Vol. 98, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1981 Pages 234-241

COMPARATIVE STUDIES OF THYROTROPIN RELEASING HORMONE DIAZOMETHYL KETONE AND CHLOROMETHYL KETONE ANALOGS

James L. Balk, Robert B. Johnston and John T. Pelton

Department of Chemistry
University of Nebraska - Lincoln
Lincoln, Nebraska 68588

Received December 1,1980

SUMMARY

Diazomethyl ketone and chloromethyl ketone analogs of thyrotropin releasing hormones have been synthesized and studied for their inhibitory effects on thyrotropin releasing hormone-induced release of radioactive ^{125}I-labelled hormones from the thyroid gland of eight-week old male Long-Evans rats. When Long-Evans rats were pretreated with thyrotropin releasing hormone diazomethyl ketone (TRH-DMK) or the chloromethyl ketone derivative (TRH-CMK), a dose-related inhibition of thyrotropin releasing hormone-induced ^{125}I release was observed which could be partially reversed by thyrotropin stimulating hormone (TSH). The diazomethyl ketone was a more effective inhibitor than the chloromethyl ketone. These compounds may act as an active-site directed antagonists whose effects are unique to the hypothalamo-pituitary-thyroid system.

INTRODUCTION

The isolation and chemical identification of hypothalamic releasing hormones has established the important biochemical role of small molecular weight peptides in the neuroendocrine system (1,2). Since thyrotropin releasing hormone (TRH) is a relatively simple molecule compared to other biological active peptides, it is an obvious model of choice for the synthesis of analogs to study the relationships between specific changes

---

Abbreviations: TRH, thyroid releasing hormone; TRH-OH, the free acid derivative of thyroid releasing hormone which the proline residue has a carboxylic acid group; TRH-OMe, the derivative of thyroid releasing hormone which the prolinamide is replaced by proline methyl ester; TRH-DMK, the derivative of thyroid releasing hormone which the amide is replaced in a diazomethyl group; TRH-CMK, the derivative of thyroid releasing hormone which the amide is replaced by a chloromethyl group.

0006-291X/81/010234-08$01.00/0

in molecular structure and of biological activity. Extensive studies have been conducted on the structure-function relationship of TRH by substituting an amino acid or amino acid analogs (3,4,5,6). These studies have established that the biological activity of TRH is highly dependent upon preserving the essential features of the side chain of the three constituent amino acids. Several analogs with agonist activity have been synthesized, including a superactive agonist ($[N^{\tau}$(3-Me)-histidine$]^2$-TRH), but antagonists of the releasing hormone are difficult to obtain (7,8,9).

The rationale of the experiments described here was to retain the structural features of the TRH molecule and to modify the molecule by the introduction of specific chemically reactive functional groups which may react specifically with the pituitary receptor site. Inhibitors for enzyme-substrate interactions (halomethyl ketone and diazomethyl ketones) are known (10,11).

The pyroglutamyl residue in TRH serves as a blocking group for the amino terminal. This unique chemical property permits the convenient synthesis of the diazomethyl ketone of TRH. The availability of diazomethyl ketone and chloromethyl ketone analogs of TRH could provide useful chemical probes for comparative studies of the two inhibitors which could be employed to investigate the mechanism by which TRH interacts at the receptor site as well as to study the possible effects these compounds may have on key enzymes involved in the synthesis and breakdown of TRH.

In this paper we report the synthesis of TRH-diazomethyl ketone and TRH-chloromethyl ketone, and present evidence that the more structurally related diazomethyl ketone of TRH is more effective than the chloromethyl ketone in the *in vivo* inhibition of TRH-induced release of ^{125}I-labelled hormones from the thyroid gland.

MATERIALS AND METHODS

Preparation of TRH-CMK and TRH-DMK:

TRH-OH (pGluHisProOH) was synthesized by coupling the dipeptide free

acid, pGluHisOH, with neutralized ProOMe with dicyclohexylcarbodiimide in dimethylformamide and triethylamine. The resulting ester was saponified (NaOH/MeOH) and purified by Dowex 50 (H^+), 8X, 50-100 mesh by the batch technique. The free acid exhibited no biological activity in the McKenzie in vivo bioassay (12), The amide ($pGluHisProNH_2$) obtained from the ester by treatment with methanolic ammonia was as active as an authenic sample of TRH in the McKenzie assay. Thin layer chromatography with silica G showed one Pauly (+), ninhydrin (-) spot (R_f = 0.53) for pGluHisProOMe; MeOH/$CHCl_3$ (2:1). R_f for the TRH-OMe = 0.38; BuOH:AcOH:H_2O:Pyr (60:12:48:40). TRH-OH (pGluHisProOH) gave a Pauly (+), ninhydrin (-) spot (R_f = 0.27) with the same system. IR spectrum (Perkin-Elmer 621 Grating IR) for TRH-OMe (pGluHisProOMe) had absorption frequences: (a) broad band 3700 cm^{-1} - 2200 cm^{-1} (NH stretch); (b) peak at 3224 cm^{-1}; (c) carbonyl stretch at 1690 cm^{-1}; and (d) a series of peaks at 1600 cm^{-1}, 1455 cm^{-1}, 1400 cm^{-1}, 1265 cm^{-1}, and 1100 cm^{-1} which are characteristic for $pGluHisProNH_2$, pGluHisProOH, and pGluHisProOMe. Analysis of a TRH-OH acid hydrolysate (Beckman Model 120°C amino acid analyzer) gave a molar ratio of the histidine:glutamic acid:proline:1.37:1.00:1.13, respectively. Electron impact low resolution mass spectrum for TRH (probe temp. 280° C) from ammonalysis of TRH-OMe showed a molecular ion at m/e 362.13 and is consistent with that reported by Boler, Chan, and Folkers (13). The molecular ion as well as the dipeptide, pGluHis, (m/e = 121.19) indicates that the chemical structure represents TRH.

TRH-diazomethyl ketone (TRH-DMK) and TRH-chloromethyl ketone were synthesized by reacting the mixed anhydride of TRH-OH (isobutyl-chloroformate/Et_3N:) with ethereal diazomethane (14). TRH-chloromethyl ketone (TRH-CMK) was prepared by reaction of TRH-DMK with 4.2 M HCl in dioxane. The IR spectrum of TRH-DMK showed an absorption frequency at 2100 cm^{-1} (diazo group) and a carbonyl stretch at 1634 cm^{-1}, whereas the IR spectrum of TRH-CMK showed a carbonyl stretch at 1742 cm^{-1}. The 60 MHz proton-NMR spectrum of TRH-CMK showed a methylene singlet ($-CH_2Cl$) at δ = 4.25 ppm (D_2O). The low resolution electron-impact mass spectrum of TRH-CMK showed m/e at 245.61 (M^+-CH_2Cl), m/e 49.50 (CH_2Cl), and a M^+ (molecular ion) at m/e 396.12 (0.15 % RA).

In Vivo Assay for TRH, TRH-DMK, and TRH-CMK Biological Activity: TRH releasing activity was determined from the amount of ^{125}I-labelled thyroid hormone released into peripheral blood in response to TRH injected by a modification of the McKenzie technique (12). Twenty-four hours before bioassay the animals (approximately 200 g male Long-Evans rats, low iodine diet (Remington diet, Nutritional Biochemical Corp., Cleveland, Ohio) with distilled water ad lib) were injected intraperitoneally (ip) with 15 μCi $Na^{125}I$, and one hour before injection of TRH, TRH-OH, or 0.85% NaCl, the animals (ether anesthesia) were pretreated with various compounds or saline. One hour following pretreatment with 0.85% NaCl, TRH-DMK, or TRH-CMK, 0.2 ml blood was taken from either the tail vein or jugular and immediately after the blood sample was withdrawn TRH, TRH-OH, or 0.85% NaCl at various doses was injected. Three hours following pretreatment a second 0.2 ml blood sample was collected from the same anatomical site. The ^{125}I content at the first and second blood samples was evaluated with a Beckman γ-spectrometer, and the net cpm released calculated by difference. The protocol was designed so that animals were challenged with compounds for which a known response was expected to serve as controls. Four animals per dose-level were used to obtain a precision index of 0.25.

RESULTS AND DISCUSSION

The TRH dose-response curve obtained from animals pretreated with

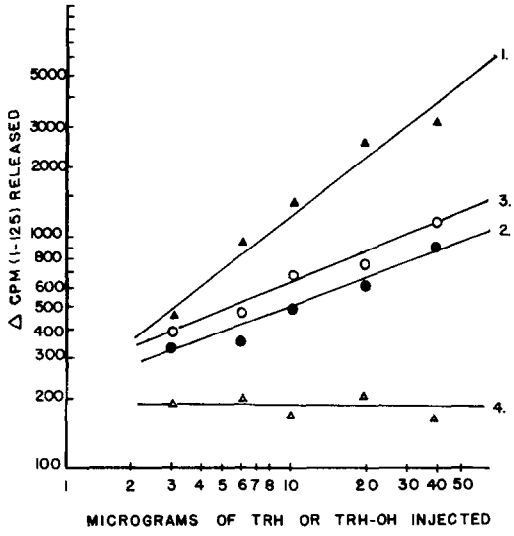

FIGURE 1: Curve 1. Approximately 200 g male Long-Evans rats (4 per dose level) were pretreated as indicated followed by treatment with 3,6, 10, 20, and 40 µg of TRH one hour later. Curve 1. Pretreated with 0.85% NaCl. Curve 2. Pretreated with 40 µg of TRH-DMK. Curve 3. Pretreated with 40 µg TRH-DMK and 5 mU TSH at one-and-one-half hours. Curve 4. Pretreated with physiological saline at "zero" time followed by treatment with 3, 6, 10, 20, and 40 µg of TRH-OH one hour later.

40 µg of TRH-DMK and given TRH (Fig. 1, line 2) has a slope less than that of animals pretreated with saline and given TRH (line 1), but greater than that of line 4 (TRH-OH) whereby animals were pretreated with saline followed by treatment with TRH-OH at one hour. Therefore, TRH-DMK at the 40 µg level caused a 73% inhibition (from 3020 cpm to 812 cpm) of the TRH-induced *in vivo* release of ^{125}I-labelled hormone (e.g., T_3 and T_4). Injection of 5 mU of TSH one-half hour following injection of the various doses of TRH at one hour into animals pretreated with 40 µg of TRH-DMK resulted in partial relief of TRH-DMK inhibition (see line 3).

Animals pretreated with 40 µg of TRH-CMK (curve 2, Figure 2) and challenged with 40 µg of TRH after one hour released a Δcpm ^{125}I (two hours after TRH treatment) of 1600 cpm as compared to 3000 cpm for animals pretreated with saline and treated similarly (curve 1, Figure 2). A net difference of 1400 cpm (or 47% decrease) in Δ cpm ^{125}I release occurred. A 37% increase (1600 cpm → 2200 cpm) resulted when TRH-CMR pretreated

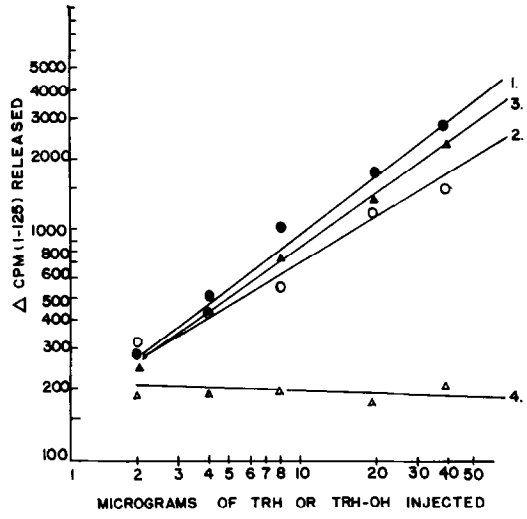

FIGURE 2: Curve 1: Approximately 200 g male Long-Evans rats (4 per dose level) were pretreated as indicated followed by treatment with 3, 6, 10,20, and 40 μg of TRH one hour later. Curve 1. Pretreated with 0.85% NaCl. Curve 2. Pretreated with 40 μg of TRH-DMK. Curve 3. Pretreated with 40 μg of TRH-DMK and 5 mU TSH at one-and-one-half hours. Curve 4. Pretreated with physiological saline at "zero" time followed by treatment with 3, 6, 10, 20, and 40 μg of TRH-OH one hour later.

animals were injected with 5 mU TSH into the jugular vein (see curve 3, Figure 2).

The results in Table I show the effect of various doses of TRH-DMK on the TRH dose-response curve. An increasing dose of TRH-DMK caused a decrease in Δcpm ^{125}I release when animals were challenged with 0.85% NaCl, 40 μg, and 80 μg TRH-DMK during pretreatment. Therefore, the inhibitory effect of increasing doses of TRH-DMK (or TRH-CMK) is dose-related.

The TRH-DMK derivative is a more potent inhibitor than the TRH-CMK derivative of the TRH-induced ^{125}I release from the thyroid gland. This may be due to its more closely related structure to TRH or to its being chemically more reactive. General methods of preparation of diazomethyl ketones of biologically active peptides have not been developed. In this special case of TRH where the diazomethyl ketone can be prepared it is of interest that it is more active than chloromethyl ketone. The observation that TSH partially relieves the inhibition of both substances suggests

Table I

TRH-INDUCED ^{125}I RELEASE AT VARIOUS DOSES OF TRH-DMK OR TRH-CMK

Pretreatment (zero time)	Treatment (at one hour)	*Δ cpm ^{125}I released at three hours)
0.85% NaCl	0.85% NaCl	29 ± 5
0.85% NaCl	4 μg TRH	129 ± 30
0.85% NaCl	40 μg TRH	567 ± 49
0.85% NaCl	80 μg TRH	698 ± 52
0.85% NaCl	40 μg TRH	479 ± 56
4 μg TRH-DMK	40 μg TRH	396 ± 49
40 μg TRH-DMK	40 μg TRH	212 ± 29
80 μg TRH-DMK	40 μg TRH	216 ± 19
0.85% NaCl	80 μg TRH	597 ± 53
4 μg TRH-DMK	80 μg TRH	476 ± 49
40 μg TRH-DMK	80 μg TRH	113 ± 16
80 μg TRH-DMK	80 μg TRH	129 ± 20
0.85% NaCl	40 μg TRH	476 ± 51
4 μg TRH-CMK	40 μg TRH	401 ± 32
40 μg TRH-CMK	40 μg TRH	296 ± 16
80 μg TRH-CMK	40 μg TRH	276 ± 21
0.85% NaCl	80 μg TRH	716 ± 32
4 μg TRH-CMK	80 μg TRH	693 ± 29
40 μg TRH-CMK	80 μg TRH	516 ± 16
80 μg TRH-CMK	80 μg TRH	501 ± 22

* Δ cpm ^{124}I released ± 1 Std. Dev.

that these inhibitors are acting at a site other than the thyroid which is presumably at the pituitary level.

The simplest interpretation of these results is that the inhibitor acts at a receptor site for TRH. Nonspecific chloromethyl ketones (e.g., tosylphenylalanine chloromethyl ketone) at this level do not show any inhibition; therefore, the inhibition observed is not due to a nonspecific interaction of the functional group. Since the inhibitor is active at very low concentrations, comparable to those at which the releasing factor acts, the inhibitor must have a high affinity for its site of action.

Although these inhibitors possess a chemical group which can potentially interact with the receptor site to form a covalent complex, our

experiments do not provide evidence that such an interaction occurs. Unlike enzymes with both affinity and catalytic residues, receptor site proteins possess only affinity type residues. With receptor proteins a covalent interaction would have to be with a functional group of an affinity side chain. Since hydrogen bonding can be important in binding of ligands to proteins, and since hydrogen bond donors are often associated with nucleophilic groups, the covalent attachment of the inhibitor to some nucleophilic group at the active site remains a possibility. The rationale of the preparation and study of this general type of inhibitor is the assumption that if sufficiently reactive chemical groups can be introduced into various positions of the active molecule, then some of these reactive groups may be in a position to interact with the protein to form covalent complexes. This would provide a powerful tool for investigating the interaction at the active site as well as provide a biochemical basis for potential effects which would result from specific control of cell communication by use of specific inhibitors at receptor sites of cell surfaces. To further investigate the effects of TRH-CMK and TRH-DMK on TRH-induced ^{125}I release, analogs such as $[N^{\tau}(3\text{-Me})His]^{2}$-TRH-DMK (or -TRH-CMK) are currently being synthesized. In vivo and in vitro studies whereby the effects of these compounds on TSH and prolactin release are being evaluated by radioimmunoassay are also in progress as well as the preparation of corresponding analogs for other biologically active peptides.

ACKNOWLEDGMENTS

We would like to acknowledge Dr. E.F. Ellington and Dr. D.R. Zimmerman of the Department of Animal Science and Dr. E.B. Barnawell, School of Life Sciences, for their assistance and advice during the course of this research. We also wish to thank Ken Wingrove for his financial support of this research endeavor and Paul Mattern, Department of Agronomy, for his help in the characterization of these compounds.

REFERENCES

1. Guillemin, R.; Burgus, R., and Vale, W. (1971), "The Hypothalamic

Hypophysiotropic Thyrotropin Releasing Factor", in Vitamins and Hormones **29**, pp 1-39.
2. Schally, A.V., Arimura, A., and Kastin, A. (1973) Science **179**, 341.
3. Sievertssoro, H., Chang, J.K., Folkers, K., and Bowers, C.Y. (1972) Journal Medicinal Chemistry **15**, 219.
4. Rivier, J. and Vale, W., et al., (1972) Journal Medicinal Chemistry **15**, 479.
5. Grant, G. and Vale, W. (1973) Endocrinology **92**, 1629.
6. Rivier, C. and Vale, W. (1974) Endocrinology **95**, 978.
7. Lybeck, H., Leppaluoto, J., Verkkunen, D., et al., (1973) Neuroendocrinology **12**, 366.
8. Sievertsson, H., Castensson, S. (1975) Biochem. Biophys. Research Communications **66**, 1401-1407.
9. Bowers, C.Y., Sievertsson, H., Chang, J., et al., (1975), In: Thyroid Research, Proceedings of the 7th Int'l. Thyroid Conference, pp. 1-3, Eds., Jacob Robbins and Lewis Braverman.
10. Mares, M. and Shaw, E. (1963) Federation Proceedings **22**, 528.
11. Shaw, E. and Ruscia, J. (1971) Archs. Biochem. Biophys. **145**, 484.
12. McKenzie, J.M. (1958) Endocrinology **63**, 372.
13. Boler, J., Chang, J.K., Folkers, K. (1971) Journal Medicinal Chem. **15**, 479.
14. Anderson, G.W. (1967) Journal Am. Chem. Soc. **89**, 5012.