Regulation of Pituitary Receptors for Thyrotropin-releasing Hormone by Thyroid Hormones*

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Thyroid hormones regulate the concentration of receptors for thyrotropin-releasing hormone (TRH) on GH₃ pituitary tumor cells. The addition of 10 nM L-triiodothyronine (T₃) to hypothryroid culture medium caused a decrease in TRH receptors from 0.8 to 0.4 pmol/mg of cell protein, and half of this decrease was obtained with 0.2 nM L-triiodothyronine. TRH receptor concentrations were significantly lower 12 h after addition of L-triiodothyronine and reached a minimum after 72 h. These effects were reversible within 72 h if L-triiodothyronine was removed. Both L-triiodothyronine and L-thyroxine caused the same loss of TRH receptors, and these effects did not depend on cell growth. Half-maximal saturation of receptors was obtained with 8 to 10 nM [³H]TRH using cultures grown in hypothryroid or L-triiodothyronine-supplemented media, but maximal [³H]TRH binding was reduced 50% in cells grown with L-triiodothyronine. Membranes prepared from cells incubated with L-triiodothyronine exhibited the same Kᵦ (5 to 6 nm at 0° C) as those prepared from hypothryroid cells, but the maximal number of TRH binding sites was decreased. The effects of thyroid hormones on TRH receptors were additive to the loss of TRH receptors caused by TRH alone. TRH stimulates the synthesis and secretion of prolactin by the GH₃ cell line. In hypothryroid cultures, TRH caused increases of 550 and 110% in prolactin synthesis and release, respectively. These responses were only 100 and 30% in L-triiodothyronine-treated cultures. In cells incubated with thyroid hormones the maximal effects of TRH on prolactin synthesis and release were reduced, but the dose-response curves for TRH effects were not shifted. The data indicate that thyroid hormones regulate the number of TRH receptors on pituitary mammotrophs without altering the affinity of receptors for TRH. These changes in the number of receptors per cell may be partially responsible for the decreased responses to TRH observed after L-triiodothyronine treatment.

The levels of thyroid hormones in tissues and plasma are tightly controlled. Release of L-thyroxine and L-triiodothyronine from the thyroid gland is stimulated by the pituitary hormone thyrotropin. Thyrotropin secretion is in turn stimulated by the hypothalamic peptide thyrotropin-releasing hormone, pGlu-His-ProNH₂ (1, 2). Thyroid hormones act as feedback regulators of the thyroid and pituitary glands.

In the adenohypophysis, L-triiodothyronine and L-thyroxine inhibit basal and TRH-stimulated secretion of thyrotropin. Hypothyroid subjects respond to TRH with large increases in plasma thyrotropin, but in hyperthyroidism the response is small or undetectable (3-5). The inhibitory actions of L-triiodothyronine and L-thyroxine are also observed in vitro in dispersed cell cultures prepared from normal pituitary tissue. If the cultures are incubated with thyroid hormones and then with TRH, thyrotropin release is not stimulated (4, 6). This blockade reportedly does not occur if inhibitors of protein or RNA synthesis are present during the incubation with L-triiodothyronine (5, 6). These observations have led to the hypothesis that thyroid hormones induce the synthesis of an inhibitor which blocks TRH actions in the pituitary (5, 6).

In addition to stimulating thyrotropin, TRH causes an increase in the release and synthesis of prolactin from the pituitary in vivo (3, 5, 7) and in vitro (4, 8). The prolactin-stimulating activity of TRH is inhibited by thyroid hormones in animals and in cell culture experiments (3, 4), although the inhibition is less marked than inhibition of thyrotropin release. Clonal lines of rat pituitary tumor cells have been used extensively to study the actions of TRH and thyroid hormones. We have used one of these lines, GH₃, which secretes prolactin and growth hormone, to study the mechanism of feedback inhibition by L-triiodothyronine and L-thyroxine; the system offers the advantage of a homogeneous cell population in which the TRH and L-triiodothyronine responses have been characterized. In GH₃ cells TRH stimulates prolactin release (9) and synthesis (8, 10). The peptide binds to a limited number of high affinity receptor sites which are found only on responsive cell lines (11-14). The affinity of TRH and structural analogs for these receptors correlates well with their ability to stimulate prolactin, suggesting that receptor binding is an early event in TRH-mediated stimulation of hormone production (12, 14). The effects of TRH on prolactin are reduced by thyroid hormones in a similar system (15). Physiological concentrations of L-triiodothyronine increase the rate of cell growth and markedly stimulate growth hormone synthesis (16-18). Thyroid hormones bind to nuclear receptor sites in pituitary tumor cells (19, 20).

In this communication we report that L-triiodothyronine and L-thyroxine control the concentration of TRH receptors in GH₃ cells and that the concentration of receptors correlates with the ability of the cells to respond to the peptide. A preliminary account of this work has appeared (21).

EXPERIMENTAL PROCEDURES

Materials—Tissue culture dishes were from Corning Glass Works. Cell culture media, horse serum, and fetal calf serum were from Grand County (New York) Cancer and Leukemia Society, Cancer Center Core Research Grant CA 11198 and Grant AM 19974 from the National Institute of Arthritis, Metabolism and Digestive Diseases. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Island Biological Co. Serum from a thyroidectomized calf was obtained from Rockland Farm, Gilbertsville, Pa. The hormone concentrations in this serum was less than 10 ng/100 ml for L-triiodothyronine and 1 μg/100 ml for L-thyroxine, as determined by radioimmunoassay; these values represent the lower limits of detection. ['H]TRH (0.2-3.0 nCi/proline; 21.8 Ci/mmol) was from New England Nuclear Corp. Synthetic TRH was a gift of Abbott Laboratories; L-triiodothyronine and L-thyroxine were purchased from Sigma Chemical Co. Radioimmunoassay kits for rat prolactin were generously supplied by Dr. Albert Parlow of the Rat Pituitary Hormone Program of the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD). Carrier-free 125I for protein iodination was obtained from Amersham/Searle. The second antibody preparations were from Calbiochem or Antibodies Inc. 

**Methods**—The methods of culture for GH3 cells have been previously described (22). Cells were maintained in Ham's F10 medium supplemented with 2.5% fetal calf serum and 15% horse serum. Cells were grown at 37°C in a humidified atmosphere of 5% CO2, 95% air. Experiments were performed using replicate 35- or 60-mm dishes inoculated with equal aliquots from a single donor culture. Either 1×106 cells or 4×105 cells were inoculated on 35- and 60-mm dishes, respectively. The cells were permitted to adhere to the dishes and grow for 48 h in this medium.

Samuels and co-workers have shown that in order to study effects of physiological concentrations of thyroid hormones it is necessary to maintain cells in medium containing serum from a thyroid hormone animal (16). To remove thyroid hormones in the culture medium, replicate dishes of GH3 cells were rinsed quickly and incubated for 1 h in serum-free Ham's F10. This medium was then replaced with Ham's F10 medium containing 10% serum from a hypothyroid calf (hypothyroid medium). In our experience each batch of hypothyroid serum must be tested; satisfactory results are obtained if the levels of L-triiodothyronine and L-thyroxine are too low to be detected by routine clinical radioimmunoassays. In order to measure effects of thyroid hormones, L-triiodothyronine or L-thyroxine was added to some cultures at the times indicated; the concentrations given in the text refer to the final concentrations of L-triiodothyronine and L-thyroxine added to the culture medium.

In experiments measuring prolactin release, replicate dishes were incubated for 72 h in hypothyroid medium with or without thyroid hormone. At this time the medium was removed and the dishes were washed twice with 3 ml of serum-free Ham's F10. Fresh hypothyroid medium containing the indicated concentrations of thyroid hormone and TRH was equilibrated at 37°C in 5% CO2, 95% air and then added to cultures for a 60- or 90-min incubation. Prolactin in the culture medium over this period comes entirely from the release of stored prolactin (9). To measure prolactin synthesis, cells were incubated in hypothyroid medium with or without L-triiodothyronine for 72 h. Fresh hypothyroid medium with L-triiodothyronine and TRH was then added for a 72-h incubation. Prolactin accumulated in the medium over a 3-day period is almost entirely the result of de novo synthesis (10). Medium samples were frozen for later immunoassay.

Prolactin was measured by specific radioimmunoassay using double antibody immunoprecipitation. Radioiodination of prolactin and the immunoassay were carried out as described by the Rat Pituitary Hormone Program of NIAMDD. The culture medium containing 10% hypothyroid serum contained no material which reacted in the immunoassay at the limits of detection, 0.19 ng/ml.

The number of available TRH receptors in intact cells was determined as previously described (11). Briefly, medium was removed and the cells were incubated with either 1 ml (35-mm dish) or 2 ml (60-mm dish) of serum-free medium (Newman-Tytell) containing 25 mm ['H]TRH for 1 h at 37°C. The cultures were then rinsed four times with ice-cold 0.15 M NaCl and scraped into distilled water; aliquots were used for determination of cell protein and ['H]TRH bound. Specific binding was determined by subtracting a blank representing the H bound in the presence of a saturating 200-fold excess of unlabeled TRH; nonspecific binding was less than 10% of the total binding in essentially the same manner as used in experiments in which the cells had previously been incubated with nonsaturating TRH, except that the cultures were washed three times with 0.15 M NaCl and prior to the addition of ['H]TRH. Under these conditions bound unlabeled TRH exchanges with ['H]TRH, as demonstrated in earlier work (23). A Millipore filter assay was used to measure the binding of ['H]TRH to membrane fragments (11).

Radioactivity in aqueous samples was determined by counting in ACS (Amersham/Searle) and radioactivity on dried filters was counted in toluene-based scintillation fluid. Protein was measured by the method of Lowry et al. (24) using bovine serum albumin as the standard.

**Results**

**Effect of Thyroid Hormones on TRH Receptors**—The concentration of TRH receptors on GH3 cells is dependent on the level of L-triiodothyronine (Fig. 1). Replicate cultures were grown for 72 h in medium containing serum from a hypothyroid medium, as described under "Experimental Procedures." Fresh hypothyroid medium containing either no addition (○) or 50 nM L-triiodothyronine (●) were then added. At the indicated times the number of available TRH receptors was determined as described under "Experimental Procedures." Each point is the average of duplicate determinations. Dishes contained 80 to 167 μg of cell protein. 

Fig. 1 (left). The effect of L-triiodothyronine on available TRH receptors. Replicate 35-mm dishes were inoculated from a single donor plate. The cultures were incubated 48 h in Ham's F-10 supplemented with 17.5% serum. The plates were washed with serum-free F-10 medium, and hypothyroid medium containing the indicated concentrations of L-triiodothyronine was added. After 72 h of incubation the medium was removed, and the cultures were incubated for 1 h with medium containing 25 nM ['H]TRH in order to determine the number of available TRH receptors. Each point represents the average and range of duplicate determinations. L-triiodothyronine stimulated cell growth so that at the end of the experiment dishes maintained in hypothyroid medium contained 114 μg of cell protein and those treated with 100 nM L-triiodothyronine contained 220 μg of protein.

Fig. 2 (center). Rate of L-triiodothyronine-induced receptor loss. Replicate cultures of GH3 cells were incubated for 72 h in hypothyroid medium, as described under "Experimental Procedures." Fresh hypothyroid medium containing either no addition (●) or 50 nM L-triiodothyronine (○) were then added. At the indicated times the number of available TRH receptors was determined as described under "Experimental Procedures." Each point is the average and range of duplicate determinations. Dishes contained 80 to 199 μg of cell protein.
Thyroid Hormones and Pituitary TRH Receptors

The GH3 cultures incubated with high concentrations of L-triiodothyronine bound only 50% as much [3H]TRH per mg of cell protein as those maintained in hypothyroid medium. This difference reflects a change in the amount of [3H]TRH bound per cell since L-triiodothyronine did not alter the cellular content of either protein or DNA, 140 and 13 pg/cell, respectively. Thyroid hormones do increase the growth rate of pituitary cells (16). The population doubling time for GH3 cells is 120 h in hypothyroid and 40 h in L-triiodothyronine-supplemented medium. However, the effects of thyroid hormones on receptor concentrations do not depend on differences in growth or total cell protein. When dense cultures were incubated with or without L-triiodothyronine for 4 days cell proteins were the same, 105 and 101 mg/dish, in hypothyroid and L-triiodothyronine-treated cultures, but TRH receptors were 0.407 and 0.179 pmol/dish, respectively. When cultures were continuously maintained in either hypothyroid or L-triiodothyronine-supplemented medium the differences in TRH receptors were observed for over 3 months.

Rates of Changes in TRH Receptor Concentration—The rate at which L-triiodothyronine causes a decrease in receptors is shown in Fig. 2. GH3 cells were grown in hypothyroid medium for 3 days. At the start of the experiment fresh medium with or without 50 nM L-triiodothyronine was added, and TRH receptors were determined at intervals. TRH receptor concentrations were significantly lower after 12 h with L-triiodothyronine and decreased gradually to 55% of hypothyroid values. This loss of receptors is reversible. When cultures were treated with L-triiodothyronine for 3 days and then switched to hypothyroid medium, receptor levels slowly increased to the level observed in hypothyroid cultures (Fig. 3). If cultures were alternately incubated with L-triiodothyronine-supplemented and hypothyroid media, the number of TRH receptors per dish increased after each 48-h period in hypothyroid medium and decreased after L-triiodothyronine treatment, while values for protein per dish followed an opposite pattern (Fig. 4). Thus L-triiodothyronine causes a decrease in the total number of receptors present while increasing the number of cells, and hypothyroid conditions cause an increase in receptors without any increase in cell number.

Effect of Thyroid Hormones on Affinity of TRH for Receptors—The effect of L-triiodothyronine on [3H]TRH binding could result from either a change in the number of available receptors or a change in their affinity for TRH. Therefore we measured the equilibrium binding of [3H]TRH to GH3 cultures which had been grown in either hypothyroid or L-triiodothyronine-supplemented medium (Fig. 5). Maximal [3H]TRH binding was reduced by 50% in L-triiodothyronine-treated cells while half-maximal binding occurred at approximately the same [3H]TRH concentrations, 8 and 10 nM for hypothyroid and L-triiodothyronine-treated cells, respectively. Similar results were obtained from equilibrium binding studies carried out using membrane fractions. Total receptor concentrations were reduced 55% by L-triiodothyronine treat-

![Fig. 5. Binding of [3H]TRH to cultures of hypothyroid and L-triiodothyronine-treated GH3 cells. Replicate 60-mm dishes of GH3 cells were incubated for 72 h in hypothyroid medium with no additions (□) or with 50 nM L-triiodothyronine (○), as described in Fig. 1. The cells were then incubated with the indicated concentrations of [3H]TRH for 75 min to determine the amount of [3H]TRH bound, as described under "Experimental Procedures." A blank representing the amount of [3H]TRH bound to the cells in the presence of 100 nM unlabeled TRH and the indicated concentration of [3H]TRH has been subtracted from each point. Each point gives the average and range of duplicate determinations. Dishes contained 142 to 326 pg of cell protein in hypothyroid and T3-supplemented medium, respectively.](http://www.jbc.org/)

![Fig. 6. Binding of [3H]TRH to membrane fractions from hypothyroid and L-triiodothyronine-treated GH3 cells. Cells were harvested from replicate 100-mm plates of GH3 cells incubated for 72 h in hypothyroid medium with no additions (□) or with 50 nM L-triiodothyronine (○). The particulate fractions were prepared and equilibrium binding studies performed using membrane fractions. Total receptor concentrations were reduced 50% by L-triiodothyronine treatment, while values for protein per dish followed an opposite pattern (Fig. 4). Thus L-triiodothyronine causes a decrease in the total number of receptors present while increasing the number of cells, and hypothyroid conditions cause an increase in receptors without any increase in cell number.](http://www.jbc.org/)

![Fig. 4. Effects of hypothyroid and L-triiodothyronine-supplemented media on cell growth and TRH receptors. Cells were incubated on 35-mm dishes from a single donor culture, as described under "Experimental Procedures." The plates were alternately incubated for 48-h periods in hypothyroid medium containing 50 nM L-triiodothyronine or in hypothyroid medium alone. Available TRH receptor number and cell protein were measured in duplicate dishes after each 48-h period. Values shown are the average and range of duplicate determinations.](http://www.jbc.org/)
Thyroid Hormones and Pituitary TRH Receptors

Additive Effects of TRH and Thyroid Hormones—Hinkle and Taichman (23) reported that exposure of C130 cells to TRH caused a slow, reversible loss of TRH receptors. To determine whether the effects of L-triiodothyronine and TRH were additive, hypothyroid or L-triiodothyronine-treated cultures were incubated with various concentrations of TRH. Available TRH receptors were then measured as described previously (23) using conditions in which [\(^{3}H\)]TRH exchanges with any bound unlaabeled TRH so that the amount of radioactivity bound is a measure of the total number of TRH binding sites (23). Each point is the average of duplicate determinations. Dishes maintained in hypothryoid or thyroid hormone-supplemented medium contained, respectively, 97 and 330 \(\mu\)g of protein (Panel A) and 324 and 381 \(\mu\)g of protein (Panel B). TRH had no effect on cell protein.

![Figure 7](http://www.jbc.org/)

**Table I**

| Serum and addition | Cell protein [\(^{3}H\)]TRH bound |
|--------------------|-----------------------------------|
| None               | 134 ± 7                           |
| TRH (100 nM)       | 120 ± 7                           |
| T3 (100 nM)        | 224 ± 12                          |
| T3 (100 nM) + TRH (100 nM) | 201 ± 10                           |
| T3 (1 \(\mu\)M)    | 235 ± 4                           |
| T3 (1 \(\mu\)M) + TRH (100 nM) | 244 ± 3                           |
| Horse + 2.5% fetal calf | 179 ± 4                           |
| TRH (100 nM)       | 178 ± 3                           |

Effects of Thyroid Hormones on TRH Responses—TRH has been shown to increase the rates of prolactin release and synthesis by GH cells. The effects of thyroid hormone on both of these responses to TRH have been tested.

TRH stimulation of prolactin release was examined in cells which had been grown in either hypothyroid or L-triiodothyronine-supplemented medium. The cultures were rinsed and then incubated for 60 to 90 min with fresh medium containing different concentrations of TRH. Under these conditions prolactin in the culture medium comes entirely from the release
The rate of TRH loss in hypothyroid tissue was measured with the use of L-triiodothyronine. The Kd value for the L-triiodothyronine-receptor complex, 0.5 nM, is close to the concentration of L-triiodothyronine that caused a half-maximal loss of TRH receptors, 0.2 nM. These data suggest that the half-maximal loss of TRH receptors is due to the half-maximal concentration of L-triiodothyronine that caused the loss. The L-triiodothyronine-mediated receptor loss seen in the hypothyroid tissue was not observed in the L-thyroxine-supplemented cultures.

The experiments described in this communication demonstrate that thyroid hormones regulate the number of TRH receptors. The results of these experiments are consistent with the hypothesis that thyroid hormones regulate the number of TRH receptors.

**DISCUSSION**

The factors that regulate the number of TRH receptors are not well understood. However, the results of these experiments suggest that thyroid hormones may regulate the number of TRH receptors. The mechanism by which thyroid hormones regulate the number of TRH receptors is not well understood. However, the experiments described in this communication demonstrate that thyroid hormones regulate the number of TRH receptors.

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