Ascorbic Acid Increases the Thyrotropin-Releasing Hormone Content of Hypothalamic Cell Cultures

Christopher C. Glembotski,* Scott Manaker,†,‡,¹ Andrew Winokur,* and Thomas R. Gibson*

Departments of *Pharmacology, †Biology, and ‡Psychiatry, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Thyrotropin-releasing hormone (TRH) is one of many COOH-terminal α -amidated neuropeptides. Recent work with the intermediate pituitary has indicated that ascorbate is a required cofactor for the COOH-terminal α -amidation of α -melanotropin. This is consistent with the ascorbate requirement of an enzyme found in pituitary and hypothalamus capable of converting peptides with a COOH-terminal glycine (-X-Gly) to α -amidated molecules (-H-NH₂). Thus, it has been proposed that COOH-terminal glycine-extended TRH (TRH-Gly) may be the direct precursor to TRH. In the present study, primary hypothalamic cultures supplemented with ascorbate for 7 d contained two- to threefold more TRH immunoactivity (amide-specific) than cultures maintained without ascorbate. A dose-response experiment indicated that 20 μ M ascorbate was capable of producing 50% of the maximum observable increase in culture TRH immunoactivity; this concentration is similar to the K_m value for ascorbate uptake obtained in adrenal chromaffin and pituitary cells. A stereoisomer of ascorbate, D-isoascorbate, was also capable of producing an increase in TRH immunoactivity, but oxidized ascorbate was not. Recent studies have shown that the amidation enzyme from pituitary is capable of utilizing both L-ascorbate and D-isoascorbate but is incapable of utilizing oxidized ascorbate. The culture extracts were analyzed further by reversed-phase high-performance liquid chromatography; the increased TRH immunoactivity observed in extracts of cultures maintained in ascorbate comigrated with standard synthetic TRH. TRH- and TRH-Gly specific antisera were used to measure the quantities of these peptides: When cultures were maintained for 7 d in the absence of ascorbate, the TRH to TRH-Gly ratio was 8-fold less than the TRH to TRH-Gly ratio in cultures maintained in the presence of 250 μ M ascorbate. These results suggest that ascorbate is a required cofactor for the COOH-terminal α -amidation of TRH and that the direct precursor to TRH in the rat hypothalamus may possess a COOH-terminal glycine residue.

During the past 10 years a large number of peptides, many of which were originally characterized in endocrine tissues, have been localized to the CNS. In order to characterize the factors that regulate their production, the biosynthetic pathways of both endocrine and neuropeptides must be determined. Over the last

15 years many aspects of endocrine peptide biosynthesis have been elucidated. In part, these studies have been facilitated by the relatively high levels of peptides found in endocrine tissues. However, since neuropeptides are present in the CNS in such low levels (1000- to 10,000-fold lower than endocrine peptides), detailed biosynthetic studies are relatively few (Bloom, 1983; Brownstein, 1983; Douglass et al., 1984; Hökfelt et al., 1984; Iverson, 1983; Krieger, 1983; Richter et al., 1984). In the study reported here, we have been able to extend some of our recent findings on endocrine peptide biosynthesis to studies of an important neuropeptide, thyrotropin-releasing hormone (TRH).

Thyrotropin-releasing hormone (pyro-Glu-His-Pro-NH₂) was the first hypothalamic releasing factor to be isolated and sequenced (Boler et al., 1969; Burgus et al., 1969). Subsequently, it was found to be widely distributed throughout the CNS (Brownstein et al., 1974; Winokur et al., 1974) and shown to exert many non-hypophysiotropic effects (Jackson, 1982). However, attempts to characterize the mechanism of biosynthesis of TRH have largely been unsuccessful for several reasons, including its low levels in the CNS, small size, and blocked NH₂- and COOH-terminals. Several early studies demonstrated relatively low levels of incorporation of radioactivity into TRH either *in vivo* or in organ culture (McKelvy, 1974, 1983; McKelvy et al., 1975; Reichlin and Mitnick, 1971). Rupnow et al. (1979) demonstrated the possible existence of a larger molecular weight substance derived from frog brain that—on trypsinization, chemical amidation, and pyro-glutamyl formation—was converted into material possessing TRH immunoactivity and bioactivity. However, studies demonstrating the incorporation of radioactivity into TRH by cultured cells have been few, and the convincing demonstration of the formation of authentic TRH from a biosynthetic precursor has not been performed.

The precursor and/or gene sequences of many neuro- and endocrine peptides have been elucidated (Douglass et al., 1984). From these studies the amino acid sequences of peptide precursors have been inferred; single or paired basic amino acids generally flank the sequence of the mature peptide, and COOH-terminal glycine extended peptides (-X-Gly) have been proposed as the direct precursors to α -amidated molecules (-X-NH₂). Recently, a partial sequence of the precursor for TRH from frog skin was reported, and these data indicate that pro-TRH has at least 3 repeats of the following sequence: -Basic-Basic-Gln-His-Pro-Gly-Basic-Basic (Richter et al., 1984). This inferred peptide sequence suggests that the direct precursor to TRH is COOH-terminally glycine extended.

Several groups have recently reported an enzymatic activity involved in the COOH-terminal α -amidation of peptides. Bradbury et al. (1982) have demonstrated the existence of an enzyme in ovine pituitary that converts model peptide substrates possessing COOH-terminal glycine residues to the corresponding α -amidated products. A similar enzyme, localized to the secretory vesicles of rat anterior, intermediate, and posterior pituitary

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Correspondence should be addressed to Christopher C. Glembotski, Department of Pharmacology/G3, School of Medicine, University of Pennsylvania, 36th Street and Hamilton Walk, Philadelphia, PA 19104.

¹ Present address: Department of Medicine, Boston City Hospital, Boston, MA.

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tissue, as well as hypothalamus, has been shown to require ascorbate, CuSO_4 , and molecular oxygen for optimal activity (Eipper et al., 1983b; Emeson, 1984). The enzyme has been named peptidyl α -amidating monooxygenase (PAM), and the pituitary enzyme has been shown *in vitro* to convert COOH-terminally glycine-extended forms of α -melanocyte-stimulating hormone (α -MSH) to the α -amidated peptides in an ascorbate-dependent fashion (Glembotski, 1985). Several recent reports have suggested that a similar activity in brain homogenates is capable of converting synthetic radiolabeled TRH-Gly to material with chromatographic properties similar to that of authentic TRH (Gomez et al., 1984; Husain and Tate, 1983; Kizer et al., 1984); the ascorbate dependence of the reaction was not determined in those studies. Recent biosynthetic experiments have shown that in primary culture, intermediate pituitary cells produce COOH-terminal glycine-extended forms of α -MSH in the absence of ascorbate, while in the presence of ascorbate, α -amidated α -MSH is produced (Eipper et al., 1985; Glembotski, 1984).

Although the α -amidation of neuropeptides is a common modification and is required in most cases for optimal bioactivity, a detailed examination of the mechanism of this modification in neurons has not been performed. Since PAM is present in neural tissue, the COOH-terminal α -amidation of many neuropeptides may occur by a mechanism similar to that observed for α -MSH in the intermediate pituitary. Thus, ascorbate would be a required cofactor for the α -amidation of neuropeptides, such as the hypothalamic releasing factors. In support of this hypothesis, we report the existence of TRH and TRH-Gly in rat hypothalamus and evaluate the effects of ascorbate on the levels of these two peptides in primary cultures of fetal rat hypothalamus.

Materials and Methods

Cell culture

Hypothalamic tissue was dissected from 20 d fetal Sprague-Dawley rats and stored in sterile air-compatible Dulbecco's modified Eagle's medium during the dissection period (maximum time, 2 hr). Each hypothalamus was bisected and incubated in a mixture of collagenase (Type 2), hyaluronidase, BSA (fraction V), and DNase with stirring, as previously described for pituitary cell dissociation (Mains and Eipper, 1979). The tissue was stirred for 20–30 min at 37°C, followed by further mechanical dissociation by trituration using a plastic automatic pipet tip. After the dissociation of the tissue, the cells were collected by sedimentation, filtered through 125 μm fiber mesh (Small Parts, Inc.), collected again by sedimentation, and resuspended in the desired volume of DMEM containing 10% fetal bovine serum and 100 $\mu\text{g}/\text{ml}$ kanamycin sulfate. The cells derived from 5 to 9 fetal animals were plated onto protamine-coated 35 mm Costar culture dishes in a total volume of 4 ml. In initial studies, cultures were prepared using a previously published mechanical dissociation procedure (Faivre-Bauman et al., 1980) or a previously published procedure involving dissociation with papain (Canick et al., 1977). Culture medium was replaced at 2 d intervals. Cells were collected from dishes by scraping into 200 μl of PBS. The dishes were then rinsed twice with 500 μl of absolute methanol and the suspensions homogenized with a ground glass homogenizer (5–10 passes by hand). The homogenate (1200 μl) was centrifuged to remove precipitated material, and the supernatant was vacuum evaporated.

TRH radioimmunoassays

TRH radioimmunoassays (RIA) were performed with an antibody prepared by the method of Bassiri and Utiger (1972). This previously characterized antibody cross-reacts less than 2% as well with TRH-OH, TRH-Gly, His-Pro-NH₂, and cyclo-His-Pro as compared with authentic TRH. After vacuum evaporation of the methanol/PBS mixture, extracts were redissolved in a total volume of 200 μl of 50 mM phosphate buffer (pH 7.4) containing 2.5 mg/ml BSA. Five serial twofold dilutions of each sample were prepared in the same buffer; each sample was then incubated for 14 hr with the TRH antiserum (1:50,000 dilution) and ^{125}I -TRH trace that had been prepared as described below. Bound TRH

was separated from free TRH using a subsequent 4 hr incubation with second antibody. The midpoint of the assay was 10–15 pg TRH/tube and allowed the detection of as little as 5 pg TRH.

Preparation of TRH-Gly antiserum

TRH-Gly was custom-synthesized by Bachem (Torrance, CA) and coupled to BSA using bis-diazotized benzidine, as previously described for TRH (Bassiri and Utiger, 1972). The linkage efficiency was established as 70% by using trace quantities of ^{125}I -TRH-Gly during cross-linking. Rabbits were injected with the equivalent of 68 μg of TRH-Gly conjugated to BSA; the initial immunization was performed with an equal-volume mixture of conjugate and complete Freund's adjuvant. Booster injections were performed at approximately 4 week intervals using incomplete instead of complete Freund's adjuvant. Serum samples were obtained 2 weeks after each boost and tested for the ability to bind ^{125}I -TRH-Gly and ^{125}I -TRH. Eight rabbits were immunized, and 2 animals displayed positive titers for TRH-Gly 2 weeks after the first boost. Only one of the animals (Glynda) had a titer sufficient for further use in radioimmunoassays. TRH-Gly radioimmunoassays were performed essentially as described for TRH utilizing ^{125}I -TRH-Gly as the trace and various TRH-related peptides for displacement.

Preparation of ^{125}I -TRH and ^{125}I -TRH-Gly

Iodinations were performed with the chloramine T method previously described for TRH (Bassiri and Utiger, 1972). The iodinated peptides were purified on ODS cartridges (Waters) as previously described (Bennett et al., 1981; Court et al., 1983). The differential elution of the unlabeled and iodinated TRH-related peptides allowed for the isolation of relatively high specific activity trace.

Reversed-phase HPLC analysis of TRH

The TRH immunoactivity in culture and tissue extracts was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) on a C_{18} $\mu\text{Bondapak}$ column (3.7 mm $\times$ 30 cm, Waters) using a 0.1% trifluoroacetic acid (TFA) buffer system and gradient elution with acetonitrile, similar to a system previously described (Bennett et al., 1981; Court et al., 1983). Briefly, buffer A was 0.1% TFA and buffer B was 0.1% TFA containing 80% acetonitrile. After evaporation, extracts were redissolved in 300 μl of buffer A, applied to the column, and chromatographed at a flow rate of 1 ml of buffer A/min. After 5 min, gradient elution was initiated; the gradient progressed from 0–35% buffer B in 60 min. Fractions (0.5 ml) were collected beginning at sample application. The following peptides eluted with the following retention times: cyclo-His-Pro, 13 min; TRH, 26 min; TRH-Gly, 28 min; TRH-OH, 30 min. Although His, His-Pro, and Glu-TRH (Glu-His-Pro-NH₂) eluted before the gradient was initiated, these peptides were separated from one another. The average recovery of TRH immunoactivity from the RP-HPLC analyses was 87%.

Results

Extraction and tissue dissociation procedures

Several methods of tissue extraction are frequently used for the preparation of TRH-containing samples. We compared one of these, the PBS/methanol extraction procedure of Winokur and Utiger (1974), with a procedure we routinely use for the extraction of pro-adrenocorticotrophic hormone/endorphin-related peptides from pituitary (Eipper et al., 1985; Glembotski, 1984). The latter procedure entails homogenization of tissue in 5 N acetic acid containing 0.3 mg/ml phenylmethylsulfonyl fluoride (PMSF) and 0.1% 2-mercaptoethanol, followed by either lyophilization or vacuum evaporation. The major disadvantage of the acetic acid extraction was the apparent TRH-like immunoactivity that was contributed by the extraction solution components. All 3 components contributed apparent immunoactivity, such that the acetic acid extraction buffer blank possessed about 5 or 6 times more apparent TRH than did the methanol extraction buffer blank. The PBS/methanol extraction buffer background contributed 25 pg of apparent TRH immunoactivity, or about one-fourth of the average value obtained with only one 20 d fetal rat hypothalamus (average, 96 pg TRH).

Figure 1. Effects of ascorbate on TRH immunoactivity in hypothalamic cultures: time course. Cells were prepared from the hypothalamic tissue from 71 (with ascorbate) and 57 (without ascorbate) fetal rats (20 d), plated, and maintained in DMEM containing 10% fetal bovine serum, as described in Materials and Methods. Culture medium was replaced at 2, 4, and 6 d; we have previously shown that reduced ascorbate is stable to auto-oxidation in the presence of the added fetal bovine serum (Glembotski, 1984; P. P. Shields et al., unpublished observations). Duplicate cultures were analyzed for TRH immunoactivity at 1, 3, and 5 d. The TRH immunoactivity in each extract was normalized to content per hypothalamus; cultures with ascorbate (○) contained cells prepared from the equivalent of 8.8 hypothalamus, whereas cultures without ascorbate (●) were prepared from 7 hypothalamus each. This data represents the results from one of 2 experiments; both produced similar results.

Thus, because of the relatively low background contribution of the PBS/methanol buffer and because methanol has been shown to inhibit TRH degradative enzymes (Bassiri and Utiger, 1974; Winokur and Utiger, 1974), this was the extraction medium chosen for further studies.

Figure 2. Effect of varied ascorbate on culture TRH immunoactivity. Each dish contained cells prepared from 5 to 6.5 fetal rats and was maintained for 7 d in medium containing 10% fetal bovine serum and various levels of ascorbate. Culture medium was replaced as described in legend to Figure 1. Each point represents the immunoreactive TRH content of a single culture; each evaporated culture extract was dissolved in phosphate buffer (pH 7.4) containing 2.5 mg/ml BSA and 5 serial 2-fold dilutions were prepared and immunoassayed.

Figure 3. Effect of various forms of ascorbate on culture TRH immunoactivity. Cultures were prepared from 5 to 6.5 fetal rats each and were maintained for 7 d in medium containing 10% fetal bovine serum and 250 μM ascorbate (AA), D-isoascorbate (ISO), or diketascorbate (DKA). The diketascorbate was prepared by bubbling oxygen through a 1 mM ascorbate solution (pH 3.6) for 6 min followed by raising the pH to 7.4 and incubating at 37°C for 60 min (Tolbert and Ward, 1982). The oxidation of the ascorbate was confirmed by RP-HPLC analysis as previously described (Diliberto et al., 1983). The number of cultures assayed after each treatment (n) is shown within each histogram. The SEM was calculated for each treatment. Analysis of variance revealed significant differences among the means ($F_{3,20} = 11.81$; $p < 0.0001$). *Post hoc* Newman-Keuls comparisons demonstrated both isoascorbate and ascorbate treatments to result in significantly greater culture TRH levels than both the control (0) and diketascorbate treatments.

Since most of the experiments required the dissection of 50–80 fetal animals, we investigated whether the tissue TRH levels were stable during storage of the tissue in medium at room temperature during the 2 hr dissection period. There was no significant difference in TRH content between hypothalamic tissue extracted immediately on removal from the animal and tissue stored in medium for 2 hr before extraction (data not shown).

We were also interested in maximizing the recovery of TRH-containing cells during the dissociation procedure. Several dissociation procedures for fetal hypothalamic tissue have been reported. These include the use of papain as a proteolytic enzyme to facilitate cell dissociation (Canick et al., 1977) and mechanical procedures that depend primarily on trituration with fire-polished pasteur pipets for dissociation (Faivre-Bauman et al., 1980). In our previous work with pituitary cell dissociation, we have used a mixture of collagenase, hyaluronidase, and DNase followed by trypsin (Glembotski, 1984; Mains and Eipper, 1979). Thus, for hypothalamic dissociations we compared these 3 procedures for recovery of TRH immunoactivity. The collagenase procedure was modified by omission of the trypsin step. The papain, mechanical, and collagenase procedures resulted in the recovery of 8, 40, and 68% of the initial TRH immunoactivity, respectively. Thus, the collagenase dissociation procedure was used in all further experiments.

The plating efficiency was determined by maintaining the cultures at 37°C in 5% CO₂ for 15 hr to allow attachment to the dish, and then extracting and comparing the culture TRH immunoactivity with that of the dissociated cells before plating. After the 15 hr incubation, 61% of the TRH immunoactivity originally plated was recovered. Thus, after 15 hr in culture, 40% (68 × 61%) of the TRH immunoactivity originally in the tissue was recovered.

Figure 4. Effect of ascorbate on culture TRH immunoreactivity: RP-HPLC analysis. Two cultures, each prepared from 8 fetal hypothalamus, were maintained for 7 d with (○) or without (●) 200 μ M ascorbate. The cultures were extracted and analyzed by RP-HPLC as described in Materials and Methods. The elution position of synthetic TRH (fraction 53, 26.5 min) was determined in a parallel analysis. The recovery of TRH immunoreactivity was approximately 85%.

TRH immunoreactivity in cultures supplemented with ascorbate

To establish the importance of ascorbate in the biosynthesis of α -amidated neuropeptides, the level of TRH immunoreactivity present in hypothalamic cultures with and without ascorbate was determined. Since the TRH antiserum cross-reacts poorly with TRH-Gly, RIA with this antiserum could be used as an initial measure of the effect of ascorbate on the levels of authentic, α -amidated TRH in cultures. Culture TRH immunoreactivity decreased during the initial 3 d in all cultures. In cultures without ascorbate, the TRH immunoreactivity continued to decrease for the subsequent 4 d (Fig. 1). In contrast, cultures supplemented with 250 μ M ascorbate possessed increasing amounts of TRH immunoreactivity as a function of time from day 3–7 (Fig. 1). Four experiments of this type have shown that at 7 d, cultures supplemented with 250 μ M ascorbate possess 2- to 3-fold more TRH immunoreactivity than those maintained in ascorbate-free conditions. In preliminary experiments the ascorbate-supplemented cultures have shown a continued increase in TRH immunoreactivity through day 9, whereas cultures lacking ascorbate possess less TRH immunoreactivity at day 9 than at day 3. Thus, since TRH immunoreactivity was steadily increasing at 7 d in culture, this time period was chosen for experiments designed to probe further the nature of this response to ascorbate.

Ascorbate dose-response

To evaluate the levels of ascorbate required for increased culture TRH immunoreactivity a dose-response experiment was carried out: cultures were maintained for 7 d in medium supplemented with various amounts of ascorbate. There was a clear ascorbate concentration dependence in culture TRH immunoreactivity showing the half-optimal ascorbate concentration to be about 20 μ M (Fig. 2). The maximal TRH immunoreactivity was observed when cultures were supplemented with 50 μ M ascorbate (3-fold greater than with no ascorbate) and seemed to remain relatively constant throughout the remainder of the concentration range up to 250 μ M ascorbate. In a previous study it has been shown that cultured intermediate pituitary cells produce α -amidated α MSH in response to medium ascorbate levels between 25 and 50 μ M (Glembotski, 1984).

Figure 5. Characterization of TRH-Gly antiserum Glynda. Immunoassays using the TRH-Gly antiserum were performed as described in Materials and Methods. In each case, approximately 18,000 dpm of 125 I-TRH-Gly was used as the trace and various unlabeled peptides related to TRH were used as competing compounds. Total binding of 125 I-TRH-Gly was between 30 and 40%, and the nonspecific binding was less than 3%.

Various forms of ascorbate

Since the fully reduced form of L-ascorbate is the major form of the cofactor in the circulation and in cells (Hornig, 1975), we were able to establish further that the increase in TRH immunoreactivity in ascorbate-supplemented cultures was physiologically relevant by testing fully oxidized ascorbate and a stereoisomer of L-ascorbate, D-isoascorbate, as cofactors. Oxidized ascorbate (diketoascorbate) at 250 μ M was not capable of producing an increase in culture TRH immunoreactivity (Fig. 3); this is consistent with the observation that the oxidized form of ascorbate is not assimilated by pituitary (P. P. Shields, T. R. Gibson, and C. C. Glembotski, unpublished observations) and adrenal medullary cells in culture (Diliberto et al., 1983). D-Isoascorbate was also capable of producing an equivalent increase in culture TRH immunoreactivity to that observed with ascorbate, consistent with the observation that D-isoascorbate can support α -amidation of α MSH in intermediate pituitary primary cells (Glembotski, 1984).

HPLC verification of culture TRH

RP-HPLC was used to characterize further the TRH immunoreactivity in culture extracts. All of the culture immunoreactivity comigrated with authentic TRH and the 2- to 3-fold increase in immunoreactivity observed in crude culture extracts was reflected by a similar increase observed after HPLC analysis (Fig. 4).

TRH-Gly antiserum Glynda

The increase in TRH immunoreactivity with ascorbate supplementation could be due to an inhibition of secretion by the cofactor, or to the ascorbate-dependent conversion of TRH-Gly to TRH. To distinguish between these possibilities, an antiserum was prepared to TRH-Gly to enable the direct determination of levels of this peptide in culture extracts. At a dilution of 1:400, the IC_{50} for antiserum Glynda was between 400 and 800 pg TRH-Gly/tube (Fig. 5). The relative cross-reactivities for various peptides and amino acids related to TRH are summarized in Table 1. As expected for an antiserum raised to

Figure 6. RP-HPLC characterization of TRH and TRH-Gly. Forty rat hypothalami from adult animals were extracted, chromatographed on a Sephadex G-75 column in 10% formic acid/0.1 mg/ml BSA, and the TRH-related material was evaporated and chromatographed by RP-HPLC as described in Materials and Methods. Fraction collection (0.5 ml) was initiated 5 min after sample application. The arrows mark the elution positions of standard synthetic TRH and TRH-Gly as localized by A_{214} in a different analysis. Synthetic TRH and TRH-Gly eluted at approximately 26 and 28 min, respectively.

a small peptide, there is a high degree of selectivity. Compared to TRH-Gly, TRH is only 0.01% as potent as TRH-Gly at displacing ^{125}I -TRH-Gly from the antiserum (Table 1). Other peptides related to TRH that are suspected metabolites were also tested (Table 1, Fig. 5). These metabolites might be present in cell and tissue extracts, but since they all have relatively low cross-reactivity with the antiserum, TRH-Gly levels could be determined using relatively crude samples. For routine TRH-Gly immunoassays, extracts were purified on ODS cartridges.

Levels of TRH and TRH-Gly immunoactivity in hypothalamic tissue extracts

A recent developmental study has demonstrated a continual increase in hypothalamic TRH content in rats as a function of both pre- and postnatal development. At 21 d gestation, 7 d postnatal, and in the adult, the hypothalamus has been reported to contain 41, 458, and 2696 pg TRH immunoactivity per structure (Lamberton et al., 1984). In the present study, rats of var-

Table 2. Levels of TRH and TRH-Gly in hypothalamic tissue

Animal age	TRH (pg/hypo)	TRH-Gly (pg/hypo)	TRH/TRH-Gly
Fetal	52.5 $\pm$ 9.0	44.5 $\pm$ 0.8	1.2
Neonatal	318.9 $\pm$ 12.5	64.5 $\pm$ 4.9	4.9
Adult	2788.9 $\pm$ 146.7	106.4 $\pm$ 3.6	26.2

Conditions: 123 fetal (20 d gestation), 13 neonatal (7 d old), and 20 adult (150–200 gm) rats were sacrificed, the hypothalamic tissue removed, and extracted as described in Materials and Methods. After ODS cartridge purification, the extracts were evaporated and analyzed for TRH and TRH-Gly immunoactivities. Three aliquots equivalent to $\frac{1}{25}$ (TRH) or $\frac{1}{5}$ (TRH-Gly) of each extract were assayed for peptide content; values shown are averages $\pm$ SEM. In a parallel group of extracts, the overall recoveries of ^{125}I -TRH and ^{125}I -TRH-Gly were 87 and 92%, respectively.

ious ages were used to determine whether the hypothalamic content of TRH-Gly varies in a similar manner with development. As previously reported, the TRH levels increased as a function of age; however, the TRH-Gly levels remained relatively unchanged. There were similar quantities of TRH and TRH-Gly immunoactivity in hypothalamic extracts from 20 d prenatal animals, while in the adult the ratio of TRH/TRH-Gly was 26/1 (Table 2). This may be due to developmental differences in the expression of the gene for TRH and the gene encoding the amidation enzyme, PAM. Alternatively, the hypothalamic cells in the fetal and newborn animal may be less ascorbate-responsive than adult hypothalamic cells, with respect to peptide amidation.

Effect of ascorbate on levels of TRH and TRH-Gly immunoactivity in cultured hypothalamic cells

In a preliminary experiment, the levels of TRH and TRH-Gly immunoactivity in cultured cells maintained with or without ascorbate were determined. Thirty cultures were prepared from a total of 120 fetal hypothalami and maintained (half with and half without ascorbate) for 7 d. Interestingly, the levels of TRH and TRH-Gly in the extracts responded oppositely to the presence of the cofactor in the medium. Cultures supplemented with ascorbate contained 106 pg TRH-Gly immunoactivity and 100 pg TRH immunoactivity. Cultures maintained without the cofactor contained 310 pg TRH-Gly immunoactivity and 35 pg of TRH immunoactivity.² These results support the hypothesis that TRH-Gly is an alternate end product that is formed in hypothalamic cells in the absence of ascorbate.

HPLC analysis of TRH and TRH-Gly immunoactivities

To verify further the identification of TRH- and TRH-Gly immunoactivities in hypothalamic extracts, reversed-phase- and ion-exchange-HPLC analyses were performed. Adult hypothalamic tissue was extracted, submitted to ODS cartridge purification, and then chromatographed on a C_{18} μ Bondapak HPLC column in a TFA/acetonitrile solvent system (see Materials and Methods). TRH and TRH-Gly immunoactivities eluted with retention times identical to the synthetic peptides (Fig. 6). The recoveries of both TRH and TRH-Gly from the tissue were greater than 75%. The ratio of TRH to TRH-Gly as determined after RP-HPLC was similar to that determined in the crude extracts by RIA. This indicated that the measurement of TRH and TRH-Gly cross-reactive material in crude extracts constitutes an accurate determination of the true levels of the two peptides. When the TRH and TRH-Gly from the RP-HPLC

Table 1. Specificity of the TRH-Gly antiserum Glynda

Peptide	Percent cross-reactivity
TRH-Gly	100
His-Pro	9
His	5
Pro	2.5
Cyclo-His-Pro	0.75
Pyro-Glu-His-Pro-Gly-NH ₂	0.09
Glu-His-Pro-NH ₂	0.09
Pro-NH ₂	0.08
TRH-OH	0.07
TRH	0.01

Immunoassays were performed as described in Materials and Methods. Various unlabeled peptides and amino acids were used to compete with the binding of ^{125}I -TRH-Gly to antiserum Glynda. Percent cross-reactivity = $[\text{IC}_{50} \text{ of TRH-Gly} / \text{IC}_{50} \text{ of competitor}] \times 100$.

² Since the analysis of 360 fetal hypothalami was not feasible, a single determination was performed. However, since the average SEM in all other culture extract RIA analyses was 8.5%, and the maximum error observed was 23%, the 300% changes in both TRH and TRH-Gly levels as a function of ascorbate supplementation are clearly greater than the inherent error in these experiments.

analysis (Fig. 6) were treated further with pyroglutamate aminopeptidase, there was a dramatic drop in the cross-reactive material in both peaks. This indicated that both immunoactive peaks contained material possessing an NH_2 -terminal pyroglutamate residue that was required for cross-reactivity. Specificity tests for both the TRH and TRH-Gly antisera indicated the requirement for the NH_2 -terminal pyroglutamate residue (Fig. 5).

Further analysis of TRH- and TRH-Gly-related peptides using RP-HPLC in a heptafluorobutyric acid/acetonitrile solvent system, and using cation-exchange HPLC in an ammonium formate solvent system, verified that the immunoreactive TRH and TRH-Gly comigrated with synthetic standard peptides (not shown).

Discussion

α -Amidation is a common posttranslational modification among peptides and is required in most cases for optimal bioactivity (Mains et al., 1983). Thus, it is important to elucidate the mechanism of α -amidation in neurons, since it is likely to be an important regulatory point in neuropeptide biosynthesis. In this paper we have presented evidence that the α -amidation of TRH in hypothalamic cells is ascorbate-dependent and that a COOH-terminally glycine-extended form of TRH (pyro-Glu-His-Pro-Gly; TRH-Gly) is a likely intermediate in the biosynthetic pathway for this neuropeptide. These present results extend our previous studies of α MSH biosynthesis in the intermediate pituitary and suggest that the α -amidation of many, if not all, neuro- and endocrine peptides is dependent on ascorbate. It is also apparent that in both endocrine and neuronal cells similar α -amidation enzyme activities are responsible for the conversion of COOH-terminally glycine-extended peptides to COOH-terminally α -amidated molecules.

Initial experiments with cultured hypothalamic cells demonstrated that TRH content in the cultures was increased by the addition of ascorbate to the medium (Fig. 1). The cultures were then maintained in medium supplemented with various amounts of ascorbate and demonstrated a half-maximal increase in TRH content at about 20 μM ascorbate (Fig. 2). This half-maximal level of ascorbate is consistent with the 20–50 μM levels of the cofactor found in the circulation (Hornig, 1975), as well as the K_m values of the ascorbate transport system in anterior pituitary (P. P. Shields, T. R. Gibson, and C. C. Glembotski, unpublished observations) and adrenal medullary cells (Diliberto et al., 1983). In intermediate pituitary cultures, the conversion of COOH-terminally glycine-extended forms of α MSH to α -amidated forms is also dependent on ascorbate with a half-maximal response at about 20 μM ascorbate (Glembotski, 1984). Similar levels of ascorbate are required to support the dopamine β -hydroxylase-mediated conversion of dopamine to norepinephrine in sympathetic cervical ganglion cells in culture (Mains and Patterson, 1973a, b). The pharmacological profile of the increase in hypothalamic culture TRH content also demonstrated the requirement for the reduced form of ascorbate (Fig. 3). D-Isoascorbate, a stereoisomer of ascorbate, was capable of supporting the increase in TRH content; this has also been demonstrated for α MSH formation by intermediate pituitary cells (Glembotski, 1984). Thus, the cultured hypothalamic cells are responsive to ascorbate forms and levels consistent with other well-characterized ascorbate-dependent cellular functions.

To demonstrate more directly that ascorbate facilitates the α -amidation of TRH by way of a COOH-terminally glycine-extended intermediate, an antibody was raised to TRH-Gly. Immunoreactive TRH-Gly was detected in hypothalamic tissue and culture extracts; RP- and ion-exchange-HPLC analysis further verified the identity of TRH-Gly immunoactivity in hypothalamic extracts. Further evidence that TRH-Gly is an intermediate in TRH formation was obtained from experiments

using hypothalamic cultures maintained with and without ascorbate (see Results). The levels of TRH and TRH-Gly immunoreactivity responded diametrically: Cultures supplemented with ascorbate contained about 3-fold more TRH and 3-fold less TRH-Gly than those maintained in medium lacking ascorbate. Since these changes are much greater than the average error of all similar experiments (see footnote, p. 1800), this result suggests that ascorbate is required for the conversion of endogenous TRH-Gly to TRH in cultured hypothalamic cells.

Immunoreactive TRH and TRH-Gly levels were also determined in hypothalamic extracts derived from rats of various ages (Table 2). The ratio of TRH/TRH-Gly immunoactivities increased as a function of animal age. The similarity in the TRH/TRH-Gly immunoactivity ratio between fetal hypothalamic extracts (TRH/TRH-Gly = 1.2) and extracts of fetal hypothalamic cells maintained in culture with ascorbate (TRH/TRH-Gly = 0.94) indicated that the cofactor supplementation *in vitro* closely simulated *in vivo* conditions. The increase in the TRH/TRH-Gly immunoactivity ratio as a function of age may be due to a corresponding developmental increase in PAM activity as a result of either a change in enzyme-specific activity or a change in the responsiveness of hypothalamic cells to ascorbate.

It has been observed that extra-hypothalamic TRH levels change in different ways during development. For example, in fetal rats (21 d gestation) the olfactory bulbs contain no detectable TRH (Lamberton et al., 1984), yet in the adult as much as 1.8 ng TRH has been localized to this CNS structure (Kreider et al., 1981; Lamberton et al., 1984). One possibility is that fetal olfactory bulbs contain TRH-Gly but are unable to convert it to TRH. We were unable to detect either TRH or TRH-Gly immunoactivity in extracts of fetal olfactory bulb. Thus, if fetal olfactory bulb cells express the gene for TRH, the lack of TRH and TRH-Gly indicate that posttranslational processing of TRH precursors is not complete. Also, neonatal rat pancreas has been reported to contain more TRH than the neonatal rat hypothalamus. However, unlike hypothalamic TRH levels, pancreatic TRH levels decline from birth throughout 28 d of age (Lamberton et al., 1984). We were able to detect TRH in fetal (20 d gestation) pancreas in quantities similar to fetal hypothalamus, but, unlike the hypothalamus, there was no detectable TRH-Gly (not shown). This may indicate that in the pancreas at 20 d gestation, the gene for TRH is no longer expressed, and all precursor forms of the peptide have been converted to the product. These interesting tissue-specific differences in the developmental profile of TRH provide a unique model system with which to characterize the various factors responsible for the expression of the same peptide (TRH) in neuroendocrine (hypothalamus), neuronal (olfactory), and endocrine (pancreatic) cells.

An important approach to further studies on TRH biosynthesis will involve the incorporation of radiolabeled amino acids into TRH-related peptides. Such studies should be performed with cultured cells in order to facilitate quantitative pulse-chase studies for verification of product-precursor relationships. The incorporation of labeled amino acids into authentic TRH by cultured hypothalamic cells obviously requires the continued formation of the peptide *in vitro*. The present data support the notion that without ascorbate in the medium, very little, if any, incorporation would take place. For example, intermediate pituitary cells maintained in culture without ascorbate lose the ability to form α -amidated α MSH with a half-time of about 15 hr (Eipper et al., 1983a; Glembotski et al., 1983); these cells produce the COOH-terminally glycine-extended form of the peptide as an alternate end product. Further studies involving the strategic use of ascorbate and the TRH-Gly antiserum described in this report will be most important in biosynthetic labeling studies designed to demonstrate the precursor-product relationship between TRH-Gly and TRH.

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