Effects of Thyrotropin-releasing Hormone and Hydrocortisone on Synthesis and Degradation of Prolactin in a Rat Pituitary Cell Strain*  

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SUMMARY

Synthesis of prolactin was measured in a clonal strain of functional pituitary cells in culture by incubating the cells with [3H]leucine; labeled prolactin was separated from the other labeled proteins by specific immunoprecipitation followed by dissociation with sodium dodecyl sulfate and disc gel electrophoresis. Accumulation of labeled prolactin in the cells was linear for at least 1 hour; labeled prolactin was released into the culture medium after 1 hour. Treatment with 28 nM thyrotropin-releasing hormone for 3 days increased prolactin accumulation in the medium 2.5-fold, and the increased synthesis of prolactin completely accounted for the increased accumulation. Treatment with 5 × 10^{-8} M hydrocortisone for 7 days decreased prolactin accumulation in the medium 2- to 5-fold, and the decrease in synthesis completely accounted for the decreased accumulation. Therefore the long term (3 to 7 days) effects on these cells of thyrotropin-releasing hormone and hydrocortisone result only in a change in the synthesis of prolactin and not in its rate of degradation. Pulse-chase experiments, using 10 μg per ml of cycloheximide or 10 mM leucine to block further incorporation of [3H]leucine into prolactin, show that there is no detectable degradation of prolactin under the usual conditions of culture. Incubation with 0.1 mM leucine does not prevent incorporation of radioactive leucine into prolactin and results in a 1.7-fold increase in labeled prolactin within 4 hours after the start of the chase. This result suggests there are two intracellular pools of leucine in GH cells, one of which does not appear to equilibrate readily with the medium.

The GH cell strains are derived from a multi-hormone-producing rat pituitary tumor which was adapted to tissue culture in 1965 (1). These clonal cell strains synthesize and secrete two hormones, growth hormone and prolactin; production of these hormones by the cells in culture is affected by several factors that influence their production in the intact animal. The effects of two of these factors, hydrocortisone and thyrotropin-releasing hormone, on the synthesis and degradation of prolactin by GH cells are reported in this paper.

Hydrocortisone at concentrations of 5 × 10^{-8} M increases the production of growth hormone by GH cells 4- to 5-fold, while that of prolactin is decreased to one-half to one-fifth of that in control cells (2). This response is not unique to GH cells; it has been shown that hydrocortisone stimulates growth hormone production in primary cultures of normal monkey pituitary glands (3).

TRH is the tripeptide pyroglutamylhistidylproline amide. This peptide causes the release of thyroid-stimulating hormone, and the isolation was based on this activity (4, 5). After the characterization of TRH, Tashjian et al. (6) demonstrated that this peptide increased production of prolactin in GH cell strains but that it did not increase growth hormone production. At concentrations of 28 nM the production of prolactin was increased 2- to 5-fold, while the production of growth hormone was decreased about 2-fold (6). Since these findings were reported, it has been shown in several laboratories that in species such as monkey, cow, and man TRH causes a release of prolactin as well as thyroid-stimulating hormone (7-9).

The amount of prolactin measured in the growth medium of the GH cells is termed production (10). There is no degradation of prolactin in the medium in the presence of GH cells (2), so this measurement represents the total amount of prolactin released by the cells. Prolactin is not stored in large amounts in the cells; the intracellular prolactin concentration changes in parallel as prolactin production changes (2). A change in production of prolactin may be the result of a change in one or both of two intracellular processes: synthesis and degradation. In this paper we find that hydrocortisone and TRH cause a change in the rate of prolactin synthesis and do not affect the rate of degradation.

Smith and Farquhar (11) have found evidence by electron microscopy for the degradation of prolactin granules in the intact pituitary gland. Although the GH cells do not store prolactin in large amounts as the pituitary gland does, intracellular prolactin degradation might occur in the cells in culture. Therefore, we also measured the rate of degradation of prolactin and found

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no detectable degradation under the usual conditions of cell culture.

MATERIALS AND METHODS

Chemicals—L-[4,5-3H]Leucine from Schwarz-Mann had a specific activity of 2 Ci per mmole. Prolactin used as a standard for complement fixation and electrophoresis was NIAMDD rat prolactin RP-1 from the National Institute of Arthritis, Metabolism and Digestive Diseases. Synthetic thyrotropin-releasing hormone was from Abbott Laboratories, and hydrocortisone sodium succinate was from the Upjohn Company.

GH Cell Strains and Methods of Culture—The establishment and propagation of GH2 cells has been described (1). The GH4 cells, which produce little or no growth hormone, developed in our laboratory by serial propagation of the GH2 cells between November 1971 and January 1972. Two cell strains were used in these experiments; GH9C8, which is a clone of the GH4 cells, and GH1 cells which had been stored in liquid nitrogen and produce both growth hormone and prolactin when thawed. Dr. I. K. Low (Harvard Medical School) found no mycoplasma or bacterial contamination.

Cells were grown in 60-mm plastic tissue culture dishes (Falcon) with 3 ml of Ham's F 10 medium (12) supplemented with 15% horse serum and 2.5% fetal calf serum at 37°C in a humidified atmosphere at 5% CO2 and 95% air.

Measurements of Prolactin and Protein—Prolactin was measured in the culture medium by microcomplement fixation (2, 13). The buffer for this assay was 0.14 M NaCl, 0.01 M Tris-HCl, pH 7.5, 1.5 x 10^-4 M CaCl2, 5 x 10^-4 M MgSO4, and 0.1% swine gelatin. Cell protein was measured by the method of Lowry et al. (14) using bovine serum albumin as a standard. Total protein synthesis was measured by incorporation of [3H]leucine into acid-precipitable radioactivity. Cells were labeled as described below, and a 0.1-ml aliquot of sonicated cells was precipitated with 2.0 ml of 5% trichloroacetic acid at 0°C for 30 min. The precipitate was collected by centrifugation at 2000 rpm as described for 30 min and the precipitate was dissolved and the antibody-antigen complex dissociated by incubation in 2% sodium dodecyl sulfate, 0.1 M sodium phosphate buffer, pH 7.1, and 1% 2-mercaptoethanol at room temperature overnight. Sucrose was added to a final concentration of 5%, and the samples layered on polyacrylamide gels (6 cm x 5 mm). Bromphenol blue was added as a marker dye. Sodium dodecyl sulfate disc gel electrophoresis was performed as described by Shapiro et al. (15) in gels that were 9% acrylamide and 0.23% bisacrylamide.

After electrophoresis the gels were frozen and sliced into 2-mm segments. Each segment was digested in 0.2 ml of 30% hydrogen peroxide at 50°C for 1 day or more. Water, 0.3 ml, was added and the radioactivity in the samples was determined by liquid scintillation counting in 5 ml of modified Bray's solution with an efficiency of 23 to 28%. Background radioactivity was estimated from the counts on either side of the prolactin peak (Fig 1); counts in the prolactin peak were summed after subtracting this background.

Precipitation of labeled prolactin by this technique was shown to be complete. After removing the original immunoprecipitate by centrifugation, a second precipitate was formed by adding 15 µg of carrier prolactin and 0.05 ml of antiprolactin. No radio-

![Fig. 1. Sodium dodecyl sulfate disc gel electrophoresis of dissociated immunoprecipitates.](http://www.jbc.org/)

A, immunoprecipitates from cell sonicates of GH1C8 cells that were labeled with [3H]leucine for 1 hour. B, immunoprecipitates from medium of GH1C8 cells that were labeled with [3H]leucine for 3 hours. The bar at the top of the figure indicates the position of standard rat prolactin which was run in separate gels and identified by staining with amido black. •—•, rat serum albumin and rabbit anti-rat serum albumin; ○--○, rat prolactin and rabbit anti-rat prolactin.
technique for an immunologically reactive precursor or a larger labeling expected for a precursor, there is no evidence by this the rate of synthesis should be the same as the increase in pro-
production is caused only by a decrease in degradation, the rate
of prolactin in the medium of TRH-treated cultures may resulted in the same background pattern in material obtained from
the cells and medium as with the prolactin-antiprolactin precipi-
tate of rabbit anti-rat prolactin plus added carrier rat prolactin is indicated by the bars at the top of the figure. A precipitate of rabbit anti-rat prolactin plus added carrier rat prolactin resulted in the appearance of a radioactive peak in material obtained from both the cells (Fig. 1A) and the medium (Fig. 1B) corresponding to the position of the rat prolactin standard. The remainder of the radioactivity in the gel appears to be nonspecific background. A control precipitation with rabbit anti-rat serum albumin and added carrier albumin resulted in the same background pattern in material obtained from the cells and medium as with the prolactin-antiprolactin precipitates, but no peak of radioactivity in the position of the prolactin standard. In other experiments, cells have been labeled from 15 min to 24 hours; we find the background pattern increases with increasing time. Since the background appears to be nonspecific and there are no peaks which have the time course of labeling expected for a precursor, there is no evidence by this technique for an immunologically reactive precursor or a larger molecular weight form of prolactin. However, small amounts of a rapidly converted precursor may not be detected by this technique.

Effect of TRH on Prolactin Synthesis—The increased accumulation of prolactin in the medium of TRH-treated cultures may be the result of increased synthesis, the result of decreased intracellular degradation, or a combination of the two processes. If the increase in prolactin produced in the presence of TRH is caused only by a change in the rate of synthesis, the increase in the rate of synthesis should be the same as the increase in prolactin accumulated in the medium. If the increase in prolactin production is caused only by a decrease in degradation, the rate of synthesis will remain unchanged in the presence of TRH. The data in Fig. 2 show that prolactin synthesis is increased in the presence of TRH. TRH had been present 3 days prior to the start of the labeling period. The effect of TRH has reached a maximum before 3 days, so the rate of prolactin production was no longer changing. The increase in synthesis of prolactin was sufficient to account completely for the increased appearance of prolactin in the medium; this is shown in Table I. The increase in prolactin in the medium in the presence of TRH corresponded to the increase in prolactin synthesis; therefore, there was no change in the rate of degradation.

Incorporation of [3H]leucine into acid-precipitable counts, a measure of total protein synthesis, was determined and remained unchanged in the presence of TRH, so the increased incorporation of label into prolactin is a specific effect of TRH and not a general effect on protein synthesis. In this experiment with GH3 cells, 0.8% of the [3H]leucine that was incorporated into total protein was incorporated into prolactin in unstimulated control cells. This amount varied from 0.5 to 1.1% in other experiments.

The increase of labeled prolactin was linear for 80 min (Fig. 2); however, in some experiments there was no further increase in the amount of labeled intracellular prolactin after about 60 min. In such an experiment, at about 60 min, labeled prolactin began to appear in substantial quantities in the medium (Fig. 3). It has been calculated indirectly from measurements by complement fixation that the amount of growth hormone in the GH3 cells is equal to the amount of growth hormone produced in 15 min, and the amount of prolactin in the cells is equal to the amount of prolactin produced in 1 to 2 hours (2, 10, 16). The detection of labeled prolactin in the medium after 1 hour (Fig. 2) and the detection of labeled growth hormone after 15 min2 agrees with the previous estimates. It is not yet understood why it takes four times as long for newly synthesized prolactin to appear in the medium as it does for newly synthesized growth hormone.

Effects of Hydrocortisone on Prolactin Synthesis and Degradation—To determine whether the reduced production of prolactin caused by hydrocortisone is the result of decreased synthesis or an increased rate of degradation, the same types of experiment were performed. If the decrease in prolactin production is the result only of a decrease in synthesis, the decrease in synthesis of prolactin will correspond to the decrease in prolactin production. If the decrease in production is the result only of increased degradation of prolactin, the rate of synthesis with hydrocortisone present will be the same as in the absence of hydrocortisone. Hydrocortisone, 5 x 10^-4 M, was added to the cultures 1 week prior to the addition of label. Prolactin production has been reduced to a minimum prior to 1 week, and the rate of production does not change further. The synthesis of prolactin was reduced by the presence of hydrocortisone (Fig. 4). In this experiment,

Table I

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<th>[3H]leucine incorporation into prolactin caused by TRH</th>
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<td>[3H]leucine in intracellular prolactin^a</td>
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<td>2.5 ± 0.02</td>
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^a Measured as [3H]leucine incorporation into prolactin in TRH-treated (+TRH) and control cells (−TRH) at four time intervals from 20 to 80 min. The data were those from the experiment shown in Fig. 2. Mean values are given ± standard errors.

^b Measured by microcomplement fixation. Medium was collected 7 hours after the start of labeling.

FIG. 2 (left). Appearance of labeled prolactin in GH3 cells with and without TRH. TRH was added to the cells 3 days before the start of the labeling experiment and [3H]leucine was added at zero time. Each point is the average of triplicates; bars indicate the range. ●, control cells; ○—○, cells treated with 28 nM TRH.

FIG. 3 (right). Appearance of labeled prolactin in the medium of GH3 cells with and without TRH. TRH was added to the cells 3 days before the start of the labeling experiment and [3H]leucine was added at zero time. Each point is the average of duplicates. Symbols are the same as those in Fig. 2.
incorporation of [3H]leucine into total acid-precipitable counts by hydrocortisone-treated cells was 92% of control cells. Prolactin synthesis was reduced 2-fold; since total protein synthesis was not affected to this extent, the decrease in prolactin synthesis was a specific effect.

Table II gives data from two experiments which demonstrate that the decrease in the synthesis of prolactin was sufficient to account for the decreased accumulation of prolactin in the medium; therefore an effect on degradation does not appear to be involved.

**Measurements of Intracellular Prolactin Degradation**—The above experiments show that hydrocortisone and TRH do not affect the rate of degradation of prolactin, but they do not show how much prolactin degradation there actually is. Several other factors in addition to TRH, such as estradiol and cyclic adenosine monophosphate, increase the production of prolactin by the GH cells. If there is no intracellular degradation of prolactin, these other factors must increase prolactin production by increasing synthesis. Therefore, we measured the rate of intracellular prolactin degradation using a pulse-chase technique. Cells were incubated with [3H]leucine for 1 hour, the radioactive medium was removed, the cells were washed, and then medium containing 10 μM leucine added to the dishes. The total amount of labeled prolactin in the cells and in the medium was measured at intervals. The use of these two techniques to prevent further incorporation of [3H]leucine into prolactin involves two different sets of assumptions. Cycloheximide at a concentration of 10 μM blocks amino acid incorporation into acid-precipitable protein 95 to 97%, and we assume prolactin synthesis is blocked to an equal extent. We also assume cycloheximide does not change the rate of degradation of prolactin. The use of 10 μM leucine, a 100-fold excess of the amount in normal medium, involved the assumption that the increased leucine does not change the rate of degradation of prolactin, and the assumption that this amount of nonradioactive leucine does inhibit the incorporation of [3H]leucine remaining in the cells. By the two techniques there was no detectable degradation of prolactin (Fig. 5). This indicates that all factors which increase prolactin production must do so by increasing synthesis.

The pulse-chase procedure was originally performed by washing the cells thoroughly and then adding culture medium with no additions; this medium contains 0.1 mM leucine. Total labeled prolactin increased 1.7-fold in the following 4 hours (Fig. 6). Total acid-precipitable radioactivity did not increase during this chase period but remained constant at 6.3 × 10^4 dpm per mg of protein. This acid-precipitable radioactivity represents [3H]leucine incorporated in protein as well as that attached to transfer RNA. To rule out the possibility that [3H]leucine actually incorporated into protein may continue to increase due to a large pool of labeled transfer RNA, and that this may account for the continued incorporation of [3H]leucine into prolactin, we measured acid-precipitable [3H]leucine after alkaline hydrolysis, which releases [3H]leucine from the transfer RNA. Total acid-precipitable radioactivity was reduced by 50% and did not increase during the chase.

Because total acid-precipitable radioactivity did not increase, there was no further net incorporation into protein. We believe the radioactive leucine that was incorporated into prolactin after the start of the chase came from the breakdown of intracellular proteins. The leucine that was released was incorporated back into protein without rapidly equilibrating with the medium; therefore total acid precipitable counts did not change. We detect the continued incorporation of this released [3H]leucine in prolactin since prolactin is secreted from the cell and is not degraded; we might not detect this continued incorporation in proteins which are being both synthesized and degraded intracellularly.

**Discussion**

Several detailed papers have recently described the reutilization of amino acids by tissues and cells (18, 19). Mortimore et al. (20) have described the existence of two intracellular pools of valine in perfused rat liver; one pool does not readily equilibrate with the perfusate and is derived from protein breakdown. They find that extracellular concentrations of 15 mM valine prevent valine reutilization. Our results are similar to their findings; at 0.1 mM leucine there is reutilization of labeled leucine (Fig. 6) and at 10 mM leucine there is apparently none (Fig. 6). Evidently the increase to 10 mM leucine extracellularly can effectively

**Table II**

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<tr>
<th>Experiment</th>
<th>[3H]Leucine in intracellular prolactin</th>
<th>Prolactin in medium</th>
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<tr>
<td>I^a</td>
<td>0.52 ± 0.012</td>
<td>0.38 ± 0.065</td>
</tr>
<tr>
<td>II^a</td>
<td>0.22 ± 0.033</td>
<td>0.19 ± 0.033</td>
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^a Measured by microcomplement fixation. Medium was collected 7 hours after the start of labeling.

^b Measured as [3H]leucine incorporation into prolactin in hydrocortisone-treated (+HC) and control cells (−HC) at three time intervals from 20 to 60 min. These data were obtained from the experiment shown in Fig. 4. Mean values are given ± standard errors.

^c Measured as [3H]leucine incorporation into prolactin in hydrocortisone-treated and control cells at 60 min. The experimental conditions were the same as those described in Fig. 4.

**Fig. 4.** Synthesis of [3H]prolactin in GH2 cells with and without hydrocortisone (HC). Hydrocortisone was added to the dishes 1 week before the addition of [3H]leucine, which was added at zero time. Each point is the average of triplicate samples; the ranges are shown. □—□, control cultures; •—•, cultures treated with 5 × 10^-5 M hydrocortisone.
samples with 10 pg per ml of cycloheximide added at zero time; 9, hour before zero time. The prolactin measured is the total in the samples with 10 mM leucine added to medium at zero time; 0, overcome incorporation of the leucine resulting from protein lactin. We can detect reutilization of leucine in prolactin moved from the processes of general intracellular protein degradation. Studies done on nonexported proteins will not necessar-

I hour pulse with \([3H]\)leucine and a chase with either excess leucine or cycloheximide. \([3H]\)Leucine was added to \(GH3\) cells for the 1 hour before zero time; at that time, the cells were washed until no radioactivity was detected in the wash and unlabeled medium added. Total \([3H]\)-prolactin was calculated by adding \([3H]\)prolactin in the cells and \([3H]\)prolactin in the medium. Each point is the average of triplicates. ■, [PH]prolactin remaining in the cells; □, [PH]prolactin in the medium; ○, total [PH]prolactin.

Acknowlegments—We would like to thank Dr. Iolanda Low for examining the cells for mycoplasma and bacteria. Misses Diane Jensen and Yolanda Santo provided expert assistance. TRH was a gift from Abbott Laboratories, N. Chicago, Ill., and the rat prolactin was provided by the Hormone Distribution Program of the National Institute of Arthritis, Metabolism and Digestive Diseases.

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