Hormonal and cellular interactions in follicular steroid biosynthesis by the sheep ovary

D. T. Armstrong, T. J. Weiss*†, G. Selstam*‡ and R. F. Seamark*

*M.R.C. Group in Reproductive Biology, University of Western Ontario, London, Canada and *Department of Obstetrics & Gynaecology, Queen Elizabeth Hospital, University of Adelaide, Australia*

Summary. Studies of isolated cell types from sheep follicles revealed several functional changes which occur during follicular maturation. Cyclic AMP production by granulosa cells from the smallest follicles studied (1–3 mm diameter) was stimulated by FSH but not by hCG, suggesting functional FSH receptors at this early stage of differentiation. Medium-sized follicles (4–6 mm) responded to both FSH and hCG. Granulosa cells were unable to synthesize androgens, but readily converted exogenous testosterone to oestradiol-17β. This conversion occurred to a limited extent in the cells from the smallest follicles, but was much greater in medium and large (>6 mm) follicles. Oestradiol production by theca preparations from small follicles was barely detectable, but increased markedly with increasing follicle size. Androgen (androstenedione and testosterone) production by theca preparations was stimulated by hCG. This stimulation was short-lived, and levels declined to below control values after 6 h of culture. This decline could not be prevented by addition of cyclic AMP. The presence of granulosa cells with thecal preparations (i.e. follicle wall tissue) enhanced production of androgen by the theca, the effect being more marked for testosterone than for androstenedione. In-vivo studies in which granulosa cells and follicular fluid were removed during the preovulatory period suggested that granulosa cells and/or follicular fluid contributed to the oestradiol secreted into the ovarian vein during this period, but did not exclude a significant contribution by the theca as well.

Introduction

Much of our present understanding of ovarian steroid biosynthesis and its regulation has been derived from investigations with laboratory rodents. In recent years, largely on the basis of in-vitro studies with isolated ovarian cell types, a hypothesis has been developed to explain the cellular basis of oestrogen synthesis by the ovarian follicle (Text-fig. 1). The experimental evidence for this hypothesis (Armstrong, Goff & Dorrington, 1979; Leung & Armstrong, 1980) includes the following. (a) Granulosa cells from immature or hypophysectomized rats produce negligible amounts of oestradiol-17β when cultured without an aromatizable substrate. (b) When cultured with testosterone, significant amounts of oestradiol are produced, and this production is markedly stimulated by FSH, but not by LH. (c) FSH, but not LH, stimulates production of cAMP by granulosa cells from immature or hypophysectomized rats. (d) Both FSH and LH are

THECA CELL   GRANULOSA CELL
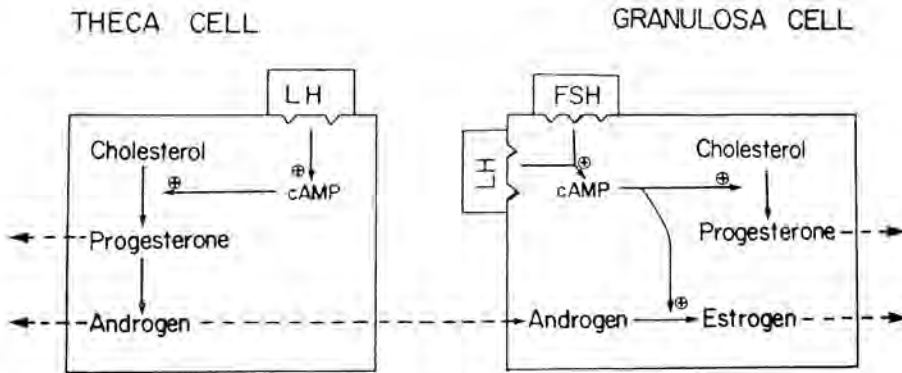
Text-fig. 1. Cellular and biochemical sites of action of gonadotrophins in regulation of oestrogen biosynthesis in the follicle: a working hypothesis (modified from Leung & Armstrong, 1980).

able to stimulate cAMP and oestrogen production by granulosa cells from pro-oestrous rats. (e) Thecal preparations from pro-oestrous rats produce substantial amounts of androgen, but very small amounts of oestrogen. (f) Androgen and cyclic AMP (cAMP) production by thecal preparations is stimulated by LH, but not by FSH, (g) LH. but not FSH, stimulates cAMP production by isolated thecal preparations.

Investigations with follicular tissues from several other mammalian species have in general supported this hypothesis, at least as far as the role of the granulosa cell is concerned. However, it has been suggested that the theca may also be an important source of oestrogen production, at least in primates, on the basis of both in-vitro (Channing, Anderson & Batta, 1978) and in-vivo (Channing & Coudert, 1976) experiments. The present studies were undertaken to investigate certain aspects of this hypothesis in the sheep, a species in which relatively pure theca preparations can easily be obtained from follicles at well-defined stages of development. In particular, we wished to determine the contributions of the theca and granulosa cells to androgen and oestrogen production by the sheep follicle.

Materials and Methods

In-vitro studies

Follicles were dissected from ovaries of sheep obtained immediately after slaughter at a local abattoir, or surgically following synchronization of oestrous cycles. They were pooled according to size: small, 1–3 mm diameter; medium, 4–6 mm diameter; and large, >6 mm diameter. Granulosa cells and thecal tissues were isolated as described previously (Weiss, Armstrong, McIntosh & Seamark, 1978a). In some experiments, portions of follicle walls were prepared without removal of granulosa from theca cells. Granulosa cells, and thecal or follicle wall preparations, were cultured for various periods in Medium 199, as described previously (Weiss *et al.*, 1978a). Tissue and culture media were frozen separately, for subsequent extraction and assay of cAMP and steroids.

For studies with follicles obtained during the preovulatory period, oestrous cycles were synchronized by the use of intravaginal progestagen sponges (Repromap: Upjohn) followed by treatment with PMSG (Ayerst) (Boland, Kelleher & Gordon, 1979). Ovaries were removed surgically, or at autopsy 28–36 h after withdrawal of the sponge and administration of 1000 i.u. PMSG. In these studies, granulosa and thecal tissues were isolated as above, but cultured for 24 h in Eagles Minimum Essential Medium, modified as described previously (Dorrington & Armstrong, 1975).

In-vivo studies

For in-vivo studies of follicles during the preovulatory period, oestrus was synchronized by the use of a luteolytic dose (125 or 250 µg) of the prostaglandin F-2α analogue, cloprostenol (Estrumate: I.C.I.), administered as a single i.m. injection between Days 8 and 13 of the oestrous cycle. Ewes were anaesthetized with pentobarbitone sodium 24–36 h after cloprostenol administration, their tracheae were intubated, and they were maintained in surgical anaesthesia with halothane in oxygen. Bilateral ovarian vein cannulations were performed via mid-ventral laparotomy, as described previously (Weiss, Janson, Porter & Seamark, 1978b). Serial blood samples were collected at approximately 15-min intervals for a control period of 30 min. Then the largest follicle in one ovary, or in some instances the largest 2–4 follicles, were slit and follicular fluid and granulosa cells were removed by flushing the follicular cavity with saline (9 g NaCl/l) while gently scraping the inner walls of the cavity by rotating a curved glass probe. In this way it was possible to remove most of the granulosa cells, leaving the basement membrane and theca layer intact. Procedures carried out on the contralateral ovaries as controls included exteriorization and handling the ovary to about the same extent without cutting or flushing the follicles. These procedures took 1–2 min. Serial blood samples were collected at approximately 15-min intervals over at least one further hour after manipulation of the ovaries. Rates of blood flow were determined by direct measurement of volume of blood collected at each period. Rates varied from 1·8 to 15·7 ml/min. Peripheral blood samples were taken, via a femoral arterial catheter, at the same time as each ovarian vein sample. Between ovarian vein samples, the ovarian venous blood was returned via a jugular venous catheter.

Analytical procedures

Cyclic AMP in tissue and culture media was determined following precipitation of protein with ethanol, using the protein-binding assay method of Brown, Albano, Ekins, Sgherzi & Tampion (1971). Steroids in ethanolic extracts of tissues and in unextracted culture media were determined by specific radioimmunoassays, as described previously (Janson, Amato, Weiss, Ralph & Seamark, 1978). In some experiments, steroid production (amount in tissue + medium at end of culture period — amount in tissue before culture) was expressed per mg tissue protein; the protein contents of tissues were determined by the method of Lowry, Rosebrough, Farr & Randall (1951). In other experiments, involving thecal or follicle wall tissue, tissues were weighed on an analytical balance after culture, and results expressed per mg tissue wet weight.

In in-vivo experiments, steroid secretion rate for each ovarian vein sample was determined from the arterio–venous difference in plasma concentrations of the steroid and the blood flow rate, as measured directly at the time of collection, by using the formula:

$$\text{Secretion rate (ng/min)} = \frac{\underset{\text{(ng/ml plasma)}}{\text{conc. in ovarian vein}} - \underset{\text{(ng/ml plasma)}}{\text{conc. in femoral artery}}}{\underset{\text{(ml plasma/min)}}{\text{Flow rate}}}$$

Results

Cyclic AMP production

Cyclic AMP production by granulosa and theca preparations from different-sized follicles was measured as a means of assessing responsiveness of the separate cell types to gonadotrophic hormone stimulation. Granulosa cells from small (1–3 mm) follicles responded to FSH, but not to hCG, with markedly increased cAMP production. In contrast, both FSH and hCG stimulated

cAMP production by granulosa cells from medium-sized (4–6 mm) follicles (Table 1). A different pattern of responsiveness of theca preparations was observed: hCG stimulated cAMP production by theca from both sizes of follicles, whereas FSH failed to cause significant stimulation in thecal preparations from follicles of either size (Table 1).

Table 1. Effect of FSH and hCG on cAMP formation by theca and granulosa preparations of sheep follicles

Tissue	Follicle size	cAMP production (pmol/mg protein)		
		Control	FSH*	hCG†
Theca	Small (1–3 mm)	0·26 (0–7·8)	17·2 (0–25·8)	157·8 (38·7–280)
	Medium (4–6 mm)	3·4 (0–9·8)	16·7 (5·8–53·0)	152·3 (141·1–300)
Granulosa	Small	8·6 (0–10·2)	75·2 (19–268)	3·6 (0–16·2)
	Medium	3·9 (0–11·0)	17·1 (9·4–133)	42·7 (11·4–268)

Values are medians (and 95% confidence limits) of cAMP production during 40 min incubations (data from Weiss *et al.*, 1978a).
* 5 μg NIH-FSH-S11/ml.
† 5 i.u. Pregnyl (Organon)/ml.

*Steroid synthesis by isolated tissues*

A comparison of the abilities of granulosa and thecal preparations from 4–6 mm follicles to synthesize androgens (testosterone and androstenedione) and oestradiol-17β is illustrated in Text-fig. 2. Isolated granulosa cells produced negligible amounts of all three steroids during culture for 6 h. Thecal preparations, on the other hand, produced significant amounts of both androgens, and negligible amounts of oestradiol. Combinations of theca and granulosa from the same follicles, i.e. follicle walls from which granulosa cells were not removed, produced androstenedione in amounts similar to those produced by isolated theca, but produced substantially more testosterone and oestradiol than did the isolated thecal preparations.

To investigate the effectiveness of LH-like hormones in stimulating steroid biosynthesis, isolated theca and follicle wall preparations were cultured for various periods in the absence or presence of hCG (5 i.u./ml). The synthesis of total androgen (testosterone + androstenedione) by both theca and follicle wall occurred in an approximately linear manner with increasing time of culture up to 6 h (Text-fig. 3) and hCG increased the production by both preparations. In experiments in which cultures were carried out for longer periods, rate of androgen synthesis by both preparations began to decline after 6 h in most experiments. This decline was greater in hCG-stimulated tissues than in controls (data not shown).

Previous studies of various steroidogenic tissues have revealed a period of 'desensitization' following gonadotrophic stimulation, indicated by decreased specific binding of the gonadotrophin to the cells (Conti, Harwood, Dufau & Catt, 1977), and by decreased cAMP production in response to the gonadotrophin (Hunzicker-Dunn, Jungmann, Derda & Birnbaumer, 1979). In order to determine whether such desensitization was responsible for the decline in androgen production observed after 6 h of stimulation with hCG, thecal preparations were cultured with dibutyryl cAMP (5 mM). The ability of this cyclic nucleotide to mimic the effects of hCG, i.e. to increase androgen production during a 6-h culture period, is illustrated in
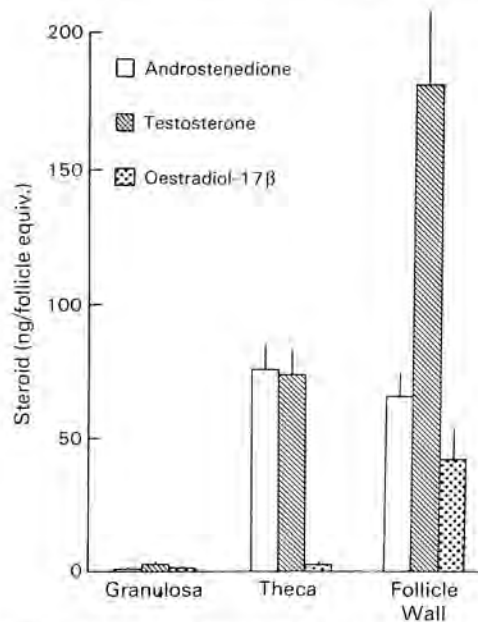
Text-fig. 2. Androgen and oestrogen synthesis during 6-h cultures of isolated granulosa, theca and follicle wall preparations from sheep follicles. Synthesis, in this and subsequent figures, refers to amount of steroid (mean $\pm$ s.e.m.) measured in tissues plus medium at end of culture period minus amount measured in non-incubated tissue. Values for testosterone may include 5α-dihydrotestosterone (DHT), because the antiserum used for the testosterone RIA cross-reacted significantly with DHT.
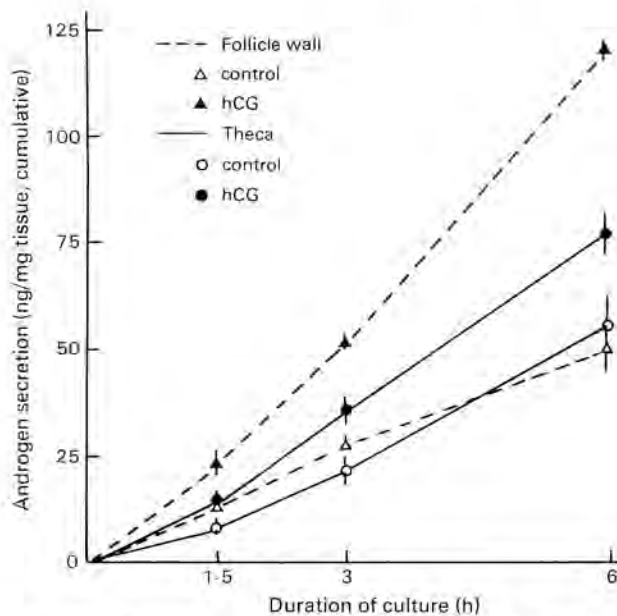
Text-fig. 3. Effect of hCG on secretion of androgens into culture media after various periods of culture of isolated theca or follicle wall preparations. Secretion here and in subsequent figures refers to release into the culture medium. Androgen refers to testosterone plus androstenedione, summed after assay separately.

Text-fig. 4. Addition of the same concentration of dibutyryl cAMP to cultures of theca for a 6-h period following an initial 6-h culture with hCG failed to prevent the decline in androgen production which occurred following hCG stimulation (data not shown). These results suggest that the short-lived responsiveness of thecal preparations to gonadotrophic stimulation cannot be attributed solely to desensitization at the level of the hormone receptor : adenylate cyclase system, and indicate an additional level of refractoriness, presumably at some step(s) in the steroidogenic pathway leading to androgen synthesis.
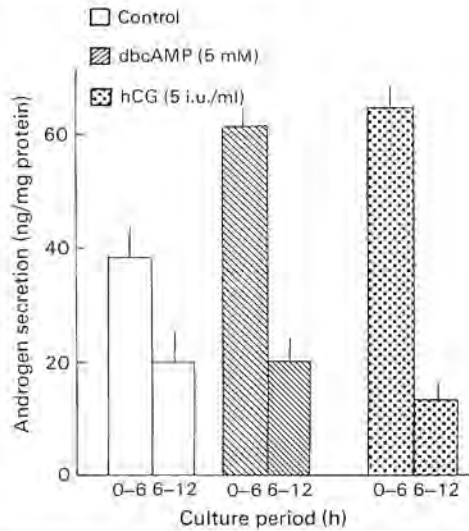

Text-fig. 4. Effect of dibutyryl cyclic AMP (dbc, 5 mM) and hCG on secretion of androgens by theca preparations during successive 6 h culture periods. The hormone or dbcAMP were present during the entire 12 h of culture.

In an attempt to determine whether thecal oestrogen synthesis can be influenced by acute gonadotrophic treatment, oestradiol-17β production by isolated thecal preparations during 6 h culture was determined (Text-fig. 5). In this experiment, oestrogen production by thecal tissue was considerably greater than in the experiment summarized in Text-fig. 2 but follicle wall preparations still produced somewhat greater amounts of oestradiol. The marginal increase in oestrogen production observed in hCG-treated tissues was not statistically significant.

As in the experiment summarized in Text-fig. 2, follicle wall preparations produced similar amounts of androstenedione, but considerably greater amounts of testosterone than did isolated theca preparations (Text-fig. 5). The production of both androgens was considerably enhanced by hCG treatment of both preparations.

Because of the observed variability in oestrogen production by thecal preparations from ovaries of sheep at unknown stages of the oestrous cycle (see Text-figs 2 and 5), experiments were undertaken with preparations from ewes under more controlled conditions. Ovaries were obtained from ewes in which oestrus was synchronized by the use of intravaginal progestagen sponges. At the time of removal of the sponges, an injection of PMSG was administered, both to provide better synchronization (Boland *et al.*, 1979), and to enable more follicles to be obtained from each ewe. Three classes (by size) of follicles were studied: small (1–3 mm), medium (4–6 mm) and large (>6 mm). Oestradiol production during 24 h culture of thecal preparations isolated from each of these size classes is summarized in Text-fig. 6. A progessive increase in thecal oestradiol production was observed with increasing follicle size when results were expressed per follicle, indicating a considerably greater contribution of the theca to oestrogen
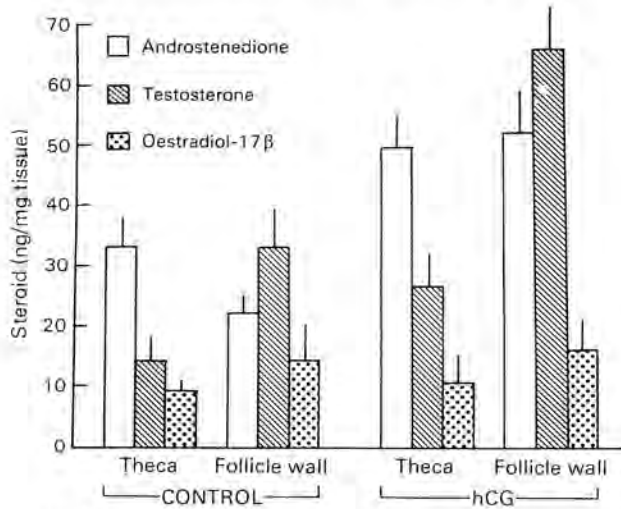
Text-fig. 5. Effect of hCG on androgen and oestrogen synthesis during 6 h culture of theca or follicle wall preparations.
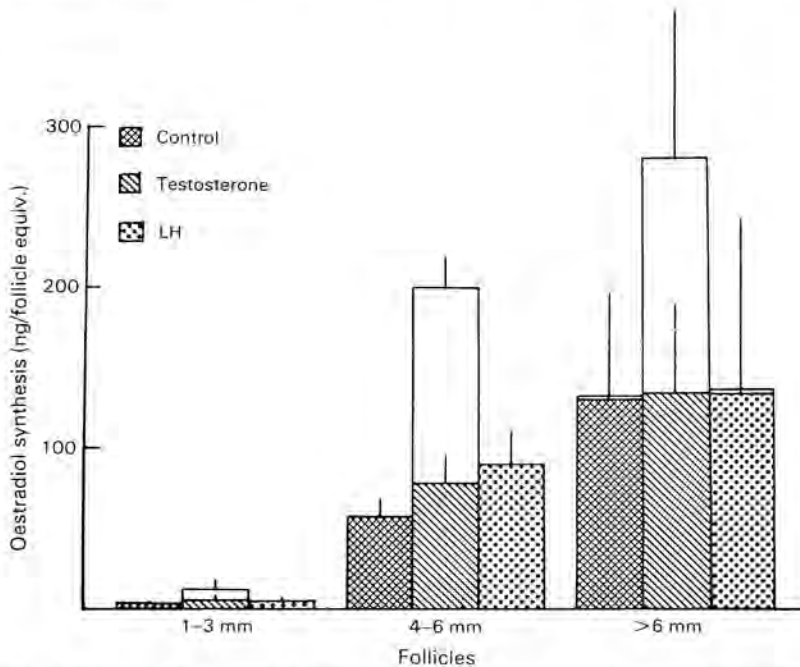

Text-fig. 6. Synthesis of oestradiol-17β during 24 h culture of theca and granulosa cell preparations from various sizes of follicles obtained during the preovulatory period. Data for theca are represented by filled lower portions of columns. Data for granulosa cells from the same treatment presented as open extensions of columns for theca preparations. Effect of testosterone (0·5 μM) and LH (NIH-LH-S11, 1 μg/ml).

production as follicles approach the preovulatory size. As in previous experiments, granulosa cells failed to produce significant amounts of oestradiol when cultured without an aromatizable substrate, irrespective of the size of follicle from which they were obtained.

Addition of testosterone (0·5 μM) to cultured thecal preparations failed to increase oestrogen production, whereas the same concentration of testosterone added to granulosa cell cultures caused very marked increases in oestradiol production, irrespective of the size of follicles from which they were obtained. Granulosa cells cultured with testosterone produced substantially more oestradiol, when expressed on a per mg tissue protein basis, than did thecal preparations, irrespective of the follicle size and whether or not exogenous androgens were added to the thecal cultures (data not shown). The most marked differences between theca and granulosa were observed with medium-sized follicles.

Oestradiol secretion in vivo

While the above in-vitro experiments suggest that both the granulosa and theca cells have the enzymic capabilities to produce substantial amounts of oestrogen, it is not possible to draw conclusions from such experiments regarding the relative contributions of the two cell types to the oestrogen which is secreted into the circulation. In an attempt to estimate the contribution of the granulosa cells to the oestradiol secreted *in vivo*, experiments were carried out in which oestradiol secretion into the ovarian vein was determined before and after removal of granulosa cells from follicles during the preovulatory period. The results of experiments performed in 7 ewes are summarized in Text-fig. 7. Removal of granulosa cells and follicular fluid resulted in an immediate and marked decline in secretion rate of oestradiol significantly below that observed during the 30-min period immediately preceding the operative procedure, and reaching a mean value of 21% of the pre-operative level at the end of 1 h. A decline was also noted by the sham-treated contralateral ovary in most experiments; this decline was less, reaching a mean value of 75% of the pre-operative level at the end of 1 h that was not statistically significant. However, the mean pre-operative secretion rate was lower for the control ovaries, reflecting the
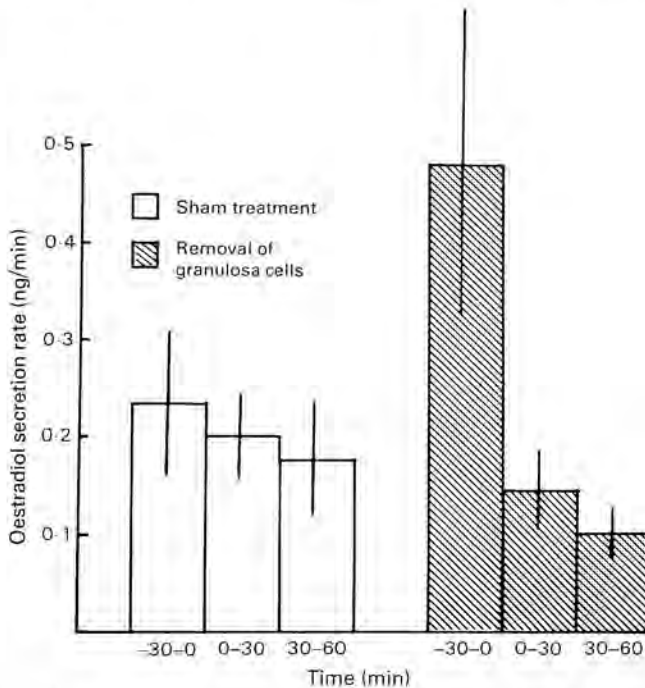
Text-fig. 7. Effect of granulosa cell removal at time zero on secretion of oestradiol-17β by sheep ovaries *in vivo*. See text for experimental design.

deliberate attempt to choose the ovary with the most advanced follicle(s) for granulosa cell removal. In two animals, this attempt was apparently unsuccessful, in that the pre-operative oestradiol secretion rate was higher for the control than for the experimental ovary. In both these instances, the decline following removal of granulosa cells was still considerably greater for the experimental than for the control ovary (data not shown). Although it is impossible to draw conclusions from this experiment concerning the quantitative contributions of the granulosa and theca cells to the oestradiol secreted into the ovarian vein, they are consistent with the results of the in-vitro experiments, suggesting that oestradiol is probably secreted by both cell types of the more advanced follicles during the preovulatory period.

Discussion

The results of these studies with isolated follicular tissues indicate a number of important changes which follicles undergo during the various stages of maturation. First, the production of cAMP in response to stimulation with FSH and hCG, taken as a measure of functional FSH and LH receptors on granulosa cells, changed with increasing follicle size (presumably representing increasing stages of maturity). Cells from small (1–3 mm) follicles responded to FSH, but not to hCG, in good agreement with the report of Carson, Findlay, Burger & Trounson (1979) that these cells bind ^{125}I-labelled FSH, but very little ^{125}I-labelled hCG. Similar good agreement was seen between the present findings and those of Carson *et al.* (1979) with granulosa cells from larger (4–6 mm) follicles, which were observed both to bind ^{125}I-labelled hCG and to respond to this LH-like hormone with increased cAMP production. Taken together, these findings with sheep granulosa cells are in general accord with the results for granulosa cells from laboratory rodents (Richards, 1979; Leung & Armstrong, 1980) and pigs (Channing & Kammerman, 1973), i.e. that FSH receptors and FSH responsiveness occur at an earlier stage of granulosa cell differentiation than do LH receptors and LH responsiveness.

A second difference observed between follicles of different sizes, suggestive of maturational changes, is the ability of granulosa cells to convert androgens to oestrogens. Although cells from small follicles carried out significant aromatization of testosterone, a very marked increase in this conversion was seen in cells from medium-sized (4–6 mm) follicles. This concidence between the ability of granulosa cells to produce oestradiol, and their acquisition of LH receptors raises the possibility that the two may be causally related. Oestrogen treatment has been reported to enhance the ability of FSH to induce LH receptors on granulosa cells of hypophysectomized rats (Richards, 1979). Perhaps the oestrogen produced by granulosa cells of the sheep follicles participates in the mechanism of induction of LH receptors on these cells.

Maturational changes were also noted in thecal tissue. Thecal cells from small follicles produced barely detectable amounts of oestradiol, whereas those from medium and large follicles produced oestradiol in substantial amounts. It is unlikely that the latter were due to contamination with granulosa cells, because addition of exogenous testosterone, which caused very marked stimulation of oestrogen production by granulosa cells from medium and large follicles, caused only small, statistically insignificant increases in oestrogen production by theca preparations from the same follicles.

Thecal preparations from large follicles cultured with or without exogenous androgen produced approximately the same amounts of oestradiol during 24 h culture as did granulosa cells from the same follicles when cultured with testosterone, suggesting approximately equal contributions by the two cell types to the oestradiol produced by follicles in the preovulatory period. Whether or not both cell types contribute equally to that which is secreted into the ovarian vein *in vivo* cannot be determined from in-vitro studies. It has been suggested that the theca may be a more important source of the secreted oestrogen, because of the closer proximity of the theca than the granulosa cells to the capillaries draining the follicle (McNatty, Makris,

DeGrazia, Osathanondh & Ryan, 1979). As a corollary of this interpretation, the oestrogen produced by the granulosa cells, tending to remain in the fluid of the follicular antrum, may be of greater importance in regulation of intrafollicular than of systemic functions. Participation in the induction of LH receptors on granulosa cells, as suggested above, may be one such intrafollicular function. Another is a possible influence on maturational processes occurring in the oocyte; Moor & Trounson (1977) have reported a beneficial effect of oestradiol, added to cultured sheep follicles, on subsequent developmental capabilities of the oocyte after fertilization.

In addition to possible effects on intrafollicular processes, the results of the in-vivo studies reported here support a role of the granulosa cells in the oestradiol secreted into the ovarian vein during the preovulatory period. A clear and marked decrease in oestradiol secretion was observed following acute removal of the granulosa cells (and follicular fluid) from the larger follicles present during this period. Although it was impossible to control these in-vivo experiments adequately, the results, taken together with the results of the in-vitro studies with isolated tissues, suggest that the theca is probably not the sole source of follicular oestrogens secreted during the preovulatory period. This suggestion differs from the interpretation of Channing & Coudert (1976) for the results of their similar studies of rhesus monkey follicles. While the difference may, indeed, indicate true species differences, there may be other explanations, such as the stage of maturity of the follicles. Channing & Coudert (1976) restricted their studies to animals in the late preovulatory stage, when the LH surge apparently had already occurred. Inhibition of oestrogen production is a well-known follicular response to LH, probably related to luteinization of the granulosa cells. Perhaps the LH surge had already completely suppressed the contribution of the granulosa cells to the preovulatory oestrogen in their studies, leaving the theca cells as the major remaining source at the time the studies were carried out.

While the theca appears to be only one of the sources of follicular oestrogen production, it emerges as the only significant source of androgen production by the follicle, and it seems evident that LH is the gonadotrophin which controls its secretion. Thecal cells from even the smallest follicles in the present studies responded to hCG with increased cAMP production, consistent with the report of Carson *et al.* (1979) that theca from all sizes of sheep follicles exhibited specific binding of ^{125}I-labelled hCG, and with our previous observations that hCG stimulates androgen secretion from 1–3 mm follicles (Weiss & Armstrong, 1979; G. Selstam, T. J. Weiss & D. T. Armstrong, unpublished observations).

Although granulosa cells are unable to produce androgens from C_{21} precursors, apparently because of lack of the $C_{17,20}$-lyase enzyme, results of the present studies suggest that granulosa cells in some way influence the production of androgens by the adjacent theca. While we cannot exclude damage to the thecal cells associated with removal of the granulosa cells as a partial explanation of the greater amount of testosterone produced by follicle wall preparations than by isolated theca, a definite effect of the presence of granulosa cells on the ratio of testosterone : androstenedione produced was observed, follicle wall preparations producing more testosterone relative to androstenedione than theca preparations. This latter effect may be due to diffusion of some unknown factor from the granulosa cells to the theca, influencing the ability of the theca to convert androstenedione to testosterone (in addition to stimulating overall androgen production). An alternative explanation may be differences in the equilibrium of the reaction, androstenedione $\rightleftharpoons$ testosterone, between the theca and granulosa cells, androstenedione being the favoured product in the theca and testosterone in the granulosa cells. Thus the greater production of testosterone by follicle wall than by theca preparations may be due to diffusion of androstenedione of thecal origin to the granulosa cells, where it is converted to testosterone (Bjersing, 1967). Further studies, e.g. involving separation and recombination of granulosa and theca cells, should help distinguish between these alternative possibilities.

The results of the research reported here may be summarized in Text-fig. 8, as a model for further testing of hypotheses concerning hormonal and cellular interactions involved in regulation of follicular steroid biosynthesis.
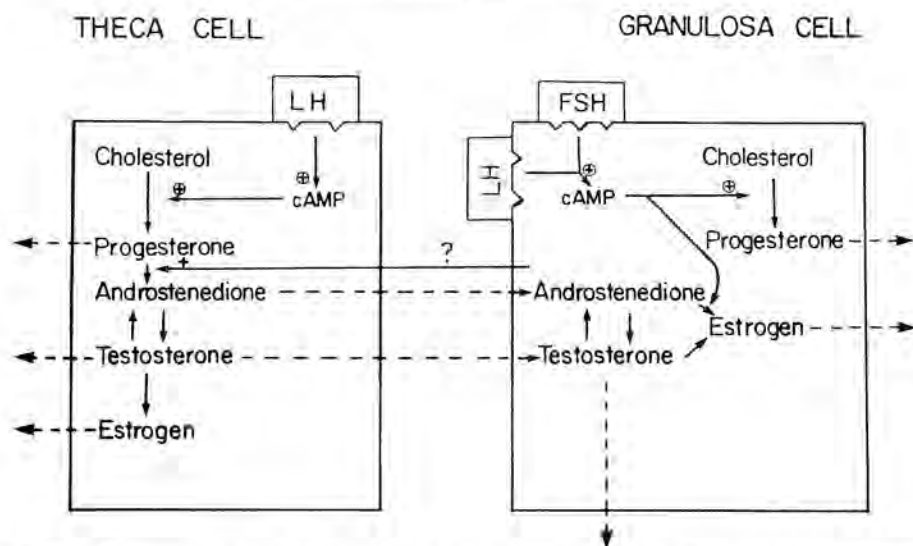
Text-fig. 8. Follicular steriod biosynthesis in the sheep: hormonal and cellular interactions.

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