

Developmental and Hormonal Regulation of Bovine Granulosa Cell Function in the Preovulatory Follicle*

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ABSTRACT. Bovine granulosa cells were isolated from small antral, medium antral, and large Graffian follicles (i.e. small, medium, and large preovulatory follicles). Serum-free cultures of granulosa cells were established and found to be viable for 3–6 days of cell culture. Radiolabeled granulosa cell-secreted proteins were obtained and analyzed electrophoretically. No major changes were detected in the protein profiles of small, medium, and large follicle granulosa cells. FSH and insulin, however, had a dramatic effect on granulosa cell-secreted proteins and increased the apparent production of 200K, 65K, 25K, and 15K proteins. The effects of these hormones on the radiolabeled secreted proteins were similar for small, medium, and large follicle granulosa cells. Aromatase activity was high for the first day of serum-free granulosa cell culture and subsequently declined to low levels. Both FSH and insulin alone stimulated aromatase activity, while a combination of hormones resulted in an additive response similar to the stimulation observed with 10% calf serum. Although the level of aromatase activity increased slightly with the size of the follicle, the effects of hormones were independent of follicle size. Progesterone production was low on days 1 and 2 of serum-free granulosa cell culture and high on days 3 and 6 of cell culture. Interestingly, FSH and insulin suppressed progesterone production on day 1 of cell culture for small and medium follicle granulosa cells, but not for large follicle cells. In contrast, hormones stimulated progesterone production on days 3 and 6 of granulosa cell culture, and the level of progesterone production increased with the size of

the follicle. The stimulatory effects of hormones on days 3 and 6 of the culture were similar for medium and large follicle granulosa cells, but were altered for small follicle cells. Results indicate that when aromatase activity is high and stimulated by hormones, progesterone levels are low and generally suppressed by the same regulatory agents. Conversely when progesterone levels are high and hormone responsive, aromatase activity is low. The inverse relationship between aromatase activity and progesterone production implies that bovine granulosa cells alter their differentiated state in culture from an estrogen-producing cell to a progesterone-producing cell. Combined observations indicate that the results obtained on day 1 of culture probably reflect the developmental and hormonal regulation of granulosa cell function in the preovulatory follicle, while data obtained at later times in culture reflect the ability of the cell to synthesize progesterone and develop a luteinization-like activity. Observations regarding the developmental regulation of granulosa cell function imply that the induction and/or suppression of the cellular functions examined does not appear to be required for preovulatory follicle development. In addition, hormonal regulation of follicle development and cellular functions appears not to be regulated through differential actions of hormones at different stages of development. However, hormones were found to have a dramatic effect on bovine granulosa cell function in both the apparent increase in production of potentially important secreted proteins and stimulation of the steroidogenic capacity of the cell. (*Endocrinology* 123: 1668–1675, 1988)

THE GRANULOSA cell has an integral role in the maintenance and control of ovarian function. This cell helps form the ovarian follicle and provides the proper microenvironment and cytoarchitectural support for the developing oocyte. A major functional parameter of granulosa cells is the biosynthesis of estrogen and progesterone. Therefore, regulation of granulosa cell function influences both local ovarian function and the endocrine status of the female. Extensive information has been obtained regarding the regulation of granulosa

cell function with the use of serum-free cultures of rat granulosa cells (1). However, due to the small size of the rat ovary the isolation of follicles at different stages of development is difficult. The bovine ovary is of sufficient size to isolate adequate quantities of purified granulosa cells from follicles at various stages of development. In addition, the bovine is similar to the human in that only one follicle is generally ovulated during each estrous cycle. For these reasons, the bovine ovary provides an attractive model system to study the developmental regulation of granulosa cell function. Bovine granulosa cells have previously been isolated and maintained in serum-supplemented culture (2). These bovine cultures have been useful in elucidating a number of events (3, 4), including the steroid interactions between thecal and granulosa cells (5). An important objective of the current study was to develop a serum-free culture system for

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bovine granulosa cells to optimize analysis of the hormonal regulation of cellular function.

The ovarian follicle has several functionally distinct developmental stages during the process of folliculogenesis. These include development of the primordial follicles to preantral, antral, Graffian, and ovulatory follicles. In addition, follicles may undergo atresia at any stage of development, and ovulated follicles luteinize to form the corpus luteum. The current investigation was restricted to an analysis of the developmental stages of small antral, medium antral, and Graffian follicles. Granulosa cells within the small and medium antral follicles undergo rapid cell proliferation as the follicle enlarges and develops an increased capacity to produce estrogen. Granulosa cells initiate the formation of the antrum and produce many components of follicular fluid. Granulosa cells in the Graffian follicle form a large antrum, produce large amounts of follicular fluid components, and provide the cytoarchitectural support for the developing oocyte. Cells in these follicles maintain a high level of estrogen production while developing an increased ability to produce progesterone. The current study was designed to determine the absence or presence of major functional differences in bovine granulosa cells obtained from various stages of preovulatory follicle development. In addition, the ability of these granulosa cells to respond to different hormones was examined. Results from these studies provide new insight into the developmental and hormonal regulation of granulosa cell function.

Materials and Methods

Cell culture

Bovine ovaries were obtained from young nonpregnant cycling heifers less than 10 min after slaughter (Bio-Resources, Inc., Irving, TX). Ovaries were delivered fresh on ice in a buffered salt solution. The stage of the estrous cycle was determined morphologically, as previously described by Ireland *et al.* (6). Under sterile conditions the follicles were removed from the ovary, cleaned of adhering interstitial tissue, and punctured by a hypodermic needle to drain the follicular fluid. Follicle diameter was determined, and follicles were separated into small (<5 mm), medium (5–8 mm), and large (>8 mm), classifications. Healthy developing follicles were assessed with the criteria previously established by Metcalf (7) for a vascularized pink theca externa and clear amber follicular fluid with no debris. The follicles were then cut into hemispheres and gently scraped under a dissecting microscope with a fine plastic loop to remove the granulosa cells. The granulosa cells were centrifuged at $400 \times g$ for 5 min, then resuspended in culture medium. Cells were plated in 24-well Linbro plates in 1 ml Ham's F-12 (Gibco, Grand Island, NY) in the absence of serum and maintained at 37°C in a 5% CO_2 atmosphere. Cells were plated at approximately 10^6 cells/ 2 cm^2 , and medium was changed every 24–48 h. Cells were treated, as outlined in *Results*, with FSH (100 ng/ml; National Pituitary Agency,

Baltimore, MD) and insulin (5 $\mu\text{g}/\text{ml}$) at the time of plating, and these were replenished every 24 h.

Gel electrophoresis and fluorography

Granulosa cell cultures were maintained in glycine- and methionine-free medium containing 5 $\mu\text{Ci}/\text{ml}$ [^{35}S]methionine and 5 $\mu\text{Ci}/\text{ml}$ [^3H]glycine. The medium was collected, centrifuged, and used for analysis. Radiolabeled proteins were electrophoretically analyzed on sodium dodecyl sulfate 5–15% polyacrylamide gradient slab gels under reducing conditions with the Laemmli (8) buffer system. The gels were fluorographed with diphenylloxazole in acetic acid, as previously described (9).

Assays for aromatase activity and progesterone levels

Aromatase activity was assessed by the release of $^3\text{H}_2\text{O}$ from aromatization of [1β - ^3H]testosterone as previously described (10) with the following modifications. At designated times during the culture of granulosa cells, 0.5 ml medium was removed from the 1-ml culture, replaced with 0.5 ml medium containing 0.3 μCi [1β - ^3H]testosterone (New England Nuclear, Boston, MA; final concentration, 0.25 μM , and incubated for 4 h at 37°C in a CO_2 atmosphere. The culture was then sonicated, and an aliquot was removed for DNA analysis. The remaining sample was transferred to a tube and incubated with dextran-coated charcoal [0.2% (wt/vol) 70K dextran (Sigma), activated 2% (wt/vol) Norit-A charcoal, 10 mM Tris, and 1 mM EDTA, pH 7.5] for 2 h at 4°C , then centrifuged at $13,000 \times g$ for 15 min. Aliquots of the supernatant containing $^3\text{H}_2\text{O}$ were then counted. Blank values were established from identical incubations in the absence of cells and contained less than 100 cpm. Aromatase activity, measured in counts per min of $^3\text{H}_2\text{O}$ released, was normalized per μg DNA. Progesterone levels were determined in the 0.5-ml medium sample removed for the aromatase assay and normalized per μg DNA. Progesterone was quantitated with a RIA, as previously described (11).

DNA and protein assays

DNA was measured fluorometrically with ethidium bromide (12). At the end of the culture period the medium was removed, ethidium bromide buffer (EBB; 20 mM sodium chloride, 5 mM EDTA, and 10 mM Tris, pH 7.8; Sigma) was added to the wells, and the cells were sonicated. Alternatively, DNA was measured in aliquots of the aromatase activity reaction solution. An aliquot of the sonicated cell suspension was added to an equal volume of ethidium bromide solution (0.25 mM ethidium bromide and 100 U/ml heparin in EBB), diluted 1:2 with EBB buffer, and allowed to incubate at room temperature for 30 min. Fluorescent emission at 585 nm with 350 nm excitation was then monitored. A standard curve with calf thymus DNA was used to quantitate DNA levels in the culture wells. This assay has a sensitivity of approximately 0.1 μg DNA and is linear up to 2.5 μg DNA. The total protein concentration was measured according to the method of Bradford (13).

Results

Freshly isolated granulosa cells were plated in serum-free conditions on a plastic substratum. Granulosa cells

in serum-free culture had the characteristic morphology, shown in Fig. 1. From this morphological analysis and considering the cell isolation procedure the granulosa cell cultures were believed to contain no major cell contaminant. Granulosa cells in serum-free culture were viable for 3–6 days in culture. Cell number remained constant for 3 days in culture, then between days 3 and 6 of culture some cells detached from the plastic substratum; thus, the total cell number dropped approximately 50%. Granulosa cells obtained from small, medium, and large follicles demonstrated similar serum-free culture characteristics. Cells isolated from large follicles periodically had a slightly lower plating efficiency and greater loss in cell number between days 3 and 6 of culture. Granulosa cells plated in the presence of serum (either 1% or 10% calf serum) had a greater plating efficiency and were viable for a longer period of culture. Cell morphology was also slightly different in serum, presumably due to the enhanced ability of the cells to attach to the plastic culture substratum (Fig. 1). Cells cultured in serum generally had a loss in cell number of approximately 25% between days 3 and 6 of culture. One of the primary differences in granulosa cells plated in the absence or presence of serum was their ability to remain attached to the plastic culture substratum. Granulosa cells cultured in the absence of serum, however, were viable and appropriate for the current investigation if used before 3–6 days of serum-free cell culture.

The proteins secreted by a cell are a qualitative measure of a wide variety of cellular functions. Examination of changes in radiolabeled secreted proteins, therefore, provides a qualitative measure of major functional alterations of a cell. Granulosa cells were isolated from small, medium, and large follicles, plated under serum-free conditions, and radiolabeled for 24 hr in the absence or presence of FSH and insulin. Examination of radiolabeled secreted protein profiles indicated no major differences between granulosa cells isolated from small, me-

dium, or large follicles. (Fig. 2). Although minor differences were observed, they were generally not detected in all of the radiolabeled secreted protein profiles examined. FSH and insulin stimulated the apparent production of a number of proteins, including 200K, 65K, 25K and 15K proteins. The effects of these hormones on secreted protein profiles were similar for cells isolated from small, medium, and large follicles (Fig. 2). Results indicated that granulosa cells isolated from these different stages of development have similar secreted protein profiles and that the effects of hormones on the cells, although dramatic, are independent of the stage of preovulatory follicle development. Further investigation of radiolabeled amino acid incorporation into secreted protein was examined by using 10% trichloroacetic acid (TCA) precipitation of radiolabeled protein. Radiolabeled secreted proteins were obtained from serum-free cultures of granulosa cells isolated from small, medium, and large follicles, then TCA precipitated. The TCA precipitate was counted and normalized per μg DNA in the cell culture or per μg protein in the radiolabeled conditioned medium. Data obtained indicated no significant difference in the amount of TCA-precipitable radiolabeled protein secreted by granulosa cells isolated from these different stages of development.

To examine more specific functions of granulosa cells the steroidogenic capacities of cells isolated from small, medium, and large follicles were examined. The first functional parameter examined was the ability of granulosa cells to aromatize testosterone to produce estrogen. This is determined through a quantitation of aromatase activity in the cells. Initially, granulosa cells from medium-size follicles were cultured, and at designated time points the cell cultures were terminated and assayed for aromatase activity. Granulosa cells for this time course were plated in the absence or presence of FSH and insulin (FI) or 10% calf serum (Fig. 3). Aromatase activity was highest on day 1 of culture, with both hormones

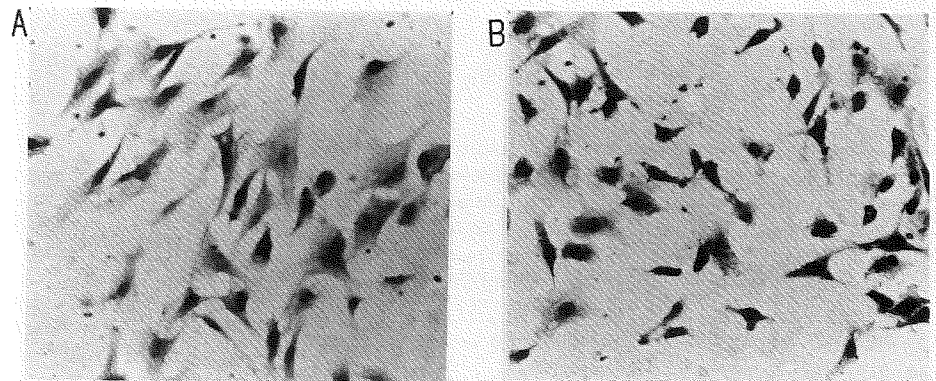


FIG. 1. Morphology of bovine granulosa cells cultured in the presence (A) or absence (B) of serum. Bovine granulosa cells were isolated from medium-sized follicles and plated in the absence or presence of serum and in the presence of FI. Cells were fixed with cold methanol on day 2 of culture, then stained with hematoxylin and eosin. Photographed at $\times 270$ magnification.

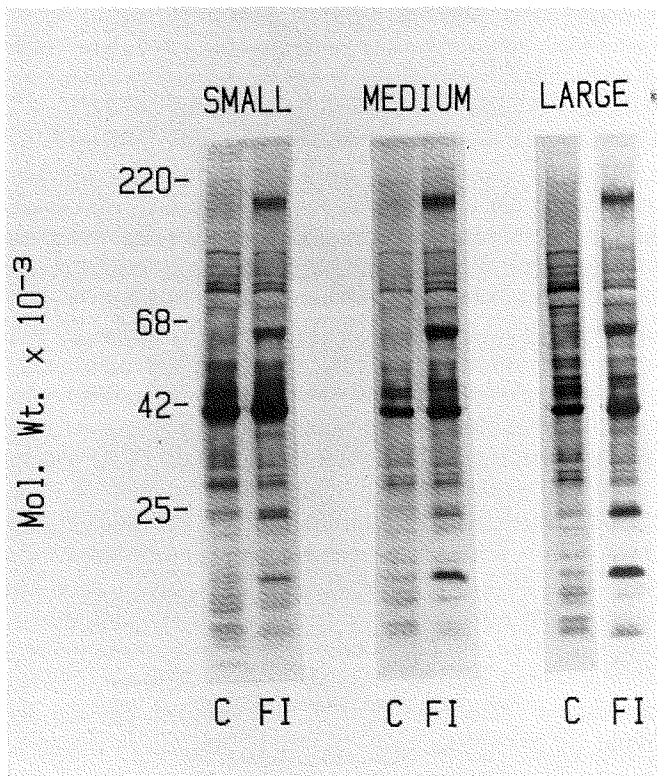


FIG. 2. Bovine granulosa cell radiolabeled secreted protein profiles. Cells were isolated from small, medium, and large follicles, then cultured in the absence of serum (C) and in the absence (control) or presence of FI. Cells were radiolabeled with [35 S]-methionine and [3 H]-glycine for 48 h, at which time the medium was removed, electrophoretically separated, and analyzed with fluorography. Data are representative of three different experiments.

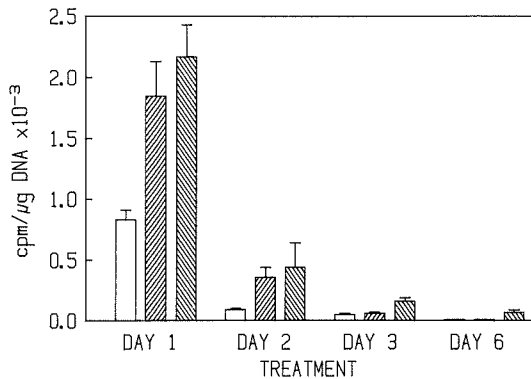


FIG. 3. Aromatase activity time course of granulosa cells cultured in the absence (□) or presence of FI (▨) or 10% calf serum (▩). Aromatase activity was determined on the day of culture indicated and normalized per μ DNA (counts per min/ μ g DNA $\times 10^{-3}$). Data are presented from medium follicle granulosa cells and are the mean \pm SEM from a triplicate determination, representative of three different experiments.

and calf serum stimulating aromatase activity. Aromatase activity decreased on day 2 of culture and approached negligible levels on days 3 and 6 of culture. However, FI and calf serum both maintained aromatase

activity above control levels on day 2, 3, and 6 of culture (Fig. 3). Similar time-course data were obtained with granulosa cells isolated from different sized follicles. Comparison of data obtained from small, medium, and large follicle granulosa cells is presented for day 1 of culture. Granulosa cells were cultured under serum-free conditions and stimulated with FSH, FI, or calf serum (Fig. 4). Aromatase activity in granulosa cells increased slightly with the size of the follicle. FI and calf serum maximally stimulated aromatase levels approximately 3-fold. Although slightly higher aromatase levels were detected as the size of the follicle increased, the fold stimulation obtained with hormones was the same, (Fig. 4). Combined results imply that aromatase activity increased during development; however, the responses of the cells to hormones were similar. To examine the effects of individual hormones on aromatase activity, granulosa cells from medium-sized follicles were cultured for 1 day under serum-free conditions in the presence of different regulatory agents (Fig. 5). Insulin had a slightly greater effect on aromatase activity than FSH, while FI produced an additive response. The response to FI was comparable to that to calf serum.

Another specific functional parameter of granulosa cells examined was progesterone production. Granulosa cells from medium-sized follicles were cultured for designated periods to collect samples of conditioned medium for progesterone analysis. The cultures shown in Fig. 3 were simultaneously analyzed for progesterone production. Progesterone production increased slightly with time in culture, and both FI and calf serum stimulated progesterone production (Fig. 6). LH and hCG had effects similar to those of FSH on days 3 and 6 of medium-sized follicle granulosa cell culture (data not shown). The data for the time course of progesterone production by

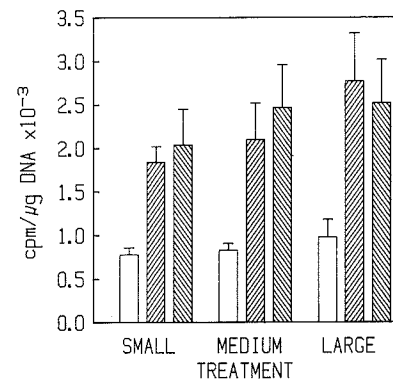


FIG. 4. Aromatase activity in granulosa cells isolated from small, medium, and large follicles that were cultured in the absence (□) or presence of FI (▨) or 10% calf serum (▩). Aromatase activity was determined on day 1 of culture and normalized per μ g DNA (counts per min/ μ g DNA $\times 10^{-3}$). Data are presented as the mean \pm SEM of triplicate determinations from three different experiments.

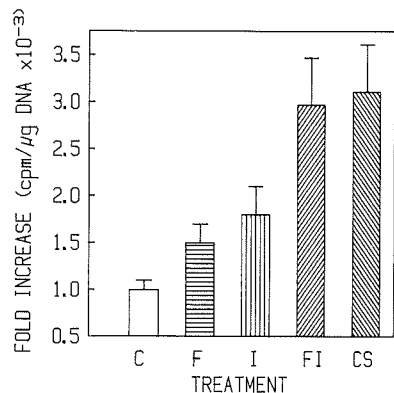


FIG. 5. Aromatase activity in granulosa cells isolated from medium follicles and cultured in the absence (C) or presence of FSH (F), insulin (I), FI, or 10% calf serum (CS). Aromatase activity was determined on day 1 of culture and normalized per μg DNA (counts per min/ μg DNA $\times 10^{-3}$), then converted to fold increase. Data are presented as the mean \pm SEM of triplicate determinations from three different experiments.

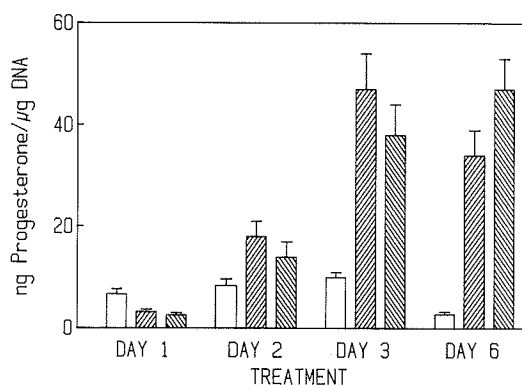


FIG. 6. Progesterone production time course of bovine granulosa cells isolated from medium follicles and cultured in the absence (□) or presence of FI (▨) or 10% calf serum (▩). Progesterone production was determined on the day of culture indicated and normalized per μg DNA (nanograms of progesterone per μg DNA). Data are presented as the mean \pm SEM from a triplicate determination and are representative of three different experiments.

granulosa cells isolated from medium-sized follicles were similar to those obtained from small and large follicle granulosa cells. Comparison of progesterone production on day 1 of culture from cells isolated from small, medium, and large follicles is shown in Fig. 7. Hormones and calf serum, which stimulated aromatase production, inhibited progesterone production on day 1 of granulosa cell culture. The inhibitory effects of hormones on progesterone production were most dramatic with small and medium follicle granulosa cells and were negligible with large follicle granulosa cells (Fig. 7). Calf serum, however, inhibited progesterone production in a similar manner for small, medium, and large follicle granulosa cells (Fig. 7). Analysis of progesterone production on day 3 of culture demonstrated that FI and calf serum had a stimulatory effect on progesterone biosynthesis (Fig. 8). FSH

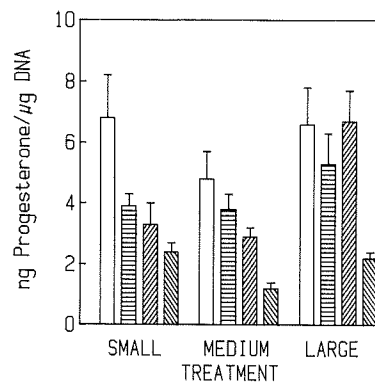


FIG. 7. Progesterone production by granulosa cells isolated from small, medium, and large follicles and cultured in the absence (□) or presence of FSH (▨), FI (▩), or 10% calf serum (▨). Progesterone production was determined on day 1 of culture and normalized per μg DNA (nanograms of progesterone per μg DNA). Data are presented as the mean \pm SEM of triplicate determinations from three different experiments.

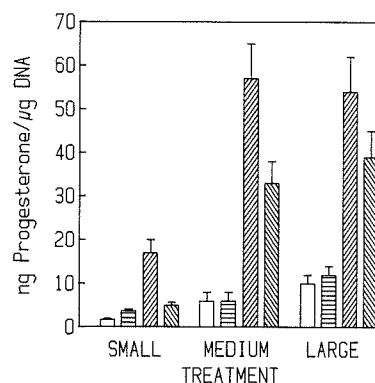


FIG. 8. Progesterone production by granulosa cells isolated from small, medium, and large follicles and cultured in the absence (□) or presence of FSH (▨), FI (▩), or 10% calf serum (▨). Progesterone production was determined on day 3 of culture and normalized per μg DNA (nanograms of progesterone per μg DNA). Data are presented as the mean \pm SEM of triplicate determinations from three different experiments.

alone had a small stimulatory effect only on small follicle granulosa cells. Both basal and hormone-stimulated progesterone production were lower in small follicle granulosa cells than in medium and large follicle granulosa cells (Fig. 8). Levels of progesterone production after FI and calf serum stimulation of medium and large follicle granulosa cells were similar. Results indicate that progesterone production increases with the size of the follicle when granulosa cells are maintained for 3–6 days of culture.

Discussion

The bovine ovary is of sufficient size to allow the isolation of adequate numbers of purified granulosa cells from follicles at various stages of development. There-

fore, the bovine ovary is quite useful in an analysis of the developmental and hormonal regulation of granulosa cell function. The current study has focused on the use of developing small antral, medium antral, and Graffian follicles. As previously described (13A), these follicles are referred to as small, medium, and large developing follicles. Comparison of functional parameters and hormonal responses of granulosa cells isolated from small, medium, and large follicles provides information on the developmental and hormonal regulation of granulosa cell functions. Although developing follicles were carefully chosen for a pink vascularized theca and clear amber follicular fluid, as previously described (7), early stage atretic follicles are difficult to identify without a detailed morphological analysis (14). Therefore, the tissue in the current study was selected for developing preovulatory follicles, but may contain some early stage atretic tissue. This is a technical limitation that will need to be considered in any data interpretation. The small, medium and large follicles obtained were used to isolate highly purified granulosa cell populations. Primary cultures of bovine granulosa cells have previously been described and used to examine a number of events (2, 5). The predominate functional parameter previously examined is the steroidogenic capacity of the granulosa cell (2, 5) and its ability to influence thecal cell steroidogenesis (2, 5). Bovine cultures have also been used to examine the production of individual protein products, such as proteoglycans (15) and inhibin (16). The majority of these studies cultured the bovine granulosa cells in the presence of serum. Serum is known to stimulate the functions of many cell types and masks the effects of regulatory agents. This is supported by the observations presented in Figs. 3-8, which demonstrate stimulation of bovine granulosa cell steroidogenesis with serum alone. For this reason a serum-free culture system was necessary in the current study to optimize analysis of the developmental and hormonal regulation of bovine granulosa cell function. Functionally viable bovine granulosa cell cultures were established and could be used for up to 3-6 days of culture. A primary limitation of the serum-free bovine cultures was the ability of cells to remain attached to the culture substratum after 6 days. Serum-free culture characteristics of the granulosa cells were essentially independent of initial follicle size. Observations indicate that this serum-free bovine granulosa cell culture system will be useful in investigating the regulation of cellular function, growth, and differentiation.

Granulosa cell radiolabeled secreted proteins were examined to provide an initial qualitative measure of major changes in granulosa cell function. The results demonstrated that major secreted proteins were similar for granulosa cells isolated from small, medium, or large follicles. No major distinct protein products were induced

during follicle development from small to large preovulatory follicles. The presence of FI, however, had a dramatic effect on the radiolabeled granulosa cell secreted protein profiles. Proteins of 200K, 65K, 25K, and 15K, were induced or dramatically stimulated with hormone treatment. Further analysis with techniques such as two-dimensional gel electrophoresis will be required to determine if multiple proteins are present within these mol wt ranges. The effects of FI were the same for granulosa cells isolated from small, medium, and large follicles. Interestingly, previous analysis of the effects of FI on rat granulosa cell radiolabeled secreted protein profiles demonstrated the induction of similar mol wt proteins (17), particularly the 200K protein (18). It is speculated that these proteins will be functionally important proteins that will provide useful markers for granulosa cell function and differentiation. Although hormones had a dramatic effect on bovine granulosa cell secreted proteins, no major differences were found in the proteins produced by cells isolated from small, medium, and large follicles. These results are supported by a previous study which analyzed the cellular proteins from secondary or subcultures of bovine granulosa cells from small and large follicles (19). Combined observations imply that no major changes occur in the proteins produced by granulosa cells during development from small to large preovulatory follicles. These results, however, do not rule out the possibility of minor secretory products having important developmental functions but not being detectable with the techniques used.

A major functional parameter of granulosa cells is the aromatization of testosterone to estrogen. Aromatase activity was dramatically reduced after the first day of granulosa cell culture. Although morphologically viable granulosa cells could be maintained for 3-6 days in culture, aromatase activity was only high on the first day of culture. These data imply that the first day of bovine granulosa cell culture may be more representative of *in vivo* granulosa cell function at the time of isolation than are longer periods of cell culture. This rapid reduction in aromatase activity indicates the possible absence of regulatory agents required to maintain estrogen production. Both insulin and FSH stimulated aromatase activity during the first day of culture. The effects of FI were additive and similar to the stimulatory actions of 10% calf serum. The actions of insulin required high concentrations of hormone and are probably due to cross-reactivity with the insulin-like growth factor (IGF) receptor. Therefore, insulin may mimic the more physiologically important actions of IGF previously described (20). Results indicate that FSH and insulin (*i.e.* IGF) are important regulators of estrogen production by bovine granulosa cells. This supports the large body of data previously obtained on the actions of FSH and insulin.

(i.e. IGF) on rat granulosa cells (1). As previously postulated, it will be through the combined actions of agents such as FSH and insulin (i.e. IGF) that optimal granulosa cell development and estrogen production will be maintained. A comparison of aromatase activity in granulosa cells from small, medium, and large follicles indicated a small increase in aromatase activity with the size of the follicle. This correlates with the increase in steroidogenic capacity of the granulosa cell during follicle development. Although absolute levels of aromatase activity increased slightly, the actions of hormones were independent of follicle size. The same fold increase was observed with FSH and insulin on granulosa cells isolated from small, medium, and large follicles. These results indicate that the actions of regulatory agents on aromatase activity are similar and independent of follicle development; however, the absolute steroidogenic capacity of the granulosa cell increases with follicle development. Short term bovine granulosa cell cultures will be useful in further examination of the regulatory agents required to promote and maintain this important granulosa cell function.

Before ovulation granulosa cells acquire LH receptors and develop the capacity to produce progesterone. After ovulation progesterone production becomes the predominant steroidogenic activity of the granulosa cell. Therefore, estrogen and progesterone production are primarily produced at different stages of follicle development. Progesterone production by cultured bovine granulosa cells increased with time in culture, with low levels on days 1 and 2 of culture and high levels on days 3 and 6 of culture. This supports previous observations of an increase in progesterone production with time in serum-supplemented bovine granulosa cell cultures (2, 4). Since progesterone is the primary biologically active progestin metabolite, other metabolites were not examined in the current study. Interestingly, the present study demonstrated that regulatory agents that stimulated aromatase activity on day 1 of granulosa cell culture inhibited progesterone production. FSH and insulin inhibited progesterone production by small and medium follicle granulosa cells on day 1 of culture, but had negligible effects on large follicle granulosa cells. This novel observation implies that these hormones may maintain development or inhibit premature luteinization of granulosa cells from small and medium follicles during the first day of culture. However, large follicle granulosa cells appear to have differentiated to a stage that is no longer responsive to this inhibition. Conversely, these same regulatory agents, FSH, insulin, and calf serum, stimulated progesterone production on days 3 and 6 of granulosa cell culture. The actions of FI or 10% calf serum, which gave optimal inhibition on day 1, resulted in a stimulation on days 3 and 6 of culture. Small follicle granulosa cells maintained a lower level of progesterone production,

which suggests their inability to develop a luteinization-like activity as efficiently as medium and large follicle granulosa cells. Comparison of granulosa cells from small, medium, and large follicles demonstrated an increase in progesterone production with the size of the follicle. The ability of FSH to stimulate progesterone production may be due to in part the 1% contaminate by LH in the FSH standard used. The demonstration of low levels of progesterone production and inhibitory actions of hormones on day 1 of granulosa cell culture are inversely related to the data obtained on aromatase activity. When aromatase activity is high and responsive to hormones, progesterone production is low and inhibited by the same hormones. These observations support the proposal that granulosa cells differentiate *in vitro* to change functional parameters from an estrogen-producing cell to a progesterone-producing cell. Whether this *in vitro* alteration in cellular differentiation is due to the promotion of a differentiation process or is the result of a spontaneous dedifferentiation (i.e. regression) remains to be investigated. However, data correlate with these two steroidogenic capacities being predominant at different stages of follicle development. Observations support the hypothesis that granulosa cells *in vivo* develop in response to FSH to produce high levels of estrogen while acquiring LH receptors. Granulosa cells then respond to LH and luteinize, which reduces estrogen production and allows high levels of progesterone to be made. The results of the present study imply that hormones that promote follicle development and estrogen production also inhibit progesterone production and may prevent premature luteinization of granulosa cells.

Information obtained in the current study regarding the developmental regulation of granulosa cell function indicates minimal changes in major functional parameters during development from a preantral follicle to a preovulatory follicle. A slight increase in the steroidogenic capacity of the cell was the only difference detected during development from a small to a large follicle. The major difference was the low level of progesterone production by small follicle granulosa cells. Hormonal regulation of granulosa cell function was similar for small, medium, and large follicles. The major difference was the inability of hormones to inhibit progesterone production by large follicle granulosa cells on day 1 of culture. Therefore, the ability of bovine granulosa cells to respond to hormones was generally independent of follicle size during development from a preantral follicle to the preovulatory follicle. Although no major differences were observed in the hormonal regulation of small, medium, and large follicle granulosa cells, dramatic effects of the hormones were detected in both the induction of potentially important secretory proteins and the stimulation of the steroidogenic activity of the cell. The serum-free

bovine granulosa cell culture used in the current study will aid in elucidating further the regulatory agents and ovarian factors required to maintain and promote granulosa cell functions and differentiation.

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