Thyrotropin-Releasing Hormone Regulates the Number of Its Own Receptors in the GH_3 Strain of Pituitary Cells in Culture†

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ABSTRACT: Thyrotropin-releasing hormone (TRH), a hypothalamic tripeptide, binds rapidly and reversibly to specific membrane receptors on GH_3 cells, a clonal strain of rat pituitary cells grown in culture. GH_3 cells were incubated for 1–72 hr with unlabeled TRH, washed, and then incubated for 1 hr with [^{3}H]TRH. Under these conditions 80% of any bound, unlabeled TRH exchanges with [^{3}H]TRH in the medium, and the amount of radioactivity bound to the cells gives a measure of the number of TRH receptors. In GH_3 cells, the number of available TRH receptors decreased from 92% of control after 1 hr to 35% after 48 or 72 hr of incubation with unlabeled TRH. Binding of [^{3}H]TRH to both intact control and TRH-treated cells was half-maximal at 8 n*M* [^{3}H]TRH, but the maximum amount of [^{3}H]TRH bound was decreased by 75% in cells previously incubated for 48 hr with unlabeled TRH. Equilibrium binding studies were performed using membrane fractions prepared from control cells and cells previously exposed to TRH for various periods. The dissociation constant of the TRH–receptor complex was the same in all cases, but the maximum amount of TRH bound decreased progressively in membrane fractions from cells incubated with TRH for 1–51 hr. TRH receptors were not found in cytoplasmic fractions of control or TRH-treated cells. The loss of TRH receptors was reversible within 4 days. In the continued presence of the tripeptide the number of receptors remained low for 12 days. After incubation for 2 days with different concentrations of TRH, the number of receptors was decreased to 33% of control at 100–300 n*M* TRH, and half of this decrease occurred at about 1 n*M* TRH; half-maximal biological responses occur at 2 n*M* TRH. The biologically active N^{τ}-methylhistidyl derivative of TRH also effected a loss of receptors, while three inactive analogs of TRH did not cause reductions in the number of TRH receptors. In cultures incubated for 40 hr with cycloheximide, protein synthesis was inhibited by 85%, but the number of TRH receptors was 76% of control suggesting that the receptor has a long half-life. When GH_3 cells were incubated with cycloheximide plus TRH, the number of TRH receptors decreased by only 23% as compared to a decrease of 73% in cells incubated with TRH alone, suggesting that receptor loss is partially dependent on active protein synthesis. We conclude that in GH_3 cells TRH regulates the number of its own receptors.

Thyrotropin-releasing hormone, a hypothalamic tripeptide L-pGlu-L-His-L-ProNH_2 (Burgus et al., 1969; Bϕler et al., 1969), stimulates the synthesis as well as the release of both thyrotropin and prolactin from the pituitary gland (Burgus et al., 1969; Bϕler et al., 1969; Wilber, 1971; Jacobs et al., 1971; Bowers et al., 1973; Schams, 1972; Convey et al., 1973; Vale et al., 1973). TRH[1] has been shown to have the following effects on GH_3 cells, a clonal strain of rat pituitary tumor cells which synthesize and secrete prolactin and growth hormone, but not thyrotropin. Within 30 min of its addition to the medium, TRH stimulates the release of previously synthesized intracellular prolactin (Dannies et al., 1974). TRH increases by 100–500% the rate of synthesis of prolactin, and decreases by 50–80% the production of growth hormone (Tashjian et al., 1971; Dannies and Tashjian, 1973; Hinkle et al., 1974). These effects on hormone synthesis are first observed 4–6 hr after TRH is added to the culture medium and reach a maximum after 24–48 hr (Tashjian et al., 1971; Dannies and Tashjian, 1974; Tashjian and Hoyt, 1972). The morphology of GH_3 cells is also altered by treatment with TRH (Tashjian and Hoyt, 1972).

The interaction between TRH and receptors for the tripeptide has been studied using whole bovine (Labrie et al., 1972) and rat (Wilber and Seibel, 1973; Eddy et al., 1973) pituitary tissue, mouse thyrotropin-producing tumors (Grant et al., 1972; Grant et al., 1973; Vale et al., 1973), and GH_3 cells (Vale et al., 1973; Hinkle et al., 1974; Hinkle and Tashjian, 1973; Gourdji et al., 1973; Brunet et al., 1974). TRH receptors have been found predominantly in plasma membrane fractions prepared from target cells (Vale et al., 1973; Poirier et al., 1972). The characteristics of the binding reaction between TRH and GH_3 cells strongly imply a functional significance for the receptors. TRH binds only to cells which respond biologically to the tripeptide (Hinkle and Tashjian, 1973). Binding occurs rapidly and precedes the biological responses, and the initial binding reaction is reversible (Hinkle and Tashjian, 1973; Gourdji et al., 1973). The concentration of TRH required to half-saturate receptors in intact cells, 11 n*M*, is reasonably close to the concentration required to elicit half-maximum changes in the rates of prolactin and growth hormone synthesis, 2 n*M* (Hinkle et al., 1974; Hinkle and Tashjian, 1973). Finally, the affinities of 26 analogs of TRH for the receptor parallel closely their biological activities in the GH_3 system (Hinkle et al., 1974).

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[1] Abbreviation used is: TRH, thyrotropin-releasing hormone.

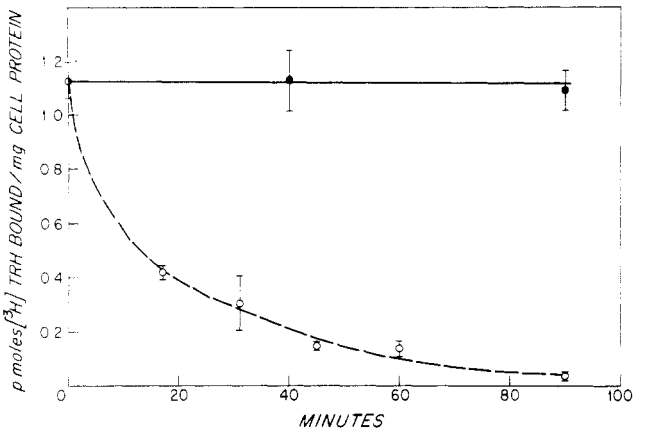

FIGURE 1: Reversibility of the TRH binding reaction. Replicate dishes of GH_3 cells were incubated for 1 hr with 100 nM [^{3}H]TRH. The amount of [^{3}H]TRH bound to receptors was then determined for duplicate dishes as described under Methods. All remaining dishes were washed three times with medium lacking TRH. Medium containing either 100 nM [^{3}]TRH (●) or 25 nM unlabeled TRH (○) was then added to each dish and incubation continued for the indicated times before measurement of the amount of [^{3}H]TRH bound. Average cell protein was 21 μg/35-mm culture dish. Values given are the mean and range of duplicate determinations.

We have previously shown (Hinkle and Tashjian, 1975) that when GH_3 cultures are incubated for long periods with [^{3}H]TRH there is a slow, intracellular degradation of the peptide to its constituent amino acids. Over a period of 24–48 hr radioactive metabolites of [^{3}H]TRH accumulate in the cell, and simultaneously the amount of intact [^{3}H]TRH associated with GH_3 cells decreases despite its continued presence in the culture medium. Since the products of TRH degradation would not be expected to bind to receptors (Hinkle et al., 1974) or to compete with [^{3}H]TRH for binding, these results suggested that the capacity of GH_3 cells to bind TRH had decreased during prolonged exposure of the cells to the tripeptide. The experiments reported in the present communication demonstrate that when GH_3 cells are exposed to TRH for long periods, the number of TRH receptors on the cells does, in fact, decrease.

Experimental Procedure

Materials. Cell culture media and sera were obtained from Grand Island Biological Co., and tissue culture dishes were from Falcon Plastics. [^{3}H]TRH (L-[2,3-[^{3}H]proline), 40 Ci/mmol, and [^{3}H]leucine, 5 Ci/mmol, were from New England Nuclear. [^{3}H]TRH has the same specific biological activity as that of synthetic, nonradioactive TRH. Synthetic TRH and TRH analogs were the gifts of Abbot Laboratories. Cycloheximide was from Nutritional Biochemicals Co.

Methods. The methods of culture of GH_3 cells have been described previously (Tashjian et al., 1968). Experiments in the present communication were carried out using either the GH_3 or GH_4C_1 strain; GH_4C_1 is a subclone of the GH_4 line (Tashjian et al., 1973) which has hormone-producing properties and responses to TRH similar to GH_3 cells except that basal growth hormone production in this strain is low.

Cells were grown at 37°, and all experiments were performed in Ham's F 10 medium supplemented with 15% horse serum and 2.5% fetal calf serum. Cell culture experiments were carried out using replicate 35- or 60-mm dishes inoculated with equal aliquots of cells from a single donor culture. Compounds to be added to the cultures were dissolved in medium and sterilized by Millipore filtration. Additions were made in a volume of 50–100 μl.

In order to measure the number of available TRH receptors in cells which had been incubated with unlabeled TRH, the following procedure was used. Cells were incubated with unlabeled TRH as described in the text. The medium was removed and the cells were washed three times with 1 ml (35-mm dishes) or 3 ml (60-mm dishes) of medium lacking TRH; 2 ml (60-mm dishes) or 0.75 ml (35-mm dishes) of medium containing 25 nM [^{3}H]TRH, except where noted, was then added to each dish and the incubation continued for 1 hr. The amount of [^{3}H]TRH bound to the cells was then determined as described previously (Hinkle and Tashjian, 1973) except that the cells were scraped into distilled water and an aliquot was used for the determination of cell protein. A blank, representing the amount of ^{3}H bound in the presence of a 200-fold or greater molar excess of unlabeled TRH, was subtracted from each point (Hinkle and Tashjian, 1973).

For measurement of the binding of TRH to cell fractions, cells from a single culture bottle (75 cm^2) or 100-mm culture dish were scraped into 2 ml of ice-cold assay buffer (20 mM Tris-HCl–2 mM $MgCl_2$ (pH 7.6)). All subsequent procedures were performed at 0°. After incubation for 10–30 min, cells were ruptured by 25 strokes in a Dounce homogenizer (pestle A) and centrifuged for 10 min at 4000g. The pellet was suspended in 0.4 ml of assay buffer. Aliquots (35 μl) of the resuspended 4000g pellet material and supernatant fluids were then incubated for 3 hr at 0° in a final volume of 50 μl containing the concentrations of [^{3}H]TRH indicated in the text. Under these conditions, equilibrium was attained. The amount of [^{3}H]TRH bound to receptors was then determined using a Millipore filter assay (Hinkle and Tashjian, 1973). Blanks containing cell fractions plus 10 μg/ml of unlabeled TRH were determined for each concentration of [^{3}H]TRH and were subtracted from each point. The blanks in the presence of excess unlabeled TRH (approximately 0.07% of the added radioactivity) were identical with those obtained using [^{3}H]TRH in the absence of added cell protein.

The rate of protein synthesis was determined by incubating dishes of cells with 5 μCi of [^{3}H]leucine for the indicated times. The incorporation of ^{3}H into a trichloroacetic acid insoluble product was measured as described previously (Dannies and Tashjian, 1973).

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

At the time these experiments were performed, the cells were found to be free of contamination by mycoplasma or bacteria by Dr. Iolanda Low, Harvard Medical School.

Results

Effect of TRH on the Number of TRH Receptors. In order to measure the number of TRH receptors on cells previously incubated with TRH, we have used an exchange assay which takes advantage of the fact that TRH binds to its receptors rapidly and reversibly. The reaction between TRH and its receptors reaches equilibrium in less than 1 hr (Hinkle and Tashjian, 1973; Gourdji et al., 1973). The reversibility of the TRH binding reaction is shown in Figure 1. Replicate cultures of GH_3 cells were incubated for 1 hr with 100 nM [^{3}H]TRH and the amount of [^{3}H]TRH

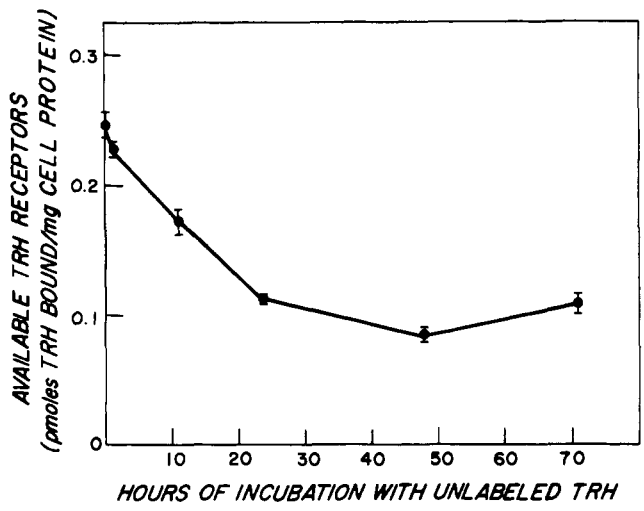

FIGURE 2: Effect of prior incubation with TRH on the number of TRH receptors. Medium was changed on replicate dishes of GH_3 cells at the start of the experiment. All dishes were incubated for 72 hr before the medium was removed, the cells were washed, and the dishes were incubated for 1 hr with 25 nM [^{3}H]TRH in order to measure the number of available TRH receptors as described under Methods. At the indicated times before measurement of available receptors, 50 μl of unlabeled TRH was added to duplicate dishes to give a final concentration of 100 nM. Average cell protein was 240 μg/dish. Values given are the mean and range of duplicate determinations.

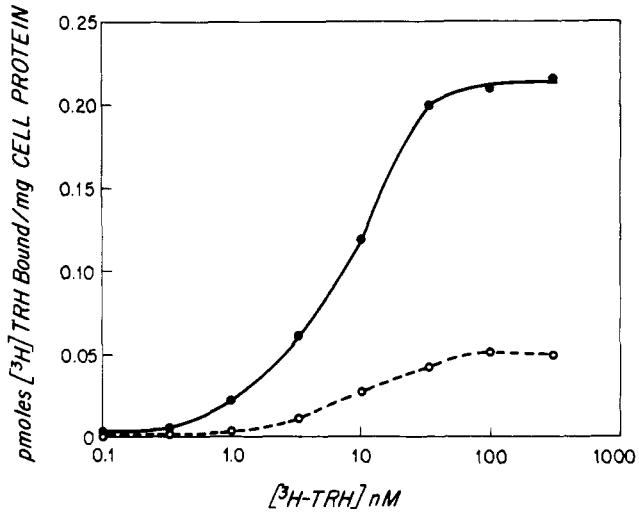

FIGURE 3: Binding of [^{3}H]TRH to intact control and TRH-treated GH_3 cells. Replicate 35-mm dishes of GH_3 cells were incubated with either medium alone (●) or 100 nM unlabeled TRH (○) for 48 hr. All dishes were then washed twice with medium lacking TRH and incubated in medium alone for 2 hr to allow bound TRH to dissociate from receptors. The medium was removed and the cells were incubated with the indicated concentrations of [^{3}H]TRH for 1 hr. The amount of [^{3}H]TRH bound to receptors was determined as described under Methods. A blank, representing the amount of ^{3}H bound to cells in the presence of the indicated concentration of [^{3}H]TRH and 10 μM unlabeled TRH, has been subtracted from each point. Average cell protein was 263 μg/dish. Each point gives the result of a single determination.

bound was determined for duplicate dishes. All remaining cultures were washed three times with medium lacking TRH; [^{3}H]TRH remains bound to receptors during washing (Hinkle and Tashjian, 1973) but free [^{3}H]TRH is removed. Medium containing either 100 nM [^{3}H]TRH or 25 nM unlabeled TRH was then added to groups of dishes, and the cultures were incubated for 15–90 min before determination of the amount of radioactivity bound to the cells. Bound [^{3}H]TRH was constant throughout the experiment in dishes incubated with 100 nM [^{3}H]TRH alone. However, when radioactive TRH was removed from the medium, [^{3}H]TRH bound to receptors exchanged rapidly with unlabeled TRH in the medium, and after 1 hr only 12% of the [^{3}H]TRH remained bound to the cells.

Essentially the same procedure was used to measure the number of available TRH receptors after prior exposure to TRH, except that cultures were initially incubated with unlabeled TRH and the number of receptors was determined after a 1-hr incubation with 25 nM [^{3}H]TRH. The concentration of [^{3}H]TRH used, 25 nM, is sufficient to saturate 75% of available receptors (Hinkle and Tashjian, 1973); higher concentrations were not used because of the expense of the labeled peptide. In the experiment shown in Figure 2, 100 nM unlabeled TRH was added to replicate dishes of GH_3 cells at different times from 1 to 72 hr before all cultures were washed and the number of available TRH receptors was measured. The number of receptors available to bind [^{3}H]TRH was 92% of control in cultures incubated with unlabeled TRH for 1 hr but decreased to 38% of control after exposure of the cells to the tripeptide for 48 hr.

It should be noted that using the procedures described under Methods, we detect only one class of TRH binding sites with a K_{diss} of 25 nM at 0° which contributes to binding over the range 0–200 nM TRH (Hinkle and Tashjian, 1973; Figure 3 of this paper). Grant et al. (1973) have detected two classes of TRH binding sites on mouse thyrotrophs, one of high affinity (K_{diss} = 20 nM) and low capacity, the other of low affinity (K_{diss} = 500 nM) and high capacity. Using procedures different from ours to measure binding, Gourdji et al. (1973) found nonsaturable binding to GH_3 cells at high TRH concentrations. We do not detect substantial nonsaturable binding of TRH to GH_3 cells at concentrations up to 200 nM, probably because extensive washing removes any [^{3}H]TRH bound to low affinity sites. Thus under the conditions used for all of the experiments reported in the present communication, only a single type of TRH receptor contributes measurably to binding; both the affinity constant and maximum amount of TRH bound to GH_3 cells determined by our procedures correspond closely with values reported by others for the high affinity TRH receptor.

Equilibrium Binding Studies on Receptors from TRH-Treated Cells. We have previously found (Hinkle and Tashjian, 1975) that the amount of intact [^{3}H]TRH associated with GH_3 cells decreases as the cells are incubated with the peptide for 1–48 hr. [^{3}H]TRH was not found either very tightly or covalently attached to cellular protein(s). The apparent loss of TRH receptors shown in Figure 2 suggests, therefore, that one of the following events occurs over a 48- to 72-hr period: (1) a decrease in the absolute number of TRH receptors; (2) a decrease in the affinity of TRH receptors resulting in only partial saturation of binding sites with [^{3}H]TRH under the assay conditions used; or (3) a migration of TRH receptors to a portion of the cell inaccessible to [^{3}H]TRH in the culture medium. In an attempt to distinguish among these possibilities, equilibrium binding studies were carried out to measure the binding of [^{3}H]TRH to intact control GH_3 cells and cells previously incubated with 100 nM unlabeled TRH for 48 hr. Before the binding studies were initiated, cultures were washed and incubated for 2 hr in medium lacking TRH, conditions which allow bound, unlabeled TRH to dissociate

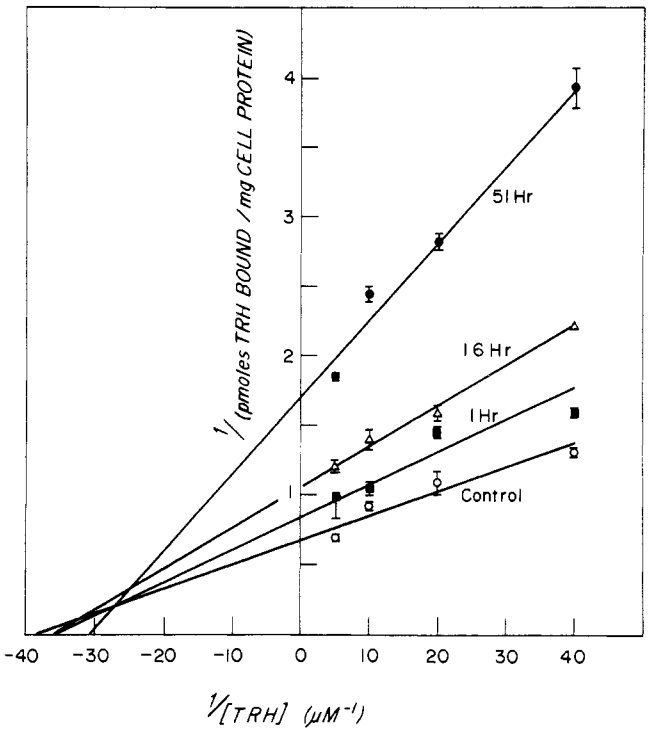

FIGURE 4: Equilibrium binding studies on particulate fractions prepared from cells previously exposed to TRH. Medium was changed on GH_4C_1 cultures at the start of the experiment. Cells were then incubated with medium alone (○), or 100 n*M* TRH was added 1 (■), 16 (△), or 51 (●) hr before the medium was removed from all cultures. All cells were then washed three times with medium lacking TRH and incubated in medium alone for 1 hr to allow bound TRH to dissociate from receptors. Cells were harvested, cell fractions were prepared, and equilibrium measurements of the amount of [^{3}H]TRH bound to the 4000*g* pellet were carried out as described under Methods. Each assay tube contained from 83 to 106 μg of protein. Values shown are the mean and range of duplicate determinations.

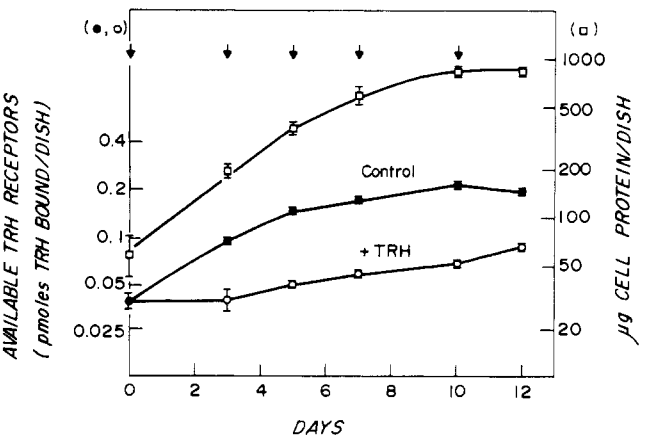

FIGURE 5: Effect of prolonged exposure to TRH on the number of TRH receptors. Replicate dishes of GH_3 cells were inoculated at low density 4 days prior to the start of the experiment. Cells were then incubated with either medium alone (●) or medium plus 100 n*M* TRH (○). At the indicated times, the number of TRH receptors on duplicate control and TRH-treated dishes was determined as described under Methods. Media changes, with or without added TRH, are shown by the arrows. Values shown for the number of receptors are the mean and range of duplicate determinations. Values for cell proteins (□) are the mean and range of control and TRH-treated dishes; TRH did not affect the amount of cell protein/dish.

from receptors. Binding to receptors was half-maximal at 8 n*M* [^{3}H]TRH in both control and TRH-treated cultures, while the maximum amount of [^{3}H]TRH bound to cells previously incubated with unlabeled TRH was reduced by 75% (Figure 3). Therefore the apparent loss of TRH receptors is due entirely to a change in the number of available binding sites and not to changes in the affinity of receptors for TRH.

Using broken cell preparations, we have carried out equilibrium binding studies on fractions from cells which had been incubated for various times with unlabeled TRH. TRH binds to particulate fractions prepared from GH_3 cells (Hinkle and Tashjian, 1973), and in other systems the TRH receptor has been shown to be located on the plasma membrane (Vale et al., 1973; Poirier et al., 1972). Particulate fractions prepared from control and TRH-treated cells have approximately the same affinity for TRH, and only the absolute number of receptors is decreased (Figure 4). These data are summarized in Table I. The decreases in the number of membrane receptors measured in broken cells correspond well to the loss of TRH receptors found at various times in intact cells. In agreement with earlier results (Hinkle and Tashjian, 1973), the apparent dissociation constant of the TRH-receptor complex measured in membrane fractions at 0°, 25-30 n*M*, is greater than the value obtained for half-maximal binding of [^{3}H]TRH to intact cells at 37°, 8 n*M*.

In addition, the ability of the 4000*g* supernatant fractions of these cells to bind TRH was determined and is shown in Table I. The amount of TRH bound by the supernatant fractions is small and is not increased in cells which had been exposed to TRH. The observed binding probably represents contamination with membrane fragments. This result indicates that the lost TRH receptors have not migrated to the cytoplasm in a form in which they can bind TRH.

Table I: Equilibrium Binding Studies on Fractions Prepared from GH_4C_1 Cells Previously Incubated with TRH.[a]

Fraction	Pretreatment with 100 n*M* TRH (hr)	K_{diss} (n*M*)	Maximum Binding pmol of TRH Bound/mg of Cell Protein
4000*g* pellet	0	26	1.48
	1	27	1.14
	16	27	0.94
	51	32	0.58
4000*g* supernatant fraction	0		0.23
	1		0.10
	16		0.11
	51		0.04

[a] Data for the binding of [^{3}H]TRH to 4000*g* pellets are taken from Figure 4. The binding of TRH to 4000*g* supernatant fractions was determined at a [^{3}H]TRH concentration of 200 n*M* using the procedure described under Methods. Supernatant fractions contained from 25 to 30 μg of protein/assay tube. The amount of [^{3}H]TRH bound to supernatant fractions is greater in this experiment than in previously published studies (Hinkle and Tashjian, 1973) because the cells were homogenized more extensively and centrifugation was at a lower speed.

Duration, Reversibility, and Concentration Dependence of the Loss of TRH Receptors. In order to determine whether the loss of TRH receptors is maintained for long periods, the number of available TRH binding sites was measured in control cultures and in cultures incubated with 100 n*M* TRH for up to 12 days (Figure 5). During this ex-

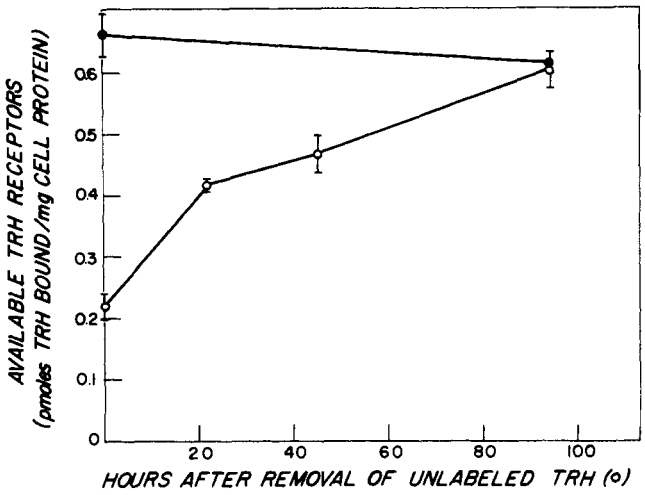

FIGURE 6: Effect of removal of TRH on the number of TRH receptors in cells previously exposed to TRH. Replicate dishes of GH_3 cells were incubated for 48 hr with medium alone (●) or medium plus 100 nM unlabeled TRH (○). At zero time the medium was removed from all dishes, the cells were washed three times with 3 ml of medium lacking TRH, and the incubation continued for the indicated times in 3 ml of medium lacking TRH before determination of the number of TRH receptors as described under Methods. Average cell protein was 780 μg/dish. Values shown are the mean and range of duplicate determinations.

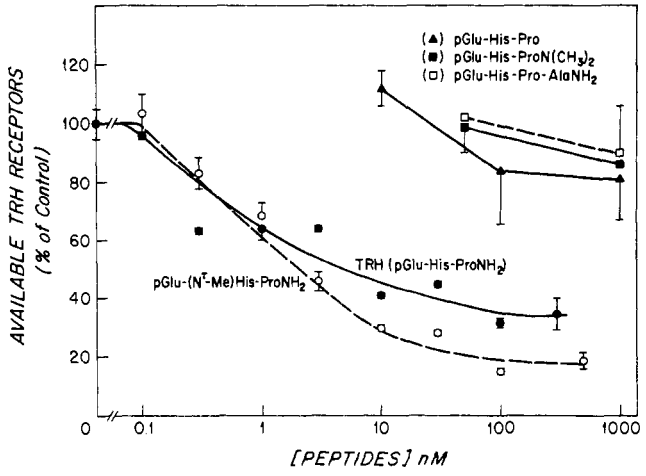

FIGURE 7: Effect of incubation with various concentrations of TRH or TRH analogs on the number of TRH receptors. Replicate dishes of GH_3 cells were incubated for 48 hr with the indicated concentrations of: TRH, pGlu-His-ProNH_2 (●); pGlu-(N^{τ}-Me)His-ProNH_2 (○); pGlu-His-Pro (▲); pGlu-His-Pro$N(CH_3)_2$ (■); or pGlu-His-Pro-AlaNH_2 (□). The number of TRH receptors was then determined as described under Methods, except that a [^{3}H]TRH concentration of 10 nM was used; under these conditions, approximately half of the TRH binding sites are occupied. Values shown are the mean and range of duplicate determinations.

periment cell protein increased 16-fold. Treatment with TRH resulted in a 54–66% loss of TRH receptors and the effect was maintained throughout, indicating that the peptide can cause receptor loss in GH_3 cells in both the exponential phase of growth and in dense cultures in which cell protein is not increasing. However, the total number of TRH receptors did increase during cell growth, so receptor synthesis does occur in the presence of TRH. It appears from the data in Figure 4 that the number of TRH receptors/mg of cell protein actually decreases in dense GH_3 cultures.

The loss of TRH receptors caused by TRH treatment is a reversible phenomenon (Figure 6). GH_3 cells were incubated for 48 hr with 100 nM TRH, at which point the number of TRH receptors was reduced to 33% of that in control cultures. The dishes were then washed and incubated in medium lacking TRH for various times before the measurement of the number of available TRH receptors. The number of TRH receptors doubles within 22 hr after TRH is removed and returns to control values by 96 hr.

The effect of incubation for 48 hr with different concentrations of TRH and TRH analogs on the number of available receptors is shown in Figure 7. Both TRH and the N^{τ}-methylhistidyl derivative, which is more active than TRH in GH_3 cells (Hinkle et al., 1974), effect receptor loss at concentrations of 0.3 nM or above. Three other analogs which have less than 0.05% of the receptor-binding and biological activities of TRH (Hinkle et al., 1974) did not cause significant loss of receptors at concentrations up to 1 μM.

Effects of Cycloheximide. In order to estimate the rate of degradation of the TRH receptor, protein synthesis was inhibited by the addition of 1 μg/ml of cycloheximide to cultures for various times before the determination of the number of receptors. As shown in Figure 8, there was only a 24% decrease in the number of TRH receptors in cultures which had been treated for 38 hr with sufficient cycloheximide to inhibit overall protein synthesis by about 85%. This result suggests that the TRH receptor has a half-life of at least several days.

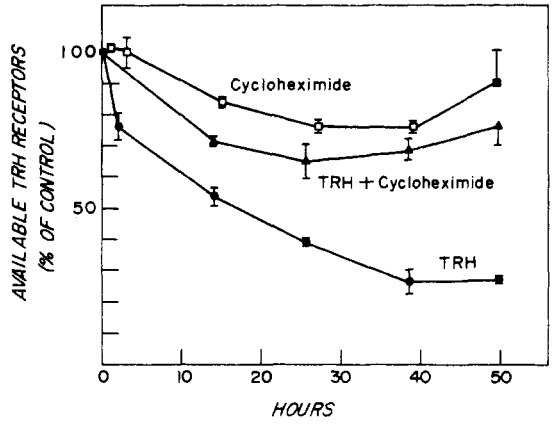

FIGURE 8: Effects of cycloheximide on the number of TRH receptors. At the start of the experiment medium was changed on replicate dishes of GH_3 cells. At the indicated times before the measurement of receptors, cycloheximide (1 μg/ml) alone (■, □), cycloheximide (1 μg/ml) plus TRH (100 nM) (▲); or TRH (100 nM) alone (●) was added to duplicate dishes. The number of TRH receptors on all dishes was then determined as described under Methods. The results of two separate experiments are shown by the open and filled symbols. Values in control dishes were: open symbols, 286 μg of cell protein/dish, 0.215 pmol of TRH bound/mg of cell protein; filled symbols, 278 μg of cell protein/dish, 0.203 pmol of TRH bound/mg of cell protein. Cell proteins were identical in control dishes and dishes treated with TRH alone. In dishes treated with cycloheximide cell proteins were: 81–110% of control at 1–27 hr; 61–78% of control at 38 hr; 42% of control at 49 hr. The rate of protein synthesis, determined as described under Methods, was 11 and 18% of control in dishes incubated with 1 μg/ml of cycloheximide for 1 or 24 hr, respectively. Values given are the mean and range of duplicate determinations.

In order to determine whether TRH causes receptor loss by accelerating the rate of receptor degradation, the number of receptors was measured in GH_3 cells incubated with both TRH and cycloheximide (Figure 8). In the presence of cycloheximide the reduction in the number of receptors normally induced by TRH is greatly diminished. Nevertheless, treatment with TRH does result in a small but reproducible

decrease in the number of receptors even in the presence of cycloheximide.

Discussion

TRH binds to specific receptors on GH_3 cells rapidly and reversibly. These features of the binding reaction make it possible to measure the total number of receptors capable of binding [^{3}H]TRH, regardless of whether or not unlabeled TRH is initially bound. The data reported in this communication demonstrate that chronic but not acute (less than 2 hr) exposure of GH_3 cells to TRH leads to a reduction in the number of TRH receptors. Loss of receptors occurs slowly, over 12–48 hr, and is reversed slowly, over a period of 4 days. It seems likely that loss of receptors is a result of TRH binding to receptors. The concentrations of TRH required to cause receptor loss correspond to those required to bind to receptors and to elicit biological responses, changes in the rates of prolactin and growth hormone synthesis. The abilities of TRH analogs to reduce the number of receptors parallel their receptor-binding and biological activities.

These results with GH_3 cells are strikingly similar to those reported by Gavin et al. (1974), who showed that chronic exposure of cultured human lymphocytes to high concentrations of insulin results in a decrease in the number of insulin receptors. The affinity of receptors for insulin is apparently not altered. Although insulin binds to receptors within 30 min, the loss of receptors in that system also occurs slowly (5–16 hr). Receptor concentration is restored to normal within 16 hr of the removal of insulin. One difference, however, is that the concentrations of insulin which cause receptor loss are several orders of magnitude greater than those necessary to produce biological effects. These effects of insulin on cultured lymphocytes correspond to the situation observed in vivo, in which chronic but not acute hyperinsulinemia leads to a decrease in the number of insulin receptors on target cells (Kahn et al., 1972, 1973; Archer et al., 1973; Goldfine et al., 1973). It has also been reported that the number of growth hormone receptors on lymphocytes decreases after chronic exposure to growth hormone (Gavin et al., 1974).

In the GH_3 system, as in cultured lympocytes (Gavin et al., 1974), loss of hormone receptors apparently depends on events subsequent to the binding of peptide to receptors. Under conditions where TRH occupies all available receptor sites within minutes, loss of receptors does not occur for several hours. Although the mechanism of receptor loss is not known, some possibilities can be eliminated by the data available. For example, receptors could migrate to a portion of the cell inaccessible to [^{3}H]TRH in the medium. However, from binding studies in broken cell preparations, it is clear that unaltered receptors do not appear in the cytoplasm, nor are any membrane receptor sites exposed after Dounce homogenization. Another possibility, that TRH becomes covalently attached to receptors, was shown not to occur in earlier studies (Hinkle and Tashjian, 1975).

Alternatively, the number of receptors would be reduced if TRH caused either a decrease in the rate of synthesis or an increase in the rate of degradation of TRH receptors. TRH does not completely block the synthesis of new receptors, because the number of receptors continues to increase in growing cultures exposed to TRH, although to a lesser extent than in control cultures (Figure 5). The TRH receptor is destroyed by treatment with proteolytic enzymes and phospholipases A and C (Hinkle and Tashjian, 1974; Barden and Labrie, 1973) and may be assumed to contain protein, and either to contain phospholipid or depend on the integrity of a phospholipid component of the plasma membrane. To estimate the rate of degradation of the TRH receptor, cycloheximide was used to inhibit the synthesis of new receptors. This approach involves two assumptions: that cycloheximide does not alter the rate of receptor degradation; and that receptor synthesis does not proceed preferentially when overall protein synthesis is inhibited. Since blocking overall protein synthesis by 85% for 40 hr led to only a 24% decrease in the number of receptors, it appears that the TRH receptor contains a stable protein with a half-life of at least several days. Exposure of GH_3 cells to TRH for 48 hr leads to a 73% reduction in the number of TRH receptors (Figure 8). Since this loss of receptors is considerably greater than that obtained when protein synthesis is blocked by cycloheximide, it is unlikely that TRH acts merely by inhibiting receptor synthesis.

The inhibitor experiments were repeated in the presence of TRH to determine whether TRH accelerates receptor degradation. Instead of finding accelerated degradation of receptors, we found that the loss of receptors induced by TRH was much less when cycloheximide was present; under conditions where TRH alone caused a 73% loss of receptors, the loss was only 23% when protein synthesis was inhibited. The simplest explanation for these data is that the TRH-mediated loss of TRH receptors is a process which requires active protein synthesis.

GH_3 cells appear to have spare, or reserve receptors for TRH, i.e., the amount of TRH required to half-saturate receptors, 11 nM, is greater than that required to yield a half-maximum biological response, 2 nM (Hinkle and Tashjian, 1973). Grant et al. (1972, 1973) have obtained similar evidence suggesting that mouse thyrotrophs also have spare receptors for TRH. On the basis of the data discussed above, we suggest that the purpose of spare TRH receptors on GH_3 cells, and possibly of spare receptors in other endocrine systems, may be to allow the target cell to regulate its sensitivity to a hormone. There are circumstances in which this would clearly be advantageous. For example, in certain clinical situations (hyperinsulinemia, medullary thyroid carcinoma, and choriocarcinoma) discussed by Gavin et al. (1974), target cells are chronically exposed to abnormal, excessive hormone concentrations. A cell might regulate its sensitivity to a hormone directly by altering the affinity constant of the hormone–receptor complex, but this would necessitate the ability to synthesize groups of receptors with different binding sites. However, if a cell has spare receptors, then by changing the number of receptors the cell can, in effect, regulate sensitivity to a hormone. When the number of receptors is reduced, higher hormone concentrations will be necessary to elicit the biological response. But, since there is initially a surplus of receptors, the maximal response obtained at high hormone concentrations need not be substantially reduced. The net effect of changes in receptor number will be similar to that which could be obtained by altering the affinity of the receptors for the hormone.

This hypothesis is difficult to test in GH_3 cells because changes in the rates of hormone synthesis induced by TRH persist for several days after the peptide is removed (Tashjian et al., 1971; Tashjian and Hoyt, 1972). Thus the number of receptors would begin to increase before responsiveness to TRH could be measured. Bowers (1971), using hemipituitary glands incubated in vitro, found that the thyrotropin response to TRH decreased after repeated TRH treatment, and that the diminished responsiveness was not

due solely to depletion of pituitary thyrotropin stores. A possible explanation for these results is that a loss of TRH receptors had occurred. Experiments to test the possibility that regulation of the number of TRH receptors occurs in vivo have not been performed. However, it should be noted that in intact animals, responsiveness to TRH is also controlled by the circulating concentrations of thyroid hormones.

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