A NEW RADIOIMMUNOASSAY OF THYROTROPIN-RELEASING HORMONE

T. J. VISSER, W. KLOOTWIJK, R. DOCTER and G. HENNEMANN
Department of Internal Medicine III and Clinical Endocrinology, Medical Faculty, Erasmus University Rotterdam, The Netherlands

Received 22 August 1977

1. Introduction

Several radioimmunoassays for the measurement of thyrotropin-releasing hormone (pGlu–His–Pro–NH_2, TRH) have been described. Antisera have been produced mainly by immunization of rabbits with TRH–protein conjugates in which coupling was effected via the imidazole of the histidyl moiety of TRH. For this purpose bis-diazotized benzidine has been most widely used [1]. In another procedure TRH is reacted with *p*-diazoniumphenylacetic acid and the derivative is coupled to protein with the aid of a carbodiimide [2,3]. An entirely different approach has been the introduction of an amide bond between pGlu–His–Pro–OH and NH_2-groups of a protein carrier resulting in the formation of a TRH-like structure, i.e., pGlu–His–Pro–NH–protein [4].

We here report the attachment of TRH to hemocyanin (HC) using the bifunctional reagent 1,5-difluoro-2,4-dinitrobenzene (DFDNB) [5]. Administration of the conjugate to rabbits elicited the production of antisera, which were used in the radioimmunoassay of the hypothalamic hormone.

2. Materials and methods

2.1. *Materials*

TRH and luteinizing hormone-releasing hormone (LH-RH) were obtained by courtesy of Dr M. von der Ohe, Hoechst, Frankfurt, FRG. Another preparation of TRH and pGlu–His–Pro–OH were donated by Professor Dr H. C. Beyerman and Dr J. L. M. Syrier, Technische Hogeschool, Delft, The Netherlands. Both tripeptides were also purchased from Beckman Bioproducts, Geneva, Switzerland. Professor Beyerman and Dr Syrier supplied us in addition with pGlu–N^{im}Bzl–His–Pro–NH_2 (Bzl, benzyl), pGlu–N^{im}Bzl–His–Pro–OH and pGlu–N^{im}Bzl–His–Pro–OCH_3. pGlu–His–OH, pGlu–His–OCH_3 and Glu–His–Pro–OH were kindly provided by Drs R. O. Studer and D. Gillessen, Hoffman-LaRoche Basle, Switzerland. His–Pro–NH_2 was a gift of Dr A. O. Geiszler, Abbott Laboratories, North Chicago, IL, USA. Glu–His–Pro–NH_2, pGlu–Phe–Pro–NH_2 and pGlu–His–Pro–Gly–NH_2 were purchased from Peninsula Laboratories, San Carlos, CA, USA; [^{3}H]TRH from New England Nuclear, Dreieichenhain, FRG; DFDNB from Sigma, St. Louis, MO, USA and keyhole limpet HC from Calbiochem, Lucerne, Switzerland.

2.2. *Preparation of conjugate*

Bridging of TRH to HC by means of a dinitrophenylene (DNP) moiety was carried out essentially according to the method described by Tager [5]. In brief, to 2.5 mg TRH in 0.2 ml 0.1 M phosphate, pH 7.2, were added 30 mg DFDNB in 1 ml methanol. After reaction for 15 min at 22°C, excess reagent was removed by several washings with ether. To the aqueous phase containing N^{im}-(5-fluoro-2,4-dinitrophenyl)TRH (FDNF–TRH) was added 25 mg HC in 0.8 ml 0.1 M borate, pH 10.0. The mixture was stirred 24 h at 22°C and the TRH-DNP–HC complex was dialyzed exhaustively against water. From the recovery of simultaneously reacted [^{3}H]TRH it was calculated that over 50% of added TRH had reacted with HC, which means that over 450 molecules TRH were attached to each molecule HC.

2.3. *Immunization*

Four New Zealand white rabbits were immunized with 0.5 mg TRH-DNP–HC in 2 ml water emulsion in complete Freund's adjuvant (1:3, v/v). Injections were given both sub- and intracutaneously at multiple sites on the back at intervals of 11 and 9 weeks.

2.4. *Radioiodination and radioimmunoassay of TRH*

Labelling of TRH with ^{125}I and radioimmunological procedures were performed essentially as previously described [3].

3. Results

3.1. *Generation of antibodies to TRH*

Two (Nos. 4094 and 4101) out of the 4 rabbits responded to the immunization by developing antibodies to TRH as demonstrated by significant binding of ^{125}I-labelled TRH to these sera at a final dilution of 1 : 1000 or higher. The antisera used in this study were obtained 2 weeks (4101) and 4 weeks (4094) after the 3rd injection. Binding of ^{125}I-labelled TRH (20 000 cpm, < 20 pg/assay tube) to the antisera amounted to approx. 30% at final dilution 1 : 7500 and 1 : 15 000, respectively.

3.2. *Radioimmunoassay of TRH and specificity of the antisera*

Figure 1 shows a typical standard curve for TRH of an assay employing antiserum 4094. Very similar results were obtained when antiserum 4101 was used. The sensitivity of the radioimmunoassays was found to be approx. 10–25 pg/assay tube (final volume, 1 ml). In fig.1 dose–response curves for several analogues are also displayed. The relative affinities of the various compounds in both assays are depicted in table 1. In addition the properties are summarized of an antiserum (No. 3158) raised against a conjugate in which TRH was coupled to bovine thyroglobulin after derivatization with *p*-diazoniumphenylacetic acid as previously described [3].

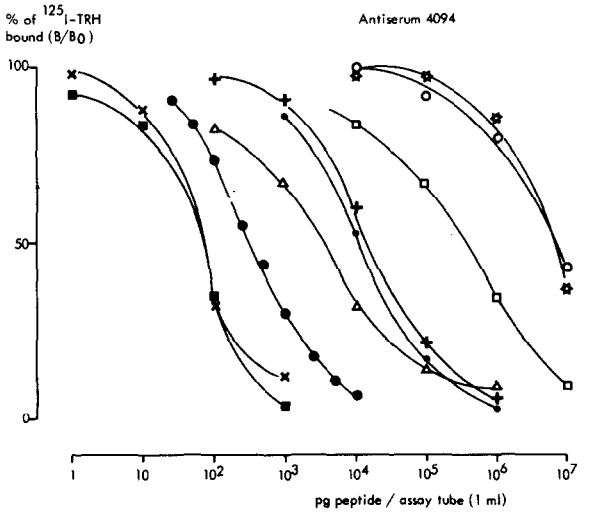

Fig.1. Displacement of ^{125}I-labelled TRH from antiserum 4094 (final dilution, 1:15 000) by increasing concentrations of TRH (•), Glu–His–Pro–NH_2 (·), His–Pro–NH_2 (○), pGlu–N^{im}Bzl–His–Pro–NH_2 (▪), pGlu–N^{im}Bzl–His–Pro–OCH_3 (△), pGlu–N^{im}Bzl–His–Pro–OH (□), LH-RH (∗), pGlu–Phe–Pro–NH_2 (×) and pGlu–His–Pro–Gly–NH_2 (+).

4. Discussion

The main feature of the coupling of TRH to proteins with DFDNB is the use of a large excess of the bifunctional reagent to convert the hormone into a N^{im}-FDNP derivative, thereby assuring a high degree of reaction and minimizing dimerization of the tripeptide [5]. Furthermore, conditions are chosen which preclude hydrolysis of the reagent [5]. Following the removal of unreacted DFDNB, the coupling of FDNP-TRH to HC is accomplished with a minimum of side reactions [5]. The method involving DFDNB has therefore some distinct advantages over hitherto described techniques [1–4] for the preparation of TRH–protein conjugates.

An important difference between the methods is that reaction of the diazonium reagents with TRH involves the carbon atoms of the imidazole [6] as is the case with iodination of the hormone [7], while reaction with DFDNB takes place at one of the nitrogens [8]. Despite this difference in site of attachment of TRH to the protein carrier, the properties of the antisera raised against the two types of conjugate are almost identical (table 1). Deamidation of TRH (pGlu–His–Pro–OH), deletion of one of the terminal residues (pGlu–His–OH and His–Pro–NH_2), hydrolysis of the lactam of the pyroglutamyl moiety (Glu–His–Pro–NH_2) and replacement of Pro–NH_2

Table 1
Cross-reactivity of various TRH analogues with anti-TRH antisera

Analogue	Cross-reactivity (%)[a]		
	Antiserum 4094[b]	Antiserum 4101[b]	Antiserum 3158[b]
pGlu–His–Pro–NH_2 (TRH)	100	100	100
Glu–His–Pro–NH_2	3.3	4.0	2.4
pGlu–His–Pro–OH	<0.005	<0.005	<0.025
Glu–His–Pro–OH	<0.005	<0.005	<0.025
His–Pro–NH_2	0.006	0.006	0.025
pGlu–His–OH	<0.005	<0.005	<0.025
pGlu–His–OCH_3	<0.005	<0.005	<0.025
pGlu–N^{im}Bzl–His–Pro–NH_2	560	1100	560
pGlu–N^{im}Bzl–His–Pro–OCH_3	11.6	0.8	3.7
pGlu–N^{im}Bzl–His–Pro–OH	0.11	0.03	0.13
pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2 (LH-RH)	0.02	0.02	<0.05
pGlu–Phe–Pro–NH_2	460	580	460
pGlu–His–Pro–Gly–NH_2	2.6	4.6	2.1

[a]Cross-reactivity is defined as the ratio (× 100) of the concentration of TRH over that of the analogue both of which displace 50% of ^{125}I-labelled TRH from the antiserum

[b]4094, antiserum raised against TRH-DNP–HC; final dilution, 1:15 000 4101, antiserum raised against TRH-DNP–HC; final dilution, 1:7500 3158, antiserum obtained as described [3]; final dilution 1:2000

by Pro–Gly–NH_2 (pGlu–His–Pro–Gly–NH_2) or by an octapeptide chain (LH-RH) result in an almost complete loss of immunoreactivity.

Substitution of the imidazole in TRH and TRH–OH with a benzyl-group (pGlu–N^{im}Bzl–His–Pro–NH_2 and pGlu–N^{im}Bzl–His–Pro–OH) and replacement of His by Phe (pGlu–Phe–Pro–NH_2) favours the interaction of the peptides with all antisera to a large extent. The cross-reactivity of pGlu–N^{im} Bzl–His–Pro–NH_2 with antiserum 3158 is higher than previously reported [3]. However, the antiserum used in the present study was obtained approx. 18 months later than that previously employed. These results seem to indicate that the relative affinity of the benzyl-derivative has increased in the course of subsequent immunizations.

The much higher activity of pGlu–N^{im}Bzl–His–Pro–NH_2 compared with TRH may be caused by its closer resemblance with the structural arrangement of the haptens in the conjugates. The aromatic moiety attached to the imidazole of TRH is therefore likely to be part of the antigenic determinant in the TRH–protein complex. It is also possible that the basicity of the imidazole in the TRH–protein conjugates is decreased because of the electron-withdrawing properties of the bridging moiety. This may at least in part explain the high affinities not only of the N^{im}Bzl derivatives but also of pGlu–Phe–Pro–NH_2.

From our findings it is expected that the N^{im}-DNP derivative of TRH will possess even higher activity towards antisera 4094 and 4101. Since this derivative can easily be obtained, studies are now in progress in our laboratory in order to increase the sensitivity of the radioimmunoassay by prior conversion of TRH into this compound and by using analogous derivatives for labelling with ^{125}I.

Acknowledgements

The authors are grateful to Drs Beyerman, Geiszler, Gillessen, Studer, Syrier and Von der Ohe for their generous supply of the various synthetic peptides. Thanks are also due to Mrs C. Boot for typing the manuscript.

References

[1] Bassiri, R. M. and Utiger, R. D. (1972) Endocrinology 90, 722–727.
[2] Koch, Y., Baram, T. and Fridkin, M. (1976) FEBS Lett. 63, 295–298.
[3] Visser, T. J., Klootwijk, W., Docter, R. and Hennemann, G. (1976) Neuroendocrinol. 21, 204–213.
[4] Visser, T. J., Docter, R. and Hennemann, G. (1974) Acta Endocrinol. 77, 417–421.
[5] Tager, H. S. (1976) Anal. Biochem. 71, 367–375.
[6] Glazer, A. N., DeLange, R. J. and Sigman, D. S. (1975) in: Laboratory techniques in biochemistry and molecular biology (Work, T. S. and Work, E. eds) Vol. 4, part I, Chemical Modifications of Proteins, pp. 157–160, Elsevier/North-Holland, Amsterdam.
[7] Ling, N., Leppäluoto, J. and Vale, W. (1976) Anal. Biochem. 76, 125–133.
[8] Glass, J. D., Schwartz, I. L. and Walter, R. (1972) J. Am. Chem. Soc. 94, 6209–6211.