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Influence of Follicular Maturation on Luteinizing Hormone-, Cyclic 3',5'-Adenosine Monophosphate-, Forskolin- and Cholesterol-Stimulated Progesterone Production in Hen Granulosa Cells[1]

E. K. ASEM and F. HERTELENDY[2]

Departments of Obstetrics and Gynecology and Physiology
St. Louis University School of Medicine
St. Louis, Missouri

ABSTRACT

The influence of follicular maturation on progesterone production by collagenase-dispersed hen granulosa cells was measured in short-term incubations. Granulosa cells of the largest follicle (F_1) produced up to ten times more progesterone than cells from smaller follicles (F_3-F_5), not only in response to luteinizing hormone (LH), but also when stimulated by exogenous cyclic AMP or forskolin, both of which raise intracellular cyclic AMP levels by nonreceptor-mediated mechanisms. Moreover, when granulosa cell progesterone synthesis was stimulated by incorporating 25-hydroxycholesterol into the incubation medium, an identical pattern was obtained. This could be attributed to a corresponding increase in the specific activity of the mitochondrial cholesterol side-chain cleavage enzyme (20,22 desmolase). An increase in the apparent V_{max} was observed without a change in the apparent K_m values. Pregnenolone substrate at concentrations which raised progesterone production to levels similar to those observed in response to LH stimulation was utilized equally by granulosa cells of mature and developing follicles. However, at high pregnenolone concentrations, granulosa cells of mature follicles converted significantly more of the precursor to progesterone. Assay of 3β-hydroxysteroid dehydrogenase (3β-HSD) showed that the enzyme has two K_ms: a low K_m present in cells of both mature and developing follicles, and a high K_m found only in granulosa cells of more mature follicles. It is concluded that LH-promoted progesterone synthesis in granulosa cells of developing chicken follicles is restricted not so much by the availability of receptors and the competence of the adenylate cyclase/cyclic AMP system, but by the activity of key enzymes, principally the cholesterol-20,22 desmolase.

INTRODUCTION

The ovary of a regularly laying hen contains a cluster of yellow, yolk-filled follicles arranged in a size hierachy. The largest follicle (F_1) is ovulated first, the second largest (F_2) the following day, and so on until an anovulatory day interrupts the sequence of ovipositions. The exceptionally rapid growth of these follicles is reflected by their corresponding weights, ranging from about 2–3 g for F_5 to 14–20 g for F_1 follicles. Granulosa cells, which can be readily prepared from such follicles in pure, uncontaminated form, are functionally characterized by producing large amounts of progesterone and, unlike mammalian granulosa cells, no estrogen (Hammond et al., 1978; Huang et al., 1979; Marrone and Hertelendy, 1983). The responsiveness of chicken granulosa cells to luteinizing hormone (LH) stimulation in terms of enhanced progesterone production shows a striking increase during the last few days of follicular maturation, peaking shortly before ovulation, while follicle-stimulating hormone (FSH)-induced progesterone synthesis decreases concomitantly (Hammond et al., 1981). These observations have been interpreted to reflect changes in LH/FSH receptors that are known to accompany follicular development in mammals (Richards, 1980; Channing et al., 1980), even though such data for avian species are unavailable. Since cyclic AMP is generally viewed as the intracellular mediator of gonadotropin-induced steroidogenesis in ovarian and testicular cells, it could be expected that the steroidogenic action of extracellular cyclic AMP (which bypasses the hormone-receptor step) or that of agonists which raise intracellular cyclic AMP by nonreceptor-mediated mechanisms is

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[2] Reprint requests: F. Hertelendy, Dept. of Obstetrics and Gynecology, St. Louis University School of Medicine, 1325 South Grand Blvd., St. Louis, MO 63104.

independent of the nature of the dominant gonadotropic receptor population and is equally effective in both mature and developing granulosa cells. The aim of the present study was to identify the critical steps in hormone-induced progesterone synthesis that may account for the striking increase in the steroidogenic capacity of these cells during the last few days of follicular maturation. The results indicate that postreceptor steps distal to cyclic AMP generation are involved.

MATERIALS AND METHODS

Hormones and Chemicals

Ovine LH (oLH; NIAMDD-LH 22; 3 NIH-LH S1 U/mg) was kindly provided by the Pituitary Hormone Distribution Program of the NIADDK. N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid (Hepes), 3-isobutyl-1-methylxanthine (IBMX), N^6,O^2-dibutyryladenosine 3',5'-cyclic monophosphate (BU_2 cAMP), bovine serum albumin (BSA, Fraction V), pregenolone, progesterone and collagenase Type I were purchased from Sigma Chemical Co. (St. Louis, MO). Lima bean trypsin inhibitor was supplied by Worthington Biochemical Corp. (Freehold, NJ). Medium 199 (M199) with Hank's salts was a product of Grand Island Biological Co. (Grand Island, NY). Forskolin was supplied by Calbiochem-Behring (LaJolla, CA) and ^{3}H-labeled steroids were obtained from New England Nuclear (Boston, MA). 25-Hydroxycholesterol was purchased from Steraloids (Wilton, NH).

Experimental Animals

White Leghorn hens in their first year of reproductive activity were caged individually, in a windowless, air-conditioned room with a light-dark cycle of 14L:10D. The birds had free access to a pelleted commercial laying ration (Purina Layena) and tap water. The time of oviposition was monitored by an electronic device (Hammond et al., 1980) and birds with at least 5 consecutive laying days were selected for the experiments.

Preparation and Incubation of Granulosa Cells

The animals were killed by cervical dislocation and the desired follicles removed and placed in cold saline. The granulosa layer was separated and the cells dissociated in M199 containing collagenase and trypsin inhibitor (Hammond et al., 1980). Cell viability as determined by the trypan blue dye exclusion method was routinely better than 90%. After appropriate dilution in M199, granulosa cells (approx. 2×10^5/ml) were incubated in open tubes (12 × 75 mm borosilicate) at 37°C with constant shaking. All reagents were diluted with M199 and were added after a 30- to 60-min preincubation of the cells.

Enzyme Assays

Cholesterol-20,22 desmolase activity was measured in a mitochondrial preparation isolated from granulosa membranes using the procedure described by Toaff et al. (1979) with some modifications. Briefly, the granulosa membranes were homogenized at 4°C in 5–7 volumes of medium containing 250 mM sucrose, 1 mM EDTA, 25 mM Tris, pH 7.4 and 10 mg/ml of fatty acid-free BSA. The homogenates were centrifuged at 600 × *g* for 10 min and the resulting supernatants were centrifuged at 15,000 × *g* for 10 min to sediment mitochondria.

Incubations were carried out using the technique described by Tanaka and Strauss (1982). The mitochondria pellets were suspended in incubation buffer which contained 200 mM sucrose, 5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 25 mM Tris and 10 mM Na_2HPO_4, pH 7.4. Aliquots containing 100–200 μg/ml protein were added to a reaction mixture composed of 10 mM sodium isocitrate and 1 mg/ml fatty acid-free BSA in 0.5 ml final volume and incubated for 30 min at 37°C in a shaking water bath opened to room air. The reaction was stopped by boiling each tube for 4 min. The samples were stored at −20°C before analysis of progestins (pregnenolone and progesterone). (The values given for steroid synthesis represent the net production of pregnenolone and progesterone obtained by subtracting the zero time value from values after the indicated incubation time.)

In preliminary studies, we observed that the progesterone content of the product mixture at the end of each incubation was less than 10% (Fig. 1), hence, it was unnecessary to include an inhibitor of 3β-hydroxysteroid dehydrogenase (3β-HSD) in the incubation mixture.

3β-HSD assay was performed by a modified procedure of Armstrong et al. (1977). Granulosa membranes were homogenized in a Dounce homogenizer at 4°C in 5–7 volumes of medium containing 200 mM sucrose, 5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 25 mM Tris, pH 7.4, 10 mM Na_2HPO_4 and 1 mg/ml fatty acid-free BSA. The homogenate was centrifuged at 600 × *g* for 10 min. The supernatant was diluted with the same medium and used for the enzyme assay. The incubation mixture consisted of 0.4 ml of enzyme preparation (100–200 μg/ml protein), 0.1 ml of NAD^+ (final concentration 1 mM) and pregnenolone substrate in 10 μl of ethanol. Blank reaction tubes contained 0.1 ml of buffer instead of NAD^+. For each measurement, duplicate reaction tubes were made with and without the addition of 1.0 mM NAD^+. The expression of enzyme activity was based on the difference between these two measurements. After 30 min incubation in a 37°C shaking water bath, the reaction was stopped by placing the tubes in boiling water for 4 min. Net progesterone synthesis was determined by subtracting the progesterone content of the complete assay mixture at zero time from the values obtained at the end of the incubation.

Hormone Assays

Pregnenolone content was determined without extraction by a sensitive radioimmunoassay (Jones et al., 1983) with specific antiserum supplied by Dr. George P. Chrousos (NICHD, Bethesda, MD). This antiserum cross-reacts <0.3% with progesterone, 11α-hydroxyprogesterone, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, estradiol and testosterone.

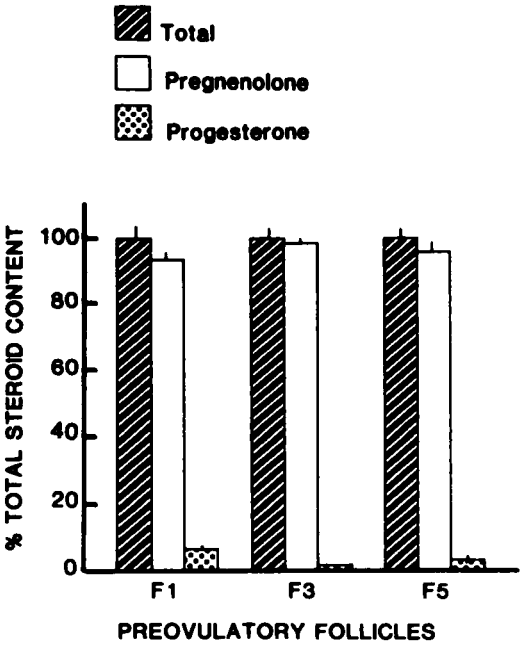

FIG. 1. Relative production of progestins by mitochondrial preparations of mature and developing follicles. Mitochondria obtained from granulosa membranes of mature and developing follicles removed 3 h prior to ovulation were incubated in the presence of 25-hydroxycholesterol (2 μM) for 30 min at 37°C. Results are expressed as percent total steroid content after the determination of net steroid synthesis as described in the text. Values represent mean ± SEM of four determinations.

Progesterone content was also analyzed by a validated radioimmunoassay (Asem et al., 1983) without extraction. Cross-reactivity of the antiserum with pregnenolone and 25-hydroxycholesterol was less than 10% and 0.02%, respectively.

Statistical Analysis

This was carried out using analysis of variance (ANOVA), and post hoc Tukey's test; differences of $P<0.05$ level were considered significant. Student's *t* test was done where applicable. Data points represent mean ± SEM of radioimmunoassay determinations of

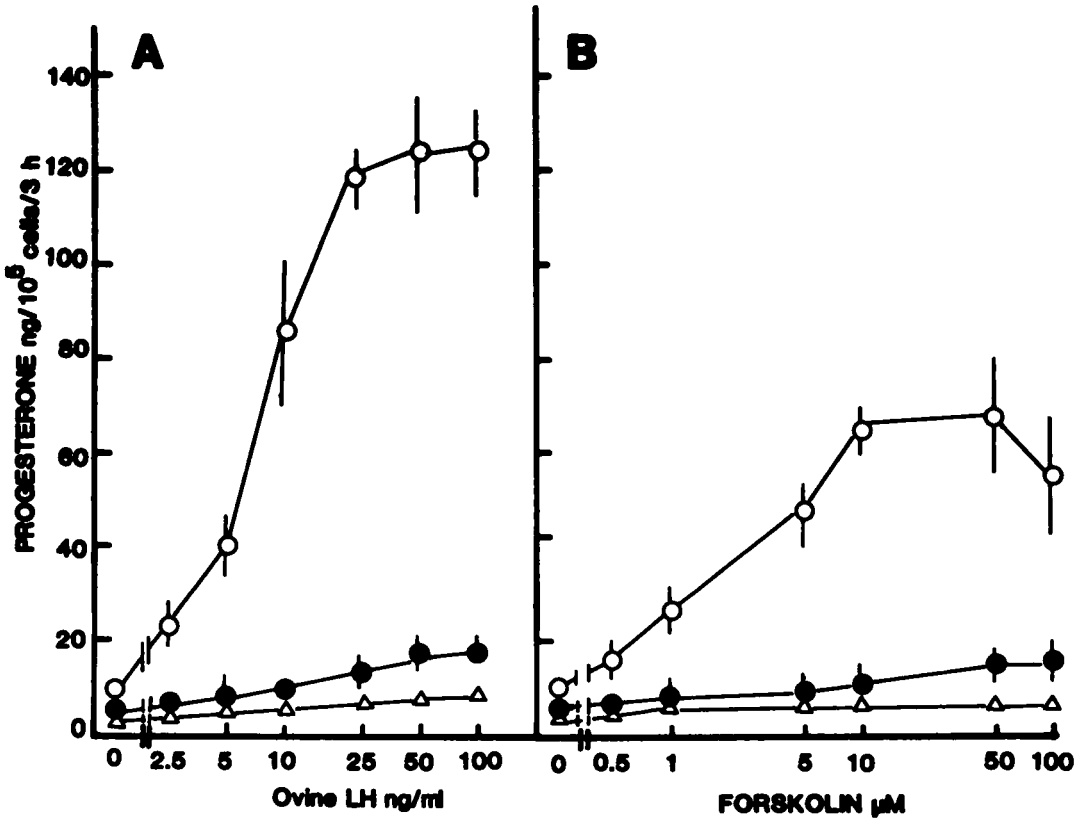

FIG. 2. Effect of ovine LH (*A*) and forskolin (*B*) on progesterone production by granulosa cells. Granulosa cells isolated from the largest (F_1 =○), third largest (F_3 =●), and fifth largest (F_5 =△) preovulatory follicles of laying hens 3 h before expected ovulation were incubated in the presence of increasing concentrations of ovine LH and forskolin for 3 h at 37°C. Results represent mean ± SEM of five experiments using pooled cells from two birds in each experiment.

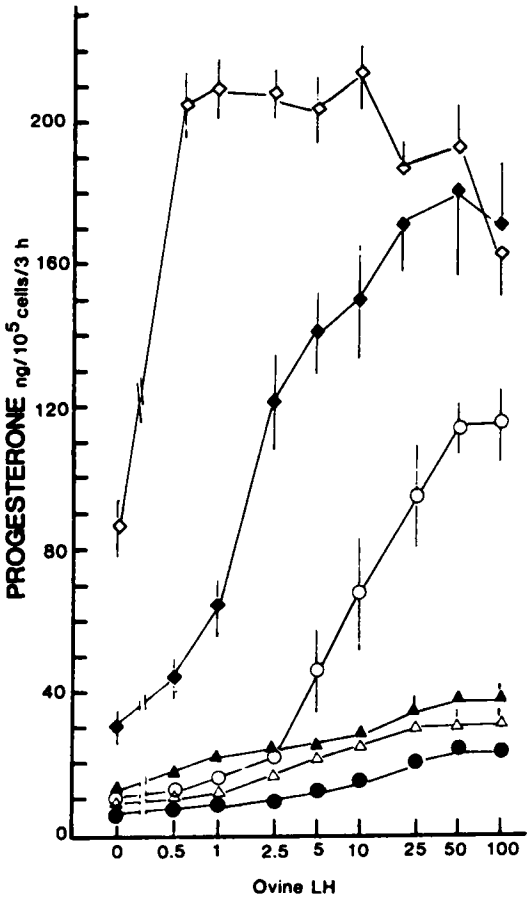

FIG. 3. Influence of follicular maturation on the potentiation by forskolin of ovine LH (oLH)-promoted steroidogenesis in granulosa cells. Conditions were the same as in Fig. 2. F_1 oLH alone (○); F_1 oLH + 1 μM forskolin (◆); F_1 oLH + 50 μM forskolin (◇); F_3 oLH alone (●); F_3 oLH + 1 μM forskolin (Δ); F_3 oLH + 50 μM forskolin (▲). Results are mean ± SEM of quadruplicate determinations. Two additional experiments of a similar design yielded results consistent with these data.

quadruplicate incubations from a number of experiments as indicated in the figure legends unless specified otherwise.

RESULTS

Stimulation of granulosa cells isolated from the most mature (F_1) and developing (F_3 and F_5) follicles with oLH and forskolin, both of which are believed to promote progesterone production by raising intracellular cyclic AMP levels although by different mechanisms, yielded results that were qualitatively similar (Fig. 2). Granulosa cells of F_1 follicles produced, respectively, 8 and 12 times more progesterone than F_3 and F_5 cells did ($P<0.001$). Quantitatively, oLH was the more potent steroidogenic agonist, raising unstimulated baseline levels of 10 ng/10^5 cells to a maximum of 125 ng/10^5 cells at 50 ng/ml concentration. More significantly, forskolin, which bypasses the hormone receptor level and activates adenylate cyclase directly (Seamon and Daly, 1981), was unable to promote steroidogenesis in F_3 and F_5 cells to the extent observed in F_1 cells. When forskolin was combined with LH to stimulate progesterone synthesis, the remarkable synergistic effect reported previously using F_1 cells (Asem and Hertelendy, 1983) was markedly reduced in F_3 cells (Fig. 3). For example, at 5 ng/ml concentration of LH, progesterone

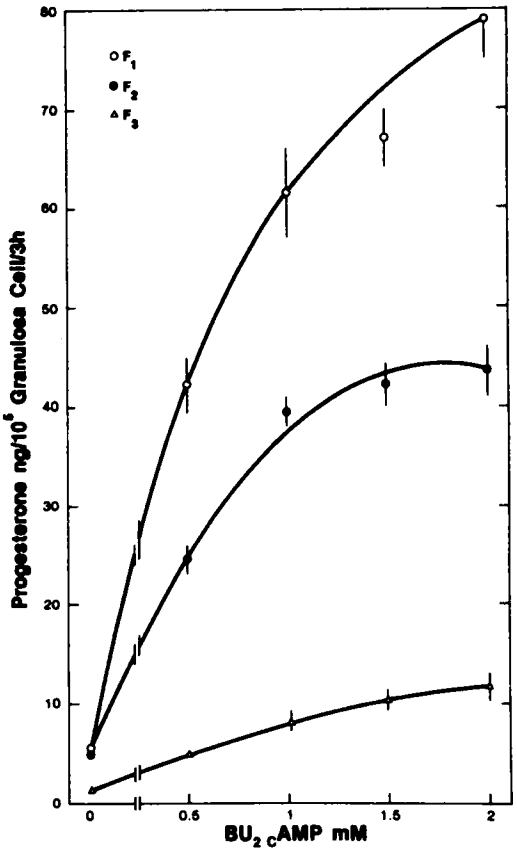

FIG. 4. Effect of dibutyryl cyclic AMP (BU_2cAMP) on progesterone production by granulosa cells. Experimental conditions were the same as in Fig. 2. Results are mean ± SEM of four observations. Such dose-response experiments were repeated using longer incubation times (5 and 8 h), as well as using 8-bromo-cAMP, with highly consistent results.

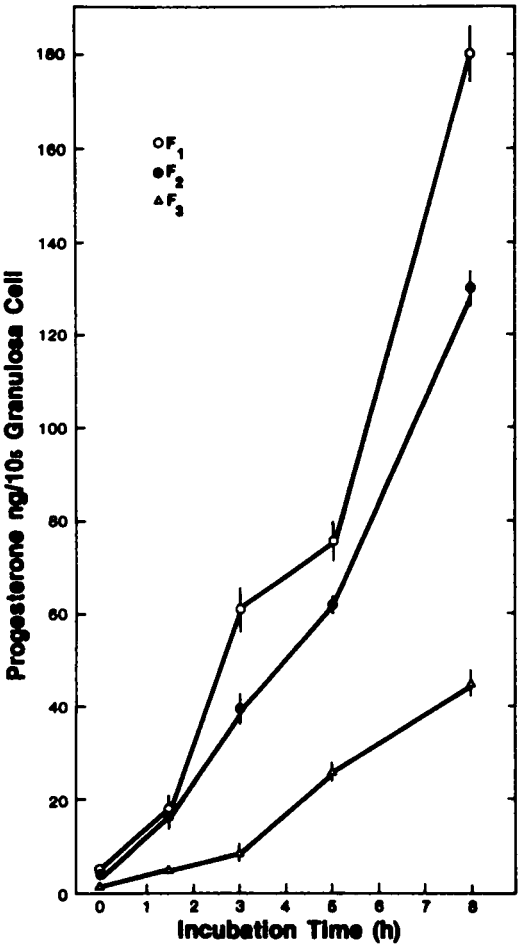

FIG. 5. Time course of progesterone production in response to dibutyryl cyclic AMP. Granulosa cells isolated from follicles removed 3 h prior to ovulation were incubated with 1 mM BU_2cAMP at 37°C. At the times indicated samples were removed and analyzed for progesterone content. Results are mean ± SEM of quadruplicate incubations.

production in F_1 cells was increased from 46 to 124 ng/10^5 cells by the addition of 1 μM forskolin and to 210 ng/10^5 cells by the addition of 50 μM forskolin. In F_3 cells, the same concentration of forskolin raised LH-stimulated progesterone output from 8.5 to 16.2 and 21.5 ng/10^5 cells, respectively.

To examine the effects of exogenous cyclic AMP on steroidogenesis in relation to follicular development, granulosa cells from the three largest follicles (F_1, F_2 and F_3) were incubated in the presence of increasing concentration of BU_2cAMP. The marked dissociation of steroidogenic capacity between the three cell populations was once again clearly manifested (Fig. 4). A time course study of BU_2cAMP-stimulated progesterone production in such cells further corroborated this observation (Fig. 5).

Because these experiments have indicated that the step(s) which restrict the response of granulosa cells from the younger developing follicles to steroidogenic agonists are located distally to cyclic AMP, we examined the conversion of cholesterol and pregnenolone to progesterone in relation to follicular maturation. Addition of pregnenolone to the medium within the concentration range (50–250 ng/ml) that yielded progesterone levels similar to those obtained in response to LH or BU_2cAMP stimulation revealed no differences between granulosa cells from large (F_1), medium (F_3) and small (F_5) follicles (Fig. 6A). However, at high pregnenolone substrate concentrations (0.5–10 μg/ml) there was a dramatic increase in progesterone output in F_1 granulosa cells, reaching a maximum of 6250 ng/10^5 cells at 15 μg/ml pregnenolone concentrations versus 1650 ng/10^5 cells in F_3 and 1400 ng/10^5 cells in F_5 (Fig. 6B). A similar rate of increase in 3β-HSD activity in a cell-free granulosa preparation of developing hen follicles has also been described (Armstrong, 1982).

Exposure of granulosa cells to 25-hydroxycholesterol, a substrate for cholesterol desmolase (Jefcoate et al., 1974) and a precursor of progestins in rat luteal cells (Toaff et al., 1982) and avian granulosa cells (Sgarlata et al., 1984), clearly differentiated the steroidogenic capacity of granulosa cells during follicular maturation (Fig. 7). It is noteworthy that F_1 cells produced significantly ($P<0.001$) more progesterone than F_3 cells did even at a low substrate concentration (0.25 μg/ml).

To elucidate further the post-cyclic AMP steps that might be involved in follicular maturation, we measured the activity of the mitochondrial cholesterol side-chain cleavage enzyme (20,22 desmolase). Figure 8 demonstrates that the activity of this enzyme increased concomitantly with follicular maturation. The specific activities were 0.12, 0.02 and 0.005 nmol steroid per mg protein per min for F_1, F_3 and F_5, respectively.

To evaluate the effect of follicular maturation on the kinetics constants of the mitochondrial 20,22 desmolase, enzyme activities of mito-

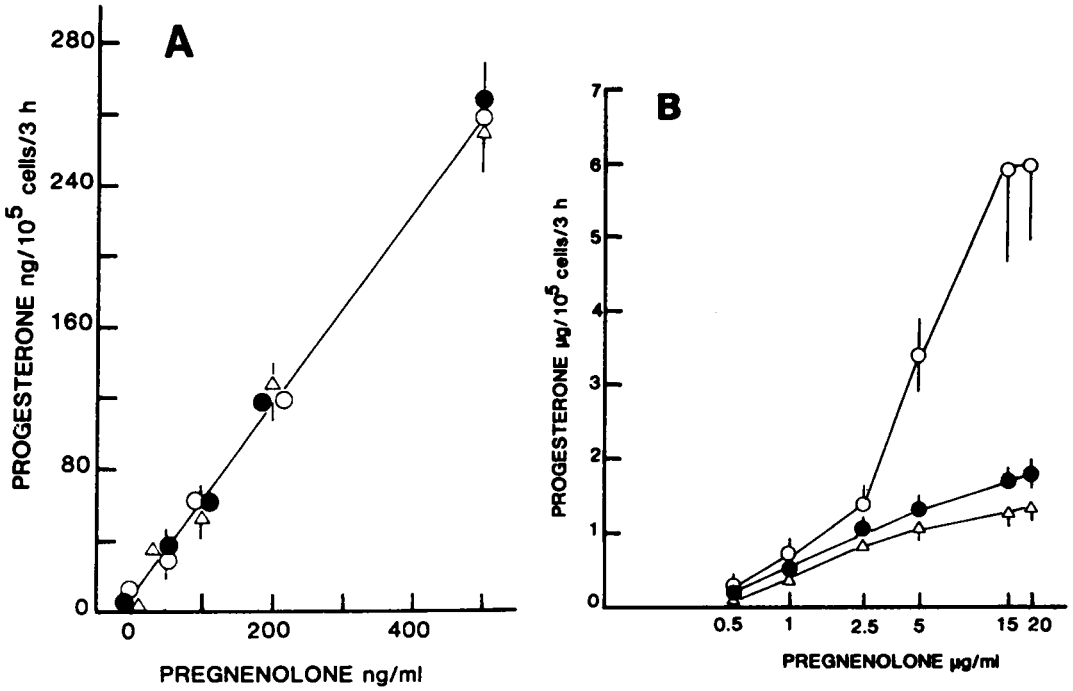

FIG. 6. Effect of follicular maturation on pregnenolone metabolism by granulosa cells. Granulosa cells isolated from F_1 (○), F_3 (●) and F_5 (△) follicles removed 3 h prior to ovulation were incubated in the presence of increasing doses of pregnenolone for 3 h at 37°C. Results are mean ± SEM of four separate experiments. *A*) Low concentrations of pregnenolone. *B*) High concentrations of pregnenolone.

chondrial pellets obtained from F_1, F_3 and F_5 follicles were determined in the presence of increasing concentrations of 25-hydroxycholesterol as described in the *Materials and Methods*.

Isolated mitochondria converted the 25-hydroxycholesterol substrate in a dose-related manner (Fig. 9). The apparent V_{max} of the enzyme increased significantly ($P<0.001$) as the follicle approached ovulation. Figure 10 shows a Lineweaver-Burk plot derived from the dose-response studies, illustrating that although the V_{max} increased with follicular maturation, the apparent K_m did not. To determine the apparent enzyme kinetics constant (K_m and V_{max}), data were calculated by a modified Lineweaver-Burk plot (Hofstee, 1952). A linear regression program was used to compute the slope (negative value of apparent K_m) and *y*-intercept (apparent V_{max}). As shown in Table 1, the apparent K_m value remained the same throughout follicular maturation. On the other hand, the V_{max} increased with follicular maturation.

Next, we assayed the activity of 3β-HSD, the next key enzyme in the steroid biosynthetic pathway. The activity of this enzyme did not differ in F_1, F_3 and F_5 follicles whenever low pregnenolone substrate concentrations (<3 μM) were used (Fig. 1). On the other hand, when substrate levels were above a critical dose (>3 μM) there was a dramatic rise in the V_{max} of the 3β-HSD in F_1, a less marked increase in F_3, and no change in F_5 (Fig. 11), indicating that the 3β-HSD has two apparent K_m's, a low K_m form that is present in granulosa cells from both young and mature follicles, and a high K_m enzyme that is present only in mature follicles. Figures 12 and 13 present double reciprocal plots of enzyme activities at low and high pregnenolone substrate levels, respectively. Table 2 summarizes the values of the kinetic constants of 3β-HSD obtained at both low and high substrate concentrations.

DISCUSSION

The enhanced responsiveness of granulosa cells to LH stimulation in terms of increased steroidogenesis has generally been attributed to an increase in membrane receptors as the

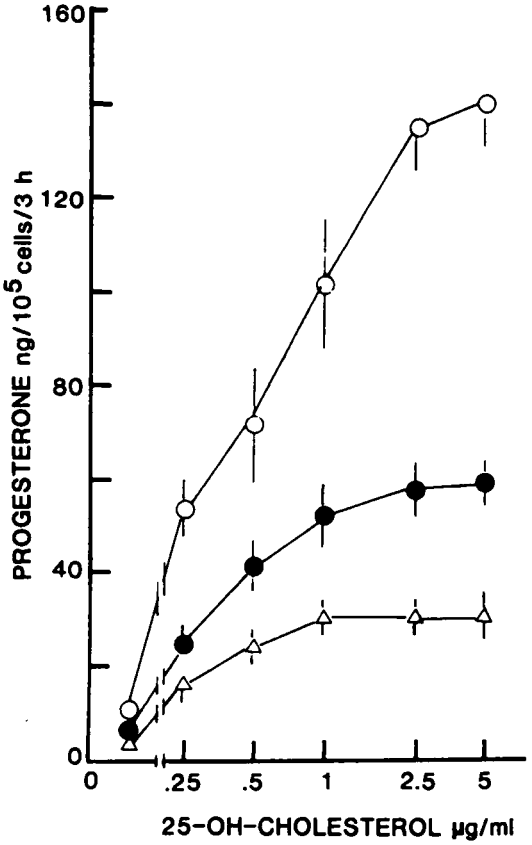

FIG. 7. Stimulation of progesterone synthesis by 25-hydroxycholesterol in granulosa cells of F_1 (○), F_3 (●) and F_5 (△) follicles. Experimental conditions were the same as in Fig. 6. Results represent mean ± SEM of four individual experiments.

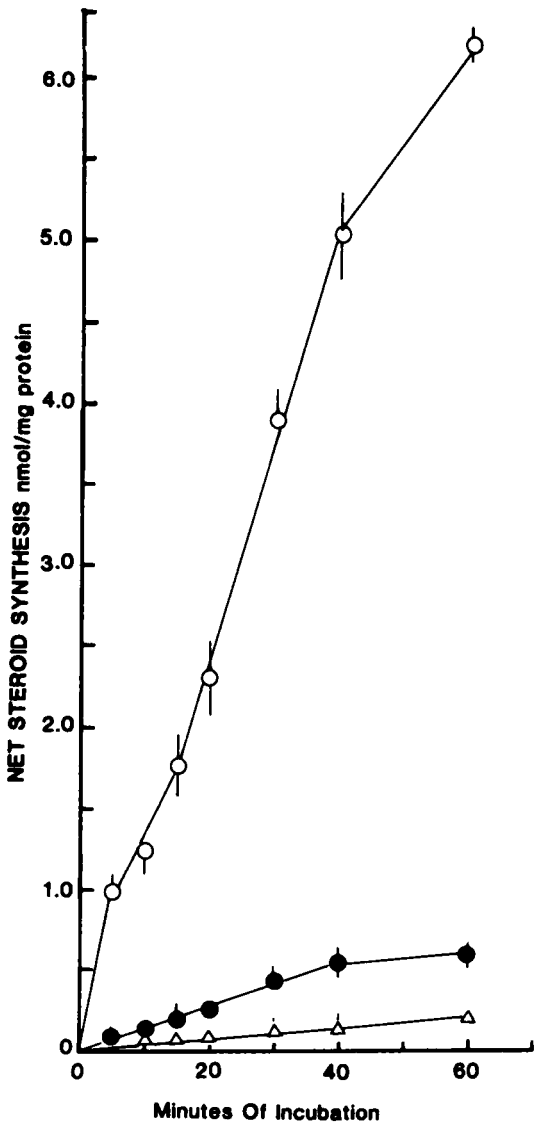

FIG. 8. Time course of metabolism of 25-hydroxycholesterol by granulosa mitochondria. Mitochondria isolated from granulosa membrane of F_1 (○), F_3 (●) and F_5 (△) follicles removed 3 h prior to ovulation were incubated with 2 µM 25-hydroxycholesterol at 37°C. At the times indicated samples were removed and analyzed for both pregnenolone and progesterone. Net steroid synthesis was determined as in the text. Values presented are the mean ± SEM of quadruplicate determinations.

follicle matures (Richards, 1980; Channing et al., 1980). The results of the present study illustrate that intracellular steps which regulate the steroidogenic potential of granulosa cells undergo a process of maturation. As in other steroidogenic cells, LH-induced progesterone production in the avian granulosa cell is the result of a series of complex biochemical reactions, many of which are still poorly understood. LH-promoted adenylate cyclase activity has been shown to increase in broken granulosa cell preparations with follicular maturation (Calvo et al., 1981) and thus parallels steroidogenesis. If one accepts the widely held view that the intracellular concentration of cyclic AMP (reflecting the respective activities of adenylate cyclase and phosphodiesterase) is a key mediator of LH-induced steroidogenesis in these cells, then by bypassing the receptor-adenylate cyclase step using exogenous cyclic AMP (BU_2cAMP), one could expect to find similar progesterone responses in granulosa cells isolated from developing follicles as well as from mature follicles. This is, however, not the case because BU_2cAMP paralleled LH-induced

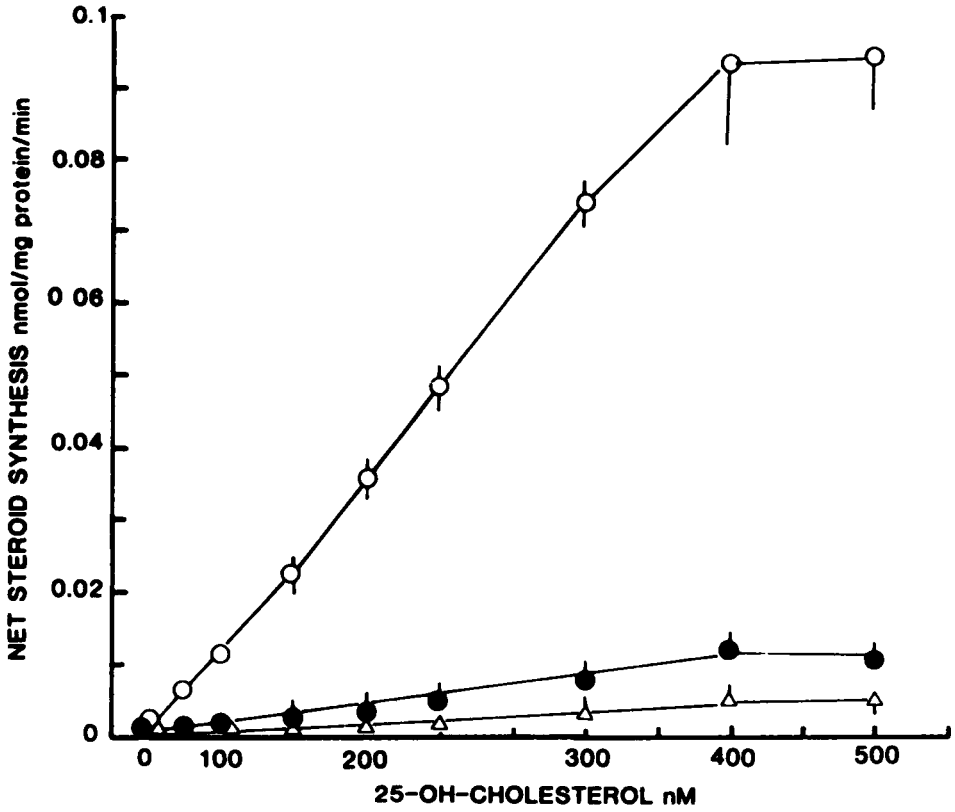

FIG. 9. Effect of follicular maturation on mitochondrial steroidogenesis. Mitochondria isolated from granulosa membranes obtained from F_1 (○), F_3 (●) and F_5 (Δ) preovulatory follicles pooled from three hens 3 h before expected ovulation were incubated with 25-hydroxycholesterol for 30 min at 37°C in a shaking water bath opened to air. Net steroid synthesis was determined as described in the text. Results are mean ± SEM of quadruplicate incubations. This experiment is a representative of three studies with similar results.

steroidogenic responses of granulosa cells from both young and mature follicles. We considered the possibility that the cell membrane of granulosa cells prepared from developing follicles (F_2-F_5) is less permeable to the cyclic AMP derivative and consequently lower intracellular concentrations of the nucleotide may explain the attenuated steroidogenesis. However, the application of the diterpene forskolin, which readily penetrates cell membranes (Seamon et al., 1981) and which has been shown to stimulate both cyclic AMP and progesterone production in hen granulosa cells (Asem and Hertelendy, 1983), resulted in a steroidogenic pattern similar to that obtained with LH and BU_2cAMP. Moreover, the remarkable synergistic effect of forskolin when combined with LH was also reduced in granulosa cells from immature follicles.

Taken collectively, these results support the notion that post-cyclic AMP events may represent the "bottleneck" in the hormone-sensitive steroidogenic mechanisms of granulosa cells during follicular development. These conclusions are supported by data from two additional experimental approaches employed in this study. In the first approach, intact granulosa cells were incubated in the presence of 25-hydroxycholesterol and the production of progesterone was measured. Granulosa cells from F_1 follicles utilized 5–6 times more of the hydroxysterol than cells from smaller F_4-F_5 follicles. Similarly, when pregnenolone, the immediate substrate of progesterone, was used at high concentrations, a highly significant difference between F_1 and the less-developed (F_3-F_5) follicles was observed. However, at lower pregnenolone concentrations, which produced pro-

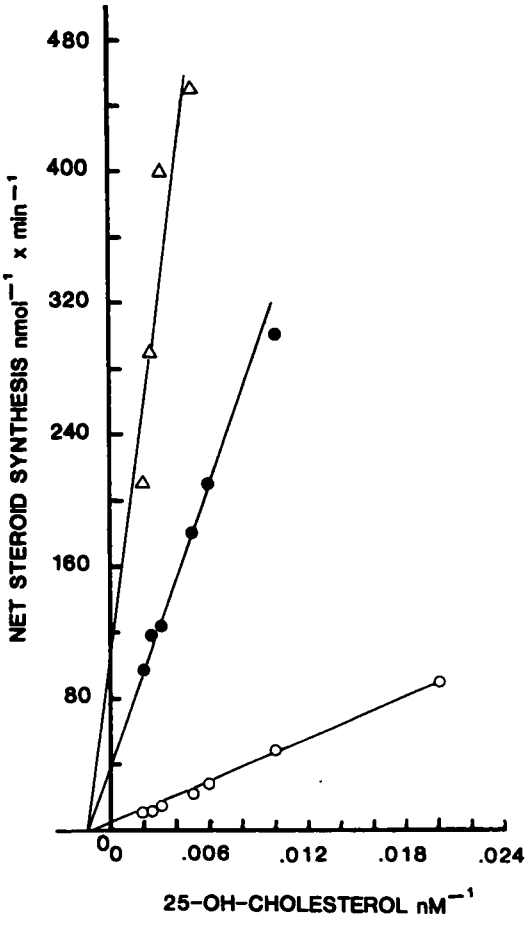

FIG. 10. Lineweaver-Burk plots of the mitochondrial desmolase activity. The values shown in Fig. 9 are presented on a Lineweaver-Burk plot. The straight lines were calculated using linear regression analysis. All three lines had correlation coefficients greater than 0.95.

gesterone levels that are routinely found in response to LH stimulation in vitro, there were no differences between the granulosa cells isolated from mature and growing follicles, suggesting that 3β-HSD activity may not represent the rate-limiting step in progesterone synthesis when it proceeds at physiological or near-physiological rates.

To corroborate these observations, we employed a second approach, i.e., direct measurement of the two key steroidogenic enzymes involved in the conversion of cholesterol and pregnenolone to progesterone. The finding that the V_{max} of 20,22 desmolase increased markedly in F_1 cells without a significant change in the apparent K_m points to quantitative changes in enzyme activity paralleling follicular maturation and is consistent with the progressively increasing ability of intact granulosa cells to utilize cholesterol for progesterone synthesis. Similarly, data obtained from the assay of 3β-HSD are in good agreement with those obtained using intact cells and show that both mature and young granulosa cells possess the enzyme with a similar low apparent K_m. As the follicles mature, granulosa cells also acquire a type of 3β-HSD with a 3- to 4-fold higher apparent K_m. Such changes in enzyme affinities may explain the differences observed between the capacity of mature and young granulosa cells to convert pregnenolone to progesterone in vitro.

In summary, the results of this study provide good evidence that during follicular maturation the steroidogenic capacity of avian granulosa cells develops through a complex sequence of

TABLE 1. Effect of follicular maturation on the kinetics parameters of mitochondrial cholesterol desmolase.[a]

Follicle	K_m[b] (μM)	V_{max}[b] (nmole steroid·mg protein^{-1}·min^{-1})	Correlation coefficient	Regression equation
F_1	0.9	0.25	0.83	$y = 0.25 - 0.9\,x$
F_3	0.5	0.02	0.96	$y = 0.02 - 0.5\,x$
F_5	0.8	0.01	0.92	$y = 0.01 - 0.8\,x$

[a]Enzyme assay was performed on mitochondrial fraction isolated from granulosa layers of 3 laying hens, 3 h before ovulation as described in *Materials and Methods*.

[b]The K_m and V_{max} values were estimated using Hofstee plots from data shown in Fig. 9.

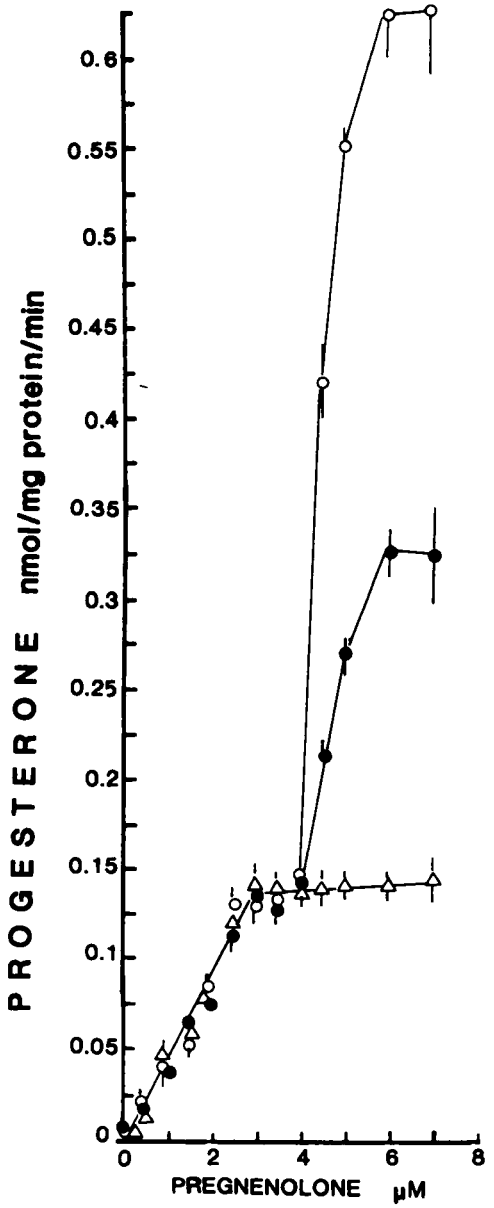

FIG. 11. Effect of follicular maturation on granulosa 3β-hydroxy-Δ^5-steroid dehydrogenase activity. A cell-free preparation was obtained from granulosa of F_1 (○), F_3 (●) and F_5 (△) preovulatory follicles removed from four laying hens 3 h prior to ovulation and was incubated with increasing concentrations of pregnenolone at 37°C for 30 min. Results represent mean ± SEM of four observations. This study was repeated once with comparable results.

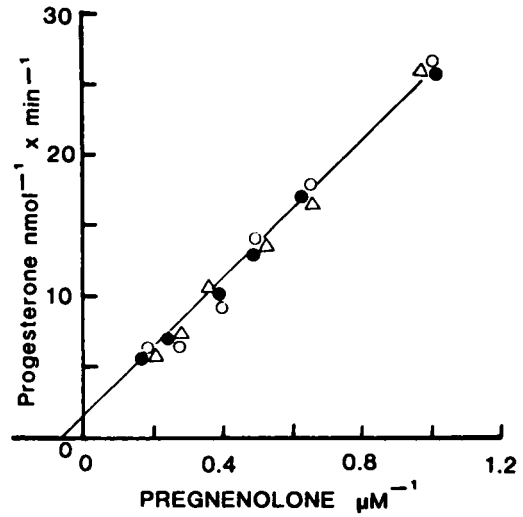

FIG. 12. Lineweaver-Burk plots of the metabolism of low-dose pregnenolone. Results shown in Fig. 11 for 0.3 μM pregnenolone served to construct the plot. The line with a correlation coefficient of 0.96 was calculated by linear regression analysis.

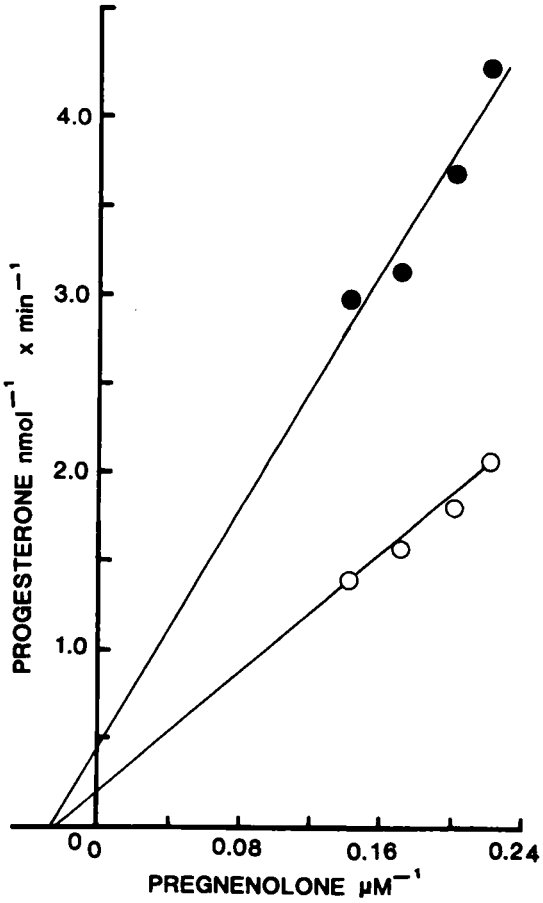

FIG. 13. Lineweaver-Burk plots of 3β-HSD activity at high concentrations of pregnenolone substrate. Results shown in Fig. 11 for 3- to 7-μM pregnenolone concentration were used to construct the double reciprocal plots. The two lines had correlation coefficients greater than 0.93 and were calculated using linear regression analysis.

TABLE 2. Effect of follicular maturation on the kinetics parameters of 3β-hydroxysteroid dehydrogenase activity.[a]

Follicle	K_m[b] (μM)	Vmax (nmole steroid·mg protein^{-1}·min^{-1})	Correlation coefficient	Regression equation
Low pregnenolone substrate dose (<3 μM)				
F_1	2.0	0.138	0.96	y=0.138−2.0x
F_3	1.8	0.135	0.94	y=0.135−1.8x
F_5	1.7	0.134	0.96	y=0.134−1.7x
High pregnenolone substrate dose (≥3 μM)				
F_1	7.9	1.34	0.94	y=1.34−7.9x
F_3	6.0	0.59	0.99	y=0.59−6.0x

[a] 3β-Hydroxysteroid dehydrogenase assay was performed on preparations from granulosa membranes of preovulatory follicles removed from four laying hens 3 h prior to expected ovulation as described in *Materials and Methods*.

[b] The K_m and V_{max} values were estimated using Hofstee plots from data shown in Fig. 11.

events. These include not only the evolution and nature of the dominant gonadotropin receptors and the activity of the functionally coupled adenylate cyclase, but the "maturation" of post-cyclic AMP steps. Moreover, our data suggest that the cholesterol desmolase activity increases during follicular maturation and that this enzymatic step restricts the provision of pregnenolone substrate in granulosa cells of younger, developing follicles. Whether or not the enzyme concentration itself is under hormonal control remains to be established. However, the present data do not provide information on the status of the intervening steps between cyclic AMP generation and the provision of cholesterol for progestin synthesis. Therefore, the possibility that additional components of the steroidogenic mechanism may also undergo "maturation" during follicular development should also be considered.

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