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THYROTROPIN-RELEASING HORMONE AND A HOMOLOGOUS PEPTIDE IN THE MALE RAT REPRODUCTIVE SYSTEM

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SUMMARY

TRH and a TRH-like peptide have been shown to occur throughout the male rat reproductive system by TRH radioimmunoassay, SP-Sephadex C25 cation exchange chromatography, high pressure liquid chromatography and parallel line analysis. The total concentration of TRH and TRH-like peptide was highest in the prostate followed by the testis, cauda epididymis and seminal vesicles. Dilution curves for extracts of prostate, testis and seminal vesicles were parallel with TRH while the corresponding curve for epididymis was nonparallel.

Thyrotropin-releasing hormone (TRH) occurs not only in the hypothalamus (1) and central nervous system (1-3) but also in the gastrointestinal tract (4), amniotic fluid (5), milk (6) and human semen (7). This nearly ubiquitous distribution of TRH led to the present search for TRH in the reproductive organs of male rats and a preliminary characterization of its molecular forms. The striking variations in the concentrations of TRH and a TRH-like peptide observed suggest that these substances have a function in the reproductive tract.

MATERIALS AND METHODS

Tissue extraction

Reproductive organs from sexually mature male Sprague-Dawley rats weighing 250 or 500 g were used in all experiments. After decapitation, decapsulated testes, seminal vesicles, prostate and both caudal epididymides were weighed and placed into glass test tubes containing 0.5 M acetic acid at 97°C in a heating block to inactivate TRH-degrading enzymes. In one experiment the contents of the seminal vesicles from 500 g rats were expressed into a graduated conical centrifuge tube and an equal volume of 0.5 M acetic acid added followed by heating at 97°C. After at least 15 min, all tubes were allowed to cool and their contents dispersed with a Polytron homogenizer. Homogenates were dried completely with a heater block and filtered air blowing into each tube. The dried residues were extracted twice with methanol. The combined methanol supernatants were dried completely and dissolved in 0.15 M NaCl-0.05 M phosphate buffer, pH 7.5 (PBS). Weighed, decapsulated testes were also placed into tubes containing 3.0 ml of 0.5 mg collagenase (Worthington, Type IV)/ml of Ham's F-10

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medium or PBS. The tubes were stoppered and placed in a shaker bath at 37°C with the long axis of the tubes parallel to the reciprocating motion of the shaker which was set for 120 cycles/min. After 15 min, the tubes were placed upright and the tubules allowed to settle for 5 min at room temperature. The supernatants were carefully removed with a Pasteur pipet and saved in separate test tubes. The sedimented tubules were washed with 2.0 ml of the corresponding collagenase-free medium. After pooling the corresponding supernatant fractions from each collagenase-treated testis, some of these supernatants were centrifused at 300 x g for 5 min at room temperature to obtain a Leydig cell-containing pellet (8). The four components thus obtained, washed tubules, Leydig cells, Leydig cell supernatant and uncentrifuged Leydig cell fraction (interstitial fraction) were dried completely and extracted for TRH if prepared in Ham's F-10 medium or measured for total protein by the biuret method (9) if in PBS.

TRH Radioimmunoassay

The TRH radioimmunoassay of 100 µl aliquots of a serial dilution of each extract was carried out with a modification (10) of the Bassiri and Utiger method (11). TRH antiserum was a generous gift from Dr. Robert Utiger, University of North Carolina. 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 (12).

Chromatography

Tissue extracts were subjected to SP-Sephadex C25 cation exchange and Sephadex G-10 exclusion chromatography using a 0.9 x 58 cm column which was equilibrated and eluted with 0.2 M ammonium acetate, pH 6.2 (10). Fractions of 1.1 ml were collected. An Altex high pressure liquid chromatographic (HPLC) system was also employed for separation of the tissue extract components. The isocratic mobile phase consisting of 6.5% acetonitrile-0.02 M acetic acid-0.1% heptane sulfonic acid (13) was pumped through a reversed phase LiChrosorb C-18 column (Altex), 10 µm particle size, at a flow rate of 2.0 ml/min. TRH radioimmunoassay measurements were carried out on the fractions collected from the SP-Sephadex C-25, Sephadex G-10 and HPLC separations of the tissue extracts without further processing.

Measurement of primary amines

The relative concentration of primary amine-containing compounds in the chromatography fractions were measured by diluting 50 µl of each fraction in 1.0 ml of 0.2 M borate buffer, pH 8.0, and then adding 0.33 ml of 28 mg fluorescamine (Sigma)/100 ml acetone with vortexing. The fluorescence was monitored with an Aminco-Bowman spectrophotofluorometer using a 390 nm excitation and a 487.5 nm emission wavelength (14).

RESULTS

Among the major reproductive organs of the male rat, the prostate contains the highest concentration of TRH immunoreactivity per gram wet weight of unfractionated tissue, as seen in Tables 1 and 2. The testis has the next highest concentration followed by cauda epididimis and seminal vesicles. Percent recoveries of added TRH from these tissues were: prostate, 99; testis, 48; epididymis,

Table 1. Distribution of TRH-like immunoreactivity in the reproductive organs of 250 gram male Sprague-Dawley rats (n = 6). Results are given as mean ± SD.

Testes		Prostate	
Wet weight (g)	TRH (ng/g)	Wet weight (mg)	TRH (ng/g)
1.31 ± 0.02	0.16 ± 0.04	150 ± 14	44 ± 22
Seminal Vesicles		**Cauda Epididymis**	
Wet weight (mg)	TRH (ng/g)	Wet weight (mg)	TRH (ng/g)
176 ± 68	0.11 ± 0.07	69 ± 8	0.14 ± 0.10

97 and seminal vesicles, 83. The dilution curves for these tissue extracts were parallel to the TRH standard curve with the exception of the epididymis (Fig. 1).

The substantially higher prostatic TRH concentration observed in the 250 g rats, Table 1, compared to that in the 500 g males, Table 2, is not due to TRH recovery errors resulting from rapid tissue degradation prior to enzyme inactivation. TRH immunoreactivity disappearance from rat prostates kept at room temperature was negligible at 2 hrs while no more than 5 min elapsed from decapitation to the boiling of tissues.

Table 2. Distribution of TRH-like immunoreactivity in the reproductive organs of 500 gram male Sprague-Dawley rats (n = 6). Results are given as mean ± SD.

Testes		Prostate	
Wet weight (g)	TRH (ng/g)	Wet weight (mg)	TRH (ng/g)
3.52 ± 0.24	0.58 ± 0.19	731 ± 187	3.9 ± 2.2
Seminal Vesicles		**Cauda Epididymis**	
Wet weight (g)	TRH (ng/g)*	Wet weight (mg)	TRH (ng/g)
1.41 ± 0.32	0.23 ± 0.09	688 ± 122	0.39 ± 0.06

* Seminal vesicular fluid pooled from 6 other rats contained 0.3 ng TRH/ml of fluid.

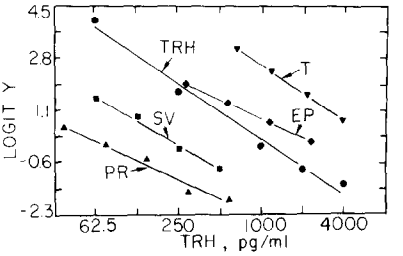

Fig. 1. Parallel line analysis of serial dilutions of tissue extracts from the reproductive system of 500 gram rats measured by TRH radioimmunoassay: T, testis; EP, cauda epididymus; SV, seminal vesicles; P, prostate. Synthetic TRH was used as the reference material.

The sum of the TRH immunoreactivity measured in the collagenase treated testicular fractions, Table 3, equalled one-half that of the intact testes of Table 2. This lower TRH content may be due to the greater processing time for the collagenase treatment and Leydig cell centrifugation. The percentage loss in TRH content for the tubules, however, is expected to be less since they were boiled before the Leydig cell-containing fractions. Thus the actual proportion of TRH and TRH-like material associated with the Leydig cell fraction is probably underestimated. The ratios of TRH immunoreactivity to protein content in each testicular fraction are given in Fig. 2.

Table 3. Distribution of TRH-like immunoreactivity in testes of 500 gram Sprague-Dawley rats.* Results are given as mean ± SD.

Leydig Cells (n = 3)		Leydig Cell Supernatant (n = 3)	
Protein (mg)	TRH (pg)	Protein (mg)	TRH (pg)
28 ± 3	70 ± 20	13.6 ± 0.2	384 ± 81
Interstitial Cell Fraction (n = 2)		**Tubules (n = 5)**	
Protein (mg)	TRH (pg)	Protein (mg)	TRH (pg)
41.6 ± 0.6	422 ± 2	189 ± 3	131 ± 20

* Decapsulated wet weight of testes: 1,760 ± 21 mg (n = 6). Biuret method was used to measure the protein content of each fraction of one testis. Ratio of protein content of each fraction to total testis weight was then used to calculate the protein content of the corresponding fractions within the other five testes used.

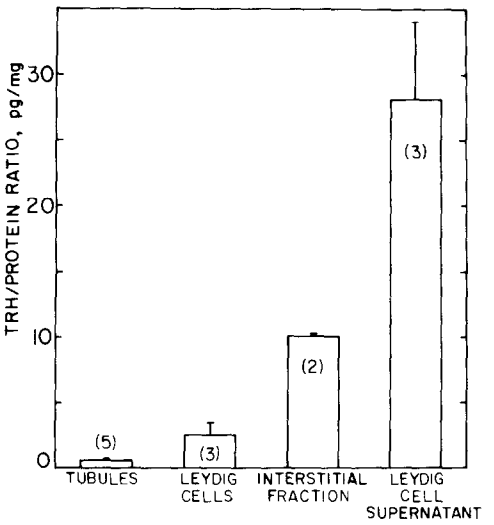

Fig. 2. Ratio of TRH immunoreactivity to total protein content (mean ± SD) of different fractions of collagenase-treated rat testes. See text and table 2 for details of testis fractionation and total TRH and protein content, respectively.

The SP-Sephadex C-25 chromatogram for TRH immunoreactivity obtained from the prostate, Fig. 3A, consists of two peaks, one of which (Peak II) coelutes with synthetic TRH. The elution positions for pGlu-His-Pro (tube 28) and His-Pro diketopiperazine (tube 70) do not correspond with Peak I (15). Moreover, the molar crossreactivities of these two compounds in the TRH radioimmunoassay was <0.01%. Among the numerous TRH analogs checked for crossreactivity, only 3-methyl-TRH gave significant crossreaction, and its elution from SP-Sephadex C-25 just ahead of TRH has been previously described (8). The fluorescamine-reactive compounds which are not removed by the TRH extraction process characteristically emerge just after peak I, as seen in the dashed profile of Fig. 3A. The molecular weight of this material is comparable to that of TRH since it coelutes with TRH during Sephadex G-10 chromatography.

Fig. 3B shows the TRH immunoreactivity and fluorescence profile for the cation exchange chromatography of the Leydig cell supernatant. To establish that Peak I is not TRH which elutes prematurely due to column overloading by

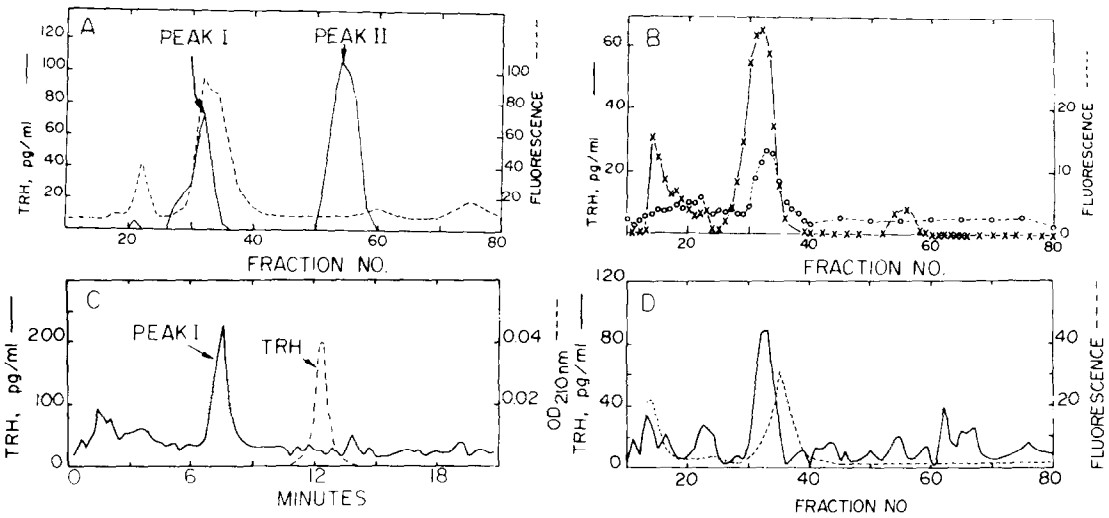

Fig. 3. SP-Sephadex C-25 cation exchange chromatography of (A) prostatic extract from 250 gram rats, (B) extract of interstitial fraction from testes of 250 gram rats, (D) epididymal extract from 500 gram rats and (C) HPLC of peak I from (B) and synthetic TRH. See Materials and Methods section for details of chromatographic procedures.

extract contaminants, the peak I fractions were pooled and analyzed by HPLC. Fig. 3C depicts the HPLC immunoreactive profile superimposed with the ultraviolet absorption profile measured following the injection of pure TRH.

A TRH-like structure for the peak I material seems likely based on the following observations: (a) the TRH antibody prepared by the Bassiri and Utiger method is highly specific for the N-terminal pyroglutamyl and C-terminal prolineamide residues. Modifications to the central histidyl residue, however, do not affect the crossreactivity markedly since the imidazole ring on this residue is the site of crosslinkage to the carrier protein used for immunization. (b) Peak I coelutes with TRH on a 0.9 x 58 cm Sephadex G-10 column and (c) Peak I remains in the aqueous phase of a water-ethyl ether extraction as does TRH.

DISCUSSION

The relatively high levels of TRH found in the prostate and Leydig cell fraction of testis relative to peripheral blood (16) are of particular interest

for several reasons. Prostatic fluid, for example, contains factors which stimulate the motility of spermatozoa following ejaculation and protects them from the deleterious effects of the seminal vesicular fluid (17,18). The prostatic TRH concentration, moreover, declines with age as does the level of TRH in the rat pancreas (19). The neonatal rat pancreas contains far more TRH than does the neonatal hypothalamus and is a major source of the high level of circulating TRH (19). It seems reasonable to conclude that TRH is biosynthesized in the pancreas and the rat prostate and testis may be among the other extrahypothalamic tissues contributing to the high blood levels of TRH in the neonate.

Preliminary immunohistochemical studies using a rabbit anti-TRH serum prepared in our laboratory by the method of Bassiri and Utiger (17) revealed a very faint staining of the Leydig cells of a 250 g rat. Since Leydig cells disappear from the rat testis soon after birth and reappear at puberty (20), their contribution to the high neonatal serum TRH concentration is worthy of further study.

The amount of TRH in the gonads of the frog, in contrast to the rat, is about equal to the blood concentration times the tissue vascular space (21). Perhaps it is the extraordinarily high blood levels of TRH in the frog (22), derived mainly from the cutaneous poison glands (23), which renders any contribution of TRH from the reproductive system itself difficult to detect and of limited physiological significance in this species. Further examination of the phylogenetic distribution of TRH and TRH-like peptides within prostatic and spermatogenic tissues may provide clues not only to the reproductive implications of this material but also to its cellular origins.

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