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A MACROMOLECULE WHICH GIVES RISE TO THYROTROPIN RELEASING-HORMONE

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SUMMARY: Chemical and enzymatic treatment of a high-molecular weight fraction from a frog brain extract resulted in formation of a "TRH-like material" (TRH-i). Sequential treatment with trypsin and carboxypeptidases A and B, acetic acid and then chemical amidation generated a quantity of TRH-i equivalent to 25% of the endogenous TRH. TRH-i was similar to TRH (pGlu•His•ProNH_2) as assessed by molecular weight estimations, radioimmunoassay and susceptibility to serum inactivation. TRH and TRH-i also competed with [^{3}H]-TRH for binding to TRH receptors, stimulated prolactin synthesis and uridine uptake, and "down-regulated" TRH receptors in pituitary cells. These results suggest the possibility that TRH may be processed from a macromolecular precursor.

Thyrotropin releasing-hormone (TRH), the smallest of the hypothalamic hormones, has been isolated from porcine (1) and ovine (2) hypothalamic fragments and its structure determined. The hormone and numerous analogues have been chemically synthesized and their biological activities investigated (3-4). Despite these advances, little is known about the biosynthesis of TRH.

Biosynthesis of small peptides such as glutathione (5) or bacterial peptide antibiotics (6) has been shown to proceed by a non-ribosomal mechanism of amino acid polymerization. Initial observations indicated that TRH was synthesized by a similar pathway (7-8), however, these reports have not been confirmed by other laboratories (9-10).

TRH is composed of only three amino acids, and if it were synthesized on ribosomes, it would probably be formed as a higher molecular weight species that would be enzymatically processed to its biologically active form. Although it is difficult to find supporting precedents for the non-ribosomal synthesis of TRH, there is extensive documentation that hormones and proteins are synthesized as larger precursors (11). In the present study we have determined that the frog brain contains a macromolecule that can be converted via enzymatic and chemical modifications to a compound which is similar to TRH as determined by physical characteristics, immunochemical properties and biological activity.

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EXPERIMENTAL PROCEDURES

Materials - (L-proline 2,3-^{3}H) TRH ([^{3}H]-TRH) used for radioimmunoassay had a specific activity of 21.8 Ci/mmole. Similar material used in receptor binding experiments had a specific activity of 115 Ci/mmole (New England Nuclear). Trypsin, carboxypeptidase A, carboxypeptidase B, bovine thyroglobulin and bovine serum albumin were purchased from Sigma. TRH and pGlu•His•Pro (free acid) were products of Bachem or gifts from Abbott Laboratories. Tissue culture media, horse serum and fetal calf serum were from Grand Island Biological Company. Hypothyroid calf serum (thyroxine < 1 µg/ml) was from Rockland Farms, Gilbertsville, PA. Carrier-free ^{125}I for protein iodination and [^{3}H] uridine (27 Ci/mmole) were from Amersham/Searle. The GH_3 and GH_4C_1 cell lines were obtained from Dr. Armen H. Tashjian, Jr., Harvard Medical School, Boston, MA. Protein concentrations were determined by the method of Lowry et al. (12) using bovine serum albumin as a standard.

Radioimmunoassay - Antibody to TRH was prepared and TRH concentrations measured by a modification of the procedure of Bryce (13). Radioimmunoassay incubation mixtures contained 10 µl of TRH antiserum (binds 5,000 cpm [^{3}H]-TRH), 5 µl [^{3}H]-TRH (12,500 cpm, 0.25 ng), 0 to 50 µl of sample of TRH standard and phosphate buffered saline (PBS, 0.01 M sodium phosphate containing 0.15 M NaCl, pH 7.4) to make a final volume of 50 to 100 µl. After overnight incubation at 4°C, 500 µg of non-immune rabbit serum in 10 µl PBS were added and the incubated mixtures were diluted with an equal volume of saturated ammonium sulfate. After 20 min, the precipitated antigen-antibody complex was collected by centrifugation at 8,000 g for 6 min. The supernatant was discarded and the pellet was washed with 300 µl and 200 µl aliquots of 50% saturated ammonium sulfate. The final pellet was dissolved in 100 µl of 1 N NaOH and transferred to a scintillation vial. Each assay tube was rinsed with 900 µl of H_2O which was combined with the NaOH solution.

The antiserum was found to be quite specific for TRH. The structural analogues ProNH_2, pGlu•His, pGlu•His•Pro, Luteinizing Hormone-Releasing Hormone and Glu•His•ProNH_2 were tested at 1000-fold molar excess and did not reduce the binding of [^{3}H]-TRH to the antibody. However, pGlu•His•Pro•GlyNH_2 cross reacted with the antibody at a 25-fold molar excess and pGlu•L-dopa•ProNH_2 showed a competition for antibody that parallels that of authentic TRH and suggests that the antibody discriminates poorly at the histidine residue. This result is not surprising since TRH is conjugated to thyroglobulin via the imidazole of histidine.

Preparation of Brain Extracts - Frogs (Rana pipiens), purchased from Mogul Ed, Oshkosh, WI, were sacrificed by decapitation and their entire brains excised. The brains from 59 frogs (3.69 g) were homogenized in 25 ml of chilled 2 N acetic acid using a Polytron at setting 5 for 30 sec. The pellet was dissolved in 10 ml of 2 N acetic acid, homogenized and sonicated as above. The supernatants from these extractions were combined and centrifuged at 40,000 g for 5 hr. The supernatant was concentrated by ultrafiltration using an Amicon UM-05 filter and passed over a 2 x 50 cm Sephadex G-15 column using 1 N acetic acid as the eluent. Fractions having molecular weights greater than TRH were pooled and concentrated by ultrafiltration (Amicon UM-05).

Enzymatic Modification - A 3.7 ml aliquot, representing 25% of the sample after removal of the endogenous TRH, was lyophilized and redissolved in 0.1 M ammonium bicarbonate buffer at pH 8.2. Two mg of trypsin were added and the reaction mixture was incubated at 37°C. After 12 hr incubation an additional 2 mg of trypsin were added and incubation continued for an additional 12 hr; then the mixture was heated at 100°C for 10 min. The pH was adjusted to 7.4 with 1 N HCl, a 100 µl aliquot of carboxypeptidase A (18.6 mg/ml) and a 240 µl aliquot of carboxypeptidase B (2 mg/ml) were added to the trypsin digest and incubation continued for 6 hr at 25°C.

Chemical Modification - In order to cyclize NH_2-terminal Glx residues the lyophilized proteolytic digest was refluxed in 2 ml glacial acetic acid for 2 min. Conversion of carboxyl terminal amino acids to their respective amides was achieved according to the method of Enzmann et al. (14). The cyclized proteolytic digest was dissolved in 2 ml of methanol containing 5 g HCl/100 ml and incubated at room temperature for 90 min. The sample was freed of HCl by resuspending in methanol followed by rotary evaporation to dryness. This procedure was repeated three times. The dried residue was then dissolved in 2 ml of methanol saturated with ammonia at -5°C and incubated at room temperature for 4 hr. The sample was freed of ammonia by rotary evaporation of the solvent. This was also repeated three times. At each step of the various modification procedures samples were removed and the "TRH-like" activity (TRH-i) determined.

Biological Assay - GH_3 and GH_4C_1 cells were grown in monolayer culture in Ham's F10 medium containing 15% horse serum and 2.5% fetal calf serum. Where noted in the text, the cells were rinsed with serum-free medium and the medium replaced with Ham's F10 containing 10% hypothyroid calf serum as previously described (15). TRH is not degraded by the culture media. For tests of receptor affinity and bioactivity, samples containing TRH-i were dissolved in 0.15 M NaCl and diluted at least

250-fold before addition to cell cultures. The affinity of unlabeled TRH and TRH-i for TRH receptors was determined using a radioreceptor assay with GH_3 membranes (16). Reactions contained in a final volume of 50 µl, 44 µg membrane protein, 0.5 pmol [^{3}H]-TRH and 10 µl unlabeled TRH or extract. After incubation to equilibrium for 2 hr at 0°C reactions were diluted, passed through a Millipore filter and rinsed in order to determine the amount of [^{3}H]-TRH bound to membrane receptors. Non-specific binding, measured in control tubes containing no membrane protein or excess unlabeled TRH, was 1%.

The rate of prolactin synthesis was determined from prolactin in the culture medium; prolactin is stable in the medium and the amount secreted is equivalent to the total synthesized over a 72 hr period (17). Prolactin was determined by radioimmunoassay using preparations and procedures from the Hormone Distribution Program of the National Institute of Arthritis, Metabolism and Digestive Diseases. Binding of [^{3}H]-TRH to GH_3 cultures was determined using 25 nM [^{3}H]-TRH in an exchange assay as previously described (18); nonspecific binding was 15% and has been subtracted from each point. The uptake of [^{3}H] uridine into GH_4C_1 cells was measured by the procedures of Martin et al. (19). In all tests of bioactivity TRH-i concentrations were determined by radioimmunoassay.

RESULTS

Formation of TRH-i - Table I shows the effect of various treatments on the appearance of TRH-like material. When the frog brain extract was chromatographed on Sephadex G-15, most of the TRH was removed, with only 11 ng of radioimmunoassayable TRH-like material (TRH-i) observed in the macromolecular fraction. TRH-i will be referred to as radioimmunoassayable material present in, or formed from, chemical or enzymatic treatment of the macromolecular fraction. One equivalent of TRH-i corresponds to 1 ng of TRH as measured by radioimmunoassy. Digestion of the macromolecular fraction by trypsin and carboxypeptidases A and B did not appreciably alter the amount of TRH-i. When the macro-

Table I

Appearance of TRH-i

	TRH-i[1] Equivalents
2 N acetic acid extract of frog brain	3360[2]
Extract after Sephadex G-15[3]	11
Trypsin	24
Trypsin + cyclization	20[4]
Trypsin + carboxypeptidases A and B	60[4]
Trypsin + carboxypeptidases A and B + cyclization	168[4]
Trypsin + cyclization + amidation	44[4]
Trypsin + carboxypeptidases A and B + cyclization + amidation	828[4]

[1] One equivalent of TRH-i is defined as the amount of TRH corresponding to one nanogram as measured by radioimmunoassay.

[2] This value corresponds to the TRH present in the 2N acetic acid extract of the frog brain as measured by radioimmunoassay.

[3] The frog brain extract was subjected to chromatography on Sephadex G-15 which removed all but 11 ng of TRH from the macromolecular fraction. Sequential treatment of the macromolecular fraction with trypsin, carboxypeptidases A and B, followed by cyclization and amidation results in formation of 828 equivalents of TRH-i. Other treatments which omit one or more of the steps described above result in smaller amounts of TRH-i.

[4] Each of these steps utilized 25% of the G-15 gel filtration sample and the equivalents of TRH-i have been multiplied by 4 to give the total amount obtained at each step.

molecular fractions digested with trypsin and carboxypeptidase were further reacted with acetic acid (which will cyclize NH_2-terminal Glx residues) and then amidated, 828 equivalents of TRH-i were formed. Although it has not been demonstrated that each of the series of steps listed in Table I is unique in forming TRH-i, several experiments have been carried out to demonstrate that the radioimmunoassayable activity did not arise from artifacts. TRH-i did not arise from trypsin, carboxypeptidases A or B, or the various treatments that might alter these proteins. In addition, when the macromolecular fraction was treated with trypsin and acetic acid and then amidated (carboxypeptidases A and B were omitted), only a small increase in the amount of TRH-i was noted (Table I). Unless, stated otherwise, all additional experiments were carried out with the macromolecular extract which had been treated with trypsin, carboxypeptidase A and B, cyclized and amidated.

Immunochemical and Physical Properties of TRH-i - The 828 equivalents of TRH-i formed as a result of the treatments outlined in Table I has a number of properties that would indicate that it is very similar to authentic TRH. The radioimmunoassay of TRH and TRH-i afford similar profiles as shown in Figure 1. The binding of [^{3}H]-TRH to antibody was also analyzed by incubating a constant amount of antibody with varying [^{3}H]-TRH concentrations using conditions similar to the TRH-radioimmunoassay procedure described previously. When the data were expressed by plotting the reciprocals of bound [^{3}H]-TRH versus free [^{3}H]-TRH, a straight line was obtained (Fig. 1 - Insert). Incuba-

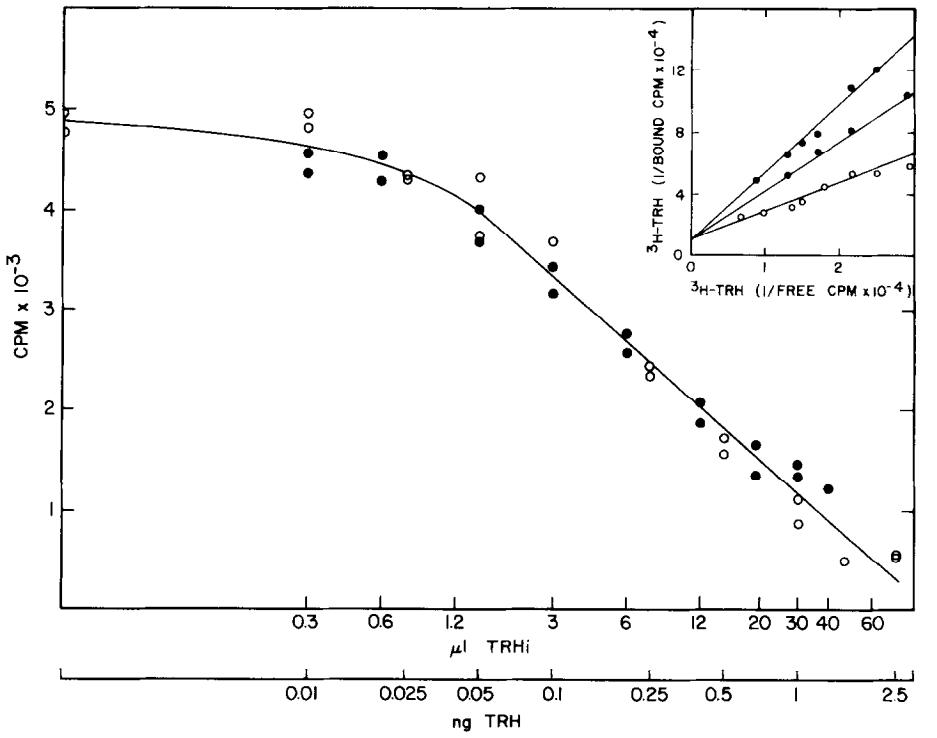

Figure 1: Radioimmunoassay of TRH (o) and TRH-i (•) (insert) Lineweaver-Burk plot. Binding of [^{3}H]-TRH (o) to anti-TRH in the absence of TRH-i and binding of [^{3}H]-TRH to anti-TRH in the presence of 0.35 (upper) and 0.175 (lower) equivalents of TRH-i (•).

tion of [^{3}H]-TRH in the presence of a constant amount of TRH-i afforded a double reciprocal plot with an increased slope and no apparent change in the y-intercept (V_{max}) indicating competitive binding between [^{3}H]-TRH and TRH-i.

In order to estimate the size of TRH-i, a 0.5 ml aliquot of the enzymatically and chemically modified material (containing 130 of the 828 equivalents of TRH-i) was passed over a 1 x 50 cm Sephadex G-15 column. A 25 µl aliquot of each 1 ml fraction was subjected to radioimmunoassay. Two peaks of cross reactivity were resolved. The first peak had the same elution volume as [^{3}H]-TRH and represented approximately 60% of the total cross reactivity. The second peak eluted with the void volume of the column. Total recovery of immunoreactive material was 94%. These results indicated that only 60% of the radioimmunoassayable TRH-i has physical properties which are identical to TRH.

Numerous reports have shown that rat serum is effective in degrading TRH (20). When rat serum was diluted (3:4) with PBS and incubated with 250 µl aliquots of authentic TRH (1.25 ng/10 µl) or TRH-i (1.25 equivalents/10 µl) at 37°C for varying times, both authentic TRH and TRH-i were degraded by the serum. Following 20 min incubation, 75% of the authentic TRH and 40% of the TRH-i were degraded as determined by radioimmunoassay.

Biological Properties of TRH-i - The affinity of TRH-i for specific TRH receptors was determined by a radioreceptor assay in which pituitary tumor cell membranes (GH_3) were incubated with tracer [^{3}H]-TRH and different concentrations of either unlabeled TRH or TRH-i (Fig. 2). Parallel competition displacement curves were obtained, and TRH-i appeared to have slightly higher affinity for TRH receptors than predicted by radioimmunoassay.

The bioactivity of TRH-i was tested using GH_3 monolayer cultures. The material did not affect cell growth at concentrations equivalent to 10 nM by radioimmunoassay, although some toxicity was

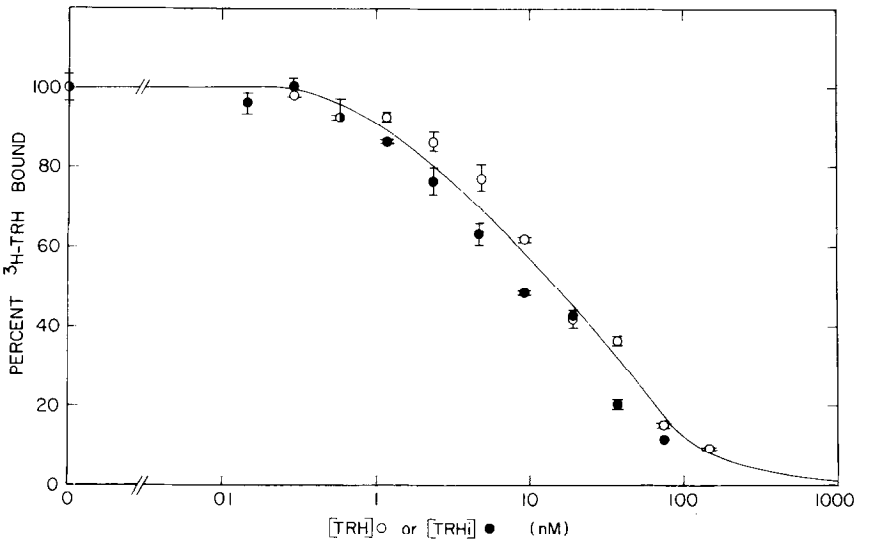

Figure 2: Affinity of TRH and TRH-i for TRH receptors. Competition displacement curves show the means and range of duplicate determinations for unlabelled TRH (o) TRH-i (●). The results are plotted using TRH-i concentrations estimated by radioimmunoassay.

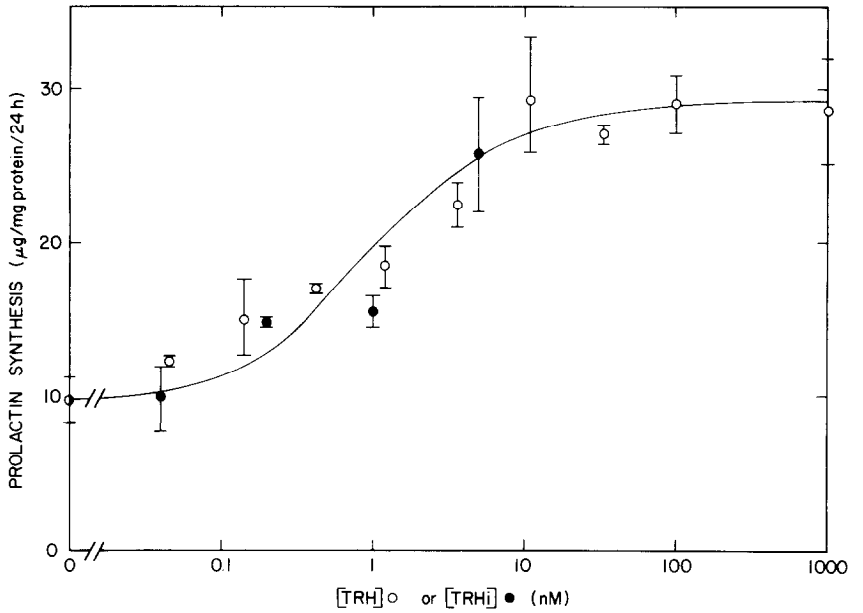

Figure 3: Prolactin stimulation by TRH and TRH-i. Replacate 35 mm dishes of GH_3 cells were incubated with 1.5 ml hypothyroid medium containing either TRH (o) or TRH-i (●). The concentrations of TRH-i were estimated by radioimmunoassay. Prolactin was measured in the culture medium after 72 hrs. Cell proteins were not affected by TRH or TRH-i and averaged 185 μg/dish. The mean and range of duplicate dishes are shown.

noted at higher levels. Both TRH and TRH-i caused a 3-fold stimulation of prolactin synthesis in a 72 hr incubation, and the dose response curves were parallel with half-maximal effects at 1 to 2 nM (Fig. 3). Growth hormone synthesis was not affected by either TRH or TRH-i (data not shown).

Another long-term action of TRH is "down regulation" of TRH receptors. After 24 to 48 hr incubation with TRH the number of receptors per cell decreased but affinity for TRH was not changed (18). TRH and TRH-i caused parallel dose-dependent depletion of TRH receptors (data not shown). A rapid effect of TRH is the stimulation of uridine uptake by a subclone of the GH_3 cell lines (19). In 90 min after its addition to cultures TRH and TRH-i increased the uptake of [^{3}H] uridine into cells, and TRH-i had slightly greater activity.

DISCUSSION: The physical, immunochemical and biological properties of TRH-i would suggest that it is very similar to TRH. The results presented in Fig. 1 and 2 demonstrate that TRH and TRH-i bind competitively with antiserum to TRH and to pituitary TRH receptors. The antiserum used in this report is highly specific. Structural requirements for binding to TRH receptors on thyrotrophs and mammotraphs are also stringent, as minor changes in any of the three amino acids can severely reduce affinity (16).

In pituitary tumor cells TRH stimulates the synthesis of prolactin by increasing the concentration of pre-prolactin mRNA (21-22), causes a loss of receptor sites by an unknown mechanism (18) and stimulates the acute uptake of uridine by increasing uridine kinase activity (23). TRH-i exerts

these TRH-like actions at equivalent concentrations based on radioimmunoassy, and dose-response curves for TRH and TRH-i are parallel. These data indicate the TRH-i interacts with TRH receptors as an agonist leading to the full spectrum of biological activities of TRH.

Results presented herein suggest that TRH-i arises from a compound having a greater molecular weight than TRH. Trypsin has been shown to convert several prohormones to their respective hormones (24). In addition, a carboxypeptidase B-like enzyme is necessary for the conversion of proinsulin to insulin (24). Carboxypeptidases A and B are also necessary for formation of 828 equivalent of TRH-i. In the absence of these enzymes 44 equivalents of TRH-i was formed (Table I). This suggests that removal of amino acids from the C-terminus is important for TRH-i formation. There is also precedent for peptides which terminate as C terminal amides to have amino acid extensions (25,26). Treatment of the frog brain extract with trypsin and carboxypeptidases A and B does not result in an appreciable increase in TRH-i. This may reflect the fact that the antibody to TRH, which was used to measure TRH-i, is highly specific for pGlu and ProNH_2. Using chemical procedures which are known to cyclize NH_2-terminal Glx residues to pGlu (14) and also result in C-terminal amidation (14), a rather dramatic increase in the concentration of TRH-i was observed. Do the chemical and enzymatic steps outlined in Table I substitute for the processing enzymes required for conversion of a macromolecule to TRH? Obviously, additional experiments are necessary to answer this question.

Tissues other than brain may also possess this or a similar macromolecule which gives rise to TRH-i. Antibodies directed toward luteinizing hormone-releasing hormone have been shown to cross-react with a macromolecule which is present in a variety of tissues (27-28). Similarly, higher molecular weight immunoreactive species of somatostatin have also been reported (29).

It should also be noted that the amino acid sequence Glx•His•Pro can be found in several proteins (30). Indeed, carboxypeptidase A possesses this sequence of amino acids but is not converted to TRH-i by the chemical and enzymatic steps outlined in Table I. Thus, the susceptibility of the precursor to specific post-translational processing enzymes must be of importance.

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