Peptides, Vol. 10, pp. 145–155. Pergamon Press plc, 1989. Printed in the U.S.A. 0196-9781/89 $3.00 + .00

Thyroid Hormone Modulation of TRH Precursor Levels in Rat Hypothalamus, Pituitary, Thyroid and Blood

MARIE SIMARD, A. EUGENE PEKARY,[1] VIERKA P. SMITH AND JEROME M. HERSHMAN

Endocrinology Research Laboratory, Medical and Research Services
Veterans Administration Wadsworth Medical Center and Department of Medicine
University of California at Los Angeles, Los Angeles, CA 90073

Received 11 July 1988

SIMARD, M., A. E. PEKARY, V. P. SMITH AND J. M. HERSHMAN. *Thyroid hormone modulation of TRH precursor levels in rat hypothalamus, pituitary, thyroid and blood.* PEPTIDES **10**(1) 145–155, 1989.—In the present study we have examined the in vivo effects of thyroid hormones and TRH on tissue and blood levels of TRH and TRH-Gly (pGlu-His-Pro-Gly), a TRH precursor. Using specific radioimmunoassays (RIAs), we measured TRH immunoreactivity (TRH-IR) and TRH-Gly-IR concentrations in blood, hypothalamus, anterior and posterior pituitary, and thyroid in euthyroid, hypothyroid and thyroxine (T4)-treated 250 g male Sprague-Dawley rats. TRH-Gly-IR and TRH-IR were detected in all of these tissues. Highly significant positive correlations between whole blood TRH-Gly-IR levels and the corresponding serum TSH values ($p<0.01$), whole blood TRH-IR versus serum TSH ($p<0.01$) and whole blood TRH-Gly-IR versus whole blood TRH-IR ($p<0.01$) are consistent with cosecretion of TRH and TRH precursor peptides into the circulation. Euthyroid rats injected with TRH IP (1 μg/100 g b.wt.) and hypothyroid rats had 4-fold higher whole blood TRH-Gly-IR levels compared to euthyroid controls ($p<0.0005$). Injection of TRH into euthyroid rats significantly increased the TRH-Gly-IR concentration in the hypothalamus, anterior and posterior pituitary and thyroid. The increase in blood TRH-Gly-IR following intravenous TRH may be due, in part, to partial saturation of TRH-degrading enzymes in blood and cell membranes. The ratio of TRH-Gly to TRH was significantly increased in the anterior pituitary by hypothyroidism and TRH injection, suggesting that thyroid hormones and TRH regulate the alpha-amidation of TRH-Gly to form TRH in this tissue. TRH-Gly levels of pooled pituitary and thyroid extracts quantitated by a combination of TRH-Gly RIA and high performance liquid chromatography (HPLC) revealed several-fold increases following incubation at 60°C. Heating at this temperature may block the alpha-amidation activity in extrahypothalamic tissues but not the "trypsin-like" enzymes which cleave prepro-TRH into TRH-Gly-immunoreactive peptides.

Thyrotropin-releasing hormone Hypothalamus Pituitary Thyroid Blood Radioimmunoassay Chromatography

THE complete mRNA sequence for the rat hypothalamic TRH precursor macromolecule, prepro-TRH, which codes for a 255 amino acid protein with 5 repeating sequences of -Lys-Arg-Gln-His-Pro-Gly-(Lys/Arg)-Arg- has recently been reported (24). This protein is rapidly cleaved to a number of precursor fragments by trypsin-like enzymes. These peptides are further processed until a free N-terminal Gln residue is produced which is cyclized to form a N-terminal pyroglutamic acid residue by one or more recently characterized enzymes (9). The C-terminal Gly, with or without C-terminal basic residue extensions, serves as the $-NH_2$ donor for the alpha-amidation of the C-terminal proline residue by a monooxygenase enzyme which requires ascorbate, copper ion, molecular oxygen and a pH of 7.5 (15). This understanding of the structure of the TRH-precursor and its enzymatic processing has suggested new experiments on the regulation of TRH biosynthesis. Recently, the level of hypothalamic TRH-Gly has been reported to remain constant in fetal, neonatal and young adult rats while the TRH level increased 66-fold (17). This observation clearly suggests a regulatory function for the alpha-amidation reaction in TRH production.

The regulation of TRH biosynthesis and release by thyroid hormones within the hypothalamus or extrahypothalamic sites may occur at a number of possible levels including the transcription and stabilization of prepro-TRH mRNA, its ribosomal transcription and enzymatic processing to form TRH, the release rate of TRH and the corresponding receptor levels in pituitary thyrotrophs.

Evidence for suppression of prepro-TRH mRNA levels in

[1]Requests for reprints should be addressed to A. Eugene Pekary, Ph.D., VA Wadsworth Medical Center, Bldg. 114, Rm. 200, Wilshire and Sawtelle Blvds., Los Angeles, CA 90073.

rat hypothalamus by thyroid hormones has been clearly demonstrated (44) which could be due to either decreased prepro-TRH mRNA transcription rate or a decrease in its intracellular stability. TRH receptor levels in pituitary cells have previously been shown to be down-regulated by both T4 and TRH (19,21). Blood levels of TRH-degrading enzymes have been shown to be thyroid hormone responsive (3). On the other hand, very little information regarding the effect of T4 or TRH on the enzymatic processing of prepro-TRH has been reported.

A large number of intermediary peptide fragments result from the action of the trypsin-like, alpha-amidating and N-Gln cyclizing enzymes on prepro-TRH. Agents known to selectively inhibit these enzymes (9,42) are cytotoxic, precluding their use in in vivo and cell culture systems. An alternative approach to the quantitation of the effects of thyroid hormones and TRH on the enzymatic processing of prepro-TRH involves the combined use of radioimmunoassays for TRH and TRH precursor peptides, high pressure liquid chromatography (30) and selective inactivation of the processing and degrading enzymes by thermal perturbation (33).

In the present studies we have examined the in vivo effects of thyroid hormones and TRH on the concentrations of TRH and TRH-Gly in various tissues and blood and have correlated the serum TSH, TRH and TRH-Gly values. The results obtained are consistent with cosecretion of TRH and TRH precursor peptides from hypothalamic and extrahypothalamic tissues which biosynthesize TRH and competitive inhibition of TRH-Gly degrading enzymes in serum and cell membranes by exogenously administered TRH. Recent evidence indicates that some of these precursor fragments may have intrinsic TSH biosynthesis-stimulating activity (10). The trypsin-like enzymes may be more thermally stable than the alpha-amidating and TRH- and TRH-Gly-degrading enzymes.

METHOD

Treatment of Animals

Eight-week-old male Sprague-Dawley rats were obtained from Simenson Laboratories (Gilroy, CA). The animals were housed, 4 per cage, in our animal facility under conditions of constant temperature (22°C) and a controlled light schedule (lights on: 6 a.m. to 6 p.m.). The animals had free access to Purina rodent chow and water. The animals were allowed a 3-week acclimation period prior to the experiment.

In Experiment 1, rats were divided into 3 groups according to thyroid status. The euthyroid control group consisted of 6 sham-thyroidectomized rats. A second group of 6 euthyroid sham-thyroidectomized rats were treated with an IP injection of 1 μg TRH/kg body weight 30 min prior to sacrifice. The rats were sham-thyroidectomized under ether anesthesia. There were 3 groups of surgically thyroidectomized hypothyroid rats: the first were untreated, the second received an IP injection of 1 μg triiodothyronine (T3)/100 g b.wt. 48 hr prior to sacrifice and the third received an IP injection 1 μg TRH/kg b.wt. 30 min prior to sacrifice. These animals were given 1% calcium lactate drinking water ad lib after thyroidectomy. The euthyroid and hypothyroid rats were maintained for 4 weeks prior to experimentation and weighed weekly. The T4-treated group consisted of 6 sham-thyroidectomed rats which received an IP injection of T4 (1.25 μg T4/100 g b.wt. in 0.01 M NaOH) to raise plasma T4 concentration quickly to the desired level, and a subcutaneously implanted constant infusion Alzet osmotic minipump (Alza Corp., Palo Alto, CA), releasing T4 in a 0.01 N NaOH-10% ethanol vehicle at a rate of 10 μg/100 g b.wt. per day. These animals were weighed weekly and sacrificed 2 weeks later.

In Experiment 2, 3 groups of 8 rats were studied. A euthyroid control group; a hypothyroid group treated by thyroidectomy followed by 0.1% PTU and 1% calcium lactate drinking water ad lib for 4 weeks prior to sacrifice; and a T4-treated group which was injected SC with T4 (25 μg/100 g b.wt./day) for a 3-week period prior to decapitation.

Blood and Tissue Extraction for TRH and TRH-Gly Radioimmunoassays

Trunk blood was collected from all rats after sacrifice. Two ml of blood was immediately transferred into 5 ml of ice-cold methanol, vortexed, centrifuged, and the supernatant was stored at −20°C. The supernatant was dried on a heater block at 60°C with filtered air blowing into each sample tube. The dried residue was extracted twice in 3 ml of methanol, the supernatant was dried completely and stored at −20°C prior to TRH and TRH-Gly radioimmunoassays. A four ml portion of whole blood was allowed to clot for 2 hr at room temperature. Serum was then obtained by centrifugation and stored at −20°C until assayed for thyrotropin (TSH), T3 and T4. The brain, anterior pituitary and posterior pituitary and thyroid gland were rapidly excised from each animal. The area encompassing the hypothalamus was removed from the brain of each animal. The excised tissue was delineated by making an anterior cut immediately behind the optic chiasm, the posterior cut just anterior to the cerebral peduncles and lateral cuts at the hypothalamic sulci. The 5 different tissues were individually weighed. The hypothalamic, anterior and posterior pituitary and thyroid specimens were placed in 1 ml of 0.15 M NaCl-0.05 M phosphate buffer, pH 7.5 (PBS) and the brain specimens were submerged into 5 ml of cold PBS. All samples were homogenized with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY). For each specimen, half of the homogenate was immediately heated on a heater block at 97°C for at least 15 min to inactivate the TRH degrading enzymes. The second half of each homogenized sample was incubated at 60°C for 20 hr to inactivate selectively (33) the alpha-amidating enzymes (15,22). All homogenates were then dried completely in a heater block with filtered air flowing into each tube and then stored at −20°C. The stored dried residues were later extracted in 5 ml of methanol. The methanol supernatants were dried completely, and the residues were redissolved in 1 ml water for TRH and TRH-Gly radioimmunoassays (RIA) and SP-Sephadex C-25 chromatography followed by TRH-Gly RIA.

In Experiment 2, pituitaries and thyroids were collected and processed as described above. However, in each animal group, the total pituitaries were pooled prior to processing. The collected samples were assayed for TRH-Gly by RIA before or after fractionation by HPLC.

Serum TSH, T3 and T4 Levels

Rat TSH, T3 and T4 levels in unextracted serum were measured by previously described RIA methods (43).

TRH Radioimmunoassay

The TRH radioimmunoassay of 100 μl aliquots of a serial dilution of each extract was carried out with a modification (32) of the method of Bassiri and Utiger (2). The TRH antiserum was produced as previously described (37). TRH concentrations were calculated with the aid of a parallel line and relative potency computer program modified for use on the Hewlett-

Packard model 9830 computer (Hewlett-Packard, Palo Alto, CA) (29). The cross-reactivity of TRH-Gly in this assay was <0.01%.

TRH-Gly Radioimmunoassay

Synthetic TRH-Gly was conjugated to keyhole limpet hemocyanin using dinitrodifluorobenzene (46). One mg of conjugate emulsified in Freund's complete adjuvant was injected intradermally and subcutaneously every 8 weeks into 3 New Zealand female white rabbits over a period of several months. All TRH-Gly RIA measurements reported in this paper were obtained using the second bleeding from rabbit No. 898 (antibody 898B2). The cross-reactivity profile relative to the TRH-Gly standard curve demonstrated that this antibody did not cross-react with TRH, TRH-OH or pGlu-His-Gly-NH_2. TRH-Gly-NH_2, a synthetic peptide which does not occur in nature, did cross-react significantly. Other TRH-like peptides such as Arg-TRH-Gly, TRH-Gly-Lys and TRH-Gly-Lys-Arg also cross-reacted significantly (Fig. 1, right panel). The relative order of cross-reactivity for the TRH-precursor peptides given in Fig. 1 for antibody 898B2 was also found for antisera obtained from the other 2 rabbits immunized with TRH-Gly conjugate. The antiserum used for the present study had the highest useful working dilution among all those tested. The minimum detectable concentration of TRH-Gly was 100 pg/ml. One hundred μl of standard or sample was added to 12×75 mm glass test tubes. Two hundred μl of ^{125}I-labeled TRH-Gly in 0.625% normal rabbit serum (NRS) + 0.05 M EDTA was added along with 100 μl of specific rabbit antiserum in PBS, pH 7.5. The samples were vortexed and then incubated at 4°C for 3 days. One hundred μl of a second antibody was then added and the incubation continued overnight at 4°C. Samples were centrifuged at 1000 × g for 30 min, aspirated and counted for 2 min.

Chromatographic Analysis

Three types of chromatography were used to characterize the TRH-Gly-IR of rat tissues and blood: SP-Sephadex C-25 cation exchange, reverse phase HPLC and Bio-Gel P-2 exclusion chromatography. In the first experiment, pooled thyroid extracts from 6 TRH-treated rats were purified by reversed phase chromatography using Sep Pak C18 cartridges (Waters Inc., Milford, MA) and then subjected to SP-Sephadex C-25 cation exchange chromatography (Pharmacia, Piscataway, NJ) using a 0.9×58 cm column which was equilibrated and eluted with 0.2 M ammonium acetate, pH 6.2 (35). Fractions of 1.1 ml were collected. TRH-Gly radioimmunoassay measurements were carried out on these fractions after drying and reconstituting with distilled water. In the second experiment, pooled thyroid extracts from 8 euthyroid and from 8 hyperthyroid rats and pooled pituitary extracts from 8 hypothyroid and 8 hyperthyroid rats, before and after incubation at 60°C, were applied to a Sep Pak C18 cartridge, washed with HPLC-grade water and the adsorbed material eluted with 50% acetonitrile (Omnisolve grade, Matheson Coleman and Bell). The combined water and 50% acetonitrile washes were dried completely and then reconstituted in HPLC-grade water. This extract was centrifugally filtered using a Bioanalytical Systems, Inc. microfiltration device (West Lafayette, IN) and then injected into an HPLC system (Altex Scientific, Inc.) equipped with a Model 421 controller, an ultraviolet detector set at 220 nm and a 4.6×25 cm Ultrosphere ODS reverse phase column (Altex, Berkeley, CA) previously equilibrated with 0.01 M trifluoroacetic acid (TFA, Sequenal grade, Pierce Chemical Co., Rockford, IL). At the time of injection a 0.5%/min linear gradient of acetonitrile was begun which was increased to 2%/min at 40 min after starting the gradient. The collected fractions (1.0 ml) were dried, stored at −20°C, reconstituted in HPLC-grade water and measured for TRH-Gly-IR. Whole blood from 50 rats was extracted with cold methanol to rapidly inactivate the TRH- and TRH-Gly-degrading enzymes, then centrifuged. The supernatants were pooled, dried completely and extracted with a TRH-Gly affinity column prepared as previously described (37). The eluate was lyophilized to dryness and redissolved in HPLC-grade water containing 6 M guanidine hydrochloride to dissociate TRH-Gly-immunoreactive peptides from binding substances in the whole blood extract. This mixture was fractionated by HPLC with and without added synthetic TRH-Gly.

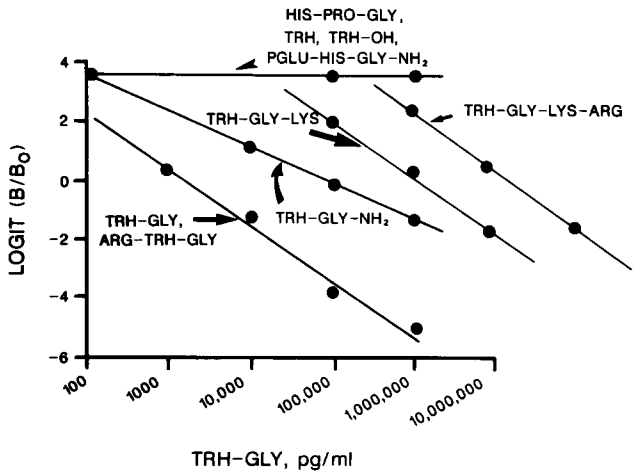

FIG. 1. Cross-reactivity curves for rabbit antiserum to TRH-Gly (898B2) at 1:2500 final dilution. Logit $(B/B_0) = \ln[(B/B_0)/(1 - B/B_0)]$ where B/B_0 is the ratio of counts bound at finite dose divided by counts bound with zero dose of unlabeled antigen. Serial dilutions of each tissue extract studied displaced ^{125}I-TRH-Gly in parallel with the TRH-Gly standard curve.

To further characterize the TRH-Gly-immunoreactive peptides in rat blood a pooled methanol extract of blood from 100 male Sprague-Dawley rats, 250 g each, was extracted with a TRH-Gly affinity column prepared as previously described (37) and fractionated by exclusion chromatography on a 0.9×112 cm column of Bio-Gel P-2 equilibrated and eluted with 0.2 M ammonium acetate, pH 6.2. Fractions of 2.5 ml were collected, dried completely and reconstituted with distilled water just prior to RIA. The excluded volume, V_0, and the salt peak were determined with bovine thyroglobulin and Na ^{125}I, respectively.

Peptide Synthesis

TRH-Gly was synthesized by Bachem Fine Chemicals, Torrance, CA. Arg-Gln-His-Pro-Gly (Arg-TRH-Gly) was synthesized by Joseph R. Reeve, Jr., Ph.D., VA Wadsworth Medical Center by standard solid phase synthesis methods. His-Pro-Gly was prepared by Incell Corp., Milwaukee, WI. All other peptides were obtained from Peninsula Laboratories, Belmont, CA.

Statistical Analysis

All data are presented as mean±SD. Comparisons between groups of animals were evaluated by the nonpaired Student's *t*-test after correcting for multiple comparison bias by the Bon-

ferroni method (27). Differences were considered significant for $p<0.05/n$ where n is the number of comparisons made.

RESULTS

Serum T4 and T3 Concentrations

In Experiment 1, hypothyroid rats had significantly lower serum T4 and T3 levels compared to euthyroid rats. Chronic T4 treatment and acute TRH stimulation did not alter significantly serum T4 and T3 concentrations compared to values of intact rats. In hypothyroid animals, acute T3 treatment significantly increased serum T3 levels but did not alter serum T4 levels. In this latter group, TRH stimulation did not affect serum T4 and T3 levels (Table 1).

In Experiment 2, hypothyroid animals had a significantly lower serum T4 and T3 concentrations compared to euthyroid controls. T4-treated animals had significantly higher serum T4 and T3 concentrations compared to euthyroid animals (Table 1).

Serum TSH and Whole Blood TRH-Gly-IR Concentrations

In the first experiment, TRH injection into euthyroid animals significantly increased both serum TSH and whole blood TRH-Gly-IR levels as did untreated hypothyroidism (Fig. 2). Euthyroid and hypothyroid rats exhibited a 5- and a 2-fold rise in serum TSH levels, respectively, 30 min following a TRH injection. TRH injection to euthyroid animals significantly increased the mean whole blood TRH-Gly-IR 3.5-fold. T4 treatment significantly lowered serum TSH levels and suppressed whole blood TRH-Gly-IR compared to values from euthyroid controls (Fig. 2, left panel). A correlation of whole blood TRH-Gly-IR values for all the various treatment groups with the corresponding serum TSH results was found to be highly significant. There was a linear relationship between whole blood TRH-Gly-IR and serum TSH concentrations: $y = 0.89x + 141$, $r=.69$, $n=30$, $p<0.01$ (Fig. 3, left panel). In Experiment 2, hypothyroid rats had significantly higher mean serum TSH levels and T4-treated rats had significantly lower mean serum TSH levels compared to euthyroid controls (Table 1).

TABLE 1

SERUM THYROID HORMONE VALUES FOR EACH TREATMENT GROUP

Group	T3 (ng/dl)	T4 (μg/dl)	TSH (μU/ml)	Body Weight (g)
		Experiment 1		
Euthyroid	49 ±12	5.0±0.8	51± 19	233±24
Euthyroid + IP TRH	55 ±12	6.0±0.7	254±174*	221±27
Hypothyroid	13 ± 4†	2.0±0.5†	234± 62†	205±44
T4-treated	49 ± 7	7 ±2	19± 5†	262±24
		Experiment 2		
Euthyroid	79.5±13	2.6±0.5	104± 25	237±15
Hypothyroid	33 ± 9†	<0.3±0†	547±102†	106±12†‡
T4-treated	161 ±21†	6.6±1.5†	34± 6†	222±17

Experiment 1, n=6; Experiment 2, n=8. *$p<0.05$; †$p<0.01$ versus the euthyroid group. ‡Animals in this group were slightly smaller at the beginning of the experiment and lost weight following thyroidectomy.

Blood TRH-IR Concentrations

Whole blood TRH-IR was significantly higher for hypothyroid rats compared to euthyroid controls in Experiment 1 (Fig. 2, upper right panel). TRH injection significantly increased whole blood TRH-IR in both euthyroid and hypothyroid animals by 3- and 9-fold increments, respectively. Simultaneous correlation of whole blood TRH-IR values from all treatment groups with the corresponding serum TSH and TRH-Gly-IR results was found to be highly significant (Fig. 3, middle and right panels, respectively).

Chromatography of Rat Blood Extracts

The profile of TRH-Gly-IR for the methanol extract of whole rat blood following reverse phase HPLC (1 ml sample in 6 M guanidine HCl) or Bio-Gel P-2 exclusion chromagraph (1 ml sample in distilled water) is given in Figs. 4 and 5, respectively. The HPLC profile reveals several broad peaks of TRH-Gly-IR which emerge at a higher acetonitrile concentration, and therefore are more hydrophobic, than synthetic TRH-Gly. The hydrophobicity of these peptides approximates that for prepro-TRH(115-151) and prepro-TRH(160-169) which are peptide sequences linking TRH precursor sequences in prepro-TRH and which contain a high proportion of hydrophobic amino acid residues such as Trp, Tyr and Phe (24). The corresponding HPLC profile for rat blood extract in distilled water (not shown) consists of a single TRH-Gly-IR peak which emerged at fractions 5 and 6, the position for unretained salts.

The profile of TRH-Gly-IR following Bio-Gel P-2 exclusion chromatography of TRH-Gly affinity column-purified rat blood extract reveals two major, partially resolved peaks. The larger of these two peaks emerged after the elution position of synthetic TRH-Gly. The tendency of Bio-Gel P-2 to interact selectively with Trp and Tyr residues (5,41) is well illustrated by the increased retention times for tyrosyl-proline diketopiperazine (cyclo(Tyr-Pro)) compared to cyclo(His-Pro), Trp^2-TRH (pGlu-Trp-Pro-NH_2) compared to TRH and prepro-TRH(115-151) and prepro-TRH(160-169) with 37 and 10 amino acid residues, respectively, compared to Lys-Arg-TRH-Gly-Lys-Arg (Lys-Arg-Gln-His-Pro-Gly-Lys-Arg) with 8 amino acid residues. Prepro-TRH(115-151) contains 4 Trp, 1 Tyr and 3 Phe residues while Prepro-TRH(160-169) has 1 Trp and 1 Phe residue (24).

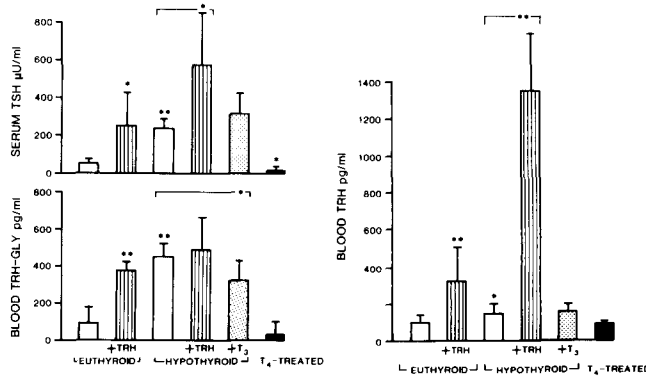

FIG. 2. Whole blood TRH-Gly-IR and serum TSH-IR (left panels and whole blood TRH-IR (right panel) in relation to thyroid status and the effects of TRH injection in euthyroid and hypothyroid rats and T3 injection in hypothyroid rats. N=6 (*$p<0.05$, **$p<0.01$).

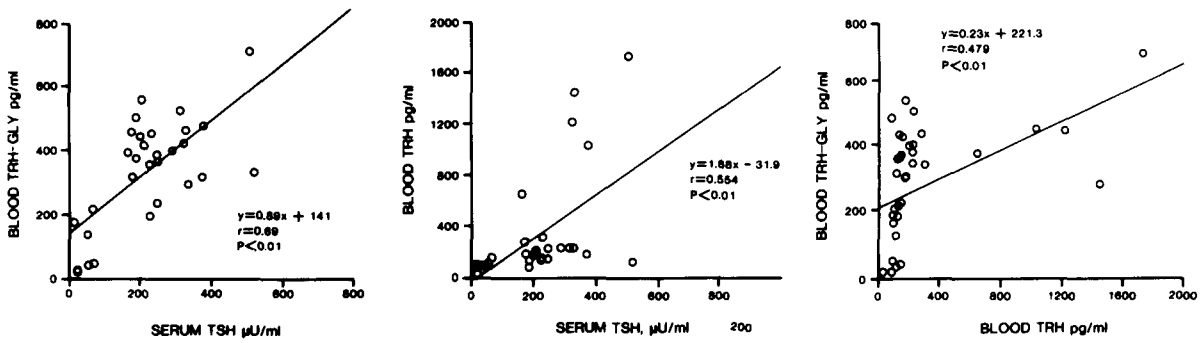

FIG. 3. Linear regression of whole blood TRH-Gly-IR, TRH-IR and serum TSH for all treatment groups.

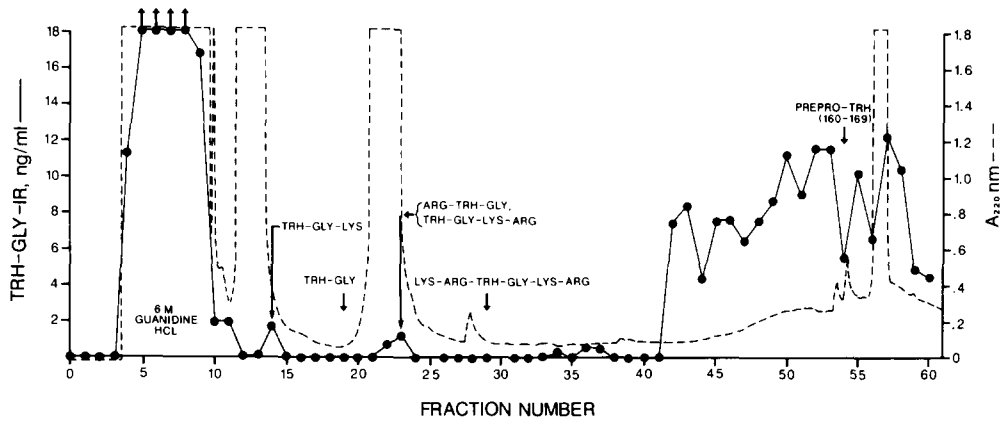

FIG. 4. TRH-Gly-IR and A_{220} nm profiles for whole rat blood extracted with methanol and subjected to reverse phase HPLC after initial purification with a TRH-Gly affinity column prepared from 10 ml of TRH-Gly antiserum obtained from the same rabbit which provided the TRH-Gly antibody used for the TRH-Gly RIA. Injected sample in 1 ml of 6 M guanidine HCl. Prepro-TRH(115-151) eluted at fraction 66 in a separate experiment. (n=50.)

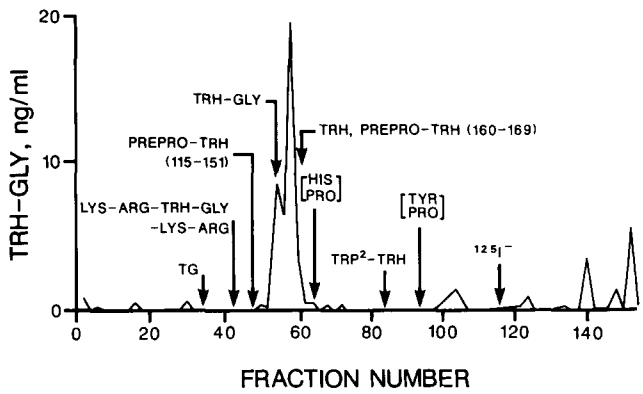

FIG. 5. TRH-Gly-IR profile for Bio-Gel P-2 exclusion chromatography of whole rat blood extract after initial purification with a TRH-Gly affinity column. TG: bovine thyroglobulin used as a marker for the excluded volume, V_o. (n=100.)

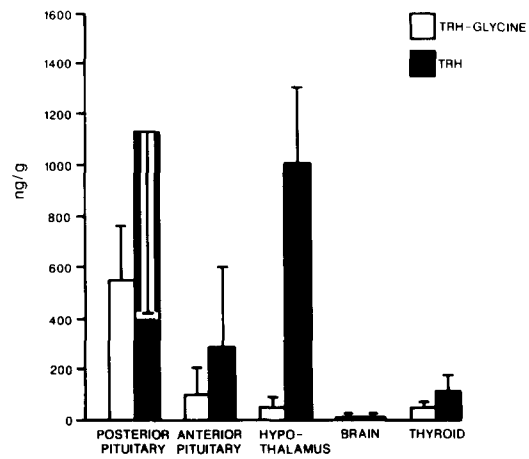

FIG. 6. TRH- and TRH-Gly-IR in posterior and anterior pituitary, hypothalamus, extrahypothalamic brain and thyroid of euthyroid rats.

Tissue TRH-IR and TRH-Gly-IR Levels in Euthyroid Animals

In Experiment 1, TRH-IR and TRH-Gly-IR were detectable in the brain, hypothalamus, anterior and posterior pituitary and thyroid gland of euthyroid rats (Fig. 6). Apart from the brain, TRH levels were higher than those of TRH-Gly-IR, particularly in the hypothalamus. Posterior pituitary and hypothalamus had similarly elevated TRH concentrations which were

respectively 3.6- and 3.3-fold higher than that of the anterior pituitary and 10-fold greater than that of the thyroid (all comparisons $p<0.01$). The highest TRH-Gly-IR concentration was observed in the posterior pituitary. It was 5.5-fold higher than in the anterior pituitary ($p<0.01$) and 11-fold greater ($p<0.01$) than in both the hypothalamus and thyroid (both $p<0.01$). The extrahypothalamic brain levels of TRH-IR and TRH-Gly-IR were much lower than in the other tissues.

Posterior Pituitary TRH-IR and TRH-Gly-IR in Relation to Thyroid Status

In Experiment 1, chronic T4 treatment did not affect the TRH-IR and TRH-Gly-IR concentrations in the posterior pituitary when compared to the euthyroid state (Fig. 7, left panel). However, the serum T3 and T4 levels were not different from that of controls, due in part, to the compensatory fall in serum TSH. TRH treatment significantly increased the posterior pituitary TRH-Gly-IR in intact control rats. Euthyroid and T4-treated animals had significantly greater TRH-IR than TRH-Gly-IR in the posterior pituitary ($p<0.05$ for both groups). Such differences were not observed in the TRH-treated euthyroid and in the hypothyroid animals.

Anterior Pituitary TRH-IR and TRH-Gly-IR in Relation to Thyroid Status

Anterior pituitary TRH-IR levels were similar for all treated and intact animals. Untreated hypothyroidism and TRH administration to euthyroid animals resulted in 5- and 9-fold increments, respectively, in the anterior pituitary TRH-Gly-IR (Fig. 7, right panel).

Hypothalamic TRH-IR and TRH-Gly-IR Levels in Relation to Thyroid Status and Incubation at 60°C for 20 hr

In Experiment 1, TRH injection into euthyroid rats significantly increased hypothalamic TRH-Gly-IR and TRH-IR (Fig. 8, upper panels). Incubation at 60°C lowered the hypothalamic TRH-Gly-IR concentrations in the TRH-treated euthyroid rats, in the untreated and T3-treated rats, and in the T4-treated rats (Fig. 8, upper left panel) and decreased hypothalamic TRH-IR in all groups (Fig. 8, upper right panel). Incubation at 60°C caused greater decrements in hypothalamic TRH-Gly-IR than in TRH-IR.

Total Pituitary TRH-Gly-IR in Relationship to Thyroid Status and Incubation at 60°C for 4 hr

In Experiment 2, pooled pituitary homogenates from euthyroid, T4-treated and hypothyroid animals, following incubation at 60°C for 4 hr, revealed marked increments in TRH-Gly-IR levels compared to those of the immediately extracted control aliquots (Fig. 8, lower left panel).

Thyroid TRH-IR and TRH-Gly-IR in Relation to Thyroid Status and Incubation at 60°C for 20 hr

In Experiment 1, TRH treatment of euthyroid rats significantly increased thyroid TRH-IR and TRH-Gly-IR. Chronic T4 treatment significantly increased thyroid TRH-IR levels compared to those of euthyroid controls. Incubation at 60°C increased TRH-Gly-IR from thyroid homogenates 5-fold in the euthyroid group, 4.5-fold in the TRH-treated euthyroid group and 5.3-fold in the T4-treated group, compared to the immediately extracted control aliquots (Fig. 8, lower right panel). The ratios of TRH-Gly-IR to TRH levels before and after incubation were 0.9 and 2.9, respectively, in the euthyroid rats; 0.8 and 3.1, respectively, in the TRH-treated euthyroid group; and 0.7 and 2.5, respectively, in the T4-treated group.

Chromatography of Pituitary Extracts

The results of HPLC fractionation of pooled pituitary extracts from 8 hypothyroid and from 8 T4-treated rats are given in Fig. 9, upper panels. High performance liquid chromatography disclosed multiple peaks of immunoreactivity. Incubation at 60°C for 4 hr of pooled pituitary extracts from hypothyroid and T4-treated rats produced a major peak of immunoreactivity which coeluted with synthetic TRH-Gly and was 39- and 6-fold greater, respectively, than the levels from the immediately extracted control aliquots.

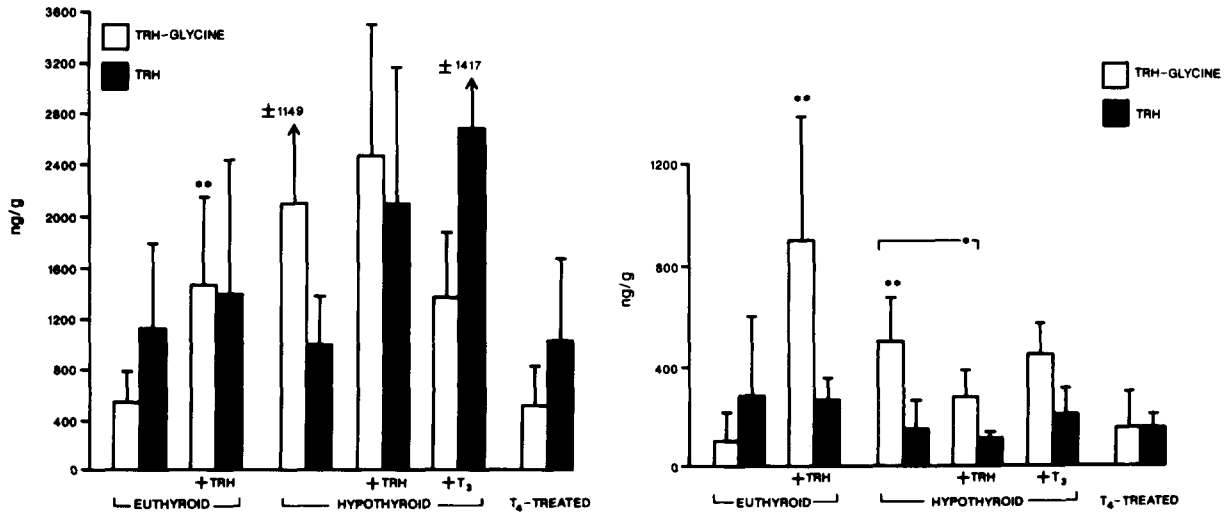

FIG. 7. TRH- and TRH-Gly-IR in the posterior (left panel) and anterior pituitary (right panel) in relation to thyroid status. N=6 for each group (*$p<0.05$, **$p<0.01$).

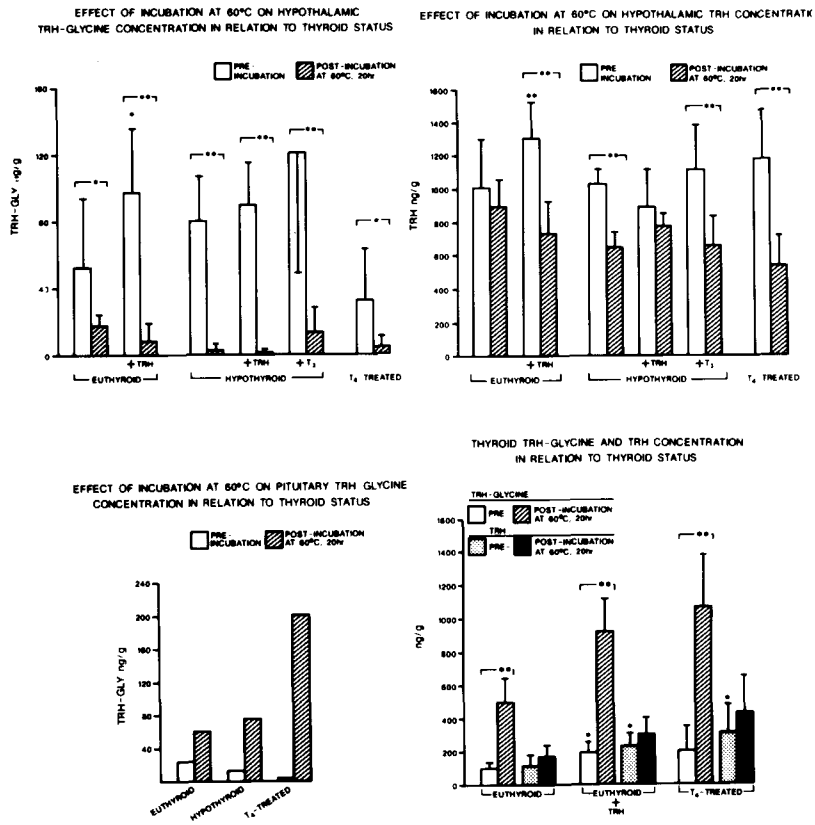

FIG. 8. Effect of thyroid status, TRH injection and incubation at 60°C on TRH-Gly- and TRH-IR in hypothalamus and thyroid (upper and right panels). N=6 for each group except for the thyroidectomized group treated with TRH, n=5. Effect of thyroid status and incubation at 60°C on TRH-Gly in pituitary, n=8 for each group.

Chromatography of Thyroid Extracts

SP-Sephadex-C25 cation exchange chromatographic profiles of pooled thyroid extracts from TRH-treated euthyroid rats, before and after incubation at 60°C for 20 hr, revealed peaks of TRH-Gly-IR which coeluted with synthetic TRH-Gly. The TRH-Gly-IR peak for homogenates incubated at 60°C was 15.5-fold greater than that of the immediately extracted control aliquots (results not shown). The results of HPLC analysis of pooled thyroid extracts from 8 euthyroid and 8 T4-treated rats are given in Fig. 9, lower panels. HPLC disclosed multiple peaks of immunoreactivity. Incubation at 60°C for 4 hr of pooled extracts from both groups produced a major TRH-Gly-IR peak coeluting with synthetic TRH-Gly which was not present in the immediately extracted control aliquots.

DISCUSSION

Biosynthesis of TRH in extrahypothalamic tissues is now a well-documented phenomenon (11–13, 16, 34). Hormonal regulation of this process has also been demonstrated (4, 6, 23, 30). Our group has, for example, recently reported that TRH and TRH precursor levels in the rat prostate are increased by thyroid hormone deficiency, that the prostatic TRH concentration decreases markedly following castration, and is restored by testosterone replacement (4,30). The present studies extend these observations by examining the effect of altered thyroidal status on the concentration of TRH and TRH precursor peptide levels in the hypothalamus, pituitary, thyroid and peripheral circulation.

Hypothyroidism and TRH injection increased serum TSH levels significantly, as shown in Fig. 2. Blood TRH levels following the 1 μg TRH injection in hypothyroid rats rose significantly higher than in euthyroid rats because of a well-characterized reduction in blood TRH-degrading enzyme levels in thyroid hormone-deficient animals (3). When the data from all treatment groups are combined, highly significant correlations between serum TSH, TRH and TRH-Gly become evident (Fig. 3). TRH-Gly (pGlu-His-Pro-Gly) is converted to TRH (pGlu-His-Pro-NH_2) by enzymes which cleave C-terminal Gly resulting in an alpha-amidated C-terminal amino acid residue and glyoxylate as reaction products (7, 12, 15, 17, 18, 22). The C-terminal amide is chemically unreactive, leading to irreversibility of the amidation reaction. Moreover, glyoxylate is a noncompetitive inhibitor of alpha-amidation (15). TRH raises the blood levels of TRH-Gly-immunoreactive peptides in euthyroid rats within 30 min of its administration. This observation may be attributable either to decreased alpha-amidation, decreased enzymatic degradation, or increased production of TRH-Gly-immunoreactive peptides derived from prepro-TRH. TRH and TRH-Gly are subject to rapid degradation by TRH-degrading enzymes in physiologic fluids (14, 33, 35, 39, 40), cytoplasmic membranes (47) and intact (38) and homogenized (36) tissues. Competitive inhibition of TRH-Gly degradation by administered synthetic TRH represents a likely explanation for

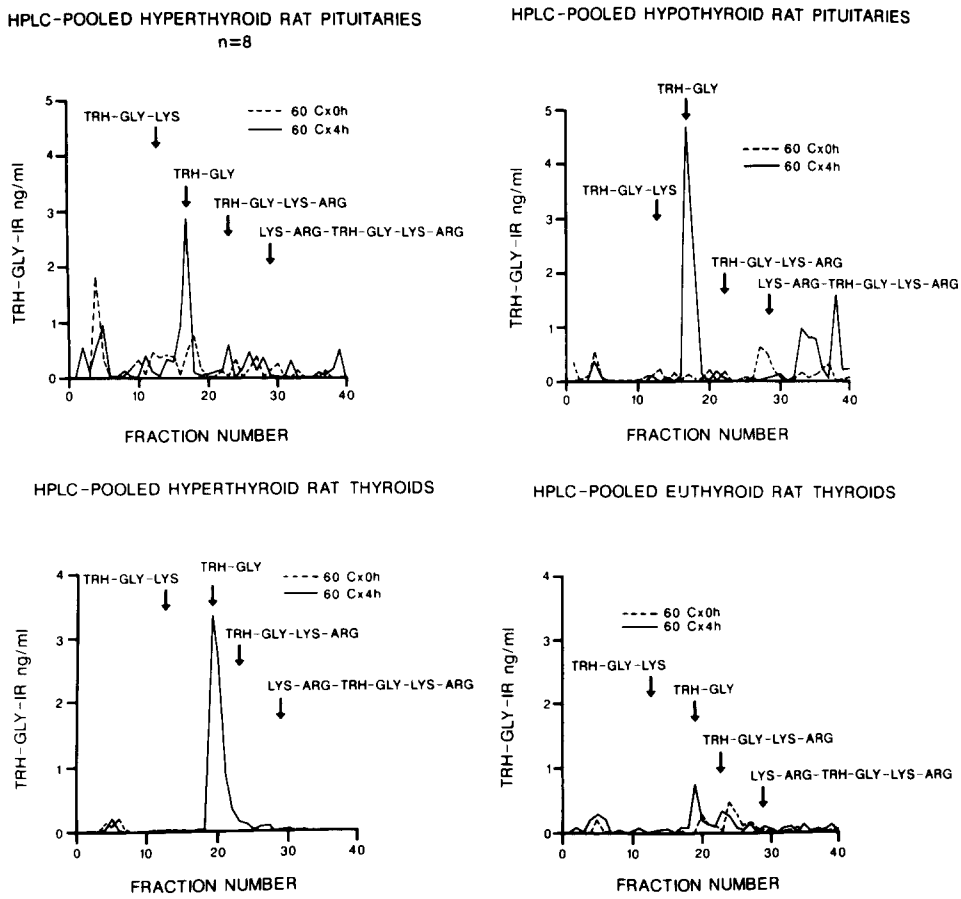

FIG. 9. Reversed phase HPLC profile of pooled pituitary extracts from 8 hypothyroid (upper right) and 8 T4-treated rats (upper left) before (dashed line) and after (solid line) incubation at 60°C for 4 hr. Similar profiles for pooled thyroid extracts from 8 T4-treated (lower left) and 8 control rats (lower right) are also presented. Elution positions for other TRH-Gly-immunoreactive peaks are located and identified at the arrows.

the prompt TRH-induced rise in serum TRH-Gly-IR levels since the in vitro and in vivo alpha-amidating activity of rat serum is low (39). Pyroglutamate amino-peptidase levels are very low in pituitary and serum, are high in brain, kidney and heart and intermediate in liver (42). Blood TRH-Gly-IR is most probably cosecreted with TRH by the hypothalamus (25) but this source is likely to be minor because of the relatively small mass of this brain region and the efficiency of TRH-Gly alpha-amidation in the postnatal period (17). The posterior pituitary (Fig. 6), reproductive organs (34), gut and other extracentral nervous system endocrine tissues [Fig. 6, (45)] in which the alpha-amidation of TRH-Gly to form TRH is more rate-limiting (30) may contribute the bulk of circulating TRH-Gly-IR.

The ratio of TRH-Gly-IR to TRH-IR in tissues of euthyroid rats decreases in the order thyroid, posterior pituitary, anterior pituitary, hypothalamus. The extrahypothalamic brain concentrations of TRH- and TRH-Gly-IR are very low and approximately equal. The corresponding absolute values for TRH-Gly decline in the order posterior pituitary, anterior pituitary, hypothalamus equal to thyroid, brain. The TRH values fell in the order posterior pituitary equal to hypothalamus, anterior pituitary, thyroid, brain (Fig. 6). The low ratio of TRH-Gly to TRH in hypothalamus is consistent with rapid alpha-amidation of TRH-Gly by this tissue, in marked contrast with the severe rate-limitation of this process in the ventral prostate (30).

Recent immunohistochemical studies have shown TRH-IR within the secretory granules of a unique multihormonal cell of the anterior pituitary (26). These cells, which represent 2% of the anterior pituitary volume, contain TRH, GnRH, LH and ACTH. The immunoreactivity within these cells was unaffected by 21 days of cell culture, suggesting that these hormones were actively synthesized during this time interval. TRH- and GnRH-immunoreactive peptides have been shown to bind to thyrotroph and gonadotroph receptors, respectively, be internalized and then incorporated into TSH- and LH-containing secretory vesicles, respectively, or into lysosomes (26). The present observations that in vivo TRH administration increases TRH-Gly levels in the anterior pituitary is subject to several possible interpretations. Least likely would be an effect of TRH on TRH processing in thyrotropic cells since current evidence indicates that this TRH derives from exogenous, TRH-receptor-bound hormone (1), rather than from proteolytic cleavage of in situ prepro-TRH. In addition, the TRH binding capacity of the rat anterior pituitary is 85±27 fmoles/pituitary (8) while the TRH content is of the order of 10 pmoles/pituitary (see Fig. 6). More likely would be TRH perturbation of prepro-TRH pro-

cessing in the plurihormonal cells of the anterior pituitary. This model implies the existence of TRH receptors on these "multipotential" cells of the pituitary.

TRH-IR within the posterior pituitary has been localized to TRH-containing nerve fibers projecting from the hypothalamus (28). Our group has previously reported TRH-IR in dog thyroid glands (36). The level of TRH-Gly-IR in both the anterior and posterior pituitary was increased by TRH injection and TRH-Gly-IR in the anterior pituitary was increased by hypothyroidism (Fig. 7).

HPLC of TRH-Gly affinity column-purified rat blood extract dissolved initially in 6 M guanidine HCl (Fig. 4) disclosed a broad series of partially-resolved peaks of TRH-Gly-IR which are all much more hydrophobic than synthetic TRH-Gly but similar in retention time to prepro-TRH linker peptides. Exclusion chromatography of this affinity-purified blood extract produced 2 partially-resolved peaks of TRH-Gly-IR, the larger being retained longer than tetrapeptide TRH-Gly (Fig. 5). On the other hand, HPLC of tissue extracts of pituitary and thyroid (Fig. 9) revealed a major peak which coeluted with synthetic TRH-Gly. The antibody to TRH-Gly has specificity for the C-terminal region of TRH-Gly but does not cross-react with His-Pro-Gly. N-terminally-extended forms of TRH-Gly do cross-react well, leading to the possibility that higher molecular weight precursor forms of TRH, which terminate with C-terminal Gly, would be detectable with the TRH-Gly RIA. From an examination of the rat prepro-TRH sequence (24) it is apparent that large fragments terminating with Gly will have considerable hydrophobic character due to the frequent occurrence of Trp, Tyr and Phe in all of the linker sequences between the repeated -Gln-His-Pro-Gly- segments. These amino acid residues interact strongly with Sephadex and Bio-Gel, leading to greater retention times than would be predicted from the molecular weight of the peptides in which they occur (5,41), as seen in Fig. 5. Given a) the constraints of the TRH-Gly-RIA specificity (Fig. 1), b) greater than expected hydrophobicity of TRH-Gly-IR from rat blood (Fig. 4), c) anomalously increased retention on Bio-Gel P-2 of linker peptides from prepro-TRH (Fig. 5), d) the rapid ($t\frac{1}{2}$=9.0 min) in vivo clearance of TRH-Gly from serum (39) and e) the complete nonretention of the TRH-Gly-IR from rat blood by reverse phase HPLC in the absence of 6 M guanidine HCl pretreatment (data not shown), it seems most likely that large prepro-TRH fragments, with a high degree of hydrophobically-stabilized secondary structure, circulate in rat blood and are not subject to rapid proteolytic degradation.

Heating at 60°C has been previously shown to greatly enhance TRH-Gly-IR levels in human semen due to the thermal inhibition of the alpha-amidation of TRH-precursor peptides (33). Several-fold increases in TRH-Gly levels were observed in thyroid and whole pituitary homogenates (Fig. 8, lower panels). Similar experiments carried out using hypothalamic homogenates, on the other hand, resulted in a marked decline in TRH-Gly levels. Hypothalamic TRH levels also fell during 20 hr at 60°C (Fig. 8, upper panels). The HPLC profile of TRH-IR for hypothalamic extracts consisted of a single peak coeluting with synthetic TRH (31). The total TRH-Gly-IR in rat hypothalami was very low (Fig. 6), making its chromatographic characterization unrealiable. Possible explanations for this marked difference in the effect of 60°C incubation on TRH-Gly levels in hypothalamic and extrahypothalamic tissues may be the substantial differences in the absolute and relative levels of the alpha-amidating and TRH- and TRH precursor-degrading enzymes and their thermal lability within the central nervous system (CNS) and extra-CNS tissues. Chromatographic analyses were carried out on whole pituitary and thyroid homogenates before and after incubation at 60°C to determine the identity of the immunoreactive species responsible for the increase in TRH-Gly-IR at this temperature. An increase in an immunoreactive peak coeluting with synthetic TRH-Gly was observed in all cases, both by SP-Sephadex-C25 cation exchange chromatography (data not shown) and by HPLC (Fig. 9).

In summary, prepro-TRH occurs in rat hypothalamus, pituitary and thyroid and is rapidly cleaved into TRH precursor peptides, which are further modified by alpha-amidating and N-terminal Gln cyclizing enzymes within secretory vesicles to produce TRH. This multistep process, however, can be interrupted by incubating tissue homogenates at 60°C, particularly those of the pituitary and thyroid, leading to highly significant increases in the level of TRH-Gly, a TRH precursor peptide. This observation implies that the trypsin-like enzymes which cleave prepro-TRH at the paired basic residues bracketing the sequence -Gln-His-Pro-Gly- are relatively stable at 60°C while the alpha-amidating enzymes are not. The N-terminal Gln cyclizing reaction, which also occurs nonenzymatically (9), should still proceed significantly at 60°C even if the corresponding enzyme is unstable at this temperature. Hypothyroidism increases TRH-Gly-IR levels in anterior pituitary (Fig. 7), whole blood (Fig. 2) and the reproductive system (30). In the latter case, the TRH-Gly levels were shown to be highly correlated with the serum T3 and T4 levels, while the corresponding tissue TRH-serum thyroid hormone regressions were not significant. This observation, and the invariance of the prostatic TRH-Gly/TRH ratio with alternations of thyroidal status, strongly suggest that thyroid hormones regulate prepro-TRH precursor biosynthesis, not alpha-amidation (30) in the eugonadal rat prostate.

Finally, administration of TRH increases anterior and posterior pituitary TRH-Gly levels and blood concentrations of TRH-Gly-immunoreactive peptides. A nonthyrotropic multihormone-containing cell of the anterior pituitary has recently been identified with secretory granules containing endogenously produced TRH (26). A preexisting intrapituitary cell-cell regulatory function for TRH may have been the direct anticedent for the cooption of this peptide for thyrotropic regulation by the hypothalamus (20). The possibility of shortloop feedback regulation of prepro-TRH processing by a nonthyrotropic "multipotential" (26) stem cell in the pituitary by its secretory product, TRH, whether arising locally or from the hypothalamus, raises new experimental questions as well as the potential complexity of the mammalian neuroendocrine system.

ACKNOWLEDGEMENTS

The authors thank Mark Knoble for his excellent technical assistance. Supported by Veterans Administration Medical Research funds.

REFERENCES

1. Antakly, T.; Zeytinoglu, F.; Pelletier, G.; Labrie, F. Autoradiographic localizaiton of thyrotropin-releasing hormone (TRH)-binding sites in cultured rat pituitary cells. Exp. Cell Res. 122:394-399; 1979.
2. Bassiri, R. M.; Utiger, R. D. The preparation and specificity of antibody to thyrotropin releasing hormone. Endocrinology 90:722-727; 1972.
3. Bauer, K. Degradation and biological inactivation of thyrotropin

releasing hormone (TRH): regulation of the membrane-bound TRH-degrading enzyme from rat anterior pituitary by estrogen and thyroid hormones. Biochemie 70:69–74; 1988.
4. Bhasin, S.; Pekary, A. E.; Brunskill, B.; Hershman, J. M.; Swerdloff, R. S. Hormonal control of prostatic thyrotropin-releasing hormone (TRH): testosterone modulates prostatic TRH concentrations. Endocrinology 114:946–950; 1984.
5. Bhat, P. G.; Krishnamoorthy, G. P.; Pattabiraman, T. N. Deviation behavior of charged and aromatic molecules on highly cross-linked dextran and polyacrylamide gels. J. Indian Inst. Sci. 62(C):13–19; 1980.
6. Bolaffi, J. L.; Jackson, I. M. D. Regulation of thyrotropin-releasing hormone secretion from frog skin. Endocrinology 110: 842–846; 1982.
7. Bradbury, A. F.; Finnie, M. D. A.; Smyth, D. G. Mechanism of C-terminal amide formation by pituitary enzymes. Nature 298:686–688; 1982.
8. Burt, D. R.; Taylor, R. L. TRH receptor binding in retina and pituitary: major species variation. Exp. Eye Res. 35:173–182; 1982.
9. Busby, W. H., Jr.; Quackenbush, G. E.; Humm, J.; Youngblood, W. W.; Kiser, J. S. An enzyme(s) that converts glutamyl-peptides into pyroglutamyul-peptides. J. Biol. Chem. 262:8532–8536; 1987.
10. Carr, F. E.; Wessendorf, M. A pre-prothyrotropin-releasing hormone fragment is a more potent stimulator than thyrotropin-releasing hormone (TRH) of thyrotropin B-subunit (TSH B) gene expression in a transcient expression assay system. Am. Thyroid Assoc., 63rd Annual Meeting, Montreal, Sept. 27–Oct. 1; 1988 (Abstract #109).
11. Cockle, S. M.; Smyth, D. G. Processing of the thyrotropin releasing hormone (TRH) precursor in Xenopus skin and bovine hypothalamus: evidence for the existence of extended forms of TRH. Regul. Pept. 14:217–227; 1986.
12. Cockle, S. M.; Smyth, D. G. Detection of TRH extended peptides in rat hypothalamus using an antibody raised to pGlu-His-Pro-Gly-Lys. Biosci. Rep. 6:519–526; 1986.
13. Dolva, L. O.; Nielsen, J. H.; Welinder, B. S.; Hanssen, K. F. Biosynthesis and release of thyrotropin-releasing hormone immunoreactivity in rat pancreatic islets in organ culture. J. Clin. Invest. 72:1867–1873; 1983.
14. Dvorak, J. C.; Utiger, R. D. Immunoreactivity and serum destruction of N^{3im}-methyl-TRH. J. Clin. Endocrinol. Metab. 44:582–585; 1977.
15. Eipper, B. A.; Park, L. P.; Dickerson, I. M.; Keutmann, H. T.; Thiele, E. A.; Rodrigues, H.; Schofield, P. R.; Mains, R. E. Structure of the precursor to an enzyme mediating COOH-terminal amidation in peptide synthesis. Mol. Endocrinol. 1:777–790; 1987.
16. Engler, D.; Scanlon, M. F.; Jackson, I. M. D. Thyrotropin-releasing hormone in the systemic circulation of the neonatal rat is derived from the pancreas and other extraneural tissue. J. Clin. Invest. 67:800–808; 1981.
17. Glembotski, C. C.; Manaker, S.; Winokur, A.; Gibson, T. R. Ascorbic acid increases the thyrotropin-releasing hormone content of hypothalamic cell cultures. J. Neurosci. 6:1796–1802; 1986.
18. Gomez, S.; diBello, C.; Hung, L. T.; Genet, R.; Morgat, J. L.; Fromageot, P.; Cohen, P. C-terminal amidation of neuropeptides: Gly-Lys-Arg extension an efficient precursor of C-terminal amide. FEBS Lett. 167:160–164; 1984.
19. Hartnell, J. M.; Pekary, A. E.; Hershman, J. M. Comparison of the effects of pulsatile and continuous TRH infusion on TSH release in men. Metabolism 36:878–882; 1987.
20. Hershman, J. M.; Pekary, A. E. Regulation of thyrotropin secretion. In: Imura, H. ed. The pituitary gland. New York: Raven Press; 1985:149–188.
21. Hinkle, P. M.; Tashjian, A. H., Jr. Thyrotropin releasing hormone regulates the number of its own receptors in the GH_3 strain of pituitary cells in culture. Biochemistry 14:3845–3851; 1975.
22. Kizer, J. S.; Busby, W. H., Jr.; Cottle, C.; Youngblood, W. W. Glycine-directed peptide amidation: presence in rat brain of two enzymes that convert p-Glu-His-Pro-Gly-OH into pGlu-His-Pro-NH_2 (thyrotropin-releasing hormone). Proc. Natl. Acad. Sci. USA 81:3228–3232; 1984.
23. Lamberton, P.; Wu, P.; Jackson, I. M. D. TRH release from rat pancreas is stimulated by serotonin but inhibited by carbacol. Endocrinology 117:1834–1838; 1985.
24. Lechan, R. M.; Wu, P.; Jackson, I. M. D.; Wolf, H.; Cooperman, S.; Mandel, G.; Goodman, R. H. Thyrotropin-releasing hormone precursor: characterization in brain. Science 231:159–161; 1986.
25. Liao, N.; Bulant, M.; Nicholas, P.; Pelletier, G. Light and electron microscopic localization of thyrotropin-releasing hormone (TRH) precursor in the rat brain. The Endocrine Society, 70th Annual Meeting, New Orleans, June 8–11; 1988 (Abstract #1011).
26. May, V.; Wilber, J. F.; U'Prichard, D. C.; Childs, G. V. Persistence of immunoreactive TRH and GnRH in long-term primary pituitary cultures. Peptides 8:543–558; 1987.
27. Miller, R. G. Simultaneous statistical inference. New York: McGraw-Hill Book Co.; 1966; 67–70.
28. Morel, G.; Leneveu, E.; Tonon, M. C.; Pelletier, G.; Vandry, H.; Dubois, P. M. Subcellular localization of thyrotropin-releasing hormone (TRH)- and neuropeptide Y (NPY)-like immunoreactivity in the neurointermediate lobe of the frog pituitary. Peptides 6:1085–1091; 1985.
29. Pekary, A. E. Parallel line and relative potency analysis of bioassay and radioimmunoassay data using a desk top computer. Comp. Biol. Med. 9:355–362; 1979.
30. Pekary, A. E.; Bhasin, S.; Smith, V.; Sugawara, M.; Swerdloff, R. S.; Hershman, J. M. Thyroid hormone modulation of thyrotropin-releasing hormone (TRH) and TRH-Gly levels in the male rat reproductive system. J. Endocrinol. 114:271–277; 1987.
31. Pekary, A. E.; Carlson, H. E.; Yamada, T.; Sharp, B.; Walfish, P. G.; Hershman, J. M. Thyrotropin-releasing hormone levels decrease in hypothalamus of aging rats. Neurobiol. Aging 5:221–226; 1984.
32. Pekary, A. E.; Morley, J. E.; Hershman, J. M. Absence of phyroglutamyl-N^{3i}-methyl-histidyl-prolineamide (methyl-thyrotropin-releasing hormone) in the rat hypothalamus. J. Endocrinol. 77: 405–408; 1978.
33. Pekary, A. E.; Reeve, J. R., Jr.; Friedman, S. In vitro production of pGlu-His-Pro-Gly, a precursor for thyrotropin-releasing hormone biosynthesis, by human semen. Pacific Coast Fertility Society, 34th Annual Meeting, San Diego, April 9–13; 1986 (Abstract #24).
34. Pekary, A. E; Reeve, J. R., Jr.; Smith, V. P. Evidence for thyrotropin-releasing hormone (TRH) biosynthesis in rat prostate. Life Sci. 39:2565–2570; 1986.
35. Pekary, A. E.; Reeve, J. R., Jr.; Smith, V. P.; Friedman, S.; Hershman, J. M. In vitro production of a TRH-homologous peptide and His-Pro diketopiperazine by human semen. J. Androl. 6: 379–385; 1985.
36. Pekary, A. E.; Richkind, M.; Hershman, J. M. Thyrotropin releasing hormone and related peptides in canine tissues. J. Endocrinol. 98:299–306; 1983.
37. Pekary, A. E.; Sharp, B.; Briggs, J.; Carlson, H. E.; Hershman, J. M.; High concentrations of pGlu-His-Pro-NH_2 (thyrotropin-releasing hormone) occur in rat prostate. Peptides 4:915–919; 1983.
38. Pekary, A. E.; Sharp, B.; Rosen, J. I.; Meyer, N. V.; Hershman, J. M. Postmortem processing of thyrotropin-releasing hormone (TRH) in rat hypothalamus and reproductive organs. The Endocrine Society, 63rd Annual Meeting, Cincinnati, OH, June 17–19; 1981 (Abstract #954).
39. Pekary, A. E.; Stephens, R.; Simard, M.; Hershman, J. M. Thyrotropin (TSH) release in rats after intracisternal injection of a thyrotropin-releasing hormone precursor. Clin. Res. 33:126A; 1988.
40. Pekary, A. E.; Stephens, R.; Tache, Y.; DiStephano, J. J., III; Landaw, E. M.; Hershman, J. M. Kinetics of in vivo TRH-Gly to TRH conversion in rat cerebrospinal fluid. The Endocrine Society, 70th Annual Meeting, New Orleans, June 8–11; 1988 (Abstract #1010).
41. Politi, L.; Nicoletti, R.; Scandurra, R. Adsorption chromatography on Bio-Gel P-2. J. Chromatogr. 267:403–408; 1983.
42. Prasad, C.; Peterkofski, A. Demonstration of pyroglutamyl-peptidase and amidase activities toward thyrotropin-releasing hormone in hamster hypothalamus extracts. J. Biol. Chem. 251:3229–3234; 1976.
43. Rookh, H. V.; Azukizawa, M.; DiStephano, J., 3rd; Ogihara, T.;

Hershman, J. Pituitary-thyroid hormone periodicities in serially sampled plasma of unanesthetized rats. Endocrinology 104:851–856; 1979.
44. Segerson, T. P.; Kauer, J.; Wolfe, H. C.; Mobtaker, H.; Wu, P.; Jackson, I. M. D.; Lechan, R. M. Thyroid hormone regulates TRH biosynthesis in the paraventricular nucleus of the rat. Science 238:78–80; 1987.
45. Simard, M.; Pekary, A. E.; Smith, V.; Hershman, J. M. Detection of biosynthetic precursor of thyrotropin-releasing hormone (TRH) in secretory tissues of the rat. Clin. Res. 35:402A; 1987.
46. Visser, T. J.; Klootwijk, W.; Doctor, R.; Henneman, G.; A new radioimmunoassay of thyrotropin-releasing hormone. FEBS Lett. 83:37–40; 1977.
47. Zlokovic, B. V.; Lipovac, M. N.; Begley, D. J.; Davson, H.; Rakic, L. Slow penetration of thyrotropin-releasing hormone across the blood-brain barrier of an in situ perfused guinea pig brain. J. Neurochem. 51:252–257; 1988.