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Differential production of steroids by dispersed granulosa and theca interna cells from developing preovulatory follicles of pigs

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Summary. Dispersed granulosa and theca interna cells were recovered from follicles of prepubertal gilts at 36, 72 and 108 h after treatment with 750 i.u. PMSG, followed 72 h later with 500 i.u. hCG to stimulate follicular growth and ovulation. In the absence of aromatizable substrate, theca interna cells produced substantially more oestrogen than did granulosa cells. Oestrogen production was increased markedly in the presence of androstenedione and testosterone in granulosa cells but only to a limited extent in theca interna cells. The ability of both cellular compartments to produce oestrogen increased up to 72 h with androstenedione being the preferred substrate. Oestrogen production by the two cell types incubated together was greater than the sum produced when incubated alone. Theca interna cells were the principal source of androgen, predominantly androstenedione. Thecal androgen production increased with follicular development and was enhanced by addition of pregnenolone or by LH 36 and 72 h after PMSG treatment. The ability of granulosa and thecal cells to produce progesterone increased with follicular development and addition of pregnenolone. After exposure of developing follicles to hCG *in vivo*, both cell types lost their ability to produce oestrogen. Thecal cells continued to produce androgen and progesterone but no longer responded to LH *in vitro*. These studies indicate that several functional changes in the steroidogenic abilities of the granulosa and theca interna compartments occur during follicular maturation.

Introduction

The control of ovarian steroid synthesis and secretion during preovulatory follicular development is a complex process involving interaction of two follicular compartments, the membrana granulosa and the theca interna, as well as a co-ordinated sequence of actions by pituitary gonadotrophins (Armstrong & Dorrington, 1977; Erickson, 1983). Follicular development involves cytodifferentiation characterized by marked biochemical and morphological changes which include acquisition of various hormone receptors and the development or alteration of the abilities for steroid synthesis and metabolism (Richards & Midgley, 1976; Bjersing, 1978; Kumari & Channing, 1979; McNatty, 1981).

Much attention has been given to the study of granulosa cell differentiation during follicular development in the pig (Channing, 1970; Channing & Kammerman, 1973; Anderson, Schaerf & Channing, 1979; Schwartz-Kripner & Channing, 1979; Haney & Schomberg, 1981). These studies

involved cell culture or incubation of granulosa cell suspensions. In contrast, parallel studies on theca differentiation have involved explant cultures or incubation of thecal fragments (Haney & Schomberg, 1981; Evans, Dobias, King & Armstrong, 1981; Tsang, Moon & Armstrong, 1982b). Therefore direct comparison of steroidogenic abilities and hormonal responsiveness could not be made with assurance that observed differences were not merely the result of the different states of tissue aggregation affecting solute and gas exchange. Interpretation of observations in these studies was also hindered by the fact that the tissues used were usually from abattoir material and so the stage of follicular development could only be estimated from the size of the follicles. Moreover, even when the follicular stage is known precisely, it may not be assumed that the metabolic profile, which characterizes the tissues at the time of their isolation from the follicle, is retained during culture periods for 24 h or more.

In previous studies we have demonstrated that prepubertal gilts treated with PMSG/hCG produce large numbers of synchronously developing follicles which will ovulate at a predictable time. In these studies we charted the sequence of changes in follicular fluid concentrations of steroids (Ainsworth, Tsang, Downey, Marcus & Armstrong, 1980), cyclic AMP and prostaglandins (Tsang, Ainsworth, Downey & Armstrong, 1979) and in meiotic maturation of the oocyte during preovulatory development (Ainsworth *et al.*, 1980). In the present studies, we have sought to characterize more precisely the steroidogenic abilities of dispersed theca interna and granulosa cells, at well-defined stages of follicular maturation until shortly before ovulation, by incubating the cells for short periods in a chemically defined medium with or without steroid precursors and gonadotrophins. A preliminary report of some of this work has been presented (Tsang, Ainsworth, Downey & Marcus, 1982a).

Materials and Methods

Animal preparation. Groups of 4–8 prepubertal Landrace gilts, 4·0–4·5 months of age and weighing 60–70 kg, were treated intramuscularly with 750 i.u. PMSG (Equinex: Ayerst Labs, Montreal, Quebec) and 72 h later with 500 i.u. hCG (APL: Ayerst) to enhance follicular growth and ovulation. Laparotomies were carried out on groups of gilts 36, 72 or 108 h after PMSG treatment and the ovaries removed as described previously (Ainsworth *et al.*, 1980). At least 3 replicate experiments were performed for each time. One group of gilts was used per replicate.

Isolation and dispersion of follicular cells. After removal, the ovaries were transferred into ice-cold Hank's balanced salt solution (HBSS, modified; Flow Laboratories, Mississauga, Ontario) and follicles of uniform diameter (36 h, 3-4 mm; 72 h, 5–6 mm; 108 h, 6–8 mm) were dissected out, trimmed of stromal tissue and transferred into fresh medium. Initially, the gross appearance of dissected follicles was examined under the stereomicroscope for evidence of atresia. Follicles with a uniform translucent appearance and extensive vascularization were considered to be non-atretic. Non-atretic follicles were further characterized by having a regular granulosa layer which could be removed from the thecal wall in translucent sheets. Follicles not conforming to these criteria were discarded. The follicles were cut into halves or quarters, depending on size, and the granulosa cells gently scraped from the thecal wall with a fine wire loop, using stereomicroscopy. The theca interna and theca externa were then separated using fine forceps. Granulosa and theca interna layers from individual follicles were pooled separately and kept in ice-cold Hank's solution. Representative samples of the intact thecal layers and of isolated theca interna were taken from several replicate experiments, fixed in Helly's fluid, dehydrated, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. Histological examination showed the absence of granulosa cells from the intact follicle wall (Pl. 1, Fig. 1) and a good dissection of theca interna (Pl. 1, Fig. 2) from theca externa.

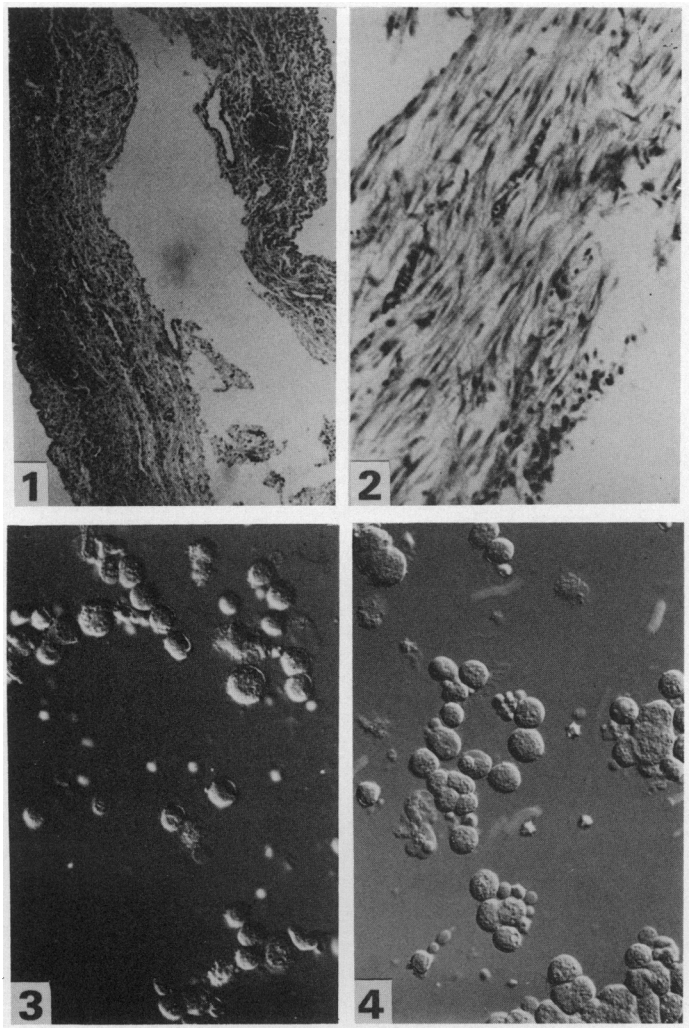


Fig. 1. Section of follicular wall after removal of granulosa cells. H and E stain, × 25.

Fig. 2. Section of theca interna layer. H and E stain, × 150.

Fig. 3. Dispersed theca interna cells. Nomarski, × 250.

Fig. 4. Dispersed granulosa cells. Nomarski, × 250.

(*Facing p.* 460)

The pools of theca interna layers and granulosa cells were subjected to enzymic dissociation, after centrifugation at 150 *g* and decanting of the Hank's solution. Tissues were suspended in 10 ml Moscona's buffer (Moscona, 1961), pH 7·4, containing 0·5% crude collagenase (Type II: Sigma Chemical Co., St Louis, MO, U.S.A.), 0·1% hyaluronidase (Type III: Sigma) and 0·1% pronase (Protease Type XIV: Sigma) and 1% chicken serum and incubated at 37°C in a shaking water bath for 45–60 min. After 15 min of incubation, ~1 mg DNase (Type I: Sigma) was added and dissociation of cells was assisted by repeated pipetting of the suspensions with a Pasteur pipette. At the end of the incubation, 2 ml fetal calf serum were added to each suspension. The cell suspensions were diluted 1 :1 with HBSS and centrifuged for 5 min at 150 *g*. The dispersed cells (Pl. 1, Figs 3 & 4) were washed 3 times in HBSS by repeated suspension and centrifugation (150 *g*, 5 min) and finally resuspended in Eagle's Minimum Essential Medium (MEM; GIBCO Laboratories, Grand Island, NY, U.S.A.) as modified by Dorrington & Armstrong (1975) but supplemented with 25 mM-Hepes buffer, and kept at 0°C until addition to incubation tubes.

The viability and concentration of the dispersed cells were determined by the fluorogenic ester hydrolysis procedure (Rotman & Papermaster, 1966). Fluorescein diacetate was used as the fluorogenic ester and the number and proportion of fluorescing cells were determined with a haemocytometer using a Zeiss fluorescence microscope with incident u.v. illumination and KP490 excitation and LP20 barrier filters. Cell viability of granulosa and theca interna cells was 90–95% and 85–95%, respectively.

Cell incubation. To examine the changes in steroidogenic abilities of ovarian follicular cells during preovulatory growth and maturation, 2·75–3·5 × 10^5 dispersed granulosa or theca interna cells prepared 36, 72 or 108 h after PMSG treatment were dispensed in triplicate into 12 × 75 mm glass test tubes. The cells were incubated separately in 0·3 ml or together in 0·6 ml modified Eagle's MEM in air at 37°C for 6 h unless otherwise indicated. In some instances, androstenedione, testosterone or pregnenolone (100 nM–100 μM) and/or highly purified ovine LH (S-1390; activity 2 × NIH-S14 containing < 0·02% oFSH (S1557BP)) or highly purified ovine FSH (S1557BP; activity 100 × NIH-S10, containing < 0·5% oLH (S-1390)) were added to the incubation medium. Both gonadotrophin preparations were kindly donated by Dr M. R. Sairam, Clinical Research Institute of Montreal, Montreal, Quebec. At the end of the incubation period, 1·0 ml absolute ethanol was added to each incubation tube to lyse the cells and precipitate cellular proteins. The supernatants were separated by centrifugation (500 *g*, 15 min) and stored at −20°C until analyses.

Assays. Progesterone was determined using the antiserum and method described by Orczyk, Hichens, Arth & Behrman (1979). This antiserum shows negligible cross-reactivity (< 0·1%) with androgens and oestrogens. Androstenedione and testosterone were assayed as described by Fortune & Eppig (1979) and by Tsang, Armstrong & Whitfield (1980), respectively. The androstenedione antiserum exhibits low cross-reactivity (< 1%) with neutral and phenolic steroids except for 5α-androstene-3,17-dione (5%) and testosterone (2%). The testosterone antiserum cross-reacts significantly with other androgens, such as 17β-hydroxy-5α-androstane-3-one (DHT; 75%), 5α-androstane-3α,17β-diol (17%), and 5α-androstane-3β,17β-diol (10%) but not with progesterone (< 0·001%), oestradiol (< 0·01%) or other androgens (< 0·02%): the assay therefore measures androgens (primarily testosterone and DHT) rather than testosterone. The antisera against oestradiol and oestrone have low cross-reactivity with other phenolic steroids (< 10%) and negligible cross-reactivity with neutral steroids (< 0·1%). The sensitivities of the assays for progesterone, androstenedione, testosterone + DHT, oestradiol and oestrone were 12·5, 25, 25, 2·5 and 5 pg, respectively. The intra- and inter-assay coefficients of variation of the assays were < 10% and < 15%, respectively. Data presented in Text-figs 1–4 have not been corrected for initial steroid content of cells.

Statistical analyses. Results were treated by analysis of variance and Duncan's new multiple range test (Steel & Torrie, 1960). When there was evidence of heterogeneity of variance, statistical analysis was performed on logarithmically transformed data. Tabulated results were analysed by a least squares procedure (Harvey, 1960) using a linear model to compare the effects of multiple variables and their interactions.

Results

Oestrogen production

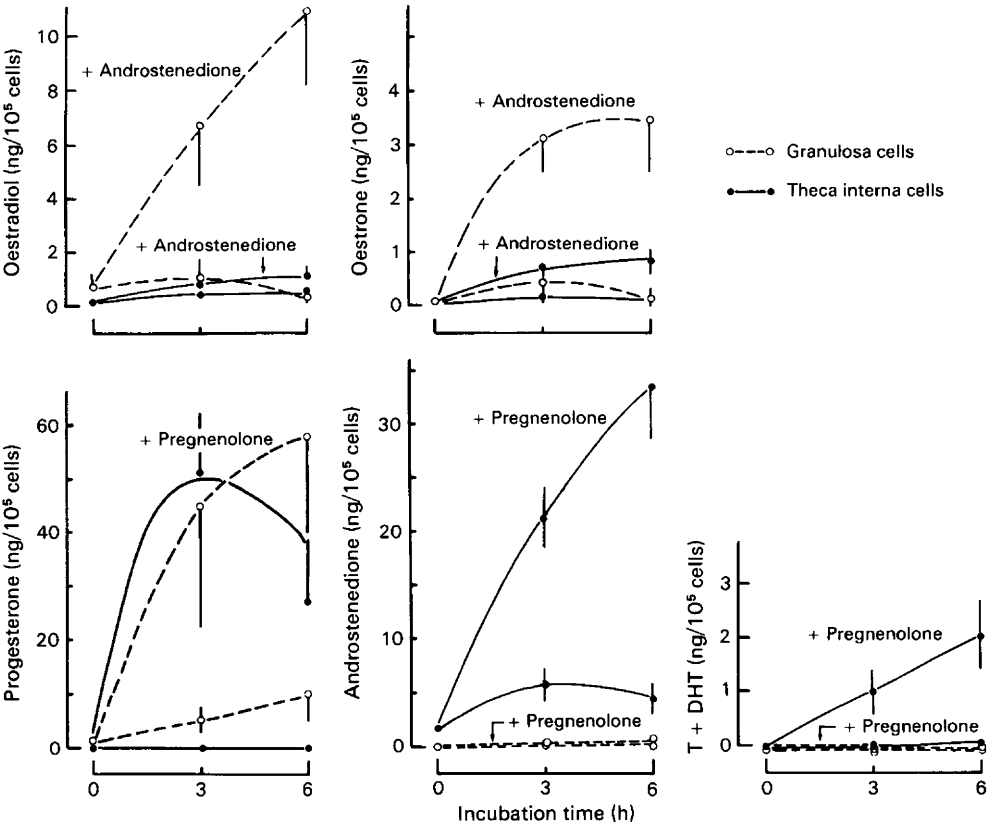
In the absence of exogenous substrate, theca interna cells produced significant quantities of oestrone and oestradiol-17β, particularly when isolated from follicles 36 and 72 h after PMSG treatment (Table 1). In contrast, granulosa cells from the same follicles produced negligible quantities of oestrogen. After exposure of the developing follicles to hCG *in vivo*, the ability of both cell preparations to produce oestrone and oestradiol was greatly diminished ($P < 0.001$).

Table 1. Steroid production *in vitro* by theca interna (TC) and granulosa (GC) cells in the absence of added substrate

		Net steroid production			Summary of ANOVA		
		Time after PMSG treatment					
Steroid	Cell type	36 h	72 h	108 h	Effect of cell type	Effect of time	Interactions
Progesterone (ng/10^5 cells)	GC	2·92 ± 0·97 (4)	6·07 ± 2·32 (4)	40·10 ± 11·80 (4)	$P < 0.01$	$P < 0.01$	$P < 0.025$
	TC	0·15 ± 0·05 (4)	0·03 ± 0·01 (4)	6·12 ± 2·28 (4)			
Androstenedione (ng/10^5 cells)	GC	0·01 ± 0·00 (5)	0·01 ± 0·00 (5)	0·03 ± 0·01 (4)	$P < 0.001$	$P < 0.001$	$P < 0.001$
	TC	0·50 ± 0·19 (4)	1·93 ± 1·33 (5)	9·21 ± 2·23 (4)			
Testosterone + DHT (pg/10^5 cells)	GC	9·77 ± 2·82 (5)	12·60 ± 3·90 (3)	8·85 ± 0·74 (4)	$P < 0.001$	$P < 0.001$	$P < 0.001$
	TC	15·60 ± 4·40 (5)	29·80 ± 13·20 (3)	88·80 ± 28·50 (4)			
Oestrone (pg/10^5 cells)	GC	14·30 ± 3·50 (3)	40·40 ± 13·40 (8)	1·90 ± 0·70 (3)	$P < 0.01$	$P < 0.001$	NS
	TC	128 ± 4 (3)	151 ± 4·90 (8)	6·30 ± 1·10 (3)			
Oestradiol (pg/10^5 cells)	GC	41·30 ± 23·30 (3)	36·90 ± 9·80 (8)	1·50 ± 0·60 (3)	$P < 0.01$	$P < 0.001$	NS
	TC	332 ± 152 (3)	359 ± 78 (8)	15·90 ± 5·20 (3)			

Values are mean ± s.e.m. Number in parentheses indicates number of replicates, each done in triplicate incubations of 300 000 cells collected at the indicated times and incubated for 6 h in 0·3 ml medium.

Addition of testosterone or androstenedione (0–100 μM) resulted in concentration-dependent increases in production of oestradiol and oestrone by granulosa cells obtained 36 and 72 h after PMSG treatment (Text-figs 1 & 2). In addition to the significant influence of the stage of follicle development ($P < 0.001$) and aromatizable substrate ($P < 0.001$), there was also significant interaction ($P < 0.001$) between these factors due to the loss in the ability of androgen to stimulate oestrogen production 108 h after PMSG. Theca interna oestrogen production also increased significantly in the presence of androstenedione and testosterone (Text-figs 1 & 2). However, this stimulation was substantially less than that in granulosa cells, at the same substrate concentrations. LH, but not FSH, stimulated ($P < 0.025$) oestrogen production *in vitro* by theca interna cells obtained 72 h after PMSG treatment (Table 2), but neither gonadotrophin affected oestrogen production by the thecal or granulosa cell in the presence of exogenous androstenedione (10 μM) (data not shown). When incubated together, granulosa and theca interna cells produced larger amounts of oestrogen per cell than the sum produced by each incubated alone (Table 2).



Text-fig. 1. Effects of incubation time and 10 μM exogenous substrate (pregnenolone for progesterone, androstenedione and testosterone (T) + DHT production; androstenedione for oestradiol-17β and oestrone production) on steroid production by dispersed granulosa (○-----○) and theca interna (●——●) cells obtained from follicles of gilts 72 h after PMSG injection. Each point represents the mean ± s.e.m. of 3–8 replicates.

Table 2. Oestrogen production by theca interna and granulosa cells incubated separately and together with and without gonadotrophin

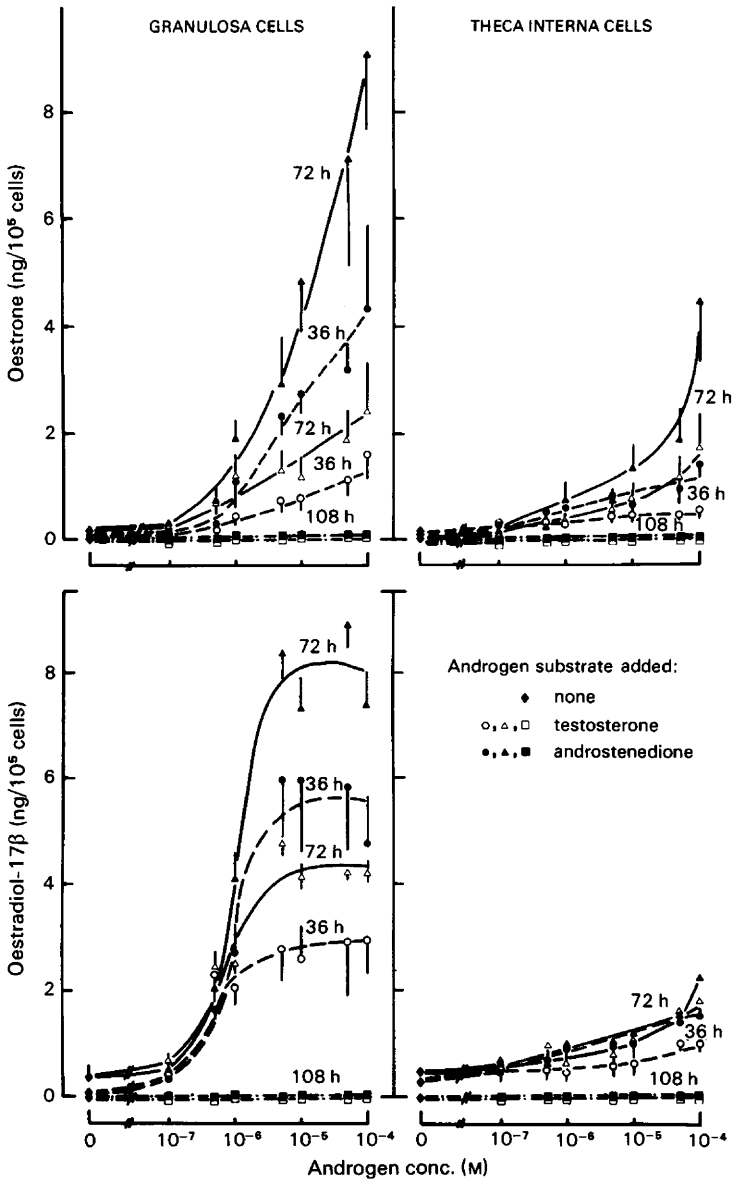
	Total oestrogen production†			
	pg/10⁵ cells		pg/2 × 10⁵ cells	
Treatment	Granulosa	Theca	Sum‡	Coincubation§
None (control)	62	553	615	2072
FSH (100 ng/ml)	38	784	820	2309
LH (10 ng/ml)	71	1022	1099	3290
Standard error	12–23	114–222	216	474

(1) Effect of coincubation (sum *vs* coincubation): $P < 0.001$; (2) effect of FSH: $P > 0.05$; (3) effect of LH: $P < 0.025$; (4) interaction (coincubation and gonadotrophin): $P > 0.05$

† Oestrone plus oestradiol produced in 6 h by cells collected 72 h after PMSG treatment (corrected for cell content before incubation).

‡ The least square means of the sum of oestrogen production by granulosa and theca interna cells incubated separately (6 replicates).

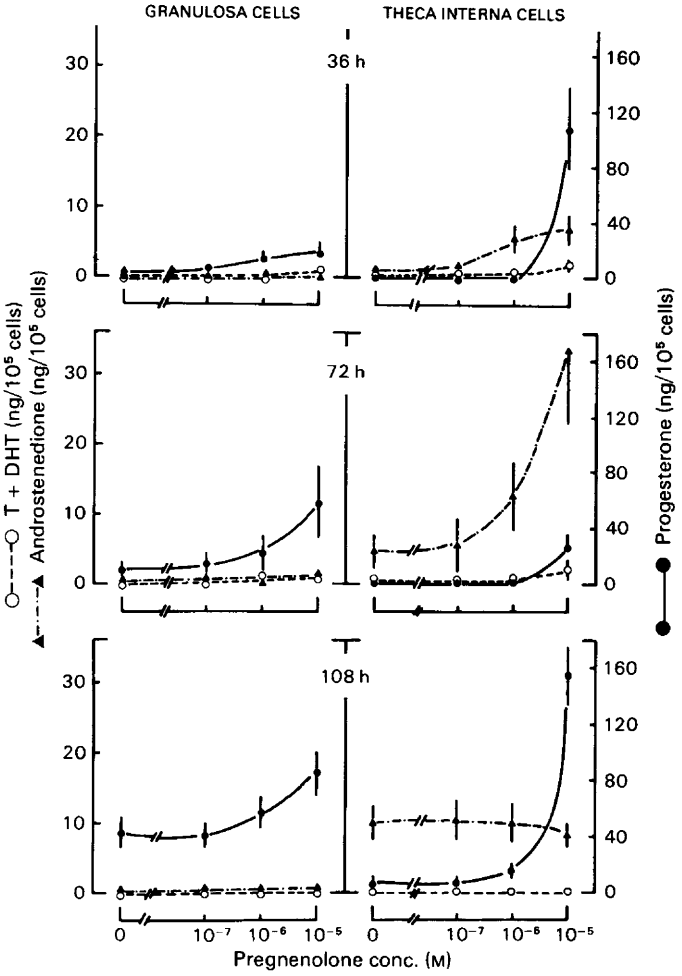
§ The least square means of oestrogen production by granulosa and theca interna cells incubated together (5 replicates).



Text-fig. 2. Oestrone and oestradiol production during a 6-h incubation in the presence of different concentrations of exogenous aromatizable substrate by dispersed pig granulosa (GC) and theca interna cells (TC) isolated from follicles of gilts 36, 72 and 108 h after PMSG/hCG treatment. The oestrogen content (pg/10^5 cells) of the cells at these times before incubation was: oestrone: 1·53 ± 0·91, 46 ± 31 and 0·69 ± 0·37 in GC; 3·11 ± 2·41, 19 ± 5 and 1·28 ± 0·75 in TC; oestradiol: 5·97 ± 2·78, 152 ± 41 and 3·50 ± 0·30 in GC; 11·4 ± 4·8, 108 ± 64 and 1·60 ± 1·00 in TC. Each point represents the mean ± s.e.m. of 3–7 replicates.

Androgen production

In the absence of exogenous substrate, theca interna cells produced significant quantities of androstenedione and testosterone + DHT, the production of androstenedione being 50 to 100 times greater than that of testosterone + DHT (Table 1). Androgen production by theca interna cells increased significantly ($P < 0.001$) with follicular development; addition of pregnenolone significantly increased androstenedione and testosterone + DHT production in a concentration-dependent manner at 36 and 72 h, but not at 108 h, after PMSG treatment (Text-figs 1 & 3).



Text-fig. 3. Progesterone, androstenedione and testosterone (T) + DHT production during a 6-h incubation in the presence of different concentrations of pregnenolone by dispersed pig granulosa (GC) and theca interna (TC) cells isolated from follicles of gilts 36,72 and 108 h after PMSG/hCG treatment. The steroid content of the cells at these times before incubation was: progesterone (ng/10^5 cells): 0·40 ± 0·26, 1·27 ± 0·49 and 2·21 ± 0·41 in GC; 0·05 ± 0·03, 0·09 ± 0·02 and 0·94 ± 0·15 in TC; androstenedione (ng/10^5 cells): 0·04 ± 0·01, 0·06 ± 0·02 and 0·02 ± 0·00 in GC; 0·74 ± 0·41, 2·76 ± 1·56 and 0·93 ± 0·38 in TC; testosterone + DHT (pg/10^5 cells): 9·85 ± 4·76, 12·1 ± 1·76 and 5·71 ± 2·01 in GC; 63·9 ± 46·0, 23·0 ± 3·49 and 12·0 ± 3·50 in TC. Each point represents the mean ± s.e.m. of 4–5 replicates.

Addition of LH *in vitro* produced a concentration-dependent stimulation ($P < 0.001$) of androstenedione (Text-fig. 4b) and testosterone + DHT production (data not shown), in the absence but not the presence (10 μM, $P > 0.05$) of pregnenolone, by theca interna cells obtained from follicles 36 and 72 h after PMSG treatment. FSH was stimulatory ($P < 0.01$) only at a concentration of 1 μg/ml. Neither gonadotrophin nor pregnenolone affected androgen production by theca interna cells obtained 108 h after PMSG treatment. In contrast, granulosa cells from the same follicles produced negligible quantities of androgen at any stage of follicular development, whether or not pregnenolone and/or gonadotrophins were added (Table 1; Text-figs 3 & 4b).

Progesterone production

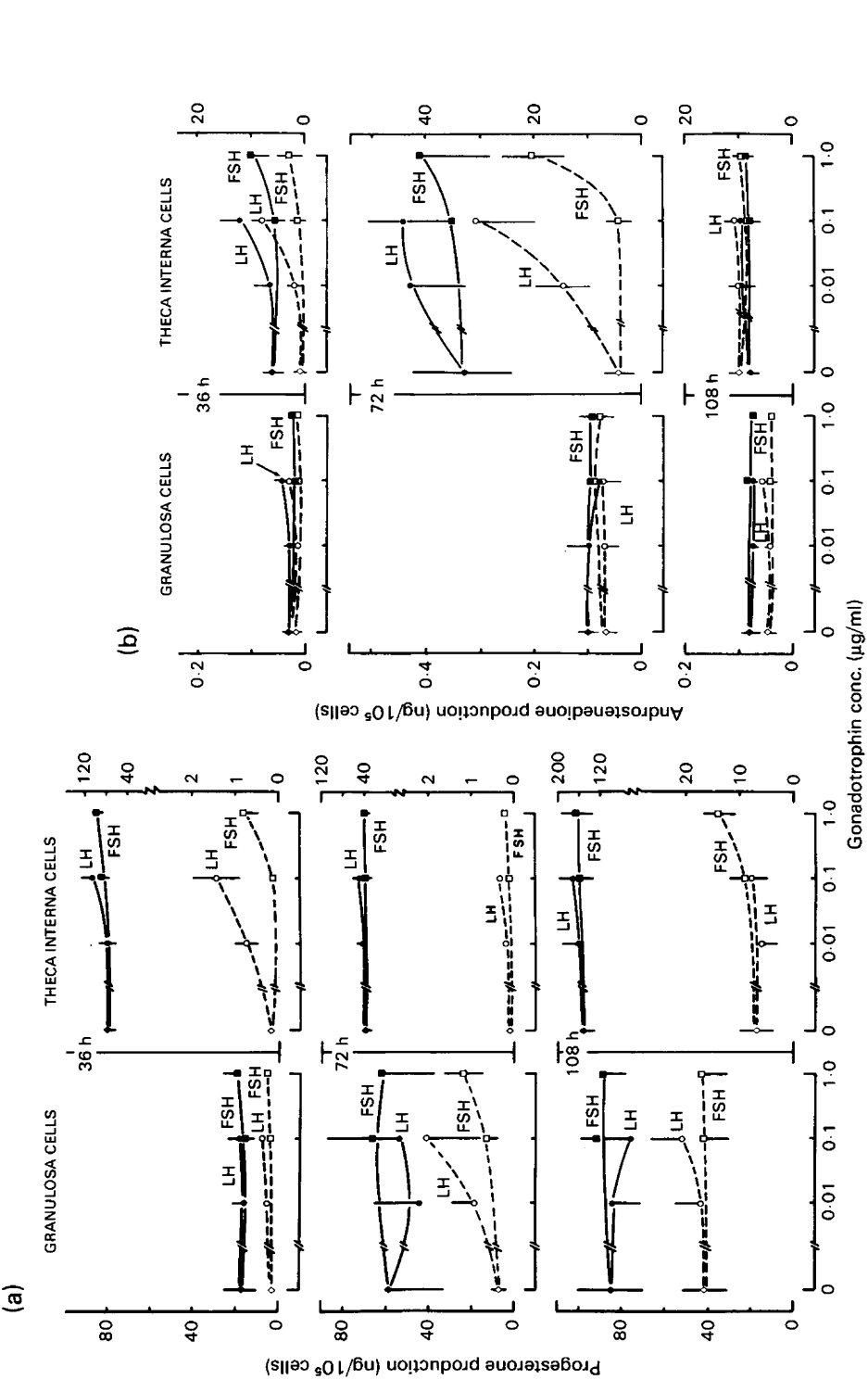
Granulosa cells produced more progesterone than did theca interna cells (Table 1; Text-fig. 1). In the presence of pregnenolone, progesterone production by both cell preparations was increased significantly ($P < 0.001$), particularly at higher substrate concentrations (Text-fig. 3). However, whereas production of progesterone by granulosa cells increased progressively with follicular development, apparent thecal progesterone production was significantly lower at 72 h than at 36 h or 108 h after PMSG treatment ($P < 0.001$).

Addition of LH, in the absence of exogenous substrate, resulted in a significant increase ($P < 0.01$) in progesterone production by theca interna cells 36 and 72 h after PMSG treatment although the response appeared to be markedly lower at the later stage (Text-fig. 4a). FSH was without effect except at high concentration (1 μg/ml). Neither gonadotrophin had a significant effect on progesterone production by theca cells collected 108 h after PMSG treatment ($P > 0.05$). There were no significant effects ($P > 0.05$) of FSH or LH on progesterone production by granulosa cells (Text-fig. 4a).

Discussion

The present study shows that pig granulosa cells have very little ability to synthesize oestrogen *in vitro* in the absence of added aromatizable substrate (Table 1). Addition of androstenedione or testosterone significantly increased their ability to produce oestrogen (Text-fig. 2). These findings are consistent with those of others for the pig (Anderson *et al.*, 1979; Schomberg, 1979; Evans *et al.*, 1981; Stoklosowa, Gregoraszczuk & Channing, 1982; Tsang *et al.*, 1982b) and for other species (Lacroix, Eechaute & Leusen, 1974; Dorrington, Moon & Armstrong, 1975; Erickson & Ryan, 1975; Moor, 1977; Fortune & Armstrong, 1978; Moon, Tsang, Simpson & Armstrong, 1978; McNatty, Makris, de Grazia, Osathanondh & Ryan, 1979a, b; Tsang *et al.*, 1980). While these observations confirm reports that the inability of granulosa cells to produce oestrogens is due to the lack of the enzymic machinery necessary to convert C_{21} steroids to androgens (Short, 1962; Bjersing & Carstensen, 1967), Johnson & Hoversland (1983) have shown that rat granulosa cells produce significant amounts of oestrogen in the absence of added androgen provided that NAD is available in the incubation medium.

In contrast, theca interna cells produced significant quantities of oestrogen in the absence of added androgen (Table 1). Although addition of androgen significantly increased thecal oestrogen production, these increases were substantially less ($\sim \times 10$) than those produced in granulosa cells ($\sim \times 200$) by the same additions (Text-fig. 2). These results are in agreement with those of Evans *et al.* (1981) and Stoklosowa *et al.* (1982) but differ from those of Haney & Schomberg (1981), who found no effect of added testosterone on thecal oestrogen production in organ culture and used this lack of response to indicate the effectiveness of separation of granulosa and thecal components. In the present work, although the possibility of granulosa cell contamination of thecal preparations cannot be completely excluded, the histological criteria (Plate 1) and the differences in oestrogen production by the two cell types when androgen is supplied, indicate that contamination would be $< 5\%$, which would be insufficient to affect the interpretation of results.



Text-fig. 4. Influence of gonadotrophins *in vitro* on the production of (a) progesterone and (b) androstenedione by granulosa and theca interna cells from pig preovulatory follicles at different stages of maturation. Dispersed granulosa cells and theca interna cells were obtained from follicles 36, 72 and 108 h after PMSG/hCG treatment and incubated for 6 h in the absence (----) and presence of 10 μM-pregnenolone (——) and in the absence (◇, ◆) and presence of two concentrations of LH (○, ●) or FSH (□, ■). For androstenedione and progesterone content of these cells before incubation, refer to legend to Text-fig. 3. Each point represents the mean ± s.e.m. of 4 replicates.

The finding that total oestrogen production by combined granulosa and thecal cells in the absence of added androgen (Table 2) was substantially greater than when the cells were incubated separately supports the two-cell hypothesis of follicular oestrogen production (Ryan, 1979) and establishes the theca interna as the principal source of androgen. This synergism between the cellular compartments indicates that the granulosa contribution to total follicular oestrogen production *in vivo* depends largely on the availability of androgen from the theca. It is therefore likely that the granulosa contribution to total follicular oestrogen *in vivo* increases significantly as the follicle matures, due to the greater number of granulosa cells per follicle (Daguet, 1978) and the progressive increase in granulosa aromatase activity and thecal androgen production.

The lack of significant androgen production by granulosa cells, even in the presence of pregnenolone or gonadotrophin (Table 1; Text-figs 3 & 4b) confirms our earlier findings (Tsang *et al.*, 1982*b*) and those of Evans *et al.* (1981). However, the results conflict with those of Stoklosowa *et al.* (1982) who regarded the granulosa compartments to be a significant source of androgen because they found only twice as much androgen was produced by thecal cells. However, the androgen measured in that study was mainly testosterone + DHT and 5α-reduced androgens. In the present work, testosterone + DHT production by thecal cells was also only about twice that of granulosa cells but total androgen production was up to 200 times greater (Table 1).

Progesterone production by granulosa cells in the absence of pregnenolone increased progressively with follicular maturation (Table 1; Text-fig. 3). The marked increase in progesterone production by granulosa cells after exposure to LH/hCG *in vivo*, with or without added pregnenolone, is consistent with the findings of others (Channing, Brinkley & Young, 1980; Haney & Schomberg, 1981; Evans *et al.*, 1981). However, progesterone production by granulosa cells was not increased by addition of LH or FSH *in vitro*, which contrasts with the marked response obtained by Evans *et al.* (1981). The reasons for this apparent discrepancy are not clear. Nevertheless, the results indicate that the steroidogenic ability of the granulosa cell after exposure to LH/hCG *in vivo* changes, losing its oestrogenic character and, with luteinization, becomes progestagenic.

The observed pattern of changes in thecal androgen and progesterone production with follicular maturation extends the results of Evans *et al.* (1981) and Haney & Schomberg (1981). In the absence of exogenous substrate, theca interna cells produced more androgen than progesterone at all stages (Table 1). Pregnenolone stimulated the production of both progesterone and androgen by thecal cells isolated 36 and 72 h after PMSG. By 72 h however, the ability of thecal cells to produce androgen was increased to the extent that there was no significant accumulation of progesterone, and androstenedione was the major steroid produced. LH stimulated progesterone production by theca only in the absence of pregnenolone, suggesting that the principal action of LH is to increase the availability of pregnenolone. This is consistent with the known action of LH on cholesterol side-chain cleavage (Channing & Tsafriri, 1977). FSH had no effect except at high concentrations, suggesting that its action may be due to contamination by LH.

By 108 h after PMSG, i.e. 36 h after treatment with hCG *in vivo*, LH no longer stimulated steroidogenesis and pregnenolone stimulated only progesterone production. Before hCG injection, both follicular compartments were capable of oestrogen production which could be enhanced by the addition of androgen. However, oestrogen production was markedly reduced by 108 h and could no longer be stimulated (Text-fig. 2; Table 1), despite continued production of significant amounts of androgen by the thecal cells (Table 1; Text-fig. 3). These steroidogenic activities are consistent with the pattern of changes in the content of steroids in follicular fluid obtained at different stages of follicular maturation in the pig (Ainsworth *et al.*, 1980).

The loss of responsiveness to LH *in vitro* after exposure to the action of this hormone *in vivo* has been noted previously with rat follicles (Lamprecht, Zor, Tsafriri & Lindner, 1973) and with human thecal tissues (Dennefors, Hamberger & Nilsson, 1983). Moreover, Webb & England (1982) have shown that, in sheep follicles, exposure to endogenous LH is followed by a sharp reduction in the concentration of LH receptors in both granulosa and thecal cells. Similarly, curtailment of

oestrogen production has been noted in rat follicles after in-vivo exposure to LH (Lieberman *et al.*, 1975; Hamberger, Hillensjo & Ahren, 1978) and in sheep follicles after exposure to LH *in vitro* (Moor, 1974) or *in vivo* (Webb & England, 1982). While the mechanism by which LH brings about these changes is not well understood, the reduction in oestrogen production in the rat ovary has been attributed to a decline in the availability of aromatizable androgen, associated with a reduction in the activity of the 17α-hydroxylase/$C_{17,20}$-lyase system which is essential for the conversion of C_{21} steroids to androgens (Hamberger *et al.*, 1978; Suzuki & Tamaoki, 1983; Weiss & Eckstein, 1983). These results are inconsistent with the present findings whereby thecal production of androgen was sustained after exposure to hCG *in vivo* (Table 1). However, there was essentially no net increase in androgen production by thecal cells in the presence of added pregnenolone (Text-fig. 3). These results suggest the possibility of end-product inhibition by androgen of the conversion of C_{21} steroids to androgens, with the result that progesterone accumulates. It is clear, nevertheless, that the decline in oestrogen production in the pig follicle is due primarily to a decrease in the aromatase activity of both cellular compartments. Whether this is accompanied by a change in activity of 17α-hydroxylase and/or $C_{17,20}$-lyase activities remains to be clarified.

In summary, our study of isolated cells from pig follicles have revealed several functional changes in steroidogenesis which occur during follicular maturation. The granulosa and theca interna cells of the developing preovulatory follicle of the pig produce oestrogen. The relative rate of production of oestrogen by each cell type is dependent on the stage of development of the follicle and availability of aromatizable substrate. The theca interna is the principal source of androgen and as such controls de-novo oestrogen production by providing aromatizable substrate. Although the granulosa cells appear to be the principal source of progesterone, theca interna cells also contribute significantly to follicular progesterone production. After exposure of the follicle to hCG *in vivo*, the aromatase activity of both cell types declines. The theca interna continues to produce progesterone and androgen whereas the granulosa cells are transformed from oestrogen-producing cells to ones producing primarily progesterone.

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