eagle, 1959), supplemented with Hepes buffer (20 mM), gentamicin (50 μg/ml) and 1.0% bovine serum albumin. Assessment of the health of each follicle and the harvesting of granulosa cells was performed as described previously (McNatty *et al.*, 1984). Follicles considered to be non-atretic were those with visible thecal capillaries when viewed at × 10 magnification under a dissecting microscope, no debris in the follicular fluid, an oocyte of healthy appearance, ≥ 26% of the maximum number of recoverable granulosa cells for a follicle of a given size, and a pink to red theca interna. Follicles were considered to be atretic when one or more of these 5 criteria was not applicable.

Granulosa cell cultures. Preliminary studies with cells from non-atretic follicles indicated that approximately 1×10^6 granulosa cells/culture were required to produce sufficient amounts of inhibin for determination by the bioassay. Therefore, for each pair of ovaries it was often necessary to pool cells from follicles of a similar size and classification. Pools were composed of cells from either non-atretic or atretic follicles of diameters 1–2.5 mm, 3–4.5 mm and ≥ 5 mm. After collecting the cells and pooling as appropriate, the cells were centrifuged at 200 *g* for 10 min and resuspended in Dulbecco's Modified Eagle Medium containing glutamine (1%), non-essential amino acids, sodium pyruvate (1 mM), sodium bicarbonate (3.7 mg/ml), gentamicin (50 μg/ml), amphotericin B (2.5 μg/ml) and penicillin (100 units/ml), pH 7.4 (Medium DM). The cells were centrifuged again (200 *g*, 10 min) and finally resuspended in Medium DM containing 10% ovariectomized sheep serum (Medium DMS). The cells were counted using a haemocytometer, and the volume of Medium DMS was adjusted to give approximately 2×10^6 cells/ml. Samples of 0.5 ml were pipetted into individual round wells (16 mm diam.) of multiwelled tissue-culture dishes (Nunc, Kamstrup, Denmark). A further 0.5 ml Medium DMS alone or Medium DMS containing 2 μg testosterone/ml was

added. The limited number of cells available meant that generally only duplicate cultures of each treatment were established. The cells were cultured at 37°C in an humidified atmosphere of 5% CO₂ in air for 48 h, with the medium being renewed after 24 h. At the end of the culture period, the cells were washed thoroughly with saline and stored frozen until assayed for protein by the method of Lowry *et al.* (1951), using bovine serum albumin as the protein standard. In some instances, samples of granulosa cells for culture were homogenized in 1 ml of Medium DMS and the media stored frozen immediately so that the endogenous cellular inhibin content could be determined. The 1-ml samples of spent granulosa cell culture medium were divided into two 0.5 ml aliquots. One aliquot was stored frozen at -20°C until subsequent determination of oestradiol-17 β , and in some instances determination of inhibin by radioimmunoassay. The other aliquot, for inhibin bioassay, was incubated with 0.5 ml of aqueous dextran T-70 (0.02% w/v)-coated charcoal (0.2% w/v) for 16 h at 4°C. Following centrifugation at 3000 g, the supernatant was stored frozen at -20°C.

Inhibin bioassay. The bioassay of inhibin activity was based on the suppression of basal FSH output by cultured anterior pituitary cells from ovariectomized ewes as described previously (Henderson *et al.*, 1989). Briefly, dispersed cells were obtained from anterior pituitaries following enzymic treatment with collagenase, dispase and deoxyribonuclease. Samples of 1×10^6 dispersed cells were cultured in 1 ml Medium DMS in multiwelled tissue culture dishes (Nunc, Kamstrup, Denmark) for 24 h at 37°C in an humidified atmosphere of 5% CO₂ in air. The medium was then replaced with fresh Medium DMS (1 ml) containing added dextran-charcoal-treated granulosa cell culture medium or the ovine follicular fluid inhibin standard. Triplicate cultures of each dose of each treatment were usually established. Incubation was continued for a further 48 h with the medium being renewed after 24 h with a fresh 1 ml medium containing the same additions of granulosa cell culture medium or inhibin standard. Spent media were stored frozen at -20°C until assayed for gonadotrophins. At the end of the culture period, the cells were washed thoroughly with saline (0.154 M) and stored frozen until assayed for protein by the method of Lowry *et al.* (1951), using bovine serum albumin as the protein standard. In the pituitary cell culture medium, the concentration of granulosa cell culture medium ranged from 2.5 to 10%. Culture medium (DMS) with and without added testosterone (1 μ g/ml) incubated in the absence of granulosa cells, and then treated with dextran-coated charcoal, served as control medium for adding to the pituitary cell culture medium. The calibrated ovine follicular fluid inhibin standard was added to the pituitary cell cultures at doses of 0.04 to 5.2 units/ml. Inhibin bioactivity of the granulosa cell culture media was expressed relative to the activity of the inhibin standard by interpolation from standard curves generated by plotting FSH output (as a percentage of that of control pituitary cultures) versus the logarithm of the concentration of the ovine follicular fluid standard. Inhibin bioactivity in the culture medium was then normalized with respect to either the number of granulosa cells initially cultured, or the protein content of the granulosa cells at the end of their 48-h culture period.

Radioimmunoassays. Gonadotrophin concentrations in pituitary cell culture medium were measured by specific radioimmunoassays and normalized with respect to the pituitary cell protein. FSH was measured using the sheep FSH radioimmunoassay kit and protocol provided by the National Hormone and Pituitary Program, NIDDK, University of Maryland, USA. The FSH antiserum was NIAMDD-anti-oFSH-1 (AFP-C5288113), the ovine FSH for iodination was NIAMDD-oFSH-1-1 (AFP-5679C) and the reference preparation was NIAMDD-oFSH-RP-1 (biopotency $75 \times$ NIH-FSH-S1). The sensitivity of the assay was 0.05–0.1 ng/assay tube and the intra- and inter-assay coefficients of variation were <10%. LH was measured using the specific radioimmunoassay described previously (McNatty *et al.*, 1981). The antiserum used was generated in a rabbit against NIH-LH-S11 and was used at an initial dilution of 1:40 000. The ovine LH reference preparation was NIADDK-oLH-24 and the iodinated tracer was NIADDK-oLH-1-3. The sensitivity of the assay was 0.025–0.05 ng per assay tube, and the intra- and interassay coefficients of variation were <11%.

Inhibin concentrations in granulosa cell culture medium were measured by radioimmunoassay using reagents provided by Dr D. M. Robertson, Department of Anatomy, Monash University, Clayton, Victoria, Australia. The methodology was as previously described and found suitable for measurement of inhibin concentrations in bovine granulosa cell culture medium (McLachlan *et al.*, 1986). The inhibin antiserum (no. 1989) was generated in a rabbit against pure bovine inhibin of M_r 31 000. The antiserum exhibited insignificant (<1%) cross-reaction with ovine LH, FSH, growth hormone and prolactin, bovine activin-A, porcine transforming growth factor- β , Müllerian inhibiting substance, the reduced and alkylated α - and β -subunits of bovine inhibin, and FSH-suppressing protein. However, the antiserum displayed considerable cross-reaction with bovine pro- α C-subunit (288%; Robertson *et al.*, 1989). Purified bovine inhibin of M_r 31 000 was used to prepare the iodinated tracer. The inhibin reference preparation was the same ovine follicular fluid pool used as a standard in the inhibin bioassay. It was calibrated in the radioimmunoassay against the pig follicular fluid reference inhibin preparation (code no. 86/690) provided by the National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, UK. Both preparations gave parallel dose-response curves in the radioimmunoassay. The potency of the ovine follicular fluid pool in the radioimmunoassay was 230 Units/ μ l. The sensitivity of the radioimmunoassay was 230 mU/tube and the intra- and interassay coefficients of variation were 7% and 13%, respectively.

Oestradiol-17 β was measured directly in aliquants of granulosa cell culture medium by a specific radioimmunoassay as described previously (McNatty *et al.*, 1984). The sensitivity of the assay (per tube) was 5 pg and the intra- and interassay coefficients of variation were <11%.

The inhibin and oestradiol-17 β values were normalized with respect to either number of granulosa cells at the start of culture, or granulosa cell protein at the end of culture.

Results

Number of corpora lutea and follicles in *FF* and *++* ewes

Table 1 shows the number of corpora lutea and the distribution of follicles in each genotype. The mean number of total and nonatretic follicles per ewe, 1–4.5 mm in diameter, did not differ significantly between the genotypes ($P > 0.05$, Student's t test). Follicles ≥ 5 mm in diameter were present in the ovaries of *++* but not *FF* ewes.

Table 1. Number of corpora lutea per ewe, and distribution of follicles with regard to health and size in *FF* and *++* ewes

Genotype	No. of corpora lutea/ewe	No. of follicles per ewe of diameter (mm)					
		1–2.5		3–4.5		≥ 5	
		Total	Nonatretic	Total	Nonatretic	Total	Nonatretic
<i>++</i>	1.6 ± 0.1	39 ± 5	16 ± 2	4.7 ± 1.0	2.6 ± 0.6	1.7 ± 0.3	1.1 ± 0.2
<i>FF</i>	6.1 ± 0.5	31 ± 4	15 ± 3	3.1 ± 0.6	2.4 ± 0.5	0	0

Values are arithmetic means $\pm$ s.e.m. for 16 ewes per genotype.

Inhibin and oestradiol-17 β production by granulosa cells

In preliminary studies, FSH output by cultured pituitary cells was suppressed by the ovine follicular fluid inhibin standard and granulosa cell culture medium to a greater extent during the second 24-h period of exposure to these agents than during the first 24 h. This confirms previous findings of the effects of ovine follicular fluid (Henderson *et al.*, 1989). Inhibin bioactivity was therefore subsequently estimated only from the effects of the inhibin standard and granulosa cell culture medium samples during the second 24-h period of their exposure to the pituitary cells.

Figure 1 shows the effect on FSH output by cultured pituitary cells of increasing amounts of the ovine follicular fluid inhibin standard, and increasing volumes of granulosa cell culture medium pooled from cultures of granulosa cells obtained from nonatretic follicles of different sizes from *++* and *FF* ewes. FSH output was suppressed in a dose-dependent fashion by the inhibin standard, and all the pools of granulosa cell culture medium, although with Pool 1 50 μ l medium was no more effective than 25 μ l in suppressing FSH output. There was insufficient medium from Pool 4 to test at 100 μ l. The slopes of straight lines fitted to the dose-response data for the effects of the pools of granulosa cell media on FSH output did not differ significantly ($P > 0.05$) from the corresponding slope for the inhibin standard. The slope values were (mean $\pm$ s.d.): Pool 1, 10.4 ± 2.4 ; Pool 2, 9.8 ± 1.6 ; Pool 3, 7.0 ± 0.9 ; Pool 4, 9.4 ± 2.0 ; inhibin standard, 8.1 ± 1.1 .

While pituitary cell FSH output could be suppressed by the inhibin standard and granulosa cell culture medium pools, there was no consistent effect on LH output, which varied from 88 to 113% of that produced by control pituitary cultures. None of the treatments had any significant effect on the mean amounts of pituitary cellular protein determined at the end of the culture period, relative to control cultures. The suppression of FSH output was therefore unlikely to be a consequence of a toxic effect of the treatments.

Although pooled medium from the first 24 h of granulosa cell culture could suppress FSH output by pituitary cells (Fig. 1), pooled medium from the second 24 h of granulosa cell culture was without effect on FSH output, at doses of up to 100 μ l culture medium per pituitary culture. Having established that the dose-response curves of the pooled granulosa cell culture media, tested at 2 or 3 doses, paralleled that of the follicular fluid inhibin standard (Fig. 1), it was considered appropriate

Fig. 1. Effect of increasing doses of inhibin standard (▲) and volumes of pooled granulosa cell culture medium on FSH output by cultured ovine pituitary cells during the second 24 h of treatment. Granulosa cell medium Pools 1 (□) and 4 (●) were derived from medium from the first 24 h of culture of cells from nonatretic follicles 3–4.5 mm and ≥ 5 mm in diameter, respectively from ++ ewes. Pools 2 (■) and 3 (○) were derived from medium from the first 24 h of culture of cells from non-atretic follicles 1–2.5 mm and 3–4.5 mm in diameter, respectively, from FF ewes. Values for the pools of medium are means $\pm$ s.e.m. for 3 replicate cultures. Values for the inhibin standard are means $\pm$ s.e.m. for 8 replicate experiments. Mean FSH output has been expressed as a percentage of the mean output by appropriate control (zero dose) cultures. Linear regression lines have been fitted to the data (50 and 100 μ l data only for Pool 1) and the associated regression equations are shown, together with the correlation coefficients (r) and significance.

to determine inhibin concentrations in individual granulosa cell cultures at a single dose-level. Triplicate 50- μ l aliquants of all medium samples from the first 24 h of culture, and representative samples from the second 24 h of culture were therefore assayed. Assaying a single volume of all the granulosa cell culture medium samples minimized the number of sheep which had to be ovariectomized and subsequently killed to provide pituitary cells for the bioassay.

Figure 2(a) shows the bioactive inhibin output, during the first 24 h of culture, by granulosa cells from nonatretic follicles of various sizes from FF and ++ ewes. Values presented have been normalized with respect to the number of granulosa cells initially cultured. However, similar results were obtained if inhibin output was normalized with respect to cellular protein determined at the end of the culture period. Within each genotype, the geometric mean bioactive inhibin output increased with increasing follicular diameter. Follicles ≥ 5 mm in diameter were not found in FF ewes. However, the geometric mean bioactive inhibin output by cells from 3–4.5 mm diameter FF follicles did not differ significantly ($P > 0.05$) from that of cells from ≥ 5 mm diameter ++ follicles, and was significantly greater ($P < 0.05$) than that of cells from 3–4.5 mm diameter ++ follicles. The bioactive inhibin output by granulosa cells from 1–2.5 mm diameter ++ follicles was undetectable (< 0.2 U/ 10^6 cells), whereas the inhibin output by cells from similarly sized FF follicles, although low, was measurable. A similar pattern of results emerged when the inhibin content of the same samples was measured by radioimmunoassay, although the absolute amounts of inhibin measured by the bioassay and radioimmunoassay differed markedly (Fig. 2b).

Inhibin bioactivity was not detectable in the medium homogenates of freshly isolated granulosa cells. Inhibin output by cultured cells probably therefore represents production of inhibin, rather than simply release of preformed intracellular inhibin. Irrespective of follicle size, or genotype,

Fig. 2. Inhibin output, measured by bioassay (a) and radioimmunoassay (b), and oestradiol-17 β production (c) by granulosa cells from non-atretic follicles of various diameters from ++ and FF ewes. Data are for the first 24 h of granulosa cell culture. Oestradiol-17 β production was determined in cultures treated with testosterone (1 μ g/ml). Inhibin output was determined in cultures not exposed to testosterone, and no detectable amounts of oestradiol-17 β were produced by these cultures. Values are geometric means of (*n*) ewes with 95% confidence limits indicated by the vertical lines. Geometric mean values with different letters are significantly different ($P < 0.05$, analysis of variance in conjunction with Newman-Keuls multiple range test on data logarithmically transformed [$\ln(x + 1)$] to equalize the variances). Bioactive inhibin output by cells from 1-2.5 mm diameter ++ follicles was not detectable (ND, i.e. < 0.2 U/10⁶ cells) and this group was excluded from the statistical comparisons.

no detectable amounts of bioactive inhibin were produced by cells from atretic follicles during the first 24 h of culture, or by cells from non-atretic or atretic follicles during the second 24-h period of culture.

Figure 2(c) shows oestradiol-17 β production during the first 24 h of culture by granulosa cells from non-atretic follicles of various diameters from ++ and FF ewes. The results are similar to those for inhibin output. The geometric mean oestradiol-17 β production by cells from 3–4.5 mm diameter follicles of FF ewes was similar to that of cells from ≥ 5 mm diameter follicles of ++ ewes. Geometric mean oestradiol-17 β production by cells from 3–4.5 mm and 1–2.5 mm diameter FF follicles was significantly higher ($P < 0.05$) than that of cells from correspondingly sized follicles from ++ ewes. Oestradiol-17 β production by cells from nonatretic follicles fell markedly during the second 24 h of culture; geometric mean values being only 20–54% of values for the first 24 h of culture. Oestradiol-17 β production during both 24-h periods of culture by cells from atretic follicles was low (< 0.2 ng oestradiol-17 β /10⁶ cells), irrespective of follicle diameter or ewe genotype.

For each genotype, there was a significant linear correlation between bioactive inhibin output and oestradiol-17 β production by granulosa cells during the first 24 h of culture (Fig. 3). There was no significant difference ($P > 0.05$) between the values for the slopes (0.7 vs. 0.6) or the intercepts (0.5 vs. 0.8), indicating that the regression lines for the 2 genotype populations were coincidental.

Fig. 3. Relationship between bioactive inhibin output and oestradiol-17 β production during the first 24 h of culture by granulosa cells from 1–4.5 mm and 3– ≥ 5 mm diameter nonatretic follicles from FF (Δ) and ++ ($\blacktriangle$) ewes, respectively. Data have been transformed to logarithms $y' = \ln(y + 1)$, $x' = \ln(x + 1)$, to equalize the variances. Linear regression lines have been fitted to the ++ and FF data separately, and the associated regression equations are shown, together with the correlation coefficients (r), significance and n values.

Effect of testosterone on inhibin production

Testosterone (1 μ g/ml) generally had a stimulatory effect on bioactive inhibin production by granulosa cells from both genotypes, during the first 24 h of culture. Overall, inhibin production by

testosterone-treated cultures was 2.3 ± 0.4 -fold (mean $\pm$ s.e.m.) and 2.4 ± 0.5 -fold higher than that of corresponding control cultures for $++$ and FF ewes, respectively ($P < 0.05$, $n = 19$ for $++$ ewes; $P < 0.01$, $n = 15$ for FF ewes; paired Student's t test).

Discussion

Follicles in FF ewes have a maximum diameter of 3–4.5 mm, whereas in $++$ ewes preovulatory follicles are ≥ 5 mm in diameter (Table 1; McNatty & Henderson, 1987). Granulosa cells from 3–4.5 mm diameter FF follicles, however, share characteristics similar to those of cells from ≥ 5 mm diameter $++$ follicles. The mean aromatase (i.e. oestradiol-17 β synthetase) activity and mean FSH- and LH-stimulatable cyclic AMP production by granulosa cells from 3–4.5 mm diameter FF follicles is greater than that of cells from $++$ follicles of similar size, but comparable to that of cells from ≥ 5 mm diameter $++$ follicles (Fig. 2c; Henderson *et al.*, 1987; McNatty & Henderson, 1987). The present study demonstrates that inhibin output by granulosa cells from the two genotypes follows a similar pattern. In both genotypes, geometric mean inhibin output by cells from non-atretic follicles increased with increasing follicular diameter. Geometric mean inhibin output by cells from 3–4.5 mm diameter FF ewes was significantly higher ($P < 0.05$) than that of cells from similarly sized $++$ follicles, but comparable to that of ≥ 5 mm diameter $++$ follicles. Oestradiol-17 β production by the cells, cultured in the presence of testosterone, followed a similar pattern, and so confirmed previous findings (Henderson *et al.*, 1987).

The observed differences in granulosa cell inhibin production between the genotypes showed a similar pattern whether inhibin was measured by radioimmunoassay or bioassay (Figs 2a & b). However, the absolute amounts of inhibin measured by the two assays differed markedly, much lower values being measured by the bioassay relative to the radioimmunoassay. Previous comparisons of the bioassay and immunoassay of inhibin have also found that inhibin concentrations measured by bioassay can be considerably lower than those measured by radioimmunoassay (McLachlan *et al.*, 1986; Robertson *et al.*, 1988; Mann *et al.*, 1989). When discrepancies between bioassay and immunoassay data occur, particularly when the discrepancies are very large as in the present study, considerable caution must be exercised when interpreting the data. The disparity between the bioassay and immunoassay measurements of inhibin may be related to differences in the specificities of the two assays (Robertson, 1990). Granulosa cells may secrete, in addition to inhibin, other proteins such as activin and gonadotrophin-releasing hormone-like proteins, which stimulate pituitary FSH production, and follistatin, which suppresses FSH production. While these proteins would not be detected by the inhibin radioimmunoassay, they could influence the inhibin bioassay through their effects on FSH production. Thus, bioactive inhibin values could be artificially high or low depending on the relative amounts of other proteins in the granulosa cell culture medium influencing pituitary FSH output. In addition, granulosa cells may secrete subunits of inhibin, notably monomeric inhibin α subunit, and inhibin subunit fragments and precursors, e.g. pro- α C-subunit, which are inactive in the inhibin bioassay, but highly reactive in the radioimmunoassay (Bicsak *et al.*, 1988; Knight *et al.*, 1989). Consequently, inhibin concentrations measured by the radioimmunoassay could be artificially high. Nevertheless, despite the differences in the absolute amounts of inhibin measured, both assays indicated that the highest mean amounts of inhibin produced by granulosa cells *in vitro* were similar for both genotypes. Moreover, it was achieved with cells from the largest non-atretic follicles in $++$ (i.e. ≥ 5 mm diameter) and FF (i.e. 3–4.5 mm diameter) ewes.

It could be argued that the high ovulation rate of FF ewes, relative to $++$ ewes, is a result of their higher plasma concentrations of FSH (McNatty *et al.*, 1987), allowing more follicles to reach ovulatory maturity in synchrony. The higher plasma FSH concentrations, in turn, being brought about by the F gene having a negative influence on ovarian inhibin production. Consistent with

this argument was the early finding of Cummins *et al.* (1983) that the bioactive inhibin content of ovaries collected from highly fecund Booroola $\times$ Merino ewes was only one-third of that of control Merino ewes. Moreover, active immunization with inhibin immunogens, to immuno-neutralize circulating inhibin, raises ovulation rates in sheep (Henderson *et al.*, 1984; Cummins *et al.*, 1986; Forage *et al.*, 1987). As granulosa cells are the principal source of follicular inhibin production (Henderson & Franchimont, 1983; Rodgers *et al.*, 1989; Torney *et al.*, 1989), one might have expected mean cellular inhibin production *in vitro* by granulosa cells from FF ewes to be lower than in cells from ++ ewes. This, however, was not the case. It is possible that cellular inhibin production *in vitro* may not reflect granulosa cell inhibin production *in vivo*. Although plasma FSH concentrations differ between the genotypes *in vivo*, the granulosa cells from follicles of both genotypes were cultured in media containing the same low concentration of endogenous FSH (0.9 ng/ml) originating from the 10% ovariectomized sheep serum. The extent to which this endogenous FSH might have influenced the functioning *in vitro* of the cells from the 2 genotypes, if at all, is uncertain. Exogenous FSH does not influence oestradiol-17 β production by ovine granulosa cells *in vitro*, while the effects on inhibin production *in vitro* have not been studied. However, previous studies with Booroola granulosa cells *in vitro* have indicated that ≥ 10 ng FSH/ml is required to stimulate production of cyclic AMP, an early event in FSH action (Henderson *et al.*, 1987). The present study was performed using ewes at Days 10–13 of the oestrous cycle. It is possible that differences in granulosa cell inhibin production between the genotypes may only occur at particular times of the oestrous cycle, e.g. during the preovulatory period, when ovulation rates can be markedly influenced by relatively small changes in plasma FSH concentrations (Henderson *et al.*, 1988). Differences in plasma FSH concentrations between the genotypes are, however, found during both the luteal and follicular phases of the oestrous cycle, and during anoestrus (McNatty *et al.*, 1987).

In sheep of both genotypes, granulosa cells from the largest nonatretic follicles produced the highest mean amounts of inhibin and oestradiol-17 β . Such large antral follicles are also the major source of circulating inhibin in sheep (Mann *et al.*, 1989; Findlay *et al.*, 1990). Previous studies have shown that in FF Booroola ewes there are on average 4.8 large oestrogenic follicles, each containing 1.1×10^6 granulosa cells (McNatty *et al.*, 1986). In ++ ewes, there are on average 1.5 large oestrogenic follicles with 3.8×10^6 cells/follicle (McNatty *et al.*, 1986). The total number of granulosa cells present in these large non-atretic follicles is therefore similar for both genotypes (5.3 vs. 5.7×10^6 cells for FF vs. ++ ewes). The mean per cell production of inhibin *in vitro* by granulosa cells from large nonatretic follicles is also similar for both genotypes (Fig. 2). One might, therefore, anticipate that the amounts of inhibin secreted by the ovaries of both genotypes would be similar, as has already been found for oestradiol-17 β (McNatty *et al.*, 1986), and *F*-gene-specific differences in plasma FSH may not be mediated through inhibin. Indeed, the ovary may not even be involved, as recent studies have demonstrated *F*-gene-specific differences in plasma FSH concentrations in ovariectomized Booroola ewes (McNatty *et al.*, 1989).

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