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Cytochrome P_{450} Side-Chain Cleavage (P_{450scc}) in the Hen Ovary. I. Regulation of P_{450scc} Messenger RNA Levels and Steroidogenesis in Theca Cells of Developing Follicles[1]

K. I. KOWALSKI,[3] J. L. TILLY,[2,3] and A. L. JOHNSON

Department of Animal Sciences, Rutgers, the State University of New Jersey
New Brunswick, New Jersey 08903-0231

ABSTRACT

We have recently shown that granulosa cells from hen ovarian follicles, collected at a stage of development 2–3 wk prior to ovulation (e.g., 6–8 mm in diameter) are steroidogenically inactive. Therefore, the hypothesis tested in the present studies was that theca cells from follicles at this stage of development must contain sufficient levels of functional cytochrome P_{450} side-chain cleavage (P_{450scc}) enzyme to produce the progestin precursor required for the synthesis of androgens and estrogens. Northern blot analysis of total theca RNA collected from 6–8-mm follicles indicated the presence of a single P_{450scc} mRNA transcript of approximately 2 kb whose expression was increased following an 8-h preincubation with 200 ng/ml ovine LH (oLH) or 10 μM forskolin. Western blot analysis of crude mitochondrial protein revealed a band of immunoreactive P_{450scc} protein of approximately 53 kDa that was determined to be capable of converting 25-hydroxycholesterol to pregnenolone in a cell-free system.

In the second set of studies, conducted to examine the cellular regulation of steroidogenesis in isolated theca cells of 6–8-mm follicles, theca cells were found to produce measurable basal levels of cAMP, progesterone, androstenedione, and estradiol following a 3-h incubation of 5×10^5 cells. Furthermore, significant dose-dependent increases in steroidogenesis were observed in response to oLH (0.2–200 ng/ml), chicken FSH (cFSH; 20–200 ng/ml), cholera toxin (0.002–20 ng/ml), and 8-bromo-cAMP (0.1–3.33 mM). Phorbol 12-myristate 13-acetate (PMA; 10–167 nM) also stimulated dose-dependent increases in basal progesterone, androstenedione, and estradiol production. In addition, while PMA had no effect on oLH (200 ng/ml)-promoted cAMP accumulation, or on oLH (20 ng/ml)- or 8-bromo-cAMP (1 mM)-stimulated progesterone production, it attenuated oLH-induced and 8-bromo-cAMP-induced androstenedione and estradiol accumulation.

We conclude that theca cells from 6–8-mm follicles possess mRNA and immunoreactive protein coding for functional P_{450scc}. Furthermore, basal steroidogenesis is increased by both the protein kinase A and protein kinase C pathways, whereas evidence suggests that protein kinase C inhibits LH-induced androstenedione production at a site distal to cAMP and progesterone production, most likely by decreasing $C_{17,20}$-lyase activity.

INTRODUCTION

The follicular arrangement within the avian ovary provides a unique model to study changes associated with discrete stages of ovarian follicular development, maturation, and ovulation in a sexually mature animal without the need for surgical manipulation and/or hormonal priming [1, 2]. Additionally, the morphological arrangement of the preovulatory follicle allows the isolation of granulosa and theca cells with essentially no contamination of one cell type with the other. Therefore, we initiated studies using the advantages of the avian ovarian model to examine changes in steroidogenic capacity that occur during follicular development.

In the domestic laying hen, the source and regulation of steroidogenesis by cellular components of mature preovulatory follicles (15–40 mm in diameter; F_6–F_1) have been well documented. However, comparatively little is known concerning the production of steroids by granulosa and/or theca cells in less mature, growing follicles (less than 12–15 mm in diameter) in the hen ovary. Studies conducted by Robinson and Etches [3] and Robinson et al. [4] demonstrated that individual whole follicles (<1, 2–3, and 5–10 mm) incubated, in vitro, are capable of producing measurable basal levels of dehydroepiandrosterone (DHEA), androstenedione, and estradiol, but not progesterone. From these data, it was suggested that a functional Δ^5 (androstenedione produced from DHEA precursor), but not Δ^4 (androstenedione produced from progesterone precursor), pathway for steroid metabolism exists in less mature follicles. In a recent study by Lee [5], it was determined that 2–5-mm whole follicles (containing granulosa plus theca cells) produce testosterone and estradiol, both of which were increased by treatment with 8-bromo-cAMP. Furthermore, these follicles were shown to be capable of metabolizing either progesterone or DHEA precursor to testosterone, indicating that both the Δ^4 and Δ^5 pathways are functional.

Although these previous studies clearly show that whole follicles incubated in vitro produce androgens and estrogens, several fundamental problems may arise with the interpretation of data generated from whole-follicle studies. For instance, the contribution of different cellular components (e.g., granulosa versus theca) to overall follicular ste-

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[2]Correspondence: J.L. Tilly, Division of Reproductive Biology, Department of GYN/OB, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, CA 94035-5317. FAX: (415) 725-7102.
[3]Current address: Division of Reproductive Biology, Department of GYN/OB, Stanford University School of Medicine, Stanford, CA 94035-5317.

roidogenesis remains unclear. Additionally, the uptake of hormones and other treatments may be markedly impeded due the presence of the follicular wall. To alleviate these problems, we initiated studies to examine the steroidogenic properties of isolated granulosa and theca cells collected from these small developing follicles in the hen ovary.

We have recently reported that granulosa cells isolated from 6–8 mm yellow follicles are steroidogenically inactive under a variety of incubation conditions known to stimulate progesterone production by granulosa cells of mature preovulatory follicles [6, 7]. In light of these findings, it was concluded that theca cells of 6–8-mm follicles are the sole source of ovarian steroids in follicles at this stage and, presumably, at less mature stages of development. Since the biosynthesis of steroids from cholesterol requires the initial action of cytochrome P_{450} side-chain cleavage (P_{450scc}), and theca cells from 6–8-mm follicles must produce their own progestin precursor for further metabolism to androgens and estrogens, we hypothesized that this enzyme is present and functional in this follicle layer. Therefore, the present study was conducted to (a) determine the existence of cytochrome P_{450scc} in theca tissue collected from 6–8-mm follicles, and (b) evaluate the hormones and second messenger pathways that regulate steroid production by isolated theca cells of these follicles.

MATERIALS AND METHODS

Animals

In these studies, single-comb white Leghorn hens (Avian Services, Frenchtown, NJ), 26–44 wk of age and laying regular sequences of at least 5 eggs, were used. Birds were housed individually in layer batteries, had free access to feed (Agway Layer Mash, Agway, Inc., Bordentown, NJ) and water, and were exposed to a photoperiod of 15L:9D with lights-on at 2300 h. Individual laying cycles were monitored daily by the timing of oviposition and, on the day of an experiment, digital palpation of the reproductive tract.

Source and Preparation of Reagents

Stock solutions of all treatments used were diluted to the desired concentration for each experiment with sterile Medium 199-HEPES incubation medium, supplemented with 0.2% α-D(+) glucose, 0.2% BSA and 0.01% trypsin inhibitor [see 8]. Ovine LH (oLH; NIAMDD-oLH-23) and ovine FSH (oFSH; NIH-FSH-S17) were gifts from the National Hormone and Pituitary Program (Baltimore, MD). Recombinant human FSH (rhFSH [9]) was generously provided by Dr. Aaron J.W. Hsueh (Stanford University School of Medicine, Stanford, CA), and highly purified chicken FSH (cFSH-I-1; potency, 3.87 × NIH-FSH-S17) was kindly donated by the USDA (Beltsville, MD). Sources of all other reagents and preparations were as previously described [10, 11].

Tissue Collection

On the morning of an experiment, ovaries were collected from hens killed by cervical dislocation 17–16 h prior to ovulation of the largest preovulatory (F_1) follicle. Nonatretic [see ref. 12 for determinants of atresia], 6–8-mm yellow follicles were removed, cleaned of adhering connective tissue and/or follicles, and pooled in incubation medium. To obtain theca tissue from 6–8-mm follicles, each follicle was slit with a scapel blade, carefully inverted with fine-tipped forceps, and swirled in medium to remove yolk. The granulosa layer was then gently teased away from the theca tissue with fine-tipped forceps [7] and the isolated theca layers were thoroughly rinsed in 1.0% saline to remove any residual granulosa cells.

Experiment 1: Northern Blot Analysis of P_{450scc} mRNA in Theca Tissue Collected from 6–8-mm Yellow Follicles

Theca layers (25–30 layers from 6–8-mm follicles/treatment) were preincubated with vehicle, 200 ng/ml oLH, or 10 μM forskolin in 20 ml M199-HEPES incubation medium at 39°C for 7–8 h at 60 rpm in a Lab-Line Orbit Environ-Shaker (Fisher Scientific, Springfield, NJ). After incubation, the theca layers were pelleted by centrifugation (200 × *g*, 20 min, 4°C) and the supernatant was discarded. Total RNA was then prepared from the tissue pellets essentially as described by Chomczynski and Sacchi [13]. The RNA pellets were resuspended in 20–50 μl of single-strength TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 7.6) and quantitated by reading the absorbance at 260 nm. Aliquots of the RNA were stored at −70°C until used for Northern blot analysis.

For Northern blot analysis, RNA samples (25 μg) were denatured for 15 min at 60°C in 50% deionized formamide, 6% formaldehyde, single-strength MOPS buffer (0.02 M morpholinopropane-sulfonic acid, 5 mM sodium acetate, 1 mM EDTA; pH 7.0), and then quickly chilled on ice. Samples were separated by electrophoresis through 1.0% agarose gels containing 6% formaldehyde using a BRL Horizontal Gel System (Model H4; Bethesda Research Labs, Gaithersburg, MD). After electrophoresis, gels were stained with ethidium bromide to allow visualization of RNA migration and integrity with a UV-transilluminator (Model TM-36; UVP, Inc., San Gabriel, CA). The separated RNA fragments were then transferred to Gene-Screen membranes (DuPont/NEN, Boston, MA) using either the Millipore MilliBlot-V Transfer System (Millipore Corp., Bedford, MA) or overnight capillary transfer with 25-mM sodium phosphate mono/dibasic (pH 6.5) as the transfer solution. The transferred RNA was then covalently cross-linked to the membranes with a Stratalinker UV Crosslinker (Stratagene, La Jolla, CA) and photographed under UV light.

The membranes were prehybridized at 42°C for 2–4 h in a shaking water bath in a sealed bag containing 10 ml of prehybridization solution (50% deionized formamide, 0.2% BSA, 0.2% polyvinyl pyrrolidone, 0.2% ficoll, 50 mM

Tris-HCl [pH 7.5], 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate, 1 M sodium chloride, and 100 μg/ml denatured, sheared, salmon sperm DNA) per membrane. After addition of 3 ml hybridization solution (prehybridization solution minus dextran sulfate, sodium chloride, and nonhomologous DNA) to each bag containing the prehybridization solution and the membrane, the membranes were hybridized for 18–22 h at 42°C in a shaking water bath to a nick-translated (according to specifications of the BRL nick-translation system), 1.2-kb cDNA probe for rat P_{450scc} (generously provided by Dr. JoAnne S. Richards, Baylor College of Medicine, Houston, TX [see 14]). Labeled probe was purified from unincorporated nucleotides using Nuctrap Push Columns (Stratagene), and subsequently used at a specific activity of 2–3 × 10^6 cpm/ml of hybridization solution. After hybridization, membranes were washed in double-strength SSC (0.3 M sodium chloride, 0.03 sodium citrate)/1.0% SDS for 10 min at room temperature, in double-strength SSC/1.0% SDS for 20 min at 42°C, and in 0.5-strength SSC/0.1% SDS for 10–20 min at 42–50°C until background counts were less than 1–2 cps. Membranes were then exposed to Kodak X-Omat AR diagnostic film (Eastman Kodak, Rochester, NY) with intensifying screens for 96–120 h at −70°C and the film was subsequently developed using conventional procedures.

To serve as a negative control, 25 μg of total RNA, prepared from chicken spleen as described above, was subjected to Northern analysis with the ovarian RNA samples. As a positive control, total RNA (1–2 μg) collected from FSH-stimulated rat granulosa cells (prepared in the laboratory of Dr. Aaron J.W. Hsueh) was simultaneously analyzed by Northern blot for the presence of P_{450scc} mRNA. Granulosa cells were isolated from diethylstilbestrol (DES)-primed, immature Sprague-Dawley rats (Johnson Laboratories, Bridgeview, IL) as previously described [15], and cultured for 48 h at 37°C under 5%CO_2:95% air in McCoy's 5a medium (7 × 10^5 cells/0.5 ml) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 100 nM androstenedione, and 100 ng/ml oFSH. After the 48-h culture period, medium was discarded and total RNA was extracted from the cells by the Nonidet P-40 method [16].

Experiment 2: Western Blot Analysis of Cytochrome P_{450scc} Immunoreactive Protein in Theca Tissue of 6–8-mm Yellow Follicles

A rabbit polyclonal antibody prepared against bovine adrenal mitochondrial P_{450scc} was obtained from OXYgene (Dallas, TX), and a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G was purchased from Organon Teknika Corp. (West Chester, PA). Crude mitochondrial protein (50 μg protein/lane) or mitochondrial protein immunoprecipitated with a rabbit polyclonal antibody to rat adrenal P_{450scc} (Dr. J. Orly, Hebrew University of Jerusalam, Israel) and Protein A-Sepharose 6MB (Pharmacia LKB Biotechnology, Piscataway, NJ) were prepared as described [17, 18] and resolved by SDS-PAGE (9% running gel, 6% stacking gel). The proteins were transferred to Immobilon membranes (Millipore Corp.) and evaluated by immunoblot analysis, essentially as described [18]. The estimated molecular weight of the avian P_{450scc} protein was confirmed by comparison of migration distance versus a P_{450scc} standard prepared from bovine adrenal mitochondria (OXYgene).

Experiment 3: Enzyme Kinetic Analysis of Cytochrome P_{450scc} Activity in Theca Tissue of 6–8-mm Yellow Follicles

Enzyme activity was measured from crude mitochondrial protein diluted in incubation buffer (25 mM Tris, 200 mM sucrose, 5 mM magnesium chloride, 20 mM potassium chloride, 10 mM sodium phosphate, 0.2 mM EDTA; pH 7.4) to a concentration of 0.5 μg protein/μl buffer. One-hundred-fifty micrograms of protein was routinely added to a reaction mixture containing 10 mM sodium isocitrate, 0.5 mg fatty acid-free BSA, and 0, 0.125, 0.25, 0.5, 1, 2, 4, or 8 μM 25-hydroxycholesterol in a final volume of 0.5 ml, and incubated for 30 min at 39°C. The reaction was stopped by boiling samples for 3 min, and the amount of pregnenolone produced was determined by RIA. Lineweaver-Burk plots were established from net pregnenolone synthesis, and the kinetic parameters, K_m and V_{max}, were calculated by regression analysis. The amount of mitochondrial protein, the doses of 25-hydroxycholesterol, and the incubation time were optimized on the basis of preliminary studies with granulosa tissues from F_1 and 6–8-mm follicles (data not shown).

Experiment 4: Regulation of cAMP and Steroid Production during Short-Term Incubation of Theca Cells Isolated from 6–8-mm Yellow Follicles

Theca cells were isolated from 6–8-mm yellow follicles by Percoll density centrifugation, essentially as described [10]. Following the isolation procedure, which yielded approximately 2–3 × 10^6 viable theca cells per follicle (as determined by trypan blue dye exclusion and a hemacytometer), cells were diluted with sterile incubation medium to a final concentration of 2.5 × 10^6 viable theca cells/ml medium. Aliquots (5 × 10^5 cells) of the theca cell suspension were pipetted into 12 × 75-mm polypropylene tubes, and incubation and/or treatments were added to obtain a final volume of 0.5 ml. Cells were then incubated for 3 h at 39°C under ambient air. Immediately following incubation, cells plus media were frozen at −70°C until assayed for cAMP or steroid concentrations by RIA.

Cyclic AMP production was measured by specific RIA as described [19]. Pregnenolone concentrations were evaluated by RIA on unextracted samples using a highly specific pregnenolone antiserum (#S-32#7; Radioassay Systems Laboratories, Carson, CA) and 7-^{3}H(*N*)-pregnenolone (spe-

cific activity of 22 Ci/mmol; DuPont) as previously described [20]. Levels of progesterone were determined by RIA using a highly specific antiserum for progesterone (R12-P2-37; Radioassay Systems Laboratories) following extraction of samples with petroleum ether [8, 18]. Androstenedione concentrations were determined without extraction by specific RIA [8]. Dehydroepiandrosterone was measured without extraction by RIA using a specific DHEA antiserum (R1#46; Radioassay Systems Laboratories; cross-reactivities of 0.32% for androstenedione, 0.02% for pregnenolone and androsterone, and <0.01% for all other steroids) and 1,2,6,7-^{3}H(*N*)-dehydroepiandrosterone (specific activity of 100 Ci/mmol; DuPont/New England Nuclear, Boston, MA) as the labeled ligand. Estradiol concentrations were determined without extraction by RIA using an E_2-antibody #244 (generously provided by Dr. G. Niswender, Colorado State University; 21) and 2,4,6,7-^{3}H(*N*)-estradiol (specific activity of 107 Ci/mmol; DuPont/New England Nuclear) as the labeled ligand. Assay sensitivity for the cAMP RIA was 5 fmol/tube, whereas sensitivity of the steroid RIAs ranged from 50 pg/tube (pregnenolone) to 5–10 pg/tube (all other steroids assayed).

Statistics

Experiments for cAMP and steroid analyses were repeated in a total of three or four separate experiments (with

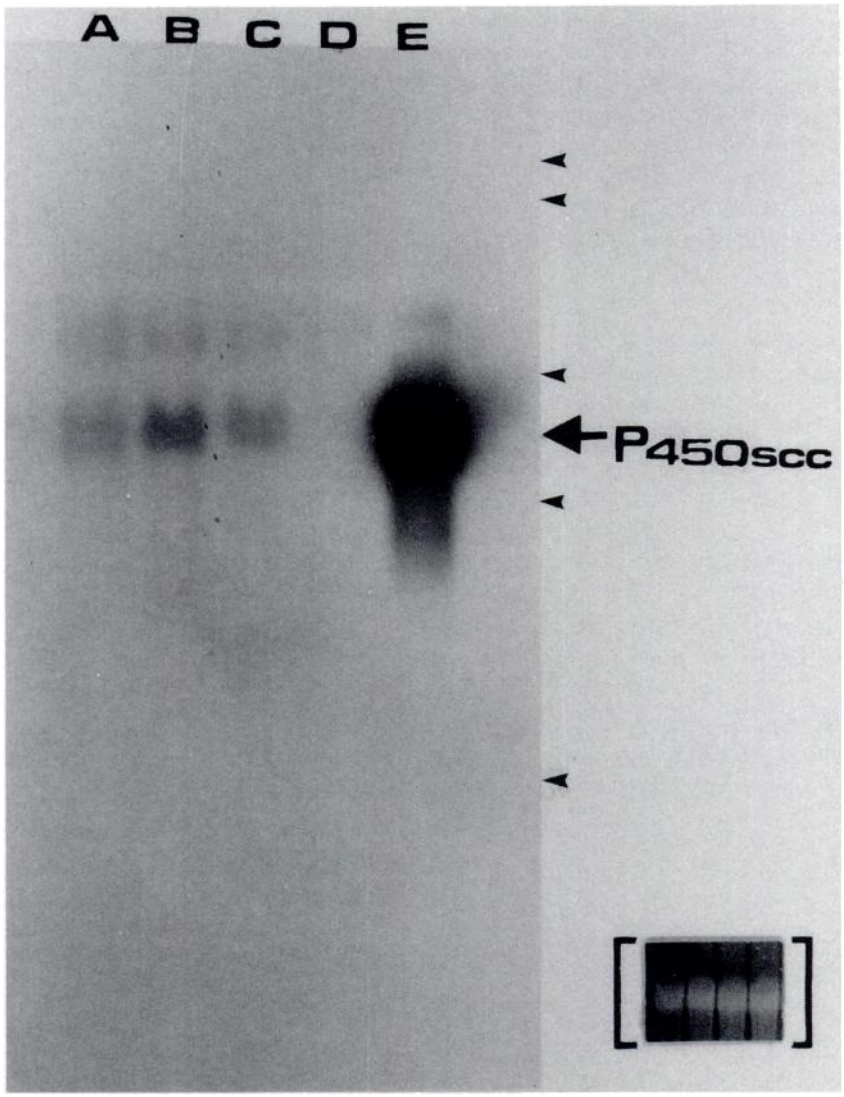

FIG. 1. Northern blot analysis of cytochrome P_{450scc} mRNA levels in theca tissue collected from 6–8-mm yellow follicles and preincubated for 8 h with vehicle (lane A), 10 μM forskolin (lane B), or 100 ng/ml oLH (lane C). No specific hybridization was observed to an equivalent amount of RNA (25 μg/lane) prepared from hen spleen (lane D), whereas lane E depicts the hybridization of the rat cDNA probe to 1–2 μg of RNA prepared from FSH-stimulated rat granulosa cells. Molecular size markers are shown as the small arrows (top to bottom: 9.4, 7.4, 2.4, 1.4 and 0.24 kb) and the ethidium bromide-stained 28S ribosomal RNA bands from lanes A–D are presented in the lower insert.

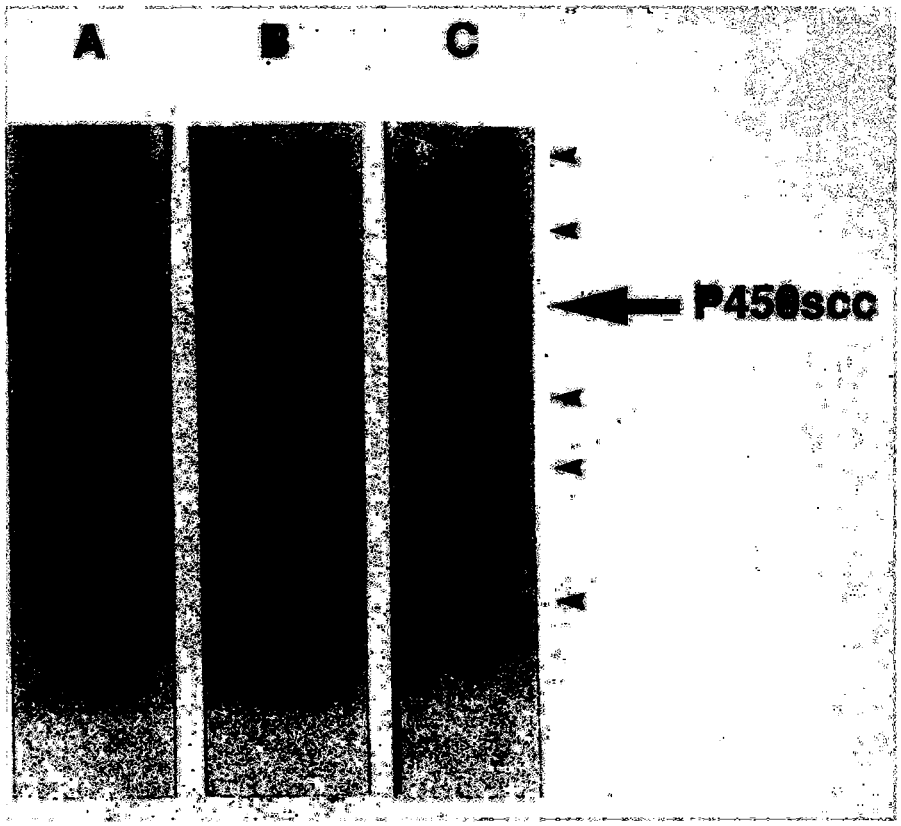

FIG. 2. Western blot analysis of immunoreactive P_{450scc} protein following resolution of crude mitochondrial protein prepared from theca tissue of 6–8-mm yellow follicles by SDS-PAGE. Lane C represents 50 μg of crude mitochondrial protein, whereas lane B is the same protein immunoprecipitated with a different P_{450scc} antibody prior to the immunoblot analysis. Molecular mass standards are shown by the small arrows (top to bottom: 97, 68, 43, 24, and 18 kDa) and the migration of a bovine P_{450scc} standard (53 kDa; lane A) is indicated.

TABLE 1. Effects of chicken (c) and recombinant human (rh) FSH on the accumulation of progesterone (P; pg/5 × 10^5 cells), androstenedione (A; pg/5 × 10^5 cells), and estradiol (E; pg/5 × 10^5 cells) from theca cells prepared from 6–8-mm follicles and incubated for 3 h.*

Treatment	P	A	E
cFSH (ng/ml)			
0	54 ± 4	153 ± 10	212 ± 10
20	52 ± 10	473 ± 36	225 ± 29
100	148 ± 30	2244 ± 506*	707 ± 134
200	220 ± 30*	4776 ± 205*	1047 ± 141*
rhFSH (IU/ml)			
0	35 ± 22	200 ± 17	194 ± 21
0.40	62 ± 13	226 ± 26	213 ± 9
1.00	83 ± 20	337 ± 63	277 ± 11*
2.00	146 ± 20*	680 ± 40*	419 ± 2*

*Data are the mean ± SEM of results from three replicate experiments.
*$p < 0.05$ versus respective control.

three replicate tubes per experiment), and data are presented as the mean ± SEM of results from the replicate experiments. Data were analyzed by a one-way analysis of variance, and significant interactions ($p < 0.05$) were determined by the Newman-Keuls multiple range test. Regression analysis was conducted on the combined data from dose-response curves where appropriate using the HSD Statistical Analysis Program (Human Systems Dynamics, Northridge, CA).

Enzyme kinetic studies were replicated a total of three times (results presented are the mean ± SEM of the replicate studies); values for K_m and V_{max} were calculated from combined data using Lineweaver-Burke plot analyses. Northern and Western blot analyses were repeated a total of three times with similar results, and a representative blot for each is presented.

RESULTS

In the first experiment, conducted to evaluate the presence and regulation of P_{450scc} mRNA in developing theca cells, Northern blot analysis indicated the existence of a single molecular species of mRNA encoding for P_{450scc} with an estimated molecular size of 2 kb in 25 μg of total RNA collected from theca tissue of 6–8-mm follicles (Fig. 1). Furthermore, preincubation of theca tissue with oLH (200 ng/ml) or forskolin (10 μM), increased the signal intensity of the band as determined by densitometric scanning (1.6- and

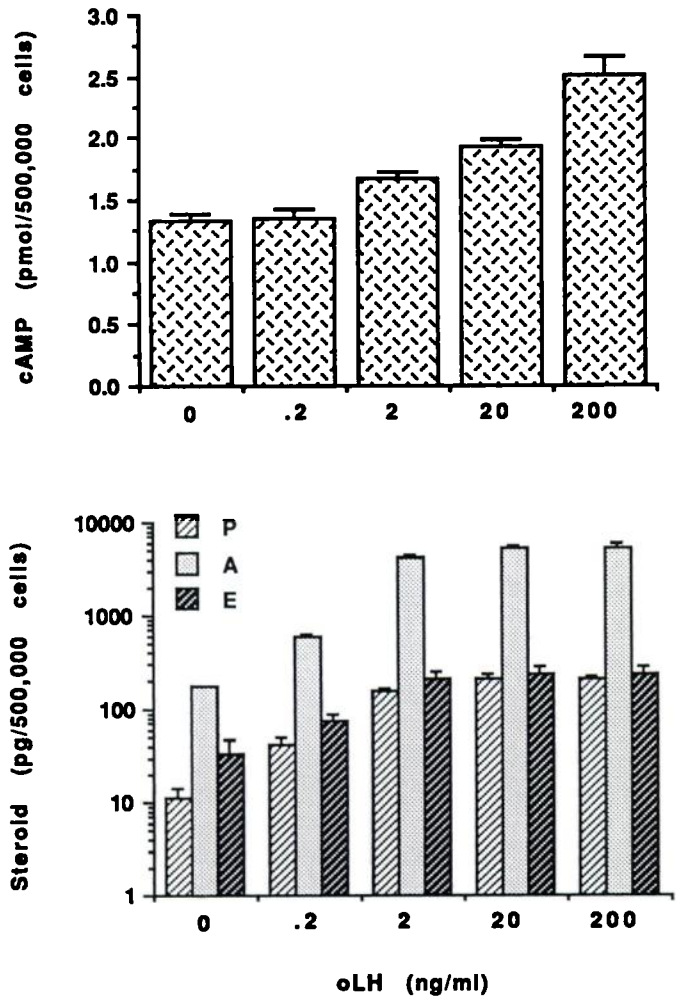

FIG. 3. Cyclic AMP (upper panel) and steroid (lower panel, progesterone [P], androstenedione [A], and estradiol [E]) production from theca cells of 6–8-mm follicles following a 3-h incubation with and without oLH. Data are the mean ± SEM of results from 3 or 4 replicate incubations (see text for discussion of significant interactions). Note log scale of abscissa for presentation of steroid data in the lower panel.

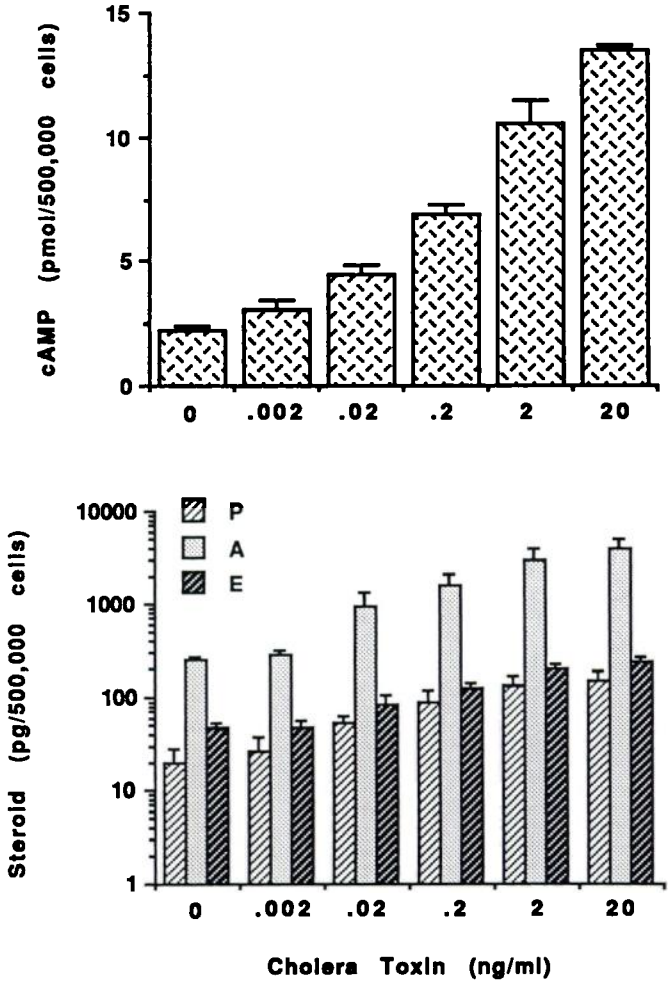

FIG. 4. Cyclic AMP (upper panel) and steroid (lower panel) response of theca cells of 6–8-mm follicles to cholera toxin following a 3-h incubation. Data are the mean ± SEM of results from three or four replicate incubations (note log scale for abscissa in lower panel). Abbreviations used: P, progesterone; A, androstenedione; E, estradiol.

2.0-fold, respectively, versus control), suggesting an increase in the amount of P_{450scc} mRNA (control, lane A; forskolin, lane B; oLH, lane C; Fig. 1). No specific hybridization was observed to total RNA prepared from hen spleen (lane D; Fig. 1), whereas a strong band of hybridization was observed at 2 kb for total RNA prepared from oFSH-stimulated rat granulosa cells (lane E; Fig. 1). In all cases (lanes A–E), low nonspecific hybridization was observed to 28S ribosomal RNA, likely due to the low stringency conditions required for maintenance of hybridization between the chicken P_{450scc} transcript and the rat cDNA probe.

In Experiment 2, Western blot analysis of crude mitochondrial protein (50 μg) prepared from theca tissues of 6–8-mm follicles (lane C; Fig. 2) revealed the presence of immunoreactive P_{450scc} protein, which migrated to an estimated molecular mass of 53 kDa (comparable to that observed for a bovine P_{450scc} standard, lane A; Fig. 2). Furthermore, immunoprecipitation of the hen mitochondrial protein (50 μg) with a different antibody directed against P_{450scc} (prepared from rat adrenal cortex mitochondria) and subsequent Western blot analysis with the bovine P_{450scc} antibody clearly demonstrated specific binding of the bovine antibody to hen theca protein at 53 kDa (lane B; Fig. 2). It is noted that while a second band of immunoreactive protein (of approximately 68 kDa) was apparent in both chicken follicle and bovine adrenal mitochondrial protein, the relative amount of this immunoreactive protein decreased following immunoprecipitation (lane B versus lane C). To determine if the immunoreactive mitochondrial protein contained active P_{450scc} enzyme (Exp. 3), enzyme kinetic analysis of 150 μg of crude mitochondrial protein prepared from theca tissue of 6–8-mm follicles indicated that the protein preparation converted exogenous 25-hydroxycholesterol to pregnenolone with a calculated K_m of 0.807 +/− 0.063 μM and V_{max} of 3.51 ± 0.01 pmol/mg protein/

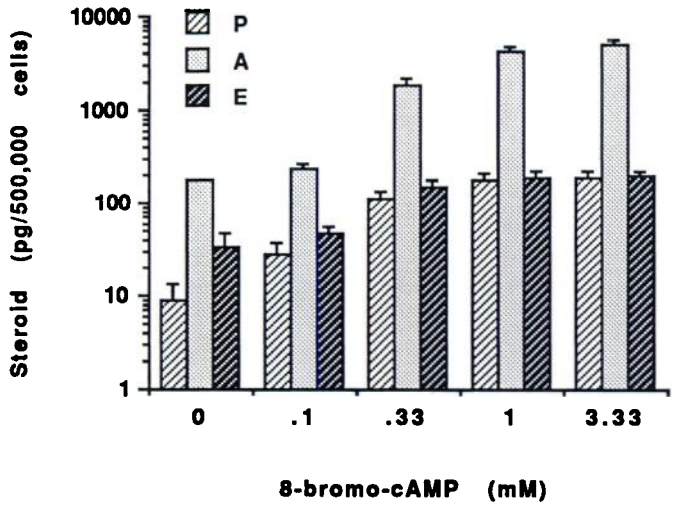

FIG. 5. Dose-dependent stimulation of progesterone (P), androstenedione (A), and estradiol (E) production by theca cells of 6–8-mm follicles in response to 8-bromo-cAMP following a 3-h incubation. Data are the mean ± SEM of results from three or four replicate incubations (note log scale for abscissa).

min (mean ± SEM of results from three replicate experiments).

To characterize the hormonal and second messenger regulation of steroidogenesis in theca cells collected from 6–8-mm follicles, dispersed cells were incubated for 3 h in the absence and presence of treatments, after which cAMP and steroid levels were evaluated (Exp. 4). Theca cells treated with oLH (0.2–200 ng/ml) increased cAMP levels in a dose-dependent manner ($r = 0.865, p < 0.001$), with significant increases observed at 2, 20, and 200 ng/ml of oLH ($p < 0.05$ versus basal; Fig. 3, upper panel). LH also stimulated dose-dependent increases in progesterone ($r = 0.533, p = 0.014$), androstenedione ($r = 0.553, p = 0.030$), and estradiol ($r = 0.414, p = 0.066$) production. Significant increases in all steroids were observed at 2, 20, and 200 ng/ml of oLH ($p < 0.05$ versus basal; Fig. 3, lower panel), with the highest dose of oLH increasing progesterone, androstenedione, and estradiol levels by 19-, 31.4-, and 7-fold, respectively.

Treatment of theca cells with increasing concentrations of cFSH or rhFSH did not significantly affect cAMP accumulation over the 3-h incubation period (2.24 ± 0.62 and 1.71 ± 0.53 pmol/5 × 10^5 cells for 200 ng/ml cFSH and 2 IU/ml rhFSH, respectively, versus basal levels of 1.73 ± 0.41 pmol/5 × 10^5 cells; $p > 0.10$, mean ± SEM from three replicate experiments). In contrast, incubation of theca cells from 6–8-mm follicles with cFSH caused a significant dose-dependent increase in the levels of progesterone ($r = 0.574$, $p < 0.05$), androstenedione ($r = 0.942$, $p < 0.001$), and estradiol ($r = 0.919$, $p < 0.001$) (Table 1). Additionally, 2 IU/ml rhFSH was found to increase progesterone (4-fold; $p < 0.05$), androstenedione (3.4-fold; $p < 0.05$), and estradiol (2.1-fold; $p < 0.05$) accumulation compared to controls (Table 1).

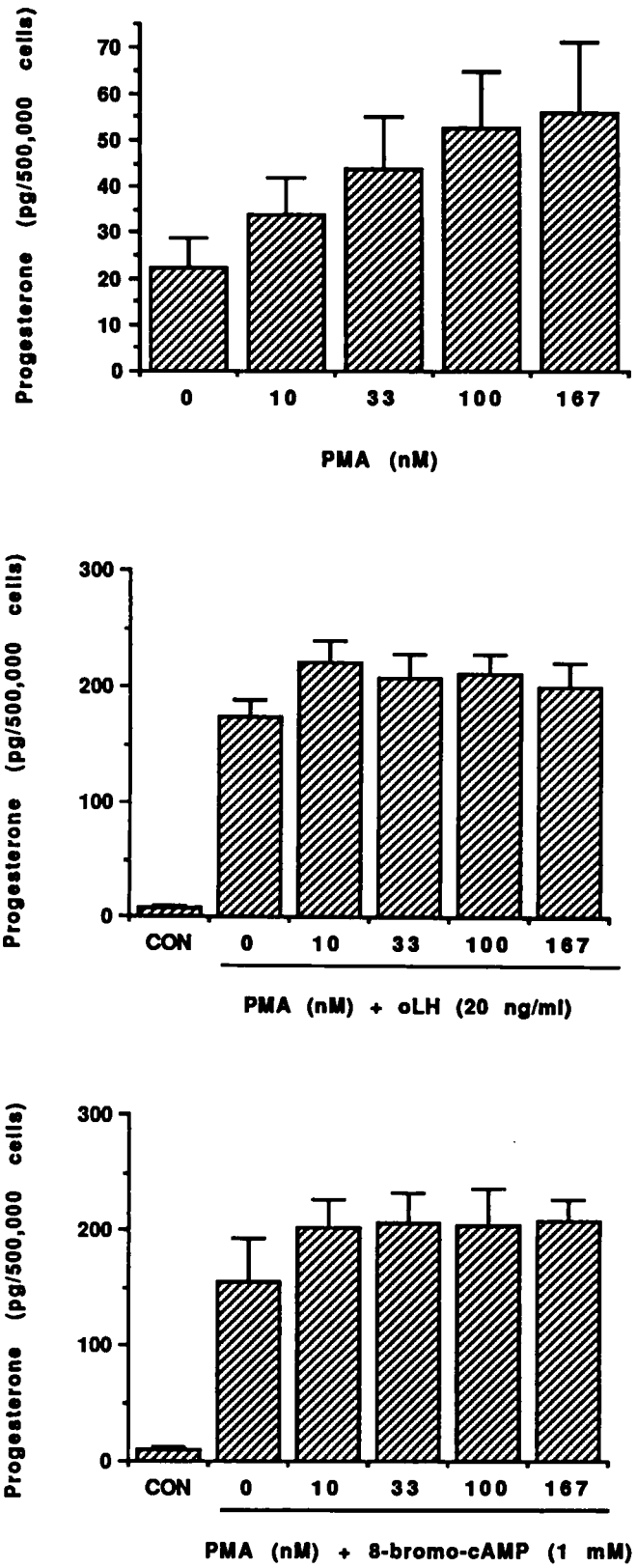

FIG. 6. Effects of PMA ± oLH or 8-bromo-cAMP on progesterone production from theca cells of 6–8-mm follicles following a 3-h incubation. Data are expressed as the mean ± SEM of results from three or four replicate incubations (see Results for discussion of statistical analysis). CON = control.

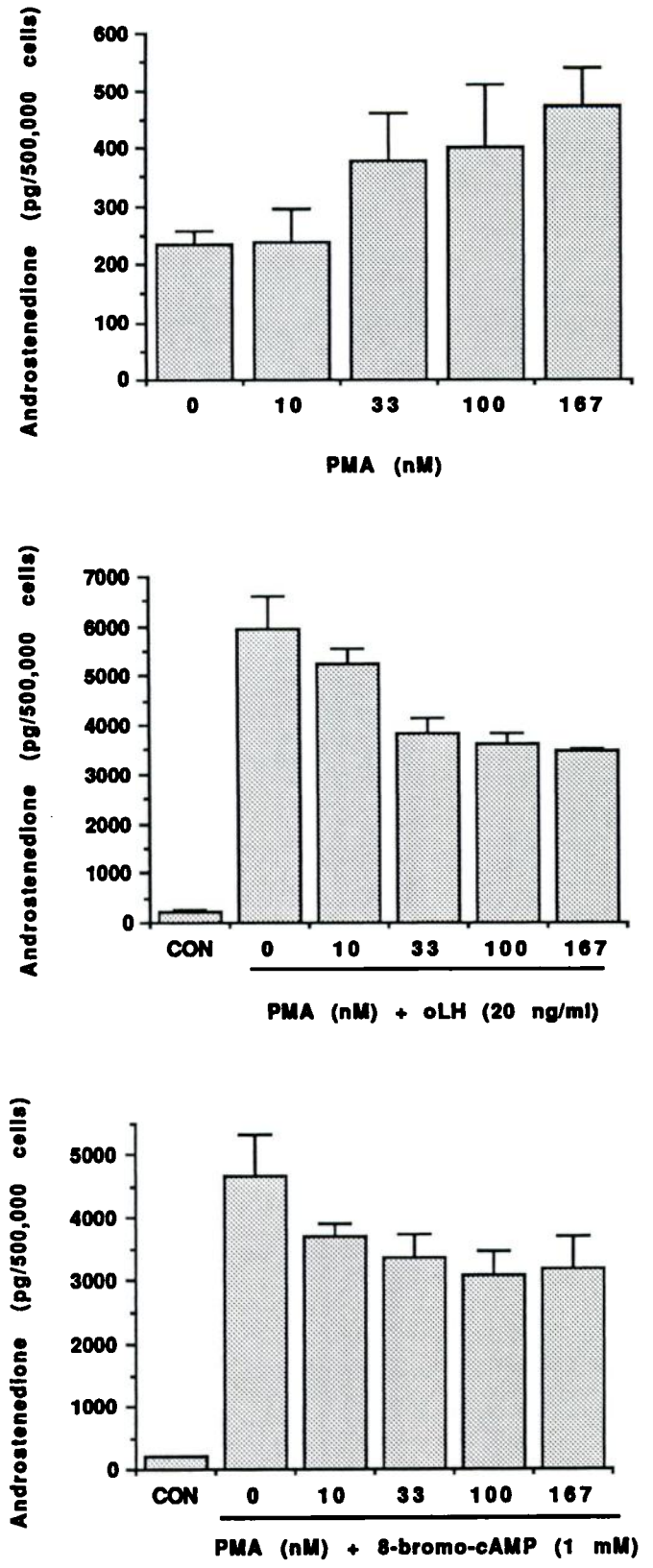

FIG. 7. Androstenedione production from theca cells of 6–8-mm follicles following a 3-h incubation with PMA ± oLH or 8-bromo-cAMP. Data are the mean ± SEM of results from three or four replicate incubations. CON = control.

Cholera toxin caused a dose-dependent increase in cAMP production ($r = 0.782, p = 0.004$), with significant stimulatory effects first observed at 0.02 ng/ml ($p < 0.05$ versus basal) and a maximum 6.2-fold increase observed at 20 ng/ml (Fig. 4, upper panel). Cholera toxin stimulated theca cells to produce significant levels of progesterone (at doses of 2 and 20 ng/ml of cholera toxin), androstenedione (at doses of 0.2, 2, and 20 ng/ml), and estradiol (at a dose of 20 ng/ml) over basal levels ($p < 0.05$; Fig. 4, lower panel). Progesterone, androstenedione, and estradiol production were increased in a dose-dependent manner ($r = 0.574, p = 0.012$; $r = 0.663, p = 0.002$; and $r = 0.691, p < 0.001$, respectively), with the highest concentration of cholera toxin (20 ng/ml) increasing progesterone, androstenedione, and estradiol levels by 7.5-, 15.9-, and 5-fold, respectively, over controls (Fig. 4, lower panel). Moreover, forskolin (0.1, 1, and 10 μM) was also found to stimulate cAMP, progesterone, androstenedione, and estradiol production in a dose-dependent fashion (data not shown).

Theca cells responded to 8-bromo-cAMP with significant dose-dependent increases in progesterone ($r = 0.717, p = 0.002$), androstenedione ($r = 0.823, p < 0.001$), and estradiol ($r = 0.605, p = 0.004$) output. Significant effects on the production of all steroids were observed at 0.33, 1, and 3.33 mM 8-bromo-cAMP ($p < 0.05$ versus basal) (Fig. 5).The highest concentration of the cAMP analog (3.33 mM) increased accumulation of progesterone by 21.4-fold, androstenedione by 28.8-fold, and estradiol by 5.9-fold, over basal levels.

Basal levels of cAMP (1.34 ± 0.05 pmol/5 × 10^5 cells) were not significantly altered by the presence of 10–167 nM PMA (1.49 ± 0.18 pmol/5 × 10^5 cells for 167 nM PMA; $p > 0.10$ for all doses of PMA versus control); however, PMA increased basal steroid accumulation in a dose-dependent manner (progesterone: $r = 0.550, p = 0.032$, Fig. 6; androstenedione: $r = 0.653, p = 0.008$, Fig. 7; estradiol: $r = 0.689, p = 0.004$, Fig. 8). Treatment of theca cells with 200 ng/ml oLH caused a significant 2-fold increase in cAMP formation (2.51 ± 0.16 pmol/5 × 10^5 cells) and the inclusion of PMA did not alter the response of theca cells to oLH (2.65 ± 0.08 pmol/5 × 10^5 cells for 167 nM PMA plus 200 ng/ml oLH; $p > 0.10$). Incubation of theca cells with 20 ng/ml oLH or 1 mM 8-bromo-cAMP stimulated progesterone production by 23.7- and 17.2-fold, respectively, over basal (Fig. 6, center and lower panels, respectively), whereas coincubation of these cells with PMA (10–167 nM) did not significantly alter progesterone output induced by either oLH or 8-bromo-cAMP ($p > 0.05$ versus either agonist alone; Fig. 6). The presence of oLH and 8-bromo-cAMP increased theca androstenedione levels by 26.7- and 22.9-fold, respectively (Fig. 7, center and lower panels); however, increasing concentrations of PMA attenuated oLH-promoted androstenedione production in a dose-dependent manner ($r = -0.677, p = 0.001$; $p < 0.05$ for 33, 100, and 167 nM PMA versus oLH alone). Similar inhibitory effects of PMA

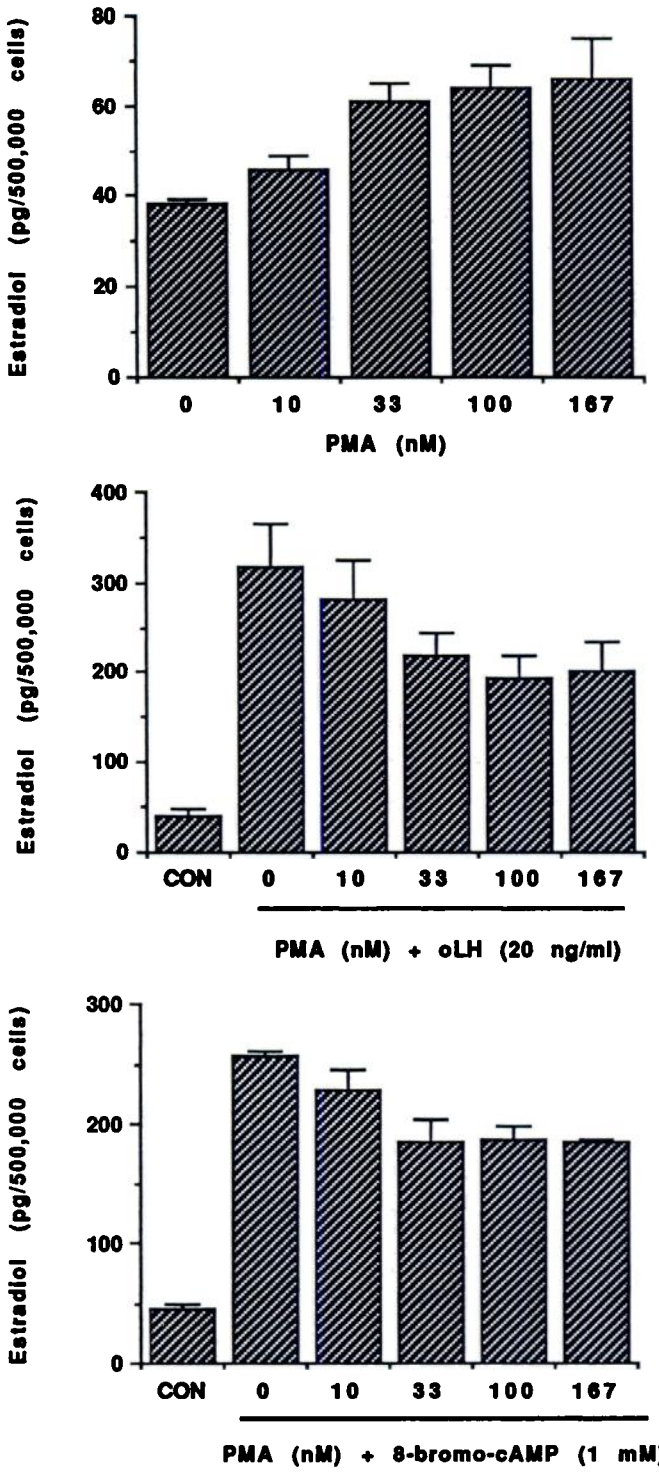

FIG. 8. Effects of PMA ± oLH or 8-bromo-cAMP on estradiol production from theca cells of 6–8-mm follicles following a 3-h incubation. Data are the mean ± SEM of results from three or four replicate incubations. CON = control.

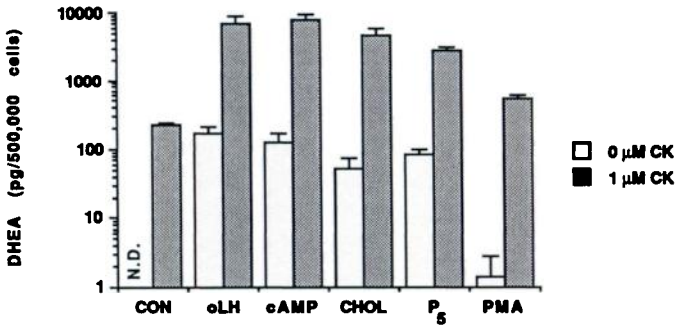

FIG. 9. DHEA production by theca cells of 6–8-mm follicles following a 3-h incubation without and with oLH (200 ng/ml), 8-bromo-cAMP (3.33 mM), 25-hydroxycholesterol (CHOL; 5 μg/ml), pregnenolone (P_5; 40 ng/ml), or PMA (167 nM) in the absence and presence of the 3β-hydroxysteroid dehydrogenase inhibitor, cyanoketone (CK). Data are the mean ± SEM of results from three replicate incubations. N.D. = nondetectable.

were observed for 8-bromo-cAMP-stimulated androstenedione production ($r = -0.474, p = 0.071$), with the three highest doses of PMA reducing the response to the cAMP analog by at least 30% (Fig. 7). Estradiol levels in theca cell incubations were increased 7.7- and 5.7-fold by oLH and 8-bromo-cAMP, respectively (Fig. 8, center and lower panels). Similar to the actions of PMA on agonist-stimulated androstenedione levels, 10–167 nM PMA caused a dose-dependent suppression of oLH-promoted estradiol production ($r = -0.485, p = 0.028$) and estradiol output stimulated by the presence of 8-bromo-cAMP ($r = -0.616, p = 0.013$; $p < 0.05$ for 33, 100, and 167 nM PMA versus 8-bromo-cAMP alone; Fig. 8).

In the absence of the 3β-hydroxysteroid dehydrogenase inhibitor, cyanoketone [20], DHEA levels in control and PMA-treated theca cells were not detectable; however, incubation of theca cells with oLH, 8-bromo-cAMP, 25-hydroxycholesterol or pregnenolone resulted in markedly increased DHEA concentrations (Fig. 9). Inclusion of 1 μM cyanoketone in the incubation medium increased basal levels of DHEA to 230 ± 15 pg/5 × 10^5 cells, and enhanced the levels of DHEA measured under all incubation conditions by at least 30-fold ($p < 0.001$ for each treatment versus the absence of cyanoketone).

DISCUSSION

We have recently determined that granulosa cells isolated from 6–8-mm, nonatretic yellow follicles (a stage of rapid follicular growth 2–3 wk prior to ovulation) contain extremely low levels of progesterone, and these levels are not increased following a short-term incubation with LH, FSH, 8-bromo-cAMP, or 25-hydroxycholesterol [6, 7]. However, these cells do effectively convert exogenous pregnenolone to progesterone, suggesting a lack of functional P_{450scc}, but not 3β-hydroxysteroid dehydrogenase, enzyme. Additional experiments indicated that the low levels of progesterone present within granulosa tissue of 6–8-mm follicles are the result of steroid diffusion from the adjacent, steroidogenically active theca layer, since acutely dispersed granulosa cells contain the same level of progesterone as cells incubated for 3 h [7]. From these initial findings, we

concluded that theca tissue from 6–8-mm follicles (and presumably smaller, less mature follicles in the hen ovary) must be the sole source of ovarian steroids until such a time that the granulosa layer becomes steroidogenically competent, providing progesterone precursor to the theca layer for further metabolism to androgens and estrogens. Therefore, the present study was conducted to (a) determine the existence of P_{450scc} mRNA, protein, and activity in theca cells of 6–8-mm follicles, since these cells apparently must synthesize their own progestin precursor needed for the production of androgens and estrogens; and (b) evaluate the cellular mechanisms that regulate steroidogenesis in theca cells of these growing follicles.

In the first experiment, Northern blot analysis using a rat P_{450scc} cDNA probe clearly demonstrated the existence of P_{450scc} mRNA within theca tissue of 6–8-mm yellow follicles. Size estimates indicated that the single mRNA transcript in hen theca tissues encoding P_{450scc} is approximately 2 kb, a size comparable to that reported for the P_{450scc} mRNA transcript of rat ([14, 22–24]; Fig. 1, lane E) and porcine [25, 26] ovarian cells. Of further interest was the finding that pretreatment of theca tissue from developing, 6–8-mm follicles with LH or forskolin stimulated an increase in the level of expression of the P_{450scc} mRNA transcript. These findings are consistent with those previously reported by Goldring et al. [14], which demonstrated an increase in P_{450scc} mRNA expression in rat granulosa cells cultured with forskolin, and suggested that a cAMP-responsive region is present within the chicken P_{450scc} gene similar to that reported for mammalian species [for review, see 27–29]. Additionally, the ability of oLH to enhance P_{450scc} mRNA levels is consistent with its ability to enhance cAMP production (Fig. 3). Aside from a companion paper demonstrating the existence of P_{450scc} mRNA in granulosa tissues of developing and preovulatory hen follicles [30], these findings are the first reported for the identification of P_{450scc} mRNA and its responsiveness to cAMP in theca tissues of avian species. Further studies are in progress to characterize the hormonal and intracellular mechanisms that modulate the level of P_{450scc} mRNA in isolated theca cells of developing and mature ovarian follicles.

The ability of theca tissue to translate the P_{450scc} mRNA to a fully active protein was evaluated in the next series of experiments by confirming the existence of immunoreactive and functional P_{450scc} protein in mitochondria of 6–8-mm follicle theca cells through protein blot and enzyme kinetic analyses, respectively. The single immunoreactive band that was more clearly observed in mitochondrial protein immunoprecipitated with a different P_{450scc} antibody prior to the Western blot migrated to an apparent molecular mass of 53 kDa, a size consistent with that reported for P_{450scc} in rat, bovine, and hen steroidogenic tissues [17, 22, 23, 31, 32]. Furthermore, the K_m reported herein for P_{450scc} activity in theca from 6–8-mm follicles (0.807 ± 0.063 μM) is not significantly different from that found in theca cells from the second largest preovulatory (F_2) follicle (0.890 ± 0.131 μM), whereas the V_{max} (3.51 ± 0.01 pmol/mg protein/min) is approximately 62% of that found for F_2 theca (5.64 ± 0.44 pmol/mg protein/min) (Kowalski KI, Tilly JL, and Johnson AL, unpublished data). Although we [30] and others [17] have previously identified immunoreactive P_{450scc} protein in hen granulosa cells, this is the first report demonstrating the presence and enzyme kinetic parameters of P_{450scc} protein in theca tissues of hen ovarian follicles. These data taken collectively indicate that theca tissue collected from developing, 6–8-mm follicles possess P_{450scc} mRNA, and immunoreactive and functional P_{450scc} protein, which is used to produce the progestin precursor needed for the synthesis of androgens and estrogens.

Considering the apparent importance of theca tissue in the maintenance of steroid hormone production in developing follicles, the remaining studies were conducted to examine the hormonal and cellular mechanisms that regulate steroidogenesis in 6–8-mm follicle theca cells. Similar to findings reported for steroid production by F_2 theca cells [10], LH was found to stimulate significant increases in progesterone, androstenedione, and estradiol accumulation, with its actions apparently mediated, at least in part, through elevation of intracellular cAMP levels. However, dramatic rises in steroid levels were apparent at low doses of LH that did not measurably alter cAMP accumulation, suggesting that LH may act via additional intracellular pathways aside from the cAMP-protein kinase A system. One possibility is the calcium/calmodulin pathway, as this signalling system has been shown to be prerequisite for gonadotropin-stimulated steroidogenesis in theca tissues of mature preovulatory follicles [33, 34].

We have previously reported that granulosa cells isolated from 6–8-mm follicles in the hen ovary respond to FSH with increased cAMP formation, and that these actions are not replicated by treatment of the cells with LH [7]. Since these data indicate a specific action exists for FSH in granulosa tissue of less developed follicles, we wished to evaluate a possible role of FSH in the regulation of cAMP and steroid production by theca cells of these follicles. Treatment with cFSH caused significant increases in steroid output, without measurably altering cAMP levels. The ability of cFSH to increase steroidogenesis in the absence of detectable changes in cAMP levels was similar to that observed for the lowest dose of oLH, and may have resulted from the activation of "cAMP-independent" second messenger pathways such as the calcium-calmodulin system [33–35]. The actions of cFSH on theca cell steroidogenesis were mimicked by a recombinant preparation of hFSH, clearly indicating that FSH serves a role in the regulation of theca cell function.

To further evaluate the involvement of the cAMP pathway in the control of theca cell steroid output, the actions of several compounds that bypass cellular receptors to stimulate the cAMP-protein kinase A system were utilized.

Treatment of theca cells with cholera toxin, forskolin, or 8-bromo-cAMP caused a significant dose-dependent increase in all steroid levels measured, indicating that all mechanisms within the cAMP-generating/steroidogenic-response system are functional within these cells. These data, which clearly demonstrate a stimulatory role for cAMP in the regulation of steroidogenesis by theca cells of 6–8-mm follicles, are comparable to those previously reported for the actions of these steroidogenic agents on androstenedione production by F_2 theca cells [10].

Our previous studies have indicated that, in addition to the cAMP-protein kinase A system, protein kinase C plays a predominant role in the regulation of basal and gonadotropin-induced androstenedione production by theca cells of the F_2 follicle [10]. To evaluate the role of protein kinase C in regulating steroid production by theca cells of 6–8-mm follicles, the tumor-promoting phorbol ester, PMA (an agonist known to activate protein kinase C [36]), was utilized. Incubation of theca cells with PMA in the absence of steroidogenic agents stimulated a dose-dependent increase in basal progesterone, androstenedione, and estradiol levels, effects comparable to those previously reported for F_2 theca cells [10]; however, PMA attenuated oLH and 8-bromo-cAMP induced androstenedione and estradiol production. In contrast, PMA had no effect on gonadotropin-induced cAMP accumulation or progesterone production. These data would indicate that protein kinase C inhibits gonadotropin-induced androstenedione and estradiol output at a site distal to cAMP and progesterone production, most likely by suppressing $C_{17,20}$-lyase activity.

Finally, data from this study demonstrate that theca cells isolated from developing follicles produce measurable basal and agonist-induced levels of progesterone, suggesting the existence of a functional Δ^4 pathway. However, the presence or absence of a functional Δ^5 pathway within these cells could not be determined by examining androstenedione as the steroid product. Therefore, the ability of these theca cells to produce DHEA (the Δ^5 steroid precursor for androstenedione production) in the absence and presence of a 3β-hydroxysteroid dehydrogenase inhibitor (cyanoketone) was evaluated. In the absence of cyanoketone, basal levels of DHEA were nondetectable. However, stimulation by receptor (LH)- and nonreceptor (8-bromo-cAMP, 25-hydroxycholesterol, pregnenolone)-mediated pathways were found to increase DHEA accumulation. These data indicate that although the conversion of DHEA to androstenedione is most likely occurring at a rapid rate (thus accounting for the nondetectable basal DHEA levels), the ability to measure DHEA following agonist stimulation in the absence of cyanoketone suggests the presence of a functional Δ^5 pathway for steroid metabolism. In the presence of cyanoketone, which effectively blocks the Δ^4 pathway and prevents the conversion of DHEA to androstenedione, basal and agonist-stimulated levels of DHEA were comparable to levels of androstenedione measured in earlier experiments (e.g. see Figs. 3 and 5). Although these findings cannot be used to assess the relative importance of the Δ^4 versus the Δ^5 pathway for androgen production in theca cells of 6–8-mm follicles, the data taken collectively clearly indicate that both the Δ^4 and Δ^5 pathways for steroid metabolism are functional in these cells. Additionally, it is tempting to speculate that this stage of follicular development may represent the transition period associated with the switch from a predominantly Δ^5 (present in relatively undifferentiated, small white follicles) to Δ^4 (found in preovulatory follicles) pathway for androgen production within theca tissue [see ref. 2].

In summary, the use of the domestic hen as our model has provided a means to begin a systematic evaluation of the changes associated with the maturation of follicles from a point at which rapid growth is initiated through to the preovulatory stage of development. Data from the present study confirm that in 6–8-mm follicles, theca tissue is competent to produce the progestin precursor required for the synthesis of androgens and estrogens. This is of critical importance in light of our earlier findings indicating that granulosa cells from these follicles do not contribute to follicular steroidogenesis [6, 7], a model that contrasts those previously reported for mature avian preovulatory follicles [37, 38]. The ability of the theca layer to maintain production of androgens and estrogens in developing follicles that have yet to be selected into the preovulatory follicular hierarchy will likely be an important consideration in the subsequent recruitment-atresia process [39, 40]. Although published data are currently lacking to fully support this contention in the avian model, the continual production of steroids is thought to play a major role in the maintenance of follicular integrity and development in mammals [41, 42]. In light of this information, studies have been initiated using tissues of developing, hen ovarian follicles to resolve this question.

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