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FOLLISTATIN SPECIFICALLY INHIBITS PITUITARY FOLLICLE STIMULATING HORMONE RELEASE IN VITRO

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SUMMARY: **Two forms of purified follistatin, a single-chain polypeptide of mol wt 35,000 (35 Kd) protein, and a related molecule of mol wt 32,000 (32 Kd), which differs from the 35 Kd form in glycosylation or carboxyl terminal truncation, specifically inhibit the release of immunoreactive FSH by primary cultures of rat pituitary cells. Both forms of follistatin and inhibin-A give similar dose-response curves, with identical slopes and maximal effects, suggesting that they may all act through the same mechanism on the pituitary cells. The median effective dose (ED_{50}) of each of the follistatins is 6.2-7.3 ng/ ml (1.8×10^{-10} M), which corresponds to ~1/3 of the potency of inhibin. The effect of 35 Kd or 32 Kd follistatin is highly specific for suppressing the release of immunoreactive FSH since there is no demonstrable concomitant effect on the secretion of other pituitary hormones. The effect of follistatins, like that of inhibins, is different from that of the hypothalamic hypophysiotropic factors, requiring $\geq$ 18 h of incubation in a pituitary monolayer culture system to demonstrate. Coincubation of inhibin and follistatin shows an additive effect in the suppression of FSH release. Pituitary cells exposed to follistatin have significantly less depletion of intracellular FSH (0.01) than those treated with inhibin, indicating that follistatin may act primarily on the suppression of FSH release rather than on both release and synthesis of FSH, as is the case with inhibin.**

In 1985, two forms of inhibin (A and B) were isolated from porcine ovarian follicular fluid (FF) (1-3). Each inhibin is comprised of a common mol wt 18,000 (18 Kd) glycosylated α-subunit and a similar but distinct β-subunit of mol wt 14,700 (β_A) or mol wt 14,000 (β_B), linked by interchain disulfide bonds. A similar inhibin of mol wt 32,000 (32 Kd) was isolated from bovine FF (4-5). In addition, an inhibin of mol wt 56,000 (56 Kd) comprised of a larger form of the α-subunit (44 Kd) and the same β-subunit of inhibin-A has also been reported (6). These heterodimeric glycoproteins were found to be specific, potent inhibitors of the secretion of FSH by the pituitary (1-6). When the messages encoding the α-, β_A-, and β_B-subunits of porcine inhibin were cloned (7), the β_A and β_B subunits were found to be structurally homologous to transforming growth factor-β (TGFβ), a homodimeric protein isolated from platelets on the basis of its ability to promote anchorage-independent growth. Furthermore, TGFβ itself was found to stimulate FSH secretion by the pituitary in vitro (8). Attempts to identify the TGFβ-like materials in porcine FF (pFF) led to the isolation and characterization of two FSH-releasing dimeric proteins of

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apparent mol wt 24,000 from pFF, activins (9-11). Activin-A is a homodimer comprising two β-subunits of inhibin A ($\beta_A\beta_A$), while activin AB, a heterodimer, consists of the β-subunits of inhibins A and B ($\beta_A\beta_B$). Activin A and activin AB are equipotent in stimulating the specific secretion of FSH (9-11).

Recently, a novel mol wt 35,000 protein comprised of a single polypeptide chain which is highly enriched in cysteines was isolated from pFF based on its ability to suppress the secretion of FSH by pituitary cells in vitro (12). This protein, named follistatin, has no sequence homology with the previously characterized inhibins (13). In addition, a mol wt 32,000 follistatin that may differ from the 35 Kd form in glycosylation or carboxyl terminal truncation was also isolated (12). We report here that both forms of follistatin are specific inhibitors of the secretion of FSH in vitro. Moreover, while follistatins and inhibin-A are qualitatively indistinguishable in their ability to suppress the basal secretion of FSH in vitro, we have observed a highly significant difference in their respective effects on the pituitary tissue content of FSH.

MATERIALS AND METHODS

Follistatin preparations: Two proteins, 35 Kd and 32 Kd, were purified from pFF using essentially the same procedures employed for the isolation of inhibins and activins (12). These proteins, with homogeneity ascertained by sequencing and showing no contamination of inhibin, migrated ahead of the activins and inhibins on the HPLC column, and were named follistatins because of their ability specifically to suppress the release of FSH (follitropin). On SDS/PAGE under non-reducing conditions, the proteins migrated as a single band at apparent mol wt 35,000 and 32,000, respectively, while under reducing conditions they migrated, still as single bands, at mol wt 42,000 and 40,000, respectively, indicating that each protein is composed of a single polypeptide chain (See 12).

Pituitary Monolayer Culture and Bioassay: The FSH release-inhibiting activity of follistatin was measured by the same in vitro bioassay used in the isolation of inhibins and activins (14). In experiments in which cell contents of FSH were measured, after the media were removed and saved, the cells were washed once with DMEM and then lysed with 1 N HOAc. The lysed mixtures were lyophilized and reconstituted with RIA buffer. To determine cell number, the cells were trypsin-digested, removed from the wells and counted by Coulter counter. The ^{51}Cr cytotoxicity test was performed according to Robertson et al. (15). In experiments in which the combined effects of inhibin and follistatin were assessed, the cells were incubated (48 h) in the presence or absence of 1 ng/ml inhibin-A with increasing doses of follistatin.

RIA: RIAs for rat pituitary hormones were performed with antiserum provided by Dr. A. Parlow through the National Hormone and Pituitary Program (National Institutes of Diabetes and Digestive and Kidney Diseases).

Statistical Analyses: The dose response curves were calculated using the best-fit program (Allfit) described by DeLean et al. (16). Potencies were determined by the Bioprog method described by Rodbard (17) and statistical significance between means was determined by the multiple comparison tests of Duncan and Dunnett (18) following analysis of variance (EXBIOL). All calculations were conducted through the Biocomputing Laboratory of the Salk Institute.

RESULTS

Dose-Response Relationships of Follistatin: Purified 35 Kd and 32 Kd follistatins significantly suppressed FSH release in an identical dose-response curve when studied at doses ranging from 0.25 to 64 ng/ ml with an ED_{50} of 6.2 and 7.3 ng/ml, respectively (Fig. 1). Statistical analyses showed that they have identical slopes and are equipotent. When compared with the dose-response curve of inhibin, follistatins and inhibin-A show identical slopes and identical maximal effect (ED_{max}). However, the potency of follistatin is 1/3 that of inhibin. In other words, follistatins and inhibins have identical effects and intrinsic activities, but with different specific activity. The purified follistatin preparations were not cytotoxic in culture as based on the failure to observe any significant change in ^{51}Cr release with increasing doses of follistatin (data not shown). Nor did it alter the cell number in culture as determined by cell counting at the end of incubation.

Specificity of Follistatin on the Suppression of Immunoreactive FSH Release: When tested in the assay described above, purified 35 Kd and 32 Kd follistatins at doses ranging from 0.25 to 64 ng/ml, suppressed the secretion of immunoreactive FSH. These proteins had no effect on the secretion of immunoreactive LH, TSH, GH, or PRL (data not shown).

Time-Course Relationship of Follistatin: Dispersed pituitary cells were incubated with follistatin for different times to determine the time period needed to exhibit activi-

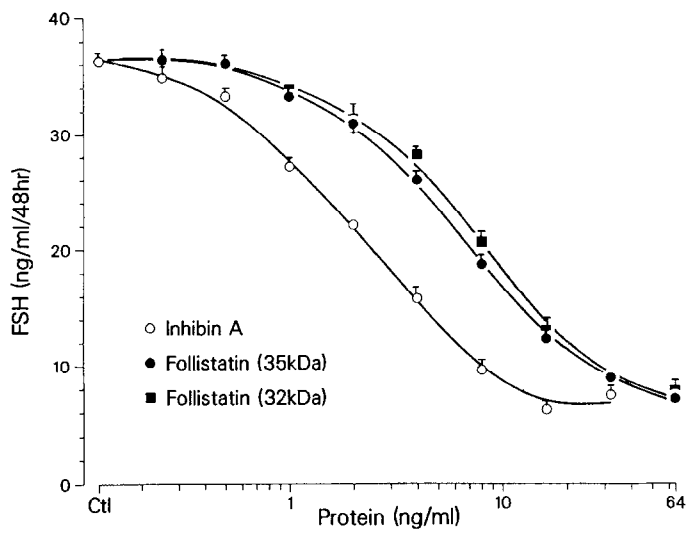

FIG. 1. Dose-response curves of 35 Kd and 32 Kd follistatins and 32 Kd inhibin on the suppression of FSH release in cultured rat pituitary cells. The vertical bar on the symbols represents SEM; when SEM is less than the height of the symbol, the vertical bar is not indicated; n=3 wells. Data shown correspond to one of two experiments with similar results.

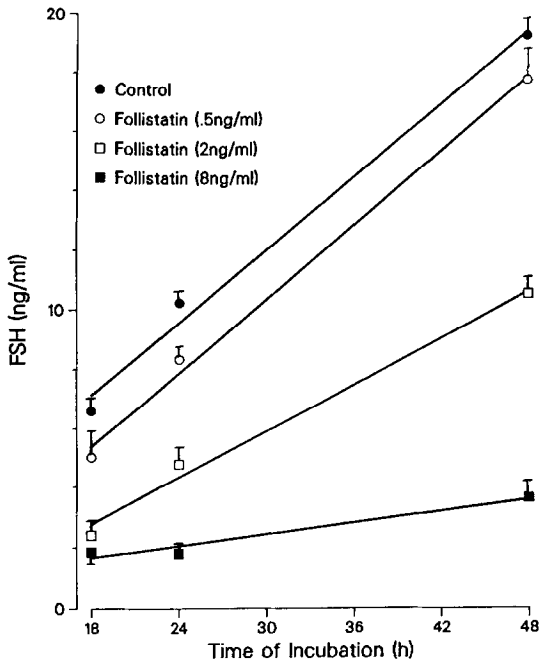

FIG. 2. Pituitary cells in triplicate wells were incubated with increasing amounts of follistatin, the medium was removed at different hours throughout the culture and basal release of FSH was measured by RIA.

ty. Suppression of immunoreactive FSH release was demonstrable 18 h after contact with the pituitary cells (Fig. 2). This suppression was further manifested after longer duration of incubation.

Follistatins primarily act on the release of FSH: The cell content of FSH in follistatin-treated cells was not significantly different from that in control except at high doses (Fig. 3), indicating that the synthesis of FSH was less affected by follistatin than by inhibin in the multiple comparison tests; the differences in the mean values for FSH content of cells treated with the higher doses of follistatin or inhibin are highly significant (≥ 0.01).

Follistatins showed no cross-reactivity in RIA specific for inhibin: The purified follistatin at doses as high as 100 ng/ tube did not cross-react with a sensitive, specific RIA for inhibin using antibodies raised against a synthetic replicate of the NH^2-terminal portion of the α-chain of inhibin, [Tyr^{30}]-IN-α(1-30) (19).

Follistatins and inhibins are additive agonists: When 1 ng/ml inhibin was added to cells treated with increasing doses of follistatins, additive suppression of FSH release was observed (Fig. 4).

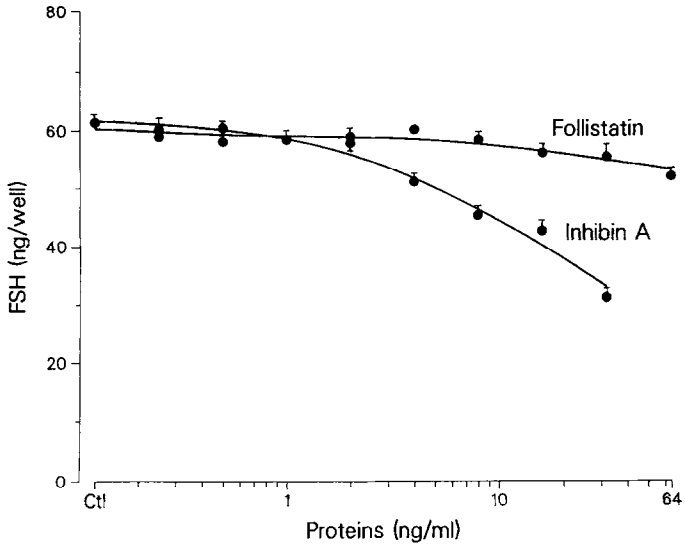

FIG. 3. Pituitary FSH content was determined in cells (~0.25 X 10^6 per well) in triplicate wells incubated with varying doses of follistatin and inhibin. Data shown correspond to one of two experiments with similar results.

DISCUSSION

The data presented here show that the biological activity of the highly purified 35 Kd follistatin is quantitatively indistinguishable from that of 32 Kd follistatin. The slopes

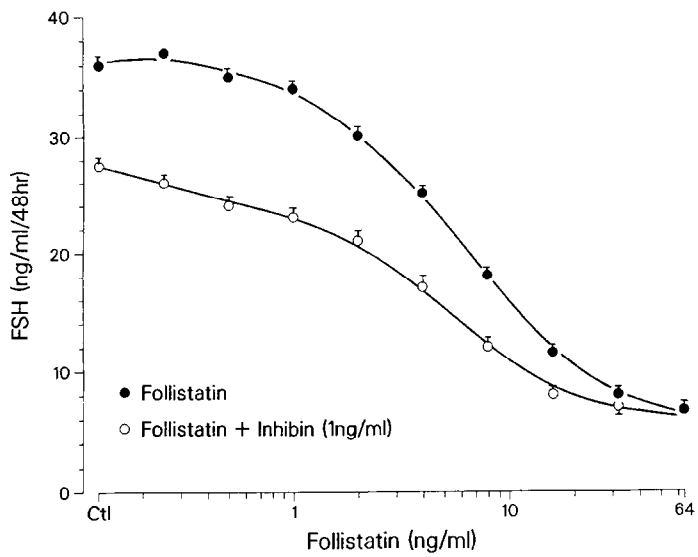

FIG. 4. Pituitary cells in duplicate or triplicate wells in each experiment were incubated in the presence or absence of an effective dose of inhibin (1 ng/ml) with increasing doses of follistatin. After 48-h incubation, immunoreactive FSH was determined as described. n=10, as pools of 4 identical experiments.

of the dose-response curves are identical as are the values for ED_{50} and ED_{max}. These novel proteins have an activity similar to that of the dimeric inhibins with identical slopes and ED_{max}, but are about 1/3 as potent as inhibin on an equimolar basis. The administration of an effective dose of inhibin with increasing doses of follistatin produces an additive effect on the suppression of FSH release. The action of follistatin, as is the case with inhibin, is demonstrable only after 18-h incubation with a latent period of 8 h. This long-delayed action is totally different from that of the hypothalamic hypophysiotropic factors which take seconds to show demonstrable response and <4 h for maximal effect.

Follistatin can be distinguished from inhibins in several ways. Inhibin is more potent than follistatin, typically exhibiting ED_{50} values <1/3 that of follistatin. The primary action of follistatins appears to be the suppression of FSH release instead of inhibition of both basal secretion and synthesis of FSH as shown by inhibin. Antibodies raised against the synthetic fragment at the N-terminal of the inhibin α-chain, which could completely neutralize the FSH-suppressing activity of inhibin, have no effect on the secretion of FSH suppressed by follistatin. Furthermore, a specific RIA for inhibin developed with the antiserum described did not cross-react with follistatin. These results indicate that follistatin is a totally new molecule that specifically suppresses the release of FSH by the pituitary.

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