EPIDERMAL GROWTH FACTOR AND THYROTROPIN-RELEASING HORMONE ACT SIMILARLY ON A CLONAL PITUITARY CELL STRAIN

Modulation of Hormone Production and Inhibition of Cell Proliferation

AGNES SCHONBRUNN, MARGO KRASNOFF, JOANNE M. WESTENDORF, and ARMEN H. TASHJIAN, JR.

From the Laboratory of Toxicology, Harvard School of Public Health, and the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT

GH_4C_1 cells are a clonal strain of rat pituitary cells that synthesize and secrete prolactin and growth hormone. Chronic treatment (longer than 24 h) of GH_4C_1 cells with epidermal growth factor (EGF) (10^{-8} M) decreased by 30–40% both the rate of cell proliferation and the plateau density reached by cultures. Inhibition of cell proliferation was accompanied by a change in cellular morphology from a spherical appearance to an elongated flattened shape and by a 40–60% increase in cell volume. These actions of EGF were qualitatively similar to those of the hypothalamic tripeptide thyrotropin-releasing hormone (TRH) (10^{-7} M) which decreased the rate of cell proliferation by 10–20% and caused a 15% increase in cell volume. The presence of supramaximal concentrations of both EGF (10^{-8} M) and TRH (10^{-7} M) resulted in greater effects on cell volume and cell multiplication than either peptide alone. EGF also altered hormone production by GH_4C_1 cells in the same manner as TRH. Treatment of cultures with 10^{-8} M EGF for 2–6 d increased prolactin synthesis five- to ninefold compared to a two- to threefold stimulation by 10^{-7} M TRH. Growth hormone production by the same cultures was inhibited 40% by EGF and 15% by TRH. The half-maximal effect of EGF to increase prolactin synthesis, decrease growth hormone production, and inhibit cell proliferation occurred at a concentration of 5×10^{-11} M. Insulin and multiplication stimulating activity, two other growth factors tested, did not alter cell proliferation, cell morphology, or hormone production by GH_4C_1 cells, indicating the specificity of the EGF effect. Fibroblast growth factor, however, had effects similar to those of EGF and TRH. Of five pituitary cell strains tested, all but one responded to chronic EGF treatment with specifically altered hormone production. Acute treatment (30 min) of GH_4C_1 cells with 10^{-8} M EGF caused a 30% enhancement of prolactin release compared to a greater than twofold increase caused by 10^{-7} M TRH. Therefore, although EGF and TRH have qualitatively similar effects on

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GH_4C_1 cells, their powers to affect hormone release acutely or hormone synthesis and cell proliferation chronically are distinct.

GH[1] cells are clonal strains of rat pituitary tumor cells that synthesize and secrete prolactin and growth hormone (28, 30). These cells have retained many of the differentiated properties of normal pituitary cells during 14 yr in continuous culture and have proved a valuable model for studying regulatory factors which control pituitary cell function in the intact animal. For example, effects of the hypothalamic tripeptide thyrotropin-releasing hormone (TRH) on the synthesis or release of pituitary hormones other than thyrotropin had not been described until Tashjian et al. (31) reported a direct stimulatory effect of TRH on prolactin production by GH_3 cells. Prolactin-releasing activity of TRH was then extensively documented in many species both in vivo (5, 21) and in vitro (35). Since the initial observation, the biological actions of TRH on GH cells have been further characterized (for a review, see reference 23). Chronic treatment of GH cells (longer than 4 h) with TRH stimulates the biosynthesis of prolactin and inhibits the rate of growth hormone production (10, 31). TRH appears to modulate the rates of hormone biosynthesis by regulating the concentrations of prolactin and growth hormone mRNA's and not by altering the rate of translation of preexisting mRNA (11, 14, 15). In addition to these chronic actions, TRH acutely (within 30 min) stimulates the release of previously synthesized intracellular prolactin and growth hormone (12, 24). In contrast to these stimulatory effects of TRH on GH cells, the hypothalamic tetradecapeptide somatostatin has been shown to inhibit acutely prolactin and growth hormone release and the production of both hormones during chronic treatment (27). Specific high-affinity receptors for both TRH and somatostatin have been characterized, and there is strong evidence that these receptors mediate the biological actions of both hypothalamic peptides on GH cells (18, 19, 27).

In this paper we demonstrate that hormone production by GH cells is also regulated by epidermal growth factor (EGF), a protein whose physiological role is not yet understood (for reviews, see references 8 and 16). The most thoroughly documented biological action of EGF is stimulation of cell proliferation in epidermal and epithelial tissues in animals and in several nontransformed and transformed cell types in culture. In contrast to the growth stimulatory effects observed with most cells, we have found that low concentrations of EGF inhibit the proliferation of GH cells. At the same time, EGF alters specifically the production of prolactin and growth hormone, and these effects are qualitatively similar but quantitatively greater than the effects of TRH. A preliminary report of some of these results has been presented (footnotes 2 and 3).

MATERIALS AND METHODS

Hormones and Growth Factors

Mouse EGF, bovine fibroblast growth factor (FGF) and multiplication stimulating activity (MSA) were purchased from Collaborative Research Inc. (Waltham, Mass.). Bovine insulin was obtained from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, Calif.). Synthetic TRH was a gift from Abbott Diagnostics, Diagnostics Products (North Chicago, Ill.).

Cell Culture

The properties of GH_4C_1 rat pituitary cells and the methods of their culture have been described (28, 33, 23). All other cell strains utilized were grown under the same conditions as GH_4C_1 cells.

Cell culture experiments were performed using replicate 35-mm dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) inoculated simultaneously with equal aliquots of cells from a single donor culture and grown under identical conditions. Cultures were generally inoculated at a density of 0.5 to 2×10^5 cells/35-mm dish and grown as monolayers in Ham's F10 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 15% horse serum and 2.5% fetal calf serum

[1] *Abbreviations used in this paper:* EGF, epidermal growth factor; FGF, fibroblast growth factor; $F10^+$, Ham's F10 medium supplemented with 15% horse serum and 2.5% fetal calf serum; F10 lh, Ham's F10 medium supplemented with 5 mg/ml lactalbumin hydrolysate; GH, growth hormone; hCG, chorionic gonadotropin; MSA, multiplication stimulating activity; TRH, thyrotropin-releasing hormone.

[2] Tashjian, A. H., Jr., A. Schonbrunn, and T. F. J. Martin. 1978. Program of the Sixth Cold Spring Harbor Conference on Cell Proliferation, Hormones and Cell Culture. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y. 35.

[3] Schonbrunn, A., M. Krasnoff, M. E. Lomedico, and A. H. Tashjian, Jr. 1979. Program of the 61st Meeting of The Endocrine Society. The Endocrine Society, Bethesda, Md. 80.

($F10^+$) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium (1.5 ml/dish) was changed every 3–4 d. Compounds to be added to cultures were diluted in $F10^+$, sterilized by filtration through Millipore filters (Millipore Corp., Bedford, Mass.), and added in a volume of 10–20 μl.

Measurement of Cell Proliferation

For growth experiments, cells were plated at a density of 10^4–10^5 cells/35-mm dish in 1.5 ml of $F10^+$. After 24–72 h, the medium was aspirated to remove unattached cells and replaced with fresh $F10^+$ containing either no additions or 10^{-8} M EGF or 10^{-7} M TRH. This protocol eliminated any effects on plating efficiency. The media and peptides were renewed every 2–4 d as described in Results. Replicate dishes were incubated with 0.05% trypsin (Worthington Biochemical Corp., Freehold, N. J.), 0.02% EDTA in Earle's basal salt solution, and the harvested cells were resuspended in $F10^+$. The number of cells in the suspension was determined with an electronic particle counter (Coulter Electronics Inc., Hialeah, Fla.; model ZBI). For each point, cells harvested from triplicate dishes were counted. The standard deviation did not exceed ±5% of the mean. In four independent experiments, 10^{-8} M EGF inhibited cell proliferation by 37 ± 11% (mean ± SD), whereas 10^{-7} M TRH inhibited cell proliferation by 18 ± 7%.

To determine the cell volume within a treatment group, cells harvested from triplicate dishes were pooled and spectra of cell volume distributions were obtained from a multichannel analyzer (Coulter Channelyzer) connected to an X-Y recorder. Total counts of $\sim 10^5$ cells were accumulated in each spectrum. The validity of cell volume measurements with a Coulter spectrometer has been demonstrated by Anderson and Petersen (2).

To study cellular morphology, photographs of living cells were taken with an inverted phase-contrast microscope (Nikon) at × 200.

Protein Determination

Alkali-soluble cell protein was determined by the method of Lowry et al. (22) using bovine serum albumin as a standard.

DNA Determination

The amount of DNA per dish was measured by a fluorometer assay modified from Thomas and Farquhar (34). Cells from each dish were suspended in 1.8 ml of 0.05 M KH_2PO_4 buffer, pH 7.5, and freeze-thawed two times to disrupt the cells. Trichloroacetic acid was added to the cell suspension to a final concentration of 10%. Aliquots containing 1–10 μg of DNA were distributed to siliconized 10 × 75-mm test tubes. Bovine serum albumin (100 μg) was added to each sample, and the pellets were collected by centrifugation at 4°C. Each pellet was washed two times with cold 5% trichloroacetic acid and once with 50 mM sodium acetate in 95% ethanol and then dried at 60°C for 2 h. Recrystallized, charcoal-purified 3,5-diaminobenzoic acid (20 μl of a 40% wt/vol aqueous solution) (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was added and the samples were incubated for 45 min at 60°C. After addition of 0.5 ml of 1.0 N HCl, fluorescence was measured on a Turner Model III fluorometer (Turner Associates, Div. of Am. Sterilizer Co., Palo Alto, Calif.) equipped with a Corning type 405 primary filter (Corning Glass Works, Science Products Div., Corning, N. Y.) and a Corning 65 A secondary filter. DNA samples from each dish were assayed at two dilutions in duplicate. Calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.) was used as standard.

Measurement of Hormone Secretion

To determine the chronic effects of EGF on hormone production, replicate 35-mm dishes were incubated at 37°C in $F10^+$ with or without EGF for 24–72 h. Medium was collected and any floating cells were removed by centrifugation for 10 min at 5,000 *g* at 4°C. The supernatant medium was stored at −20°C for subsequent measurements of prolactin and growth hormone. No significant degradation of prolactin occurs either intracellularly or in the medium, and the amount of prolactin accumulated in the medium during these long-term incubations is a measure of *de novo* synthesis of the hormone (23).

To determine the acute effects of EGF on the release of presynthesized prolactin, each dish was washed two times with 1.5 ml of Ham's F10 medium supplemented with 5 mg/ml lactalbumin hydrolysate (F10 lh), then 2 ml of preequilibrated F10 lh medium with or without EGF was added. After incubation of cultures at 37°C in a CO_2-air incubator for 30 min, medium was collected, any floating cells were removed by centrifugation, and the supernatant medium was stored at −20°C. Pulse-labeling experiments with GH cell cultures have shown that newly synthesized prolactin exhibits a lag of ~1 h before appearing in the medium (10), indicating that the bulk of the hormone collected during a 30-min incubation period consists of previously stored and not newly synthesized hormone.

The amount of prolactin and growth hormone accumulated in medium was measured by specific quantitative microcomplement fixation immunoassays using baboon antiserum to rat GH and rabbit antiserum to rat prolactin (29, 30).

RESULTS

Action of EGF on Cell Proliferation

The data in Fig. 1 illustrate the effect of EGF on the growth of GH_4C_1 cells. 3 d after plating, any unattached cells were removed and fresh medium without or with 10^{-8} M EGF was added. Cells were grown without further medium changes until cell proliferation ceased as a result of exhaustion of medium nutrients. Cells in control medium increased in number 2.8-fold during the 4-d incubation, whereas EGF-treated cells increased only 1.7 fold (Fig. 1, top). Net DNA synthesis was decreased in EGF-treated cells in parallel with cell multiplication (Fig. 1, bottom). Average DNA per cell was 11 pg for both control and EGF-pretreated cultures.

The results shown in Fig. 2 demonstrate that EGF not only inhibited the rate of cell proliferation but also decreased the plateau cell density. Cells were plated at two densities differing by a factor of ten, and allowed to attach to culture dishes. Half the cultures were then treated with 10^{-8} M EGF, the remaining cultures were incubated with medium lacking EGF. Cells grown in the absence of EGF had an average population doubling time of 36 h, whereas cells grown in the presence of EGF had an average doubling time of

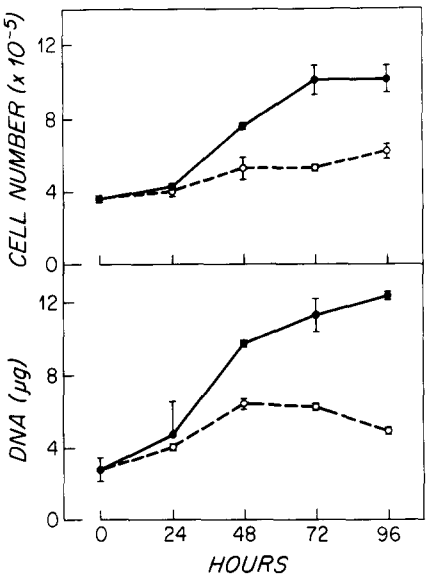

FIGURE 1 Effects of EGF on cell proliferation and DNA content. GH_4C_1 cells were plated at a density of 5 × 10^4 cells/35-mm dish in 1.5 ml $F10^+$. 3 d after plating, cells were fed with fresh medium with (○) or without (●) 10^{-8} M EGF. Cultures were incubated without further medium changes and dishes were harvested every 24 h. Cell number/dish (top panel) and cellular DNA/dish (bottom panel) were determined for replicate dishes as described in Materials and Methods. Each point represents the mean of duplicate dishes and the bars show the range.

46 h. In the presence of EGF, cultures reached a plateau density of 3 × 10^6 cells/dish, 66% of the final density reached by control cultures (4.5 × 10^6 cells/dish). The plateau density reached was not limited by the depletion of nutrients from the medium, since no net increase in cell number resulted when fresh growth medium was added.

It is important to emphasize that these inhibitory effects of EGF were not caused by a toxic effect on the cells. Addition of EGF to GH_4C_1 cells never resulted in a loss of cells; only the rate of increase of the cell population was altered. When EGF was added to cultures in the plateau phase of growth, no change in cell number was observed.

When cells grown in the presence of EGF were transferred to medium lacking EGF, the rate of cell proliferation increased and EGF-pretreated cells attained the same density as cultures never previously exposed to EGF (Fig. 2). Therefore, the inhibitory effect of EGF was reversible both for cultures that had reached a plateau density (Fig. 2, open triangles) and for cultures that were still in the log phase of cell division (Fig. 2, open circles).

EGF Increases Cell Size and Alters Cellular Morphology

Fig. 3 shows the appearance of cultures grown to high density in the presence or absence of EGF. Cells grown in the absence of EGF were spherical and loosely adherent to the substratum (Fig. 3*A*). Two types of cellular morphology were observed in cultures grown in the presence of EGF. Most of the cells were elongated and flattened with angular borders (Fig. 3*B*) while some, although larger than control cells, retained their rounded shape (Fig. 3*C*). The appearance of EGF-treated cells was reminiscent of the previously described effects of TRH on GH cell morphology (32), and Fig. 3*D* and *E* show the appearance of TRH-treated cells in the same experiment for comparison. Again, both enlarged rounded cells and elongated, flattened, angular cells were observed.

The presence of two types of cellular morphology in GH cell cultures is not a reflection of cell heterogeneity within the population. Time-lapse

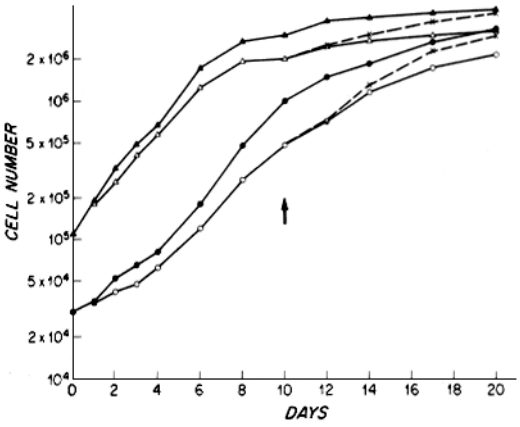

FIGURE 2 Effects of EGF on the proliferation of GH_4C_1 cells. GH_4C_1 cells were plated at a density of 10^5 cells/35-mm dish (▲, △), or 10^4 cells/35-mm dish (●, ○) in 1.5 ml of $F10^+$. 1 d after plating, cultures were fed with fresh medium with (△, ○) or without (▲, ●) 10^{-8} M EGF. Replicate dishes were harvested every 48 or 72 h as shown and fresh medium with or without EGF was added to the remaining dishes. After 10 d (shown by the arrow) some of the cultures treated with EGF were switched to control medium (×). Cell number was determined as described in Materials and Methods. Each point represents the mean value of triplicate dishes. The standard deviations did not exceed ±5% of the mean values shown.

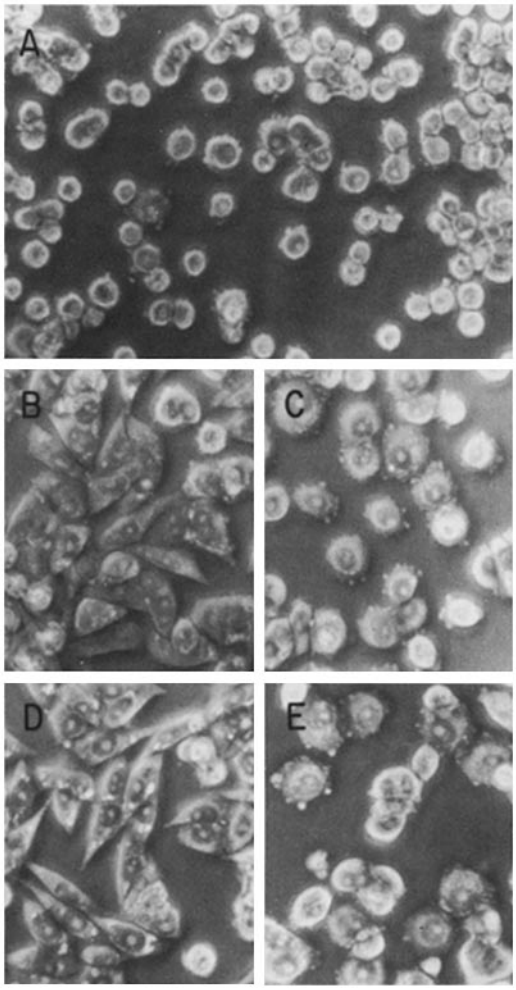

FIGURE 3 Appearance of GH_4C_1 cells grown in the presence and absence of EGF or TRH. GH_4C_1 cells were plated at a density of 5×10^4 cells/35-mm dish in 1.5 ml of $F10^+$. 3 d after plating, cultures were fed with fresh media without any additions (*A*) or with 10^{-9} M EGF (*B* and *C*) or 10^{-7} M TRH (*D* and *E*) added. The media were renewed every 3 d, and fresh EGF and TRH were added. On day 10, the living cells were photographed under phase contrast (× 200). Control dishes contained $1.3 \pm 0.04 \times 10^6$ (mean ± SD) cells/dish. EGF-treated dishes contained $8.2 \pm 0.03 \times 10^5$ cells/dish. TRH-treated dishes contained $8.3 \pm 0.12 \times 10^5$ cells/dish. The data in Fig. 4 give the cell size distribution for this experiment.

photography of living GH cells under phase-contrast optics has shown that a single cell can spontaneously change from a rounded morphology to a flattened, angular shape. In control cultures, cells are rounded most of the time; therefore a still photograph of a culture shows >90% of the cells to be spherical. In contrast, EGF- and TRH-treated cells remain flat and angular most of the time. Therefore a photograph of treated cultures shows most of the cells to be stretched. GH cells are thus able to assume two different morphologies. EGF and TRH shift the prevalent morphology of the population presumably by altering the fraction of time each cell assumes a particular shape.

To determine whether the apparent increase in cell size detected by microscope observation was caused by a real increase in cell volume or merely the result of cell stretching, cells were detached from dishes, and cell volume was determined by electronic counting. The results, shown in Fig. 4, demonstrate that EGF, and to a lesser extent TRH, produce highly significant increases in mean cell volume.

The effect of EGF on cell volume was reversible as was its effect on cell proliferation. 4 d after cessation of EGF treatment, mean cell volume had decreased substantially although cell number was the same as for cultures continued in the presence of EGF (Table I, Day 14). 7 d after cessation of EGF treatment, mean cell volume was indistinguishable from that of control cells even though cell number had not yet reached the same value as cells never previously exposed to EGF (Table I, Day 17).

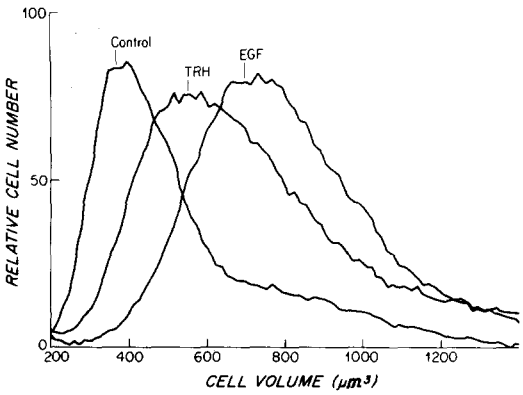

FIGURE 4 Effect of EGF and TRH on cell volume. Cells from the experiment described in Fig. 3 were detached from dishes with trypsin as described in Materials and Methods. Cell volume distribution was determined by electronic counting. The mean cell volume of control, TRH-treated, and EGF-treated cells was 380, 560, and 740 μm^3, respectively.

TABLE I

Reversibility of EGF Action on Cell Volume and Cell Number

Treatment	Cell volume	Cell No./dish*
	μm^3	$\times 10^6$
Day 14		
Control	320	1.8 ± 0.07
EGF	510	1.2 ± 0.03
EGF → control	430	1.3 ± 0.05
Day 17		
Control	320	2.6 ± 0.05
EGF	500	1.7 ± 0.03
EGF → control	320	2.3 ± 0.05

GH_4C_1 cells plated at a density of 10^4 cells/35-mm dish were treated as described in Fig. 2. On days 14 and 17, cell volume and cell number were determined for control cultures, cultures treated with 10^{-8} M EGF for the entire experiment (EGF), and cultures treated for 10 d with 10^{-8} M EGF and then transferred to control medium (EGF → control).

* Mean ± SD of triplicate dishes.

TRH Also Decreases Cell Proliferation

The qualitative similarity between the effects of EGF and TRH on cellular morphology and size prompted us to reexamine the effect of TRH on cell proliferation. Tashjian et al. (31) had previously shown that chronic TRH treatment did not affect total cell protein per dish in cultures of GH_3 cells. The results in Fig. 5 demonstrate that neither TRH nor EGF significantly altered total cell protein per dish in cultures of GH_4C_1 cells. However, in the same experiment, EGF caused a 30% inhibition and TRH caused a 10% inhibition in cell number per dish after 6 d. Therefore, a net increase in protein content per cell occurred with both EGF- and TRH-treated cultures.

TRH and EGF together had a greater effect on cell proliferation than either peptide alone, resulting in a 45% decrease in the number of cells per dish (Fig. 5). This additive effect was also observed when cell volume was measured. The results in Table II demonstrate that treatment of GH_4C_1 cells for 3 d with TRH resulted in a 15% increase in cell volume, treatment with EGF caused a 70% increase, while both peptides together caused a 94% increase.

EGF Modulates Hormone Production

TRH has previously been shown to stimulate prolactin synthesis and inhibit GH production by GH cells (23). Because the effects of EGF and TRH on cell proliferation and morphology were similar, we examined the effects of EGF on hormone production. The results in Fig. 6 demonstrate that EGF stimulated prolactin synthesis markedly and inhibited growth hormone production. The amount of prolactin that accumulated in the medium of EGF-treated cultures was eightfold greater than in control cultures after 3 d of treatment. In the same experiment, growth hormone was reduced to 35% of control after 3 d.

We next examined whether EGF and TRH had additive effects on hormone production as they did on cell proliferation and cell volume. GH_4C_1 cells were incubated in control medium or medium containing 10^{-8} M EGF alone, 10^{-7} M TRH alone, or both peptides. Medium samples were collected

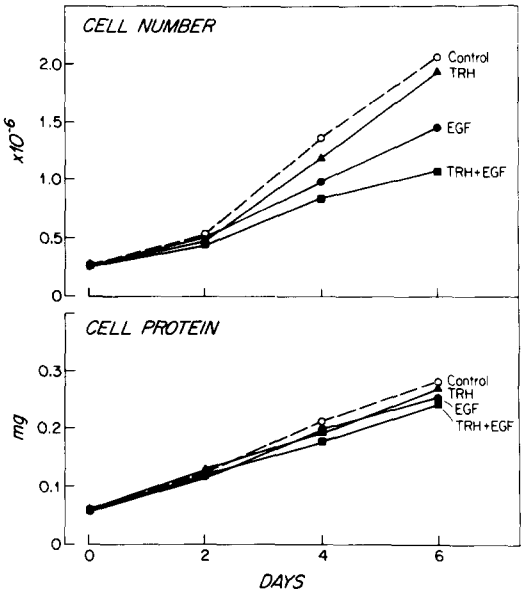

FIGURE 5 Effect of EGF and TRH on cell proliferation and cell protein. GH_4C_1 cells were plated at a density of 10^5 cells/35-mm dish. 3 d after plating, cultures were fed with fresh media lacking any additions (○) or containing 10^{-7} M TRH (▲), 10^{-8} M EGF (●), or both peptides (■). Replicate dishes were harvested every 2 d, and fresh medium with or without the peptides was added to the remaining dishes. Cell number (top panel) and total cell protein (bottom panel) per dish were determined as described in Materials and Methods. Each point gives the mean value of triplicate dishes. The standard deviation did not exceed ±5% of the mean for cell number determinations and ±10% of the mean for protein measurement. The data in Fig. 7 show hormone production in this experiment.

TABLE II

Additivity of Effects of Supramaximal Concentrations of EGF and TRH on Cell Volume and Cell Number

Treatment	Cell volume	Cell No./dish*
	μm^3	$\times 10^5$
Control	340	10.3 ± 0.08
EGF, 10^{-8} *M*	570	7.4 ± 0.12
TRH, 10^{-7} *M*	390	7.6 ± 0.04
EGF and TRH	660	6.8 ± 0.17

GH_4C_1 cells were plated at a density of 1.4×10^5 cells/35-mm dish in 1.5 ml of $F10^+$. 3 d after plating, cells were fed with fresh medium containing the peptides shown. After 3 d of treatment, cell volume and cell number were determined as described in Materials and Methods.

* Mean ± SD of triplicate dishes.

every 48 h and assayed for prolactin and growth hormone. The effects of EGF on hormone production were much greater than those of TRH (Fig. 7). Prolactin synthesis per culture was stimulated two- to threefold by TRH, whereas EGF increased the synthesis of prolactin five- to ninefold. These effects were even greater when calculated on a per cell basis. After 6 d of treatment with EGF, prolactin synthesis per cell was increased 12-fold. When TRH and EGF were present simultaneously for 6 d, prolactin synthesis per culture was increased 12-fold whereas synthesis per cell was 23-fold higher than for untreated control cells. Thus, the effects of TRH and EGF on prolactin synthesis were additive.

In the experiment described in Fig. 7, TRH alone caused a 15% decrease in growth hormone production after 6 d, whereas EGF caused a 40% decrease. These inhibitory effects on growth hormone production were similar in magnitude to the inhibition of cell proliferation (Fig. 5), such that growth hormone per cell was only marginally altered by TRH or EGF in this experiment. In most experiments, however, EGF did cause a net decrease in growth hormone production per cell (e.g., Fig. 8). Because TRH treatment caused a rather small decrease in growth hormone production in the experiment shown in Fig. 7, it is not clear whether the effects of TRH and EGF on growth hormone were additive, as they were on prolactin production.

The concentration dependence of the effects of EGF on cell proliferation and hormone production by GH_4C_1 cells is shown in Fig. 8. The dose-response curves for inhibition of cell proliferation, stimulation of prolactin synthesis, and inhibition of GH production are similar with half-maximal effects occurring at $\sim 5 \times 10^{-11}$ M. In three independent experiments the Kapp observed for these biological effects of EGF was $4.0 \pm 2.3 \times 10^{-11}$ M (mean ± SD).

Effects of Growth Factors Other Than EGF on GH_4C_1 Cells

To determine whether the effect of EGF on hormone production and cell proliferation by GH_4C_1 cells was specific to this growth factor, the experiments described in Fig. 9 and Table III were performed. Although neither insulin nor multiplication stimulating activity (MSA) altered prolactin synthesis after 3 d of treatment, both EGF and

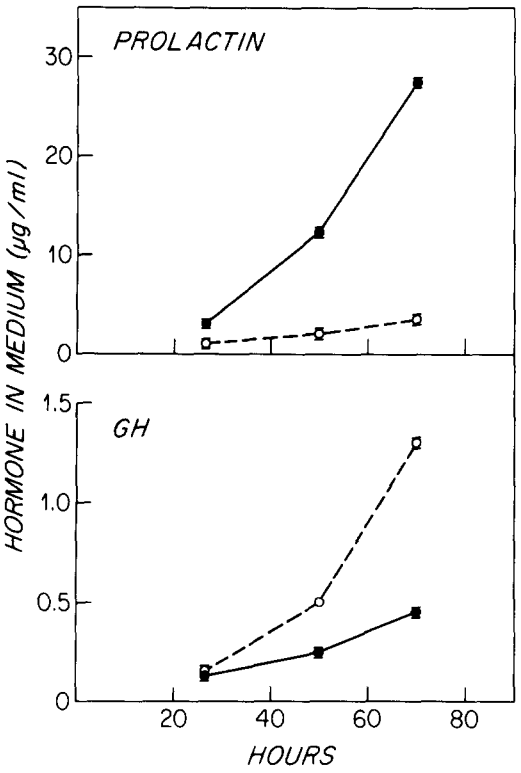

FIGURE 6 Effects of EGF on prolactin and growth hormone production. GH_4C_1 cells were plated at a density of 10^5 cells/35-mm dish in 1.5 ml of $F10^+$. 5 d after plating, cultures were fed with fresh $F10^+$ with (○) or without (●) 10^{-8} M EGF. Cells were grown without further medium changes. Media from replicate dishes were harvested at the times shown and the content of prolactin (top panel) and growth hormone (bottom panel) was determined as described in Materials and Methods. Each point gives the mean value of duplicate dishes and the bars show the range.

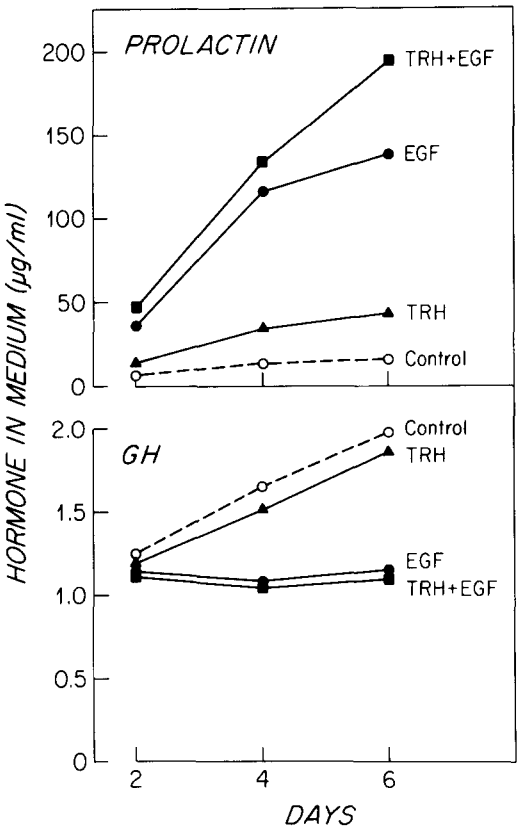

FIGURE 7 Effect of EGF alone, TRH alone, and EGF plus TRH on hormone production. GH_4C_1 cells were plated at a density of 10^5 cells/35-mm dish. 3 d after plating, cultures were fed with fresh media without any additions (○) or containing 10^{-7} M TRH (▲), 10^{-8} M EGF (●), or both peptides (■). Medium from replicate dishes was harvested every 48 h, and fresh medium with or without the peptides was added to the remaining dishes. The content of prolactin (top panel) and growth hormone (bottom panel) was determined for each 48-h collection as described in Materials and Methods. Each point gives the mean value of duplicate dishes. The range did not exceed ±10% of the mean value. Fig. 5 shows cell number and total cell protein for this experiment.

FGF caused a significant stimulation (Fig. 9). In the same experiment, FGF decreased GH production by 16% while EGF caused a 25% inhibition.

Table III shows that FGF also increased cell volume and decreased cell proliferation and that the magnitude of these changes was again smaller than that caused by EGF. Insulin and MSA had no effect on cell growth or cell volume. Therefore the actions of EGF on GH_4C_1 cells are not entirely unique to this growth factor, as FGF had qualitatively similar effects, though of smaller magnitude. However, not all growth factors mimic the actions of EGF.

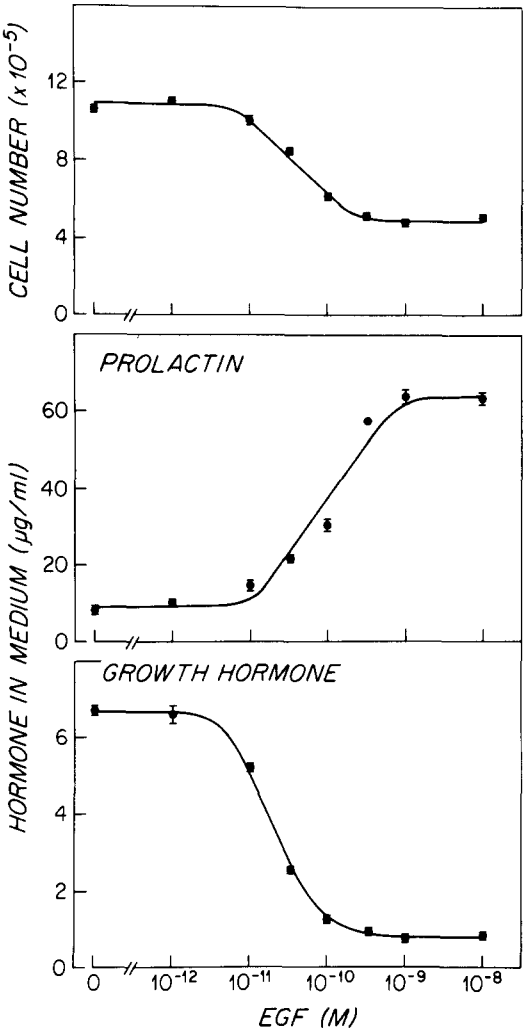

FIGURE 8 Concentration dependence of EGF effects on cell proliferation and prolactin and growth hormone production by GH_4C_1 cells. GH_4C_1 cells were plated at a density of 3×10^4 cells/35-mm dish. 4 d after plating, fresh $F10^+$ containing the indicated concentrations of EGF was added. After 3 d of incubation, the medium was renewed and fresh EGF was added. After three additional days of incubation, cell number per dish (top panel) was determined as described in Materials and Methods. Media from the last 3 d of incubation were collected and assayed for prolactin (middle panel) and growth hormone (bottom panel). Each point gives the mean value of triplicate dishes and the bars show the standard deviation.

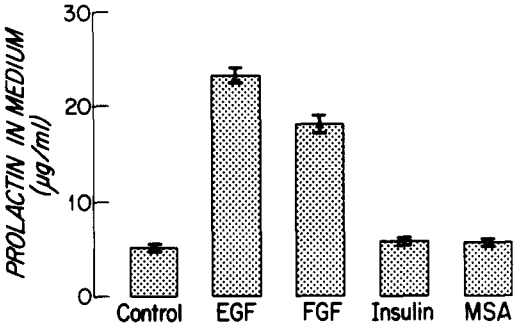

FIGURE 9 Effects of different growth factors on prolactin production by GH_4C_1 cells. GH_4C_1 cells were plated at a density of 1.4×10^5 cells/35-mm dish. 3 days after plating, cultures were fed with fresh medium containing either no additions or EGF (10^{-8} M), FGF (10^{-8} M), insulin (10^{-7} M), or MSA (1.5×10^{-8} M). After 3 d, media were collected and prolactin was determined as described in Materials and Methods. Each bar gives the mean value of triplicate dishes and the brackets show the standard deviation. Table III gives the cell numbers per dish for this experiment.

Cell Specificity of EGF Effect on Hormone Production

To test the specificity of EGF action on GH_4C_1 cells, we examined the ability of EGF to modulate hormone production by several clonal pituitary tumor cell strains (Table IV). GH_3, GH_12C_1, and GC are clonal strains of pituitary cells which were derived from the same tumor (MtT/W5) as the GH_4C_1 cell strain (23). These strains secrete different relative amounts of growth hormone and prolactin. GC and GH_12C_1 cells secrete large amounts of growth hormone but no detectable prolactin, whereas GH_4C_1 and GH_3 cells secrete large amounts of prolactin and lesser amounts of growth hormone. F_4C_1 cells were derived from a different pituitary tumor (MtT/F_4) than the GH cell strains and synthesize large amounts of prolactin and relatively little growth hormone (25). For all the strains tested, EGF stimulated prolactin synthesis and decreased growth hormone production. However, not all pituitary cells responded to EGF with altered hormone production. AtT20/D16 cells are a clonal strain of mouse pituitary cells that secrete ACTH (26) and endorphin (1). EGF had no effect on ACTH production by AtT20/D16 cells.

We have examined each of these pituitary cell strains for the presence of specific EGF receptors and have found that only the AtT20/D16 cells lack receptors: all the other pituitary cell strains shown in Table IV have saturable, high-affinity binding sites for ^{125}I-EGF (A. Schonbrunn and A. H. Tashjian, Jr., manuscript in preparation). Therefore, there is a close correlation between the presence of EGF receptors and the ability of EGF to modulate hormone production.

Effect of EGF on Prolactin Release

Because TRH has been shown to stimulate acutely the release of stored intracellular prolactin from GH cells, as well as to increase prolactin synthesis during chronic treatment (12), the ability of EGF to modulate prolactin release was studied. The results in Fig. 10 show that EGF caused a small but significant stimulation of prolactin release during a 30-min incubation. However, in contrast to the relative magnitude of the TRH and EGF effects in modulating chronic hormone production, EGF was less effective at stimulating prolactin release than TRH. In fact, in experiments in which the TRH effect on release was small (less than a 50% increase), we often were unable to detect a significant effect of EGF on prolactin release. Therefore, although the effects of EGF and TRH on GH_4C_1 cells are qualitatively similar, the power of their different biological actions is not identical.

DISCUSSION

EGF stimulates the proliferation of epidermal and epithelial tissues in animals and enhances cell proliferation in culture for a variety of transformed

TABLE III

Effects of Different Growth Factors on Proliferation and Cell Volume of GH_4C_1 Cells

Treatment	Cell volume	Cell No./dish*
	μm^3	$\times 10^6$
Control	340	10.5 ± 0.31
EGF, 10^{-8} M	460	8.0 ± 0.02
FGF, 10^{-8} M	400	9.2 ± 0.20
Insulin, 10^{-7} M	340	10.0 ± 0.30
MSA, 1.5×10^{-8} M	330	9.8 ± 0.36

GH_4C_1 cells were plated at a density of 1.4×10^5 cells/35-mm dish. 3 d after plating, cultures were fed with fresh medium containing the peptides shown. After three additional days of incubation, cell number and cell volume were determined as described in Materials and Methods. Fig. 9 shows prolactin production for these cultures.

* Mean ± SD of triplicate dishes.

TABLE IV

Effects of EGF on Hormone Production by Different Pituitary Cell Strains

Cell type	Hormone assayed	Hormone production*		P value
		Control	EGF	
		μg/ml/72 h		
GH_4C_1	Prolactin	20 ± 0.4	36 ± 1.3	<0.001
	GH	3.5 ± 0.06	1.7 ± 0.07	<0.001
GH_3	Prolactin	22 ± 0.5	39 ± 0.9	<0.001
	GH	2.2 ± 0.07	1.3 ± 0.02	<0.01
F_4C_1	Prolactin	19 ± 1.0	25 ± 0.40	<0.001
GH_12C_1	GH	7.5 ± 0.40	5.5 ± 0.39	<0.001
GC	GH	13 ± 0.6	11 ± 0.5	<0.001
AtT20/D16	ACTH‡	0.10 ± 0.010	0.10 ± 0.008	>0.05

Cells at high density were treated for 3 d without or with 2×10^{-8} M EGF. At the end of the incubation, media were collected and assayed for hormones as described in Materials and Methods. Statistical significance was determined from a *t* test of paired data using a pooled residual error for the entire experiment.

* Mean ± SD of triplicate dishes.

‡ ACTH was measured by radioimmunoassay following the procedure of Eipper and Mains (13).

and nontransformed cell types (8). The new results presented in this paper raise the possibility that EGF may have important biological actions not related directly to mitogenesis. EGF and TRH, a hypothalamic-releasing hormone, act similarly on GH_4C_1 cells, a clonal strain of functional rat pituitary cells. Both peptides increased the release and synthesis of prolactin and inhibited growth hormone production. TRH was more powerful at stimulating acute prolactin release. EGF had larger effects on chronic hormone production. These actions of EGF occurred at low concentrations of the polypeptide; half-maximal stimulation of prolactin synthesis and inhibition of growth hormone production were observed with 5×10^{-11} M EGF (Fig. 8). The plasma concentration of EGF in the adult mouse is ~1.5 ng/ml (7) and the concentration of hEGF or urogastrone in human plasma is 2–4 ng/ml (9). Comparable concentrations of EGF are likely to be present in rat body fluids as well. Thus, the concentrations of EGF in the circulation ($\sim 2.5 \times 10^{-10}$ M) are sufficiently high that EGF could play a role in regulation of pituitary hormone synthesis and secretion in the intact animal. In an entirely different tissue, Benveniste et al. (4) have also found effects of EGF on hormone production. These workers showed that addition of EGF to cultured human choriocarcinoma cells stimulated the production of chorionic gonadotropin (hCG) and to a lesser extent the production of free hCG-α subunits.

The striking similarity between the actions of EGF and TRH on GH_4C_1 cells raises the question as to whether EGF is also synthesized in the hypothalamus. The tissue distribution of immunoreactive EGF has been studied in both the mouse (6) and the human (20). In both studies, EGF concentration was below detectable levels in brain and pituitary tissue. However, even if the hypothalamus was a major source of EGF, its presence might not have been detected in studies in which the content of whole brain was measured. EGF has been found throughout the gut and, because many hypothalamic peptides have been shown to be present in gastrointestinal tissue, it would not be unexpected to discover a similar distribution for EGF.

The inhibitory effect of EGF on the multiplication of GH_4C_1 cells was surprising because with most cell types it either increased the rate of cell proliferation or had no effect (8). The concentration of EGF that caused a half-maximal inhibition of cell proliferation by GH_4C_1 cells (5×10^{-11} M) was in the same range that caused stimulation of

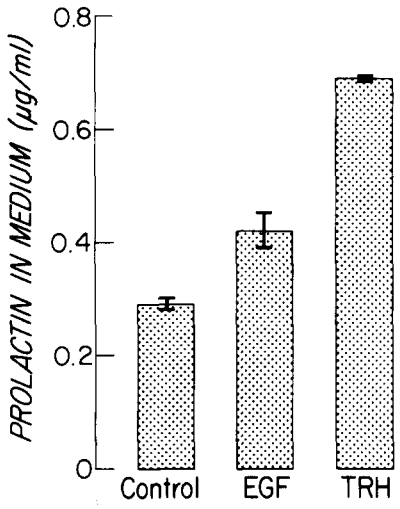

FIGURE 10 Effect of EGF on prolactin release. GH_4C_1 cells were incubated with 2 ml of F10 lh containing either no additions (control) or 10^{-8} M EGF or 10^{-7} M TRH. Media were collected after 30 min of incubation at 37°C, and prolactin was determined as described in Materials and Methods. Each bar gives the mean value of duplicate dishes and the brackets show the ranges. Cell protein was 0.40 mg/60-mm dish.

fibroblast cell proliferation (8, 16). It is not known whether this inhibition of cell proliferation or the effect on hormone production constitutes the primary physiological action of EGF on pituitary cells. It is interesting in this regard that TRH also inhibited the proliferation of GH_4C_1 cells. Although we found that TRH had no effect on total cell protein per culture, in agreement with the original observations of Tashjian et al. (31), this result gives an incomplete view of effects on cell proliferation. In fact, treatment of cultures with TRH does result in a small but significant decrease in cell number and DNA. Therefore, changes in cell size and protein content per cell can result in incorrect conclusions when protein content of cultures alone is used as the measure of cell growth.

Not all growth factors affect GH_4C_1 function: neither MSA nor insulin altered prolactin production, cell proliferation, or cellular morphology. However, the results in Table III demonstrate that FGF, also a potent mitogen for certain cell types (16), had qualitatively similar effects on GH_4C_1 cells as EGF and TRH. The inhibitory effect of FGF on cell proliferation was particularly surprising since Hayashi et al. (17) have reported that FGF increased the proliferation of GH_3 cells, the cell line from which GH_4C_1 cells were originally derived. The experiments of Hayashi et al. were, however, performed in chemically defined medium lacking serum. The role of serum factors in determining the proliferative and hormonal responses of GH cells to FGF and to EGF clearly requires further investigation.

Almost 10 yr ago, Tashjian et al. reported the unexpected observation that crude acid extracts of bovine liver, kidney and cerebral cortex, as well as hypothalamus, caused a 6- to 10-fold increase in prolactin synthesis by GH_3 cells, accompanied by a 50% decrease in growth hormone production (30, 32). There was no concomitant change in total cell protein per dish; however, the tissue extracts did alter the morphology of the cells from rounded to highly stretched. The active material in liver extracts had a mol wt >10,000 and was heat-labile, losing most of its biological activity after 10 min at 60°C (3). The identity of the active principle remains to be defined; however, the similarity between its properties and those of FGF would indicate that this growth factor is a possible candidate molecule.

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