The TRH-like peptides in rabbit testis are different from the TRH-like peptide in the prostate

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Abstract Human seminal fluid contains a number of tripeptide amides with similar structures to thyrotropin releasing hormone (TRH), two of which have been identified as pGlu-Glu-Pro amide and pGlu-Phe-Pro amide. To determine whether these peptides originate in the same tissues and have the same molecular origin, TRH-immunoreactive peptides were extracted from the prostate and testis of the rabbit, purified by ion exchange chromatography and HPLC, and identified by co-chromatography with ^{3}H-labelled marker peptides. In addition, trypsin digestion was used to release TRH-like tripeptides from N-extended forms of these peptides. The sole TRH-like peptide in the prostate was shown to be pGlu-Glu-Pro amide; it was not accompanied by a detectable amount of pGlu-Phe-Pro amide. The prostate also appeared to contain a very small amount of N-extended forms of these peptides. In contrast to the prostate, the testis contained high concentrations of N-extended forms of pGlu-Phe-Pro amide but essentially no tripeptide. The testis also contained N-extended forms of two other neutral TRH-like peptides which were less hydrophobic than pGlu-Phe-Pro amide. Neither the prostate nor the testis contained a significant amount of TRH. The results show that in the rabbit the TRH-like peptides pGlu-Glu-Pro amide and pGlu-Phe-Pro amide occur in different tissues and appear to be formed from different precursors.

Key words: Thyrotropin releasing hormone; Tripeptide amide; TRH-immunoreactive peptide; Testis; Prostate

1. Introduction

Initial reports that rat prostate and human seminal fluid contain peptides with TRH-immunoreactivity [1,2] led to the identification of two novel TRH-like tripeptides [3,4]. These peptides were related in structure to TRH, the histidine residue at position 2 of the hormone being by replaced by either glutamic acid or phenylalanine. Since the TRH-like peptides were present in the seminal fluid, it was anticipated that they might be important for fertility. Recently a potent activity was demonstrated for pGlu-Glu-Pro amide in increasing the capacitation of sperm [5,6] and this peptide has also been found to increase the motility of sperm from certain infertile males [7]. Similar studies have yet to be reported with the phenylalanine containing tripeptide but the presence of a C-terminal amide group in these peptides, a structure possessed by a multiplicity of peptide hormones, indicates that the TRH-like peptides will be found to fulfil physiological functions.

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When pGlu-Glu-Pro amide and pGlu-Phe-Pro amide were isolated from seminal fluid, it was not known whether they originated from the same or from different tissues. Furthermore, since their sequences do not occur in the TRH-prohormone [8,9], it is clear that these TRH-like peptides must be formed by the processing of precursors distinct from that of TRH. In this communication we present evidence that pGlu-Glu-Pro amide and pGlu-Phe-Pro amide originate in different tissues and seem likely to be formed from different precursors.

2. Experimental

2.1. Dissection and extraction of tissues

Prostate and testis were removed from a New Zealand White rabbit (3.1 kg) immediately after sacrifice. The tissues were frozen at −70°C and stored at this temperature until they were extracted. Each tissue was weighed and homogenised at 4°C for 3 min in 10 ml of acid acetone (40 ml of acetone: 1 ml of concentrated HCl: 5 ml of H_2O) using an Ultra-Turrax homogenizer. To each homogenate was added ^{125}I-labelled TRH (10^4 counts, prepared by labelling synthetic TRH with [^{125}I]iodine using the chloramine-T method) together with [^{3}H]pGlu-Glu-Pro amide and [^{3}H]pGlu-Phe-Pro amide (approximately 5.10^3 counts of each peptide, generously donated by Dr. R. Bilek) which served as markers to indicate the elution positions of the corresponding endogenous peptides. The ^{3}H-labelled peptides also provided internal standards to calculate the recoveries of the endogenous peptides [10]. The suspensions were centrifuged at $2.10^4 \times g$, 4°C for 30 min, the supernatant solutions decanted and the solvents removed in vacuo on a rotary evaporator.

2.2. Trypsin digestion of N-extended forms of TRH-like peptides

The dried tissue extracts were taken up in 10 ml of 50% acetic acid and divided into two equal parts, the first for trypsin digestion to release TRH-like peptides from their N-extended forms and the second for direct RIA to determine the free TRH-like tripeptides. Each fraction was evaporated in vacuo. Trypsin digestion was carried out in 2 ml of 100 mM Tris hydrochloride at pH 8 in the presence of TPCK-trypsin (40 μl of 1 mg/ml); the digest was incubated at 37°C for 16 h and then was maintained at 100°C for 1 h to complete the cyclization of N-terminal glutaminyl residues to pyroglutamic acid. The solutions were dried in vacuo and the peptides extracted into methanol (5 ml). The methanol solution was decanted, the solvent evaporated in vacuo and the residual peptides dissolved in 1 ml of 50% acetic acid in preparation for mini-column cation exchange chromatography. The remaining half of the initial tissue extracts, which would contain free TRH-like tripeptides, was not digested with trypsin but like the trypsin digested fraction was purified by methanol extraction prior to mini-column chromatography.

2.3. Separation of TRH-like peptides from TRH by chromatography on cation exchange mini-columns

Briefly the procedure involved addition of the methanol extracted peptide mixtures in 50% acetic acid (0.9 ml) to a mini-column (6 × 0.5 cm) of SP-Sephadex C25, prepared in the pyridinium form by washing with 1 M-HCl and then successively with H_2O, 1 M-pyridine, H_2O, and 50% acetic acid (2 ml each). After addition of the sample, the column

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was eluted in 50% acetic acid (8 × 0.5 ml fractions) and then in 0.4 M pyridine in 50% acetic acid (12 × 0.5 ml fractions). Using this method each peptide mixture added to the column was divided into 2 fractions, the first containing peptides that were neutral or acidic and the second peptides that carried a positive charge. Thus TRH was retained during the elution with 50% acetic acid while the TRH-like peptides emerged without retention. To confirm the retention of TRH in each application of the mini-column, the [^{125}I]TRH present in each sample was measured by γ-counting of the eluted fractions. Aliquots (50 μl) of each fraction were then dried in a vacuum centrifuge (V.A. Howe, Banbury, Oxfordshire, UK) and the TRH-like peptides determined by RIA with a TRH-antiserum. The TRH-immunoreactive peptides in the column fractions were combined in two groups: non-retained (Fractions 4–8) and retained (fractions 12–17). The resulting solutions were concentrated in vacuo and the residual peptides dissolved in 1 ml of 10 mM-HCl in preparation for HPLC.

2.4. Resolution of TRH-like peptides by HPLC

High performance liquid chromatography was carried out on a C18 μBondapak column (0.39 × 30 cm, 10 μm particle size, Millipore-Waters, UK, Watford, Herts., UK) with 10 mM HCl as the stationary phase and methanol as the mobile phase. A linear gradient of 0.5% methanol/min was applied during collection of the first 20 fractions (6 min), after which the gradient was increased to 2%/min for the remaining 80 fractions (24 min). The flow rate used was 1.5 ml/min. Included with the sample added to the HPLC column was ^{3}H-labelled TRH (Du Pont Ltd., NEN Products, Stevenage, Herts., UK, 119 mCi/μmol, approximately 5×10^{3} counts) in addition to the two H^{3}-labelled TRH-like peptides that were added to the initial tissue extracts. In this way each sample added to the HPLC column included three ^{3}H-labelled markers, pGlu-His-Pro amide (TRH), pGlu-Glu-Pro amide, and pGlu-Phe-Pro amide. Aliquots (50 μl) of each fraction obtained by HPLC were removed to locate the ^{3}H-labelled peptide markers; they were detected by addition to a scintillation cocktail (4 ml, Ready Safe, Beckman Instruments, USA) before counting on a Beckman LS 7000 instrument. The endogenous peptides were located and determined by RIA with TRH-antiserum after removal of solvent in vacuo. The fractions containing each peak of TRH-immunoreactivity were combined.

2.5. Resolution of acidic from neutral and basic TRH-immunoreactive peptides by analytical mini-column chromatography on DEAE-Sephadex

Mini-columns (6 × 0.5 cm) were prepared by pouring a suspension of DEAE-Sephadex A25 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) in 400 mM sodium phosphate at pH 7.5 and the columns were washed with 1 ml of H_2O and 2 ml of 20 mM sodium phosphate pH 7.5. Aliquots from each TRH-like peptide resolved by HPLC were dried in vacuo and the residues dissolved in 1 ml of 20 mM sodium phosphate for application to the DEAE-column. Ten fractions (0.5 ml) were collected in 20 mM sodium phosphate and a further 10 fractions were collected in 200 mM sodium phosphate. To detect the peptides, aliquots were removed for RIA with TRH-antibody, the salt concentration in each aliquot being adjusted to 200 mM before assay.

2.6. RIA of TRH-immunoreactive peptides

The concentrations of TRH-like peptides were determined by RIA using a sheep antiserum raised against synthetic TRH [11] and quantitation was by comparison with a synthetic TRH standard (Peninsula Laboratories, St. Helens, Merseyside, UK). The concentrations of the peptides are given in terms of synthetic TRH; pGlu-Glu-Pro amide possesses approximately 50% of the immunoreactivity of TRH [3] and pGlu-Phe-Pro amide is equally reactive [4]. The procedure employed for RIA has been described [12] except that the separation of bound from free ligand was accomplished by using 20% (w/v) polyethylene glycol (PEG) in place of activated charcoal. After incubation with antibody, 100 μl of heat inactivated horse serum (Flow Laboratories Ltd., Uxbridge, Mddx., UK) and 500 μl of 20% PEG (Sigma, St. Louis, MO, USA) were added. The suspensions were mixed and centrifuged at 2×10^{3} g for 20 min at 4°C using a Beckman GPR centrifuge. The supernatants were removed cautiously and the radioactivity in the pellets was measured with an LKB γ-counter Model 1282, Pharmacia LKB, Uppsala, Sweden).

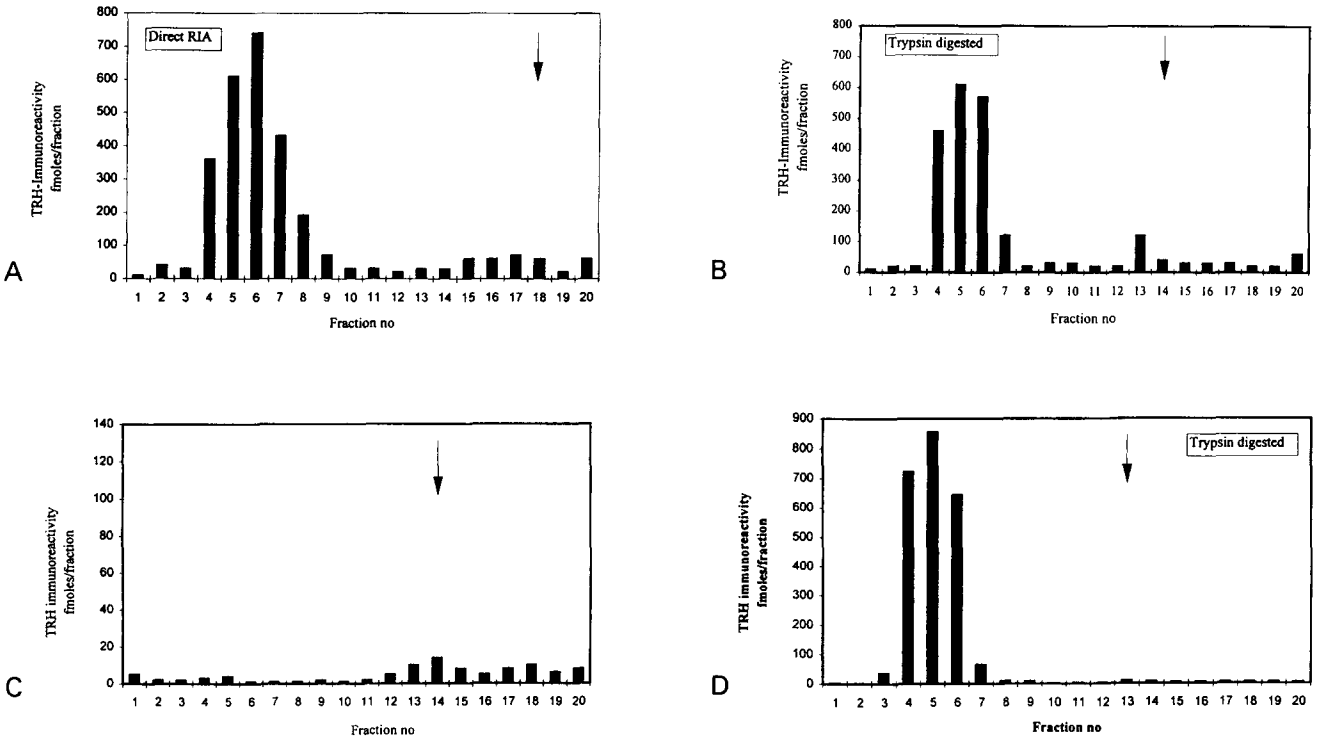

Fig. 1. Mini-column cation exchange chromatography of TRH-immunoreactive peptides from rabbit prostate (a) without trypsin digestion, (b) with trypsin digestion; and from rabbit testis (c) without trypsin digestion, and (d) with trypsin digestion. The vertical arrow in each figure indicates the elution position of [^{125}I]TRH.

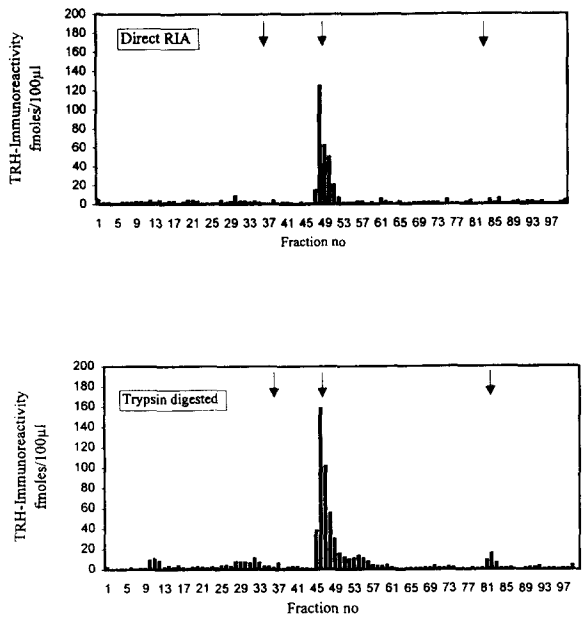

Fig. 2. Resolution of TRH-immunoreactive peptides from rabbit prostate by HPLC (a) without trypsin digestion and (b) with trypsin digestion. The arrows from L to R mark the elution positions of [^{3}H]pGlu-His-Pro amide, [^{3}H]pGlu-Glu-Pro amide, and [^{3}H]pGlu-Phe-Pro amide.

3. Results

3.1. Determination of TRH-like peptides in rabbit prostate

The initial purification of TRH-like peptides from rabbit prostate (0.73 g) was by methanol extraction and mini-column cation exchange chromatography (Fig. 1a). As shown in Table 1, more than 90% of the total TRH-immunoreactivity (2.45 pmol) passed through the mini-column without retention. Thus the majority of the TRH-like peptides in the prostate were neutral or acidic and little or none of the basic hormone (TRH) appeared to be present, in accord with our previous observation that mRNA for the TRH-prohormone could not be detected in rat prostate [14]. When the trypsin digested extract from the prostate was applied to the mini-column (Fig. 1b), essentially the same result was obtained. This extract would contain TRH-like peptides released from N-extended forms in addition to free TRH-like tripeptides, but the TRH-immunoreactivity in this fraction was found to be marginally less than was observed in the extract that had not been exposed to trypsin. It therefore appears that negligible TRH-immunoreactive peptide was released by the digestion with trypsin. The results show that the rabbit prostate contained acidic or neutral TRH-like tripeptides (3.17 pmol TRH-immunoreactivity/g) but no significant N-extended forms of these peptides.

Further chromatography of the unretained fraction from the cation exchange column was carried out by HPLC (Fig. 2a), revealing a single TRH-immunoreactive peptide. It co-chromatographed with ^{3}H-labelled pGlu-Glu-Pro amide. The trypsin digested peptide mixture, after mini-column chromatography, showed a very similar pattern to the non-digested fraction (Fig. 2b), indicating that little or no N-extended forms of TRH-like peptides were present in the prostate. Since the TRH-immunoreactivity of pGlu-Glu-Pro amide was 50% that of TRH, the concentration of this peptide in the prostate was calculated to be 6.34 pmol/g. As expected, the acidic properties of the prostate peptide were confirmed by anion exchange mini-column chromatography: the peptide was completely retained on DEAE-Sephadex.

3.2. Determination of TRH-like peptides in rabbit testis

The first stage in the purification of TRH-like peptides from rabbit testis was carried out in parallel with the purification of the prostate peptides, involving methanol extraction and mini-column cation exchange chromatography. In the case of the extract that had not been exposed to trypsin, virtually no TRH-immunoreactivity was seen in either the non-retained or retained fractions from the mini-column (Fig. 1c). In contrast, when the trypsin digested peptides from the testis (1.26 g) were fractionated on the mini-column, substantial TRH-immunoreactivity (23 pmol) was observed which was not retained on the column; but again very little basic TRH-immunoreactivity was seen in the retained fractions (Fig. 1d). Since the TRH-immunoreactivity was observed only in the trypsin digested fraction, it can be attributed to TRH-like peptides released from N-terminally extended forms. Further chromatography of these peptides, which were not retained on the mini-column, was carried out by HPLC. Three clearly resolved peaks of TRH-immunoreactivity were seen (Fig. 3), of which the most hydrophobic co-chromatographed with [^{3}H]pGlu-Phe-Pro amide. These TRH-immunoreactive peptides were examined by anion exchange mini-column chromatography: each of the peptides passed through the DEAE- column without retention and behaved as a neutral peptide. The structure of the two TRH-like peptides that were less retained on HPLC than pGlu-Phe-Pro amide have not so far been elucidated.

4. Discussion

The two TRH-like peptides pGlu-Glu-Pro amide and pGlu-Phe-Pro amide have been identified previously in human semen [4,15] but since the seminal fluid includes the secretions of a number of glands it was not known whether these peptides were produced together or whether they originated from different tissues. The acidic TRH-like peptide pGlu-Glu-Pro amide was first isolated from rabbit prostate [3] and since that time it has been shown to occur in the prostate of a number of other

Table 1
Determination of TRH, TRH-like peptides and their N-extended forms in rabbit prostate and testis by mini-column cation exchange chromatography

Tissue	Molecular form	Non-retained (pmol/g)	Retained (pmol/g)
Prostate	Tripeptides	3.17	0.25
	Tripeptides + N-extended	2.44	< 0.17
Testis	Tripeptides	–	0.11
	Tripeptides + N-extended	18.2	0.10

The tripeptides in the tissue extracts were determined by TRH-RIA of aliquots of the column fractions without trypsin digestion; the N-extended forms of the tripeptides in the tissue extracts were digested with trypsin prior to chromatography and the released TRH-immunoreactive peptides together with the endogenous tripeptides were chromatographed and determined by TRH-RIA.

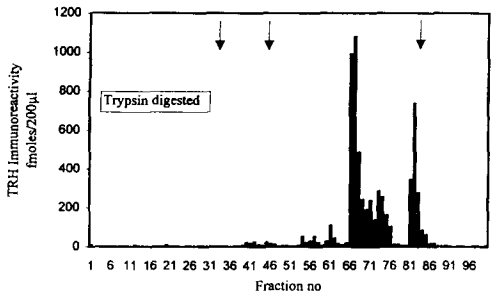

Fig. 3. Chromatography of TRH-immunoreactive peptides released by trypsin from N-extended forms present in rabbit testis by HPLC. The arrows from L to R mark the elution positions of ^{3}H-labelled marker peptides as in Fig. 2.

species [14,16]. The neutral TRH-like peptide pGlu-Phe-Pro amide, on the other hand, was first isolated from human semen; it has not been reported to be present in rabbit prostate though it does appear to occur as a minor component in rat prostate [14] and possibly as a trace component in human prostate [16]. It should be mentioned that this peptide appears more prominent when its presence is revealed by the use of an antibody with a higher reactivity for neutral peptides such as pGlu-Phe-Pro amide than for acidic peptides such as pGlu-Glu-Pro amide [14]. In general, however, it is clear that the principal TRH-like peptide in the prostate of most species is pGlu-Glu-Pro amide.

The present experiments confirm that rabbit prostate contains pGlu-Glu-Pro amide but show that it does not contain a detectable amount of pGlu-Phe-Pro amide. The major tissue where this peptide was found was the testis; however it occurred there exclusively in an N-extended form from which the tripeptide could be released by digestion with trypsin. This would indicate that pGlu-Phe-Pro amide is not derived from a precursor that contains a paired basic residue sequence on the N-terminal side of the tripeptide, since this would be expected to offer a favourable site for proteolytic processing. It is more likely that this tripeptide is preceded in its precursor by a single basic residue which would render the precursor sensitive to cleavage by trypsin but not to intracellular processing [17]. Unlike pGlu-Phe-Pro amide, the acidic peptide pGlu-Glu-Pro amide which was present in the prostate and not the testis occurred almost exclusively as the tripeptide. This peptide, therefore, is likely to be formed by a classical processing mechanism involving cleavage of the precursor at paired basic residues followed by removal of the basic amino acids to release Gln-Glu-Pro-Gly, reactions that generally go to completion. The TRH-like tripeptide pGlu-Glu-Pro amide would be generated from the tetrapeptide by cyclisation of its N-terminal glutamine [18,19] and amidation at the C-terminal glycine [20].

That pGlu-Glu-Pro amide and pGlu-Phe-Pro amide were present in different tissues implies that they are formed from different precursors. This conclusion, however, will have to be confirmed by identification of the precursor sequences. The differential distribution of the two peptides certainly suggests that their physiological roles are unlikely to be the same, though their activities could prove to be complementary since both peptides are present or are generated in the seminal fluid. It has been reported that pGlu-Glu-Pro amide can increase the capacitation of mouse sperm [5], with a potential for increasing fertility. The interaction of this peptide with sperm in vivo would take place when the prostate secretions containing the peptide are added to the seminal fluid. On the other hand, the neutral TRH-like peptide pGlu-Phe-Pro amide is elaborated principally in an N-extended form and it is to be expected that this tripeptide would be released from its precursor by the action of the trypsin-like enzymes derived from the prostate after ejaculation has taken place. This would be consistent with the possibility that the tripeptide pGlu-Phe-Pro amide is generated over a period of time. Indeed it has been reported that incubation of human semen in vitro leads to the progressive appearance of a hydrophobic TRH-immunoreactive peptide [21]. It is likely that this peptide is pGlu-Phe-Pro amide.

In conclusion, the main finding of the present study is that rabbit prostate contains pGlu-Glu-Pro amide but not pGlu-Phe-Pro amide whereas the testis contains an N-extended form of pGlu-Phe-Pro amide but no pGlu-Glu-Pro amide. This implies that the two TRH-like peptides are generated from different precursors and are formed by different processing mechanisms.

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