Regulation of inhibin production by bovine ovarian cells *in vitro*

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Summary. The regulation of ovarian inhibin production was investigated using a rat pituitary cell culture system as a bioassay for inhibin activity. Bovine follicular granulosa cells produced inhibin *in vitro* provided that the culture medium contained serum. The stimulatory factor(s) present in serum is unlikely to be gonadotrophins, because bovine LH and/or FSH failed to stimulate inhibin production when added to medium devoid of serum. Luteinization of granulosa cells in culture was accompanied by a reduction in their inhibin production and an inverse relationship existed between inhibin and progesterone production by granulosa cells. Bovine corpus luteum cells in culture failed to produce detectable amounts of inhibin.

Androgens stimulated granulosa cell inhibin production with testosterone and 5 α -dihydrotestosterone being more potent than androstenedione. The androgens did not stimulate inhibin production by luteal cells. Progesterone inhibited granulosa cell inhibin production but oestrogens had no effect. Measurement of steroids and inhibin in fluid from individual follicles indicated that as follicle size increased, concentrations of oestradiol-17 β increased, testosterone and inhibin decreased and progesterone remained unchanged. The stimulatory effect of testosterone on inhibin production *in vitro* together with the parallel changes in follicular fluid concentrations of testosterone and inhibin suggest that ovarian inhibin production *in vivo* may be controlled, at least in part, through androgens modifying granulosa cell inhibin production. The inhibitory effect of progesterone on granulosa cell inhibin production may be more important in regulating ovarian inhibin production at the time of granulosa cell luteinization and CL formation. The stimulatory effect of androgens on granulosa cell inhibin production might also be a means by which androgens promote follicular atresia.

Introduction

The ovary, like the testis, may produce a non-steroidal compound, inhibin, which is capable of inhibiting pituitary FSH secretion (Franchimont *et al.*, 1979b). Steroid free bovine (de Jong, Welschen, Hermans, Smith & van der Molen, 1978), equine (Miller, Wesson & Ginther, 1979), human (Chappel, Holt & Spies, 1980), monkey (Channing, Anderson & Hodgen, 1980) and porcine (de Paolo, Wise, Anderson, Barraclough & Channing, 1979b; Schander, Anderson, Barraclough & Channing, 1980) follicular fluid selectively reduce FSH levels *in vivo* and/or selectively inhibit FSH production by anterior pituitary cells *in vitro*. Erickson & Hsueh (1978) indicated that granulosa cells are the probable source of this follicular inhibin, while studies of the rat (de Paolo, Shander, Wise, Barraclough & Channing, 1979a) and monkey (Channing *et*

et al., 1980) have shown that inhibin is secreted into the ovarian vein, thus strengthening the concept that ovarian inhibin may be physiologically important in the regulation of FSH secretion. Inhibin activity in follicular fluid is related to follicle size (Welschen, Hermans, Dullart & de Jong, 1977) and ovarian inhibin production changes throughout the oestrous/menstrual cycle (de Paolo *et al.*, 1979a; Chappel *et al.*, 1980). However, there is little information about the regulation of ovarian inhibin production. The aim of the present study was to investigate (a) the regulation of inhibin production by bovine ovarian cells *in vitro* and (b) the relationship between the inhibin concentration in individual bovine follicles to their hormonal environment.

Materials and Methods

Reagents

Purified bovine gonadotrophins were provided by Bioproducts, Peptide Department (UCB, Brussels, Belgium). The bovine FSH had a potency 60 times that of NIH-FSH-B1 (Steelman–Pohley assay) and LH contamination of < 3% (RIA) or < 0.45% (RRA). The bovine LH had a biological activity of 2.3 i.u./mg (Parlow assay) with FSH contamination of < 0.05% (by bioassay with NIH-FSH-S9 as standard). Steroids were obtained from Sigma (London) Chemical Co. Ltd, Poole, Dorset, U.K.

Bovine follicular fluid

Ovaries were obtained from healthy adult cows within 1 h of their slaughter at a local abattoir. Follicular fluid was aspirated from all visible antral follicles and that from follicles containing ≤ 0.1 ml fluid was pooled for follicles from the same ovary or each pair of ovaries. Aliquots taken for inhibin determination were freeze-dried while the remaining follicular fluid was stored frozen for subsequent steroid determination.

Ovarian cell cultures

Granulosa cells. Granulosa cells harvested and pooled from antral follicles containing ≥ 0.3 ml follicular fluid were cultured as described previously (Henderson & Moon, 1979). Briefly, a minimum of 10^5 'live' cells were cultured in multi-welled tissue culture Petri dishes (Sterilin, Middlesex, U.K.) at 37°C in 1 ml culture medium consisting of 10% donor calf serum and 90% Eagle's Minimum Essential Medium (Modified) with Earle's Salts (Eagle, 1959) and supplemented with Hepes buffer (20 mM), glutamine (2 mM), penicillin (50 units/ml), streptomycin (50 µg/ml), amphotericin B (0.625 µg/ml) and non-essential amino acids (Eagle, 1959) (all reagents obtained from Flow Laboratories, Irvine, Scotland). The gas phase was air. The medium was replaced at selected times and aliquots taken and freeze-dried for subsequent inhibin determination while the remainder was stored frozen until assayed for steroids. At the end of the culture period the cells were washed thoroughly with culture medium devoid of calf serum and assayed for protein by the method of Lowry, Rosebrough, Farr & Randall (1951) as modified by Patterson (1979). In some preliminary studies, granulosa cells were cultured in the above medium devoid of calf serum. Steroids (see below) were added to the culture media in ethanol, with control cultures receiving ethanol alone. The ethanol concentration in the culture media never exceeded 1%.

Luteal cells. Dispersed cells were obtained as described previously (Henderson & Moon, 1979). Briefly, corpora lutea were freed of adherent connective tissue, cut into small pieces approximately 2 mm in diameter and washed in Hanks' Balanced Salt Solution (Flow Laboratories) devoid of magnesium and calcium but supplemented with Hepes, glutamine and antibiotics as described above (HBS–HGA). Tissue dissociation was achieved by stirring at

37°C in HBS-HGA containing 0.2% collagenase (Type II, Sigma), the released cells being collected after incubation for 20 and 40 min. Final dispersal of remaining tissue fragments was achieved by drawing through a syringe tip and a series of needles (18–22 gauge). The freed cells were pooled, filtered through sterile gauze, washed 4 times and their viability determined using nigrosin dye. The procedure for the culture of the luteal cells was exactly as described above for the granulosa cells.

Inhibin assay

The assay of inhibin activity was based on the selective inhibition of basal and LH-RH-induced FSH secretion by cultured anterior pituitary cells as described by Franchimont, Demoulin, Verstraelen-Proyard, Hazee-Hagelstein & Tunbridge (1979a). Dispersed anterior pituitary cells from adult male Wistar rats were obtained by enzymic digestion using the technique described by Hopkins & Farquhar (1973). The dispersed cells (1×10^6) were cultured at 37°C on Petri dishes (Falcon Plastics) in 3 ml Dulbecco's Modified Eagles Medium (Dulbecco & Freeman, 1959) supplemented with 5% horse serum, 2.5% fetal calf serum, 1% glutamine and 1% non-essential amino acids (Flow Laboratories). The gas phase was a water-saturated atmosphere of 95% air and 5% CO₂. After 3 days of culture, the medium was discarded and replaced with 1.6 ml of medium containing the test material or a reference standard preparation of inhibin. Incubation was continued for a further 3 days and then the medium was removed and stored frozen until assayed for gonadotrophins. The culture plates were washed and incubation was continued with 1.6 ml medium containing standards or test material and LH-RH at a final concentration of 10^{-8} M. After incubation for 6 h the medium was removed and stored frozen until assayed for gonadotrophins.

The freeze-dried aliquots of follicular fluid and cell culture medium taken for inhibin assay were reconstituted with distilled water. Steroids were removed by mixing for 16 h at 4°C with activated charcoal (Norit A, 1%) pretreated with dextran (0.1%) followed by centrifugation at 3000 g. The supernatants were filter-sterilized by passing through a 0.45 µm cellulose millipore filter before use. Such dextran-charcoal treatment removed >99.8% of exogenous steroids added to culture medium while endogenous steroids present in follicular fluid were undetectable after this treatment. In the pituitary cell culture medium, the concentration of follicular fluid ranged from 0.5 to 5% (v/v) and that of granulosa or luteal cell culture medium ranged from 3 to 60% (v/v). Culture medium with and without added steroids incubated in the absence of granulosa or luteal cells served as control medium for adding to the pituitary cell culture medium. The standard reference preparation of inhibin, kindly supplied by Professor B. Hudson (Howard Florey Institute of Experimental Physiology and Medicine, Melbourne, Australia) was derived from ovine testicular lymph (OTLP6) and has been given an arbitrary potency of 1 U/mg (Eddie, Baker, Higginson & Hudson, 1979). This inhibin standard was added to the pituitary cell cultures at doses of 250 to 2000 µg/ml. Each standard and aliquot of test material was added to 5 replicate pituitary cell cultures. Inhibin activity in follicular fluid or culture media was expressed relative to the activity of the OTLP6 standard.

Radioimmunoassays

The gonadotrophin concentrations in media from the pituitary cell cultures were measured in duplicate by double-antibody methods using NIAMDD rat pituitary gonadotrophin reagents supplied by the pituitary agency of NIH. The concentrations are expressed in terms of NIAMDD rat-FSH-RP1 for FSH and rat-LH-RP1 for LH. The sensitivities of the assays were 5 ng FSH/ml and 10 ng LH/ml. The inter- and intra-assay coefficients of variation were 3 and 8% for the FSH assay and 5 and 12% for the LH assay respectively.

Steroid concentrations in follicular fluid and granulosa and luteal cell culture media were

determined using specific radioimmunoassays described previously (Neal, Baker, McNatty & Scaramuzzi, 1975; Van Look, Hunter, Corker & Baird, 1977; Corker & Davidson, 1978). The limits of sensitivity of the assays (per tube) were 25 pg for progesterone, 5 pg for oestradiol-17 β and 10 pg for testosterone. The intra- and inter-assay coefficients of variation of all the steroid assays were each < 10% and < 16% respectively.

Statistics

Unless otherwise stated, data were subjected to analyses by paired or unpaired Student's *t* test.

Results

Specificity and validity of inhibin assay

As shown in Text-fig. 1, each preparation caused a dose-dependent inhibition of FSH secretion in basal and LH-RH stimulated conditions. The significance of regression (Finney's *Q*) was always higher than 0.01. Variations in the slopes were not significant (G^2 , $P > 0.05$; Finney, 1964) and the curves were therefore considered to run parallel. LH secretion was reduced only in LH-RH treated cultures and only by much higher amounts of the test substances than those required to impair FSH secretion. The mean lowest detectable doses of inhibin capable of decreasing LH-RH-induced FSH and LH secretion, calculated at 95% confidence limit of gonadotrophin secretion in the absence of inhibin, were $86 \pm 25 \mu\text{g}$ and $337 \pm 59 \mu\text{g}$ respectively. In basal conditions, the FSH inhibition curves were less sensitive (lowest detectable dose of inhibin $315 \pm 62 \mu\text{g}$) and less precise (index of precision, λ , range 0.15–0.41) than when the secretion was stimulated by LH-RH addition to cultures ($86 \pm 25 \mu\text{g}$ inhibin and λ 0.06–0.19). The inhibition of LH-RH-induced FSH secretion by pituitary cells was therefore considered to be the better method for determining inhibin activity and was used for comparison to the standard.

Influence of culture conditions on inhibin production by granulosa cells

Granulosa cells cultured in medium devoid of calf serum failed to secrete inhibin. Only medium from granulosa cells cultured in medium with 10% calf serum selectively inhibited basal FSH production (as demonstrated in Text-fig. 1) and preferentially inhibited LH-RH stimulated FSH production (Table 1) by pituitary cells. Granulosa cells cultured in medium with 10% calf serum underwent morphological and functional changes indicative of luteinization, i.e. cellular hyperplasia and hypertrophy and increased progesterone production (Table 1). Addition of bovine gonadotrophins to the culture medium mimicked the effect of serum in stimulating granulosa cell progesterone production but not the effect of serum in stimulating inhibin production (Table 1).

Relative production of inhibin by granulosa and luteal cells

To test the possibility that inhibin production by granulosa cells might be related to their luteinization, inhibin production by luteinizing granulosa cells and luteal cells was compared. Results in Text-fig. 2 show that as granulosa cell progesterone production increased, inhibin production decreased. Although dispersed luteal cells do not survive well in culture (Henderson & Moon, 1979), as indicated by a steady decline in their production of progesterone, substantial amounts of progesterone were produced during the 2 days of culture, indicative of some functional viability. No detectable amounts of inhibin, however, were produced by the luteal cells.

Text-fig. 1. Effect of progressive amount of the inhibin standard (●) and volumes of bovine follicular fluid (■) and granulosa cell culture medium (○) on basal FSH (a) and LH (b) secretion and on LH-RH-induced release of FSH (c) and LH (d). FSH and LH concentrations are expressed as % of control (100%, no added inhibin preparations). Each is the mean curve of 17 individual curves. Each point represents the mean $\pm$ s.d. Broken lines represent 2 s.d. from the 100% control value.

Relationship between progesterone and inhibin production by granulosa cells

The analysis of several granulosa cell culture experiments revealed an inverse relationship between progesterone and inhibin production (Text-fig. 3).

Table 1. Relative efficiency of calf serum and bovine gonadotrophins in stimulating inhibin and progesterone production by bovine granulosa cells cultured for 24 h

Material added to pituitary cell cultures (50 µl/ml culture medium)	Granulosa cells present	LH-RH-stimulated gonadotrophin production by pituitary cells (ng/ml)		Progesterone production by granulosa cells (µg/mg protein)
		FSH	LH	
Granulosa cell culture medium	—	2047 ± 51	5236 ± 161	—
with 10% calf serum after incubation	+	1303 ± 54**	4430 ± 102*	3.0 ± 0.4
Granulosa cell culture medium without 10% calf serum after incubation				
Alone	—	1845 ± 120	4794 ± 43	—
	+	1930 ± 119	4896 ± 130	1.1 ± 0.3†
+ FSH (500 ng)	—	1799 ± 65	4843 ± 83	—
	+	1772 ± 103	4980 ± 22	2.2 ± 0.3
+ LH (500 ng)	—	1806 ± 21	4748 ± 36	—
	+	1831 ± 48	4878 ± 80	3.5 ± 0.6
+ FSH + LH (500 ng of each)	—	1858 ± 91	4645 ± 80	—
	+	1975 ± 67	4958 ± 213	3.3 ± 0.4

Values are mean ± s.e.m. for 5 replicate cultures.
Values significantly different from effect of culture medium with 10% calf serum but no granulosa cells; **P* < 0.01, ***P* < 0.005.
† Significantly different from other values in same column: *P* < 0.05.

Text-fig. 2. Progesterone (a, b) and inhibin (c, d) production by 10⁶ granulosa (a, c) and 10⁶ luteal (b, d) cells on Days 1 and 2 of culture. Values are means ± s.d. for 6 replicate cultures; ND = not detectable. *Significantly different from Day 1 value (*P* < 0.01).

Effect of exogenous steroids on inhibin production and steroidogenesis by granulosa and luteal cells

The results in Table 2 demonstrate that androstenedione at 5 µg/ml and testosterone at 1 and 5 µg/ml significantly stimulated granulosa cell inhibin production. In contrast, progesterone at 1 and 5 µg/ml significantly inhibited inhibin production. Oestrogens had no significant effect (*P* > 0.05) on inhibin production. All the granulosa cell cultures produced significantly less inhibin during the 2nd day of culture than during the 1st day (*P* < 0.01). Progesterone production by granulosa cells was significantly inhibited by oestrogens and androgens during the 2nd day, but not during the 1st day of culture. Oestradiol-17β production by the granulosa cells increased during the 1st day of culture in response to the exogenous androgens and then decreased 5–10-fold during the 2nd day of culture, relative to Day 1.

The effects of the same doses of an aromatizable and non-aromatizable androgen (testosterone and 5α-dihydrotestosterone (DHT) respectively) on inhibin production by

Text-fig. 3. Relationship between progesterone and inhibin production by bovine granulosa cells in culture. The coefficient of linear correlation (r) = 0.83 with $P < 0.001$ ($n = 18$).

Table 2. Effect of steroids on inhibin production and steroidogenesis by bovine granulosa cells in culture

Additions to culture medium (dose/ml)	Inhibin production (U/mg protein)		Steroid production (µg/mg protein)			
			Progesterone		Oestradiol-17β	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
None	375 ± 16	205 ± 7	1.0 ± 0.08	5.5 ± 0.28	0.3 ± 0.08	0.3 ± 0.09
Oestrone						
1 µg	386 ± 23	194 ± 7	0.9 ± 0.02	2.1 ± 0.07 ^d	33 ± 3 ^d	79 ± 8 ^d
5 µg	391 ± 16	222 ± 10	0.8 ± 0.06	1.1 ± 0.08 ^d	166 ± 12 ^d	152 ± 10 ^d
Oestradiol-17β						
1 µg	386 ± 9	204 ± 11	0.9 ± 0.04	2.2 ± 0.04 ^d	—	—
5 µg	360 ± 13	218 ± 8	0.8 ± 0.07	1.1 ± 0.09 ^d	—	—
Androstenedione						
1 µg	437 ± 24	211 ± 5	1.1 ± 0.07	3.4 ± 0.17 ^d	3.0 ± 0.3 ^d	0.3 ± 0.01
5 µg	477 ± 13 ^c	267 ± 17 ^c	1.0 ± 0.05	1.3 ± 0.11 ^d	5.5 ± 0.8 ^d	1.3 ± 0.3 ^c
Testosterone						
1 µg	597 ± 22 ^d	249 ± 7 ^c	1.2 ± 0.09	3.5 ± 0.42 ^c	4.9 ± 0.4 ^d	0.6 ± 0.06 ^a
5 µg	681 ± 37 ^d	277 ± 11 ^d	1.0 ± 0.04	2.3 ± 0.34 ^d	6.4 ± 0.3 ^d	0.5 ± 0.04
Progesterone						
1 µg	170 ± 9 ^d	ND	—	—	ND	ND
5 µg	284 ± 33 ^a	ND	—	—	ND	ND

Values are mean ± s.e.m. for 5 replicate cultures. ND, not detectable.

Values significantly different from those for cultures with no additions: ^a $P < 0.05$; ^b $P < 0.02$; ^c $P < 0.01$; ^d $P < 0.001$.

granulosa cells in a separate experiment is shown in Table 3. Both androgens caused a significant (except for 1 µg DHT/ml during the 2nd day of culture) and dose-related increase in inhibin production during both days of culture. Consistent with the results in Table 2, both androgens significantly inhibited progesterone production. Oestradiol-17β production was again stimulated by testosterone with less oestradiol-17β being produced during the 2nd than during the 1st day of culture. A small, but significant, amount of oestradiol-17β was also produced in response to exogenous DHT. This may be the result of slight (<1%) testosterone contamination of the commercial preparation of DHT which was not repurified before use.

Table 3. Effect of androgens on inhibin and steroidogenesis by bovine granulosa cells in culture

Addition to culture medium (dose/ml)	Inhibin production (U/mg protein)		Steroid production (µg/mg protein)			
			Progesterone		Oestradiol-17β	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
None	427 ± 19	260 ± 10	2.9 ± 0.3	5.4 ± 0.3	ND	ND
Testosterone						
1 µg	1436 ± 51 ^d	435 ± 21 ^d	2.4 ± 0.2	3.3 ± 0.3 ^c	23 ± 3	2.0 ± 0.3
5 µg	2205 ± 68 ^{d*}	742 ± 28 ^{d*}	1.6 ± 0.4 ^a	2.4 ± 0.4 ^d	21 ± 3	2.0 ± 0.1
5α-Dihydrotestosterone						
1 µg	1362 ± 41 ^d	285 ± 8	2.2 ± 0.4	3.5 ± 0.7 ^a	0.3 ± 0.06	0.1 ± 0.01
5 µg	2848 ± 89 ^{d*}	478 ± 15 ^{d*}	1.7 ± 0.2 ^b	3.0 ± 0.4 ^c	0.2 ± 0.04	0.1 ± 0.03

Values are mean ± s.e.m. for 6 replicate cultures. ND, not detectable.

Values significantly different from those for cultures with no additions: ^a*P* < 0.05; ^b*P* < 0.02; ^c*P* < 0.01; ^d*P* < 0.001.

* Significantly different from the lower concentration value: *P* < 0.001.

Table 4. Effect of testosterone on inhibin production and steroidogenesis by bovine granulosa and luteal cells in culture

Addition to culture media	Inhibin production (U/mg protein)		Steroid production (µg/mg protein)			
			Progesterone		Oestradiol-17β	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
Granulosa cells						
None	470 ± 21	293 ± 11	0.9 ± 0.1	2.1 ± 0.1	ND	ND
Testosterone, 1 µg/ml	850 ± 36 [*]	483 ± 13 [*]	0.7 ± 0.2	0.9 ± 0.3 [*]	15 ± 2	1.0 ± 0.3
Luteal cells						
None	ND	ND	160 ± 29	17 ± 2	ND	ND
Testosterone, 1 µg/ml	ND	ND	115 ± 15	20 ± 1	ND	ND

Values are mean ± s.e.m. for 6 replicate cultures. ND, not detectable.

* Significantly different from value for culture with no addition: *P* < 0.001.

While testosterone stimulated inhibin production by granulosa cells, it had no effect on luteal cells which failed to produce any detectable amounts of inhibin in the presence or absence of testosterone (Table 4).

Although the granulosa cell culture media were treated with dextran-charcoal before being assayed for inhibin, it could be argued that the effects of the steroids on granulosa cell inhibin production might be artefacts caused by steroids, still present following dextran-charcoal treatment of the media, modifying pituitary FSH production. Addition to pituitary cell cultures of equivalent volumes (50 µl) of dextran-charcoal treated culture medium containing added steroids but not exposed to granulosa cells revealed, however, that only at the higher concentration tested (5 µg/ml) did any of the steroids themselves significantly modify FSH production. Testosterone and progesterone significantly increased (*P* < 0.01) FSH production (mean ± s.e.m. for *n* = 5) from 1597 ± 38 to 1790 ± 30 and 1839 ± 33 ng/ml respectively, and oestrone significantly reduced (*P* < 0.05) FSH production from 1597 ± 38 to 1459 ± 41 ng/ml. The other steroids had no effect on FSH production.

Inhibin and steroid concentrations in follicular fluid

Inhibin and testosterone concentrations in follicular fluid fell significantly ($P < 0.01$) as follicle size increased while the concentration of oestradiol-17 β rose significantly ($P < 0.01$) (Table 5). There was no significant relationship between follicle size and progesterone concentrations ($P > 0.05$).

Table 5. Relationship between follicle size and the inhibin and steroid concentrations in bovine follicular fluid

	Follicle size (ml antral fluid/follicle)		
	< 0.3 (17)*	≥ 0.3 –< 0.8 (25)*	≥ 0.8 (21)*
Inhibin (U/ml)	308 $\pm$ 32	181 $\pm$ 17	155 $\pm$ 15
Oestradiol-17 β (ng/ml)	9 $\pm$ 2	133 $\pm$ 39	181 $\pm$ 45
Testosterone (ng/ml)	43 $\pm$ 5	29 $\pm$ 10	16 $\pm$ 5
Progesterone (ng/ml)	44 $\pm$ 4	55 $\pm$ 11	63 $\pm$ 14

* Minimum number of determinations in each group. Values are mean $\pm$ s.e.m.

There was a significant relationship between follicle size and concentrations of inhibin, oestradiol-17 β and testosterone ($P < 0.01$; Kruskal–Wallis test).

Discussion

Dispersed pituitary cell culture has already been used as a method for assaying inhibin (de Jong *et al.*, 1978; Eddie *et al.*, 1979; Franchimont *et al.*, 1979a, b; Scott, Burger & Quigg, 1980). In our experimental conditions, inhibin preparations extracted from rete testis (Franchimont *et al.*, 1979a), from testicular lymph and produced by granulosa cells (Text-fig. 1) specifically decrease basal FSH secretion, and they preferentially inhibit LH-RH-induced FSH secretion. Low doses decrease only LH-RH-induced FSH release whereas higher doses reduce both FSH and LH release under the influence of LH-RH. This preferential effect is due to the fact that inhibin specifically reduces FSH synthesis, but reduces FSH and LH release, although LH release is reduced to a lesser extent (Franchimont *et al.*, 1979b). As inhibition of LH-RH-induced FSH secretion is more precise and sensitive than the specific inhibition of basal FSH secretion, this response was chosen as the end point of the inhibin bioassay (Text-fig. 1). The criteria for the quality of a parallel line assay are fulfilled (Borth, 1976). The specificity of the inhibin assay was ascertained by the absence of an effect on prolactin and thyrotrophin secretion in basal and LH-RH-stimulated conditions, by the absence of inhibition of basal LH secretion and by a more marked inhibition of LH-RH-induced FSH secretion than that of LH-RH-stimulated LH release (Franchimont *et al.*, 1979a).

Granulosa cells cultured in medium devoid of calf serum failed to produce inhibin. The nature of the factor(s) present in serum and responsible for stimulating granulosa cell inhibin production is unknown but is unlikely to be endogenous gonadotrophins. Bovine LH and/or FSH added to medium devoid of serum, although mimicking the effect of serum in stimulating granulosa cell progesterone production, failed to stimulate inhibin production. No effect on inhibin production by rat granulosa cells cultured in medium containing 10% calf serum was observed when PMSG, a predominantly FSH-like gonadotrophin, was added at increasing doses (0.02 to 1 i.u.) to the culture medium (F. Crooze & P. Franchimont, unpublished observations).

Luteinization of granulosa cells, as indicated in culture by cellular hyperplasia and

hypertrophy and increased progesterone production, was accompanied by a reduction in inhibin production. There was an inverse relationship between granulosa cell progesterone and inhibin production. This is unlikely to be an artefact of the culture system because bovine luteal tissue, of which luteinized granulosa cells constitute a major portion, failed to produce any detectable amounts of inhibin *in vitro*. Thus, luteinization of granulosa cells and corpus luteum formation *in vivo* may be accompanied by a reduction in ovarian inhibin production. The finding that exogenous progesterone inhibited inhibin production by granulosa cells suggests that the reduction in inhibin production accompanying granulosa cell luteinization may be due to the increased progesterone produced having a negative feedback effect on cellular inhibin production.

Whatever the dose and the day of culture, exogenous oestrogens failed to modify granulosa cell inhibin production. They did, however, significantly reduce progesterone production by the cells as previously demonstrated (Fortune & Hansel, 1979). A stimulatory effect of androstenedione, testosterone and 5 α -dihydrotestosterone on inhibin production by granulosa cells was evident and was dose-dependent for testosterone and dihydrotestosterone. This stimulatory effect was less marked during the 2nd day of culture and may be due to an inhibitory action of the increased progesterone produced by the granulosa cells over-riding the stimulatory effect of the androgens. The extent by which inhibin production was stimulated by testosterone (1 μ g/ml) during the 1st day of culture varied between experiments from 1.6- to 3.4-fold (Tables 2, 3 and 4). This probably reflects different sensitivities to testosterone of the pools of granulosa cells used in each study. Further studies to compare the responsiveness to androgens of granulosa cells from individual follicles therefore seem worthwhile. The stimulatory effect of androstenedione and testosterone on inhibin production was associated with their aromatization to oestradiol-17 β during the 1st day of culture. During the 2nd day of culture, luteinization of the granulosa cells was accompanied by a reduction in their capacity to aromatize androgens (Henderson & Moon, 1979). The stimulatory effect of the androgens on inhibin production is a direct one, however, and not secondary to their capacity to be aromatized to oestrogens. Exogenous oestrogens failed to modify granulosa cell inhibin production, and 5 α -dihydrotestosterone, essentially a non-aromatizable androgen, stimulated inhibin production. Although stimulating inhibin production by granulosa cells, testosterone did not induce inhibin production by luteal cells. In addition to their effect on inhibin production, the androgens also inhibited progesterone production by granulosa cells.

Measurement of steroids and inhibin concentrations in bovine follicular fluid indicated that as follicle size increased there was a significant fall in fluid concentrations of testosterone and inhibin. The fall in testosterone may be due to its increased aromatization by follicular granulosa cells because the follicular fluid concentrations of oestradiol-17 β rose as those of the testosterone fell. Taking together the observation that follicular fluid concentrations of testosterone and inhibin both fell as follicle size increased, and the finding that androgens stimulated granulosa cell inhibin production *in vitro*, androgens may modify, at least in part, granulosa cell inhibin production *in vivo*. There was no relationship between follicle size and follicular fluid concentrations of progesterone. The inhibitory effect of progesterone on granulosa cell inhibin production may therefore be more important in regulating ovarian inhibin production during the period of granulosa cell luteinization and corpus luteum formation, when progesterone levels become elevated.

Androgens acting locally within the ovary may be involved in the process of follicular atresia (Louvét, Harman, Schreiber & Ross, 1975; Hillier & Ross, 1979). Androgens may also promote follicular atresia through stimulating granulosa cell inhibin production which would act to inhibit pituitary FSH production. FSH stimulates ovarian androgen aromatization (Armstrong & Papkoff, 1976) and so a decrease in FSH levels would produce an accumulation of follicular androgen from LH action on the theca (Leung & Armstrong, 1979). A closed cycle would thus be formed with the excess androgen driving the follicle further into atresia.

Materials for the RIA of rat gonadotrophins were provided by the NIAMDD rat pituitary hormone distribution programme. Antisera to steroids were provided by the M.R.C. Reproductive Biology Unit, Edinburgh, Scotland. This work was supported by grant No. 3.4501.80 from the Belgium Foundation for Medical Research (F.R.S.M.), by grant No. 74.039 of the World Health Organisation and by the Australian Research Grants Committee. K.M.H. is presently in receipt of an Australian Queen Elizabeth II Research Fellowship.

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Received 16 January 1981