GENERATION OF SPECIFIC ANTISERUM TO THYROTROPIN RELEASING-HORMONE AND ITS USE IN A RADIOIMMUNOASSAY

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Received 29 January 1976

1. Introduction

Thyrotropin releasing-hormone (TRH), identified as the tripeptide pyroglutamyl–histidyl–proline amide, was isolated from hypothalamic tissue [1,2] and found to have both TSH and prolactin releasing activities [3]. Several reports described the generation of antisera to the synthetic tripeptide after rendering it antigenic by coupling to a protein carrier, either through the proline residue [4] or through the histidine residue, using a symmetrical bifunctional coupling reagent in a one step reaction [5]. This paper describes the synthesis of an immunogenic protein conjugate of TRH using an asymmetrical bifunctional reagent in a two-step coupling procedure. Using antisera generated against this conjugate, a radioimmunoassay procedure for the peptide was developed, which permits the study of its distribution in biological tissues.

2. Materials and methods

2.1. *Preparation of TRH–BSA conjugate*

p-Diazonium phenylacetic acid was attached to synthetic TRH and the resulting azo-derivative (PAPA-TRH) was coupled to bovine serum albumin (BSA). *p*-Aminophenylacetic acid (0.094 mmol) in 0.4 ml of cold 2 N HCl was diazotized by sodium nitrite (0.1 mmol in 50 μl of water). Fifteen min later 0.094 mmol TRH (Calbiochem USA) in 0.4 ml of 3 N $KHCO_3$ was added. The reaction mixture, which gradually turned pinkish-red, was stirred for 10 h at 4°C. After acidification (pH 2.0) with 2 N HCl, the solution was extracted five times with ethylacetate, and the organic phase discarded; the aqueous phase was adjusted to pH 5.0 with $KHCO_3$ and lyophilized. The brownish product was extracted into absolute methanol, leaving insoluble white inorganic salts, and then precipitated by ether; the powder thus obtained was dissolved in absolute methanol and reprecipitated ether. Electrophoresis of the resultant brownish powder was carried out on Whatman No. 3 paper (pyridine acetate buffer, pH 6.5; 60 V/cm). Two components were detected with Pauli's reagent, one corresponding in mobility to unreacted TRH (30–50%, moving slightly towards the anode), the other (50–70%), moved towards the cathode. The latter component had a broad absorption peak in dimethylforinamide at 370–390 nm (λ_{max} = 380 nm; ϵ_{380}^{DMF} = 4.8×10^3), consistent with its identification as an azo-histidyl derivative [6,7].

The PAPA-TRH derivative was attached to BSA (Crystalline grade, Miles Labs., Kankakee, Ill.), using 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide HCl at pH 5.0 [8]. Spectral analysis of the TRH–BSA

* In partial fulfillment of the requirements for the Ph. D. degree of the Graduate School of the Weizmann Institute of Science.

conjugate at 380 nm indicated that on the average 24 residues of TRH were attached to each molecule of the carrier protein.

2.2. *Immunization*

Four mature male rabbits were injected subcutaneously in several sites with an emulsion of TRH–BSA (0.25 mg/rabbit) in complete Freund's adjuvant supplemented with 1.6 units of a vaccine against Hemophilus pertussis (Pertussis Vaccine Fluid U.S.P., Eli Lilly & Co. Indianapolis). After two booster injections at intervals of two weeks, the rabbits were bled and the sera were examined for presence of antibodies.

A conjugate of TRH and a multichain synthetic copolymer of L-lysine and DL-alanine: poly-DL-Ala–poly-L-lys, (mol. wt. = 142 500; Ala/Lys ratio = 20/1; [9]) containing approximately 14% w/w of TRH was prepared by coupling the free carboxyl derivative of TRH (pGlu–His–Pro-OH) to the free amino groups of alanine, using *N,N'*-dicyclohexyl-carbodiimide in DMF as the coupling agent [10]. This preparation, when administered to rabbits, failed to produce anti TRH sera.

2.3. *Iodination and radioimmunoassay (RIA) of TRH*

Labelling and RIA procedures for TRH were carried out as described previously for LH–RH [11].

2.4. *Dissection of various brain regions, and extraction of TRH*

Tissue fragments corresponding to different brain areas were excised according to de Groot [12]. TRH was extracted with 90% methanol, taken to dryness under a stream of air, and redissolved in 0.01 M phosphate-buffered saline (PBS), pH 6.9.

3. Results

3.1. *Formation of antibodies to TRH*

Antibodies binding [^{125}I]TRH (≈ 1 mCi/μg) appeared in the serum of the immunized rabbits two months after the first immunization. Three months later the serum of one immunized rabbit (No. 23) bound 50% of the labelled hormone (≈ 50 pg per assay tube) at 1:20 000 final dilution; the corresponding titers of the sera from the other immunized rabbits ranged from 1:500 to 1:8000.

3.2. *Sensitivity and specificity of the antisera to TRH*

The sensitivity of the radioimmunoassay for TRH was found to be approx. 10 pg (fig.1). A reference preparation of rat hypothalamic extract with TSH releasing activity (NIAMD-Rat HE-RP-1) inhibited the binding of [^{125}I]TRH by the antiserum to TRH–BSA. The inhibition curve was parallel to that produced by synthetic TRH (fig.1). The extent to which the antiserum to TRH cross-reacted with various analogues of TRH or other peptide hormones is summarized in table 1.

3.3. *Distribution of TRH*

The distribution of TRH in various regions of the rat brain is summarized in table 2.

4. Discussion

Two approaches have recently been used to render TRH antigenic: (i) Conjugation of TRH to BSA through the carboxy-terminal of pGlu–His–Pro-OH (TRH-OH) [4]. A radioimmunoassay system based on antibodies generated against this antigen were able to detect only high concentrations (above 100 pg) of TRH and cross-reacted extensively with

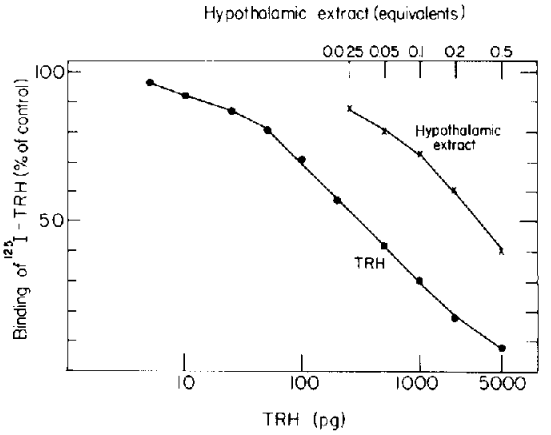

Fig.1. Inhibition of binding of [^{125}I]TRH to antiserum by unlabelled TRH or by rat hypothalamic extract (NIAMD-Rat HE-RP-1). Anti-TRH serum used at 1:20 000 dilution.

Table 1
Specificity of anti-TRH serum

No.	Peptide	Inhibition of [^{125}I]TRH-binding (%)
1	pGlu–His–Pro-NH_2 (TRH)	100
2	pGlu–His–Pro-OH (TRH-OH)	0.1
3	pGlu–His–Trp-OH	0.01
4	pGlu–His-OH	0.004
5	His–Pro–NH_2	< 0.001
6	Pro–Leu–Gly-NH_2 (MIF)	< 0.001
7	Vasopressin	< 0.001
8	Oxytocin	< 0.001
9	LHRH	< 0.001

Peptides were tested in doses ranging from 10 ng to 100 μg. Anti TRH was used at a final dilution of 1:20 000. Lysine–vasopressin and oxytocin were the product of Sigma Chemical Co., St. Louis. LH–RH was kindly provided by Hoechst, AG, Frankfurt. Compounds No. 3 and 4 were gifts from Dr W. F. White. Compounds No. 2, 5 and 6 were synthetized in our laboratory.

Table 2
Distribution of TRH in rat brain

Brain Region	Wet weight (mg)	TRH pg/mg
Median eminence[a]	3.9 ± 0.2[d]	387.5 ± 21.2
Preoptic area	16.7 ± 1.8	92.7 ± 9.8
Residual hypothalamus[b]	19.7 ± 1.7	125.3 ± 7.4
Cerebral cortex	39.6 ± 4.9	0.3 ± 0.02
Posterior pituitary	1.1 ± 0.02	167.8 ± 15
Amygdala	25.6 ± 1.1	6.1 ± 1.9
Hippocampus (dorsal)	45.4 ± 1.4	4.8 ± 0.4
Olfactory tubercle + anterior olfactory nucleus	47.4 ± 2.0	21.8 ± 3.5
Pineal gland[c]	5.0 ± 0.1	4.2 ± 0.1

[a] The 'median eminence' includes parts of the medial basal hypothalamic nuclei.
[b] Excluding the preoptic area, the median eminence, and the mammillary bodies.
[c] Pools of five glands.
[d] Mean and standard error of the mean for 6–12 determinations.

the pGlu–His–Pro-OMe and pGlu–His–Pro-OH analogues of TRH. Since TRH is rapidly inactivated in blood by conversion of the amide to a carboxyl group [13], results obtained with such antisera require careful interpretation. (ii) Coupling of TRH to BSA [5] or to thyroglobulin [14,15] via the histidyl moiety by the bis-diazotized benzidine technique. Visser et al. [4] however have failed to produce antiserum to TRH by this method. The reason for this may be that the one-step reaction utilizing a symmetrical bifunctional reagent can result in dimerization and oligomerization of TRH. We, therefore, used an asymmetrical bifunctional reagent in a two-step coupling procedure. The antigen thus produced (PAPA-TRH) generated antisera with titers of up to 1:20 000, and showed negligible cross reaction with four unrelated hypothalamic hormones (<0.001%) or with closely related oligopeptides (table 1). Thus the TRH metabolite (pGlu–His–Pro-OH) showed little competition (0.1%) with intact

labelled TRH for antibodies binding sites when tested in a radioimmunoassay system (table 1). A rat hypothalamic extract (NIAMD-Rat HE-RP-1) with TSH-releasing activity displayed inhibition curves parallel to that of synthetic TRH (fig.1). These characteristics, combined with high sensitivity (limit of detection approx. 10 pg), render this radioimmunoassay system capable of providing meaningful quantitative assessment of TRH in biological tissues.

The highest concentration of TRH in rat brain was found in the median eminence (387 pg/mg); high concentrations were also found in the rest of the hypothalamus (125 pg/mg) and in the posterior pituitary. These results are in accord with those obtained by others [15–17]. Lower concentrations were found in the preoptic area (93 pg/mg) and in the olfactory nucleus (22 pg/mg). In contrast to a recent report [18], only low TRH levels (≈ 4 pg/gland) were detected in the rat pineal gland.

The high concentration of TRH in the rat hypothalamus is consistent with the biological role of this hormone in the regulation of TSH secretion from the pituitary. The extra-hypothalamic localization of TRH, however, may suggest further activities of this tripeptide, possibly exerted on neural tissue [19].

Acknowledgements

We are grateful to Miss N. Ben Aroya for excellent technical assistance, to Dr A. F. Parlow of the National Institute of Arthritis and Metabolic Diseases for reference preparation of rat hypothalamic extract and to Dr H. R. Lindner for the interest taken in this study and his assistance in the preparation of this manuscript. The work was supported by a grant (to H.R.L.) of the Ford Foundation and the Population Council Inc., N.Y.

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