Two Novel Thioamide Analogues of TRH with Selective Activity on CNS

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Abstract. TRH analogues containing C-terminal tioamide group and norvaline ([Nva[2], Prot[3]] TRH) or norleucine ([Nle[2], Prot[3]] TRH) in position 2 were synthesized and tested for hormonal and central nervous system (CNS) activities. Receptor binding studies revealed that the analogues neither bind to pituitary nor to brain TRH receptors. Accordingly, no TSH releasing activity was recorded. However, both analogues significantly affected sleeping time and breathing frequency. Dissociation of endocrine effects from those on the CNS of [Prot[3]] TRH was achieved with the replacement of histidine[2] by aliphatic amino acids. The presence of central histidine is not essential for the analogues to be active on the CNS.

Key words: TRH analogues — Synthesis — Receptor binding — Biological activity

Introduction

The hypothalamic tripeptide, thyrotropin-releasing hormone (TRH) was originally characterized through its stimulatory actions on anterior pituitary hormone secretion (Bowers et al. 1970; Schally and Redding 1965). Later, this hormone was found to be distributed in the central nervous system (CNS) and to function as a synaptic neuromodulator or neurotransmitter (Plotnikoff et al. 1974; Prange et al. 1972). Most likely, the full spectrum of the TRH biological activity on the CNS remains unknown as yet. Of a number of TRH actions, the antidepressant and psychostimulant activity are of interest because of their possible uses in human medicine. With the aim of disscociating hormonal activity from CNS effects, a large number of TRH analogues have been synthesized (Nutt et al. 1981; Ward et al. 1987). Strong activity on the CNS without

any significant effect on the endocrine system show TRH analogues containing aliphatic amino acids such as norleucine, norvaline or leucine in the central position[2] (Szirtes et al. 1984, 1986).

As we could show previously, a TRH analogue containing C-terminal thioamide group (L-pyroglutamyl-L-histidyl-L-proline thioamide), [Prot^3] TRH, was active on both the endocrine system and the CNS similarly as the natural TRH (Kruszynski et al. 1985; Alexandrová et al. 1987). In this paper we report results obtained with two newly synthesized [Prot^3] TRH derivatives in which the central histidine was replaced by norleucine ([Nle^2, Prot^3] TRH) or norvaline ([Nva^2, Prot^3] TRH). Also, two reference compounds, [Nle^2] TRH and [Nva^2] TRH, were synthesized. Biological and binding activities of the TRH analogues on the endocrine system and the CNS were evaluated.

Materials and Methods

Chemistry. L-Proline thioamide (Prot-NH_2) was synthesized as described elsewhere (Kruszynski 1986) using Lawesson's Reagent (Scheibye 1978) for thionation of Boc-Pro-NH_2. L-pyroglutamyl-L-norvalyl-L-proline thioamide ([Nva^2, Prot^3] TRH), L-pyroglutamyl-L-norleucyl-L-proline thioamide ([Nle^2, Prot^3] TRH), and two reference compounds: L-pyroglutamyl-L-norvalyl-L-proline amide ([Nva^2] TRH: Szirtes et al. 1984) and L-pyroglutamyl-L-norleucyl-L-proline amide ([Nva^2] TRH: Szirtes et al. 1984) were prepared by stepwise solution chemistry as described previously (Kruszynski 1986). The synthesis of Glp-Nva-Prot-NH_2 proceeds in several steps:

$$\text{Boc-Nva} + \text{Prot-NH}_2 \xrightarrow{\text{DCCI HOBt}} \text{Boc-Nva-Prot-NH}_2 \xrightarrow{\text{HCl/AcOH}} \text{Nva-Prot-NH}_2\,.\,\text{HCl}(+ \text{Glp-OTcp}) \xrightarrow{\text{NMM}} \text{Glp-Nva-Prot-NH}_2.$$

Acidolytic removal of the Boc protecting group from dipeptide thioamides with HCl/AcOH at 0 °C or by TFA to receive pure products presented some difficulties. Therefore, the crude salt of the dipeptide thioamides were used immediately to prepare peptide 1 and 2.

The crude tripeptides thioamides (1,2) and tripeptides amides (3,4) were purified by column chromatography on silica gel 60, eluted with a methanol-ethyl acetate (3:1, v/v) mixture and then filtered through Sephadex G—10 with 5% acetic acid. The fractions were monitored for absorbance at 252 nm. Those containing the product were examined by TLC and HPLC, pooled and lyophilized. The homogeneity of the products was demonstrated by TLC and HPLC (HPLC showed 98—97 % purity of the four peptides obtained). The correct structure of the products was checked by amino acid analysis and mass spectrometry (molecular ions). The physicochemical properties of fragments and peptides 1—4 are summarized in Table 1.

Optical rotation was measured with a Perkin-Elmer model 141 polarimeter. Molecular ions were obtained with a Varian MAT 711 high resolution mass spectrometer using FD technique. For amino acid analysis, the peptides were hydrolyzed in 6N HCl for 18 h at 115 °C, and the hydrolysates were analyzed on a Mikrotechna model AAA 881 amino acid analyzer. For TLC, precoated plates (silica gel 60, 0.25 mm, Merck) were used in the following solvent systems (all v/v): A, butanol-l-acetic acid-water-ethyl acetate (1:1:1:1); B, butanol-l -acetic acid-water (4:1:5, upper phase); C, ethanol — (0.02 mol.l^{-1} acetic acid — 0.02 mol.l^{-1} pyridine, aq.) (4:1); D, methanol — ethyl acetate

(3:1); E, methylene chloride-acetone-acetic acid (40:10:1); F, chloroform — methanol — acetic acid (19:5:1); G, methylene chloride-methanol (15:1). Spots were visualized after exposure to vaporized iodine, minhydrine or chlorine — potasium iodide-starch. Column chromatography was carried out on silica gel 60 (Merck, 70—230 mesh ASTM). HPLC was performed on a Beckman (System Gold) apparatus with a UV detector set at 210 nm. The separation column, 250 × 4.6 mm, packed with Bondapak, C_{18}, 10 μm as chromatographic support for reversed-phase HPLC was preceded by a short (45 × 4.6 mm) precolumn (Bondapak, C_{18}/Covasil, 37—50 μm). Chromatography was conducted at room temperature with a solvent flow rate of 2.0 ml/min; methanol-water (1:3, v/v) was used as the eluent.

Abbreviations. Symbols of amino acids and peptides are in accordance with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (Eur. J. Biochem. 138, 9—37, 1984). Symbol Prot is used to indicate the thiocarbonyl analogue of the proline residue as proposed by Jones et al. (1973). DCCI: N,N′-dicyclohexylcarbodiimide; OTcp: 2,4,5-trichlorphenol; NMM: N-methyl morpholine; TFA: Trifluoroacetic acid.

Biology ^{3}H—TRH 110 Ci/mmol was a gift from Dr. J. L. Morgat (Service de Biochimie, C.E.N. Saclay, Gif-sur-Yvette, France).

Adult male Wistar rats of specific pathogen free colony (SPF) were used. The animals were kept under SPF conditions in temperature (24 ± 1.1 °C) and light (6.00—18.00 h) controlled room, and received rat chow and water ad libitum.

Tissue preparation and binding studies: The membrane fraction of the rat anterior pituitary, the hypothalamus, the amygdala, the frontal cortex or the striatum was used for ^{3}H—TRH binding studies. Tissue preparation and binding procedure were described previously (Alexandrová et al. 1987). The reaction mixture for competition studies contained 50 nmol.l^{-1} ^{3}H—TRH. The concentration of the analogues ranged from 10^{-9} to 10^{-5} mol.l^{-1}. The binding affinities of the analogues to TRH receptor are expressed in terms of IC_{50} (the peptide concentration which inhibits specific binding by 50 %).

Effects of peptides on the CNS: Groups of 6 adult male rats were anesthetized with penthobarbital (40 mg/kg/i.p.) and injected i.p., 20 min later, with saline (1 ml/kg) or the analogues in a dose of 1.0 or 10.0 mg/kg in the same volume of saline (dissolved before the injection). The frequency of breathing and body temperature were measured.

TSH-releasing activity: Adult male rats were injected i.p. with 5 μg/100 g b.w. TRH or peptide. The peptides were dissolved in saline and administered in a volume of 0.1 ml per 100 g b.w. At 0 and 20 min after administration, blood was taken transcutaneously from the jugular vein under ether anesthesia. The plasma was stored at −20 °C until assayed. Serum levels of TSH were determined by double antibody radioimmunoassay using material for rat TSH kindly provided by the Rat Pituitary Hormone Distribution Program of the NIACHD (Bethesda, MD).

Student's t-test was used for statistical evaluation.

Table 1. Physicochemical characteristics of the TRH analogues tested

Nr.	Peptide	Yield[a] (%)	$(\alpha)D^{25}$, deg (C = 0.5 Methanol)	TLC (R_F)		Amino acid analysis[c]			molecular ion (M^+)	molecular formula
						Glu	Nle/Nva	Pro		
a	Boc-Nva-Prot-NH_2	96	− 63.2	0.55 0.65	(E) (F)		—		329.5	$C_{15}H_{17}N_3O_3S$ (329.5)
b.	Boc-Nle-Prot-NH_2	85	− 84.2	0.33 0.60 0.63	(G) (E) (F)		—		343.5	$C_{16}H_{29}N_3O_3S$ (343.5)
c.	Boc-Nva-Pro-NH_2	87	− 18.0	0.24 0.71 0.36	(E) (F) (G)		–		313.4	$C_{15}H_{27}N_3O_4$ (314.4)
d.	Boc-Nle-Pro-NH_2	96	− 50.6	0.30 0.73 0.43	(E) (F) (G)		–		327.3	$C_{16}H_{29}N_3O_4$ (327.4)
1.	Glp-Nva-Prot-NH_2	6	− 125.9 c = 0.1	0.72 0.29 0.76 0.70	(A) (B) (C) (D)	0.98	1.02	0.97	341.2	$C_{15}H_{25}O_3N_4S$ (341.5)
2.	Glp-Nle-Prot-NH_2	8	− 167.9 c = 0.1	0.71 0.37 0.60	A B C	0.96	0.98	1.05	354.9	$C_{16}H_{26}N_4O_3S$ (354.9)

3.	Glp-Nva-Pro-NH_2	43	$-27.8^{b)}$	0.65 0.25 0.59 0.53	A B C D	0.97	1.00	1.03	325.4	$C_{15}H_{25}O_4N_4$ (325.4)
4.	Glp-Nle-Pro-NH_2	50	$-66.5^{b)}$	0.66 0.34 0.56 0.55	A B C D	0.95	0.98	1.06	339.3	$C_{16}H_{27}O_4N_4$ (339.4)

All amino acids are L-configuration

a) Yields based on the quantity of N-protected derivatives used for acidolytic deprotection step.

b) Szirtes et al. (1984): (α) D^{25} (c = 1, AcOH): Nr. 3 −87.0; Nr. 4 −77.1;

c) Calculated as the average value for all the three amino acids, taken as the standard value.

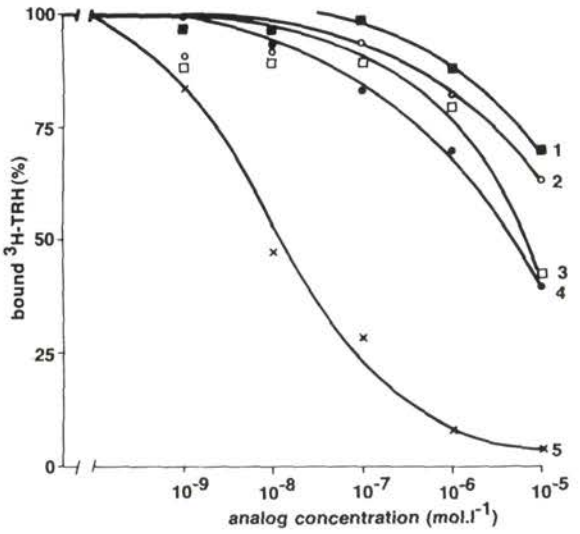

Fig. 1. Competition of TRH with the analogues tested for specific ^{3}H—TRH binding to rat anterior pituitary membrane fraction. Each point represents the mean of three experiments. S. E. of the mean below 10%. 1, [Nva^2] TRH; 2, [Nle^2, Prot^3] TRH; 3, [Nva^2, Prot^3] TRH; 4, [Nle^2] TRH; 5, TRH.

Results

Binding studies:

The relative ability of the TRH analogues tested to compete with ^{3}H—TRH for binding to the anterior pituitary membrane receptor was very weak in comparison with natural TRH (Fig. 1). The displacement of ^{3}H—TRH bound to the receptor, with the [Nle^2] TRH and [Nva^2, Prot^3] TRH analogue at 10^{-5} mol l^{-1} gave similar results. However, only [Nle^2] TRH competed for binding in a concentration dependent fashion, the IC_{50} value being more than 200 times higher than that for TRH (7500 nmol l^{-1} vs. 32 nmol l^{-1}).

The binding affinities of the thioanalogues to TRH receptors in the hypothalamus, the frontal cortex, the amygdala and the striatum were also measured. However, none of the analogues competed with ^{3}H—TRH for binding in these areas of the brain (amygdala, Fig. 2, other tissues not shown).

TSH releasing activity:

TSH-releasing activity of [Nva^2, Prot^3] TRH was approximately 80 % lower in

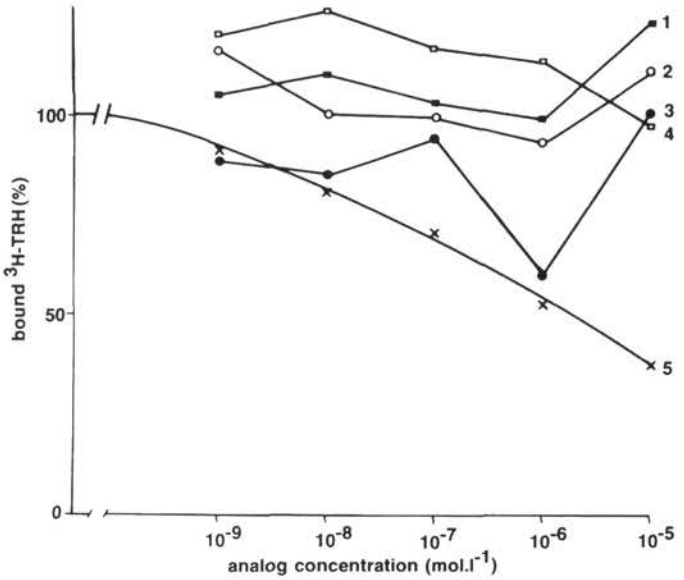

Fig. 2. Competition of TRH and the analogues tested for specific ^{3}H—TRH binding to rat amygdala membrane fraction. Each point represents the mean of three experiments. S. E. of the mean below 10 %. 1, [Nva^2] TRH; 2, [Nle^2, Prot^3] TRH; 3, [Nle^2] TRH; 4, [Nva^2, Prot^3] TRH; 5, TRH.

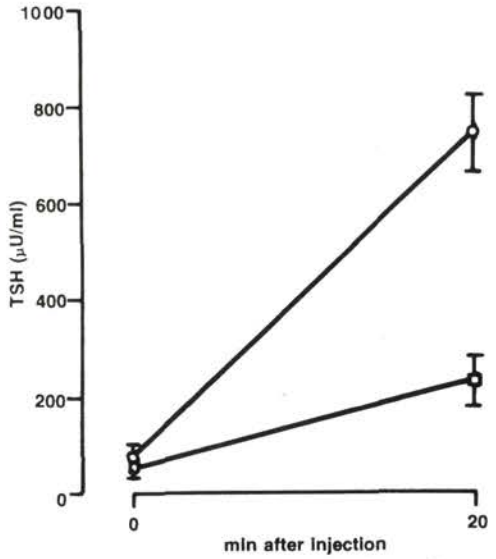

Fig. 3. Induction of TSH-release in rats by TRH and [Nva^2, Prot^3] TRH. Adult male rats were injected i. p. with 5 μg/100 g b. w. TRH (○) or [Nva^2, Prot^3] TRH (□). Blood was taken before (time 0, control sample) and 20 min after the administration of the peptides. Details, see Materials and Methods. Mean ± S. E. for six animals. 1, TRH; 2, [Nva^2, Prot^3] TRH.

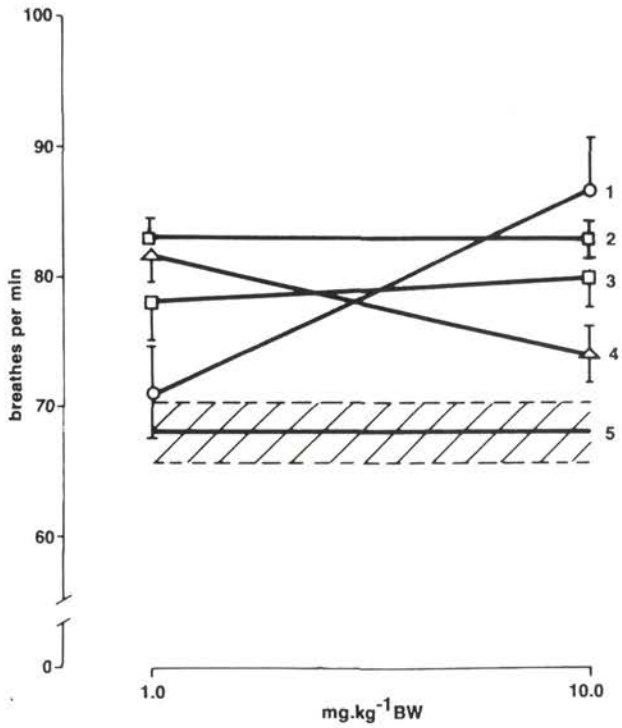

Fig. 4. Effects of TRH, the analogues tested and saline (control group) on breathing frequency. Mean ± S. E. for five animals. 1, TRH; 2, [Nle^2, Prot^3] TRH; 3, [Nva^2, Prot^3] TRH; 4, [Nle^2] TRH; 5, NaCl.

comparison with natural TRH (Fig. 3).
[Nle^2, Prot^3] TRH was devoid of any TSH-releasing potency (not shown).

Effects on CNS:

Lower dose of the [Nle^2] TRH analogue and both thioanalogues significantly increased the breathing frequency as compared to the control group, while the same dose of natural TRH was without any effect (Fig. 4), ten times higher dose of natural TRH and thioanalogues significantly increased the breathing frequency, [Nle^2] TRH analogue was not effective.

Similar results were observed concerning the effects of the analogues on body temperature (Fig. 5). TRH was effective only when administered in the higher dose (10 mg/kg). On the contrary, the thioanalogue [Nva^2, Prot^3] TRH in

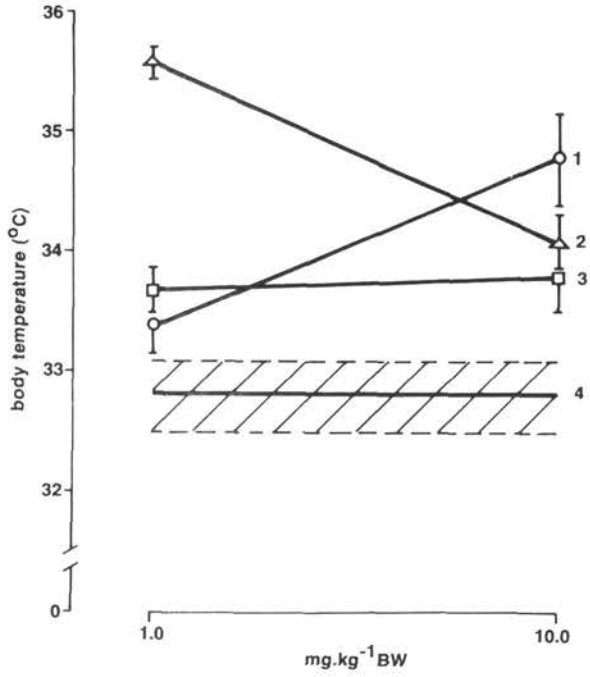

Fig. 5. Effects of TRH, the analogues tested and saline (control group) on body temperature. Mean ± S. E. for five animals. 1, TRH; 2, [Nle^2] TRH; 3, [Nva^2, Prot^3] TRH; 4, NaCl.

both doses used (1 and 10 mg/kg) and [Nle^2, Prot^3] TRH in the lower dose (1 mg/kg, the higher dose was not administered because of shortage of material) produced significant increases in body temperature as compared to control group (34.2 °C vs. 32.8 °C, not shown). Increasing concentrations of the [Nle^2] TRH analogue had decreasing effects (Fig. 5). The effects of the [Nva^2] TRH analogue on the breathing frequency and body temperature were not tested.

Discussion

As reported previously, [Prót^3] TRH has the same activity on the endocrine system as does natural TRH, with a direct correlation between the binding to the anterior pituitary TRH receptors and the TSH-releasing activity (Kruszynski et al. 1985; Alexandrová et al. 1987). The replacement of histidine in the [Prot^3] TRH molecule by aliphatic amino acids, norleucine or norvaline drastically reduced the binding activity of both [Nle^2, Prot^3] TRH and [Nva^2, Prot^3]

TRH, and concomitantly reduced TSH-releasing activity (Figs. 1,3). Elimination of TSH-releasing activity by replacing of histidine^2 in TRH molecule by norleucine or norvaline was already reported by Szirtes et al. (1984). No binding of these analogues to pituitary TRH receptors could be detected in the present study (Fig. 1) Our results emphasize again the important role of the central histidine in the hormonal activity of TRH.

The thioanalogue [Prot^3] TRH as well as natural TRH bind to TRH receptors in the CNS and affect the sleeping time and the motoric activity when applied intracerebroventricularly to the rat (Alexandrová et al. 1987). On the other hand, replacement of histidine^2 of the TRH thioanalogue by Nle or Nva disabled the binding of the thioanalogues to TRH receptors in different areas of the brain (Fig. 3). Nevertheless, they could act centrally to affect the sleeping time and the breathing frequency (Figs. 4,5), with an even higher efficacy than that of natural TRH. However, the effects are not dose dependent. This discrepancy could be explained by a fast degradation of the [Prot^3] TRH thioanalogues in the plasma (Angyal et al. 1985). The problem can be eliminated by administering the compounds into the cerebral ventricles directly. In conclusion, replacement of central histidine^2 in the [Prot^3] TRH analogue by norleucine or norvaline provides analogues with a selective effect on the CNS without any remarkable hormonal activity. The presence of histidine^2 is not necessary for TRH thioanalogues to maintain activity on the CNS. The results suggest that the action of TRH and the TRH thioanalogues, at least concerning some of the CNS effects, are not mediated via TRH receptors.

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