Binding characteristics of ^{125}I -labelled human FSH to granulosa cells from Booroola ewes which were homozygous, heterozygous or non-carriers of a major gene(s) influencing their ovulation rate

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Summary. At 37°C ^{125}I -labelled human (h) FSH (NIAMDD-hFSH-I-3) bound rapidly to granulosa cells from Booroola and Romney ewes with 50% maximum binding achieved after 3 min and equilibrium being reached within 45 min, irrespective of whether the cells were obtained from the FF, F+ or ++ Booroola genotypes or from Romney ewes. Binding of ^{125}I -labelled FSH followed second order kinetics and there was no effect of follicle diameter ($1\text{--}2.5\text{ mm}$ vs $\geq 3\text{ mm}$). Irrespective of breed, genotype or follicle size, the mean ($\pm$ s.e.m.) calculated association rate constant, (k_a) was $7.3 (\pm 0.8) \times 10^5 \text{ litres mol}^{-1} \text{ sec}^{-1}$ ($n = 12$). Dissociation of receptor bound ^{125}I -labelled hFSH was $< 5\%$ after 30 min and low but variable (i.e. between 0 and 30%) after 2–6 h irrespective of breed, genotype or follicle size. No gene-specific differences were noted in binding specificity between F+ and ++ genotypes: studies were not performed with cells from FF ewes because of insufficient cells. The binding of ^{125}I -labelled hFSH could be displaced with sheep FSH (NIH-FSH-S16; 10% cross-reaction) and FSH-P (2.5% cross-reaction) but other sheep pituitary hormones and hCG showed little or no cross-reaction ($\leq 0.1\%$).

The calculated binding capacities (B_{max}) and equilibrium dissociation constants (K_d) for ^{125}I -labelled hFSH binding to granulosa cells did not differ between the Booroola genotypes or between Booroola or Romney follicles of different diameter (i.e. $1\text{--}2.5\text{ mm}$; or $\geq 3\text{ mm}$). The overall mean $\pm$ s.e.m. ($n = 24$) B_{max} and K_d values were $16.7 \pm 0.8 \text{ fm/mg protein}$ (i.e. ~ 800 available receptor binding sites/cell) and $1.1 \pm 0.1 \text{ nM}$ respectively.

Collectively, these findings suggest that the earlier maturation of follicles in FF or F+ ewes compared to ++ ewes is unlikely to be due to gene-specific differences in the FSH binding characteristics of the granulosa cells.

Keywords: Booroola ewes; FSH binding; granulosa cells

Introduction

High fecundity Booroola ewes contain a major gene(s) which influences their ovulation rate (see Bindon, 1984, for review). Homozygous (FF), heterozygous (F+) and non-carriers (++) of the F gene(s) have been segregated by pedigree and by ovulation rate recordings of ≥ 5 , 3 or 4 and 1 or 2 respectively (Davis *et al.*, 1982). The endocrine basis for the high ovulation rate remains unknown but it has been the subject of considerable interest. A characteristic feature of animals with the F gene is that their ovarian follicles ovulate at a significantly smaller diameter than do those in ++

ewes (McNatty *et al.*, 1986a). This seems to be a consequence of a greater proportion of small follicles (0.1–1.0 mm diam.) in F gene carriers responding to follicle stimulating hormone (FSH) and luteinizing hormone (LH) to synthesize high levels of adenosine cyclic 3',5'-monophosphate (cAMP) and of granulosa cells in F gene carriers synthesizing oestradiol and acquiring functional LH receptors at a smaller follicle diameter relative to those in ++ animals (Henderson *et al.*, 1985, 1987; McNatty *et al.*, 1985a, b, 1986a, b). This so-called earlier maturation of antral follicles might be due to the ovaries of ewes with the F gene being more often exposed to high concentrations of FSH and LH than are those of ++ ewes (McNatty *et al.*, 1987) and/or to genotypic differences in gonadal sensitivity to gonadotrophins (Fry *et al.*, 1988).

The aim of this study was to examine whether F gene-specific differences in Booroola ewes were detectable at the level of the FSH receptor in granulosa cells. Some binding studies were also done with granulosa cells from slaughterhouse Romney ewes for comparative purposes.

Materials and Methods

Animals. The parous Booroola Merino ewes that were used were between 4 and 8 years of age. All the animals (66 ++ ewes, 66 F+ ewes and 31 FF ewes) were offspring of progeny tested FF rams mated with FF ewes, ++ rams mated with ++ ewes and F+ rams mated with ++ or F+ ewes (i.e. to generate the F+ ewes). Ovaries of all Booroola ewes were recovered between Days 6 and 14 of the oestrous cycle. The Romney ewes were of mixed age and of unknown reproductive status and the ovaries were recovered from the slaughterhouse. To test the effects of increased or decreased plasma FSH concentrations on FSH binding characteristics in granulosa cells, ovaries were recovered from: (I) anoestrous Romney ewes (N = 8) which had been pretreated with two injections of charcoal-treated bovine follicular fluid (bFF; 5 ml s.c. per injection) at 12-h intervals before ovariectomy; or (II) anoestrous Romney ewes (N = 8) which had been pretreated for 48 h (i.e. 4 × 12 h injections s.c.) with FSH-P (Burns-Biotech, Omaha, NE, USA; batch 518G85; 3 mg per i.m. injection). Treatment of Romney ewes with bFF led to a 70% reduction in plasma FSH concentration and a marked increase in atresia in all sizes of antral follicles ≥ 1 mm diameter (McNatty *et al.*, 1985b; Henderson *et al.*, 1986). Injections of FSH-P to anoestrous Romney ewes increase the number of non-atretic follicles ≥ 3 mm diameter from 3–8 to 5–40 (unpublished observation).

Recovery and preparation of granulosa cells for binding studies. Ovaries were collected into chilled (i.e. 4–6°C) saline (0.9% NaCl) containing 20 mM-Hepes buffer (pH 7.4) which was also used as the dissection medium. The numbers of corpora lutea (CL) in the ovaries of the Booroola but not Romney ewes were recorded. As many follicles as possible (≥ 1 mm diam.) were dissected from the ovaries. Preliminary studies with granulosa cells from Romney ewes revealed that to measure appreciable FSH binding (i.e. 1–2%) a minimum of 5 × 10⁶ granulosa cells (i.e. ~400 µg protein) was required per assay tube. To achieve this it was necessary to pool granulosa cells from 4–10 ewes. In several instances, cells from small (1–2.5 mm diam.) and large (≥ 3 mm diam.) follicles were pooled separately from ++ and F+ ewes and from Romney ewes. In FF ewes there were fewer follicles ≥ 3 mm diameter compared to ++ and F+ ewes ($P < 0.01$) and follicles > 4.5 mm diameter were never observed. Therefore, for all FF ewes, granulosa cells from all follicles ≥ 1 mm diameter were pooled. At the level of the dissecting microscope (× 64), there was no evidence that the granulosa cells were contaminated with thecal tissue. We have previously reported that theca but not granulosa cells from 1–2.5 mm diameter follicles in ++ Booroola ewes or from Romney ewes bind ¹²⁵I-labelled human chorionic gonadotrophin (McNatty *et al.*, 1986b). Moreover, we have repeatedly shown that granulosa cells from the above-mentioned 1–2.5 mm diameter follicles do not synthesize cAMP after exposure to LH (Henderson *et al.*, 1985, 1986, 1987), suggesting that our technique for recovering granulosa cells is unlikely to introduce significant thecal contamination. Once pools of granulosa cells were prepared they were centrifuged at 450 g at 4–6°C for 20 min and the cellular pellets were then resuspended in Tris buffer (i.e. 0.05 M-Tris-HCl buffer containing 0.02 M-sucrose and 5 mM-MgCl₂ (pH 7.5)) to a final concentration of 25 × 10⁶ cells/ml. The cell suspensions were added in 0.2 ml aliquants to assay tubes, capped and stored at –70°C until the binding studies were performed. The only exception to the above was with respect to a study of the relationship between the number of granulosa cells and the amount of ¹²⁵I-labelled hFSH specifically bound to the cells. In this instance granulosa cells were stored in the Tris buffer at doubling concentrations from 3.125 × 10⁶ cells/0.2 ml to 200 × 10⁶ cells/0.2 ml. Aliquants of each pool of granulosa cells were assayed for protein by the method of Lowry *et al.* (1951).

Recovery and preparation of granulosa cells for cAMP studies. Ovaries from Booroola Merino FF or ++ ewes were collected into chilled (4–6°C) minimum essential medium (MEM) containing 20 mM-Hepes buffer (pH 7.4) and 1% (w/v) bovine serum albumin (BSA; 98% purity; ICP Ltd, Auckland, NZ). Granulosa cells were isolated, pooled and washed as described for the binding studies. After washing the cellular pellets were resuspended in Dulbecco's phosphate-buffered saline with 1-methyl-3-isobutyl xanthine (0.2 mM, MIX) and 1% (w/v) BSA (i.e. DPBS; pH 7.4) at a concentration of 250 000 cells/ml. The cell suspensions were then added in 0.5 ml aliquants to 12 × 75 mm screw

topped plastic tubes semi-immersed in ice-water and containing 0.5 ml DPBS or 0.5 ml DPBS + 500 ng hFSH (NIAMDD-hFSH-I-3). The tubes were incubated at 37°C for 0 or 45 min and then heated to 80°C for 15 min before being stored at -20°C until assayed for cAMP by radioimmunoassay as described by McNatty *et al.* (1985b).

The cAMP antiserum was kindly provided by Dr A. R. La Barbera (Northwestern University, Chicago, IL, USA). The lyophilized material was made up in assay buffer and 100 µl aliquants were added to the acetylated samples to a final dilution of 1:30 000. The cross-reactions of AMP, ADP, ATP and cGMP with the antiserum in our assays were all <0.00004% while that for dibutyl cAMP was 0.0049%. About 10 000 c.p.m. iodinated cAMP derivative in sodium acetate buffer (0.05 M, pH 6.5) containing 3% (w/v) BSA was added to each assay tube in 100 µl. The radio-activated label was prepared by iodinating 2',O-monosuccinyl adenosine 3',5'-cyclic monophosphate tyrosyl methyl ester (Sigma Chemicals, St Louis, MO, USA) to a specific activity of 120–150 µCi/µg.

The assay tubes were incubated overnight at 4°C and the free and bound fractions were separated using 0.2% (w/v) Norit A charcoal in 0.1 M-potassium phosphate buffer (pH 6.3) with 0.25% BSA (w/v). The cAMP results were expressed as pmol cAMP/10⁶ cells. The intra- and inter-assay coefficients of variation were 8 and 14% respectively. The limit of detection was ≤0.1 pmol/10⁶ cells.

FSH iodination and binding studies with ¹²⁵I-labelled hFSH. Human FSH (hFSH; 25 µg; NIAMDD-hFSH-I-3; 6887 i.u./mg by RIA; contamination with hLH, hTSH, hGH and human prolactin was 420, 0.12, ≤0.01 and <0.01 i.u./mg respectively as determined by RIA) was iodinated using 1.0 mCi Na¹²⁵I (Amersham, Bucks, UK) and lactoperoxidase (Myachi *et al.*, 1972) and the products were purified on a column of Sephadex (G-75, 30 × 1 cm). The specific activities of the ¹²⁵I-labelled hFSH preparations were calculated to be 30–42 µCi/µg as described by Bolton (1977) and confirmed using the self-displacement method (Diekman *et al.*, 1978). The mass of label added to each binding assay was standardized for each iodination. Each ¹²⁵I-labelled hFSH preparation was stored at 4°C and used within 14 days of iodination. The bindability of each preparation of ¹²⁵I-labelled hFSH was not checked. However, the coefficient of variation between different preparations of ¹²⁵I-hFSH binding to granulosa cells from Romney ewes was 12.6% (n = 4).

For the binding studies, aliquants of granulosa cells were incubated in polystyrene tubes (12 × 75 mm) with ¹²⁵I-labelled hFSH (0.14–120 ng) in a final volume of 350 µl Tris buffer with 5 mM-MgCl₂, 20 mM-sucrose and 0.5% egg albumin (Sigma). Incubations were performed at 37°C for up to 16 h; specific incubation times for the various studies are indicated in the 'Results' section. The receptor-bound ¹²⁵I-labelled hFSH was separated from free ¹²⁵I-labelled hFSH by centrifugation at 3000 g for 20 min at 4°C after the addition of 1 ml ice-cold incubation buffer. The pellets containing the bound ¹²⁵I-labelled hFSH were washed with 1 ml ice-cold incubation buffer and the radioactivity in the pellets was determined after a second centrifugation at 3000 g. Non-specific binding was determined by co-incubating the cells or homogenate with excess unlabelled gonadotrophin (i.e. Gonadotrophon F.S.H.; Paines & Byrne Ltd, Greenford, UK). For every 1 ng of ¹²⁵I-labelled hFSH per assay tube 10 i.u. Gonadotrophon was added for non-specific binding; dose-response studies indicated that this ratio of Gonadotrophon to ¹²⁵I-labelled hFSH was 1–2 times the minimum amount necessary to displace the maximum amount of ¹²⁵I-labelled hFSH. Gonadotrophon is a serum extract from pregnant mares and 10 i.u. were equivalent to 100 ng hFSH-I-3 in their ability to displace ¹²⁵I-labelled hFSH from a testicular receptor homogenate prepared as described by Abou-Issa & Reichert (1977). The amount of specifically bound ¹²⁵I-labelled hFSH in c.p.m. was calculated by subtracting non-specific binding from the total amount of ¹²⁵I-labelled hFSH bound. The counting efficiency of the gamma counter (Crystal Multidetector, Packard, IL, USA) was 75%. Woolf plots (McNatty *et al.*, 1985b) were generated by using a standard amount of ¹²⁵I-labelled hFSH (3–5 ng) and increasing amounts of unlabelled hFSH (NIADDK-hFSH-I-3). In one instance the results from these studies were compared to that from a Woolf plot obtained from progressively increasing the amounts of ¹²⁵I-labelled hFSH (3.75–120 ng).

Triplicate or in most instances, duplicate measurements were made at each binding point with the coefficient of variation between replicate values being <8%.

The Woolf plots and analysis of results. From the equilibrium binding studies, the specific binding data were fitted to the Woolf equation, i.e.

$$\frac{F}{B} = \frac{K_d}{B_{\max}} + \frac{1}{B_{\max}} F,$$

where F = the concentration of free hormone and B = the concentration of bound hormone (Keightley & Cressie, 1980). The binding characteristics ($B_{\max}$, K_d) with respect to follicle size or genotype were examined by Student's *t* test or analysis of variance in conjunction with the Newman-Keuls test.

Results

The mean ± s.e.m. number of CL recovered from FF, F+ and ++ ewes were 5.32 ± 0.27 (range 4–11; N = 31 animals), 2.83 ± 0.09 (range 2–4; N = 66) and 1.09 ± 0.07 (range 0–2; N = 66) respectively.

Effect of number of granulosa cells on 'bindability' of ^{125}I -labelled hFSH

Specific ^{125}I -labelled hFSH (~ 0.14 ng or 9600 c.p.m.; $41.4 \mu\text{Ci}/\mu\text{g}$) binding to ovine granulosa cells varied with the number of cells (Fig. 1). Maximum 'bindability' was achieved with 13.5% of the ^{125}I -labelled hFSH being specifically bound to the granulosa cell preparations.

Fig. 1. Specific (●—●) and non-specific (○—○) binding of ^{125}I -labelled hFSH (0.14 ng/tube; 9600 c.p.m.) to granulosa cells from Romney ewes. The granulosa cells were incubated at 37°C for 75 min. All values are means of duplicates.

Effect of incubation time on binding of ^{125}I -labelled hFSH to granulosa cells

The specific binding of ^{125}I -labelled hFSH was a time dependent process (Fig. 2). At 37°C , specific binding increased rapidly for the first 10 min and thereafter proceeded more slowly to reach a maximum after 20–45 min. Approximately 50% maximum ^{125}I -labelled hFSH binding was achieved for cells from ewes of FF, F+ and ++ genotypes and for cells from Romney ewes at 3 min.

The rate of association of ^{125}I -labelled hFSH to granulosa cells at 37°C as shown in Fig. 2 followed second-order kinetics using the equation:

$$k_a t = \frac{1}{a - b} \ln \left[\frac{b(a - x)}{a(b - x)} \right]$$

where the initial hormone concentration, receptor concentration, hormone–receptor complex concentration and time are represented by the terms a , b , x and t respectively. The association rates (i.e. k_a) were obtained from regression lines fitted through 5–6 datum points over the first 5 min (Fig. 3). In these studies $a = 1 \times 10^{-9} \text{ M}$, $b = 0.016 \times 10^{-9} \text{ M}$ and the initial total counts added were 900 000 c.p.m. These data were obtained from pools of cells different from those described in Fig. 2. The k_a values were calculated for the FF, F+ and ++ Booroola ewes to be 9.6×10^5 (1 pool tested; Fig. 3), 8.2×10^5 [2 pools; 5.8×10^5 (data not shown) and 10.6×10^5 (see Fig. 3)] and 8.9×10^5 (1 pool; Fig. 3) litres $\text{mol}^{-1} \text{ sec}^{-1}$ respectively. The calculated mean k_a values ($\pm$ s.e.m.) for ^{125}I -labelled hFSH binding to granulosa cells from small (1–2.5 mm diam.; 4 pools tested) and large (≥ 3 mm diam.; 4 pools) follicles from Romney ewes were $5.2 (\pm 0.7) \times 10^5$ and $8.1 (\pm 1.5) \times 10^5$ litres $\text{mol}^{-1} \text{ sec}^{-1}$ respectively; these k_a values were not significantly different from one another. Figure 3 shows an example of binding with respect to time for a single pool of cells from small or large follicles. The overall k_a value ($\pm$ s.e.m.) irrespective of breed (genotype) and follicular diameter was $7.3 (\pm 0.8) \times 10^5$ litres $\text{mol}^{-1} \text{ sec}^{-1}$ (12 pools).

Fig. 2. Time dependence of specific ^{125}I -labelled hFSH (12–20 ng/tube; sp. act. = 36–41 $\mu\text{Ci}/\mu\text{g}$) binding to granulosa cells (5×10^6 cells/tube) from Booroola FF ($\triangle$ — $\triangle$), F+ ($\circ$ — $\circ$), ++ ($\bullet$ — $\bullet$) and Romney ($\square$ — $\square$, 1–2.5 mm diam. follicles; $\blacktriangle$ — $\blacktriangle$; ≥ 3 mm diam. follicles) ewes. Cells from the Booroola ewes were pooled from all follicles ≥ 1 mm diam.). The incubations were performed at 37°C and the values are means of duplicates.

To test for dissociation, 5×10^6 granulosa cells/tube were preincubated with ^{125}I -labelled hFSH (12–20 ng; 36–42 $\mu\text{Ci}/\mu\text{g}$) for 75 min at 37°C . The bound ^{125}I -labelled hFSH was pelleted by centrifugation and resuspended in 350 μl Tris-HCl buffer at 37°C for up to 6 h before the final centrifugation. Pools of cells from Booroola F+ (2 pools) and ++ (N = 1 pool) ewes and from Romney ewes (6 pools from large follicles, 6 pools from small follicles) were tested. Overall, the dissociation of specifically bound ^{125}I -labelled hFSH appeared to be low: after 30 min, < 5% of the label had dissociated whereas after 2–6 h, the amount dissociated varied between 0 and 30%. The variability was unrelated to either sheep breed (genotype) or follicular diameter.

Specificity studies

Since hFSH (NIAMDD-hFSH-I-3) contained an appreciable amount of hLH contamination (i.e. 420 i.u./mg), the ability of human chorionic gonadotrophin (hCG; CR121; 13 450 i.u./mg) to displace ^{125}I -labelled hFSH (3 ng, 40 $\mu\text{Ci}/\text{mg}$) binding was tested using a pool of granulosa cells from 1–6 mm diameter follicles from Romney ewes (5×10^6 cells/tube). The degree of cross-reaction with hCG was measured at 0.03%.

The specificity data for the ++ and F+ ewes are summarized in Table 1. Insufficient cells were available to test binding specificity for FF ewes. The % binding figures were obtained by dividing the amount of unlabelled hFSH (1.8×10^{-9} M) required to displace 50% of the ^{125}I -labelled hFSH by the amount of the other cross-reacting hormones required to displace 50% of the ^{125}I -labelled hFSH. No obvious gene-specific differences in binding specificity were noted between granulosa cells from F+ and ++ ewes. The binding of ^{125}I -labelled hFSH (~ 3 ng) to granulosa cells from F+ or ++ ewes was displaced to some extent with oFSH and to lesser extent with FSH-P but other ovine pituitary hormones (supplied by the National Pituitary Agency, Maryland, USA) displayed little or no cross reaction.

Fig. 3. Rates of ¹²⁵I-labelled hFSH binding to granulosa cells (5×10^6 cells/tube from (a) FF (Δ ; 1–4 mm diam. follicles), F+ ($\bigcirc$; 1–4.5 mm diam. follicles) and ++ ($\bullet$; 1–6 mm diam. follicles) ewes and (b) small ($\square$ — $\square$; 1–2.5 mm diam.) and large ($\blacktriangle$ — $\blacktriangle$; ≥ 3 mm diam.) follicles from Romney ewes as a function of time at 37°C. Each value is a mean of duplicate measurements. For clarity a single line is drawn through all points for Booroola ewes although the fitted lines for each genotype differ slightly. The terms a, b and x refer to the initial hormone concentration, receptor concentration and hormone-receptor complex concentration respectively.

Table 1. Specificity of ¹²⁵I-labelled hFSH (~3 ng) to granulosa cells from Booroola ewes

Hormone	% Binding with respect to genotype	
	++	F+
hFSH (NIAMDD-hFSH-I-3)	100	100
oFSH (NIH-FSH-S16)	10.00	10.00
oLH (NIADDK-oLH-S25)	0.12	0.13
oTSH (NIH-TSH-S8)	0.08	0.06
oGH (NIH-GH-S11)	<0.01	<0.01
oProlactin (NIH-P-S13)	<0.01	<0.01
FSH-P (Lot 518G85)	2.50	2.50

Granulosa cells (5×10^6) were incubated at 37°C for 75 min with ¹²⁵I-labelled hFSH (NIAMDD-hFSH-I-3; ~3 ng; 40 μ Ci/ μ g) and with increasing amounts of one of the above-mentioned unlabelled hormones. Binding in the absence of unlabelled hFSH was regarded as 100%.

Equilibrium binding studies

The incubations of ^{125}I -labelled hFSH with granulosa cells were performed at 37°C for 75 min to achieve equilibrium. The specific binding of ^{125}I -labelled hFSH to granulosa cells from Booroola FF or ++ ewes with respect to increasing concentrations of unlabelled hFSH is shown in Fig. 4(a, b). Typically, linear plots were obtained when the specific binding data were fitted to the Woolf equation (Keightley & Cressie, 1980; Fig. 4c, d). The calculated binding capacities ($B_{\max}$) and equilibrium dissociation constants (K_d) from the Woolf plots were 21.5 fmol/mg protein and 1.2 nM for the cells from FF ewes and 17.2 fmol/mg protein and 1.2 nM for the cells from ++ ewes. Binding of ^{125}I -labelled hFSH to granulosa cells from Romney ewes as a function of increasing ^{125}I -labelled hFSH concentration is shown in Fig. 5(a). A linear plot was obtained when the specific binding data were fitted to the Woolf equation. The calculated $B_{\max}$ and K_d values were 19.9 fmol/mg protein and 1.2 nM respectively.

Fig. 4. Specific binding of ^{125}I -labelled hFSH (~ 3 ng, $36 \mu\text{Ci}/\mu\text{g}$) to granulosa cells from ++ (a) and FF (b) ewes with respect to increasing concentrations of unlabelled hFSH. For the ++ and FF ewes, the granulosa cells were recovered from 1–2.5 mm diameter and 1–4.5 mm diameter follicles respectively. Binding tubes (5×10^6 granulosa cells/tube) were incubated at 37°C for 75 min. Woolf plots of the data which included the mass of ^{125}I -labelled hFSH for the ++ and FF specific binding data are shown in (c) and (d) respectively. The binding characteristic $B_{\max}$ was derived from the slope of the fitted line (i.e. slope = $1/B_{\max}$) and the K_d from the $B_{\max} \times$ intercept.

The characteristics of the FSH receptor in granulosa cells with respect to breed, genotype, and follicle size are summarized in Table 2. No F gene-specific effects, breed or follicle size effects were noted with respect to the FSH binding characteristics in granulosa cells. When all $B_{\max}$ and K_d values (i.e. from the self-displacement assays) were pooled irrespective of follicle size, breed or genotype, the respective mean $\pm$ s.e.m. ($n = 24$) values were 16.7 ± 0.8 fmol/mg protein and 1.1 ± 0.1 nM. Assuming that $400 \mu\text{g}$ protein is equivalent to 5×10^6 cells, the number of available

Fig. 5. Effect of addition of increasing ^{125}I -labelled hFSH (ng) on specific binding of ^{125}I -labelled hFSH to granulosa cells (5×10^6 cells/tube) from Romney ewes with respect to ^{125}I -labelled hFSH concentration (a: vertical bars are s.e.m. of triplicate measurements at each point, and granulosa cells were recovered from follicles of 1–6 mm in diameter) and Woolf plot of the above data (b: see Fig. 4 for further details).

FSH receptors per sheep granulosa cell was estimated to be 800. This number of receptors per cell was essentially unchanged even after Romney ewes had been pretreated with bFF or FSH-P. The respective $B_{\max}$ and K_d values for cells pooled from the 1–6 mm diameter follicles from all ewes after bFF treatment were 14.1 fmol/mg protein and 1.1 nM. The respective $B_{\max}$ and K_d values after FSH-P pretreatment were 13.6 fmol/mg protein and 1.1 nM.

Effect of human FSH on cAMP synthesis by granulosa cells

These results with respect to follicle diameter and Booroola genotype are summarized in Table 3. Human FSH significantly stimulated more cAMP compared to that produced in control cultures ($P < 0.01$ for both genotypes). In turn the control cultures produced no further cAMP above that present in the cells at the start of the incubation period (i.e. the T_0 value). In cells from FF but not ++ follicles FSH stimulated more cAMP in large follicles (≥ 3 mm diam.) than in small follicles (1–2.5 mm diam.) ($P < 0.01$). In addition, FSH stimulated significantly more cAMP in cells from both small and large follicles from FF compared to those from ++ ewes ($P < 0.05$ for small and large follicles).

Table 2. Characteristics of the FSH receptor in granulosa cells of Booroola and Romney ewes with reference to Booroola genotype, sheep breed and follicle size

Breed (genotype)	Follicle diameter (mm)	B _{max} (fmol/mg protein)	K _d (nM)	n
Booroola Merino (++)	1-2.5	15.7 ± 1.7	1.2 ± 0.3	3
	3-6	17.7 ± 1.7	1.2 ± 0.1	5
Booroola Merino (F+)	1-2.5	17.8 ± 2.2	1.0 ± 0.1	3
	3-4.5	15.8 ± 2.0	1.3 ± 0.3	3
Booroola Merino (FF)	1-4.0	17.1 ± 3.4	1.1 ± 0.2	3
Romney	1-2.5	16.3 ± 1.4	0.9 ± 0.1	3
	3-7	16.9 ± 1.0	1.1 ± 0.1	3
	1-7	19.1	1.3	1

Values are means ± s.e.m; n = number of pools of granulosa cells tested.

Each pool of granulosa cells was derived from the ovaries of 5-10 sheep.

Table 3. Effect of human FSH (NIAMDD-hFSH-I-3, 250 ng/ml) on cAMP synthesis (pmol/10⁶ cells) by granulosa cells with respect to follicle diameter and Booroola genotype

Treatment	Follicle diameter (mm)	Booroola genotype	
		++ (n = 9)	FF (n = 8)
T ₀ †	1-2.5	0.4 (0.3, 0.6)	0.5 (0.3, 0.6)
Control		0.4 (0.3, 0.6)	0.5 (0.3, 0.7)
FSH		2.3 (1.5, 3.3)	4.3* (2.9, 6.0)
T ₀ †	≥ 3	0.6 (0.4, 0.8)	0.4 (0.3, 0.7)
Control		0.6 (0.4, 0.8)	0.4 (0.2, 0.7)
FSH		3.9 (2.1, 6.7)	8.9* (6.2, 14.8)

Values are geometric means (and 95% confidence limits);

n = number of pools of granulosa cells tested.

†cAMP values in cells at time of harvest.

*P < 0.05 compared to values for FSH treated cells from ++ ewes.

Discussion

The principal aim of the present study was to examine the FSH binding characteristics in granulosa cells from the FF, F+ and ++ Booroola genotypes. The ovulation rates as assessed from the number of corpora lutea indicated that the FSH-binding studies were being performed in ovaries which were being influenced by F gene expression. The major findings were that no gene-specific differences were noted with respect to the specificity, capacity ($B_{\max}$) or dissociation constant (K_d) of ^{125}I -labelled hFSH binding to granulosa cells and, moreover, no obvious differences were noted with respect to the rate of hFSH binding (k_a). It could be argued that the lack of a gene-specific difference was masked by the necessity to pool cells from different-sized healthy and atretic follicles. Nevertheless, significant F gene-specific differences are observed in the level of FSH-induced cAMP production after pooling cells as described above (Henderson *et al.*, 1987; McNatty *et al.*, 1988). Also, it could be argued that the lack of a gene-specific difference in FSH binding was due to the use of a human FSH preparation whereas the FSH-induced cAMP differences reported by Henderson *et al.* (1985, 1987; McNatty *et al.*, 1988) were found using an ovine FSH preparation. However, the present study reports that a gene-specific difference in cAMP production also occurs in granulosa cells when the same human FSH preparation as that used in the ^{125}I -labelled FSH binding studies was employed. Collectively these findings suggest that the F gene difference in FSH-induced cAMP synthesis is the result of an event(s) distal to the FSH receptor.

In the present study, the FSH binding characteristics of low total binding (<2%) and high specificity (i.e. >60%) with a ^{125}I -labelled FSH probe of 13.5% 'bindability' are similar to those reported in several previous studies with rat testis (Bhalla & Reichert, 1974), rat granulosa cells (Louvét & Vaitukaitis, 1976; Nimrod *et al.*, 1976), bovine granulosa cells (Darga & Reichert, 1978), human corpora lutea (McNeilly *et al.*, 1980; Bramley *et al.*, 1986) and hamster ovaries (Kin & Greenwald, 1986).

This study confirms that specific receptors for ^{125}I -labelled hFSH are present in sheep granulosa cells (Carson *et al.*, 1979). In the earlier study of Carson *et al.* (1979), intact follicles were preincubated at 37°C for 27–36 h with ^{125}I -labelled hFSH before isolation of granulosa cells and measurement of FSH binding activity. This technique precluded any accurate assessment of the FSH binding characteristics of granulosa cells (i.e. k_a , K_d , $B_{\max}$) because the studies were made in the presence of follicular fluid which is known to inhibit FSH binding (Darga & Reichert, 1978). For example, any of the differences in the amount of ^{125}I -labelled hFSH bound to granulosa cells in small/large, healthy/atretic follicles could have been related to the relative amounts of inhibitor(s) in follicular fluid (Sluss *et al.*, 1983).

In the present study with isolated granulosa cells, ~50% of the ^{125}I -labelled hFSH was bound to the receptor after 3 min and maximum binding was achieved after 45 min. Similarly, in bovine granulosa cells, the binding of ^{125}I -labelled hFSH was rapid with maximum uptake being recorded within 2 h (Darga & Reichert, 1978). The rapid binding of FSH to its receptor is consistent with the finding of a ≥ 3 -fold increase in cAMP accumulation within 10 min of exposing ovine or bovine granulosa cells to FSH *in vitro* (K. P. McNatty, S. Lun, D. Heath & N. Hudson, unpublished observations). There was no significant effect of follicle diameter or sheep breed on the rate of uptake of ^{125}I -labelled hFSH to granulosa cells. These findings suggest that the timing of the cAMP response to FSH stimulation *in vitro* is unlikely to be influenced by follicular diameter or sheep breed. *In vivo*, however, the granulosa cell cAMP response to FSH is likely to be affected by other factors such as the extent of the blood supply.

We were unable to measure the rates of dissociation of ^{125}I -labelled hFSH from granulosa cells at 37°C. The dissociation of hFSH appeared to be minimal as has been shown for the rat, rabbit and hamster (Nimrod *et al.*, 1976; Kim & Greenwald, 1986). The variability of dissociation (i.e. 0–30%) of ligand within the first 6 h was a problem which remained unresolved. In bovine granulosa cells, the specific binding of ^{125}I -labelled hFSH at 37°C declined steadily after 4 h (Darga & Reichert, 1978); this decline was thought to be due to the thermal lability of the granulosa

cell receptor as no such decline in binding was noted at 4°C. Perhaps in the present study the variable loss in ligand was due to the long-term (i.e. hours) stability/lability of the receptor at 37°C.

The calculated K_d value of 1.1 nM for sheep granulosa cells was similar to that recorded for the cow (Darga & Reichert, 1978). Likewise, with bovine granulosa cells no significant change in the B_{max} was noted with follicle diameter. We estimate that, for the sheep, the number of available FSH binding sites per cell is about 800 and that this remains relatively unchanged between sheep breeds (Merino and Romney), follicle diameter or following modification of ovarian follicular development *in vivo* by pretreating sheep with bovine follicular fluid or supplementary FSH. Louvet & Vaitukaitis (1976) showed that oestrogen pretreatment of rat granulosa cells did not alter the specific number of available FSH receptors per cell. Therefore, the findings from the sheep, cow and rat suggest that as follicles mature any increased FSH uptake would occur solely as a consequence of an increased number of granulosa cells as has been demonstrated in the present study (see Fig. 1). Despite no change in the FSH receptor binding characteristics (i.e. B_{max} , K_d , k_a) with increasing follicular diameter, there is an increase in the mean level of FSH-induced cyclic AMP synthesis with respect to follicular diameter (Henderson *et al.*, 1985, 1987; McNatty *et al.*, 1985c; Table 3). This difference in FSH-stimulatable cAMP synthesis with respect to follicular diameter is not due to any change in cAMP-phosphodiesterase enzyme activity (McNatty *et al.*, 1988) but might be due, in part, to increased activity or altered composition of the regulatory or catalytic components of the adenylate cyclase system (Richards & Hedin, 1988). In addition, part of the increase in cAMP synthesis could be due to LH contamination in the various FSH preparations (e.g. 6% LH contamination of NIAMDD-hFSH-I-3 by RIA). In large follicles (i.e. ≥ 5 mm diam. in Romney or Booroola ++ ewes or > 2.5 mm diam. in FF ewes) the level of LH contamination is sufficient to stimulate at least a proportion of the increase in cAMP via the LH receptor (see Henderson *et al.*, 1985, 1987).

In summary, the greater FSH-induced cAMP responses in granulosa cells in antral follicles, the earlier induction of granulosa cell aromatase and also the earlier maturation of follicles at a small diameter in FF ewes compared to ++ ewes (see McNatty & Henderson, 1988, for review) are all unlikely to be due to gene-specific differences in the k_a , K_d or B_{max} FSH binding characteristics of the granulosa cells.

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