Bihormonal Regulation of the Thyrotropin-Releasing Hormone Receptor in Mouse Pituitary Thyrotropic Tumor Cells in Culture

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ABSTRACT Receptors for thyrotropin-releasing hormone (TRH) are present on mouse pituitary thyrotropic tumor cells. Incubation of thyrotropes with 100 nM TRH or 4 nM L-triiodothyronine (T_3) for 48 h decreased the number of TRH receptors to ≅50 and 20% of control, respectively. There was no effect on the equilibrium dissociation constant which was 3–5 nM. The depletion in the number of available TRH receptors was time- and dose-dependent. TRH, 100 nM, decreased the receptor number to 70% after 24 h, 50% after 48 h, and 45% of control after 72 h. T_3, 4 nM, decreased the receptor number to 52% after 24 h, 20% after 48 h, and 17% of control after 72 h. After 48 h, half-maximal depletion occurred with 1–2 nM TRH and ≅0.15 nM T_3. Incubation with 100 nM TRH and 4 nM T_3 caused a significantly greater reduction in the receptor level than either hormone alone. The decrease in the receptor level was reversible within 72 h after removal of TRH, 100 nM, but was only partially reversed, from 20 to 40% of control, after removal of T_3, 4 nM, after 120 h. By regulating the number of available TRH receptors on the thyrotrope, TRH and T_3 interact to control thyrotropin release.

INTRODUCTION

A number of studies have demonstrated that thyrotropin-releasing hormone (TRH)[1] stimulates synthesis and release of thyrotropin (TSH) and prolactin in vitro (1–8), and release of TSH and prolactin in intact animals (9–11). TRH has been shown to bind to receptors on the plasma membrane of cells derived from whole anterior pituitary (12), to mouse pituitary thyrotropic tumor cells that produce TSH but not prolactin (13–15), and to growth hormone (GH) cells, clonal strains of rat pituitary tumor cells that produce prolactin and growth hormone but not TSH (16, 17). Recent studies indicate that the number of plasma membrane receptors for a variety of hormones (18–20), including TRH (21–23), can be modulated, and it has been suggested that this may serve as an important site of regulation of hormone action.

In this report we describe modulation of the level of TRH receptors but not their affinity for TRH in suspension cultures of mouse pituitary thyrotropic tumor cells which we have shown to be comprised only of thyrotropes (24). These experiments demonstrate time- and dose-dependent regulation of the thyrotrope receptor for TRH by TRH and L-triiodothyronine (T_3). The TRH receptor may be an important site for regulation of TSH release.

METHODS

Dulbecco's modified Eagle's medium was obtained from Grand Island Biological Co., Grand Island, N. Y. Hypothyroid calf serum was purchased from Rockland Farms, Gilbertsville, Pa. The calf serum was shown to contain <5 ng T_3/dl and <0.1 μg L-thyroxine/dl. [^{3}H]TRH (L-[2,3-^{3}H]proline, 40 Ci/mmol or L-[2,3,4,5-^{3}H]proline, 115 Ci/mmol) was from New England Nuclear, Boston, Mass. and unlabeled TRH was from Beckman, Instruments, Inc., Fullerton, Calif. [^{125}I]T_3 (1,090 μCi/μg) was from New England Nuclear and unlabeled T_3 was from Calbiochem, San Diego, Calif.

Short-term suspension cultures of thyrotropes were established with cells derived from mouse pituitary thyrotropic tumors by selective attachment techniques from primary cultures comprised of several cell types (25). TSH-producing cells were grown in medium supplemented with 10% hypothyroid calf serum at 37°C in a humidified atmosphere at 5% CO_2-95% air. These cells have been shown to produce TSH at a constant rate for up to 9 days in culture (24).

Binding of TRH to thyrotropes was performed in fresh medium without serum containing [^{3}H]TRH at 37°C in a shaking water bath (≅100 oscillations/min). Experiments were carried

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[1] *Abbreviations used in this paper*: T_3, L-triiodothyronine; TRH, thyrotropin-releasing hormone; TSH, thyrotropin.

out in 0.5 ml of medium in 12 × 75 polypropylene test tubes with 0.1 to 0.5 × 10^6 cells that were all derived from a single tumor. After incubation the cells were centrifuged at 650 *g* for 5 min at 4°C, the medium was removed and the cells were washed three times with 1 ml of 0.1 M NaCl, 0.05 M Na phosphate, pH 7.5. The cell pellets were dissolved in 0.4 N NaOH; aliquots were used to measure radioactivity by liquid scintillation counting and for measurement of cell protein by the method of Lowry et al. (26) using bovine serum albumin as standard. Nonspecific binding, that is, [^{3}H]TRH bound in the presence of a 200-fold molar excess of unlabeled TRH, was subtracted from each value. Nonspecific binding represented <8% of the total bound radioactivity.

Binding of [^{3}H]TRH to thyrotropes was determined after culture for 48 h in the presence of 4 nM T_3 or 100 nM TRH and compared to controls. For these experiments, 1–2 × 10^6 cells were incubated in 3 ml of medium containing 10% hypothyroid calf serum in 25 cm^2 flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Cells were usually used between 6 and 9 days in culture, a time during which there was little change in cell number. After 48 h, the cells were collected, centrifuged at 650 *g* for 5 min, and washed three times with 2 ml of medium at 37°C. After the second wash, the cells were kept suspended in medium at 37°C in a shaking water bath for 15 min to increase dissociation of TRH from those cells exposed to TRH. Binding of [^{3}H]TRH to equal aliquots of cells from each flask was performed as above.

The number of available TRH receptors and "basal" and TRH-stimulated TSH production were measured in cells exposed to varying concentrations of T_3 or TRH for 48 h. For these experiments, cells were incubated in medium supplemented with 10% hypothyroid calf serum. After 48 h, the cells were collected, washed as above, and divided into two aliquots. One aliquot was incubated in serum-free medium containing 20–40 nM [^{3}H]TRH for 90 min to estimate the number of available TRH receptors. The other aliquot was cultured again, in duplicate tubes, in serum-containing medium, in the presence of 10 nM TRH, or in its absence for 24 h. TSH in the medium was measured by a heterologous, double-antibody radioimmunoassay using a pool of medium from cultures of mouse thyrotropic tumor cells containing 0.54 μeq of rat TSH/ml as standard (10, 25).

Nuclear binding of ^{125}I-T_3 was measured after incubation in medium containing 10% hypothyroid calf serum at 37°C for 2.5 h as described (25).

Statistical analysis was performed by unpaired *t* test.

RESULTS

Binding of TRH to thyrotropic tumor cells. [^{3}H]TRH added to the medium of thyrotropic tumor cell cultures bound rapidly to the cells. The specific binding was 47% of maximum after 10 min, 59% after 15 min, 89% after 30 min, maximum after 60 min, and remained constant for longer than 3 h. Therefore, in all other binding studies incubation with [^{3}H]TRH was for 90 or 120 min. To demonstrate reversibility of binding, aliquots of cells from a single culture were incubated with 25 nM [^{3}H]TRH for 90 min, the cells were washed, and the amount of specifically bound radioactivity was measured in triplicate (Fig. 1, zero time). The remaining cell aliquots were resuspended in medium containing the same concentration of [^{3}H]TRH, unlabeled TRH, or no TRH and the amount of [^{3}H]TRH bound

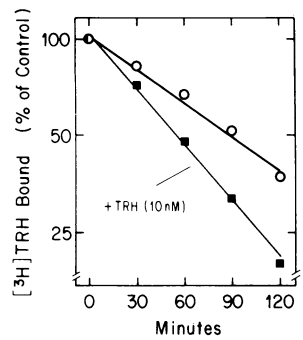

FIGURE 1 Reversibility of [^{3}H]TRH binding. Identical samples of cells from a single culture were incubated with 25 nM [^{3}H]TRH. After 90 min (zero time) the cells were washed, resuspended in fresh medium with 25 nM [^{3}H]TRH (control cells), with no added TRH (0), or with 10 nM unlabeled TRH (■) and the amount of [^{3}H]TRH bound measured at the times indicated. Control cells bound 0.60 pmol [^{3}H]TRH per milligram protein. The points represent the mean of duplicate values whose average variation was ±13%.

to the cells was measured at 30-min intervals for 2 h. The amount of bound [^{3}H]TRH remained constant in the cells re-exposed to [^{3}H]TRH. In contrast, as shown in Fig. 1, the radioactivity rapidly dissociated from cells exposed to no TRH or unlabeled TRH. The rate constant of dissociation of [^{3}H]TRH from cells was greater in the presence of unlabeled TRH, 2.3 × 10^{-4}/s, than in its absence, 1.3 × 10^{-4}/s. The ^{3}H radioactivity which dissociated from the cells was able to bind to other cells to the same extent as authentic [^{3}H]TRH suggesting that it was unaltered [^{3}H]TRH.

The characteristics of binding of TRH to its receptors at equilibrium were defined by incubating cells with increasing concentrations of [^{3}H]TRH, up to 100 nM, for 90 min. Fig. 2 shows the analysis of [^{3}H]TRH binding to thyrotropes by the method of Scatchard (27). A linear plot of the data was found consistent with a single class of noninteracting binding sites. The dissociation constant was 3–5 nM. There were 1.1 pmol [^{3}H]TRH bound/mg cell protein at saturation, corresponding to 99,000 binding sites/cell.

Binding of TRH to TRH and T_3-treated cells. Before study of the binding of [^{3}H]TRH to cells exposed to TRH or T_3, it was necessary to show that these hormones did not interfere with the subsequent binding reaction under the experimental conditions employed. Binding of [^{3}H]TRH, 25 nM, to control cells was performed in triplicate in the presence of 10 nM T_3 and compared to that in medium alone (control). The amount of [^{3}H]TRH bound in the presence of T_3 was 94±12% of control (mean±SD). In a parallel set of experiments, the effect of preincubation with TRH for 90 min was determined. Equal aliquots of cells, in triplicate, were incubated with 100 nM unlabeled TRH, 25 nM [^{3}H]TRH, or medium alone (control). The cells were washed three

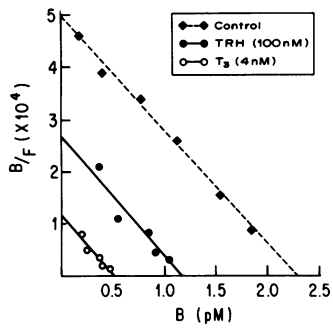

FIGURE 2 [^{3}H]TRH binding to cells incubated with TRH or T_3 for 48 h. Equal portions of cells were incubated with medium alone, 100 nM TRH, or 4 nM T_3. After 48 h the cells were washed and samples containing an average of 27 μg of protein were incubated with [^{3}H]TRH, 1 to 100 nM, for 90 min after which bound [^{3}H]TRH was measured. There was no difference in the amount of cell protein in the three cultures. The amount of [^{3}H]TRH bound at saturation was 1.1 pmol/mg protein in control cells, 0.55 pmol/mg protein in TRH-treated cells, and 0.22 pmol/mg protein in T_3-treated cells. The points represent the mean of duplicate determinations whose average variation was ±12%.

times as described in Methods. After washing, the amount of [^{3}H]TRH bound was decreased to 63±5% of the initial level. Binding of [^{3}H]TRH, 25 nM, for 90 min was then performed with the washed cells. The cells previously exposed to unlabeled TRH bound 93±3% of the amount of [^{3}H]TRH bound by controls. Therefore, it was possible to accurately measure [^{3}H]TRH binding to cells exposed to T_3 or TRH.

Because TRH may be degraded during incubation, its stability was determined by incubating 20 nM [^{3}H]-TRH at 37°C in medium containing 10% hypothyroid calf serum. After 48 h, the incubated [^{3}H]TRH was able to bind specifically to thyrotropes to the same extent as fresh [^{3}H]TRH (104±3% of control). Therefore, there was virtually no degradation of TRH in the medium under the conditions of incubation.

Cells from a single suspension culture were incubated with 100 nM TRH or 4 nM T_3 for 48 h; control cultures were incubated with no added hormone. After 48 h, the cells were washed and binding of [^{3}H]TRH was studied. There was no difference in binding affinity of the TRH receptor after incubation with TRH or T_3 as compared to controls (Fig. 2). In contrast, there was a marked decrease in the number of available receptors to 50 and 22% of control after a 48-h exposure to TRH and T_3, respectively. When cells were incubated with 100 nM TRH plus 4 nM T_3, the number of available receptors decreased further to 12% of control ($P < 0.01$).

The time-course of depletion of available TRH receptors was studied in cells from a single tumor exposed to 100 mM TRH or 4 nM T_3. The number of TRH receptors was estimated after 24, 48, and 72 h by incubating the cells with 25 nM [^{3}H]TRH, a concentration sufficient to saturate 83–89% of the receptors. The number of receptors available to bind [^{3}H]TRH was 70% of control after 24 h ($P < 0.05$), decreased further to 50% after 48 h ($P < 0.01$), and to 45% after 72 h ($P < 0.01$ vs. control; $P > 0.1$ vs. 48 h) exposure to 100 nM TRH. The depletion of TRH receptors after incubation with 4 nM T_3 was more profound. After 24 h there were 52% ($P < 0.001$) of the control number of receptors, after 48 h the level decreased further to 20% ($P < 0.001$) and after 72 h was 17% of control ($P < 0.001$ vs. control; $P > 0.1$ vs. 48 h).

As shown in Fig. 3, depletion of the number of available TRH receptors was dose dependent for TRH and T_3. After 48 h, there were 79% of the control level of receptors present in cells exposed to 1 nM TRH ($P < 0.05$), 65% in cells exposed to 10 nM TRH and 55% in cells exposed to 100 nM TRH or greater; half-maximal depletion occurred at ≅1–2 nM TRH. T_3, 0.1 nM, lowered the number of receptors to 62% of control ($P < 0.05$) and maximal inhibition was achieved with T_3 concentrations >4 nM; half-maximal inhibition occurred at ≅0.15 nM T_3.

To determine the reversibility of the loss of TRH receptors, cells from a single tumor were incubated with 100 nM TRH and 4 nM T_3 for 48 h which reduced the TRH receptor number to 52 and 21%, respectively. After 48 h, the cells were washed three times, resuspended in fresh TRH- and T_3-free medium and incubated for up to 120 h. During this experiment cell protein increased <20%. After removal of TRH, progressive repletion of the TRH receptor level occurred. After 24 h the level had returned to 65% of control ($P < 0.05$), after 48 h to 74% of control, and by 72 h

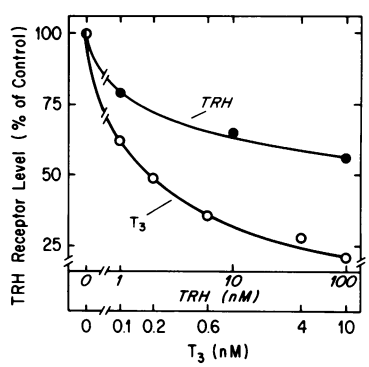

FIGURE 3 Dose-response effect of TRH or T_3 on the number of TRH receptors. Equal aliquots of cells were incubated with medium alone (control cells), with TRH, 1 to 100 nM, or with T_3 0.1–10 nM. After 48 h the cells were washed, incubated with 40 nM [^{3}H]TRH and bound [^{3}H]TRH measured after 120 min. There was ≅65 μg of cell protein in each culture. The points represent the mean of duplicate determinations whose average variation was ±6%. Control cells bound 0.91 pmol [^{3}H]TRH /mg protein. Half-maximal receptor depletion occurred at ≅1–2 nM TRH and 0.15 nM T_3.

to control levels. In contrast, there was a much more delayed repletion of TRH receptor number after removal of T_3. There was no change in the number of receptors after 24 h ($P > 0.1$), an increase to 30% of control after 48 h ($P < 0.05$), followed by a gradual rise to only 40% of control after 120 h.

To determine whether the differences in the rates of repletion of the TRH receptor after removal of TRH and T_3 from the medium was a result, in part, of differences in the amount of residual hormone remaining bound to the respective receptor, the time-course of disappearance of [^{3}H]TRH from the cell was compared to the disappearance of ^{125}I-T_3 from the nucleus. Cells from a single tumor were incubated in medium supplemented with 10% hypothyroid calf serum and 5 nM or 100 nM [^{3}H]TRH or 0.2 nM or 4 nM ^{125}I-T_3 for 2.5 h at 37°C. After 2.5 h, the cells were washed three times with medium, resuspended in TRH- and T_3-free medium and incubated again at 37°C. As shown in Fig. 4A, the rate of dissociation of [^{3}H]TRH was independent of the initial level of bound hormone and was best approximated by a simple rate constant of 1.4×10^{-4}/s. In contrast, the rate of dissociation of ^{125}I-T_3 from the nucleus, although similar to that of [^{3}H]TRH from the cell in that it too was independent of the initial level of bound hormone, was best approximated by a two-component dissociation curve (Fig. 4B). The first component in which the amount of bound ^{125}I-T_3 decreased to ≅50% of the initial level, had a dissociation rate constant of 1.9×10^{-4}/s, similar to that observed for [^{3}H]TRH, whereas the rate of dissociation of the second component was only one-seventh as fast, 2.6×10^{-5}/s.

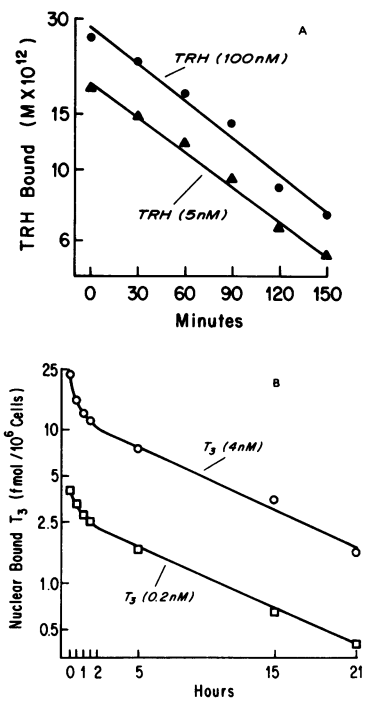

FIGURE 4 Rate of dissociation of TRH and T_3 from their receptors. (*A*) Equal aliquots of cells were incubated with 5 or 100 nM [^{3}H]TRH for 150 min after which the cells were washed and incubated in fresh medium alone. At the indicated times the amount of [^{3}H]TRH specifically bound to the cell was measured. The points represent the mean of duplicate determinations; average variation was ±6%. There was 60 μg of cell protein in each tube. (*B*) Equal aliquots of cells were incubated with 0.2 or 4 nM ^{125}I-T_3 for 150 min after which the cells were washed and incubated in fresh medium alone. At the indicated times the amount of ^{125}I-T_3 specifically bound to the cell nucleus was measured. The points represent the mean of duplicate determinations; average variation was ±4%. There were 0.4×10^6 cells in each tube.

Regulation of TRH release by TRH and T_3. Release of TSH from thyrotropic tumor cells in short-term suspension culture was inhibited by physiological levels of T_3 and stimulated by TRH (Fig. 5). Release of TSH from control cells was 3.6 μg/mg cell protein per 24 h and was stimulated to 6.0 μg by 10 nM TRH. In cells exposed to varying concentrations of T_3 for 48 h, TSH release was progressively inhibited during the subsequent 24 h with maximum inhibition to 1.4 μg/mg protein by concentrations of T_3 1 nM or greater; half-maximal inhibition occurred at ≅0.2 nM T_3. Stimulation of TSH release by TRH was progressively blunted by increasing T_3 with complete abolition of response to TRH at T_3 concentrations of 1 nM or greater.

DISCUSSION

The mouse pituitary thyrotropic tumor cell culture system employed in these experiments (24, 25) appeared to be a suitable model in which to study physiological

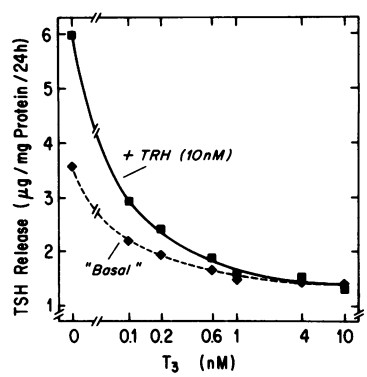

FIGURE 5 Effect of T_3 on basal and TRH-stimulated TSH release. Equal aliquots of cells were incubated with medium alone or T_3, 0.1 to 10 nM. After 48 h the cells were centrifuged, resuspended in fresh medium and divided equally into four tubes. Two tubes were incubated in medium alone (basal) and two tubes in 10 nM TRH for an additional 24 h after which TSH release was measured. Average cell protein was 42 μg/tube. Points represent the mean of duplicate determinations whose mean SD was ±10%.

regulation of thyrotrope function. In these cells, release of TSH was inhibited in a dose-dependent fashion by T_3; half-maximal inhibition occurred at a total medium T_3 concentration of ≅0.2 nM which corresponds to a concentration of unbound or free T_3 within the physiological range (25). Moreover, TRH stimulated release of TSH and this stimulation could be progressively blunted by increasing concentrations of T_3 until, at T_3 levels of 1 nM or greater, the effect of TRH was completely abolished. This interaction between TRH and T_3 is quantitatively very similar to that observed in humans (28, 29).

Putative TRH receptors on mouse thyrotropic tumor cells were originally described by Grant et al. (13, 15) and Eddy et al. (14). The characteristics of the interaction of TRH with its receptors described by Grant and his colleagues differ from those reported here. They concluded, from Scatchard analysis of their data, that there were two receptors for TRH with apparent equilibrium dissociation constants of 20 and ≅500 nM (15). Subtle differences in the kinetics of [^{3}H]TRH displacement by a series of TRH analogues were interpreted as further evidence in support of two binding sites. The number of TRH receptors per cell could not be calculated from the data presented in their report. In contrast, we have observed only a single class of binding sites for TRH, i.e., a Scatchard plot of our binding data was linear, using up to 100 nM [^{3}H]TRH, with an apparent dissociation constant of 3–5 nM. At saturation in control cultures there were 99,000 molecules of TRH bound per cell. Although we are unable to offer a conclusive explanation for the differences in binding characteristics of TRH to these cells observed in these two studies, there were major differences in the conditions under which the binding experiments were performed, in that Grant et al. (13, 15) employed half-isotonic medium at 0°C, whereas we used the more physiological conditions of incubation in isotonic medium at 37°C.

A second aspect of the binding of TRH to control thyrotropes is of interest. We observed an increased rate of dissociation of [^{3}H]TRH from its receptor when cells with previously bound [^{3}H]TRH were incubated in the presence of unlabeled TRH ($k_d = 2.3 \times 10^{-4}$/s) as compared to incubation of fresh medium alone ($k_d = 1.3 \times 10^{-4}$/s). This enhanced dissociation of [^{3}H]TRH from its binding site in the presence of unlabeled TRH may be a result of prevention of reassociation of [^{3}H]TRH. DeMeyts et al. (30, 31), using insulin binding to lymphocytes as a model, suggested that enhanced dissociation of labeled ligand in the presence of unlabeled ligand is consistant also with negatively cooperative site-site interactions within a homogeneous population of binding sites. However, the model of site-site interaction proposed by DeMeyts et al. is usually associated with a concave upward, curvilinear Scatchard plot of the equilibrium binding date, whereas our data fit best with a linear Scatchard plot. Moreover, because there was no difference in the rates of dissociation of [^{3}H]TRH from its receptor after binding at medium concentrations of 5 and 100 nM, levels that would occupy ≅60 and 95% of the receptors, there appeared to be no site-site interactions among TRH receptors. Recently, Pollet et al. (32) presented a very interesting re-analysis of the binding of insulin to lymphocytes which suggested also that interactions within a group of homogeneous binding sites was not the explanation for the increased rate of dissociation observed in the presence of unlabeled ligand. At the present time, the explanation for this phenomenon remains unclear.

The data presented here demonstrated that the number of receptors for TRH on TSH-producing cells, but not their affinity for TRH, was regulated by TRH and T_3. The depletion of the TRH receptor number was shown to be time- and dose-dependent. After 48 h of exposure, half-maximal receptor depletion occurred at 1–2 nM TRH and ≅0.15 nM T_3. These findings with TRH are similar to those reported by Hinkle and Tashjian (21) in the GH_3 clonal strain of prolactin and growth hormone producing rat pituitary tumor cells and with T_3 by Perrone and Hinkle (22) in GH_3 cells and by DeLéan et al. (23) in homogenates of whole anterior pituitary glands after in vivo administration of L-thyroxine to hypothyroid rats.

The time-course of repletion of the TRH receptor number was very different after cells were exposed to maximally effective doses of TRH or T_3. After removal of TRH the receptor number had returned to control levels by 72 h, whereas after removal of T_3 there was only a very gradual rise up to 40% of the control level after 120 h. To determine whether the more prolonged effect of T_3 compared to TRH could be a result of the amount of hormone remaining bound to its respective receptor, after their removal from the incubation medium, we compared the rate of disappearance of TRH from the cell to that of T_3 from the nucleus. We have demonstrated (25) putative nuclear receptors for thyroid hormones in these cells. The half-life of the receptor-TRH complex was ≅85 min. Therefore, after 9 h there was virtually no TRH remaining on the receptor. T_3 disappearance from its nuclear receptor was much slower. After a rapid decline to ≅50% of its initial level within 1.5 h, there was a more gradual decrease of bound T_3 ($t_{1/2}$ = 7 h). Therefore, even 12 h after exposure to 4 nM T_3 there was still as much T_3 associated with the nuclear receptor as was initially present after incubation with 0.2 nM T_3, a level which yields a half-maximal T_3 effect. The more prolonged binding of T_3 to its receptor after removal of T_3 from the medium may be a result of the high concentration of T_3 found in the cytoplasm of the cell, a compartment with which the nucleus equilibrates. It seems, therefore, that the

differences in the residual hormone bound after removal of TRH and T_3 may account, to some extent, for the different durations of their affects on TRH receptor number. However, although there was still a profound effect on the number of TRH receptors 120 h after removal of T_3 or >72 h after virtually no T_3 would be bound to its receptor, T_3 must induce a long-lived message for this effect.

Modulation of the number of TRH receptors may be an important mechanism by which thyrotropes autoregulate their sensitivity to TRH and control TSH release. For example, in the intact animal an increase in the circulating level of thyroid hormones would decrease the number of TRH receptors. Thyrotropes would then be less sensitive to TRH, TSH release would decline, thyroidal secretion of T_3 and thyroxine would diminish, and the level of circulating thyroid hormones would return towards normal. Because the receptors for several other polypeptide hormones have been shown also to be regulated under physiological conditions, it has been suggested that modulation of the number of hormone receptors might be a common mechanism by which endocrine target cells autoregulate their sensitivity to tropic hormones (18–23).

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