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9. Retinal ganglion cells were identified by the presence of a fluorescent dye, granular blue, which 2 days before enucleation had been injected into the superior colliculi and transported retrogradely to the ganglion cells [for a color photograph see (*8*)]. The presence of dye permitted the unequivocal identification of the ganglion cells after dissociation of the retina with the enzyme papain. In previous studies the retinal ganglion cells were also labeled after dissociation by means of immunocytochemical techniques with monoclonal antibodies against Thy-1 (*8*). The same population of retinal ganglion cells was identified by Thy-1 antibodies and by retrograde transport of fluorescent dye (*8*), ensuring the reliability of either technique. For convenience, the retrograde transport method was used routinely. By postnatal day 5 all of the central (axonal) connections of pigmented rat retinal ganglion cells have been formed. Thus, axonal processes that grow in culture from ganglion cells obtained from animals at this stage or later must represent regeneration rather than initial outgrowth. Electron micrographs of the ganglion cells in culture have confirmed that at least some of the neurites are axonal (S. A. Lipton and R. D. Madison, unpublished observations), indicating that they have regenerated. In contrast, many of the dendritic connections within the retina are still forming at the age of the animals used in this study. Hence, dendritic processes that grow in culture may represent the initial developmental outgrowth of these structures.
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11. About half of the clustered retinal ganglion cells had spontaneous electrical activity presumably due to synaptic input from other cells in the dish [S. A. Lipton, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9774 (1986)]. Together the solitary and clustered populations of ganglion cells accounted for approximately 1% of the total cells in a typical culture dish; this proportion resembles the composition of the intact postnatal retina.
12. Outgrowth from clustered retinal ganglion cells could not be adequately assessed because processes were more difficult to visualize within the clusters (*8*).
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16. As shown in (*6, 14*), there is a wide range in the electrical responsiveness among retinal ganglion cells to nicotinic agonists, probably reflecting the variation in cholinoreceptive sites among the cells. Thus, the fact that about a third of the ganglion cells bear processes in the absence of nicotinic inhibitors and another third do not exhibit outgrowth even in the presence of these antagonists (Table 1) does not necessarily indicate that these cells are totally refractory to the effects of ACh. Rather, different ganglion cells are possibly more or less sensitive to the presence of ACh depending on the number of receptor sites, producing a fair degree of heterogeneity in response to ACh both in electrophysiological properties and in growth characteristics.
17. As described previously (*13*), the lengths of processes did not form a normal distribution, but the logarithm of the lengths did. Therefore, the logarithmic distribution was analyzed by normal statistics. This showed that the lengths of the processes were significantly longer when the incubation medium included *d*-tubocurarine.
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19. Of the ganglion cells in cultures containing endogenous ACh, 37.4% grew processes (Table 1); in contrast, in three experiments in cultures without detectable endogenous ACh, 58.0% grew processes. This difference is highly significant ($P < 0.001$) by Fisher's exact test, as performed in (*8*). Also, for cultures without endogenous ACh, *d*-tubocurarine (10 μM) had no significant effect on process outgrowth compared with controls; 63% of the ganglion cells grew processes in medium containing *d*-tubocurarine while, as stated above, 58% grew processes in the control dishes.
20. If nicotinic agents are indeed inhibitory to the growth of processes by retinal ganglion cells, then it should be possible to recreate this effect in cultures that lack substantial levels of endogenous ACh by adding exogenous nicotinic agonists. After submission of our manuscript, a preliminary report of this experiment appeared. Monitored with AVEC-DIC microscopy, direct application of nicotine (100 μM) onto growth cones of presumptive ganglion cells from the chick retina resulted in the inhibition of activity or even retraction of filopodia and neurites [K. L. Lankford, M. I. Fonseca, W. L. Klein, *Soc. Neurosci. Abstr.* **13**, 258 (1987)].
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Dynamic Changes in Inhibin Messenger RNAs in Rat Ovarian Follicles During the Reproductive Cycle

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The alterations in morphology and function of the ovarian follicle as it matures, ovulates, and becomes a corpus luteum are dramatic. A variety of steroid and polypeptide hormones influence these processes, and the ovary in turn produces specific hormonal signals for endocrine regulation. One such signal is inhibin, a heterodimeric protein that suppresses the secretion of follicle-stimulating hormone from pituitary gonadotrophs. Rat inhibin complementary DNA probes have been used to examine the levels and distribution of inhibin α- and β_A-subunit messenger RNAs in the ovaries of cycling animals. Striking, dynamic changes have been found in inhibin messenger RNA accumulation during the developmental maturation of the ovarian follicle.

THE PROGRESSIVE GROWTH, OVULATION, and luteinization of ovarian follicles are highly integrated processes coordinated by regulatory signals including steroid and peptide hormones from the brain, anterior pituitary, adrenals, and ovaries. The two primary regulators of ovarian function are the pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (*1*). FSH and LH are usually secreted in tandem under the influence of the hypothalamic peptide gonadotropin-releasing hormone (GnRH) (*2*). However, normal physiological situations exist where the secretion of FSH and LH are dissociable (*3*).

FSH secretion can be specifically suppressed by the steroid-free portion of ovarian follicular fluid in many species (*4, 5*). Follicular fluid can inhibit both the primary and secondary FSH surges, as well as the rise in serum FSH concentrations that follows unilateral or bilateral ovariectomy (*5, 6*). Purification of this activity, termed inhibin, from follicular fluid resulted in the identification of a heterodimeric glycoprotein,

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composed of α (18-kD) and β (14-kD) subunits, that suppresses the release of FSH, but not LH, from cultured pituitary cells (*7*). Two related forms of the smaller β subunit (β_A and β_B) have been identified, and these subunits can form dimers that are potent stimulators of FSH secretion (*8*). Inhibin subunit complementary DNAs (cDNAs) have recently been characterized from several species, including rat (*9, 10*). To clarify the role of inhibin in reproductive processes, we have identified the points in follicular maturation during which the inhibin α and β_A messenger RNAs (mRNAs) are synthesized.

Ovaries were removed from four sets of animals at times during the 4-day estrous cycle that coincided with the preovulatory LH and FSH surges (afternoon of proestrus), the secondary FSH surge (proestrus to estrus), ovulation (early estrus), follicular recruitment (early estrus), and luteinization (estrus) (*11–15*). Total RNA isolated from the ovaries was used for solution hybridization to determine overall changes in inhibin mRNA levels during the course of the reproductive cycle. Serum FSH and LH were also measured for each animal.

Both inhibin α- and β_A-subunit mRNAs were detected on all days of the estrous cycle; the level of α mRNA steadily increased during the early portion of the cycle and peaked late in the afternoon of proestrus (Fig. 1A). Although the level of β_A mRNA was more variable between animals, it also reached peak values at 1830 hours on proestrus (Fig. 1B). After the preovulatory surges of LH and FSH in late proestrus (Fig. 1C), a marked decrease in expression of both mRNAs was observed by 2400 hours proestrus. By the morning of estrus (0400 to 0700 hours), inhibin mRNA levels began to increase from this nadir.

The ovary is a heterogeneous organ consisting of follicles in many different stages of growth and atresia and of corpora lutea of varying age. To obtain a more detailed and dynamic picture of inhibin biosynthesis in individual follicles, inhibin mRNAs were localized histologically. Animals were killed at the time points described, and one ovary was used for in situ hybridization histochemistry (Fig. 2) while serum was collected for gonadotropin measurements. Probes to the inhibin α and β_A subunits hybridized specifically to the mural granulosa cells of healthy, maturing follicles. During metestrus (Fig. 2, A to C) and diestrus (Fig. 2, D to F) both mRNAs were produced in low levels, and serum LH (3.3 and 1.1 ng/ml, respectively) and FSH (158 and 120 ng/ml, respectively) were also low. By 1000 hours on proestrus, Graafian follicles were formed, and a dramatic increase in inhibin mRNA levels was observed (Fig. 2, G to I). Serum LH (0.8 ng/ml) and FSH (122 ng/ml) remained low at this time. At 1600 hours proestrus, serum gonadotropin levels were markedly elevated (LH, 52.8 ng/ml and FSH, 373 ng/ml), and preovulatory follicles, which were beginning to show dispersement of cumulus cells in response to early gonadotropin surge stimulation, continued to hybridize intensely. A dramatic decrease in hybridization to both α and β_A probes was detected on the evening of proestrus in follicles displaying characteristics of complete gonadotropin stimulation (Fig. 2, J to L). In this animal, serum concentrations of LH had returned to presurge values, while serum FSH levels remained elevated (3.8 and 435 ng/ml, respectively). After ovulation, hybridization to new corpora lutea (1000 hours estrus, Fig. 2, M to O) was not detected, but inhibin mRNA levels began to increase in newly recruited follicles responding to the secondary FSH surge (*12*).

Previous studies have reported inhibin mRNA production (*9, 16*) or protein localization (*17*) in the corpus luteum, but our results show that at the cellular level little or no inhibin α- or β_A-subunit mRNA is detected in preantral follicles, oocytes, theca, stroma, cumulus cells, or corpora lutea of any age from cycling animals (Fig. 2).

Our measurements of inhibin mRNA levels throughout the estrous cycle showed that both α- and β_A-subunit mRNAs were expressed in newly recruited follicles, increased during the early portion of the rat estrous cycle, presumably in response to basal LH and FSH concentrations, and reached peak levels on the day of proestrus. The GnRH-driven preovulatory LH and FSH surges that occur during the afternoon of proestrus cause a resumption of oocyte meiosis, suppression of estradiol secretion, and increased progesterone secretion (*14*). We found that these primary gonadotropin surges preceded a dramatic decline in inhibin mRNA levels, and, even though the precise timing of this decline in inhibin mRNA levels during late proestrus was somewhat variable, in all cases it coincided with histological observation of complete gonadotropin stimulation. We have recently shown, by in situ hybridization, that in animals treated at 1200 hours proestrus

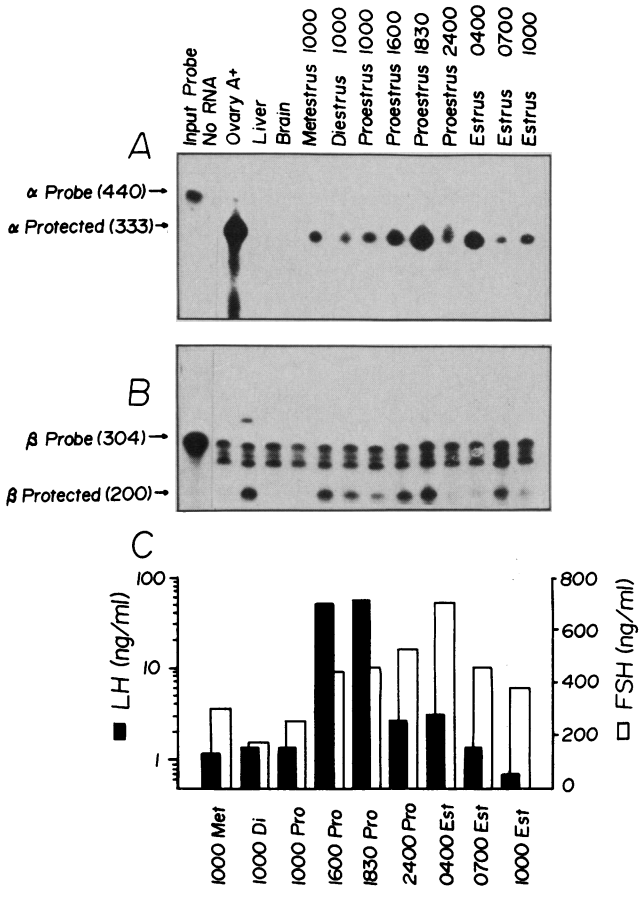

Fig. 1. Inhibin α- and β_A-subunit mRNA levels and serum gonadotropin levels during the rat estrous cycle. (**A**) Inhibin α-subunit mRNA; (**B**) inhibin β_A-subunit mRNA; (**C**) serum LH and FSH levels. A solution hybridization–ribonuclease (RNase) protection assay was used to determine inhibin mRNA levels (*21*). Arrows indicate the size of the input antisense RNA probe, as well as the size of the expected RNase-protected fragment based on the known cDNA sequences (*9*). The triplet of bands seen in the upper part of B are nonspecific and are seen in all lanes, including the control, which lacked RNA. Total RNA was prepared from one ovary of each animal killed at the indicated times (*22*), and 20 μg of total RNA was used in each hybridization reaction. Antisense RNA probes were synthesized as described, with rat inhibin α- and β_A-subunit cDNAs used in the vector pGEM3 (*9, 21*). Solution hybridization was performed for 16 hours at 42°C, the samples were digested with RNase A (40 μg/ml) and T1 (2 μg/ml) for 1 hour at 30°C, and analyzed on denaturing 6% polyacrylamide–urea gels (*21*). Negative controls included probe alone and total RNA from rat liver and brain. The positive control was 1 μg of polyadenylated RNA from the ovary of a gonadotropin-stimulated animal. The same general pattern of changes in inhibin mRNA levels shown here was observed in three additional experiments on independent sets of cycling rats. Hormone values were determined by radioimmunoassay (*23*).

with a GnRH antagonist, which blocks the subsequent gonadotropin surges, inhibin mRNA levels fail to decrease by 2400 hours proestrus (*18*).

Our findings on the modulation of inhibin mRNA biosynthesis correlate with earlier physiological observations that inhibin regulates FSH and is also influenced by the gonadotropins (*11*). Direct gonadotropin regulation of inhibin mRNA levels and inhibin production have been observed both in the animal and in granulosa cell cultures (*9, 19*). The modulation of inhibin mRNAs seen in this study is consistent with recent findings demonstrating that circulating inhibin increases throughout the metestrous, diestrous, and early proestrous stages of the rat estrous cycle, decreases during the evening of proestrus, and rebounds on the morning of estrus (*20*).

The dramatic reduction in inhibin mRNA levels seen between the afternoon of proestrus in the slightly stimulated follicles and the evening of proestrus in follicles fully

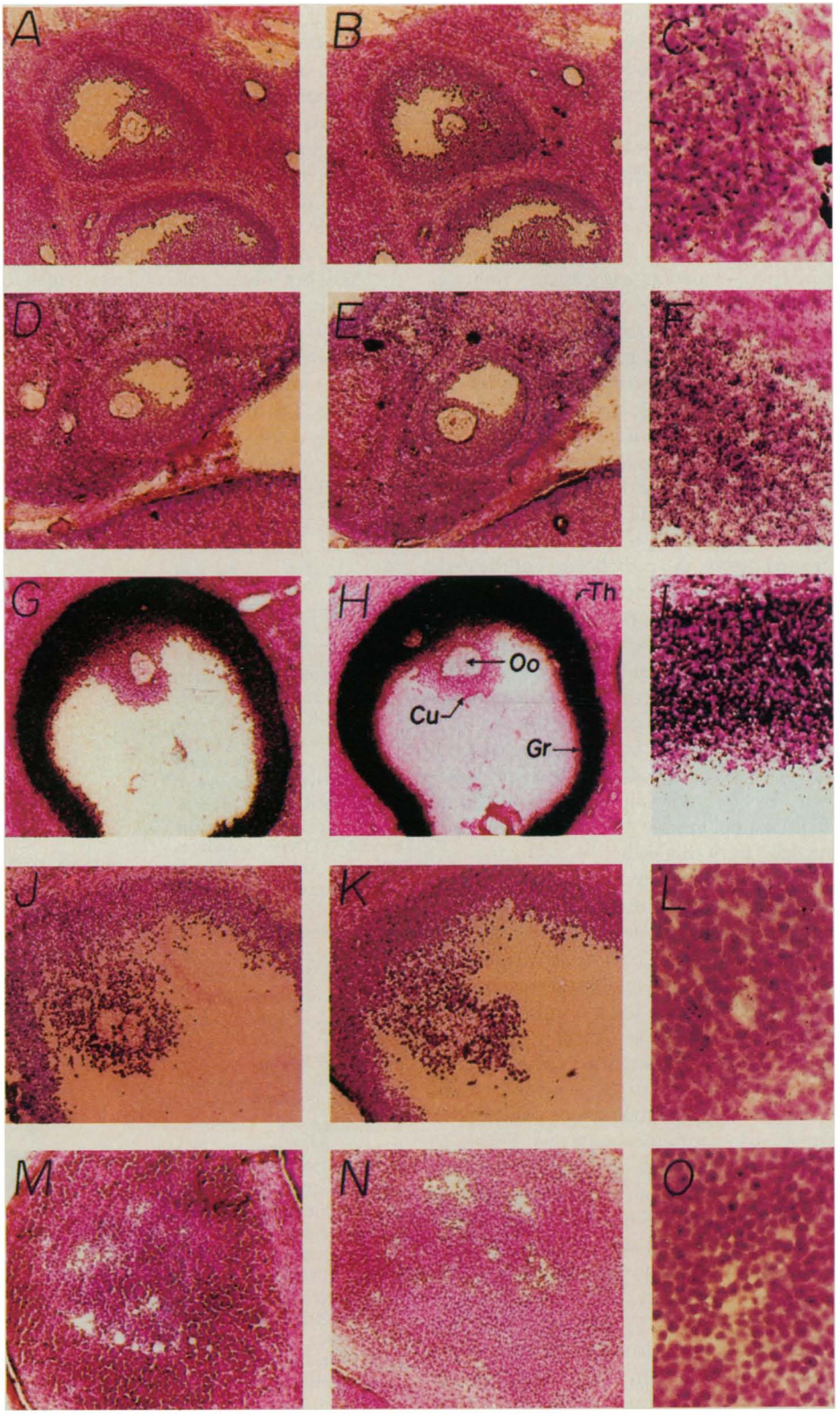

Fig. 2. In situ hybridization analysis of inhibin α- and β_A-subunit mRNAs in rat ovary. (Column 1) Localization of α-subunit mRNA; (columns 2 and 3) localization of β_A-subunit mRNA. Photographs in columns 1 and 2 are at a magnification of ×100; the entire maturing follicles can be seen and represent adjacent sections hybridized to either inhibin-α or inhibin-β_A probes. The photographs in column 3 are at a magnification of ×500; individual silver grains can be seen. (**A** to **C**), 1000 hours metestrus; (**D** to **F**), 1000 hours diestrus; (**G** to **I**) 1000 hours proestrus; (**J** to **L**), 2400 hours proestrus; (**M** to **O**), 1000 hours estrus. Relevant structures in the Graafian follicle (Th, thecal cells; Oo, oocyte; Cu, cumulus cells; Gr, granulosa cells) are labeled in (H). The structure shown at 1000 hours estrus is a fresh corpus luteum. Frozen ovaries were oriented with respect to the attached oviduct, mounted, and sequential 20-μm sections were cut on a Reichert 840 cryostat. Sections were mounted on microscope slides pretreated by incubation in Denhardt's medium and acetylation (*24*), and were fixed in a mixture of ethanol and acetic acid (3:1) at room temperature for 15 minutes. The slides were hybridized to ^{32}P-labeled antisense inhibin α- and β_A-subunit probes as previously described (*9*), processed for autoradiography with Kodak NTB-2 emulsion, exposed for 9 days at 4°C, and developed. All sections were hybridized and processed simultaneously with a single preparation of inhibin α- or β_A-subunit probe. Approximately 100 sections from one ovary of each animal were stained with hematoxylin and eosin and analyzed; two complete sets of cycling animals were analyzed. Follicles were categorized according to size class, degree of stimulation or atresia, and the relative level of hybridization. Photography was performed on a Nikon Optiphot microscope. Control hybridizations to liver sections, or to ovaries by means of sense-strand inhibin probes, showed no detectable signal.

stimulated by the preovulatory gonadotropin surge suggests that the half-lives of the inhibin mRNAs are likely to be very short. This is consistent with inhibin production being tightly regulated in response to transient conditions during the estrous cycle, and suggests that the major site of control of inhibin biosynthesis is at the level of transcription. Our results indicate that one component of this control is likely to be the gonadotropin surges. Further elucidation of the molecular mechanisms by which gonadotropins and other hormonal and developmental cues modulate inhibin biosynthesis promises to enhance our understanding of the control of the mammalian reproductive system.

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Alternative Mechanisms for Activation of Human Immunodeficiency Virus Enhancer in T Cells

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The expression of human immunodeficiency virus (HIV) after T cell activation is regulated by NF-κB, an inducible DNA-binding protein that stimulates transcription. Proteins encoded by a variety of DNA viruses are also able to activate expression from the HIV enhancer. To determine how this activation occurs, specific genes from herpes simplex virus type 1 and adenovirus that activate HIV in T lymphoma cells have been identified. The cis-acting regulatory sequences in the HIV enhancer that mediate their effect have also been characterized. The relevant genes are those for ICP0—an immediate-early product of herpes simplex virus type 1—and the form of E1A encoded by the 13S messenger RNA of adenovirus. Activation of HIV by adenovirus E1A was found to depend on the TATA box, whereas herpesvirus ICP0 did not work through a single defined cis-acting element. These findings suggest multiple pathways that can be used to bypass normal cellular activation of HIV, and they raise the possibility that infection by herpes simplex virus or adenovirus may directly contribute to the activation of HIV in acquired immunodeficiency syndrome by mechanisms independent of antigenic stimulation in T cells.

EXPRESSION OF HUMAN IMMUNODEFICIENCY virus (HIV) increases after activation of inducer T cells by phorbol esters and lectins (*1, 2*). This stimulation is mediated by NF-κB (*3*), a factor that regulates transcription and binds to the twice-repeated 11-bp κB motif in the HIV enhancer (Fig. 1). This 11-bp motif is also found in the immunoglobulin light chain enhancer (*4*). Mutations of nucleotides within these sites that eliminate the binding of NF-κB also abolish the increase in HIV gene expression seen in activated T cells (*3*). DNA from primate viruses induced HIV expression when cotransfected into fibroblasts with a plasmid containing the HIV enhancer linked to the chloramphenicol acetyltransferase (CAT) gene (*5–7*), but the

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