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 \times 10^{-8} \text{M} hydrocortisone for 7 days decreased prolactin accumulation in the medium 2-5-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 \mu g of [3H]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 1.5-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 effects 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 \times 10^{-8} \text{M} increases the production of growth hormone by GH cells 4- to 8-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 25 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 that prolactin 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.

The abbreviations used are: TRH, thyrotropin-releasing hormone; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
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 as a standard for complement fixation and electrophoresis was NIAMD R 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; GH1C3, which is a clone of the GH4 cells, and GI14 cells which had been stored in liquid nitrogen and propagated between November 1971 and January 1972. The cells were subjected to sonic oscillation for 2 min. at maximum power output in a Raytheon model DF 101 sonic oscillator. The suspension was centrifuged at 2000 rpm in an International Centrifuge model 18 with a 250 head for 30 min at room temperature, and aliquots of the supernatant solution were used for immunoprecipitation and precipitation with trichloroacetic acid.

To measure labeled prolactin in the medium, medium samples were first centrifuged at 2000 rpm as described for 30 min and aliquots of the supernatant used. For some experiments labeled prolactin in cells and medium were measured together. The cells were scraped from the dish directly into the labeling medium; the mixture was then treated by sonic oscillation and centrifuged as described.

Labeled prolactin was precipitated by mixing 1.0 ml of sample, 0.05 ml of rabbit anti-rat prolactin, and an aliquot of medium from GH cells which contained 15 µg of unlabeled prolactin (determined by complement fixation). Medium containing un-labeled prolactin was added as a carrier; 15 µg were sufficient to produce the serological reaction in slight antibody excess (determined by quantitative immunoprecipitation). The mixture was incubated at 37° for 30 min followed by incubation at 2-4° for at least 20 hours. The immunoprecipitate was collected by centrifugation at 2000 rpm as described for 30 min; the precipitate was dissolved and the antibody-antigen complex dissociated by incubation in 2% sodium dodecyl sulfate, 0.1 mM 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 × 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° 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 radioactivity was found in the supernatant.
The patterns of radioactivity in immunoprecipitates after electrophoresis are shown in Fig. 1. The migration of standard 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 min 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, $5 \times 10^{-4}$ 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,
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 either 10 μg per ml of cycloheximide or 10 mM leucine was 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 μg per ml 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 × 10^6 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 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 5% 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
overcome incorporation of the leucine resulting from protein degradation. We can detect reutilization of leucine in prolactin because the prolactin is secreted from the cell and therefore removed from the processes of general intracellular protein degradation. Studies done on nonexported proteins will not necessarily detect reutilization of label. As has been emphasized by Schimke (21), it is important to consider reutilization of amino acids when using radioactive precursors to determine degradation rates.

The effect of TRH on prolactin that has been observed in the intact animal and in isolated pituitary fragments is an immediate release of stored prolactin. We have shown in this paper that TRH causes an increase in the synthesis of prolactin. GH cells do not store large amounts of prolactin as the pituitary gland does; to detect the effect, if any, of TRH solely on the release of prolactin by GH cells will require methods not used in this report. If TRH causes an increase in the synthesis of prolactin in the intact animal as well, TRH will have two effects, an immediate effect on release and a longer term trophic effect on the synthesis of prolactin. It has already been shown that TRH causes an increase in the synthesis as well as the release of thyroid-stimulating hormone (22). The mechanisms by which TRH causes these two effects, release and synthesis, need not be the same. Likewise, the effects of TRH on prolactin and thyroid-stimulating hormone may or may not be mediated by the same mechanisms.

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