RELATIONSHIP BETWEEN RECEPTOR-BINDING AFFINITIES
AND TSH-RELEASING ACTIVITIES OF NOVEL TRH
ANALOGS IN THE RAT

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Accepted June 5, 1982

Abstract—Specific bindings of 3H-TRH to rat pituitary homogenate and apparent affinities of TRH analogs for binding sites were studied. The dissociation constant for 3H-TRH binding to rat pituitary homogenate was 30 nM at 0°C, and the number of the binding sites was 120 fmol/mg protein. The apparent affinities of TRH analogs for the binding sites, which were estimated from the ability to displace 3H-TRH from those binding sites, were found to correlate well with their TSH releasing activities. These findings support the idea that the TSH releasing activities of TRH analogs depend almost entirely upon their binding abilities to the TRH receptor in the pituitary.

Thyrotropin-releasing hormone (TRH), which stimulates the release of thyrotropin (TSH) and prolactin from the anterior pituitary gland, also has behavioral and biochemical effects on the central nervous system (1). A number of newly synthesized TRH analogs were biologically tested by us for obtaining a drug acting on the central nervous system with less endocrine side effects. Among them, γ-butyrolactone-γ-carbonyl-histidyl-proline amide citrate (DN-1417) was found to act more powerfully and persistently on the central nervous system than TRH in various behavioral paradigms in rats and mice (2, 3). On the other hand, the TSH-releasing activity of DN-1417 was approximately 40 times less potent than that of TRH (2). As the endocrinological activities of TRH analogs studied by Grant et al. and Hinkle et al. are reported to correlate well with their affinities to the pituitary receptor (4, 5), we have studied whether DN-1417 binds weakly to the pituitary receptor but more strongly to the brain receptor than TRH. In this paper, we further study the endocrinological properties of DN-1417 and compare the binding affinities of DN-1417 and other TRH analogs to rat pituitary homogenate with that of TRH. The results are discussed with respect to their TSH-releasing activities.

MATERIALS AND METHODS

Materials
TRH tartrate monohydrate (TRH), γ-butyrolactone-γ-carbonyl(Blc)-His-Pro-NH2 citrate (DN-1417), Blc-3MeHis-Pro-NH2 acetate (DN-1788), 3-oxoperhydro-1,4-thiazine-5-carbonyl(Otc)-His-Pro-NH2 (DN-1103), Otc-His-Pro-NHBu acetate (DN-1344), 2-hydroxy-4-carboxy-butanoyl(HCB)

Amino acids, peptides, and their derivatives mentioned in this paper are of the L-configuration; and γ-butyrolactone-γ-carboxylic acid is of the S-configuration. Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature.
His-ProNH$_2$, and His-Pro-diketopiperazine (DKP) were synthesized in the Chemistry Laboratories, Central Research Division, Takeda Chemical Industries (2). $^3$H-TRH (100 Ci/m mol) was purchased from the New England Nuclear Corp.

Methods

1) TSH-releasing activity: Various doses of each peptide were intravenously administered via the tail vein to male Sprague-Dawley (Jcl:SD) rats weighing 170-260 g at 6-7 weeks of age. The test peptide was dissolved in 0.9% saline containing 0.1% bovine serum albumin (BSA) and administered in a volume of 0.5 ml per animal. Most of the peptides were given in appropriate salt forms. Control animals were given saline containing BSA alone. At the indicated time after the administration, blood was taken from the abdominal aorta under urethane anesthesia (800 mg/kg, i.p.), and the serum TSH level was determined by the double antibody radioimmunoassay method using NIAMDD-Rat-TSH RIA materials. Results were expressed in terms of NIAMDD-Rat-TSH RP-1, and the TSH releasing potency of a test peptide was calculated by the parallel line assay method (6).

2) Prolactin-releasing activity: Male Jcl:SD rats weighing 240-260 g at 7-8 weeks of age were used. Since male rats did not respond clearly to TRH, they received a single subcutaneous administration of estradiol benzoate (E.B., 50 µg/rat, Schering AG) 3 days before the experiment. Estrogens are reported to increase TRH receptor concentrations in pituitary and hence prolactin secretion in rats (7). An hour after urethane anesthesia (1200-1500 mg/kg, i.p.), each peptide was administered via the femoral vein. One ml of blood was successively taken from the jugular vein before and at the indicated time after peptide administration. We found these conditions appropriate to get reliable results with less variation among experiments. The serum prolactin level was determined by the RIA method using NIAMDD-Rat-prolactin RIA materials. Results were expressed in terms of NIAMDD-Rat-prolactin RP-1.

3) Binding assay to pituitary homogenate: Male Jcl:SD rats weighing 200-300 g at 7-8 weeks of age were used. Immediately after decapitation, the adenohypophyses were freed of the posterior and intermediate lobes, rinsed in ice-cold buffer (50 mM Tris-HCl, pH 7.4), and homogenized in 30-50 vol. ice-cold buffer using a Teflon-glass homogenizer. After being filtered through twice-folded gauze, the filtrate was diluted to a concentration of 3-5 mg protein per ml. For the binding assay, 100 µl of fresh pituitary homogenate and 50 µl of $^3$H-TRH were incubated in a small tube with or without unlabeled TRH or TRH analogs in a final volume of 200 µl. After incubation at 0°C for the indicated periods, 2 ml of cold buffer was added to each tube, and with gentle suction the bound fraction was immediately separated by filtration through GF/B glass fiber filters (Whatman, Inc., Clifton, NJ). The filters were then washed 3 times with 2 ml of cold buffer. Radioactivity of the residue on the filter was counted at an efficiency of about 40% in 5 ml of scintillation fluid (ACS-II, Amersham). Each experiment included control tubes to which 250 nM (more than three thousand times excess of added $^3$H-TRH) of unlabeled TRH was added. The non specific binding (usually 10-15% of the total binding) was subtracted from each value to give the specific binding. All assays were done in triplicate.

The affinity of the TRH analog for the TRH binding site was expressed as the inhibition constant (Ki) calculated according to the formula:
where C and Kd represent the concentration and the dissociation constant of $^3$H-TRH, respectively. The IC50 of the TRH analog was determined from the concentration-response line in which the values of logit B/Bo (B and Bo denote the amount of $^3$H-TRH bound in the presence and absence of TRH analog, respectively) were plotted as a function of the log concentration of the TRH analog. The IC50 was then obtained as the concentration which satisfied logit B/Bo=0.

RESULTS

1) TRH binding to pituitary homogenate: The time-course of $^3$H-TRH binding to pituitary homogenate is shown in Fig. 1. After an hour of incubation at 0°C, almost maximal binding of $^3$H-TRH was obtained, a
plateau being reached at 1.5–2 hr. Therefore, the reaction mixture was incubated for 1.5 or 2 hr at 0°C in subsequent experiments. Under these conditions, no significant degradation of \(^3\)H-TRH was detected; and the amount of \(^3\)H-TRH bound was linearly related to the protein concentration up to 5 mg/ml (not shown). Figure 2(A) demonstrates the saturability of \(^3\)H-TRH binding to pituitary homogenate. Double-reciprocal plot analysis of the data showed a single class of binding sites with the following characteristics: the number of binding sites (Bmax) was 120 femtomoles/mg protein and the dissociation constant (Kd) was 30 nM (Fig. 2(B)). When pituitary homogenate and 11 nM of \(^3\)H-TRH were incubated with 1–500 nM of unlabeled TRH, unlabeled TRH competed with \(^3\)H-TRH for binding in a concentration-dependent fashion (Fig. 3(A)). The logit B/Bo–log concentration curve of TRH was found to be linear (Fig. 3(B)).

2) Binding of TRH analogs to pituitary homogenate: DN-1417 was also able to compete with \(^3\)H-TRH in binding. The logit B/Bo–log concentration curve fitted a straight line which was parallel to that of TRH (Fig. 4). All other TRH analogs examined also competed with \(^3\)H-TRH for binding with various efficiencies, and their Ki values are listed in Table 1.

3) TSH- and prolactin-releasing activities of TRH and DN-1417: We reported previously that the serum TSH level increased dose-dependently at 15 min after an intravenous administration of TRH or DN-1417 (2). The TSH-releasing responses at all equivalent doses of TRH and DN-1417 were parallel to each other (Fig. 4). The duration of the action did not differ between TRH and DN-1417 (Fig. 5). After an intravenous administration of TRH or DN-1417, the serum prolactin level increased rapidly and reached the maximum at 3 min. Then it returned to the basal level by 30 min, showing similar response curves between TRH and

![Fig. 3. (A) Competition of unlabeled TRH with \(^3\)H-TRH in binding to rat pituitary homogenate. Pituitary homogenate was incubated with 11 nM \(^3\)H-TRH and the indicated amount of unlabeled TRH for 1.5 hr at 0°C. The amount of \(^3\)H-TRH bound to pituitary homogenate was determined as described in the text. (B) \(^3\)H-TRH displacing activity of unlabeled TRH. The values of -logit (B/Bo), where B denotes the amount of \(^3\)H-TRH bound in the presence of unlabeled TRH and Bo denotes that in the absence of unlabeled TRH, were plotted as a function of the amount of unlabeled TRH.](image-url)
Fig. 4. Competition with $^3$H-TRH in binding to rat pituitary homogenate and TSH releasing activity of DN-1417. Pituitary homogenate was incubated with 9.3 nM $^3$H-TRH and the indicated amount of DN-1417 for 1.5 hr at 0°C. The amount of $^3$H-TRH bound to pituitary homogenate was determined as described in the text. The data were expressed as the $^3$H-TRH displacing activity as shown in Fig. 3(B). TSH releasing activity was determined at 15 min after a single i.v. injection of TRH or DN-1417. ●—● $^3$H-TRH displacing activity of TRH, △—△ $^3$H-TRH displacing activity of DN-1417, ○—○ TSH releasing activity of TRH, △—△ TSH releasing activity of DN-1417.

Table 1. TSH releasing activity of TRH analogs in the male rat and their inhibition constant (Ki) for $^3$H-TRH binding to rat pituitary homogenate

| Structure | TSH releasing activity | Ki (nM) |
|-----------|-----------------------|---------|
| pGlu-His-ProNH$_2$ tartrate (TRH) | 1 | 18.1 ± 1.7 (13) |
| Otc-His-ProNH$_2$ (DN-1103) | 1.72 | 24.9 ± 0.3 (3) |
| Otc-His-ProNH$_2$Bu acetate (DN-1344) | 1.48 | 35.3 ± 7.6 (3) |
| Bic-3MeHic-ProNH$_2$ acetate (DN-1786) | 0.204 | 609 ± 76 (3) |
| Bic-His-ProNH$_2$ citrate (DN-1417) | 0.024 | 3110 ± 170 (5) |
| HCB-His-ProNH$_2$ | 0.0009 | 307000 ± 58000 (3) |
| His-Pro-diketopiperazine (DKP) | no activity | more than $10^9$ (2) |

a) Bic: γ-butyrolactone-γ-carbonyl, HCB: 2-hydroxy-4-carboxy butanoyl, Otc: 3-oxoperhydro-1,4-thiazine-5-carbonyl, Me: methyl, Bu: n-butyl.

b) TSH releasing activity is expressed as the molar ratio of the i.v. dose of TRH to that of analog required to give the same increase in serum TSH level in the rat. Part of these results were reported by us in Ref. 2. Data for DKP were cited from published papers (Refs. 16–18.)

c) Mean±S.E.M. The number of experiments is indicated in parentheses.
DN-1417 (not shown). The prolactin-releasing responses to TRH and DN-1417 were also parallel to each other at all equivalent doses (Fig. 6). The ratio of prolactin-releasing activity of DN-1417 to TRH was 0.074 (95% confidence limits: 0.023–0.287), which was not significantly different from that of TSH-releasing activity, 0.024 (95% confidence limits: 0.017–0.036) (2).

4) Relationship of the binding abilities of TRH and TRH analogs to their TSH-releasing activities: As seen in the TSH-releasing responses to TRH and DN-1417, all other TRH analogs also showed similarly parallel curves of TSH-releasing activities, having various potencies in comparison to TRH (Table 1). The substitution of pyroglutamyl residue in the TRH structure by the Bic-, Otc-, or HCB residue produced TSH-releasing activities varying from equal to much less potent than TRH. The binding abilities of these peptides to pituitary, which were

Fig. 5. Time-course of the TSH-releasing actions of TRH and DN-1417. Serum TSH level was determined at the indicated time after a single i.v. injection of TRH (0.5 μg/rat; ○) or DN-1417 (16 μg/rat; △) in the male rat. Each point indicates the mean±S.E.M. of 6 rats.

Fig. 6. Prolactin-releasing activity of TRH and DN-1417. Increment of serum prolactin level from the control level (ΔPRL) was determined at 3 min after a single i.v. injection of TRH (○), DN-1417 (△), or vehicle (□) in the E.B.-treated male rat. Each point indicates the mean±S.E.M. of 8 rats.

Fig. 7. Comparison of the relative biological activities and the relative affinities for the TRH binding site of TRH analogs. Biological activity is expressed as the molar ratio of the i.v. dose of TRH to that of analog required to give the same increase in serum TSH level in the rat. Affinity for the TRH binding site is expressed as the ratio of the inhibition constant (Ki) of TRH to that of the analog determined in rat pituitary homogenate as described in the text.
measured as the abilities to compete with \(^{3}\text{H}-\text{TRH}\) in binding, were found to correlate well with their TSH-releasing abilities (Fig. 7). That is to say, DN-1103 and DN-1344, which were equipotent to TRH in releasing TSH from the pituitary gland, showed Ki values comparable to TRH. Moreover, DN-1786 having a methyl group at the 3-N position of the imidazole ring of histidine was several times more potent than DN-1417 in TSH-releasing activity and showed a considerably smaller Ki value than DN-1417. In contrast, one of the metabolites of DN-1417, HCB-His-ProNH\(_{2}\), possessed the least TSH-releasing activity among the peptides examined and showed the largest value of Ki with the exception of the TRH metabolite DKP.

**DISCUSSION**

It is generally accepted that binding of TRH to the TRH receptors on the plasma membrane of pituitary cells mediates its endocrine actions. Biochemical studies on the TRH receptor in the pituitary cell using mouse thyrotropic pituitary tumors; rat prolactin-producing pituitary cells; and rat, sheep, and bovine pituitary homogenate or pituitary plasma membrane fraction have demonstrated the presence of specific binding sites for \(^{3}\text{H}-\text{TRH}\) (4, 7, 9-12).

In the present study, we clearly demonstrate that there exists a single class of binding site for \(^{3}\text{H}-\text{TRH}\) using rat pituitary homogenate as a membrane containing preparation. The characteristics of this binding site (Kd of 30 nM and Bmax of 120 fmoles/mg protein) are in good agreement with the results obtained by other investigators. Although Grant et al. (4) reported 2 classes of binding sites on mouse thyrotropic pituitary tumor (one with a Kd of 20 nM, the other with a Kd of about 500 nM), other authors reported only one class of binding site with a Kd of 20-40 nM in the rat, sheep, and bovine pituitary gland. In response to TRH, normal rat pituitary gland releases prolactin as well as TSH, especially under estrogen sensitization (Fig. 6, Ref. 7, 13). This suggests that there may be at least two kinds of receptors for TRH, one on thyrotropic cells and the other on mammatropic cells. In our binding study, however, the binding site for TRH was detected as a single uniform class. Our results are consistent with those reported by De Lean et al. (7). Martin and Tashjian, Jr. (14) also suggested the possibility of multiple TRH receptors on the prolactin-producing pituitary cell (GH\(_{3}\)) because TRH showed diverse actions on the cell, and the TRH concentrations for the half-maximal response differed among these actions. They detected, however, only a single class of binding site on the GH\(_{3}\) cell (10) and discussed the possibility that there may be receptors with very high affinity and very low capacity, which might be impossible to detect by the usual binding method (14).

TRH and DN-1417 competed with \(^{3}\text{H}-\text{TRH}\) in binding to pituitary homogenate with efficiencies corresponding to their in vivo TSH-releasing activities (Fig. 7). We confirmed also that the actions of DN-1417 on the endocrine system were qualitatively similar to that of TRH, both in the dose-response pattern and in the duration of the action. From the results of the binding affinities and the biological actions, we concluded that DN-1417 acted on the endocrine system through the same mechanism as those of TRH; and the difference in activities between these two peptides was largely derived from the difference in affinities to the TRH receptor.

The TRH analog which has potent binding ability but no or nominal TSH-releasing activity might be a TRH antagonist. So far, no TRH analogs have been found to be antagonists. DN-1417 and all other TRH analogs examined in the present study are
also agonists because their in vitro binding activities have been demonstrated to correlate well with the in vivo TSH-releasing activities. The fact that the dose-response curves of the analogs paralleled those of TRH, both in TSH-releasing action and receptor-binding action, also strongly indicates that our analogs are all agonists to TRH. Recently, the TRH metabolite DKP (15) was reported to inhibit prolactin release, but not TSH release, from the pituitary gland under both basal and TRH-stimulated conditions (16–18). We found that this peptide did not bind to the TRH binding site. Therefore, we consider that this peptide would not act through the TRH-receptor.

As the dissociation of central nervous actions from endocrine actions of TRH has been observed in DN-1417, it is interesting to presume that the brain receptor might differ from the pituitary receptor in recognizing TRH and DN-1417. Relationships of the activities of TRH and TRH analogs on the central nervous systems to their binding abilities to the brain receptor are under investigation.

Acknowledgements: The authors wish to express their cordial thanks to Dr. A.F. Parlow for providing the Rat-TSH kit under the NIAMDD Rat Pituitary Hormone Distribution Program and Drs. M. Fujino, C. Hatanaka, and O. Nishimura for providing the TRH analogs. We also thank Dr. R. Nakayama for helpful advice and Mr. T. Masaki for technical assistance. This work was supported in part by a grant for new drug development from the Ministry of Health and Welfare of Japan.

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