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Hormonal Regulation of Prolactin Storage in a Clonal Strain of Rat Pituitary Tumor Cells

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GH_4C_1 cells (GH cells) are a clonal strain of rat pituitary tumor cells which secrete prolactin. GH cells have been used to study hormone secretion, but they store relatively little prolactin compared to normal prolactin-secreting cells. They are not suitable, therefore, for studying some aspects of pituitary function. We have found that the amount of prolactin GH cells store can be regulated. When GH cells were plated at 10^6 cells/well and treated for six days with 180 nM insulin or 1 nM estradiol, there was a 60 percent increase in prolactin storage compared to control cells. Insulin and estradiol in combination acted synergistically to cause a 190 percent increase in prolactin storage. In contrast, they were additive in increasing extracellular prolactin; there was a 40 percent increase in extracellular prolactin after insulin, a 20 percent increase after estradiol, and a 50 percent increase after insulin plus estradiol. The increases in prolactin storage were always greater than the increases in extracellular prolactin. The increases in prolactin storage were dose-dependent and reached maximal levels after four days of treatment with 180 nM insulin plus 1 nM estradiol. Reducing the plating density to 10^3 cells/well increased the response to insulin and estradiol to nineteenfold. Epidermal growth factor (10 nM) acted synergistically with estradiol and insulin in combination to increase prolactin storage 27-fold.

The insulin- and estradiol-induced increase in extracellular prolactin was caused by a specific increase in the rate of prolactin synthesis. The fractional increase in prolactin storage above the increase in prolactin production could not be explained by an increase in prolactin synthesis, an increase in intracellular transit time, or a change in the cell-cycle distribution of the population. Hormone storage can, therefore, be regulated independently from other processes which control hormone production.

The prolactin stored in response to insulin and estradiol was releasable by potassium depolarization. Following depletion of intracellular prolactin by depolarization, the cells retained their increased capacity for prolactin storage. The ability to increase prolactin storage will make GH cells a more useful system in which to study pituitary function.

INTRODUCTION

The GH_3 rat pituitary tumor cell line was established in 1968 and has retained the ability to synthesize and secrete prolactin and growth hormone *in vitro* [1,2]. Since that time, GH_3 cells (GH cells) have been used extensively to study prolactin secretion *in vitro*. GH cells are a clonal strain and offer the advantage of consisting of a single cell type which can be propagated indefinitely *in vitro*. GH cells, however, store a much smaller proportion of the prolactin than they secrete compared to normal prolactin-secreting cells; they also lack the typical dense secretory granules seen in electron micrographs of normal mammotrophs [3,4]. Although GH cells have re-

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tained some properties of the differentiated cell, i.e., prolactin synthesis and secretion, the lack of mature secretory granules and the relatively low intracellular levels of prolactin indicate that they have a reduced capacity for prolactin storage and that transformation has disturbed the normal pathways involved in the storage process. No studies have focused on changes in prolactin storage and very few have even reported intracellular prolactin levels. Increasing GH cell prolactin storage may make them suitable for studying pituitary function.

We tested the classical differentiating agents dimethylsulfoxide, isobutylmethylxanthine, butyrate, and insulin for their ability to induce increases in prolactin storage in GH cells. In addition, estradiol was tested because Haug et al. [5,6] demonstrated that it increases intracellular prolactin and prolactin production in GH cells. Of these agents, insulin and estradiol increased prolactin storage in our studies. The following report is a review of our characterization of this effect. In addition to previously reported data, we include recent results which have clarified our former findings.

METHODS

Culture medium and serum were from Grand Island Biological Co. (Grand Island, NY). Dr. A.F. Parlow of the hormone distribution program of the National Institute of Arthritis, Metabolism, and Digestive Disease (Bethesda, MD) supplied rat prolactin used for production of rabbit antiserum and as a standard for prolactin assays. Bovine pancreatic insulin and 17β-estradiol were from Sigma Chemical Co. (St. Louis, MO). Protein A bacterial adsorbant was from Miles Laboratories, Inc. (Elkhart, IN). L-[4,5-^{3}H]leucine was from New England Nuclear and L-[^{14}C]leucine was from Schwarz/Mann (Orangeburg, NY).

Cell Culture

GH_4C_1 cells were grown as monolayers in Ham's F10 medium supplemented with 15 percent horse serum and 2.5 percent fetal bovine serum (F10+). Cultures were plated in 35 mm wells in a volume of 1 or 1.5 ml. We obtained the same results with either volume. Two days after plating, control cells received fresh medium and treated cells received fresh medium containing the indicated concentrations of insulin and estradiol. Fresh medium and hormones were applied every two days for six or eight days unless otherwise indicated.

Measurement of Prolactin and Cell Number

GH cells were collected and cell number was determined using a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL) as previously described [7]. We measured prolactin released into the medium and intracellular prolactin in cell sonicates by microcomplement fixation [2,8]. Prolactin is reported in $\mu g/10^6$ cells or as the ratio of hormone-treated levels to control levels after normalization to cell number. Each treatment was performed in triplicate; the variation among samples was less than 10 percent. We used analysis of variance followed by the protected least significant difference to analyze the data statistically; all comparisons of hormone-treated to control mentioned in the text are significant ($p > 0.05$) unless otherwise specified.

Double-Isotopic Measurement of Insulin- and Estradiol-Induced Changes in Prolactin Synthesis

GH cells were grown six days in the presence or absence of 300 nM insulin plus 1 nM estradiol. One to two hours before addition of radiolabeled leucine, fresh F10+

containing 15 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and fresh hormones was added to the cells. Hormone-treated cells were incubated three hours with 10 μCi [^{14}C]leucine (312 mCi/mmole) and control cells were incubated with 10 μCi [^{3}H]leucine (2 Ci/mmole). Cells from control wells and hormone-treated wells were combined and sonicated. Incorporation of radiolabeled leucine into prolactin was determined by immunoadsorption onto *S. aureus* protein A as described by Kessler [9] followed by electrophoresis on SDS 13 percent polyacrylamide cylindrical gels [10]. The incorporation of radiolabeled leucine into acid-precipitable proteins was determined on 0.2 ml fractions of the sonicates which were precipitated in 10 percent trichloroacetic acid (TCA). The ratio of ^{14}C to ^{3}H was determined for the prolactin and acid-precipitable proteins distributed along each gel.

Potassium Depolarization of GH Cells

GH cells were grown six days in the presence and absence of 300 nM insulin plus 1 nM estradiol. We replaced the medium with 1 ml of fresh F10+ with and without hormones plus 50 mM K^+ or an equimolar excess of Na^+. After a 20-minute incubation at 37°C, prolactin released into the medium and remaining in the cells was measured.

Recovery of Intracellular Prolactin after Potassium Depolarization

GH cells were grown and depolarized for 20 minutes as described above. Following the depolarization period, we replaced the medium with fresh medium with and without hormones containing normal Na^+ and K^+ concentrations. Intracellular prolactin was measured two and four hours later.

RESULTS

Insulin and Estradiol Increase Intracellular and Extracellular Prolactin in GH Cells

GH cells grown six days in the presence of insulin, estradiol, or insulin and estradiol together showed increases in intracellular and extracellular prolactin compared to control cells grown in Ham's F10+ medium alone. The results of several experiments are summarized and shown in Fig. 1. There was a 60 percent increase in intracellular prolactin after 180 nM insulin, a 60 percent increase after 1 nM estradiol, and a 190 percent increase above control values after insulin and estradiol together. Extracellular prolactin increased 40 percent with insulin, 20 percent with estradiol, and 50 percent with insulin and estradiol together. The effects of insulin or estradiol alone on intracellular and extracellular prolactin were not statistically significant in every experiment; when insulin and estradiol were used in combination, the increases in intracellular and extracellular prolactin were always significant. The effects of insulin and estradiol together on extracellular prolactin were additive, but insulin and estradiol acted synergistically to increase intracellular prolactin.

Insulin and Estradiol Slow GH Cell Growth

There were fewer cells per tissue-culture well after six days of treatment with insulin, estradiol, or insulin plus estradiol (Fig. 1). Insulin or estradiol alone decreased cell number 10 to 20 percent and the two hormones together decreased cell number 25 to 30 percent. The decreases seen with either hormone alone were not always statistically significant. The reduced cell number after hormone treatment resulted

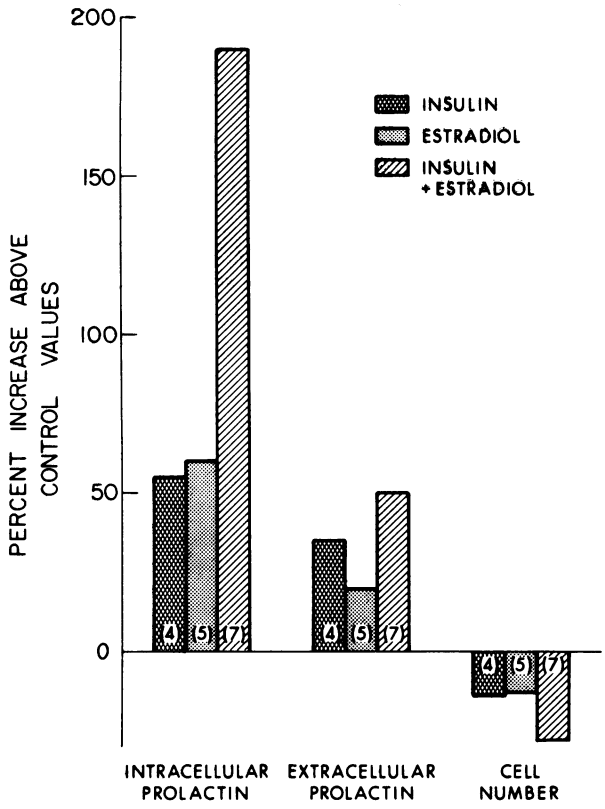

FIG. 1. Effects of insulin, estradiol, and insulin plus estradiol on prolactin and cell number. GH cells were plated at 10^6 cells/35 mm tissue-culture well and treated for six days with 180 nM insulin, 1 nM estradiol, or insulin and estradiol together. Each bar represents the mean of several independent experiments, the number of which are indicated within each bar. Measurements of intracellular and extracellular prolactin were normalized to cell number before the ratio of hormone-treated to controls was calculated. Control values averaged 0.86 $\mu g/10^6$ cells for intracellular prolactin, 15.7 $\mu g/(10^6$ cells 48 hours) for extracellular prolactin, and 2.75×10^6 cells/well.

from both a decreased rate of proliferation and a reduction in final density that the cells reach at plateau [7]. GH cells do not become confluent at plateau density; they grow in small clumps, piled on top of each other, that never completely cover the plates.

The Effects of Insulin and Estradiol Are Concentration-Dependent

The insulin- and estradiol-induced increases in intracellular and extracellular prolactin and the decrease in cell number are concentration-dependent [7]. The dose responses for all three effects are similar. The half-maximal concentration of insulin is about 2×10^{-8} M and the half-maximal concentration of estradiol is about 5×10^{-11} M. The half-maximal concentration of insulin is not affected by the presence of estradiol and the half-maximal concentration of estradiol is not affected by the presence of insulin.

Time Courses of the Effects of Insulin and Estradiol

Figure 2 shows that maximal storage and growth responses to insulin plus estradiol occurred after four days of treatment. Maximal extracellular responses occurred after six days. When insulin and estradiol were used alone, similar time courses were seen.

Insulin and Estradiol Increase the Rate of Prolactin Synthesis

Insulin- and estradiol-induced increases in intracellular and extracellular prolactin could be due to a hormone-induced increase in the rate of prolactin synthesis or a

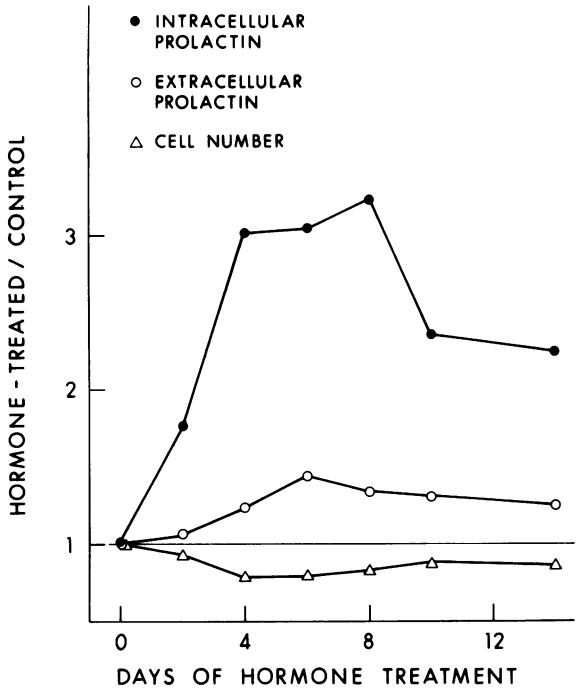

FIG. 2. Cell growth (△), extracellular prolactin (○), and intracellular prolactin (●) as a function of the duration of treatment with insulin and estradiol. GH cells were plated at 10^6 cells/35 mm tissue-culture well and treated the indicated number of days with 180 nM insulin + 1 nM estradiol. Measurements of intracellular and extracellular prolactin were normalized to cell number before the ratio of hormone-treated to controls was calculated.

decrease in its rate of degradation. Previous reports showed that GH cells do not degrade prolactin under normal conditions [11] so it was likely that insulin and estradiol were increasing prolactin synthesis. We measured the rate of [^{3}H]leucine incorporation into prolactin over a 180-minute period in GH cells which had been treated six days with estradiol plus insulin [7]. The incorporation of [^{3}H]leucine into prolactin was increased 60 percent by insulin and estradiol. If this increase reflects an increase in prolactin synthesis, it would account for the 40 percent increase in extracellular prolactin measured in the same experiment. The increased rate of [^{3}H]leucine incorporation into prolactin, however, may not have accurately reflected a specific increase in prolactin synthesis. In those studies, free intracellular [^{3}H]leucine and the rate of its incorporation into acid-precipitable proteins was also increased by hormone treatment about 50 percent. Since insulin has been shown to increase cellular amino-acid transport in other systems [12], it was possible that the increased rate of [^{3}H]leucine incorporation into prolactin was due to an increase in the specific activity of intracellular leucine rather than an increase in the rate of prolactin synthesis. It is also possible that, if there was an increase in prolactin synthesis, it was a result of an overall increase in protein synthesis and not an effect specific for prolactin.

We used double-isotopic labeling to determine whether insulin and estradiol treatment specifically increase prolactin synthesis. After six days of hormone treatment, [^{3}H]leucine was added to control cells and [^{14}C]leucine was added to hormone-treated cells for a 180-minute period. Control cells and treated cells were combined after the labeling period. Prolactin immunoprecipitates and acid-precipitable proteins from the combined samples were subjected to SDS polyacrylamide gel electrophoresis. The gels were sliced and the ^{14}C/^{3}H ratio determined for prolactin from the immunoprecipitates and for cellular proteins distributed along the gels of the acid precipitates. Any protein whose synthesis is preferentially increased by hor-

mone treatment should show an increase in the $^{14}C/^{3}H$ ratio compared to other proteins. A representative gel is shown in Fig. 3; the $^{14}C/^{3}H$ ratio is specifically increased for prolactin as well as for a few other unidentified cellular proteins. Similar results are obtained for prolactin immunoprecipitates and acid precipitates from GH cells treated with insulin or estradiol alone. When the isotopes were reversed, i.e., control cells received [^{14}C]leucine and treated cells received [^{3}H]leucine, the $^{14}C/^{3}H$ ratio is specifically decreased for prolactin (not shown). We conclude from these studies insulin and estradiol specifically increase prolactin synthesis. The increase in prolactin synthesis is enough to account for the increase in extracellular prolactin but is not enough to account for the increase in prolactin storage.

Insulin and Estradiol Do Not Increase the Intracellular Transit Time of Prolactin

The intracellular transit time for prolactin is the time it takes for newly synthesized prolactin to be processed for release from the cell. If insulin and estradiol treatment increased prolactin intracellular transit time, the cells would contain more prolactin. Insulin and estradiol did not affect the time it took for [^{3}H]prolactin to appear in the medium after the addition of [^{3}H]leucine (45 to 60 minutes) and so do not increase prolactin storage by increasing its intracellular transit time [7].

Insulin and Estradiol Do Not Affect the Cell-Cycle Distribution of the Cell Population

We stained GH cells specifically for prolactin using an indirect immunofluorescent technique and for DNA using the fluorescent dye propidium iodide. Flow microfluorometric analysis of the doubly stained cells indicated that prolactin storage increased as GH cells passed from the cell-cycle compartments G_2 to G_2 + M [13]. This was consistent with work by Hoyt and Tashjian [14] showing that GH cells are heterogeneous with respect to prolactin content. Insulin and

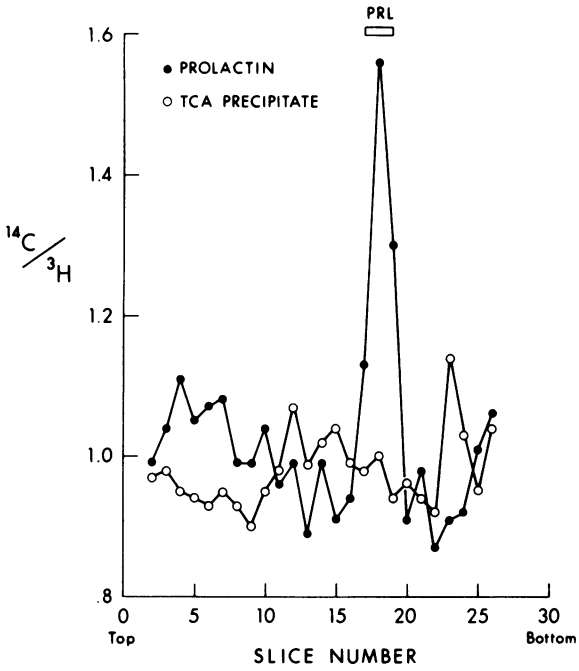

FIG. 3. The ratio of ^{14}C to ^{3}H along SDS 13 percent polyacrylamide gels of acid-precipitable proteins (○) and a prolactin immunoprecipitate (●). GH cells plated at 10^6 cells/well and treated six days with 300 nM insulin plus 1 nM estradiol were incubated for 180 minutes with 10 μCi [^{14}C]leucine and their controls were incubated with 10 μCi [^{3}H]leucine. Control and treated cells were then combined. We prepared trichloroacetic acid precipitates and prolactin immunoprecipitates from the pooled sonicates and ran each on a separate SDS gel. The position of the prolactin peak in the immunoprecipitate is indicated by the bar.

estradiol could increase prolactin storage by increasing the proportion of cells in G_2 + M. The cell-cycle distributions of GH cells treated with insulin, estradiol, or insulin and estradiol in combination were, however, identical to untreated GH cells [13]. This confirms findings by Clausen and co-workers [15] that estradiol does not influence GH cell cell-cycle distribution.

Hormone-Induced Increases in Prolactin Storage Are Cell-Density Dependent

In most of our studies, cells were routinely plated at initial densities of 10^6 or 10^5 cells/35 mm tissue-culture well. We have since found that an enhanced response to insulin and estradiol occurs in cells plated at lower initial densities (Table 1). Prolactin storage increased twofold in cells plated at 2×10^6 cells/well and nineteenfold in cells plated at 10^3 cells/well. The effect of hormone treatment on extracellular prolactin was less than the effect on prolactin storage at all cell densities tested producing twofold increases at most (not shown). We do not know the basis for the decreased response at high cell density but it does not appear to be caused by depletion of hormones or nutrients from the medium or the production of an inhibitory factor by the cells because the response to hormones is not affected by growing cells in volumes of medium ranging from 0.5 to 5.0 ml.

Hormone-Induced Stored Prolactin Is Released by Stimulation

High extracellular concentrations of K^+ depolarize GH cells [16] and result in a calcium-dependent stimulation of prolactin release [17,18]. Figure 4A shows that prolactin stored in response to insulin and estradiol was also released by K^+ depolarization. Incubation for 20 minutes in medium containing 50 mM K^+ stimulated release from untreated cells about 60 percent and from hormone-treated cells about 140 percent compared to cells incubated in medium containing an equimolar excess of Na^+. This extra amount of Na^+ did not stimulate release compared to medium containing normal Na^+ concentrations (data not shown). The amount of prolactin remaining in the cells after depolarization is reduced 60 percent in control cells and 70 percent in hormone-treated cells, compared to nondepolarized cells.

Insulin- and Estradiol-Treated GH Cells Retain Their Increased Capacity for Prolactin after Its Depletion by Stimulation

Data in Fig. 4B show the recovery of intracellular prolactin levels after release was stimulated by depolarization. After incubation with excess K^+ for 20 minutes, intra-

TABLE 1
Effect of Initial Plating Density on Hormone-Induced Prolactin Storage

Initial Density (cells per well)	Intracellular Prolactin ($\mu g/10^7$ cells ± SE)		
	Control	Insulin + E_2	Hormone-Treated/ Control
10^3	4.8 ± 0.24	93 ± 7.4	19
10^4	2.2 ± 0.11	31 ± 1.9	14
10^5	5.0 ± 0.18	12 ± 0.4	2.4
2×10^6	6.1 ± 0.03	12 ± 1.1	2.0

Note: GH cells were plated at the indicated initial densities in 35 mm tissue-culture wells. After eight days of treatment with 300 nM insulin plus 1 nM estradiol, intracellular prolactin was measured as described in "Materials and Methods."

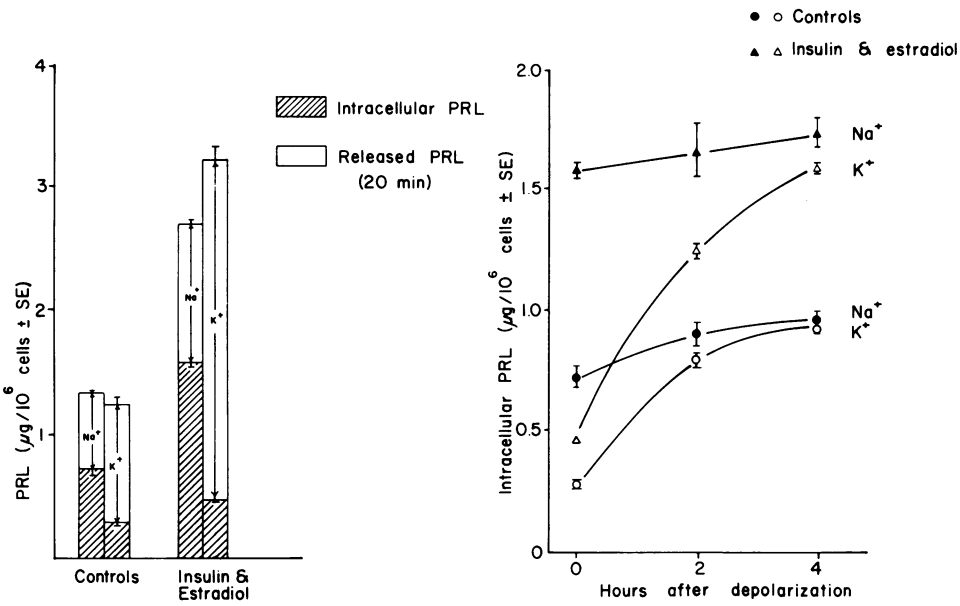

FIG. 4. Basal and potassium-stimulated prolactin release from control and hormone-treated GH cells. GH cells plated at 10^5 cells/well were grown six days with and without 300 nM insulin + 1 nM estradiol. A. Medium was replaced with F10+ with and without hormones containing 45 mM excess Na^+ (Na^+) or 45 mM excess K^+ (K^+). We measured the amount of prolactin released into the medium during the subsequent 20 minutes and the amount of prolactin remaining in the cells. B. After the 20-minute incubation, fresh medium containing normal concentrations of Na^+ and K^+ replaced the medium containing excess Na^+ or K^+. Intracellular prolactin was measured during the subsequent four hours for control cultures (no hormones, ●, ○) and hormone-treated cultures (▲, △). Closed symbols represent cultures which were previously incubated with excess Na^+; open symbols represent cultures which were incubated with excess K^+. Each point is the mean of triplicate wells ± SE.

cellular hormone was depleted, compared to cells treated with excess Na^+ (0 time in Fig. 4). Prolactin storage returned to 90 percent of unstimulated levels by four hours in hormone-treated cells. This is in contrast to the four days of hormone treatment required to reach this level of storage initially (Fig. 2). This indicates that hormone-treated GH cells retain their increased capacity for prolactin storage even after it has been depleted by acute stimulation with high concentrations of K^+.

The Presence of the Hormones Is Required to Maintain the Increased Storage

Data in Fig. 5 show that removing insulin and estradiol from the medium after eight days of hormone treatment results in a decrease in induced prolactin storage. Intracellular prolactin levels drop 50 percent two days after hormone removal. Eight days after hormone removal, however, prolactin storage remains increased twofold over cells which received control medium for the entire 16-day period. Longer time periods were not tested so we do not know if prolactin storage would have eventually reached control levels. Induced prolactin storage in cells treated the entire 16 days with insulin and estradiol also decreased during the last eight days but not as rapidly as in cultures from which hormones were removed.

Epidermal Growth Factor Enhances the Response of Prolactin Storage to Insulin and Estradiol

A report by Schonbrunn and co-workers [19] showed that epidermal growth factor (EGF) increased prolactin synthesis, decreased cell growth, and increased cell

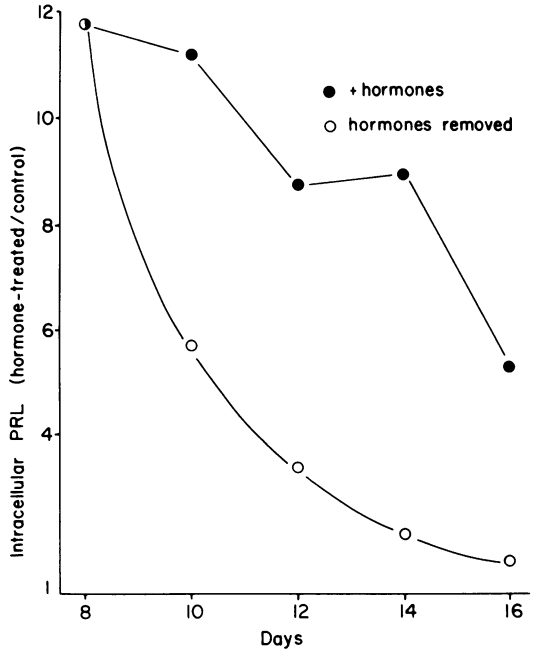

FIG. 5. Effect of removing hormones from the medium of cells treated eight days with insulin + estradiol. We plated GH cells at 10^3 cells/well and treated them for eight days with 300 nM insulin + 1 nM estradiol. Control cells received medium without added hormones. After eight days of treatment, hormones were removed from the medium of treated cells and intracellular prolactin was determined during the subsequent eight days (○). A parallel set of cultures continued to receive hormone-containing medium (●). Control cultures continued to receive medium without hormones. Intracellular prolactin was normalized to cell number before the ratio of hormone-treated/controls was determined. Each point represents the mean of triplicate wells.

volume in GH cells. These effects are qualitatively similar to effects of insulin and estradiol which we have observed, but the effects of EGF on intracellular prolactin were not reported. We measured prolactin storage in cells treated with EGF alone and in combination with insulin and estradiol. Data in Fig. 6 show that 10 nM EGF alone increased intracellular prolactin fivefold. Insulin did not affect the response to EGF. In contrast, estradiol and EGF acted synergistically to increase prolactin storage twentyfold. Although insulin did not affect the response to EGF alone, it enhanced the response to EGF and estradiol in combination producing a twenty-seven-fold increase. These synergistic responses were specific for intracellular prolactin since EGF alone increased extracellular prolactin fivefold, but insulin, estradiol, or insulin and estradiol in combination did not enhance this effect, producing fivefold increases in each case.

DISCUSSION

GH cells, like many human adenomas, synthesize and secrete large amounts of prolactin but store very little [20]. We have shown that the amount of prolactin stored can be affected by at least three hormones. We are not aware of any other studies that show the amount of hormone a cell contains is not just a function of the synthesis and release of that hormone but can be independently regulated. This regulation is obviously complex, since we found three hormones can affect storage, and some combinations of hormones act synergistically. The synergy suggests each hormone may affect a separate step to increase prolactin storage and production. It is not known what determines the storage capacity of prolactin cells. There are macromolecules other than prolactin associated with prolactin storage granules in normal mammotrophs [21,22] and it is possible that effects on these molecules may influence prolactin storage.

Both insulin and estradiol inhibited cell growth to a small extent. This effect is surprising since insulin is a mitogen in most cell cultures [23] and estrogens ad-

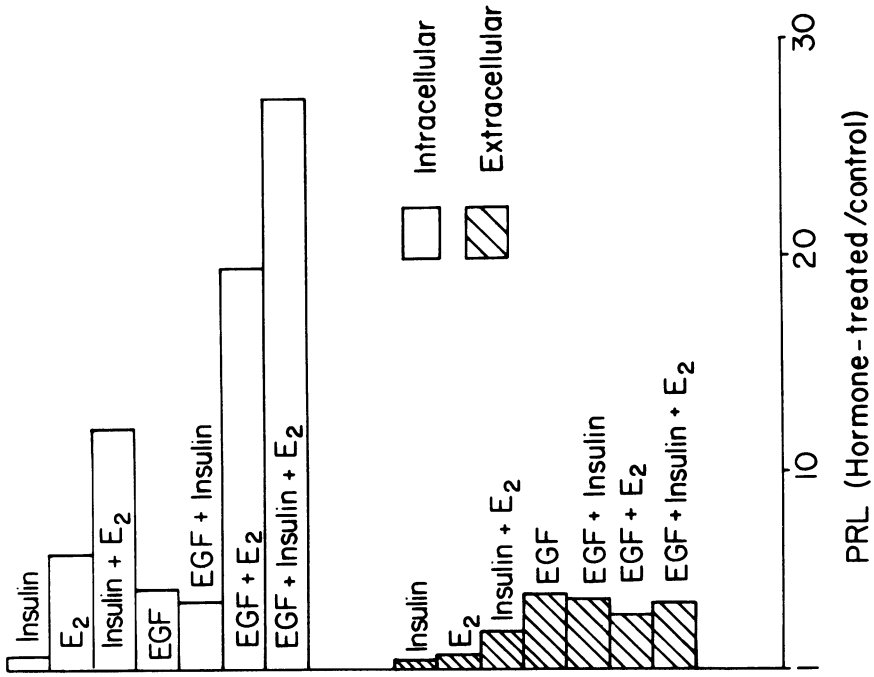

FIG. 6. The effect of epidermal growth factor (EGF) on intracellular and extracellular prolactin. We plated GH cells at 10^3 cells/well and treated them for eight days with 300 nM insulin, 1 nM estradiol (E_2), 10 nM EGF, or various combinations of these hormones. Prolactin was normalized to cell number before the ratio of hormone-treated/controls was calculated. Control values averaged 1.1 ± 0.01 $\mu g/10^6$ cells for intracellular prolactin and 58 ± 1.5 $\mu g/(10^6$ cells 48 hours) for extracellular prolactin. Each value is the mean of triplicate wells.

ministered to rats *in vivo* increase pituitary weight, mitotic index, and thymidine incorporation into mammotrophs [24–26]. Growth inhibition by agents which usually stimulate cellular growth may be a property peculiar to GH cells. EGF is also a mitogen for many transformed cells in culture [27] but inhibits growth of GH cells [19]. Other workers have studied the effects of estradiol on GH-cell growth but reported that cellular growth was not affected [5,6,15]. In those studies, cellular growth was measured as cellular protein per tissue-culture dish. Cellular protein may not be a reliable indicator of cellular proliferation. Schonbrunn et al. [19] showed that EGF decreased cellular growth when measured by cell number but not when measured as cellular protein per tissue-culture dish. EGF, therefore, increased the protein content of GH cells, indicating that determining cellular protein per dish is not a reliable measure of cellular proliferation.

Our original objective was to increase prolactin storage in GH cells to make them more suitable for studying pituitary function. By plating cells at low densities we are able to increase prolactin storage up to nineteenfold. Prolactin stored in response to hormone treatment is released by potassium stimulation. Using this system, we have recently determined that GH cells maintain functionally distinct prolactin pools

similar to those demonstrated in normal mammotrophs [28]. These pools are affected differently by potassium stimulation. Similar investigations should be possible using prolactin-release inhibitory agents and may elucidate mechanisms involved in prolactin regulation.

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