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TRH-extended peptides in the olfactory lobe are formed by incomplete cleavage at pairs of arginine residues in the TRH prohormone

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High concentrations of thyrotrophin-releasing hormone (TRH) are known to be present in the olfactory lobe, and the processing of the TRH prohormone in this region of the brain has been examined in this study. TRH-extended peptides have been detected in the rat olfactory lobe: these peptides accounted for approximately 11% of the total TRH immunoreactivity present in the tissue and contained the sequence pGlu-His-Pro-Gly-Arg exclusively at their N-termini. Extended peptides containing pGlu-His-Pro-Gly-Lys at their N-termini were not detected suggesting that incomplete cleavage occurs only at Arg-Arg residues in the TRH-prohormone. In view of the highly specific processing of the prohormone, it is likely that the TRH-extended peptides play important physiological roles.

Olfactory lobe; Thyrotrophin-releasing hormone; Prohormone; Processing

1. INTRODUCTION

The TRH prohormone in the rat contains 5 copies of the tetrapeptide sequence Gln-His-Pro-Gly, each flanked by pairs of basic residues and separated by intervening sequences of varying size and composition [1]. The basic pair, which is the recognition site for proteolytic processing, is always Lys-Arg on the N-terminal side of the tetrapeptide sequence but can be either Lys-Arg or Arg-Arg on the C-terminal side. Cleavage at all the processing sites, followed by exopeptidase trimming of the basic residues and α-amidation of the C-terminal proline using glycine as nitrogen donor, will yield 5 copies of TRH (pGlu-His-ProNH_2). In addition 7 peptides can be formed which are unrelated to the TRH sequence: several of these peptides have been detected as processed products in rat CNS, pancreas and thyroid [2–10]. However, partial processing of the prohormone to produce TRH-extended peptides can occur under some conditions [5,9,11]; these processing reactions appear to be highly specific because only C-terminally extended forms of TRH are observed in rat hypothalamus and spinal cord [11].

In this study, TRH-extended peptides have been detected in substantial concentrations in the rat olfactory lobe. These peptides contain the sequence pGlu-His-Pro-Gly-Arg at their N-termini and must be formed by incomplete cleavage at pairs of arginine residues in the TRH prohormone.

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2. EXPERIMENTAL

2.1. *Peptide synthesis*

The structures of the 43, 28 and 16 residue C-terminally extended TRH-related peptides, where each represents TRHgly attached to an intervening sequence, were predicted from the sequence of the rat prohormone [1]. These TRH-related peptides, together with pGlu-His-Pro-Gly-Lys and pGlu-His-Pro-Gly-Arg, were synthesised by conventional fluorenylmethoxycarbonyl methodology and purified as described previously [11,12]. The sequences of the C-terminally extended peptides were as follows:

16 residue peptide:
pGlu-His-Pro-Gly-Arg-Arg-Ser-Phe-Pro-Trp-Met-Glu-Ser-Asp-Val-Thr

28 residue peptide:
pGlu-His-Pro-Gly-Arg-Arg-Phe-Ile-Asp-Pro-Glu-Leu-Gln-Arg-Ser-Trp-Glu-Glu-Lys-Glu-Gly-Glu-Gly-Val-Leu-Met-Pro-Glu

43 residue peptide:
pGlu-His-Pro-Gly-Arg-Arg-Ala-Asn-Gln-Asp-Lys-Tyr-Ser-Trp-Ala-Asp-Glu-Glu-Asp-Ser-Asp-Trp-Met-Pro-Arg-Ser-Trp-Leu-Pro-Asp-Phe-Phe-Leu-Asp-Ser-Trp-Phe-Ser-Asp-Val-Pro-Gln-Val

2.2. *Detection of TRH-related peptides in olfactory lobe*

Olfactory lobe (2.5 g wet weight) was obtained from 40 female Sprague-Dawley rats (average weight, 200 g). The tissue was homogenised in ice-cold acidified acetone and the supernatant dried in vacuo before dissolving in 25% acetic acid [11]. The peptides were resolved by gel exclusion chromatography on Sephadex G-50 superfine (1 × 100 cm) in 25% acetic acid. The column was previously calibrated with the iodinated 43, 28, 16 and 3 residue TRH-related peptides. Aliquots of each fraction were dried in vacuo and the immunoreactive TRH detected by radioimmunoassay (RIA) as described previously [11]. C-Terminally TRH-extended peptides were detected by RIA with a TRH pentapeptide antibody which was raised to pGlu-His-Pro-Gly-Lys [13], after excision of the immunoreactive fragment with trypsin (20 μg of enzyme in 50 μl of 0.05 M sodium phosphate, pH 8.2, 37°C, 6 h). N-Terminally TRH-extended peptides were assayed by RIA with the TRH antibody after tryptic cleavage followed by cyclisation of the N-terminal glutamine (50% acetic acid, 100°C, 20 min).

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2.3. *Cation-exchange chromatography*

In order to confirm the identity of the low molecular weight TRH immunoreactive peptide and the tryptic fragment from the C-terminally extended peptides, both were subjected to ion-exchange chromatography on a column (0.7 × 60 cm) of SP-Sephadex C-25 (sodium form) in 25% acetic acid with a linear gradient from 0 to 0.5 M NaCl in 25% acetic acid. Radio-iodinated synthetic TRH and pGlu-His-Pro-Gly-Lys (TRHglylys) were added to mark the positions of the authentic peptides at levels that did not interfere with the RIAs used to detect the endogenous peptides.

2.4. *Anion-exchange chromatography*

The antibody raised to TRHglylys cross-reacts equally with the pentapeptides ending with lysine and arginine [13]. In order to determine the structure of the TRH pentapeptide at the N-terminus of the TRH-extended peptides, it was necessary to develop a chromatographic procedure to separate TRHglylys and TRHglyarg. Both synthetic and endogenous peptides were treated with citriconic anhydride to citriconylate the lysine residues as described previously [14]. The peptides were then subjected to ion-exchange chromatography on a column (0.7 × 60 cm) of QAE-Sephadex A-25 in 0.05 M Tris-HCl, pH 7.6, with a linear gradient from 0 to 0.5 M NaCl in 0.05 M Tris-HCl, pH 7.6. Radio-iodinated synthetic TRHglylys was included during citriconylation of endogenous peptides in order to follow the reaction.

3. RESULTS AND DISCUSSION

The TRH-related peptides from extracts of rat olfactory lobe were separated by gel exclusion chromatography (Fig. 1). Before enzymic digestion, RIA with the TRH antibody revealed only low molecular weight TRH immunoreactivity (68.4 pmol/g wet weight); whereas after treatment with trypsin, two groups of TRH-extended peptides (peak 1 = 5.5 pmol/g; peak 2 = 2.1 pmol/g) were detected in addition to low molecular weight TRH. One of the groups had a molecular size corresponding to the 16 residue marker, while the other group of higher molecular weight emerged in fractions close to the elution positions of the 43 and 28 residue markers.

The TRH-extended peptides, which represented approximately 11.0% of the total TRH immunoreactivity, must contain the sequence pGlu-His-Pro-Gly-Lys(Arg)... at their N-termini because tryptic cleavage released fragments that were immunoreactive with the TRH pentapeptide antibody. The molecular sizes of the peptides are consistent with each C-terminal extension consisting of one intervening sequence. N-Terminally extended peptides with the TRH immunoreactive tryptic fragment at their C-termini were not detected in extracts of the olfactory lobe.

The TRH-related peptides present in the olfactory lobe are similar to those that we have found previously in rat central nervous system both in molecular size and direction of the extension sequence [11,15]. However, it is of interest that a high molecular weight fraction containing internal ...Gln-His-Pro-Gly-Lys(Arg)... sequences in hypothalamus and spinal cord could not be detected in the olfactory lobe; this suggests that

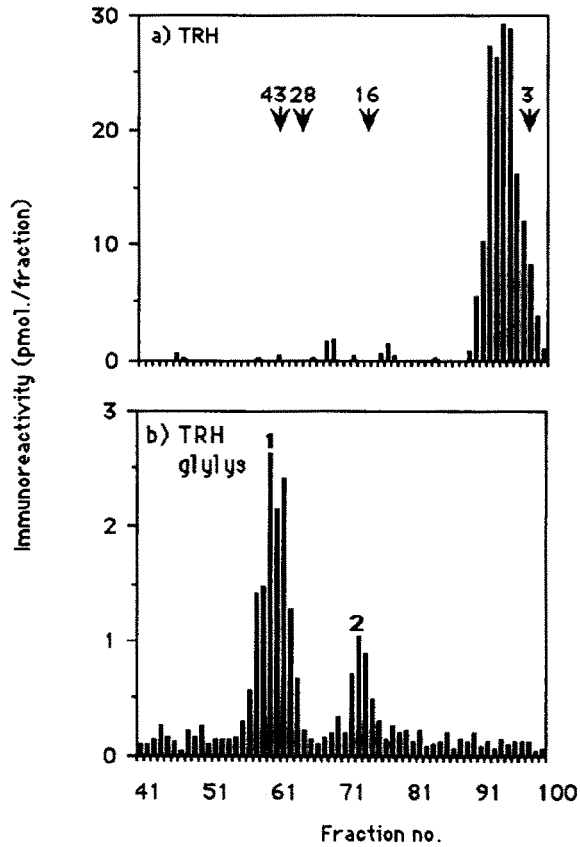

Fig. 1. TRH-related peptides in rat olfactory lobe. Peptides were resolved by gel exclusion chromatography (Sephadex G50 superfine). The arrows indicate the elution positions of the radio-iodinated TRH (3 residues) and the TRH-related peptides (43, 28 and 16 residues) used as molecular markers to calibrate the column. Aliquots of each fraction were assayed for (a) TRH and (b) TRH pentapeptide after enzymic digestion as described in section 2.

tissue-specific processing of the TRH prohormone may occur under some conditions. In this context, others have found different ratios of several of the peptides derived from the TRH prohormone in rat hypothalamus and olfactory lobe [3,5].

A TRH immunoreactive peptide non-identical with authentic TRH (pGlu-His-ProNH$_2$) has recently been identified in rabbit prostate [16] and human semen [17]. The new peptide contains a glutamic acid substitution for histidine at position 2 and is negatively charged at physiological pH. The TRH immunoreactive peptide from rat olfactory lobe was found in this study to co-elute with synthetic TRH during cation-exchange chromatography (Fig. 2a) and is thus likely to be identical to authentic TRH. In contrast, a peptide with an acidic substitution at position 2 of TRH will not bind to a cation-exchange column and will emerge before TRH close to the void volume of the column. TRH immunoreactivity was not detected in early fractions from the ion-exchange column (Fig. 2a); although low levels of the peptide may be difficult to detect by RIA because the peptide, pGlu-Glu-ProNH$_2$, cross-reacts only 50% with the TRH antibody [16].

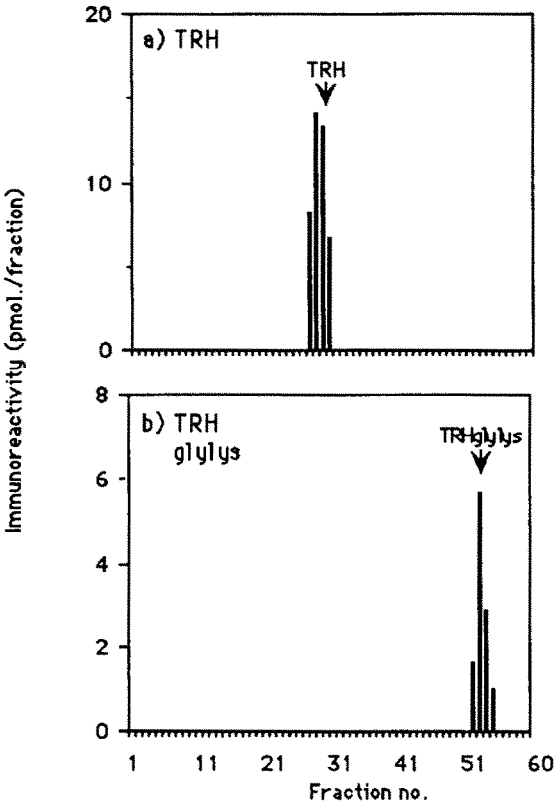

Fig. 2. Cation-exchange chromatography of the TRH-related peptides in rat olfactory lobe. (a) Low molecular weight TRH immunoreactivity (10 lobes) and (b) the tryptic fragment from the TRH-extended peptides (40 lobes) were subjected to chromatography as described in section 2. Aliquots of each fraction were assayed for TRH or TRH pentapeptide where appropriate. The position of the arrows indicate the elution positions of synthetic TRH and TRHglylys. It should be noted that radio-iodinated TRH co-elutes with cold TRH whereas radio-iodinated TRHglylys elutes 2 fractions ahead of cold TRHglylys.

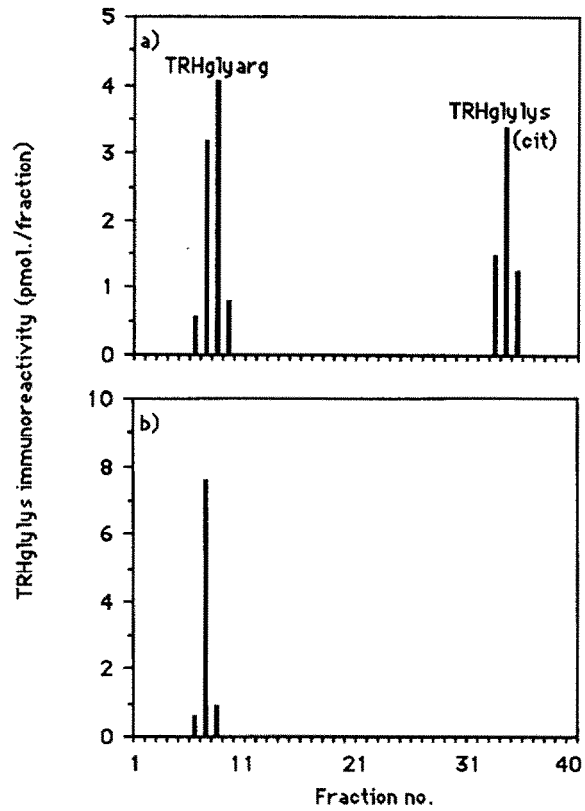

Fig. 3. Anion-exchange chromatography of the tryptic fragment from the TRH-extended peptides in rat olfactory lobe. (a) Synthetic TRHglyarg and TRHglylys and (b) the tryptic fragment from the C-terminally extended peptides were treated with citraconic anhydride and then subjected to chromatography as described in section 2.

The pair of basic amino acids on the C-terminal side of the TRH tetrapeptide sequence (...Gln-His-Pro-Gly...) in the prohormone can be either Lys-Arg (2 cases) or Arg-Arg (3 cases) [1]. Tryptic cleavage of the C-terminally extended peptides possessing the Lys-Arg pair will release pGlu-His-Pro-Gly-Lys, whereas the peptides with the Arg-Arg pair will produce pGlu-His-Pro-Gly-Arg. The fractions from the gel filtration column (Fig. 1) containing the C-terminally extended TRH-related peptides (peaks 1 and 2) were pooled, dried, trypsinised and then subjected to cation-exchange chromatography (Fig. 2b). The tryptic fragment which was immunoreactive with the TRH pentapeptide antibody was found to co-elute with synthetic TRHglylys. This experiment could not distinguish between the pentapeptides ending in lysine and arginine because the synthetic peptides were previously found to co-elute during chromatography under the conditions used in Fig. 2 (data not shown).

In order to distinguish pGlu-His-Pro-Gly-Lys from pGlu-His-Pro-Gly-Arg and to characterise the intact processing site in the C-terminally extended peptides, a chromatographic procedure was developed to separate the 2 peptides. The peptides were treated with citraconic anhydride, to citraconylate the side chain epsilon amino-group of lysine, and then they were separated by anion chromatography at pH 7.6. Fig. 3a illustrates the separation by anion-exchange chromatography of the synthetic peptides which had been treated with citraconic anhydride; by virtue of its overall negative charge at pH 7.6, the citraconylated TRHglylys bound to the column whereas the neutral TRHglyarg eluted in the void volume.

The fractions from the cation-exchange column (Fig. 2b) which contained the TRH pentapeptide immunoreactive peptides from olfactory lobe were pooled, dried and desalted by gel exclusion chromatography (Sephadex G10). The endogenous peptides were then treated with citraconic anhydride: radio-iodinated synthetic TRHglylys (10^3 dpm) was included in the reaction at a level that would not interfere with the RIA. During anion-exchange chromatography, all of the radio-iodinated TRHglylys bound to the column whereas the TRH pentapeptide immunoreactivity eluted in the void volume (Fig. 3b). Thus tryptic cleavage of the C-terminally extended peptides appears

to yield TRHglyarg in the absence of any detectable TRHglylys indicating that the unprocessed cleavage site is exclusively Arg-Arg in these peptides.

The TRH prohormone can yield three C-terminally extended TRH-related peptides containing the Arg-Arg basic pair; these peptides contain 43, 28 and 16 residues and have the structures shown in section 2. However, partial processing at the Arg-Arg sites must occur because the extended peptides represent only 11% of the total TRH immunoreactivity. Differential cleavage at Lys-Arg and Arg-Arg sites in the TRH prohormone may suggest that the endoprotease involved in processing exhibits a preference for the Lys-Arg site. Alternatively two distinct enzymes may be required for complete processing at both sites, and tissues which produce TRH-extended peptides may express the enzyme that cleaves mainly at Lys-Arg residues. Recently two Ca-dependent acidic endoproteases have been isolated from the pancreatic β-cell [18]. One protease cleaves only on the C-terminal side of Arg-Arg in proinsulin while the other cleaves preferentially on the C-terminal side of Lys-Arg.

In summary, processing at Lys-Arg residues flanking the TRH tetrapeptide sequence in the prohormone appears to be complete but incomplete processing at the Arg-Arg residues leads to the formation of a series of C-terminally TRH extended peptides in the rat olfactory lobe. Although C-terminally TRH extended peptides have been observed previously in the rat central nervous system [5,11,15], this is the first study to indicate that they are formed exclusively by incomplete cleavage at pairs of arginine residues. Such highly specific processing of the TRH prohormone suggests that the extended peptides may play important physiological roles.

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