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Inhibin and Activin Locally Regulate Rat Ovarian Folliculogenesis

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ABSTRACT. The role of inhibin and activin in the initiation of follicular development, growth, and atresia was examined. Human recombinant inhibin (1 μg) was unilaterally injected into the ovarian intrabursal space of 25-day-old rats. The contralateral ovary served as a control. Recruited growing follicles (350–500 μm) were observed 24 h after injection. The accumulation of follicles was greater in the inhibin-treated ovaries than in contralateral control ovaries. Moreover, the size distribution of the follicles was similar to the distribution of follicles recruited by systemic exogenous PMSG treatment. The effect of inhibin plus PMSG on follicular development was not different from that of PMSG treatment alone. Injection of human recombinant activin (1 μg) into the ovarian bursa caused follicular atresia. Activin therapy blocked the follicular development caused by PMSG treatment. The effect of inhibin and activin on follicular development was further characterized by measuring the incorporation of [^{3}H]thymidine into dividing cells. Inhibin enhanced follicular thymidine incorporation, while activin decreased granulosa cell proliferation. Furthermore, receptors for inhibin-A (6.4×10^3 receptors/cell) and activin-A (2.3×10^4 receptors/cell) were identified on granulosa cells. The evidence suggests that inhibin and activin act in a paracrine manner to regulate follicular development, inhibin as a follicular growth signal and activin as an atretagenic signal. (*Endocrinology* **127:** 3196–3205, 1990)

INHIBIN and activin are multifunctional dimeric protein hormones related structurally by a common β-subunit [inhibin-A (α-βA), inhibin-B (α-βB), activin-A (βA-βA), activin-AB (βA-βB), activin-B (βB-βB)] (1, 2). The subunit mRNAs and proteins are produced in diverse tissues, including the gonads, pituitary, brain, bone marrow, placenta, and adrenal (3–9). The inhibin family of hormones was originally described as gonadally derived regulators of pituitary FSH secretion; inhibin suppresses FSH release, while activin stimulates FSH release (reviewed in Ref. 10). Recent evidence suggests that inhibin and activin may have more diverse roles, including the regulation of oxytocin secretion, erythroid differentiation, and GH biosynthesis (6, 7, 11).

The integrative feedback relationship between ovarian inhibin and activin and pituitary FSH has been partially elucidated in the rodent (12–17). The ovary produces low levels of inhibin on the evening of proestrus and morning of estrus, which allows FSH to remain elevated throughout this period (the secondary FSH surge) (12–14, 18–20). The secondary FSH surge recruits a new set of follicles into the ovulatory pool and is responsible for the initiation of inhibin subunit mRNA expression (21). As a consequence of inhibin production, pituitary FSH secretion is down-regulated (14, 20). Inhibin mRNA levels increase in maturing follicles as they progress through the cycle. Follicles that become atretic (nonovulatory and highly steroidogenic) have little or no inhibin mRNA (22–24). Inhibin subunit mRNA accumulation climaxes on the afternoon of proestrus in healthy follicles simultaneously with the primary LH and FSH surges (12). The concurrent elevation of inhibin mRNA levels and serum FSH suggests a phase of the estrous cycle when inhibin is acting independent of FSH regulation. As previously noted, inhibin acts in a classically defined endocrinological manner (pituitary-ovarian axis) during the proestrus to estrus transition. We propose an additional paracrine role (intraovarian) during the transition from metestrus to proestrus. This is supported by experiments conducted *in vitro* showing that inhibin and activin can act on oocytes, granulosa cells, and thecal cells (25–31).

We tested whether inhibin and/or activin were capable of directing follicular development via local ovarian action by unilateral injection of human recombinant inhibin or activin into the intrabursal space of immature

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female rat ovaries. The contralateral ovary served as an internal control. Maturation of follicles was analyzed for morphology, follicular diameter, and mitotic activity in both the hormonally treated ovary and its contralateral control ovary. As additional support for the paracrine and/or autocrine action of these hormones, we investigated the presence and abundance of inhibin-A and activin-A receptors on ovarian granulosa cells.

Materials and Methods

Hormones and reagents

Purified human recombinant inhibin and activin were obtained from Drs. Gene Burton and Charles Schmelzer (Genentech, Inc.). Purification of the hormones has been previously described (32, 33). PMSG and fluorescein isothiocyanate (FITC) were purchased from Sigma (St. Louis, MO). Ketamine HCL (Vetlalar, Aveco Co., Fort Dodge, IA) and xylazine (Gemini, Rugby Laboratories, Rockville Centre, NY) were used for animal anesthesia. [^{3}H]Thymidine was purchased from New England Nuclear (Boston, MA). Tissue culture grade insulin, transferrin, hydrocortisone, fibronectin, penicillin, and streptomycin (Gibco, Grand Island, NY) were used.

Animals

Female Sprague-Dawley rats were obtained from Charles River Laboratories, Inc. (Wilmington, MA). They were housed on a 12-h light, 12-h dark lighting schedule, with free access to laboratory rodent chow and water. The rats used for ovarian injection were 25 days old on the day of treatment. Animals were weighed before surgery and at autopsy. Normal weight gain was noted for each group. Animals used for granulosa cell isolation were 21 days old at the time of autopsy.

Ovarian injections

Before the intrabursal injection, the animals were anesthetized with a combination of ketamine HCl (80 mg/kg) and xylazine (4 mg/kg). The left dorsal lumbar region of each rat was then clipped and prepped with 70% isopropyl alcohol and an iodine scrub solution. A 5- to 7-mm incision was made through the dermal layers caudal to the last rib, followed by a 3- to 5-mm cut into the peritoneum. The ovary was gently exteriorized by grasping the surrounding fat. A pair of straight eye dressing forceps was placed between the left horn of the uterus and the surrounding fat and then allowed to open, presenting the ovary for injection. Approximately 10 μl of the appropriate hormone (1 μg/ovary) were injected intrabursally (ib) caudal to the oviduct through a 28-gauge needle attached to a tuberculin syringe. PMSG (25 IU) was injected ip. [^{3}H] Thymidine (~5 μCi) was coinjected ib unilaterally in one set of animals and bilaterally in a total of four additional animals.

Animals were divided into the following groups: saline (n = 5), PMSG (n = 6), inhibin (n = 8), inhibin plus PMSG (n = 7), activin (n = 7), activin plus PMSG (n = 8), [^{3}H]thymidine in saline (n = 5), [^{3}H]thymidine plus PMSG (n = 5), [^{3}H] thymidine plus inhibin (n = 5), [^{3}H]thymidine, inhibin, and PMSG (n = 5), [^{3}H]thymidine plus activin (n = 5), and [^{3}H] thymidine, activin, and PMSG (n = 5). The total number of animals used in each experimental group is given in parentheses and represents at least two independent repetitions.

For each animal, one ovary was injected with hormone, and the contralateral ovary served as a control. The ovary was then replaced, the incision was closed with two 9-mm wound clips, and the animal was returned to its cage and observed until it had recovered its righting reflex. Necropsy was performed 24 h after treatment. Tissue samples were collected and placed in 4% formalin in preparation for histological analysis.

Cardiac blood samples were collected from each animal and allowed to clot at room temperature. After centrifugation, sera were stored at −20 C until analyzed by RIA.

Histological and image analysis

Ovaries were allowed to fix for 12 h in 10% neutral buffered formalin and embedded in parafin. Three-micron step sections were mounted at 50-μm intervals onto microscope slides. Fifty-micron intervals were chosen based on an average oocyte diameter of 80 μm (Woodruff, T. K., unpublished observation), such that we would be confident that we had analyzed every follicle without redundancy. Sections were stained with hemotoxylin-eosin.

Follicular diameter was determined in ovarian sections quantitatively using a Bioquant image analysis system (R & M Biometrics, Inc., Nashville, TN) or qualitatively by visual inspection. Two measurements were taken at right angles to each other at the level of the granulosa cell bridge to the cumulus and oocyte using the image analysis system or with a calibrated ocular micrometer. To avoid duplication of measurements when quantifying follicular development using the image analysis system, the first section video image was held in computer memory and then juxtaposed over the subsequent section. Follicles that had been measured in one section were not considered in the next section. At least 100 follicles were counted/ovary. Follicles were distributed into 1 of 3 categories: prerecruited follicles (<350 μm), recruited follicles (350–500 μm), and large antral follicles (>500 μm) (21 and 34). Follicles were further characterized as healthy or atretic by 2 criteria: oocyte appearance and health of the granulosa cells (22, 23).

We present the image analysis data from an animal chosen at random from each group. This is representative of the other ovaries analyzed by image analysis (an additional animal per group) or by visual inspection (the remaining three to six ovaries). Statistical comparisons between treated and contralateral ovaries for individual rats were performed using ordered categories for follicular diameter (<350, 350–500, and 500 μm). Statistical computation was aided by Exact Inference for Ordered RXC Contingency Tables (Harvard School of Public Health, Boston, MA).

Ovaries treated with [^{3}H]thymidine were fixed analogously to nonradioactive samples and sectioned at 3 μm. Slides were dipped in Ilford K-2 liquid autoradiographic emulsion (Ilford, Ltd., Mobberley Cheshire, UK), exposed for 2–4 weeks at 4 C, developed, and stained. Photomicrography was performed on a Nikon Microphot-FX microscope (Nikon, Ltd., Japan).

Serum tritium measurement and FSH RIA

Serum tritium levels were measured by scintillation counting. Four animals were used in this assay. Serum FSH concentrations were measured using the rat-rat NIDDK system, with FSH RP-2 as standard and S-11 antirat antibody. Serum samples were measured in triplicate within the same RIA. All statistics were computed using Statview (Abacus Concepts, Inc., Berkley, CA). Values are reported as the mean ± SE.

Granulosa cell culture

The rats were killed by decapitation after anesthesia with CO_2. The ovaries were collected and placed directly in Dulbecco's Modified Eagle's Medium-Ham's F-12 (DMEM:F12; 1:1) plus penicillin and streptomycin for subsequent cell isolation. Ovaries were dissected free of fat, bursa, and oviduct and transferred to DMEM:F12 (1:1) supplemented with 0.5 M sucrose and 10 μM EGTA at 37 C for 45 min (35). The ovaries were then placed in DMEM:F12 (1:1) at 37 C for an additional 45 min. The granulosa cells were manually expressed from the ovaries with the aid of a 25-gauge needle. The cells were washed twice and plated in 4 factor granulosa cell medium [DMEM:F12 (1:1) supplemented with insulin (2 μg/ml), transferrin (5 μg/ml), hydrocortisone (40 ng/ml), and fibronectin (1.5 $\mu g/cm^2$)] (36–37).

Conjugation of inhibin-A and activin-A and fluorescence-activated cell sorting (FACS) analysis

FITC labeling of inhibin and activin was performed by the method of Chatelier *et al.* (38). Briefly, 10 μg inhibin-A or activin-A were mixed with 1 μg FITC and allowed to react for 1 h at room temperature. Unconjugated FITC was removed from the reaction by size-exclusion filtration. The FITC-conjugated hormones were bioactive, as determined by rat anterior pituitary bioassay (39). Granulosa cells (3.6×10^6) were suspended in binding medium (4F granulosa cell medium plus 1 mg/ml BSA) with 2.6 pmol FITC-labeled inhibin-A or 3.6 pmol FITC-labeled activin-A. After incubation at 4 C for 30 min, the cells plus bound ligand were washed twice with PBS-BSA. Cellular fluorescence was measured using a Coulter Elite flow cytometer (Hialeah, FL). Argon ion laser excitation was at 488 nm, and emission was collected using a 525-nm (+/−25) band pass filter. The number of receptors per cell was calculated from a log fluorescence calibration standard curve using beads of known equivalent soluble fluorescent molecules, the average free to bound ratio of the conjugated material, and subtraction of nonspecific binding in the presence of a 100-fold excess of unconjugated ligand. It was necessary to use FITC-conjugated inhibin and FACS analysis because iodination resulted in a loss of the binding capacity of the hormone for its receptor (40). We validated the method using FITC-conjugated activin, for which the number of receptors per granulosa cell has been characterized (41).

Results

The intrabursal delivery of hormone was assessed using crystal violet injection into one ovary, as shown in Fig. 1A. Dye remained in the injected ovary for 24 h. Moreover, gross size differences were seen in the inhibin- and activin-treated ovaries (Fig. 1, *inset*). Ovary I was treated with inhibin-A, ovary A was treated with activin-A, and ovary C was the contralateral control ovary for the activin-treated ovary. The large size of the inhibin-A-treated ovary is apparent, and developing follicles are noted protruding from the right edge of the ovary (*arrow*). The activin-treated ovary is noticeably smaller than the normal prepubertal size of the contralateral control ovary. The inhibin contralateral ovary was similar to the activin contralateral control ovary (not shown). Thus, the hormonal effect was largely confined to the locally treated ovary.

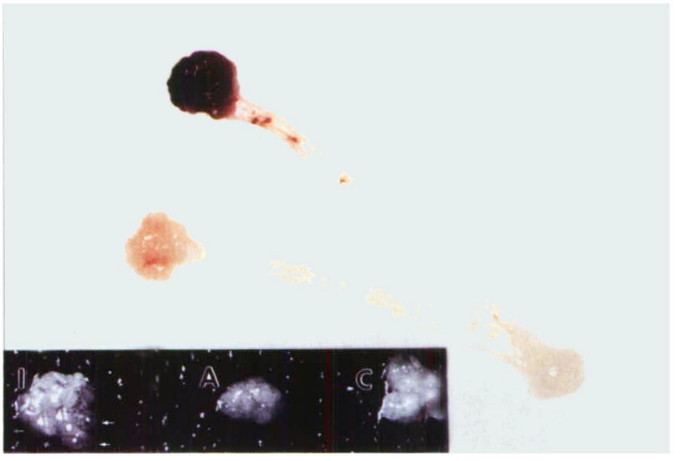


FIG. 1. Unilateral injection of crystal violet dye, inhibin, and activin into the subbursal space of an immature rat ovary. Reproductive tract of a rat after injection of crystal violet dye into one ovary. The animal was killed 24 h later. *Inset*, Ovaries treated with inhibin (I) and activin (A), and the control ovary (C; activin-treated animal). Animals were killed, and ovaries with attached oviducts were removed 24 h after hormone injection. In the inhibin-treated ovary, developing follicles can be seen on the *right side* (*arrow*).

Serum tritium levels were measured as an indication of escape of locally injected [^{3}H]thymidine and hormone. Low levels of tritium (range, 4200–9240 cpm/ml serum) were detected, indicating that less than 1% of the hormone injected into the animal was recoverable in the serum. This was further evaluated by measuring alterations in serum FSH (Fig. 2). The PMSG-treated animals showed significantly lower endogenous FSH levels ($P < 0.002$). This is due to negative feedback inhibition of pituitary FSH secretion via inhibin, as demonstrated by Lee *et al.* (16). Neither inhibin nor activin effectively modulated serum FSH levels in the presence or absence of systemic PMSG ($P > 0.2$ in all cases).

Table 1 shows the percentage of total follicles in each of the three size classes. The size classes represent prerecruited follicles (<350 μm), recruited follicles (350–500 μm), and large antral follicles (>500 μm). One set of ovaries was chosen randomly for quantification. These

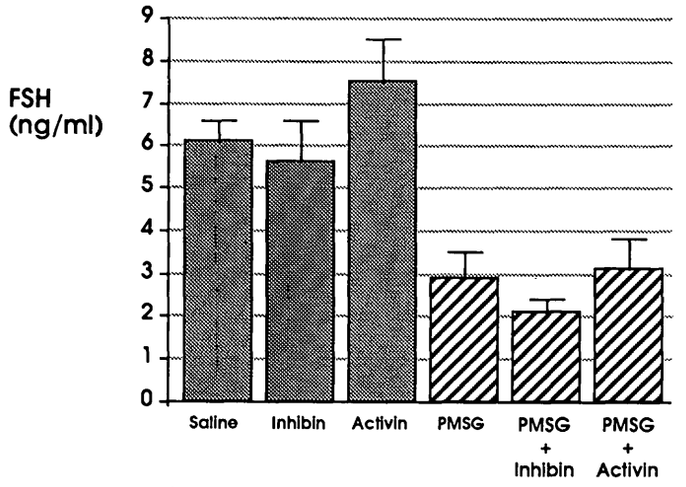


FIG. 2. Serum FSH levels were measured by RIA. Values are presented as the mean ± SEM for eight animals. The rat antibody does not cross-react with mare-derived FSH. Significance was determined for two separate groups: without PMSG and with PMSG. No statistically significant difference was noted, except between saline and PMSG ($P < 0.002$).

TABLE 1. Percent follicular distribution within treatment groups

Treatment group	Follicular diameter		
	<350 μm	350–500 μm	>500 μm
Saline	94.1	2.9	2.9
Inhibin			
Treated	76.6	16.6	6.6
Contralateral	91.8	9.1	0.0
Activin			
Treated	92.7	7.2	0.0
Contralateral	86.8	10.5	2.6
PMSG	67.0	9.0	24.0
Inhibin + PMSG			
Treated	70.7	21.1	8.1
Contralateral	76.8	9.4	13.6
Activin + PMSG			
Treated	95.9	2.4	0.8
Contralateral	76.0	14.8	9.3

Follicular classification was determined by image analysis for saline-treated, PMSG-treated, inhibin-treated, inhibin- plus PMSG-treated, activin-treated, and activin- plus FSH-treated ovaries and the contralateral control for each ovary. One animal was chosen at random to represent each group. Only healthy follicles were counted.

ovaries reflected the distribution and health of follicles in the other animals in their respective groups. The saline-injected ovary represents the untreated developmental distribution of follicles in 25-day-old animals. Small, nonrecruited-preantral or small, antral follicles predominate in the control ovaries. Systemic PMSG treatment caused recruitment and growth of follicles into the large antral size class (>500 μm). Inhibin treatment resulted in growth of follicles primarily into the intermediate size class (350–500 μm; Table 1A and Fig. 3). Inhibin did not cause the follicles to reach the size of

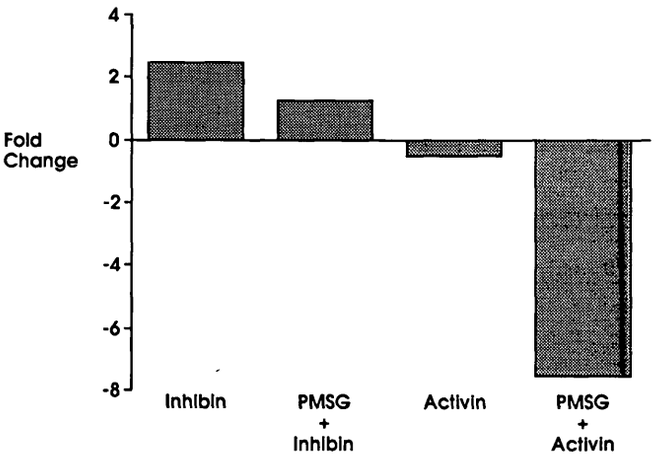


FIG. 3. Fold change in number of follicles in total recruited population (>350 μm) of treated *vs.* contralateral control ovaries.

follicles in PMSG-treated animals. Treatment with activin resulted in minimal follicular development; however, there was an increase in the number of atretic follicles in all size classes (Table 2). Inhibin treatment slightly enhanced PMSG-induced follicular development compared with that in contralateral control (PMSG-treated) ovaries (Table 1). Activin therapy completely blocked the action of systemic PMSG administration, resulting in follicular development similar to that in the saline control ovaries (Table 1). Comparison with the contralateral ovary was used to control for systemic hormonal effects. Statistical analysis of the treated and contralateral ovaries reveals that inhibin and activin plus PMSG treatment significantly alters the follicular development pattern ($P \leq 0.005$), while development after activin and inhibin plus PMSG treatment is not statistically different from that in controls ($P > 0.005$).

Activin-induced atresia was classified by the effects on various aspects of follicular and oocyte development. As shown in Table 2, activin treatment resulted in elevated levels of morphologically defined aspects of atresia in both antral and preantral follicles. Figure 4 shows an activin-treated atretic follicle whose granulosa cell layer is atrophied and oocyte is fragmented. Activin-induced oocyte degeneration is also shown in Fig. 6E. An overall increase in follicular atresia was not seen in the other treatment groups (data not shown).

Follicular development was also monitored by incorporation of [^{3}H]thymidine into growing follicles. Follicles from saline-treated ovaries had low levels of [^{3}H]thymidine incorporation in mural granulosa cells, thecal cells, and cells of the discus proligerus (cumulus and adjoining cells; Fig. 5, A and B). Not all follicles in this group were labeled. PMSG treatment resulted in a stimulation of follicular growth, with concomitant enhancement of [^{3}H] thymidine incorporation into dividing granulosa and thecal cells (Fig. 5, C and D).

TABLE 2. Percent follicular abnormalities in atretic follicle population

Treatment group	Oocyte degeneration	Thin granulosa cell layer	Beaded basement membrane	Lacy antral granulosa cells	Pyknotic nuclei
Antral follicles (>350 μm)					
Activin					
Treated	18.1	6.0	6.0	6.0	6.0
Contralateral	6.4	3.2	3.2	6.4	0
Activin + PMSG					
Treated	1.2	2.4	2.4	4.9	1.2
Contralateral	1.0	0	0	5.6	0
Preantral follicles (<350 μm)					
Activin					
Treated	6.8	0	2.4	1.7	0
Contralateral	7.1	0	0	0	0
Activin + PMSG					
Treated	1.8	3.7	1.8	0	0
Contralateral	0	0	0	0	0

Morphological levels of atresia for a representative activin-treated and the contralateral ovary, and an activin- plus PMSG-treated and the contralateral ovary for recruited and nonrecruited follicles.

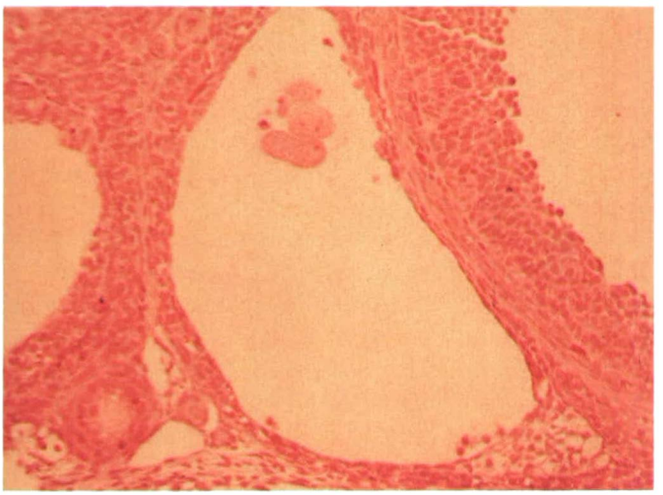


FIG. 4. Thin section analysis of granulosa cell layer of activin-treated ovary. The granulosa and thecal cell layers are visibly atrophied, and the oocyte is highly fragmented.

Injection of inhibin or inhibin plus PMSG resulted in a pattern of labeling similar to that found with PMSG treatment alone (Fig. 6, A–D). The atretic activin-treated follicles showed little or no [^{3}H]thymidine incorporation into granulosa or thecal cells (Fig. 6, E and F). Activin plus PMSG therapy resulted in follicles that displayed some morphological signs of atresia and whose thymidine incorporation was reduced to saline-injected control levels (Fig. 6, C and H).

Because of the dramatic effect of inhibin and activin on follicular development, it was of interest to identify the number of receptors for inhibin and activin on granulosa cells. FACS analysis of FITC-conjugated inhibin-A or activin-A bound to granulosa cells showed 6.4×10^3 receptors/cell for inhibin-A and 2.3×10^4 receptors/cell for activin-A (Fig. 7, *top panel*). Both peaks were competed by a 100-fold excess of unconjugated inhibin (Fig. 7, *bottom panel*) or activin (data not shown), respectively. Neither inhibin nor activin was able to compete for binding of the activin receptor or inhibin receptor, respectively, at a 100-fold excess of competitor. The data are representative of multiple repetitions of binding of inhibin or activin to granulosa cells.

Discussion

Inhibin and activin were originally identified as gonadally derived peptide hormones that modulate FSH secretion from the anterior pituitary (for reviews, see Refs. 1 and 10). As functional antagonists produced by the differential assembly of common subunits, the modulation of inhibin subunit mRNA expression and the subsequent protein production during the rat estrous cycle are of great interest. We have examined the modulation of ovarian inhibin subunit mRNA levels, ovarian protein content, and circulating inhibin levels and have correlated these findings to FSH secretion. Notably, serum inhibin levels do not reflect the dramatic rise in ovarian inhibin content or in inhibin subunit mRNA levels during the metestrus to proestrus transition. During the primary FSH surge, ovarian inhibin content and mRNA subunit levels are at their highest levels. Furthermore, follicular health is coincident with expression of inhibin mRNAs. Taken together, this evidence suggests that inhibin and activin might have a role in the local regulation of follicular development. To address this question, we injected recombinant inhibin and activin directly into the ovary of immature female rats in the presence or absence of systemic PMSG and report major effects on follicular development and cell proliferation. We support these findings with the identification of specific receptors on the granulosa cells for both of these hormones.

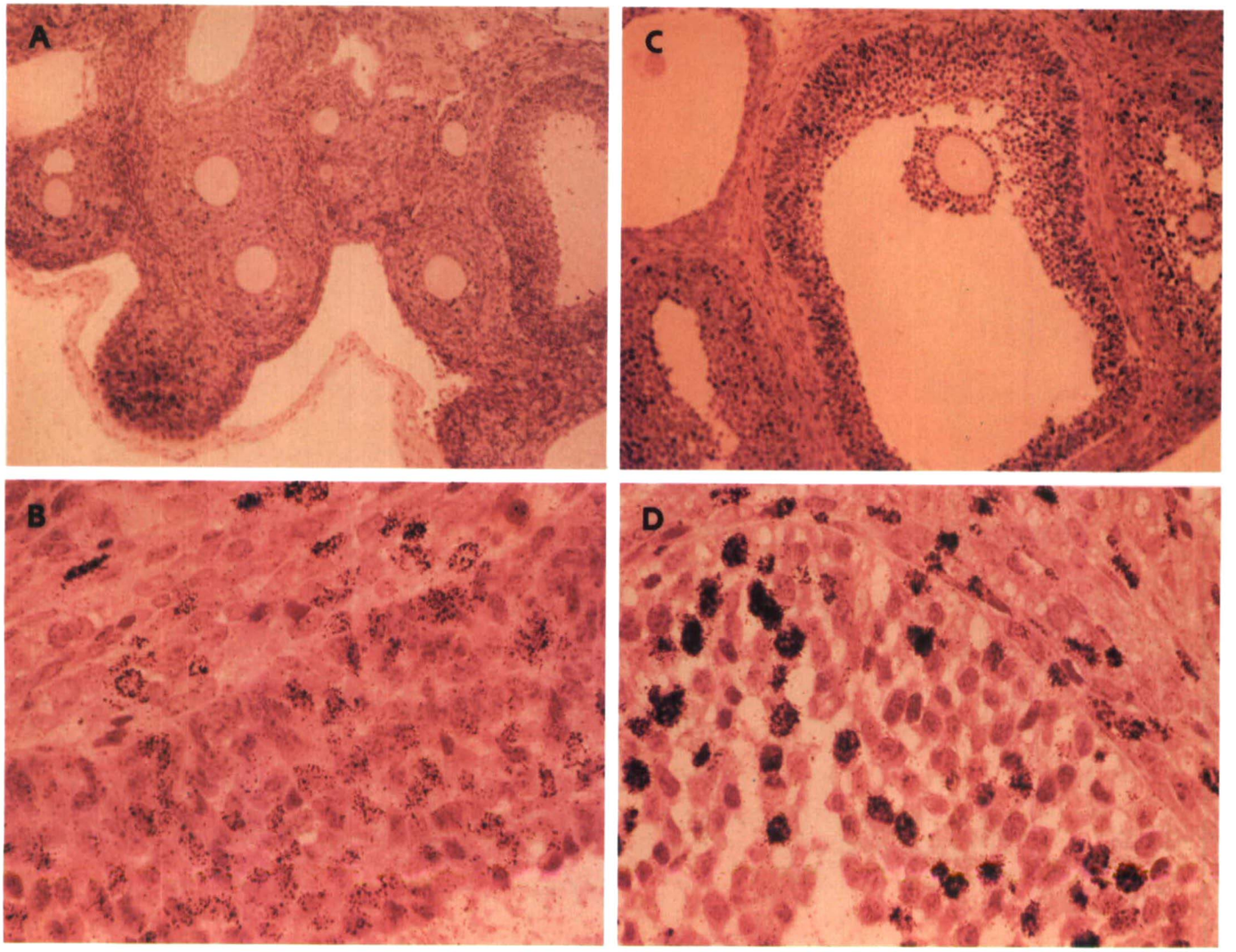
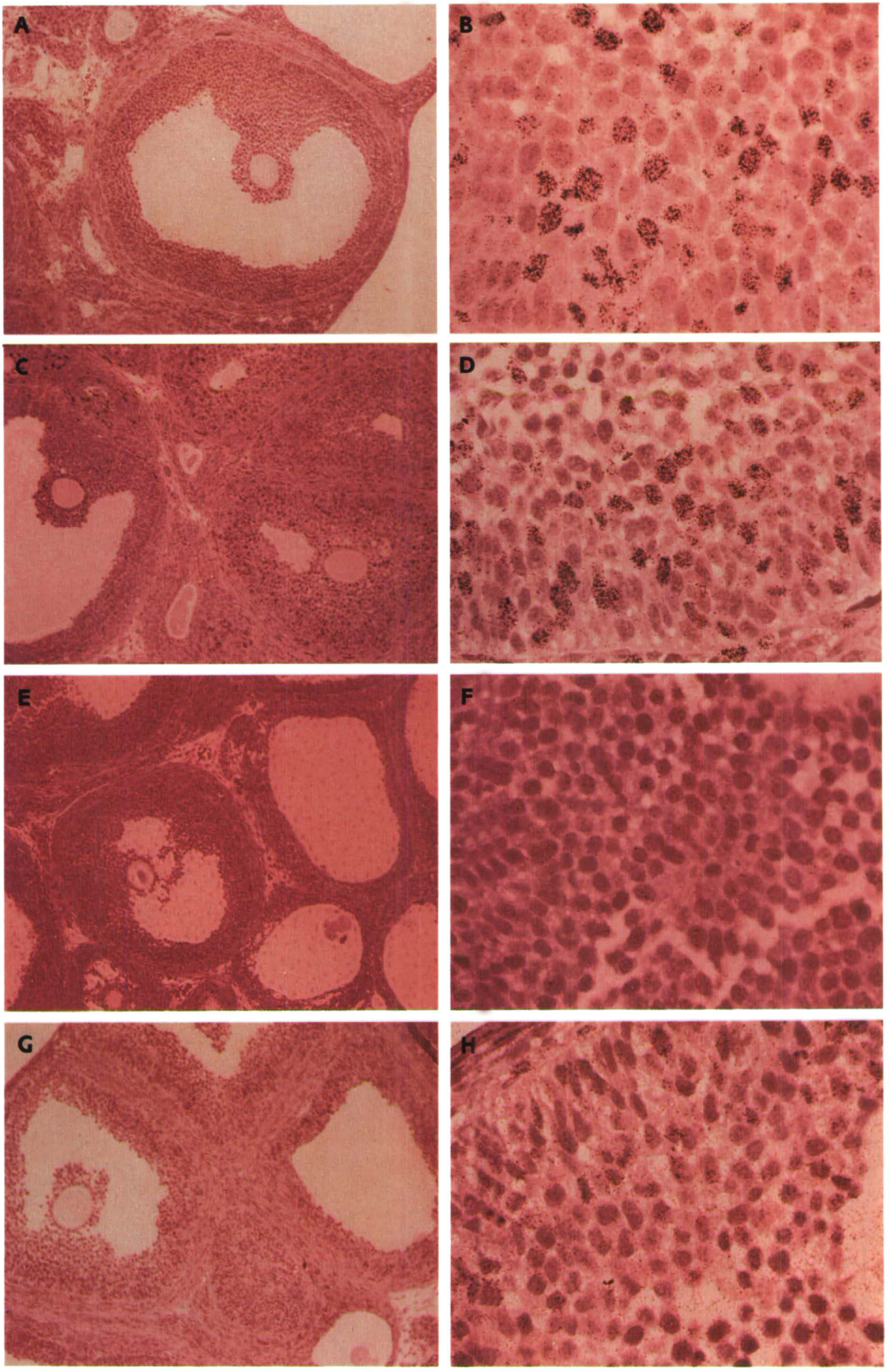


FIG. 5. Thin section analysis of follicular growth and [^{3}H]thymidine incorporation into cells of control rat ovaries. A and B, Saline-treated ovary; C and D, PMSG-treated ovary. Row 1 photographs are at ×100 magnification. The relative sizes of the follicles can be seen. Row 2 photographs are at ×500 magnification. Granulosa cells, thecal cells, and individual silver grains are visible at this magnification. Preantral and small antral follicles (<350 μm size class) are seen in A. The ovarian bursa is also distinct in this section. A large antral follicle (>500 μm) is seen is C.

The model used to analyze the ovarian role of inhibin and activin has four important features. First, the intact immature animal is not under the cyclic influence of LH, FSH, estradiol, and progesterone. We were, therefore, able to manipulate the hormonal environment exogenously while minimizing the influence of endogenous hormones. Secondly, because inhibin and activin affect a variety of other organs, we created an essentially closed system by using intrabursal injection. Third, by using [^{3}H]thymidine as a marker for ovarian mitotic activity, we were also able to verify the efficacy of the delivery system. Lastly, the effect of endogenous FSH or other circulating hormones on follicular development was controlled internally by comparing treated ovaries with the contralateral control ovary. Thus, any alteration in follicular development that we demonstrate between treated and contralateral control ovaries is the result of local inhibin or activin administration and not gonadotropin-dependent events.

We found that inhibin influenced folliculogenesis by causing growth of follicles into the recruited (350–500 μm) size class. We presume that this is a direct effect on granulosa cells based on our finding of approximately 2.3 × 10^3 inhibin receptors/cell. We cannot exclude the possibility that inhibin acts on or binds to other cells of the ovary which may produce factors mediating the effect on follicular growth. The increase in follicular diameter is similar to that found in the adult rat during the estrus to metestrus transition (12). Thus, the addition of inhibin to the immature rat ovary causes a follicular response similar to adult follicular growth (this report). The exogenous inhibin may replace the secreted inhibin in



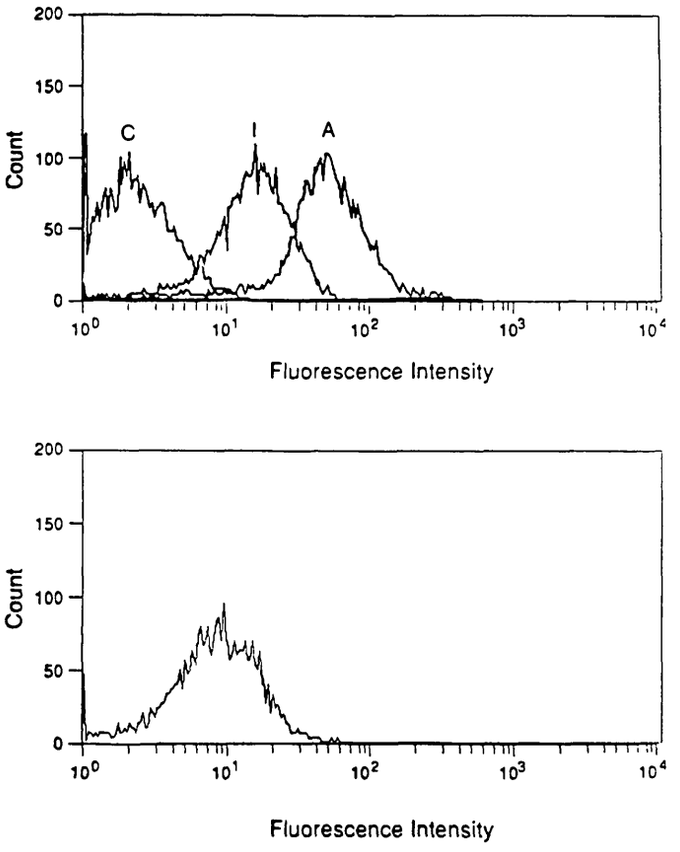


FIG. 7. FACS analysis of relative amount of FITC-conjugated inhibin or activin binding to granulosa cells. *Top panel*, Peak C (control) autofluorescence of granulosa cells; peak I, granulosa cells labeled with inhibin-A-FITC; peak A, granulosa cells labeled with activin-A-FITC. *Bottom panel*, Granulosa cells labeled with inhibin-FITC preincubated with a 100-fold excess of nonconjugated inhibin. The peak has shifted to the left, indicating 70% competition (log scale). Similar competition for activin was noted (data not shown).

developing follicles in the adult (42–44). We propose the following FSH-inhibin interactive model for folliculogenesis. The secondary FSH surge is sufficient for follicular selection (45) and initiating inhibin expression (21). As a consequence of inhibin subunit mRNA translation (14), the protein is available to drive follicular development in a paracrine manner from metestrus through proestrus. This results in the maintenance of a follicle in the ovulatory pool.

The rigorous maintenance of a certain number of follicles (12 in the rat and 1 in the human) probably involves a specific time course of expression and activation of many hormones and growth factors. The cyclic recruitment of follicles is necessary to allow delivery of the oocyte at an appropriate time for fertilization. In our observations of inhibin-stimulated follicular development, we noticed a high incidence of internal or medullary large follicles. Whether these follicles would continue to grow and develop in a normal manner after extended treatment with inhibin and result in successful ovulation is being investigated.

Reciprocally, activin caused an advancement of atresia. We cannot directly address this phenomenon mechanistically at this time, but, as with inhibin, we suggest that one way in which activin may act is to bind directly to the over 20,000 activin receptors/cell. We again cannot exclude extragranulosal binding and action. As detailed earlier, activin is known to be a functional antagonist to inhibin in regulating pituitary FSH secretion, hematopoesis, and corticosterone production (6–8, 46). Therefore, a similar antagonistic action may be operative in directing folliculogenesis.

The process of follicular selection and atresia has been investigated by Hirshfield in the immature animal (34, 47). She suggests that atresia in large follicles is caused by the decline in FSH levels on metestrus. We would extend this observation by suggesting that these follicles are also responding to elevated levels of activin, which ultimately lead to their demise. Whether the activin is produced in the follicles itself or synthesized by interstitial tissue (43) is not known. Furthermore, investigations in the immature rat by Davis *et al.* (48) showed that inhibin levels reflected follicular maturation. Based on our studies, we suggest that inhibin is also intimately involved in driving follicular development toward the first ovulation (48).

The direct binding of inhibin-A to granulosa cells has not been shown previous to this report. However, activin-A has been shown to bind the human leukemia cell line K562 with approximately 600 binding sites/cell (49). Additionally, Sugino *et al.* (41) have described the binding of erythroid differentiation factor (or activin-A) to granulosa cells. They report 1.3×10^4 sites/cell, which is similar to our finding of 2.3×10^4 receptors/cell.

LaPolt *et al.* (50) have recently demonstrated that activin has a direct effect on inhibin production and mRNA levels. In the present experiment we have no way of controlling for endogenous inhibin or activin, nor can we control for the additional production, secretion, or action of the hormones produced locally. Additionally, a plethora of regulatory factors must exist and be expressed in a timely fashion to ensure normal ovulation. These

FIG. 6. Thin section analysis of follicular growth and [^{3}H]thymidine incorporation into cells of treated rat ovaries. A and B, Inhibin-treated ovary; C and D, PMSG- plus inhibin-treated ovary; E and F, activin-treated ovary; G and H, PMSG- plus activin-treated ovary. Column 1 is photographed at ×100 magnification, and column 2 is photographed at ×500 magnification. Follicular size and follicular health can be seen at the lower magnification. The abundance of silver grains representing [^{3}H]thymidine incorporated into DNA are seen over nuclei at the higher magnification. The follicles are in the following size classes: A, large antral follicle (>500 μm); C, large antral follicle (9 o'clock; 350–500 μm) and antral follicle (3 o'clock; 350–500 μm); E, antral follicle (*center*; 350–500 μm), atretic follicle (5 o'clock; 350–500 μm), preantral follicle (6 o'clock; <350 μm), and small antral follicle (7 o'clock; <350 μm); G, antral follicle (9 o'clock; 350–500 μm) and atretic follicle (5 o'clock; <350 μm).

are all important parameters that will be examined in future experiments.

In conclusion, we have defined novel regulatory roles for inhibin and activin in the ovary as a follicular maturation factor (inhibin) or inhibitor (activin) and suggest that further studies should consider the important local actions of these hormones. Moreover, studies involving the systemic effects of inhibin and activin therapy should not neglect the effects of altered follicular growth (thereby alterations in ovarian steroid and peptide hormone production) in the interpretation of systemic responses.

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