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Estrogen and Progesterone Secretion by Isolated Cultured Porcine Thecal and Granuolsa Cells[1]

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ABSTRACT

Porcine follicular granulosa and theca interna cells were cultured singly and in combination. Both cell types were isolated from a pool of the same proestrous large follicles. Cultures were maintained for 8 days in culture medium 199 enriched with 10% calf serum and the culture medium was changed every second day. In half of the cultures, testosterone, in a concentration of 150 ng/ml (0.5×10^{-7} M), was added to the culture medium. The conditioned culture media subsequently were assayed for estrogen, progestogen and androgen using radioimmunoassay. Unless stated otherwise, data are expressed as pg/10^5 cells/48 h in the case of estrogen and androgen and ng/10^5 cells/48 h in the case of progesterone. Each culture was replicated 4 times and the data shown is the mean ± SEM of 4 determinations. Theca interna cells cultured alone secreted more estrogen 473 ± 43.9 pg than granulosa cells which secreted 34 ± 3.4 pg, ($P<0.001$). Mixed cultures of theca and granulosa secreted the largest amount of estrogen, 754 ± 5.8 pg, ($P<0.001$) versus theca alone and ($P<0.001$) versus granulosa alone. Theca and granulosa cultured alone secreted 81.8 ± 4.7 pg and 40.9 ± 1.6 pg androgen, respectively, and the mixed culture of theca and granulosa secreted 110 ± 5.7 pg androgen. These differences were true also if the secretion rate was estimated over the entire 8-day culture period.

Granulosa cells cultured alone secreted the greatest amount of progesterone and the elevated secretion rate was maintained during the entire 8-day culture period (1222 ± 125, 1033 ± 79, 894 ± 43, 234 ± 26 ng per culture/day on Days 2,4,6 and 8, respectively). After 48 h granulosa cells secreted 113.9 ± ng progesterone, whereas thecal cells secreted only 0.7 ng progesterone/10^5 cells.

The addition of testosterone to the culture medium brought about a marked increase of estrogen secretion not only by theca interna cells but also by granulosa and by granulosa plus thecal cell cultures as well. Testosterone also led to an increase in progesterone secretion by granulosa cells cultured alone and by theca cultured alone. In cultures of theca plus granulosa, addition of testosterone led to suppression ($P<0.001$) of progesterone secretion in comparison with granulosa cells cultured alone.

INTRODUCTION

The site of steroid hormone biosynthesis within the ovarian follicle has not yet been completely elucidated and 1 reason for this is the lack of satisfactory techniques for isolating and investigating follicular tissue elements. In order to examine their steroidogenic potential, granulosa cells have been successfully isolated as individual cells from porcine follicles and first cultured by Bjersing (1962), Channing (1970c) and Schomberg (1969). In contrast, however, theca interna cells have been examined only as fragments rather than isolated cells and studied both in vivo (Falck, 1959), in culture (Channing, 1966, 1969c), and in short-term incubation experiments (see Ryan, 1979, and Channing et al. 1980 for review). Tissue culture of isolated follicular cell types offers an opportunity to examine which follicular cell types are the source of a particular hormone. Stoklosowa et al. (1978) have developed a method for

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dispersal of thecal cells into individual cell suspensions so that thecal, as well as granulosa cells, can both be cultured as isolated and recombined cell suspensions. In previous studies the thecal cells were examined as fragments, whereas the granulosa cells were examined as isolated cells, making employment of a heterogeneous system necessary.

In the present study the homogeneous cell preparation of porcine, theca and granulosa cells cultured alone and mixed were used to examine the interaction of these cell types in estrogen, androgen and progesterone biosynthesis. In addition the effect of added testosterone substrate upon the steroidogenic potential of these cultured follicular cell types was examined.

MATERIALS AND METHODS

Ovarian Material and Culture Procedure

Porcine ovaries were obtained at a nearby slaughterhouse. The approximate stage of the estrous cycle of the ovaries was determined using criteria described by Akins and Morissette (1954) and Channing and Ledwitz-Rigby (1975). Only proestrous ovaries containing large (0.8–1.2 cm external diameter) vascular follicles and either no corpora lutea or corpora albicantia only, were selected as the source of tissue for culture. The large 0.8–1.2 cm follicles were used for this study. For each culture 80 follicles were aseptically removed. Granulosa cells were isolated by the technique described by Channing and Ledwitz-Rigby (1975). Subsequently, the cells were centrifuged for 5 min at 500 × *g* and the pellet was resuspended in 5 ml of culture medium and the process repeated, followed by storage at 4°C for 2–3 h until culture. In the meantime, theca interna layers from the same follicles from which granulosa cells had been removed under the binocular dissecting microscope were isolated and brought into suspension employing the technique of Stoklosowa et al. (1978). This method involves digestion of the washed thecal layer with trypsin. Viable thecal cells are recovered by this procedure. Viable cells were counted in a hemocytometer using typan blue dye (0.06%). In a series of 8 experiments the average concentration of granulosa cells collected from 80 follicles was 2,378,000 ± 222,000 cells/ml, while that of theca interna cells only 483,000 ± 39,880 cells/ml. In order to inoculate the cells at a concentration comparable to that observed in vivo, granulosa cells and thecal cells were inoculated at a concentration of 0.75×10^5 and 0.175×10^5 cells/ml culture media, respectively. When the cultures were mixed, the thecal and granulosa cells were inoculated at a concentration of 0.17×10^5 and 0.75×10^5 cells/ml culture media, respectively. The following mixtures of cell types were cultured: granulosa alone, theca interna alone and granulosa plus theca. Cells were grown as monolayers in Leighton tubes for 8 days. Daily observation of the morphology of cells was made using a Zeiss inverted microscope. Each culture was replicated 4 times and the steroid secretion data expressed as the mean ± SEM of 4 replicate cultures.

Half of the cultures were grown in Medium 199 supplemented with 10% calf serum (purchased from the Laboratory of Sera and Vaccines, Lublin, Poland) and antibiotics (penicillin, 100 U/ml; streptomycin 10 μg/ml and mycostatin 10 μg/ml). Additional cultures were cultured in medium 199 + 10% calf serum supplemented with testosterone (Sigma) in a concentration of 150 ng (0.5×10^{-7} M) on Day 0 of culture and at each medium change. Before use each batch of calf serum was assayed for estrogen, progestogen and androgen content. In each instance the levels of these steroids were less than the lower limit of the assay (<8 pg/ml for estrogen and androgen and <50 pg/ml for progesterone). An additional control experiment was performed on each batch of serum: porcine granulosa cells were cultured in medium containing serum alone and in medium containing 10 ng of NIH ovine FSH or LH (NIH-LH-S17, NIH-FSH-S11). In all instances the stimulation of progestogen and estrogen secretion by these hormones was significant ($P<0.01$ vs. control) compared to control cultures (Table 1). Although LH and FSH levels were not measured in the calf serum used in these studies it has been shown by others that LH and FSH levels are low in calf serum (Gospodarowicz, 1980).

Culture medium was changed every second day, frozen and stored at −20°C for further steroid analysis. At the end of the culture period, monolayers were fixed with absolute methanol and stained according to May-Gruwald-Giemsa technique.

A number of cultures were stained histochemically for Δ^5, 3β-hydroxysteroid dehydrogenase (Δ^5, 3β-OH SD) using the technique of Fischer and Kahn (1972). The cultures were grown as monolayers for 2 or 4 days and the growth medium removed. For the histo-

TABLE 1. Effect of LH and FSH upon progesterone and estrogen secretion by cultured porcine granulosa and theca cells.

Addition to culture	Progestogens (ng/culture/2 days)[a]	
	Theca	Granulosa
Control	3.4 ± 0.3	2445 ± 250
10 ng FSH	33.8 ± 2.3	4378 ± 280
10 ng LH	9.3 ± 0.4	5718 ± 217
	—Estrogen (ng/culture/2 days)—	
Control	1562.5 ± 46	500 ± 10
10 ng FSH	45425.0 ± 443	1337 ± 227
10 ng LH	7662.0 ± 170	3675 ± 600

[a]Granulosa and theca cells were cultured for 2 days in the presence of either ovine LH or FSH and estrogen and progesterone measured in the conditioned medium. Each value is the mean ± SE of 4 cultures.

chemical test, simple medium without serum was used and the cells were incubated with or without dehydroepiandrosterone for 3 h. Medium without substrate (dehydroepiandrosterone) was used for Δ^5-control. Cell growth was estimated at daily intervals according to the method of Paul (1965). Each day the monolayers were treated with 0.1 M citric acid and the nuclei stained with crystal violet and counted in a hemocytometer.

Steroid Analysis

Each medium sample was analyzed for estrogen, progestogen and androgen using 3 separate radioimmunoassays. No chromatography of steroids was performed. Cross-reactivity of 10 and 500 ng of testosterone in the estrogen and progesterone radioimmunoassay was negligible.

Estrogen was estimated according to the method by Hotchkiss et al. (1971). Antiserum generated in rabbit against estradiol-17β-6-oxime-BSA (bovine serum albumin) was used. The specificity of the antiserum for estrogen was high. It cross-reacted with estradiol (100%) and cross-reacted significantly (66% and 2.1%) with estrone and estriol, respectively. A series of other steroids were tested for cross-reactivity and found to cross-react less than 0.1%. These other steroids were: cholesterol, progesterone, pregnenolone, androsterone, dehydroepiandrosterone, Δ^4, androstenedione, testosterone 0.03%, and dihydrotestosterone 0.1%. The limits of the standard curve were within the range between 5–200 pg. The antiserum was used in a dilution of 1:50000. In a series of 15 experiments the coefficients of variation between and within assays were estimated to be 8% and 7.5%, respectively. Tritiated [2,4,6,7–^{3}H] estradiol (Amersham), specific activity 104 Ci/mMol, was used as a tracer. Data obtained from unextracted samples were compared with corresponding data obtained from the same media extracted with diethyl ether. There was no significant difference between estimates of extracted and unextracted samples and therefore estrogen was measured routinely in unextracted culture media.

Progestogen was analyzed using radioimmunoassay according to the method of Abraham et al. (1971). Aliquots (100 or 200 μl) of culture media were extracted in duplicate with 2.5 ml hexane each. Antiserum was raised in rabbit against 11α-OH-progesterone-succinyl-BSA as the antigen and was used at a dilution of 1:5000. Tritiated [1,2,6,7–^{3}H] progesterone (Amersham), specific activity 85 Ci/mMol, was used as a tracer. The sensitivity of the assay was 50 pg. In a series of 15 experiments the coefficients of variation between and within assays were estimated to be 7.5% and 8.0%, respectively. The antiserum exhibited 5% cross-reactivity with pregnenolone, 1.8% with 20α-dihydroprogesterone, 1.1% with 17β hydroxyprogesterone, 1.2% with estradiol 17β and less than 1% with 7 other steroids which were cholesterol, 20β-dihydroprogesterone, testosterone, dehydroepiandrosterone, estradiol 17β, estrone, androsterone.

Androgen was determined according to Dufau et al. (1972) using antiserum raised in rabbit against testosterone-3-9-carboxy-methyl-oxime-BSA as antigen and used at a dilution of 1:15000. Tritiated testosterone [1,2,6,7–^{3}H] testosterone (Amersham), specific activity 81 Ci/mMol, was used as a tracer. The sensitivity of the assay was 5 pg. The antisera exhibited 100% binding with testosterone and cross-reacted 100% with 5α-androstane, 20% with dihydrotestosterone, 15.7% with Δ^4, androstenedione, 3% with dehydroepiandrosterone, 7.4% with androsterone. The cross-reactivity of the antiserum with other steroids was below 0.01%. These other steroids were: pregnenolone, 20α–, 20β–(OH), progesterone, progesterone, estradiol, 17β estrone and cholesterol. In a series of 10 experiments the 17α hydroxyprogesterone, coefficients of variation within and between assays were estimated to be 7.5% and 9.5%, respectively. Differences between data obtained from extracted and unextracted samples were insignificant and therefore androgen was measured using unextracted culture media. Culture media without cells were used as a blank in all assays used. Media supplemented with testosterone (150 ng/ml) were not assayed for androgen content since the total concentration of those hormones was beyond the limits of the assay used. In instances where the cells were cultured for 48 h, hormone secretion rates were normalized per 1×10^5 cells during 48 h in culture. In additional experiments, when the cultures were grown for an 8-day period, the hormone secretion rate was normalized per culture per day. Data were analyzed by Student's t test.

Reagents

All reagents used were analytical grade. Steroids used as standards, and chemicals used in histochemical work were purchased from the Sigma Chemical Company St. Louis, MO. Ethanol, diethylether, hexane and toluene were distilled before use.

Antisera

Antisera to estradiol and progesterone were donated by Professor Dr. Brian Cook, Dept. of Steroid Biochemistry, University of Glasgow, Glasgow, Scotland. Antiserum to testosterone was a gift from Dr. Bela Voracova, Dept. of Molecular Biology and Genetics, Czechoslovakia Academy of Sciences, Prague, Czechoslovakia.

RESULTS

Morphology

Theca interna and granulosa cells cultured alone exhibited satisfactory growth and morphological characteristics similar to those previously observed by Stoklosowa et al. (1978). (Figs. 1 to 6). Morphology of a mixed granulosa and thecal culture is shown in Fig. 3. It was interesting to note that even in mixed cultures it was possible to distinguish between granulosa and theca interna cells; the former maintained their typical luteal-like morphology and usually formed compact colonies surrounded by more elongated theca interna cells. The latter showed an interesting tendency to grow on the outside of granulosa cell colonies, more or less concentrically, in a manner resembling their in vivo organization

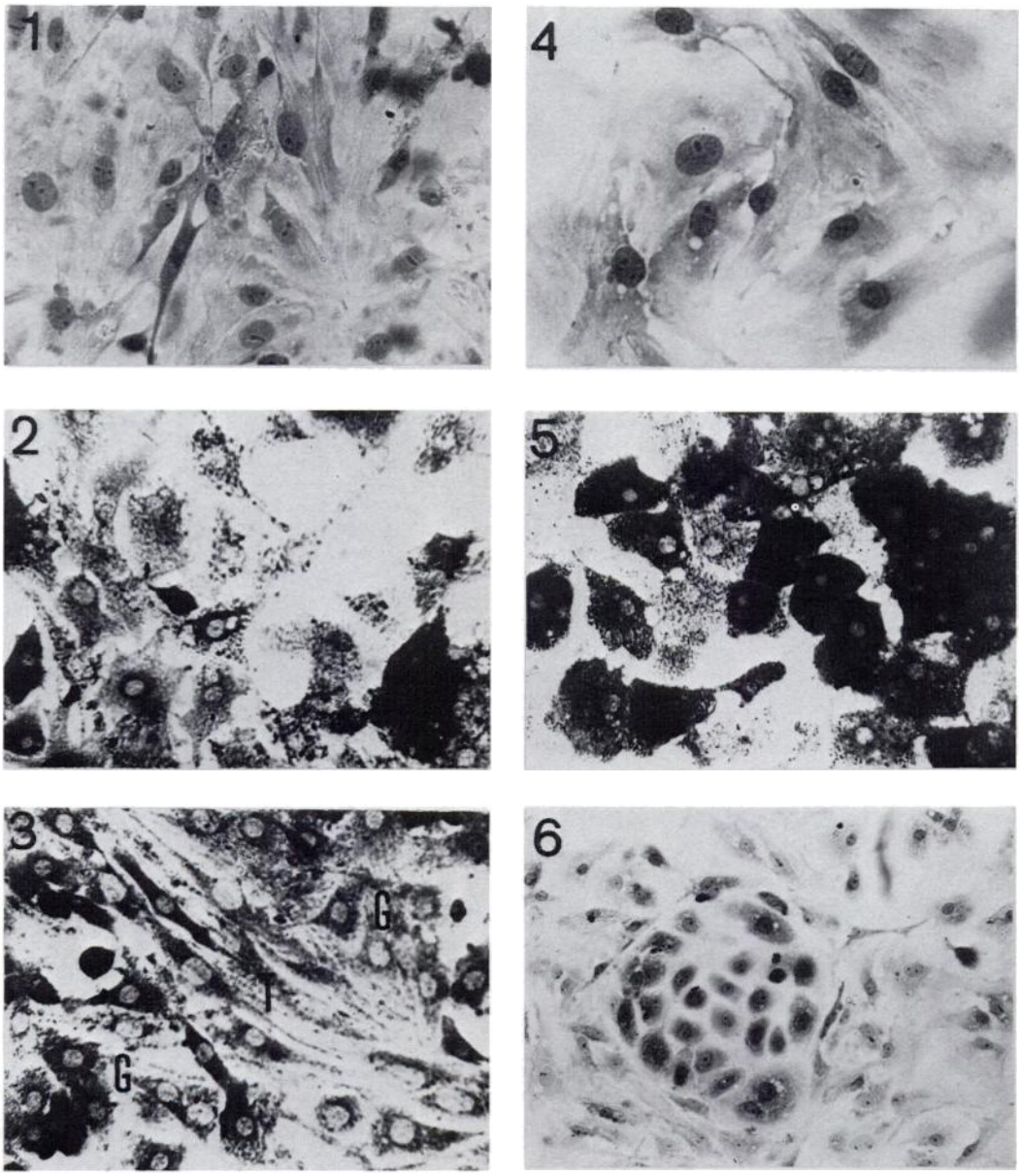

FIG. 1. A 4-day monolayer of theca interna cells cultured in control medium without testosterone. Stained according to May-Grunwald-Giemsa. X170

FIG. 2. Monolayer of granulosa cells after 4 days in culture in control medium. Cells were stained histochemically for activity of Δ^5, 3β-OH steroid dehydrogenase. X170

FIG. 3. Monolayer of a mixed thecal and granulosa cell culture after 4 days in culture histochemically examined for activity of Δ^5, 3β-OH steroid dehydrogenase. *G*—granulosa cells, *T*—theca cells. X170 The 3β-OH steroid dehydrogenase positive material is shown as the black deposit in the cytoplasm.

FIG. 4. Monolayer of theca cells cultured for 4 days in medium supplemented with 0.5×10^{-7} M testosterone. Note the difference in cell size and shape in comparison with Fig. 1. Stained according to May-Grunwald-Giemsa. X170

FIG. 5. Monolayer of granulosa cells after 4 days of culture in medium containing 5×10^{-7} M testosterone and stained for Δ^5, 3β-OH steroid dehydrogenase. Note the increased activity compared to Fig. 2. X170

FIG. 6. Culture of a mixture of theca and granulosa cells grown for 4 days. Note the arrangement of granulosa cells in the center and theca cells visible as a circle on the periphery. Stained according to May-Grunwald-Giemsa and cultured without testosterone. X85

TABLE 2. Growth of cultured granulosa and thecal cells.

Day of culture	Number of cells in culture[a]			
	Granulosa		Theca	
	Mean	SEM	Mean	SEM
1	1,100,000	1370	356,200	470
2	1,325,000	219	500,000	428
3	1,571,000	2710	866,300	550
4	2,148,000	701	531,000	943
5	2,125,000	1515	568,800	119
6	2,380,000	1931	687,500	875
7	1,962,000	2380	693,800	665
8	1,425,000	...	587,500	251

[a]Granulosa and thecal cells were harvested from large porcine follicles and cultured for varying time periods. At daily intervals cultures were terminated and the nuclei counted with crystal violet after treatment with 0.1 M citric acid. Data for granulosa and thecal cells represent the mean ± SEM of 6 and 4 observations, respectively. The number of granulosa and thecal cells in the initial inoculum was 2,100,000–400,000–438,000 cells, respectively.

(Figs. 3 and 6). Granulosa cells exhibited stronger histochemical reaction for activity of Δ^5, 3β-OH SD than theca cells (Fig. 3). Cultures treated with testosterone showed an altered morphology. Theca cells became less elongated and more epithelial in shape (Fig. 4). Granulosa cells showed increased vacuolization. In testosterone-treated cultures the activity of histochemically demonstrated Δ^5, 3β-OH SD increased markedly (Figs. 2 and 5).

Cell Growth

As shown in Table 2 there was about a 2-fold increase in the number of granulosa and thecal cells during the culture period. This increase was observed within the first 4 days of culture, thereafter cell numbers remained relatively constant.

Estrogen Secretion

Granulosa and theca interna cells cultured alone were able to secrete estrogen into the culture medium. Theca interna cells cultured alone in aliquots of 10^5 cells secreted 12-fold more estrogen ($P<0.001$) compared to the same numbers of granulosa cells. In mixed cultures, secretion of estrogen by the same number of cells increased 24-fold in comparison to theca interna cells alone ($P<0.001$) (Fig. 7). A similar relationship was observed when estrogen secretion was expressed as pg per culture per day over an 8-day period (Fig. 8). This was true however, only during 2 days in culture, while in later days a marked decrease of estrogen secretion by all cell types was seen.

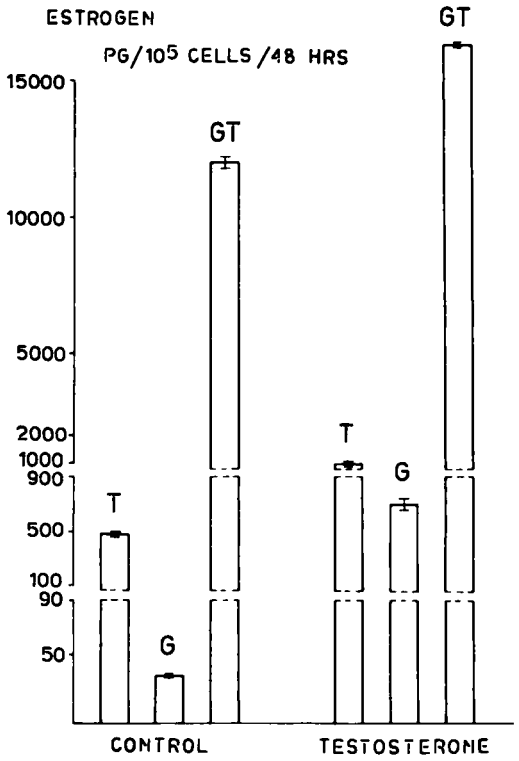

FIG. 7. Estrogen secretion by cultured theca interna alone (*T*), granulosa alone (*G*) and mixed granulosa plus theca (*GT*). Estrogen secretion is shown in pg per 1 × 10^5 cells per 2 days. The *right side* of the graph represents cultures supplemented with 0.5 × 10^{-7} M testosterone. Granulosa cell cultures were inoculated with 0.75 × 10^5 cells and thecal cell cultures were inoculated with 0.175 × 10^5 cells and mixed cultures contained 0.75 × 10^5 granulosa + 0.175 × 10^5 thecal cells. Data shown are the mean ± SEM of 4 replicates per point.

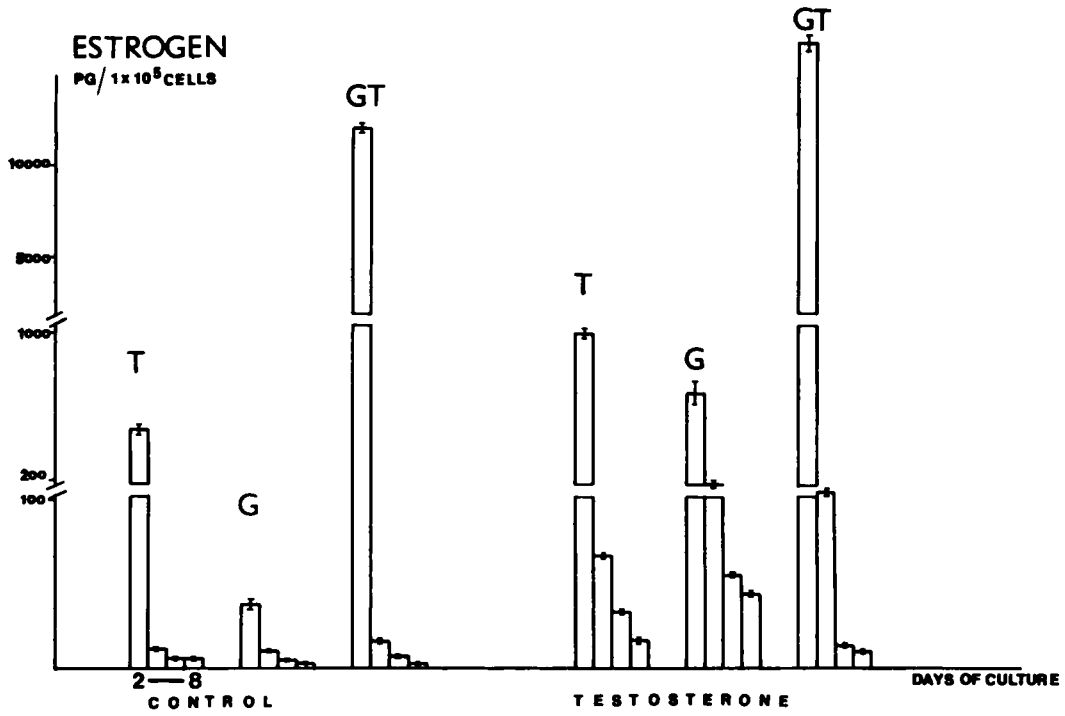

FIG. 8. Estrogen secretion by cultured theca interna alone (*T*), granulosa alone (*G*), and granulosa plus theca (*GT*). Estrogen secretion is shown on the y axis (log scale) as pg/10^5 cells/day. The cultures were grown for 8 days with medium changes being made every other day and the secretion rate estimated at 2-day intervals (days shown in bar on left of the figure). Other details are described in the legend of Fig. 7.

Effect of testosterone on estrogen secretion. The addition of 150 ng/ml (0.5×10^{-7} M) testosterone to cultures of granulosa and theca cells made either singly or in combination increased estrogen secretion. The secretion of estrogen by 10^5 granulosa cells grown for 48 h in the presence of testosterone increased 16-fold as compared to control cultures ($P<0.001$, Fig. 7). Estrogen secretion was also increased above the control secretion rate throughout the whole 8-day culture period (29–, 21–, 12– and 15-fold on Day 2,4,6 and 8 of culture, respectively). In all instances the increase was statistically significant, $P<0.0001$, (Fig. 8). After a 2-day culture period, secretion by 1×10^5 theca cells in the presence of exogenous testosterone was about 100% higher than theca cells grown in the absence of testosterone (Fig. 7, *right side* of the graph). A similar pattern was observed if the cultures were grown for 8 days (Fig. 8, *right side* of the graph). After time in culture, estrogen secretion by theca cells markedly decreased (Fig. 8). During the entire culture period estrogen secretion by mixed theca and granulosa cells grown in the presence of testosterone versus mixed control cultures increased by 1.4 fold on Day 2 ($P<0.05$), 10-fold on Day 4 of culture ($P<0.001$), 2.5-fold on Day 6 ($P<0.05$), and 5-fold on Day 8 ($P<0.001$), respectively (Fig. 8).

In comparison to theca cultured alone, the testosterone-dependent increase of estrogen secretion of mixed culture was 11.6-fold on Day 2 ($P<0.001$), 18.5-fold on Day 4, ($P<0.001$), 5.5-fold ($P<0.05$) on Day 6, and 6-fold on Day 8 ($P<0.001$) (Fig. 8).

Progesterone Secretion

Cultured granulosa cells compared to cultured thecal cells had the greatest ability to secrete progesterone (Figs. 9 and 10). Theca cultured alone secreted 0.7 ng progesterone/10^5 cells/48 h, whereas granulosa cultured alone secreted 114 ng progesterone/10^5 cells/48 h ($P<0.001$). Progesterone secretion by granulosa during the 8-day culture period was higher than that of theca throughout the entire period: a 719-fold

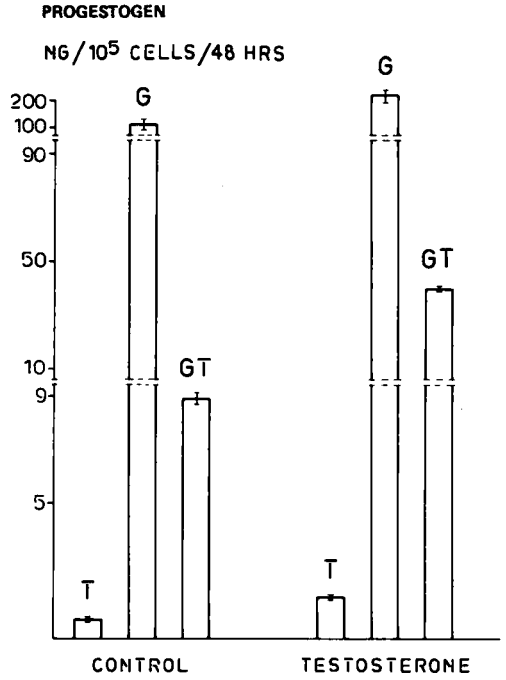

FIG. 9. Progesterone secretion by cultured theca interna alone (*T*), granulosa alone (*G*), and mixed thecal plus granulosa (*GT*). Progesterone secretion is expressed as ng/1 X 10^5 cells/2 days and is shown on the y axis in a log scale. Additional details are given in the legend of Fig. 7.

increase on Day 2, a 271-fold increase on Day 4, a 203-fold increase on Day 6 and a 66-fold increase on Day 8 of culture (in all cases $P<0.001$ vs. theca). In cultures of mixed theca and granulosa, however, a decline of progesterone secretion was observed (Figs. 9 and 10).

Effect of testosterone on progesterone secretion. The addition of 150 ng of testosterone to the culture medium caused a small but significant increase ($P<0.02$) of progesterone secretion by cultured theca cells and a 274% increase by granulosa cells after 2 days of culture ($P<0.01$) (Fig. 9). In the presence of 150 ng of testerone, progesterone secretion differed in cultured theca and granulosa cells during the 8-day culture (7.5 ng vs. 6711 ng on Day 2, $P<0.010$; 6 ng vs. 6924 on Day 4 $P<0.001$; 6.7 ng vs. 5665 ng on Day 6, $P<0.001$; and 6.1 ng vs. 5325 ng on Day 8, $P<0.001$) (Fig. 10). The secretion rate was maintained at an elevated rate throughout the entire 8-day culture period. Cultures of mixed granulosa and theca cells exhibited a decline in progesterone secretion in comparison with granulosa cells cultured alone ($P<0.001$) (Figs. 9 and 10).

Androgen Secretion

Both theca and granulosa cells were able to secrete androgen but theca cells produced almost 2.1-fold more compared to granulosa cells ($P<0.001$), (Fig. 11). Cultures of mixed granulosa plus theca produced androgen which was the sum of theca (81.8 ± 4.7 pg) and granulosa (40.9 ± 1.6 pg) secretion per 10^5 cells during 48 h (110 ± 5.7 pg, $P<0.05$) versus theca and ($P<0.001$) versus granulosa. In general the pattern of androgen secretion during the 8-day culture period was similar when compared to the 2-day culture period (Fig. 12). In this case, however, androgen secretion by the theca was lower than that of granulosa. This discrepancy might result from larger amounts of granulosa cells being present in each culture (0.75 X 10^5) rather than from the number of theca cells (0.175 X 10^5) present in the initial inoculum.

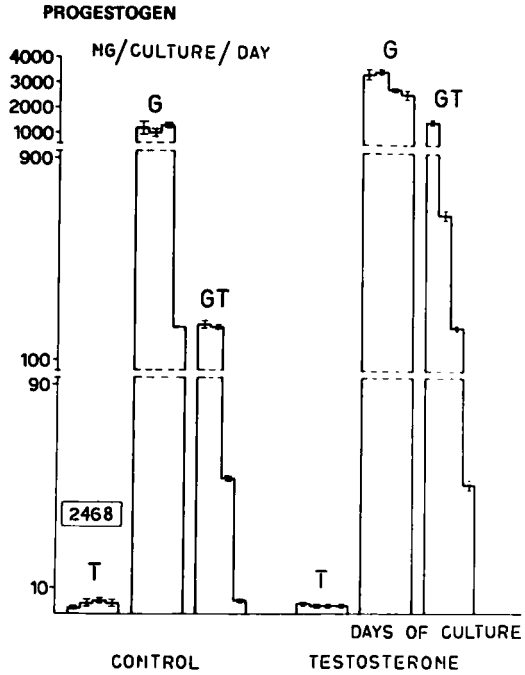

FIG. 10. Progesterone secretion by cultured theca interna alone (*T*), granulosa cells (*G*), and mixed theca plus granulosa (*GT*). Progesterone secretion is expressed an ng progesterone/culture/day throughout an 8-day culture period and is shown in a log scale in the y axis. Additional details are given in the legend to Fig. 7.

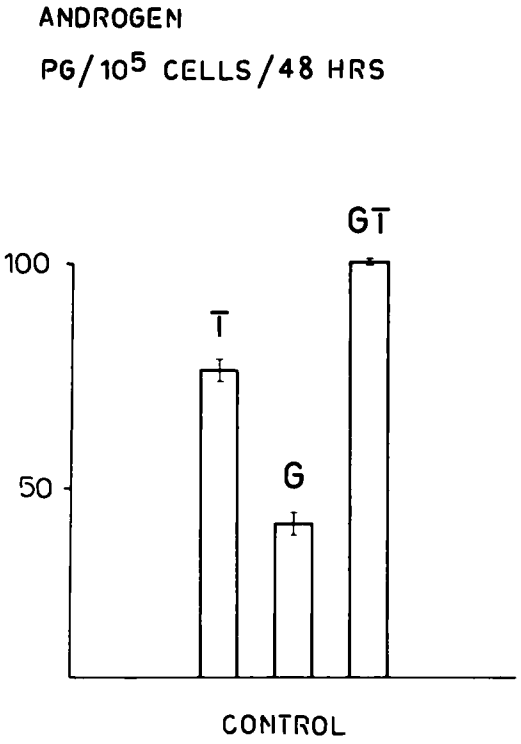

FIG. 11. Androgen secretion by cultured theca interna alone (*T*), granulosa alone (*G*) and theca plus granulosa (*GT*). Androgen secretion is expressed as pg/10^5 cells/2 days.

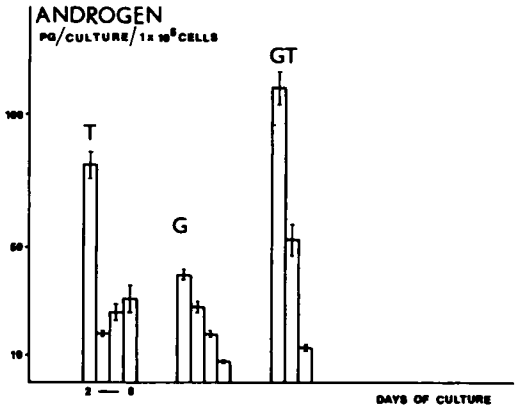

FIG. 12. Androgen secretion by cultured theca interna alone (*T*), granulosa alone (*G*) and theca plus granulosa (*GT*). Androgen secretion is expressed as pg/10^5 cells/day after 2, 4, 6 and 8 days of culture, and is shown in a log scale on the y axis.

DISCUSSION

These data are supportive of the concept that in the porcine preovulatory follicle the theca has the potential to secrete estrogen in the absence of additional androgen substrate. The potential of granulosa to secrete estrogen in the absence of androgen substrate is much less. This observation was carried out with dispersed cells where both cells were "equivalent" by the fact that both were dispersed. Electron microscopic observations by Krzysztofowicz and Stoklosowa (1977) on pig theca are supportive of the concept that pig theca has a high degree of steroidogenic potential. Studies with isolated human theca (Batta et al. 1980) and isolated monkey theca (Channing, 1980) revealed a similar situation. In vivo studies in the rhesus monkey by Channing and Coudert (1970) also demonstrated that theca is the principle source of ovarian vein estrogen.

The addition of testosterone vastly increased the ability of granulosa cells to secrete estrogen, demonstrating that the granulosa has a high potential to secrete estrogen if androgen substrate is provided. It has been shown by Channing et al. (1980) and by Anderson et al. (1979) that the potential for cultured porcine granulosa cells to secrete estrogen in the presence of androgen substrate increases as the follicle matures. The increased estrogen levels in follicular fluid observed as follicles mature could result from an increase in the ability of granulosa cells to aromatize testosterone and other androgens. In the present experiments the mixture of granulosa and theca led to a greater secretion of estrogen compared to the cell type cultured alone. This was also observed in the isolated monkey (Channing, 1980) and human (Batta et al, 1980) ovarian cell types. The fact that theca can secrete more androgen compared to granulosa and that granulosa can readily convert androgen to estrogen, would be supportive of the concept that thecal androgen serves as substrate for granulosa estrogen which ends up in the follicular fluid.

Observation of a lack of thecal estrogen secretion in the rat (Fortune and Armstrong, 1978) and in the pig (Tsang, et al., 1978) could be due to the fact that the investigators used clumps of thecal tissue rather than isolated cell types. The rat theca could potentially secrete less estrogen when compared to other species due to a species difference. It is possible that species with smaller follicles, such as the rat, have less requirement for thecal secretion of estrogen and instead have granulosa secretion of

estrogen with subsequent secretion of this intrafollicular estrogen to the blood stream since there is a shorter distance for the estrogen to diffuse out of the follicle.

Progesterone, on the other hand, was secreted mainly by granulosa cells which secreted elevated amounts throughout the culture period. Thecal cells secreted many-fold less the amount of progesterone compared to granulosa cells.

Testosterone added to the culture medium increased progesterone secretion by granulosa cells without significant decrease during 8 days in culture. The decline of progesterone production observed in mixed thecal plus granulosa cell cultures was probably the result of a suppressive action of estrogen on progesterone synthesis that has already been shown by Thanki and Channing (1978). The concentration of estrogen in culture media of mixed cultures was sufficiently high to cause inhibition of progesterone secretion. However, we observed no suppression of thecal androgen in mixed cultures, which is disparate with the data of Tsang et al. (1979), who found that estradiol inhibited androgen synthesis by porcine theca fragments. The fact that Tsang used fragments and we used isolated thecal cells could account for this discrepancy. Recently, Bellino and his colleagues observed that androgens can stimulate hemin biosynthesis which is a component of cytochrome P450 (Bellino, 1980). This could account for a stimulatory effect of androgen on progesterone secretion. Variable amounts of hemin in various culture conditions could account for variable stimulatory effects of androgen on progesterone biosynthesis.

The observation that estrogen secretion dropped after 2 days in culture, in both the thecal and granulosa cells (whether or not testosterone was added), is not unexpected. It is a common feature of differentiated tissue to lose its differentiated function in culture. Furthermore, no gonadotropins or follicular fluid steroidogenic enhancers were added to the cultures to maintain their ability to secrete estrogen. Ledwitz-Rigby and Rigby (1981) have found that addition of charcoal-treated follicular fluid from large, but not small, follicles enhances granulosa cell estrogen secretion in the presence of androgen substrate. The theca and granulosa cells could have only a short life if they secrete elevated estrogen in vitro as well as in vivo. In the present studies follicles were obtained immediately prior to ovulation. If they were allowed to go 2 days in vivo then estrogen secretion would decline. Perhaps the culture behavior could potentially reflect the in vivo situation. Further studies will be required to answer this perplexing question. In the rat, estrogen secretion by the preovulatory follicle declines drastically at 2000 h on the day of proestrus. This decline in potential to secrete estrogen is also observed in cultured follicles (Szoltys, 1976; Stoklosowa and Szoltys, 1978).

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