A nonmitogenic pituitary function of fibroblast growth factor: Regulation of thyrotropin and prolactin secretion

(releasing factors/cell culture/pituitary hormones/cell growth)

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ABSTRACT The addition of fibroblast growth factor (FGF) to primary cultures of rat anterior pituitary cells modifies their response to thyrotropin-releasing factor in a dose-dependent manner. While the pituitary response to the other releasing factors (corticotropin-releasing factor, growth hormone-releasing factor, and gonadotropin-releasing factor) is not altered, FGF increases both the sensitivity of the cells to thyrotropin-releasing factor and the amounts of prolactin and thyrotropin released. A minimum of 24 hr of preincubation with FGF is required to modify the pituitary response, and maximal effects were observed with 48 and 72 hr of preincubation. The effective doses of FGF are similar to those described for its mitogenic activity (i.e., 1–100 pM), but inhibition of cell growth with 5-fluorodeoxyuridine does not modify the effect of FGF on thyrotropin and prolactin release. These results suggest a novel paracrine, if not autocrine, role of pituitary FGF in the homeostatic mechanisms that regulate the secretion of prolactin and thyrotropin. They also suggest that the biological significance of the presence of FGF in various tissues may not be directly related to its *in vitro* mitogenic activity.

The widespread distribution of fibroblast growth factor (FGF) in several tissues as diverse as pituitary, brain, adrenal, ovary, kidney, macrophage, retina, and chondrosarcoma (1–6,*) was recently established with the availability of sequence-specific antisera (7) and the amino acid sequence information obtained from its structural characterization (2). These observations suggested that the pituitary was not the source of FGF for other tissues (6) and that the diverse target-cell population stimulated by FGF *in vitro* (8) is correlated with paracrine, if not autocrine, activities *in vivo*. Thus, the capacity of FGF to induce angiogenesis, induce neovascularization, and promote injury repair (9–11) may reflect its local presence in tumors, placenta, and macrophage, respectively. Similarly, its mitogenic effect on normal adrenal and granulosa cells may reflect an *in situ*, local function that would explain its presence in adrenal, corpus luteum, and ovary. It was with this possibility in mind that we tested the hypothesis that the physiological function of the FGF detected, isolated, and characterized from the pituitary is in the regulation of pituitary hormone secretion.

MATERIALS AND METHODS

Preparation of Pituitary Cell Cultures. Rat anterior pituitaries were collected from male Sprague–Dawley rats (125 g) and processed for cell culture by collagenase digestion (7, 12). Cells were seeded at a density of $0.5–1 \times 10^5$ cells per ml into miniwell plates and incubated at 37°C in a humidity-controlled atmosphere containing 8% CO_2. On day 5 of

culture, the cells were washed with Dulbecco's modified Eagle's medium (DMEM) and incubated with 900 μl of fresh DMEM supplemented with 0.1% bovine serum albumin. Releasing factors, in the presence or absence of FGF, were added to the cells in a 100-μl aliquot for a period of 4 hr. In each instance, as described elsewhere (12), the response to each releasing factor was the same whether they were added to the cells individually or together. The medium was collected and diluted, and the appropriate aliquots were used for the measurement of growth hormone (GH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), corticotropin (ACTH), thyrotropin (TSH), and prolactin (PRL).

Radioimmunoassays. LH, FSH, TSH, PRL, and GH concentrations were all measured with reagents provided by the National Pituitary Agency of the National Institutes of Health, except for GH, in which case an antibody raised against mouse GH was used (13). ACTH was measured by using an antibody (Ac1) raised against $ACTH_{1-24}$ (14). Proteins and peptides were radioiodinated by the method of Greenwood *et al.* (15).

Peptide and Protein Preparation. Synthetic rat $ACTH_{1-39}$, rat ACTH (corticotropin)-releasing factor (CRF), rat GH-releasing factor (GRF), LH-releasing factor (LRF), and TSH-releasing factor (TRF) were prepared in this laboratory by solid-phase synthesis (16, 17). The FGF used in these studies was prepared from bovine pituitaries by the method described by Gospodarowicz *et al.* (3). Homogeneity of the mitogen was established in this laboratory by NaDodSO$_4$/gel electrophoresis, amino acid analysis, reverse-phase HPLC, and amino-terminal sequencing. Quality control data is available on request.

Cell Number Determination. The primary cultures of rat pituitary cells were treated with 1 ml of trypsin-EDTA containing 0.9% NaCl, 0.01 M sodium phosphate, 0.05% trypsin, and 0.02% EDTA. The cells were triturated to obtain a single-cell suspension, and cell number was determined with a Coulter Counter.

Statistical Analysis. The dose–response curves were calculated according to the best-fit program (Allfit) described by De Lean *et al.* (18). Potencies were determined by the "Bioprog" method described by Rodbard (19), and statistical significance was established by the multiple comparison tests of Duncan and Dunnett. All calculations were performed through the Biocomputing Laboratory of the Salk Institute.

RESULTS

Effect of FGF on Pituitary Hormone Release. The incubation of rat anterior pituitary cells for 4 hr with FGF did not modify basal or stimulated release of pituitary hormones (Fig. 1A). As expected, FGF also had no effect on cell number during the 4-hr incubation period (not shown). When the cells were preincubated with FGF for 48 hr prior to addition of releasing factors (Fig. 1B), there was a significant increase in the amounts of PRL ($P < 0.01$) and TSH ($P < 0.01$) released from the TRF-stimulated cells. None of the other pituitary hormones was affected by the addition of FGF. Although basal PRL release was increased ($P < 0.05$), there was no effect on the basal release of TSH. The number of cells in the wells treated with FGF increased from $1.88 \pm 0.05 \times 10^5$ to $2.04 \pm 0.05 \times 10^5$ cells per well ($P < 0.05$).

Effect of FGF on the Dose–Response to TRF. The dose–response to TRF on the release of PRL and TSH is significantly modified by FGF (Fig. 2). In this instance, cells were treated for two periods of 24 hr with 2 ng of FGF, followed by an acute (4 hr) incubation with various concentrations of TRF. Potency analysis of the dose–response curves (18, 19) established that TRF was 5 times more potent on cells that had been pretreated with FGF. It was also clear, however, that the effect of FGF on PRL and TSH release was considerably different. Basal PRL release (Fig. 2 *Upper*) was increased by pretreatment of cells with FGF ($P < 0.01$), whereas basal TSH release (Fig. 2 *Lower*) was not affected. The PRL response in cells pretreated with FGF appears to be the result of a complete phase shift rather than an increase in potency

per se. As such, the PRL response to TRF in these cells, while quantitatively greater at any dose of TRF when compared to untreated cells, is in fact qualitatively identical when each is compared to its controls. In contrast, FGF pretreatment of pituitary cells increased both the TSH response and sensitivity to the secretagogue.

Potency and Time Course of the Effect of FGF. The concentrations of FGF required to elicit the modified response to TRF are comparable to those reported by ourselves and others for its *in vitro* mitogenic effects (Fig. 3). Basal TSH release was unaffected by the preincubation with any of the doses of FGF tested (Fig. 3 *Middle*). Basal PRL was significantly increased by treatment with the 2-ng dose of FGF ($P < 0.01$) (Fig. 3 *Top*). The TSH and PRL response to TRF was modified by FGF at 0.2 and 2 ng/ml ($P < 0.01$) (Fig. 3 *Middle*). PRL release was also changed by the pretreatment with FGF at 0.02 ng/ml ($P < 0.05$) (Fig. 3 *Top*). Cell growth was also modified by FGF (Fig. 3 *Bottom*) as a function of the concentration of mitogen.

The effect of FGF is time-dependent, with a minimum of 24 hr of treatment required to observe the modified pituitary response (Fig. 4). There was no effect of FGF on basal TSH release at any of the times of preincubation (Fig. 4 *Middle*). Basal PRL release was increased with the 48- and 72-hr treatments ($P < 0.05$), and both stimulated PRL ($P < 0.05$) and TSH ($P < 0.01$) releases were significantly increased after a 24-hr treatment with FGF (Fig. 4 *Top* and *Middle*). The growth of cells was also time-dependent (Fig. 4 *Bottom*). FGF increased basal cell growth as early as 24 hr after the initiation of the experiment ($P < 0.05$).
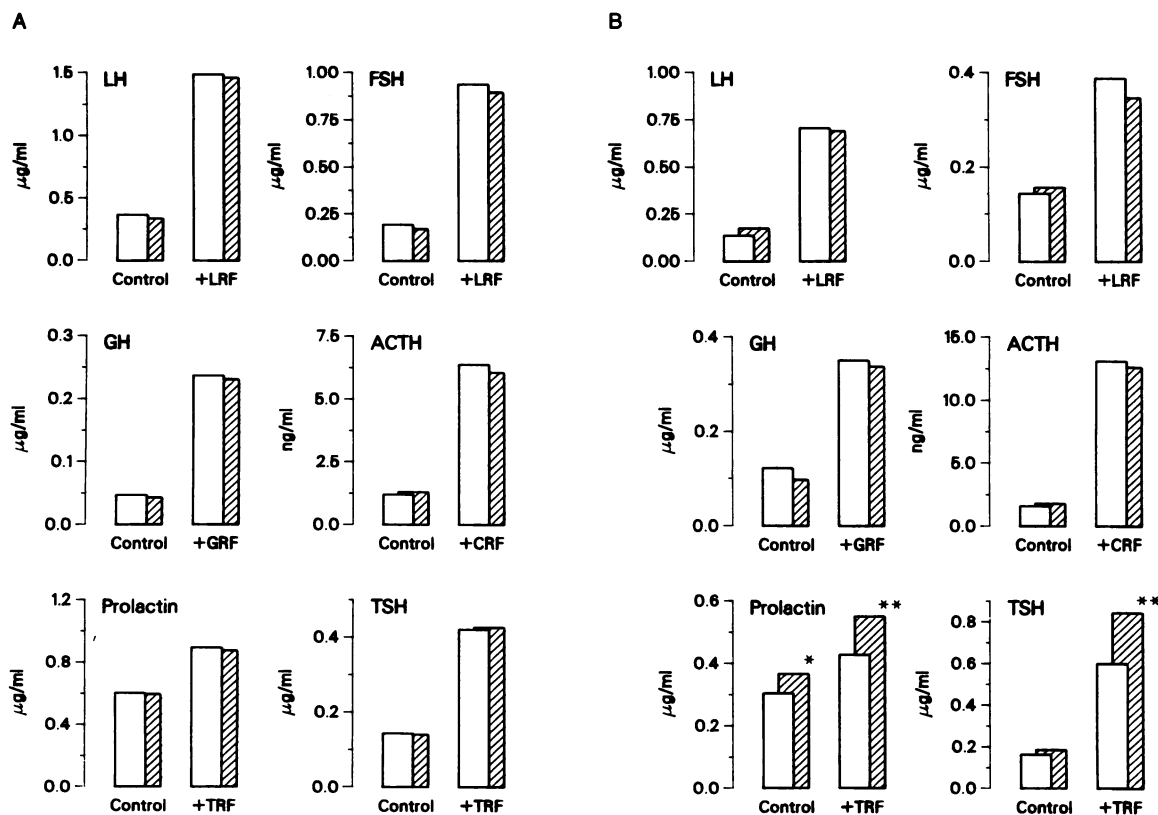

FIG. 1.   (A) Acute effects of FGF on pituitary function. Control medium or medium containing the releasing factors was added to the cells in a 100-μl aliquot with (hatched bars) or without (clear bars) 2 ng of FGF. Cells were either incubated with one releasing factor or a mixture of LRF (1 nM), GRF (0.1 nM), CRF (1 nM), or TRF (10 nM). After 4 hr, the medium was removed, and the appropriate aliquots were taken for the measurement of LH, FSH, GH, ACTH, PRL, and TSH. The standard errors were all within 10% of the mean ($n = 6$). (B) Long-term effects of FGF on pituitary function. On day 3 of culture, 2 ng of FGF was added in 10-μl aliquots to the fresh medium. The treatment was repeated the following day and on day 5 of culture, untreated (open bars) and FGF-treated (hatched bars) cells were washed and incubated with medium alone (control) or with GRF (0.1 nM), CRF (1 nM), LRF (1 nM), and TRF (10 nM). The standard errors were all within 10% of the mean ($n = 6$). *, $P < 0.05$; **, $P < 0.01$.
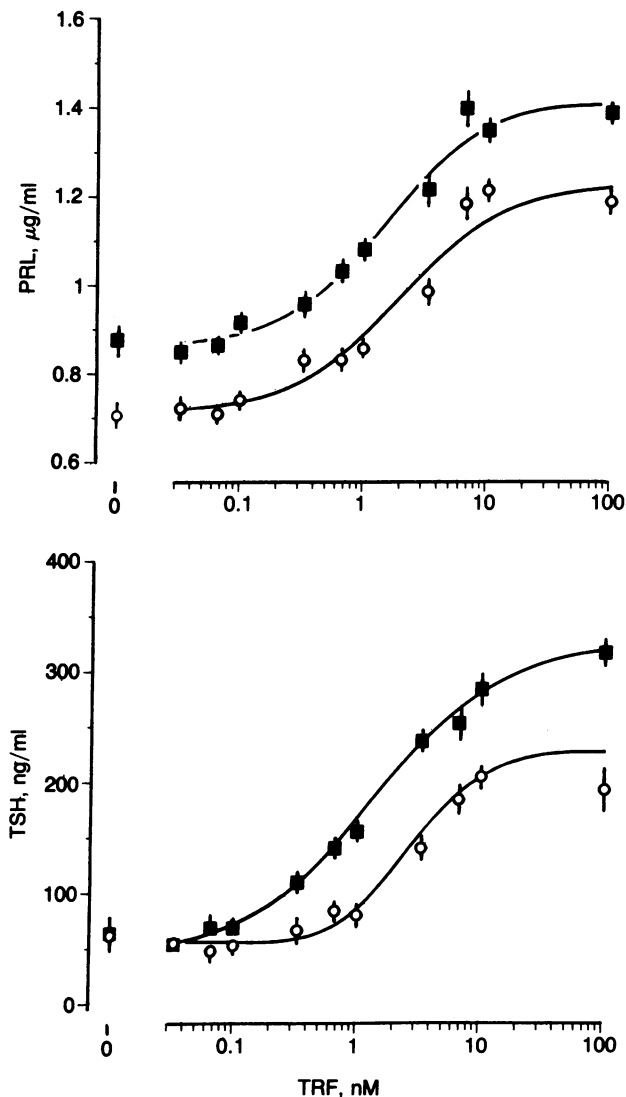
FIG. 2. TRF dose–response curve in FGF-treated cells. On day 3 of culture, pituitary cells were incubated with 2 ng of FGF for two successive periods of 24 hr. On day 5 of culture, the cells were incubated with various doses of TRF. ○, Results from control cells; ■, results from FGF-treated cells. The curves were generated from the best-fit analysis of the data (Allfit; ref. 18), and each point is the mean of seven replicates.

FIG. 3. Dose–response effect of FGF. On day 3 of culture, pituitary cells were incubated for two periods of 24 hr with 0, 0.02, 0.2, or 2.0 ng of FGF. On day 5 of culture, basal PRL (*Top*) and TSH (*Middle*) release (●) was measured in some wells, and the response to a maximal dose of TRF (10 nM) (○) was measured in others (*Top* and *Middle*). At the end of the experiment, the cell number (*Bottom*) was determined after trypsinization by using a Coulter particle counter. Each point is the mean of six replicate wells. *, $P < 0.05$; **, $P < 0.01$.

Effect of the Inhibition of Cell Growth. Because FGF is a potent mitogen for several cell types, including the mixed population of pituitary cells used here as well as other endocrine cells (9,*), we investigated the possibility that FGF acts by stimulating the proliferation of lactotrophs and thyrotrophs. The approach used was to prevent basal and FGF-stimulated cell growth with 5-fluorodeoxyuridine (5-FdU) (Fig. 5). The treatment with 5-FdU effectively inhibited the growth of control cells and completely blocked the effect of FGF on cell number ($P < 0.01$) (Fig. 5 *Bottom*). The density of cells prior to the 48-hr treatment with 5-FdU (0.62 $\times$ 10^5 cells per well) remained the same throughout the incubation time. There was no evidence to suggest cell death as determined by trypan blue exclusion. At the same time, 5-FdU had no effect of basal TSH release (Fig. 5 *Middle*), and pretreatment of either control or 5-FdU-treated cells with FGF still gave an increased TSH response to TRF (10 nM; $P < 0.01$). Consistent with the demonstration by Pasteels (20) that lactotrophs do grow in culture, the treatment of cells with 5-FdU decreased significantly ($P < 0.01$) basal PRL release
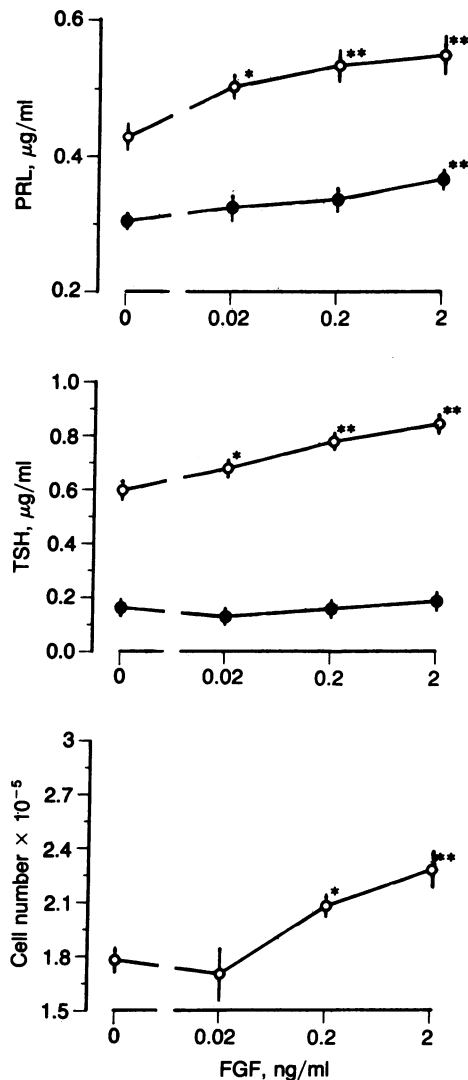
(Fig. 5 *Top*). In spite of this inhibition, FGF (2 ng/ml) remained effective in increasing both basal and TRF-stimulated PRL release by the 5-FdU-treated cells ($P < 0.01$). Therefore, after a 48-hr treatment with FGF, the magnitude of the response to TRF is the same with or without the growth inhibitor, even though throughout this period there was no cell growth. The purity of the growth factor used and the potency (10 pM) of its pituitary effects preclude the possibility that the activity reported here is not an intrinsic activity of FGF itself. Therefore, we concluded that the effect of FGF on pituitary function occurs, at least in part, independently of its activity on cell growth.

DISCUSSION

Although at this time it is difficult to ascribe a mechanism of action of FGF on pituitary function, a possible explanation would be a direct effect of FGF on the intracellular pools of releasable PRL and TSH. Increased synthesis of PRL would account for the ability of TRF to release larger amounts of the
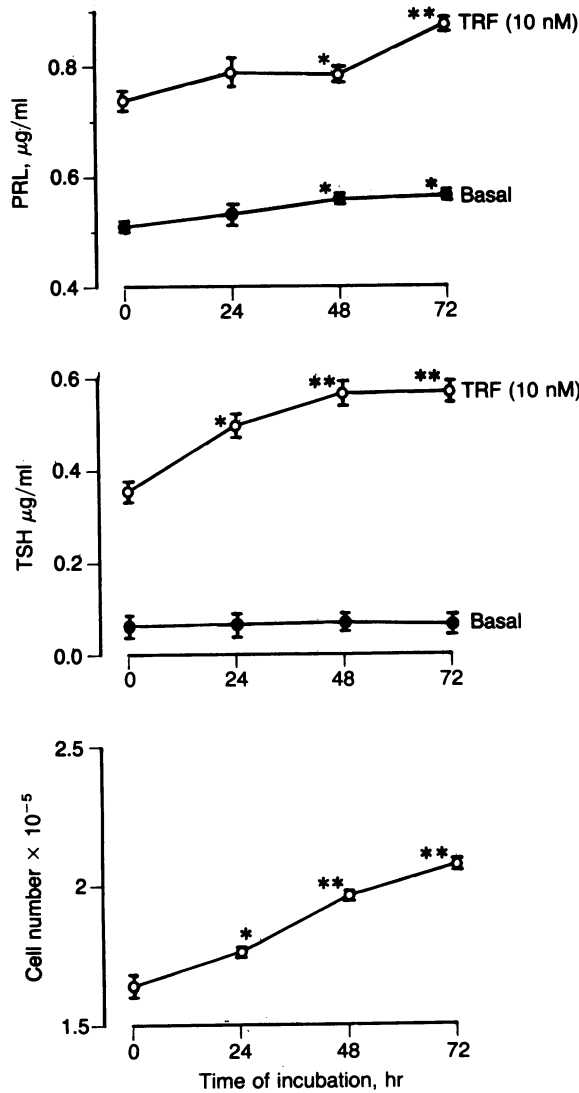
FIG. 4.   Time course of the effect of FGF. On day 3 of culture, 2 ng of FGF was added to six wells (labeled 72 hr). The following day, this exact treatment was repeated to these same cells along with a second set of six wells (labeled 48 hr). Twenty-four hours later, FGF was added again to these cells as well as to a third set of dishes (labeled 24 hr). The following day, cells that had either not seen any FGF (o) or cells that had been preincubated for 1 day (24 hr), 2 days (48 hr), or 3 days (72 hr) with FGF were washed and incubated with medium alone (●) or with medium containing 10 nM TRF (o). At the end of the experiment, cell number (*Bottom*) was determined after trypsinization by using a Coulter particle counter. Each point is the mean of six determinations.

FIG. 5.   Effect of 5-FdU on the response to FGF. On day 3 of culture, pituitary cells were incubated with or without ("Control") 10 μM 5-FdU and with (hatched bars) or without (open bars) 2 ng of FGF for two successive periods of 24 hr. On day 5 of culture, cells were washed, and cells untreated with FGF and FGF-treated cells were assessed for their ability to release PRL (*Top*) and TSH (*Middle*) without ("Basal") or with ("+TRF") stimulation by 10 nM TRF. Cell number (*Bottom*) was determined at the end of the experiment by using a Coulter particle counter. Bars are the mean of six replicates.

pituitary hormone at all effective doses. It would also explain the effect of FGF on basal PRL release; the removal of the normal dopaminergic inhibition of PRL (21) *in vitro*, coupled with the FGF-mediated increase in PRL synthesis, would have the net effect of increasing both basal and stimulated PRL release. TSH secretion, which is exclusively under stimulatory rather than inhibitory control, would not show the effect on basal release except when stimulated with TRF. It is also possible, however, that FGF acts on thyrotrophs and lactotrophs by modifying the functional heterogeneity in their responsive cell populations (22–25). In this paradigm, the specific subsets of TSH- and PRL-releasing cells that are not responsive to TRF under control conditions would be directed by FGF into a functional synchronicity in hormone release. The net result would be the activation of a subpopulation of nonresponsive cells to become sensitive to the
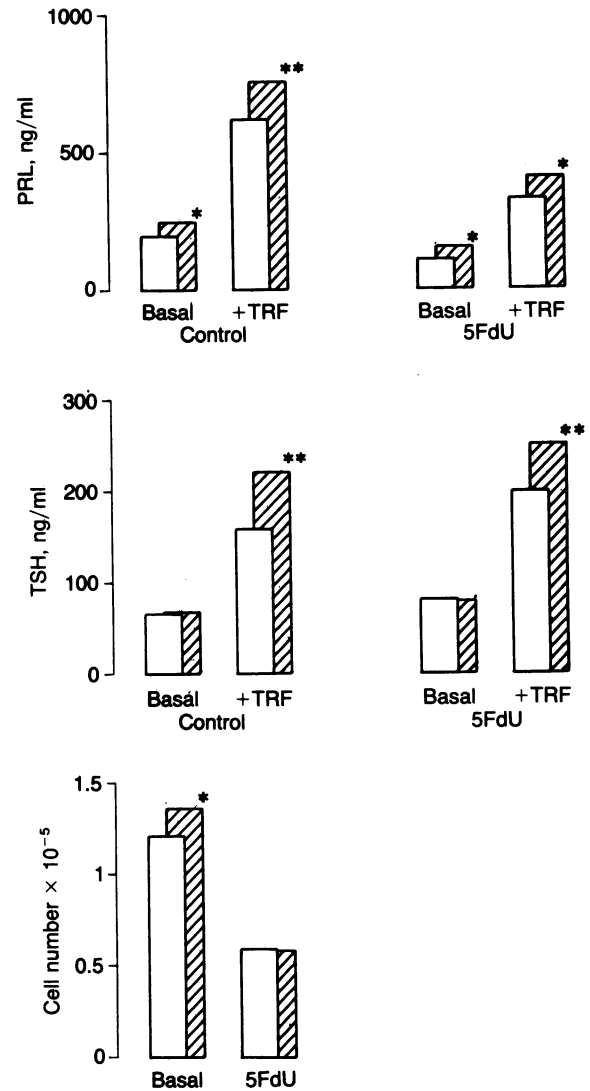
hypothalamic secretagogue. These mechanisms have several implications, particularly in understanding the homeostatic processes that regulate the release of PRL and TSH *in vivo*. In particular, it will be necessary to reconsider the possible role FGF may play during pregnancy, lactation, and fetal development. Moreover, it will be of particular importance to determine the role of factors, like estrogens, that can regulate the expression of FGF at the pituitary level (7).

The results presented here are supported by the early observations of Schonbrunn *et al.* (26) using clonal (GH$_4$C$_1$) cells derived from a rat pituitary tumor. In these experiments, high concentrations (10 nM) of a commercial preparation of FGF were found to increase the release of PRL over a 3-day incubation. Although the effect of adding TRF was not examined, this preparation of FGF was also reported to decrease cell number, increase cell volume, and decrease basal GH release; a similar but quantitatively greater effect was obtained with epidermal growth factor. From the results

presented here, it is clear that pure FGF can increase basal PRL release by normal rat anterior pituitary cells *in vitro* but that its major effect is on increasing the pituitary response to TRF. There is also no acute effect on the release of any pituitary hormone; in long-term incubations, basal and stimulated GH release is not modified by FGF. Identical experiments with epidermal growth factor on normal pituitary cells (results not shown) demonstrated that this growth factor had no effect on basal or TRF-stimulated PRL release by normal rat pituitary cells *in vitro*.

The observations reported here raise totally new questions pertaining to the physiological role of FGF in various tissues. They offer the possibility that the *in situ* function of FGF may not necessarily be exclusively linked to the stimulation of cell growth. This is of particular importance since FGF is widely distributed with apparent ubiquity in several tissues of distinct functional, embryological, and neurophysiological origin. Moreover, it can be detected in considerable quantities (1–6,*). On the basis of the results presented here, we propose that pituitary FGF participates in an intrapituitary mechanism regulating normal PRL/TSH secretion and actually may be involved in the pathophysiological expression of these hormones. The discovery that a potent growth factor like FGF, classically considered in events such as wound healing, tissue repair, and angiogenesis (9–11), can modulate cell function independently of its effects as a growth factor *per se* opens another dimension in the understanding of the normal physiology and pathophysiology of growth factors. Its local presence in the adrenal, ovary, corpus luteum, liver, and kidney (6,*) may well reflect, as in the pituitary, an intraglandular regulation of function as well as growth. Its presence in tissues such as macrophages (4) suggests that, as a paracrine activity, it may be involved in wound healing and, possibly, in pathophysiological states and atherosclerosis. In this model, the *in situ* physiological role of FGF in any given tissue would be a direct function of the local cellular milieu in which it is released. Moreover, the respective mechanisms of regulating growth and function need not be mutually exclusive.

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