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Decreased Inhibin Gene Expression in Preovulatory Follicles Requires Primary Gonadotropin Surges*

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ABSTRACT. We have examined the role of the primary gonadotropin surges in regulating inhibin α- and β_A-subunit mRNA levels in rat ovarian follicles. Inhibin subunit mRNA levels decline dramatically on the evening of proestrus in follicles of the ovulatory pool. Because this decline is temporally associated with primary gonadotropin surges, we investigated the contribution of LH and FSH to this process. The primary gonadotropin surges were blocked by injection of a GnRH antagonist (WY45760) at 1200 h on proestrus. This resulted in sustained elevation of inhibin mRNA levels through 0700 h of the subsequent day, a time when inhibin mRNA levels would normally be very low. Replacement of either exogenous LH or FSH in ovulatory doses to an antagonist-treated animal at 1530 h on proestrus resulted in a decrease in inhibin mRNA levels by 4–5 h postreplacement. We conclude that LH and FSH act via a common mechanism to repress inhibin mRNA levels in stimulated preovulatory follicles. (*Endocrinology* **124:** 2193–2199, 1989)

THE STIMULATION of mature follicles, ovulation, and recruitment of new follicles require precise interactions between hormones and target cells of the hypothalamic-pituitary-gonadal axis. Inhibin is a major regulator of pituitary FSH secretion and, thus, is an integral part of this reproductive network (1–6). We have previously shown that inhibin α- and β_A-subunit mRNA levels decline on the evening of proestrus between 1600 and 2400 h (7). It is during this same time that the primary gonadotropin surges are involved in inducing events leading to ovulation. Loss of cumulus-oocyte gap junctions, germinal vesicle breakdown, dramatic alterations in vascularization, an increase in plasminogen activator, decreases in the steroid enzymes $P450_{17\alpha}$ and the cAMP-dependent protein kinase subunits, and an increase in cytochrome $P\text{-}450_{ssc}$ enzyme are some of the events associated with the dramatic alterations in the follicle on the evening of proestrus (8–11). We have examined inhibin gene expression during this period of changing follicular structure and function.

Levels of ovarian inhibin mRNA vary with the maturational status of the antral follicle (7). Both α and β_A mRNA's are detectable in follicles committed to the ovulatory pool on the morning of estrus (12). As they progress to large Graafian follicles, the levels of inhibin mRNAs increase dramatically. By the afternoon of proestrus, maximal levels of inhibin subunit mRNAs are attained in mature preovulatory follicles. During this same period, the hypothalamic hormone GnRH stimulates secretion of pituitary gonadotropins (13). LH and FSH then act in concert to stimulate mature follicles and trigger events that will lead to ovulation and luteinization. After stimulation of preovulatory follicles on the evening of proestrus, inhibin mRNA levels drop precipitously.

We investigated the precise timing of the decline in inhibin mRNA levels on the evening of proestrus in intact animals by measuring inhibin α and β_A mRNAs using *in situ* hybridization. We then blocked primary gonadotropin surges with a GnRH antagonist and analyzed inhibin mRNA levels to test the role of gonadotropins in controlling the decrease in inhibin mRNA levels during late proestrus. Aditionally, antagonist-treated animals received replacement therapy of either LH or FSH so that the independent contributions of the two gonadotropins to the regulation of inhibin gene expression could be identified.

Materials and Methods

Animals

Adult (60-day-old) female Sprague-Dawley CD rats were obtained from Charles River (Portage, MI). Animals were maintained on a 14-h light, 10-h dark schedule (lights on, 0500

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h) in temperature-controlled quarters. Laboratory chow and water were provided *ad libitum*. Cycle stage was determined by daily inspection of vaginal cytology. Animals were used after at least two consecutive 4-day estrous cycles.

Experimental groups and hormones

Proestrous animals were divided into four experimental groups: group I, untreated control; group II, GnRH antagonist plus vehicle (Antag/Veh); group III, GnRH antagonist plus LH (Antag/LH); and group IV, GnRH antagonist plus FSH (Antag/FSH). Groups II, III, and IV received 100 μg GnRH antagonist at 1200 h on proestrus. The GnRH antagonist WY45760 ([Ac-β(2)-D-Nal[1],4-F-D-Phe[2],D-Trp[3],D-Arg[6]]LHRH) was obtained from Wyeth Laboratories (Philadelphia, PA). The antagonist (100 μg) was suspended in sesame oil (0.25 ml) and injected sc. At 1530 h on proestrus, rats received the following injections: group II, saline; group III, LH; and group IV, FSH. Ovine (O) LH (G3-330BR) and oFSH (G4211B) were kindly provided by Dr. Harold Papkoff, University of California, San Francisco. The LH and FSH preparations contain less than 0.1% contamination of FSH or LH, respectively. LH (16 μg) and FSH (10 μg) were dissolved in sterile saline (0.25 ml) and injected into the tail vein under light ether anesthesia. These ovulatory gonadotropin doses resulted in full stimulation of mature follicles, recruitment of a new set of follicles, ovulation, and luteinization by 0700 h on estrus in antagonist-treated animals (Table 1). The total number of animals used per group (n) is given in *Results* and was obtained by multiple repetitions of experiments.

Autopsy and collection of tissue

Animals were decapitated at 1830, 1930, 2030, and 2400 h on proestrus and at 0700 h on estrus. Ovaries were immediately excised, mounted on their oviducts on dry ice, and stored at −70 C. One liver lobe was also removed, frozen on dry ice, and stored at −70 C. Trunk blood was collected and allowed to clot overnight at 4 C. Whole blood was centrifuged, and serum was collected, aliquoted, and stored at −20 C for subsequent RIA.

In situ hybridization

In situ hybridization to ovarian sections has been described previously (7, 14). Briefly, 20-μm frozen sections from one ovary per animal were obtained using a Reichert 840 cryostat (Buffalo, NY). Sections were mounted alternately between two microscope slides and fixed in 5% paraformaldehyde. [^{32}P] UTP-labeled antisense and sense (control) α and β_A inhibin riboprobes were prepared and hybridized to the sections at 42 C for 18 h. After RNAse treatment and washing, slides were exposed to Kodak XAR x-ray film and Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY). After development, the sections were stained with hematoxylin and eosin for histological analysis. Photography was performed on a Nikon Optiphot microscope. Background was assessed by analyzing ovarian sections hybridized with sense probes and liver sections hybridized with antisense probes. Sections were analyzed using an IM 3000 image analysis system (Analytical Imaging Concept, Irvine, CA) and a Dage model 70 camera (Michigan City, IN). Grains were counted at ×1125 magnification in an area of 3266.61 μm^2 over the granulosa cell layer.

Hormone RIA

Serum was assayed for LH by the ovine-rat RIA using NIH LH-S16 as standard and S-10 antirat antibody. Serum FSH concentrations were measured using the rat-rat NIDDK system with FSH RP-1 standard and S-11 antirat antibody. The oLH, but not oFSH, used for replacement was detectable in the respective RIAs used. For both LH and FSH, serum samples were measured at various dilutions within the same RIA. The intraassay coefficients of variation were 2% and 3% for LH and 1% and 2% for FSH for low and high pools, respectively. Two-way analysis of variance (ANOVA) was performed to determine the effects of treatment (untreated *vs.* Antag/Veh *vs.* Antag/LH *vs.* Antag/FSH) and time of autopsy (1830 *vs.* 1930 *vs.* 2030 *vs.* 2400 *vs.* 0700 h) on serum FSH and LH concentrations. All statistics were computed using CRISP (Crunch Interactive Statistical Package, Crunch Software, San Francisco, CA). Values are reported as the mean ± SE.

Results

Animals were killed at the times indicated in Table 1. Intact animals (group I) analyzed on the evening of the proestrus displayed typical serum LH and FSH concentrations (Fig. 1). LH concentrations were high at 1830 h on proestrus (24.1 ± 8.4 ng/ml) and had returned to basal levels by 2400 h on proestrus (2.15 ± 1.36 ng/ml). FSH remained elevated throughout the course of the experiment (1830 h, 369.8 ± 61 ng/ml; 0700 h, 427.0 ± 47.6). Dispersal of the cumulus cells within ovarian follicles, an early indication of follicular stimulation by LH and/or FSH, was observed between 1830 and 1930 h proestrus in these animals (Table 1). Inhibin α and β mRNA levels were high in mature follicles that had not been stimulated

TABLE 1. Summary of autopsy time, follicle type, and hybridization to inhibin α- and β_A-subunit probes

	1830 h	1930 h	2030 h	2400 h	0700 h
Intact	++/SSF	−/SF	−/SF	−/SF	+/NR;−/CL
Antag	+++/LF	+++/LF	+++/LF	+++/LF	+++/LF
Antag/LH	++/LF	++/SSF	+/SF	+/SF	+/NR;−/CL
Antag/FSH	++/LF	++/LF	+/SF	+/SF	+/NR;−/CL

+ or − indicates relative levels of inhibin α and β_A hybridization in the follicle class indicated. +++, Very intense hybridization; ++, intense hybridization; +, little hybridization; −, hybridization not greater than background. Follicle class was determined by visual inspection of autoradiographic films and exposed slides, and size classification. SSF, Slightly stimulated follicle; SF, stimulated follicle; NR, newly recruited follicle; CL, fresh corpora lutea; LF, large follicle.

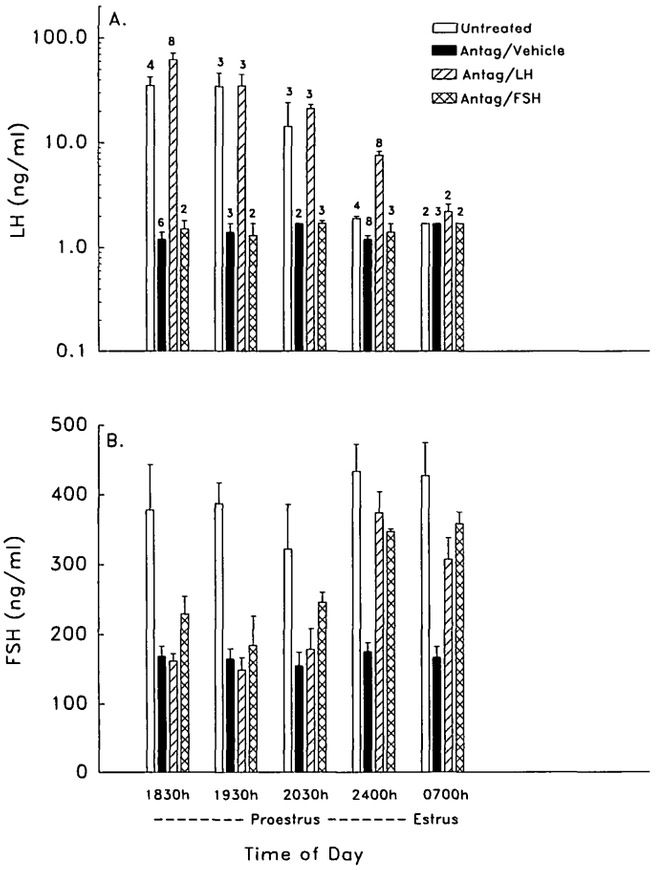

FIG. 1. Serum gonadotropin concentrations. Serum LH and FSH values are given for intact (group I), Antag/Veh (group II), Antag/FSH (group III), and Antag/LH (group IV) animals. Animals were killed at the times indicated. The numbers in A are the total number of animals per group. Values are given as the mean ± SE By two-way ANOVA: FSH, treatment group ($P < 0.0001$) and kill time ($P < 0.0015$); LH, treatment group ($P < 0.0025$) and kill time ($P < 0.0289$).

by gonadotropins (1830 h) and were dramatically lower in follicles that had received gonadotropin stimulation (1930, 2030, and 2400 h; Table 1 and Fig. 2). Fresh corpora lutea were detected on the following morning (0700 h on estrus) and did not hybridize above background levels to the inhibin probes. Background levels were assessed by hybridizing sense probes to ovarian sections or hybridizing antisense probes to liver sections (Fig. 3). Newly recruited follicles (>350 μm) expressing both inhibin mRNAs were also detected at this time.

In Antag/Veh animals (group II) the preovulatory increase in both serum LH and FSH was suppressed (Fig. 1). Unlike follicles observed in untreated rats, follicles from Antag-treated animals remained large and showed no histological signs of stimulation 19 h after injection (0700 h on estrus). Significantly, the levels of inhibin mRNA remained elevated at all autopsy times analyzed (Table 1 and Fig. 2). Neither newly recruited follicles, nor fresh corpora lutea were observed at 0700 h on estrus in Antag/Veh-treated animals (Table 1).

The contribution of the individual gonadotropins to lowering inhibin mRNA levels was determined by a replacement experiment. Animals treated at 1200 h on proestrus with the GnRH antagonist received exogenous oLH (group III) or oFSH (group IV) in ovulatory doses at 1530 h on the same day. Serum LH concentrations were elevated at 1830 h on proestrus in LH-treated rats and then declined in a pattern similar to that observed in intact animals (Fig. 1). There was no change in serum LH after the injection of oFSH. The antibody used to detect serum FSH does not cross-react with the oFSH used for replacement (15). We, therefore, measured the contribution of endogenous FSH only. We found that endogenous FSH increased in both oLH-treated animals (2400 h) and oFSH-treated animals (2030 h), confirming the ability of the gonadotropins to restore the secondary FSH surge (Fig. 1).

Both LH and FSH was capable of causing follicular stimulation, ovulation, and recruitment of new follicles by 0700 h on the following morning (Table 1). Importantly, either LH or FSH caused a decline in inhibin mRNA levels between 1930 and 2030 h proestrus (4 h after replacement injections) in the mature follicles (Table 1 and Fig. 4).

Relative inhibin mRNA levels in selected follicles were quantitated by densitometric analysis of sections after autoradiography. Silver grains were counted in three nonoverlapping fields from the same follicles shown in Figs. 2 and 4, column 1, and the data are summarized in Table 2. ANOVA indicated that treatment groups and autopsy times both showed significant differences; the interaction between group and time was also significant due to the failure of the Antag/Veh group to show grain depletion at 2400 h (Table 2). The underlying SD of the measurement, calculated from the within-group variance, indicates that the inherent error of measurement within a follicle is ±256 grains/3286.6 μm^2.

Discussion

We have previously shown that inhibin mRNA levels increase during the early portion of the estrous cycle and then quickly decline on the evening of proestrus. This study focused on the decline in inhibin mRNA levels during late proestrus. By following inhibin mRNA levels throughout the evening of proestrus we have shown that the decrease in inhibin mRNAs is a precisely timed process. Inhibin mRNA levels decline between 1830 and 1930 h on proestrus. This time period corresponds to surging LH concentrations, rising FSH levels, and the stimulation of preovulatory follicles. When the LH and FSH surges were blocked by a GnRH antagonist, inhibin mRNA levels remained elevated well into the following morning. This result demonstrates that the primary gonadotropin surges are important in directing the decline in inhibin levels on the evening of proestrus. We further

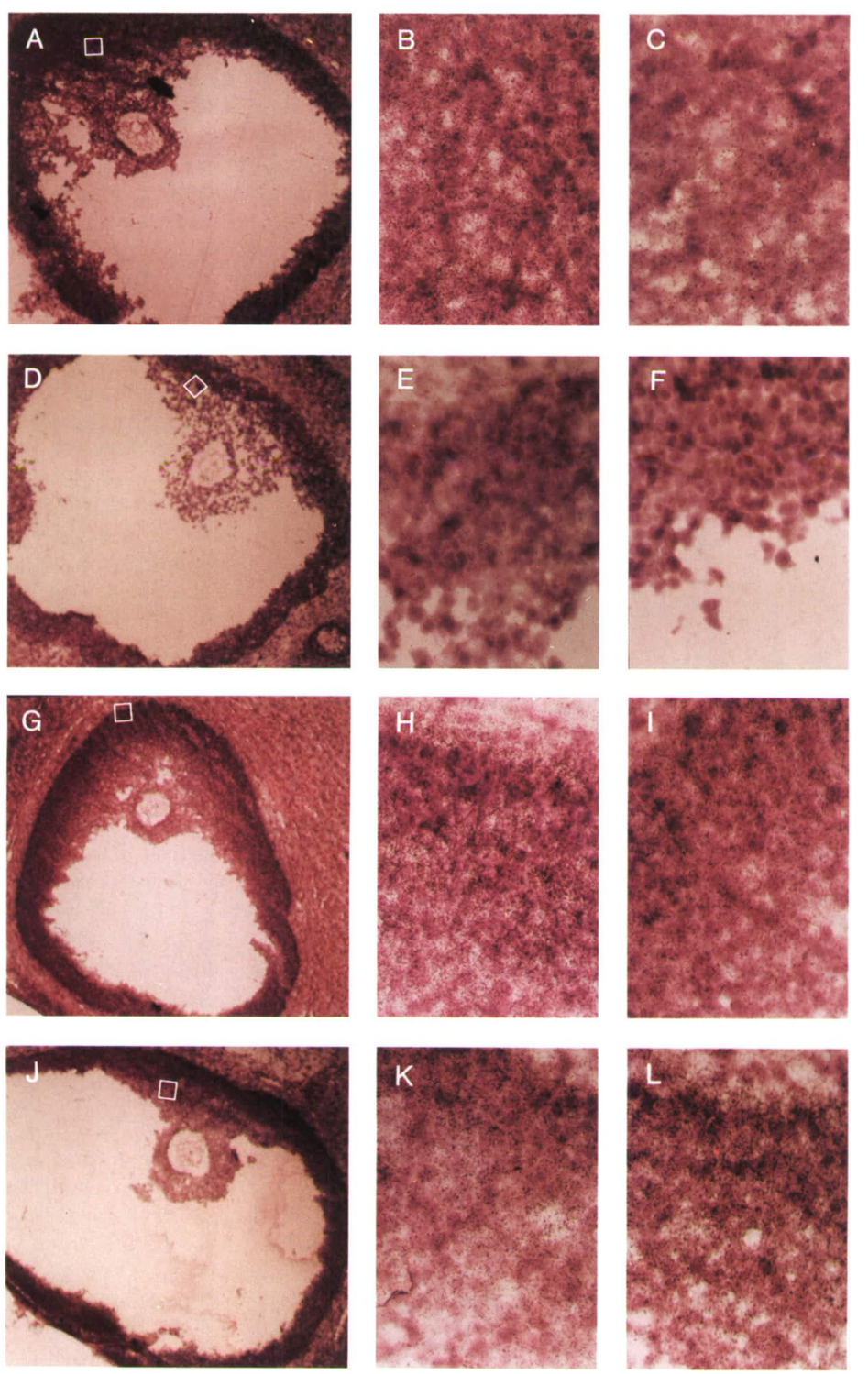

FIG. 2. *In situ* hybridization analysis of follicles in the intact and Antag/Veh-treated ovaries. Column 1 is a ×100 magnification showing the entire follicle. Columns 2 and 3 are ×500 magnifications of the granulosa cells probed with the inhibin α- or β_A-subunit probes, respectively. A–C, Intact, 1830 h on proestrus; D–F, intact, 2400 h on proestrus; G–I, Antag/Veh, 1830 h on proestrus; J–L, Antag/Veh, 2400 h on proestrus.

tested this finding by restoring ovulatory doses of LH or FSH to animals treated with the GnRH antagonist. Using either gonadotropin, inhibin levels decreased to near control levels within 5 h after gonadotropin replacement. This observation suggests that LH and FSH initiate similar events, leading to the decline of inhibin mRNA levels on the evening of proestrus.

Further support of an inverse causal relationship be-

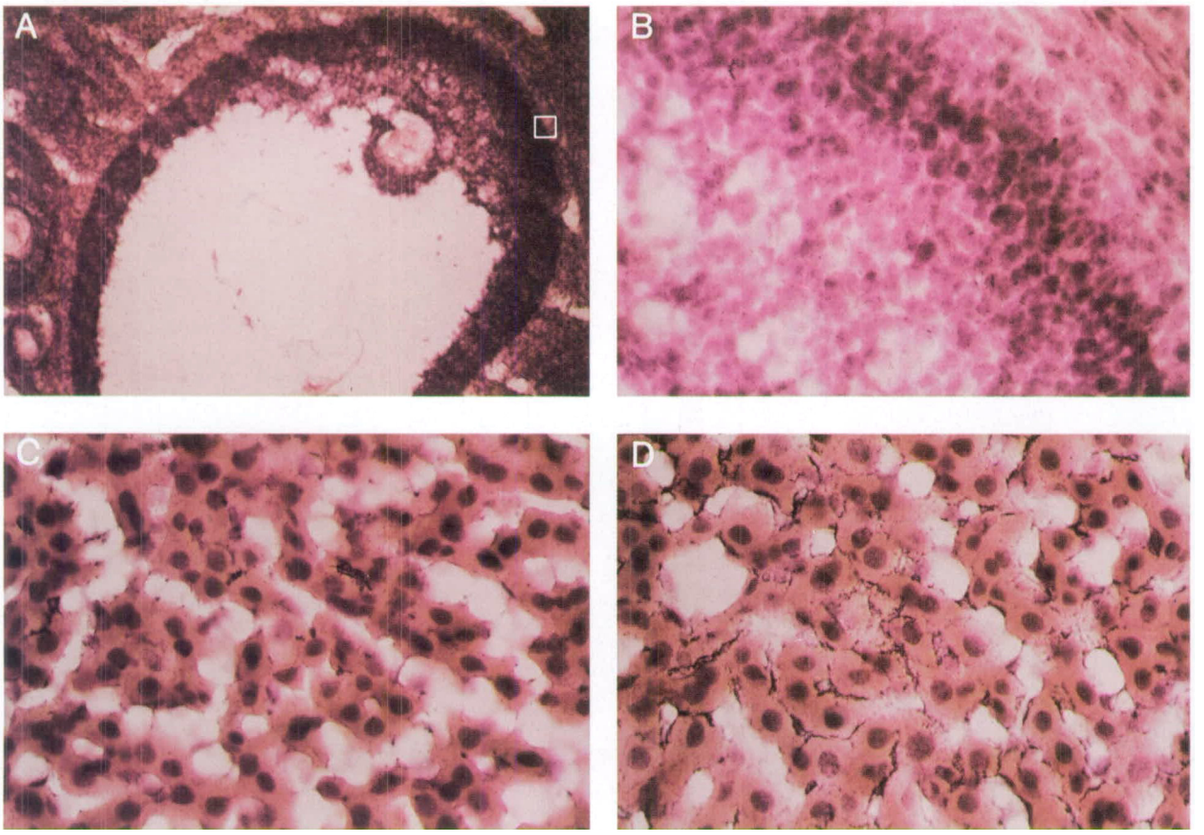

FIG. 3. *In situ* hybridization controls. A and B show hybridization of a sense inhibin probe to ovary sections at ×100 and ×500 magnifications, respectively. C and D show hybridization of antisense α and β_A inhibin probes, respectively, to liver sections (×500 magnification).

tween inhibin mRNA levels and FSH is the increase in endogenous serum FSH levels in Antag-treated gonadotropin-replaced animals. As has been previously reported (15), we found an increase in endogenous serum FSH by 2030 h in Antag/FSH-replaced animals and by 2400 h in Antag/LH-replaced animals. We infer that FSH levels and serum inhibin levels must be precisely regulated, such that serum FSH levels rise 2–3 h after the decline in ovarian inhibin mRNA levels. The importance of inhibin in regulating FSH levels has been demonstrated by a number of physiological observations. First, after bilateral ovariectomy, serum FSH concentrations increase dramatically and cannot be lowered to basal levels by steroid hormone treatment (16, 17). We have shown that inhibin subunit mRNAs are present in the rat ovary throughout the estrous cycle (7). The loss of ovarian inhibin mRNA after gonadectomy, and therefore inhibin feedback, could account for the rapid rise in serum FSH. Furthermore, this suggests that extragonadal sources of inhibin (18) are not major regulators of pituitary FSH secretion because of their inability to lower circulating FSH in gonadectomized rats. Secondly, in the unilaterally ovariectomized rat, serum FSH concentrations increase and return to basal levels 24 h later (19, 20). This transient rise in FSH is responsible for both recruitment of a new cohort of follicles and enhanced expression of the inhibin subunit mRNAs (12). The translation products of this inhibin mRNA presumably act on the pituitary to cause the decline in serum FSH levels observed by 24 h after unilateral ovariectomy. Finally, in untreated animals, serum FSH remains elevated through the morning of estrus (21). The secondary FSH surge is GnRH independent and returns to basal levels after recruitment of a new set of follicles (22, 23). We have shown that inhibin mRNA levels decline between 1830 and 1930 h on proestrus; presumably, this is followed by a decrease in the secretion of inhibin. The subsequent lack of ovarian inhibin feedback would allow FSH to remain elevated through the morning of estrus, the time at which a new set of follicles is recruited. A recent report (25) suggests that the β_A-subunit mRNA reappears at 0200 h of estrus, at a time when α-subunit mRNA is low. However, we have not observed expression of either inhibin mRNA at 0200 h estrus in gonadotropin-stimulated preovulatory follicles.

While further studies will be necessary to resolve the interactive mechanisms by which inhibin and FSH biosynthesis are regulated, we propose the following regulatory model. The secondary FSH surge between proestrus and estrus recruits follicles into the ovulatory pool. These follicles express inhibin subunit mRNA, and serum inhibin levels rise (24). Inhibin then acts to restore

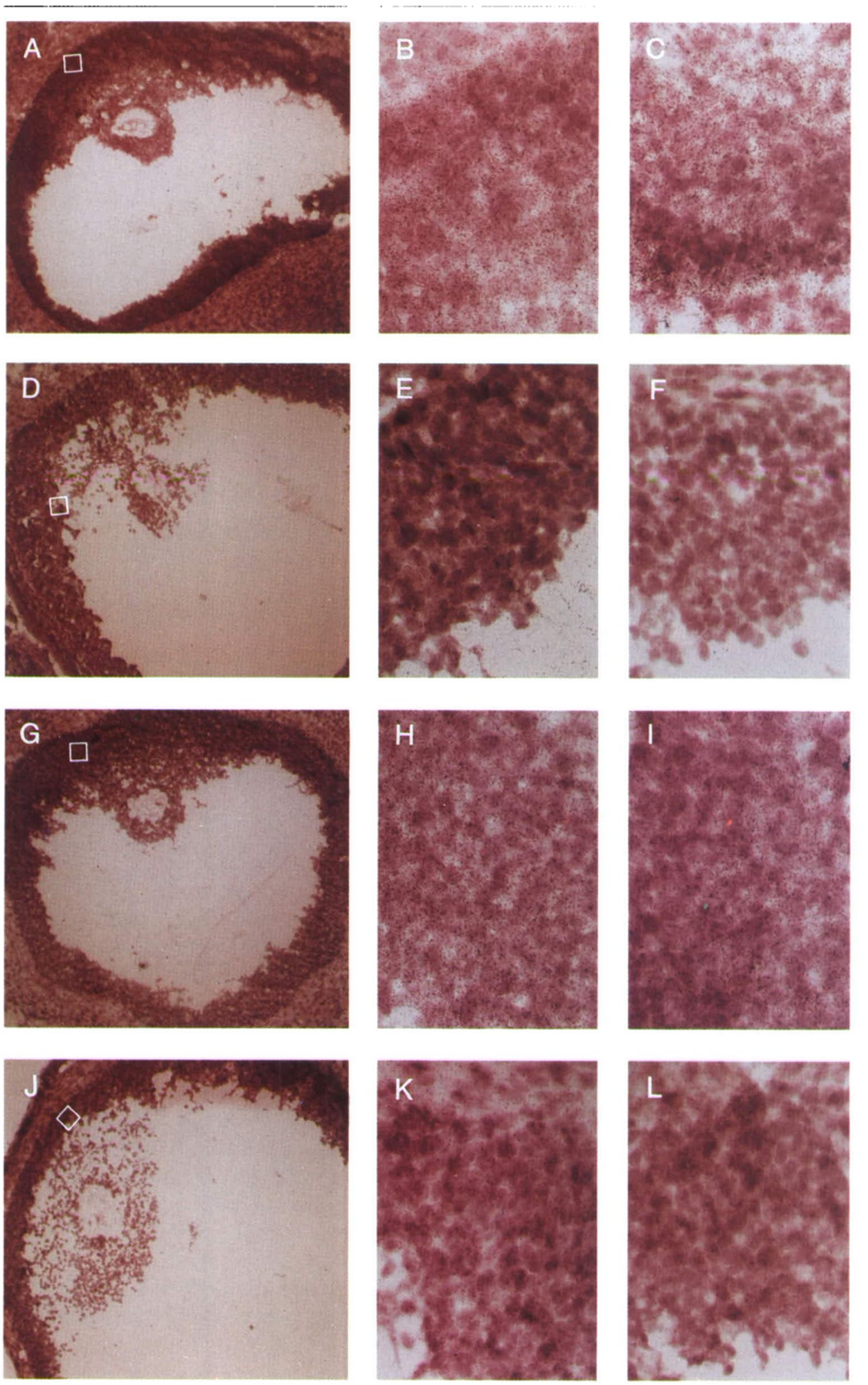

FIG. 4. *In situ* hybridization analysis of the inhibin α- and β_A-subunit mRNAs in ovaries from Antag/LH- and Antag/FSH-treated rats. Column 1 is a ×100 magnification showing the entire follicle. Columns 2 and 3 are ×500 magnifications of the granulosa cells probed with the inhibin α- or β_A-subunit probes, respectively. A–C, Antag/LH, 1830 h on proestrus; D–F, Antag/LH, 2400 h on proestrus; G–I, Antag/FSH, 1830 h on proestrus; J–L, Antag/FSH, 2400 h on proestrus.

TABLE 2. Image analysis of 1830 and 2400 h follicles probed with the inhibin α-subunit probe

Treatment group	No. of grains/3286.6 μm^2	
	1830 h	2400 h
Intact	8334 ± 919	278 ± 46
Antag/Veh	8115 ± 1285	9072 ± 336
Antag/LH	4247 ± 245	461 ± 181
Antag/FSH	2382 ± 180	804 ± 258

Values are the mean ± SE (n = 3).

basal FSH levels on the afternoon of estrus. Maturing follicles begin to secrete estradiol and a number of growth factors. Inhibin mRNA levels steadily increase in these follicles. The purpose of increased inhibin mRNA is unknown, but it presumably acts to supply inhibin to keep FSH at low levels until proestrus. On the afternoon of proestrus gonadotropins are released under the influence of GnRH. Our data show that either LH or FSH will cause a decline in inhibin mRNA levels in mature preovulatory follicles. The lack of circulating ovarian inhibin results in prolonged secretion of FSH and initiation of the next cycle. This model does not include the role of other ovarian factors that regulate FSH, but it does provide a framework for further studies directed at the mechanisms involved in the control of FSH.

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