Inhibin, Activin, and Follistatin: Regulation of Follicle-Stimulating Hormone Messenger Ribonucleic Acid Levels

Rona S. Carroll, Anne Z. Corrigan, Soheyla D. Gharib, Wylie Vale, and William W. Chin

Division of Genetics
Department of Medicine
Brigham and Women's Hospital
Howard Hughes Medical Institute
and Harvard Medical School
Boston, Massachusetts 02115

Clayton Foundation Laboratories
for Peptide Biology, (A.Z.C., W.V.)
The Salk Institute
La Jolla, CA 92037

Primary pituitary cell cultures derived from adult male rats were used to explore the direct effects of purified porcine inhibin and follistatin, and recombinant human activin A on FSHβ, as well as LHβ and α-subunit mRNA levels. Subunit mRNAs were determined by blot hybridization using α, LHβ, and FSHβ cDNA and genomic fragments. Treatment with inhibin for 72 h significantly suppressed α and FSHβ mRNA levels with parallel changes in FSH secretion. No change in LHβ mRNA levels was observed. A decrease in FSHβ mRNA to undetectable levels was seen 4 h after inhibin administration. Recombinant human Activin A caused dose-dependent and parallel increases in FSHβ mRNA levels and FSH secretion. This increase was evident at 4 h after activin administration and maintained at longer times. α and LHβ mRNA levels remained unchanged. Follistatin addition to cultures for 72 h significantly reduced FSHβ mRNA levels. In a time-course experiment, a reduction in FSHβ mRNA to undetectable levels was observed 24 h after follistatin administration. There were no changes in α or LHβ mRNA levels. These data demonstrate that the actions of these gonadal peptides on FSH secretion may be accounted for, at least in part at the level of biosynthesis, by reductions in FSHβ mRNA levels directly at the level of the anterior pituitary gland. (Molecular Endocrinology 3: 1969–1976, 1989)

INTRODUCTION

Secretion of the gonadotropins, FSH and LH, by the pituitary is controlled by multiple factors including hypothalamic GnRH and gonadal steroids (1). Recently, the purification of inhibin (2–5), activin (6, 7) and follistatin (8) from follicular fluid have made possible detailed studies of their effects on FSH secretion. It has been shown that inhibin and follistatin decrease FSH, whereas activin stimulates FSH secretion.

Little is known about the regulation of synthesis of FSH at the pretranslational level. Our laboratory has shown previously that LHβ and FSHβ mRNA levels are differentially regulated by sex steroids (9, 10). In castrate male rats, estrogens negatively regulate α, LHβ, and FSHβ mRNA levels; androgens negatively regulate α and LHβ, but not FSHβ mRNA levels (11). Others have recently reported (12) that replacement of testosterone to castrate males suppressed all three subunit mRNAs to intact levels. In ovariectomized female rats, estrogen suppresses the mRNA levels of all three subunits, whereas androgens negatively regulate α and LHβ, but have little or not effect on FSHβ mRNA levels (13).

Although inhibin and activin have been shown to decrease or increase, respectively, total FSH content in pituitary cell cultures and presumably modulate biosynthesis of gonadotropins (6), there have been few studies examining the effects of these peptides on gonadotropin subunit mRNA levels. Mercer *et al.* (14) have shown that bovine follicular fluid inhibin suppresses FSHβ mRNA levels directly at the level of the anterior pituitary gland in the hypothalamic-pituitary-disconnected GnRH-pulsed ewes. Attardi *et al.* (15) have shown that partially purified inhibin from monkey Sertoli cells culture medium significantly suppresses steady-state FSHβ mRNA levels in dispersed rat pituitary cells. We have extended these studies to examine the time-course and dose-response curves of highly

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purified inhibin, recombinant human activin (rhActivin) and follistatin on α, LHβ, and FSHβ steady-state mRNA levels in rat primary anterior pituitary cell cultures.

RESULTS

Single Peptide Studies

Effects of Purified Porcine Inhibin on Gonadotropin mRNA Subunit Levels The dose-response and time-course of the effects of purified inhibin on α, LHβ, and FSHβ subunit mRNA levels as well as serum FSH levels were examined in primary pituitary cell cultures. Treatment with inhibin for 72 h significantly suppressed FSHβ mRNA to undetectable levels (Fig. 1A). Significant reductions ($P \leq 0.05$) in α mRNA levels were also observed; the 2.0 and 10.0 ng/ml doses resulted in reductions to 55% and 14% of controls, respectively (Table 1). There were no changes in LHβ mRNA levels. A similar pattern of inhibition was observed for FSH secretion in the media (Fig. 1B). The 2.0 and 10.0 ng/ml doses of inhibin suppressed FSH secretion, to 17% and 13% of controls, respectively.

Further studies were performed to examine the time course of the inhibin response using a dose of 2.0 ng/ml. As shown above, this dose suppressed FSHβ mRNA to undetectable levels after a 72-h incubation. FSHβ mRNA levels were undetectable 4 h after inhibin administration and the levels remained undetectable at the longer time points (Fig. 1C). α mRNA levels were slightly reduced after inhibin administration. No changes in LHβ mRNA levels were observed. A significant reduction ($P \leq 0.05$) in the amount of FSH released into the media after the administration of inhibin was also observed (Fig. 1D). No changes in media LH levels were observed.

Effects of rhActivin on Gonadotropin Subunit mRNA Levels The addition of rhActivin A to primary pituitary cell cultures for 72 h stimulated FSHβ mRNA levels. Recombinant hActivin A caused dose-dependent and parallel increases in FSHβ mRNA levels and FSH secretion in primary anterior pituitary cells. A significant ($P \leq 0.05$) dose-dependent rise in FSHβ mRNA levels was observed. A similar pattern of stimulation was noted for FSH secretion (Fig. 2, A and B).

A time-course study using the 20.0 ng/ml dose of rhActivin A showed a profound increase (4-fold) in the mRNA encoding the FSHβ subunit (Fig. 2C). FSH secretion was also significantly increased ($P \leq 0.05$) and levels remained elevated for up to 72 h (Fig. 2D). Recombinant hActivin A did not affect steady-state α or LHβ mRNA levels.

Effects of Purified Porcine Follistatin on Gonadotropin mRNA Subunits Levels Purified porcine follistatin, a single-chain glycosylated protein, was found to be an inhibitor of FSHβ mRNA in primary pituitary cell cultures. When added to cultures for 72 h, a significant ($P \leq 0.05$) decrease in FSHβ mRNA was observed (Fig. 3A). FSHβ mRNA levels were reduced to undetectable levels at the 50.0 ng/ml dose. The pattern of FSH secretion was similar to the observed changes in FSHβ mRNA levels (Fig. 3B). There were no significant reductions in α or LHβ mRNA levels.

In a time-course experiment, using the 20.0 ng/ml dose of follistatin which reduced FSHβ mRNA levels to undetectable levels, a significant decrease ($P \leq 0.05$) in FSHβ mRNA levels from controls was noted (Fig. 3C). By 24 h, FSHβ mRNA levels were not detectable. A significant reduction in FSH secretion was also observed (Fig. 3D). No changes in α or LHβ mRNA levels were observed.

Combination Studies

Effects of Inhibin on Gonadotropin Subunit mRNA Levels in the Presence of Activin To assess the interaction of inhibin and activin on gonadotropin mRNA levels at the pretranslational level, pituitary cell cultures were treated with rhActivin A at a dose of 20.0 ng/ml for 72 h. This dose has previously shown to stimulate FSHβ mRNA levels 3 to 4-fold over control values. Simultaneously, cells were treated with increasing concentrations of inhibin ranging from 0.016–10.0 ng/ml. Inhibin at a dose of 2.0 ng/ml was able to significantly ($P \leq 0.05$) reduce FSHβ mRNA levels (Fig. 4A). A higher dose of 10.0 ng/ml reduced FSHβ mRNA to nondetectable levels. A significant reduction in α mRNA levels was also noted at the higher doses of inhibin (Table 2). The pattern of FSH secretion was similar to the observed changes in FSHβ mRNA levels (Fig. 4B); the only difference was that the inhibin and activin groups had a higher baseline level.

Effects of Activin on Gonadotropin Subunit mRNA Levels in the Presence of Follistatin In order to explore the interactions of rhActivin A and follistatin, cells were treated with follistatin at a dose of 20.0 ng/ml for 72 h. This dose, as shown earlier, suppressed FSHβ mRNA to undetectable levels. Only when rhActivin A was administered in nearly equimolar concentration (20.0 ng/ml) was it able to override the inhibitory action of follistatin (Fig. 5A). As seen in the earlier studies, the changes in FSH secretion paralleled the changes in FSHβ mRNA levels (Fig. 5B). There were no changes in α or LHβ mRNA levels.

DISCUSSION

In this study, male rat primary anterior pituitary cell cultures were used as a sensitive assay system to study the effects of purified inhibin, rhActivin A, and follistatin on steady-state gonadotropin subunit mRNA levels. All three peptides had dramatic effects on FSHβ mRNA levels. In this system, the purified 32K inhibin is a potent suppressor of FSHβ mRNA levels. The reduction in FSHβ mRNA was associated with a parallel decrease in FSHβ secretion. We found that inhibin

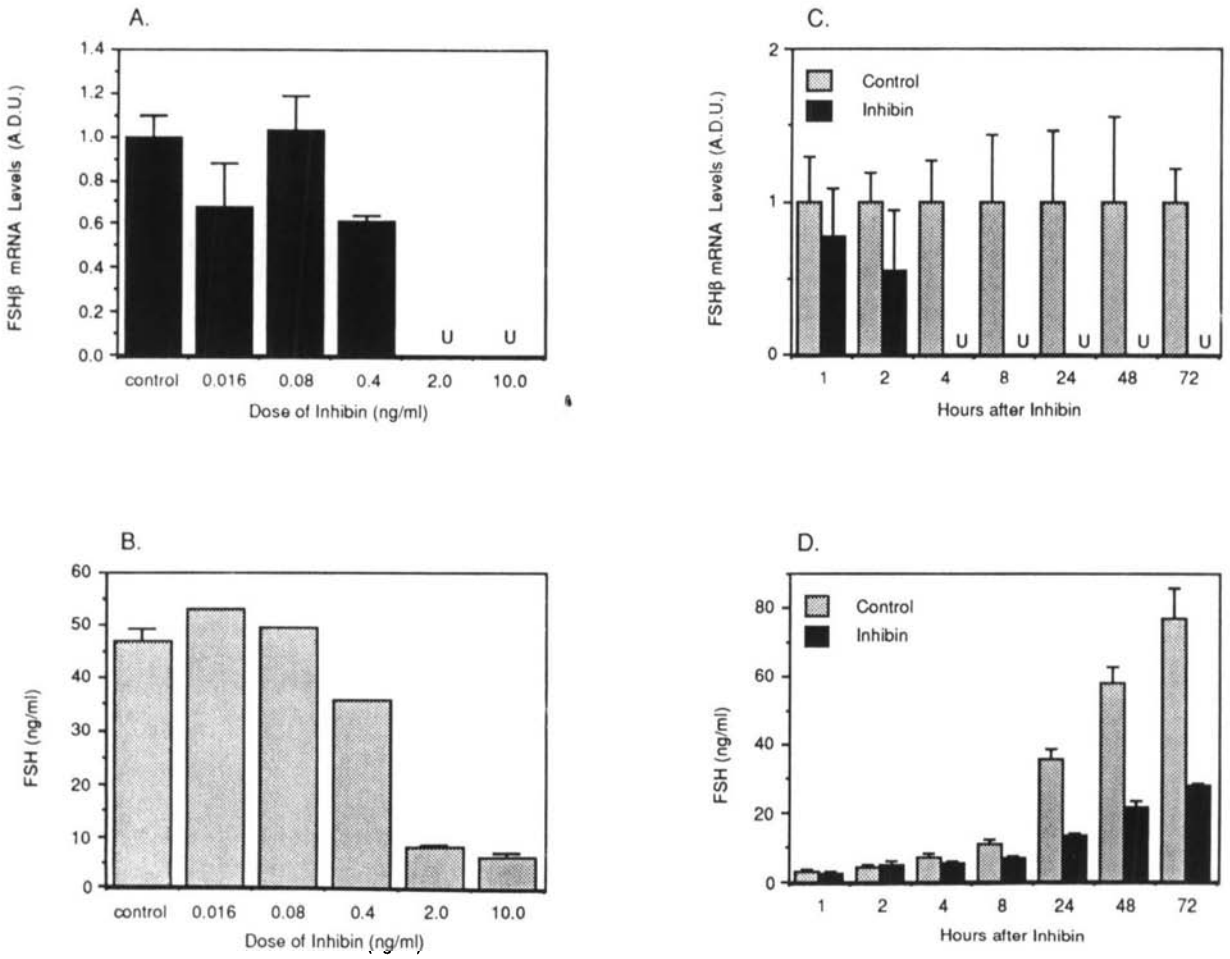

Fig. 1. Effects of Purified Porcine Inhibin on FSHβ Subunit mRNA Levels and FSH Secretion

Primary pituitary cell cultures were treated 3 days after dispersion with varying concentrations (A, B) of inhibin (ng/ml) for 72 h and for varying times with inhibin (2.0 ng/ml) (C, D). Cells were harvested and FSHβ mRNA levels were determined (A, C). The mRNA levels are standardized to the levels observed in untreated controls and reported as arbitrary densitometric units (ADU). Media levels of FSH were determined by RIA (B, D). Values represent the mean ± SD, n = 2; U = undetectable.

Table 1. Inhibin Dose-Response Curve

Inhibin Dose (ng/ml)	α mRNA Levels (ADU)	LHβ mRNA Levels (ADU)
0.00	1.0 ± 0.09	1.0 ± 0.11
0.016	0.67 ± 0.29	1.24 ± 0.28
0.08	0.77 ± 0.04	1.23 ± 0.32
0.40	0.95 ± 0.07	0.95 ± 0.12
2.0	0.55 ± 0.18	1.0 ± 0.24
10.0	0.14 ± 0.04	1.23 ± 0.21

ADU, Arbitrary density units.

reduced FSHβ mRNA levels within 4 h of administration and levels remained suppressed at longer times. FSH secretion was not significantly suppressed until 24 h. This dramatic reduction of the FSHβ subunit at the pretranslational level is consistent with previous reports. Using inhibin isolated from monkey Sertoli cell culture medium, Attardi *et al.* (15) showed a rapid (within 2 h) inhibition of FSHβ mRNA levels in rat pituitary cell cultures. In hypothalamic-pituitary disconnected sheep receiving pulses of GnRH, a decrease in FSHβ mRNA levels was observed 30 h after the administration of bovine follicular fluid (14).

The ability of inhibin to regulate LH secretion in rat pituitary cells has been an area of controversy in the literature. In our studies, inhibin did not affect LHβ mRNA subunit levels or LH secretion (data not shown). Attardi *et al.* (15) observed a slight decrease in LHβ mRNA levels (to 84% of controls) along with a small decrease in cell content. Campen and Vale (16) reported that purified ovine inhibin had no effect on basal LH secretion, but did inhibit GnRH-stimulated LH release. Kotsuji *et al.* (17) showed a decline in GnRH stimulated LH release by primate Sertoli cells (partially purified inhibin). Farnworth *et al.* (18) reported that high concentrations of 31Kd bovine inhibin suppressed basal LH and promoted intracellular degradation. In contrast, Fukuda *et al.* (19) observed no change in basal secretion or cell content of LH by pure porcine inhibin. The

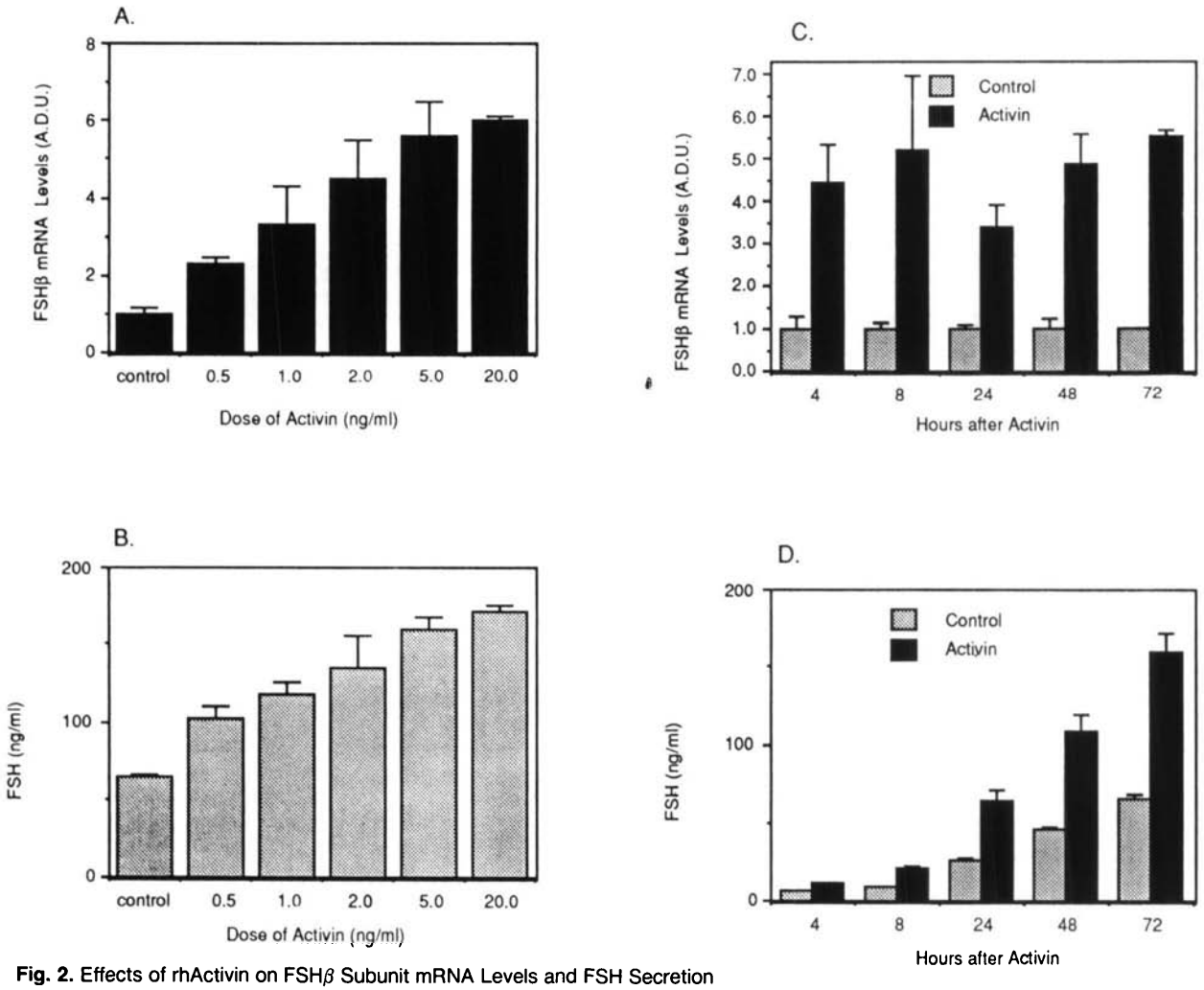

Fig. 2. Effects of rhActivin on FSHβ Subunit mRNA Levels and FSH Secretion

Primary pituitary cell cultures were treated 3 days after dispersion with varying concentrations (A, B) of activin (ng/ml) for 72 h and for varying times with activin (20.0 ng/ml) (C, D). Cells were harvested and FSHβ mRNA levels were determined (A, C). The mRNA levels are standardized to the levels observed in untreated controls and reported as arbitrary densitometric unit (ADU). Media levels of FSH were determined by RIA (B,D). All values represent the mean ± SD, n = 2.

magnitude of the effects of inhibin on FSH and LH may be dependent on the inhibin preparation, dose, duration of treatment, and the species from which the inhibin is derived.

We observed earlier that activin increased total intracellular levels of FSH in pituitary cell cultures (6) and proposed that this might reflect an increase in FSH biosynthetic rates. It has also been reported that rhActivin A-induced FSH release can be blocked by the administration of actinomycin D (20). These results taken together suggest that rhActivin A stimulates the synthesis of FSH. We examined the dose response and time course of the effects of rhActivin A on steady-state gonadotropin subunit mRNA levels. Recombinant hActivin A at a dose of 1.0 ng/ml was able to significantly stimulate FSHβ mRNA levels and FSH secretion. This is a dose range similar to that previously reported to stimulate FSH secretion. In the time-course experiment, an effect on FSH at the pretranslational and secretory levels were seen as early as 4 h after rhActivin A administration. In the initial report of activin isolation from follicular fluid, an increase in FSH secretion was described at 4 h, with a maximal effect after 24 h (6). Other reports using rhActivin A have not observed an increase in FSH secretion until 14 h after its administration (20). These discrepancies may be due to differences in the age and/or sex of the animals used. In our studies, pituitaries from adult male rats were dispersed; in contrast, Schwall *et al.* (20) used anterior pituitary glands from 21-day-old female rats. One possible explanation is that there may be age differences in the number of receptors through which activin exerts its action.

Follistatin, the glycosylated single-chain protein, has been shown to specifically inhibit the secretion of FSH from primary pituitary cell cultures (8). Ying *et al.* (21) has reported that follistatin does not decrease total cell content; in contrast, our laboratory has observed significant reductions in FSH total cell content (unpublished results). We have demonstrated that purified porcine

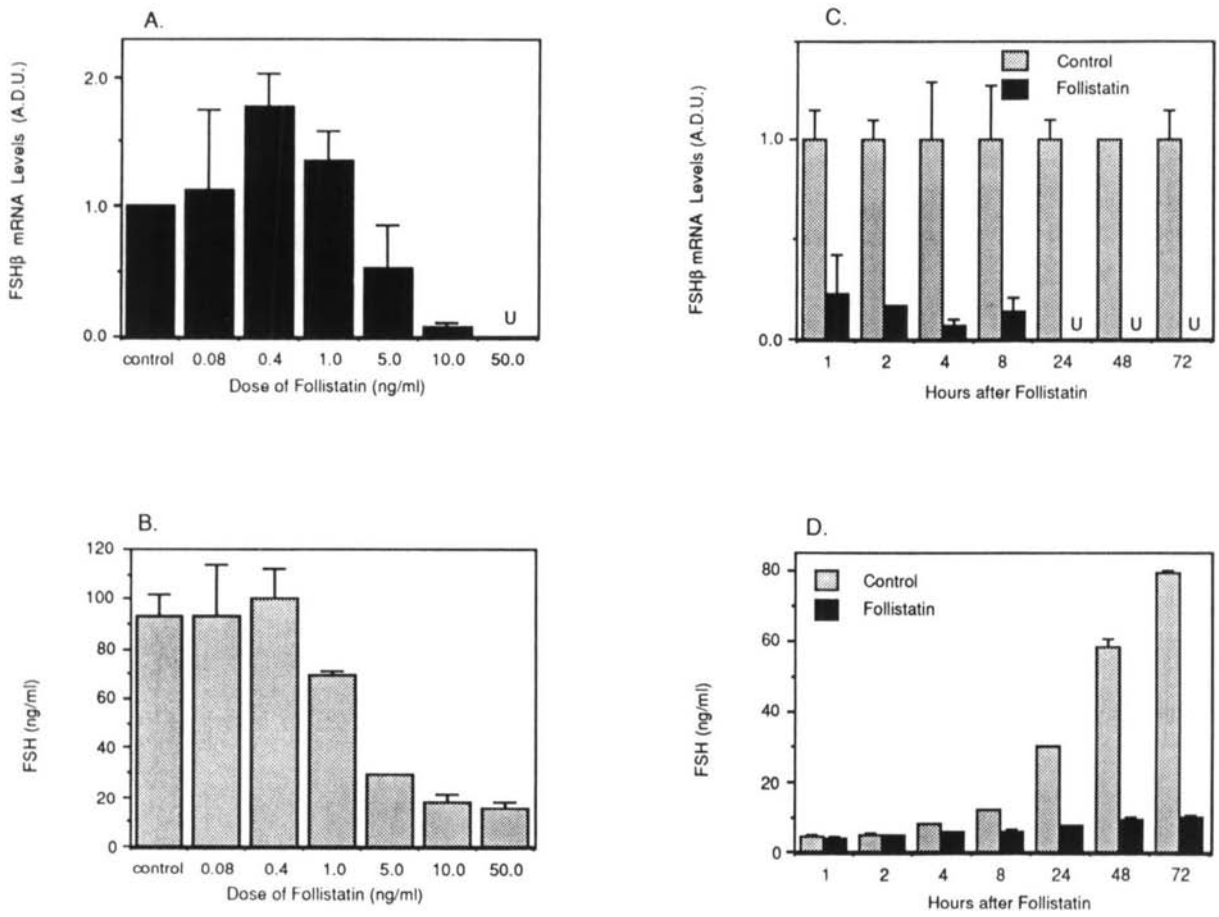

Fig. 3. Effects of Purified Porcine Follistatin on FSHβ Subunit mRNA Levels and FSH Secretion

Primary pituitary cell cultures were treated 3 days after dispersion with varying concentrations (A, B) of follistatin (ng/ml) for 72 h and for varying times with follistatin (20.0 ng/ml) (C, D). Cells were harvested and FSHβ mRNA levels were determined (A, C). The mRNA levels are standardized to the levels observed in untreated controls and reported as arbitrary densitometric units (ADU). Media levels of FSH were determined by RIA (B, D). All values represent mean ± SD, n = 2; U = undetectable.

follistatin caused a rapid, dose-dependent inhibition of FSHβ mRNA levels. A similar dose-dependent reduction in FSH secretion was observed. In a time-course study using a dose of follistatin that suppresses FSHβ mRNA levels, an effect was observed as early as 2 h after administration. In contrast, a significant decrease in FSH secretion was not seen until 8 h. The kinetics of suppression of FSH synthesis did not parallel the suppression of FSH secretion in all three time-course experiments that we performed. This may be due to the action of other factors that regulate FSH secretion preferentially.

The competition studies that we have performed suggest that the regulation of FSHβ mRNA levels and FSH secretion may in part be determined by a combination of effects of these peptides. When rhActivin A is present there is a shift to the right of the inhibition curves for inhibin. In the absence of activin, inhibin is able to reduce FSHβ mRNA levels to undetectable levels at the 2.0 ng/ml dose. In contrast, in the presence of activin A, a reduction to undetectable levels was not seen until the 10.0 ng/ml dose was reached. Follistatin also caused a shift to the right of the dose-response curve for activin. Follistatin at a dose of 20.0 ng/ml suppressed FSH to nondetectable levels. Only when rhActivin A was present at an equimolar concentration to that of follistatin (20.0 ng/ml) were FSHβ mRNA and FSH peptide levels even detectable.

Although it is unclear how these peptides exert their effects, we found that they have direct effects at the level of the pituitary gland. We do not know whether the observed effects of these peptides are due to regulation of gene transcription or effects on mRNA stability. Nuclear run-on assays, determinations of mRNA half-lives, and studies using metabolic inhibitors are in progress to answer these questions.

MATERIALS AND METHODS

Cell Culture

Anterior pituitary glands were dissected from mature male rats (Sprague-Dawley, 200–250 g; Bantin-Kingman, Fremont, CA)

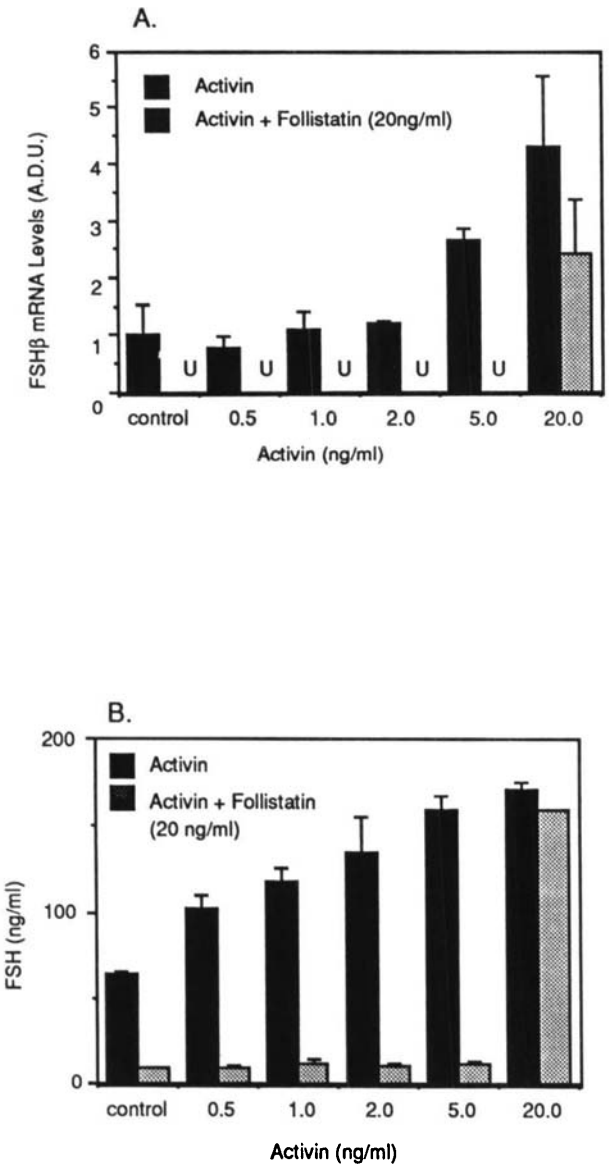

Fig. 4. Effects of rhActivin A and Inhibin on FSH Subunit mRNA Levels and FSH Secretion

All pituitary cell cultures were treated 3 days after dispersion with rhActivin (20.0 ng/ml) and increasing concentrations of inhibin for 72 h. Cells were harvested and FSHβ mRNA levels were determined (A). The mRNA levels are standardized to the observed levels in untreated controls and reported as arbitrary densitometric units (ADU). Media levels of FSH were determined by RIA (B). All values represent mean ± SD, n = 2; U = undetectable.

after decapitation (performed at the Salk Institute). After extensive rinsing in HEPES buffer [25 mM HEPES, 137 mM NaCl, 5 mM KCl, and 0.7 mM Na_2HPO4 (pH 7.3)], hemissected pituitaries were dissociated as described previously (6, 22). Briefly, tissues were incubated with 0.4% (wt/vol) collagenase (type II, Worthington, Freehold, NJ) and DNase II (8000 U/ml; Sigma, St. Louis, MO) at 37 C for approximately 2 h in a 100 ml jacketed spinner flask, followed by incubation with 0.25% (wt/vol) Viokase (GIBCO, Grand Island, NY) at 37 C for 8 min. Dissociated cells were then washed and plated in β-PJ culture medium (Scientific Services, Salk Institute) with nystatin, Sato's cocktail [insulin (5mg/liter), transferrin (5 mg/liter), PTH

Table 2. Effect of Inhibin in the Presence of Activin

Activin Dose (ng/ml)	Inhibin Dose (ng/ml)	α mRNA Levels (ADU)	LHβ mRNA Levels (ADU)
20.0	0.00	1.0 ± 0.05	1.0 ± 0.18
20.0	0.016	1.19 ± 0.10	0.83 ± 0.12
20.0	0.08	1.81 ± 0.10	1.28 ± 0.23
20.0	0.40	0.78 ± 0.05	1.31 ± 0.14
20.0	2.0	1.08 ± 0.07	1.05 ± 0.16
20.0	10.0	0.34 ± 0.05	1.11 ± 0.15

ADU, Arbitrary density units.

(0.5 mg liter), T_3 (30 pM), and fibroblast growth factor (1 μg/liter), and 2% (wt/vol) fetal bovine serum (HyClone). Cells were plated at a density of 5.0×10^6 cells in 60-mm tissue culture dishes (Falcon Plastics, Los Angeles, CA) and were allowed to attach for 3 days at 37 C in an incubator (5% CO_2-95% air) before any treatment was initiated. On the third day, the cultures were washed with fresh medium supplemented with 2% fetal bovine serum, followed by the addition of purified porcine inhibin (23), rhActivin A (courtesy of Genentech) (20), and purified porcine follistatin. The purity of the follistatin was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with silver staining. Protein content was determined by amino acid analysis. Composition was determined by Edman degradation in a gas phase protein sequencer (Applied Biosystems 470 A) and Western blot analysis. Follistatin was a mixture of the 35,000/31,000 dalton forms in a 75/25 weight ratio.

Gonadotropin RIAs

FSH and LH values were measured by RIA with materials provided by the NIDDK using the FSH and LH RP-2 standards. In the LH RIA, separation of the antigen-antibody complexes was achieved by the addition of Staphylococcal protein-A (Pansorbin, Calbiochem-Behring Corp., La Jolla, CA).

DNA Fragments

The probes used for hybridization described previously (9, 10) were as follows: a 495 base pair (bp) *NcoI-NcoI* rat α cDNA fragment, a 350 bp *PstI* rat LHβ cDNA fragment, a 650 bp *PstI* fragment of a mouse β-actin cDNA clone (courtesy of Dr. Bruce Spiegelman), and a 1000 bp rat FSHβ genomic fragment (9, 10). The cDNA and genomic fragments were labeled using random primer translation (24) to achieve a specific activity of $0.5–1 \times 10^9$ cpm/μg DNA.

Subunit mRNA Determinations

Total RNA was extracted from dispersed rat pituitary cells and subunit levels mRNAs were determined using blot hybridization analysis. 5×10^6 cells per well were harvested in 1 ml 4 M guanidinium thiocyanate. The guanidinium thiocyanate was layered over 5.7 M CsCl and spun at 100,000 × *g* in a tabletop TL100 (Beckman Centrifuge, Fullerton, CA) for 16 h at 20 C. The RNA pellets were dissolved in 0.3 M sterile sodium acetate and the RNA was ethanol precipitated. Ten micrograms (OD_{260}) of total RNA for each sample were subjected to electrophoresis and diffusion blotting onto nitrocellulose (25). Each blot was sequentially hybridized with FSHβ, α, LHβ, and finally, the β-actin probes using conditions previously described (9, 10). Blots were washed and subjected to autoradiography, and the band densities were determined by scanning densitometric analysis using a laser densitometer (Molecular Dynamics, Sunnyvale, CA).

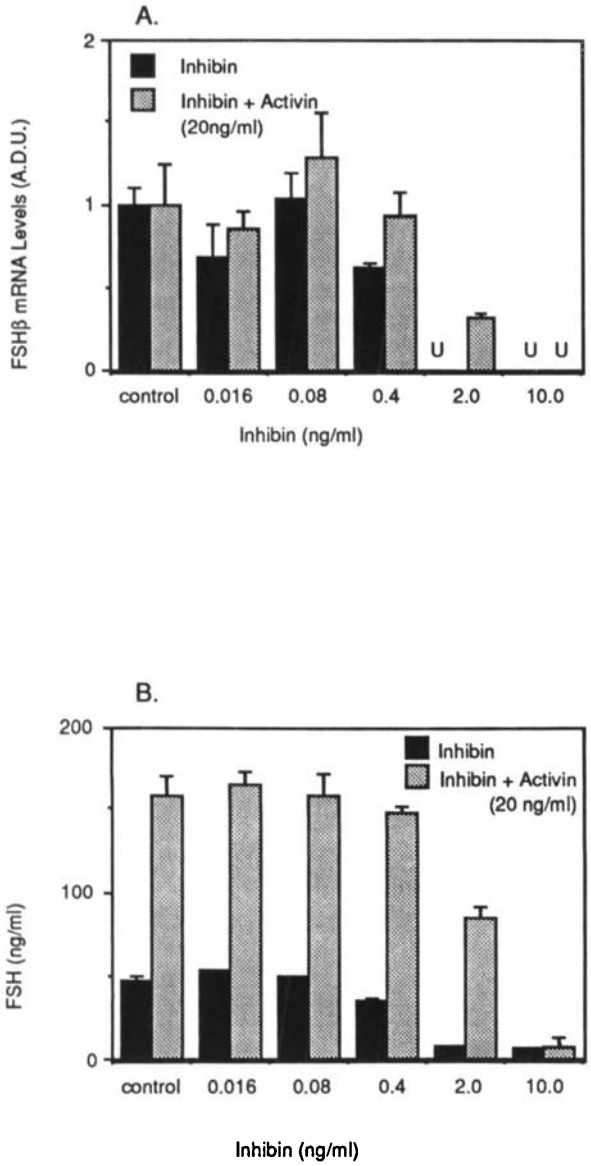

Fig. 5. Effects of Purified Follistatin and rhActivin A on FSH Subunit mRNA Levels and FSH Secretion

All pituitary cell cultures were treated 3 days after dispersion with follistatin (20.0 ng/ml) and increasing concentrations of rhActivin A for 72 h. Cells were harvested and FSHβ mRNA levels were determined (A). The mRNA levels are standardized to the observed levels in untreated controls and reported as arbitrary densitometric units (ADU). Media levels of FSH were determined by RIA (B). All values represent mean ± SD, n = 2; U = undetectable.

Standardization of Data

The amount of RNA in each lane of each blot (10 μg by OD_{260}) was internally standardized within a blot by assessing the amount of β-actin mRNA per lane and correcting the α, LHβ, and FSHβ mRNA levels accordingly, as described previously (9, 10).

Statistical Analysis

Linear regression analysis was used to assess the statistical significance of changes in mRNA and gonadotropin levels (26).

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Address requests for reprints to: Dr. Rona S. Carroll, Brigham and Women's Hospital, G. W. Thorn Building, Room 917, 20 Shattuck Street, Boston, Massachusetts 02115.

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