Low Affinity Analogs of Thyrotropin-releasing Hormone Are Super-agonists*

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We show that several analogs of thyrotropin-releasing hormone (TRH) are more efficacious agonists at TRH receptors R1 and R2 than TRH itself. The apparent efficacies of the analogs were inversely related to their potencies and were independent of the nature of the modifications in TRH structure. In studies in intact cells, we showed that the differences in apparent efficacies were not due to differences in G-protein coupling, receptor desensitization, or recycling. Moreover, the differences in efficacies persisted in experiments using accessory protein-free membranes. We conclude that the efficacy differences of TRH analogs originated from the enhanced ability of TRH-R complexes to activate G-protein(s), and not by a modulation of the activity of accessory proteins, and propose possible mechanisms for this phenomenon.

Thyrotropin-releasing hormone (TRH) receptors (TRH-Rs) are members of the rhodopsin-like family (family A) of G-protein coupled receptors. TRH-Rs couple primarily to the Gq/11 subfamily of G-proteins and mediate the intracellular release of Ca2+ through the activation of the inositol phosphate (IP) pathway (1). There are two subtypes of TRH-Rs, TRH-R1 and TRH-R2, that share about 50% sequence homology (2, 3). The physiological significance of the existence of two kinds of TRH-Rs remains unknown. TRH-R1 and TRH-R2 exhibit sub-tle functional differences varying in the level of stimulated and basal signaling, the rate of internalization, and the ability to couple to G-proteins other than Gq/11 (2–4). The ligand binding affinities of TRH analogs to the two receptors, however, are very similar (2). TRH is the natural agonist for both TRH-Rs, and numerous synthetic analogs of TRH have been shown to stimulate both receptors (2). Except for substitution of His by 1-methyl-His, all substitutions within TRH result in analogs with reduced affinities, but all are agonists.

The nature of the molecular changes that are responsible for TRH-R activation remain mainly unknown. The absence of a working hypothesis for the mechanism of TRH-R activation precludes the use of a rational approach to develop new agonists for these receptors. Therefore, study of structure-activity relationships of the known TRH-R1/R2 agonists may contribute to a better understanding of the structural basis of the efficacies of TRH-R-agonist complexes, which is necessary for development of more efficient (specific) modulators of TRH-R activity. In this work, we demonstrate a unique pharmacological profile of a series of TRH analogs in which affinities (potencies) of the compounds are related in an inverse mode to their ability to activate TRH-R1/R2. A corollary of these observations is that certain TRH analogs act as “super-agonists” for TRH-R1/R2. We propose possible mechanisms for this effect.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium and fetal bovine serum were purchased from BIOSOURCE (Rockville, MD). TRH (pGlu-His-ProNH2), MeTRH (pGlu-His(1(3)-methyl)-ProNH2), luciferin, pertussis toxin, and ammonium chloride were purchased from Sigma. Folimycin (concanamycin A), okadaic acid, Ro-31-8425 (2-[8-(aminomethyl)-6,7,8,9-tetrahydropyrido[1,2-a][1,4]indol-3-yl]-3-[1-methyl-1H-indol-3-yl]maleimide, HCl), and HA-1077 (1-[(5-isouquinolinesulf-

The abbreviations used are: TRH, thyrotropin-releasing hormone; TRH-R, TRH receptor; IP, inositol phosphate; HBSS, Hank’s balanced salt solution; CHAPS, 3-[3-cholamidopropyl(dimethylammonio)-1-propanesulfonic acid; GDP, guanosine 5′-3-O-(thio) triphosphate; GPCR, G-protein-coupled receptor.

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N. Kaur, V. Monga, J. S. Josan, and R. Jain, unpublished data.
**TRH Analogs Are Super-agonists at TRH-R**

**TABLE 1**

| Agonist          | R1 IC50 | R1 EC50 | R1 IC50/EC50 | R2 IC50 | R2 EC50 | R2 IC50/EC50 |
|------------------|---------|---------|--------------|---------|---------|--------------|
| TRH              | 4.4 ± 0.42 | 3.2 ± 0.88 | 1.4           | 2.7 ± 0.2 | 0.40 ± 0.057 | 6.8          |
| NP-654           | 3000 ± 230 | 300 ± 63  | 10.0          | 2020 ± 110 | 61.7 ± 10.6 | 32.7         |
| S-Desaza-TRH     | 1470 ± 120 | 210 ± 39  | 7.0           | 340 ± 22  | 9.4 ± 1.3  | 36.2         |
| R-Desaza-TRH     | 6150 ± 540 | 1000 ± 220 | 6.2           | 1960 ± 110 | 41.6 ± 5.7 | 47.1         |
| TRH 4.4          | 0.42     | 3.2       | 6.8           | 0.88     | 1.4       | 2.7          |
| NP-654 3000      | 3.2      | 3.2       | 1.0           | 1.3      | 3.6       | 3.6          |
| S-Desaza-TRH 3000| 22       | 22        | 1.0           | 340      | 15.0      | 22.0         |
| R-Desaza-TRH 110 | 41.6     | 41.6      | 1.0           | 47.1     | 47.1      | 1.0          |

**Internalization Assay**—The cells were incubated at 37 °C with 50 nM TRH or 50 μM R-Desaza-TRH for varied times. The cells were washed twice with 2 ml of Hanks' balanced salt solution (HBSS), 10 mM Hepes, pH 7.4, and incubated at 4 °C for 1 min with ice-cold acid solution (0.2 M acetic acid, 0.5 M NaCl, pH 2.5). This treatment removed 96% of the [3H]TRH specifically bound at the cell surface (8). The cells were washed twice with 2 ml of ice-cold HBSS, 10 mM Hepes and incubated at 4 °C for 2 h with 10 nM [3H]MeTRH. After washing three times with 2 ml of ice-cold HBSS, 10 mM Hepes, the cell-associated radioactivity was measured by dissolving the cells with 1 ml of 0.4 N NaOH and mixing with scintillation liquid.

**Luciferase Assay**—After 33 h of transfection, the cells were incubated for an additional 15 h in the absence or presence of 100 ng/ml pertussis toxin and then stimulated for 5 h with or without ligand. The cells were washed with phosphate-buffered saline and incubated in ice for 15 min with 0.5 ml of lysis buffer (25 mM Gly-Gly, 15 mM MgSO4, 4 mM EGTA, 1 mM dithiothreitol, 1% Triton X-100, pH 7.8). One hundred microliters of lysate were mixed automatically with 125 Ci/ml [3H]IP/([3H]lipids) and the luminescence was measured for 3 s in a Victor^TM^2 multilabel counter 1420 (PerkinElmer Life Sciences). Data are expressed as relative light units.

**Measurement of Phosphoinositide Hydrolysis**—The cells were labeled for 24 h by incubation with 2 μCi/ml myo-[3H]inositol (PerkinElmer Life Sciences). The cells were washed with HBSS, 10 mM Hepes, pH 7.4, and incubated at 37 °C in 10 mM LiCl HBSS solution in the absence or presence of agonist. The cells were lysed, and accumulated IPs were measured using ion-exchange chromatography as described (9). IPs conversion was calculated as [3H]IP% = 100 × [3H]IP/([3H]lipids + [3H]IP).

**Membrane Preparation**—Membranes were prepared from HEK293 cells expressing a stably transfected mouse TRH-R2. The cells in 15-cm dishes were washed twice with 10 ml of phosphate-buffered saline at room temperature and incubated at 4 °C for 5 min at 4 °C. The purified membranes were harvested by scraping and homogenized in a Dounce homogenizer (20 strokes), and the nuclei and cell debris were removed by centrifugation at 1500 × g for 5 min at 4 °C. The postnuclear membrane fraction was collected from the supernatant by centrifugation at 30,000 × g for 1 h at 4 °C. The purified membranes were extracted in the presence of 7 M urea to remove accessory proteins as described previously for BALB/c fibroblasts expressing bombesin receptors (10), frozen, and stored at −80 °C.

**Purification of G-protein Subunits and GDP/GTPγS Exchange Assay**—Gαq was isolated from cuttlefish (Sepia officinalis) retinas essentially as described by Hartman and Northup for squid Gαq (11). Cholate extracts from microvillus membrane fractions of Sepia retina were sequentially chromatographed over DEAE-Sepharose and Ultrogel AcA44. The peak of Gαq was exchanged into a solution containing 4 mM CHAPS by chromatography over Sephadex G-50 prior to storage at −80 °C. Recombinant human β1γ2 expressed in SF9 cells was purified as described previously (12), except that the viral infections were carried out at multiplicity of infection 2 for β1 and 10 for γ2. The purified β1γ2 was additionally chromatographed over Superdex HR75 in a solution containing 8 mM CHAPS prior to storage at −80 °C. The TRH-R2-catalyzed GDP/GTPγS exchange on Gαq was determined as described previously for bombesin receptors (10) with the reaction solutions containing bovine serum albumin at final concentration of 1 mg/ml.

**Data Analysis**—All data were analyzed by linear or non-linear regression using the Prism software version 4 (GraphPad Inc., San Diego, CA).

**RESULTS**

Functional characterization of a number of TRH analogs revealed that the IC50/EC50 ratio, the ratio of the concentration of an analog that causes 50% inhibition of [3H]MeTRH binding divided by the concentration that causes 50% stimulation, for certain TRH analogs is higher than that for TRH in HEK293 cells stably expressing TRH-R1 or TRH-R2 (Table 1, Fig. 1). Although the absolute values of IC50/EC50 ratios in cells expressing TRH-R2 were higher than in cells expressing TRH-R1, the
TRH Analogs Are Super-agonists at TRH-R in the legend for Fig. 2. We found that the more potent compounds exhibited poorer efficacies to stimulate IP formation. Indeed, His(1-methyl)-TRH (MeTRH), the only known TRH analog with an affinity higher than TRH, stimulated IP formation less than TRH. The linear relationship between pEC₅₀ values and intrinsic efficacies shown in Fig. 2B is limited because there are no TRH analogs with potencies between Phe²-TRH and TRH.

To obtain additional evidence to support the idea that MeTRH is a less effective agonist than R-Desaza-TRH, we measured the effect of MeTRH on IP formation stimulated by R-Desaza-TRH. As predicted, MeTRH inhibited R-Desaza-TRH-stimulated IP formation in a dose-dependent manner, behaving as a classical partial agonist (Fig. 3). In this experiment, the apparent inhibitory constant for MeTRH at TRH-R1 (0.32 ± 0.16 nM), estimated from the IC₅₀ of inhibition, is similar to the dissociation constant determined in saturation binding experiments (0.61 ± 0.10 nM), consistent with a competition between the two analogs for the same site on TRH-R1.

We also measured the efficacies of TRH analogs using luciferase gene transcription as a reporter of TRH-R signaling. In this system, luciferase expression is mediated by AP1 (activating protein 1) transcription factor, which is activated by protein kinase C during TRH-R signaling (2, 14). Luciferase gene transcription stimulated by TRH is partially inhibited by pertussis toxin,⁴ indicating the ability of TRH-R to couple to pertussis toxin-sensitive G-proteins, most likely of the Gₛ family, in addition to Gₛ₁₁. As shown in Fig. 4, the efficacy profiles of TRH analogs measured by luciferase activity were similar to those obtained when measuring IP formation. Moreover, the responses stimulated by the different agonists showed similar levels of pertussis toxin sensitivity, indicating a similar ability to activate Gₛ and/or Gₛ₁₁. In separate experiments, in which we measured accumulation of cAMP, we showed that TRH-R stimulated with both high and low affinity compounds was unable to activate Gₛ protein (not shown). These results suggest that all analogs activate the same signal transduction pathways and do not exhibit pathway (or G-protein) selectivity.

Signaling by GPCRs is rapidly desensitized by uncoupling of receptors from their signaling pathways. Therefore, it was possible that the increased agonist efficacy of some analogs may have been due to decreased desensitization. Because desensitization is usually mediated by phosphorylation by one or more protein kinases (15–17), we tested the possibility that the interaction of the activated TRH-R with a specific protein kinase is ligand-specific and contributes to generation of the

⁴ S. Neumann, in preparation.
TRH Analogs Are Super-agonists at TRH-R

FIGURE 4. Stimulation of AP1 response by TRH and TRH analogs. HEK293 cells stably expressing TRH-R1 (SN-R1-17 clone) were transfected with 0.8 μg/ml AP1-Luc DNA for 33 h followed by 15 h of incubation without (control) or with 100 ng/ml pertussis toxin (PTX). The cells were stimulated by 50 nM MeTRH, 50 nM TRH, 50 μM NP 654, or 50 μM R-Desaza-TRH (R-Des-TRH) for 5 h, and luciferase activity was measured as described under “Experimental Procedures.” Results are expressed as mean ± S.D. of assays performed in triplicate in a representative experiment.

efficacy differences between the TRH analogs. As shown in Fig. 5A, the specific protein kinase C inhibitor Ro-31–8425 (18), as well as the broad range protein kinase inhibitor HA-1077 (19), had no effect on the ligand specificity of the TRH-R1 response.

Upon prolonged stimulation, the balance between the rates of receptor desensitization and resensitization determines the steady state level of active receptors on the cell surface and thus the efficacy of response (15). To answer the question whether the internalization (and recycling) of the agonist-TRH-R complex, as part of the desensitization/resensitization pathway, is affected by the nature of agonist, we compared the ability of TRH and R-Desaza-TRH to trigger TRH-R1 loss from the cell surface. As shown in Fig. 5B, no differences in the rates or extents of TRH-R1 internalization were observed using these agonists. During the course of resensitization, a phosphorylation(s) introduced by protein kinase(s) is reversed by the action of distinct endosomal phosphatases (15, 20), and the unphosphorylated receptor is returned to the cell surface. We have used the pharmacological agents preferentially affecting the activity of endosomal phosphatases to test the possibility that the efficiency of dephosphorylation is dependent on the nature of the agonist-receptor complex internalized and contributes to the efficacy differences between TRH analogs. Okadaic acid, the selective inhibitor of the activity of PP1 and PP2A phosphatases (21), as well as the specific inhibitor of vacuolar-type H⁺-ATPase folimycin (concanamycin A) (22) and ammonium chloride, both preventing the endosomal acidification required for phosphatase activity, had no significant effect on the relative ability of MeTRH and R-Desaza-TRH to stimulate IP formation (Fig. 5C).

Whatever the mechanism responsible for the observed differences in the efficacies of agonists is, the phenomenon reflects variations in the ability of an agonist-receptor complex to activate the corresponding G-protein. A possible reason behind this effect can be an altered affinity of the activated receptor for the subunits of G-protein. If this were correct, it would be possible to decrease the efficacy differences by overexpression of a corresponding G-protein, pushing the thermodynamic equilibrium toward complex formation. As shown in Fig. 6, the overexpression of human Goαq enhanced the overall rate of IP formation, indicating that in this system, the availability of G-protein is a limiting factor in TRH-R1 signaling. However, the increase in the fraction of the receptors coupled to G-protein had no effect on the ligand specificity of the response.

In intact cells assays, in which a signal is measured downstream in a signal transduction pathway, a number of factors can influence the output of receptor stimulation (efficacy of a receptor-agonist pair), whereas the effect of only a few of them can be tested directly (e.g., using pharmacological agents). Thus, we decided to analyze the functional properties of the TRH analogs using a membrane-based assay in which the ability of a receptor to catalyze GDP/GTPγS exchange is tested using a purified exogenous G-protein. This assay monitors the initial steps in a signal-transduction cascade and has no complications related to the presence of spare receptors inherent for most cellular systems employing overexpressed receptors.

Because we were unable to achieve high levels of TRH-R1 expression, we used cells stably expressing TRH-R2 at a high level (SN-R2-114.
TRH Analogs Are Super-agonists at TRH-R

FIGURE 6. G, overexpression does not affect the differences in apparent efficacies of TRH analogs. HEK293 cells stably expressing TRHR-1 (SN-R1-17 clone) were used in this experiment. The effect of overexpression of G, on the ability of MeTRH and R-Desaza-TRH (R-Des-TRH) to stimulate IP formation was determined after 48 h of transfection with 0.8 μg/ml DNA encoding human G,. The cells were stimulated for 45 min with 50 nM MeTRH or 50 nM R-Desaza-TRH. IP formation was determined as described under “Experimental Procedures.” Results are expressed as mean ± S.D. of assays performed in triplicate in a representative experiment.

As noted above, the nature of the agonist may affect the recognition (affinity) of effector G-protein by agonist-receptor complex. Under non-saturating conditions with G-protein, this would result in different efficacies for agonists. To test this idea, we performed a G, saturation analysis of TRH-R-mediated GDP/GTP exchange in the presence of MeTRH and R-Desaza-TRH (Fig. 7C). The similar K, for activation for both compounds, as well as the inability of the saturating concentrations of G, to abolish the differences in the maximal activation, indicated that the recognition of G-protein by the agonist-TRHR complex is not affected by the nature of agonist and cannot explain the efficacy differences.

The dependence of GTP-S binding to purified G-protein. A, the dependence of GTP-S binding on the concentration of MeTRH and R-Desaza-TRH (R-Des-TRH) was determined in urea-extracted TRHR-containing membranes from SN-R2-114 clone (3.5 μg of protein corresponding to ~5 pmol of receptor binding sites), reconstituted at 4 °C for 10 min with 5 pmol of human Gβγ1γ2 (assay concentration 0.1 μmol), and 50 pmol of human Gβγ1γ2 (assay concentration 1 μmol), in 30 μl of buffer solution containing the indicated concentrations of MeTRH or R-Desaza-TRH. B, the binding was started by the addition of 20 μl of the reaction mixture containing 2.5 nm [35S]GTP-S (assay concentration 1 μmol). The GTP-S binding was determined after a 5-min reaction at 30 °C, as described under “Experimental Procedures.” C, the time course of GTP-S binding to purified G-protein was measured in the absence (×) or presence of 50 nM MeTRH or 50 nM R-Desaza-TRH (○ or ×), in the reaction volume scaled up to 150 μl. Aliquots of 10 μl were removed at the indicated times, and GTP-S binding was determined. The lines represent the non-linear regression analysis of the data using a sigmoidal dose-response function. D, the time course of GTP-S binding to purified G-protein was measured in the absence (×) or presence of 50 nM MeTRH or 50 nM R-Desaza-TRH (○ or ×). The GTP-S binding was determined after a 5-min reaction at 30 °C, as described under “Experimental Procedures.” The lines represent the non-linear regression analysis of the data using a sigmoidal dose-response function. All results are expressed as mean ± S.D. of assays performed in duplicate in a representative experiment.
**TRH Analogs Are Super-agonists at TRH-R**

differences observed for TRH analogs. This is consistent with the fact that Gaq, overexpressed in TRH-R1-expressing cells is unable to decrease the difference in the rates of IP formation stimulated by MeTRH and R-Desaza-TRH (Fig. 6).

**DISCUSSION**

We showed for the first time that certain TRH analogs are more efficacious agonists at TRH-R1 and TRH-R2 than the cognate ligand TRH. We found that decreases in the affinities caused by changes in TRH structure correlate inversely with the ability of the analog to activate TRH-Rs. Thus, the low affinity analogs are super-agonists for these receptors.

To investigate the mechanism of this phenomenon, we attempted to determine the part of the signaling process responsible for the observed effect. The "multiple active state" theory of GPCR activation suggests the existence of multiple efficacies for a ligand (24). Besides the direct activation of G-protein, a ligand can selectively alter the interactions of a receptor with other intra- or extracellular components, for example components of the desensitization machinery, resulting in ligand-specific differences in receptor stimulation. We have tested the possible involvement of receptor recycling as well as a role of specific protein kinases in establishment of the efficacy differences among TRH analogs.

The results of these experiments in which agents that affect desensitization and resensitization had no effect on the efficacies of TRH analogs suggest that the observed variations in the efficacies of TRH analogs most probably originate from the altered ability of an agonist-receptor complex to directly activate G-protein rather than affecting the functions of accessory proteins. This conclusion was further supported by the use of isolated membranes to monitor TRH-R activation of G-protein directly using GTPγS binding assays. In these experiments, in which the concentrations of receptor and G-proteins can be controlled and their direct interaction monitored, the different efficacies between TRH analogs persisted. In interpreting the results of GTPγS binding experiments, we have assumed that the urea treatment of the membranes eliminates the majority of the peripheral accessory proteins; however, we cannot exclude the possibility that some integral proteins may affect binding.

Variations in efficacy ultimately reflect an altered ability of an agonist-receptor complex to activate G-protein, that is, to increase the catalytic rate of GDP/GTPγS exchange. The catalytic rate depends on both the affinity of interaction of the agonist-receptor complex with G-protein, that is, on the concentration of catalytically active agonist/TRH-R/G-protein complexes, and the catalytic efficiency of the activated complexes in the exchange reaction. Both of these may depend on the agonist. We showed that at saturating concentrations of agonists, increasing the concentration of Gaq did not change the activity of the MeTRH/TRH-R complex relative to that of the R-Desaza-TRH/TRH-R complex and, therefore, concluded that the affinity of interaction of the agonist/TRH-R complex with Gaq subunit is not dependent on the nature of agonists. However, it has been shown by Clark et al. (25) that Gβγ subunits can interact with an agonist-receptor complex independently of Gaq, providing a scaffold facilitating the subsequent interaction of the complex with Gaq and thereby affecting the fraction of activated complexes. Thus, it is possible that altered interactions of the receptor with Gβγ are responsible for the efficacy differences between TRH analogs. Although the Gβγ, γdimer used in our experiments was effective in promoting TRH-R-mediated GTPγS binding, its low apparent affinity of interaction with TRH-R2 (not shown) made it impractical to test its effect by saturation analysis. Because of the uncertainty in the concentrations of effective agonist/TRH-R/G-protein complexes, we cannot compare the catalytic efficiency of the different agonist-receptor complexes nor draw any conclusion about the contribution of this factor to the efficacy differences between TRH analogs.

TRH is a tripeptide, and alteration of any of its constituting amino acids results, in general, in decreased affinity for both TRH-R1 and TRH-R2; the only known exception is MeTRH, which has a higher affinity than TRH. We have shown that the decreases in affinities are accompanied by increases in the efficacies of the analogs. Since this effect is not dependent on the alteration of a specific residue of TRH (we have tested modifications at all three constituting amino acids, Fig. 1), it seems unlikely that specific interactions involved in the receptor activation are affected by these changes. Rather, this phenomenon may reflect a general property of the receptor active state, somehow promoted by the low affinity agonists.

In the classical two-state model of GPCR activation, the efficacy of an agonist is proportional to the difference between its affinity to the active and inactive states of a receptor (26, 27). This model can explain the inverse correlation observed between the affinities and efficacies of TRH analogs assuming that the structural changes leading to the affinity loss decrease the ability of the agonist to bind to the inactive state of a receptor more than to the active state. A weak point in this explanation is that the nonspecific structural changes have a specific effect on the binding, selectively targeting one of the two conformation states.

In an alternative view, receptors may attain a number of active (or inactive) conformations, each with distinct functional characteristics. This hypothesis allows rationalizing the increasing number of experimental observations that cannot be easily explained by the two-state model (32). From the known kon of TRH association with TRH-R1 (28) and the dissociation constants for TRH analogs, the half-lives of receptor residency (t1/2) for these agonists can be estimated, making an assumption that similar compounds have similar kon. The first-order dissociation kinetics gives t1/2 of ~50 min, ~20 min, ~2 s, and ~<1 s for MeTRH, TRH, NP 654, and R-Desaza-TRH, respectively. It appears that in the time frame in which a signal is measured, the receptor complex with a low affinity agonist undergoes multiple cycles of association-dissociation. Considering a non-zero relaxation time, this process would result in the formation of a distinct state for the receptor (for the binding pocket), which can be regarded as a "dynamic" conformation. This is in contrast to the receptor complex with a high affinity agonist, in which the ligand "spends" most of its time tightly bound to the receptor ("static" state). We speculate that this dynamic conformation represents a more active state of the receptor than the static state.

Ramsdell and Tashjian (13) showed previously that the EC50 of TRH-stimulated inositol 1,4,5-trisphosphate formation in GH4C1 cells (~100 x 10^(-10) receptors/cell) over a short period of time (5 s) is about 2 orders of magnitude higher than those observed for sustained TRH actions, such as stimulated prolactin release, prolactin synthesis, and equilibrium receptor binding. In the absence of spare receptors, this was interpreted by the authors as being caused by the ability of TRH to induce decrease in the rate of its dissociation with longer duration of receptor occupancy. They proposed that rapid actions of TRH occur with a form of the receptor that exhibits rapid dissociation kinetics and requires multiple ligand-receptor interactions for a maximal response. Moreover, they showed that at this stage, the receptor does not discriminate between TRH and MeTRH. This finding is consistent with the data of Hinkle and Kinsella (39), indicating that the dissociation rate of TRH from TRH-R decreases from a t1/2 of less than 1 min to 18 min with increasing time of exposure. Biphasic binding kinetics have been demonstrated for agonists in several GPCRs (30–32). The data from these experiments were modeled by a similar assumption of the existence of a
sequential process in which a fast equilibrium bimolecular step is followed by a slow monomolecular “isomerization” of the complex (32, 33). It was also shown that the rapid phase of binding corresponds to a primary receptor activation and the slow phase correlates with secondary effects, such as internalization, as exemplified in β2-adrenergic receptors (30). Thus, rapid and slower kinetics of signaling or binding have been observed in several GPCR systems.

Taking the above described observations into consideration, we can propose an alternative to the two-state model mechanism of TRH-R activation, to account for the inverse correlation between potencies and efficacies of TRH analogs described in this study. TRH-R activation is a sequential process starting with fast initial binding of an agonist to a low affinity binding site. This step results in the formation of a highly active dynamic conformation of the receptor. In the next slower step, the agonist-receptor complex undergoes an induced fit to achieve a less active state characterized by a static conformation of the receptor. Transition from the dynamic to static states can be associated with movement of the ligand deeper into the transmembrane domain, as was suggested by Colson and co-workers (23, 28) to explain the apparent existence of surface and transmembrane binding pockets for TRH. The relative abundance of the agonist-receptor complexes attaining this final conformation is related to the binding energy available to stabilize it (affinity of agonist). This step is mainly responsible for the discrimination of ligands by the receptor. In the presence of a low affinity agonist, such as R-Desaza-TRH, the receptor does not effectively convert to the static conformation and remains highly active for the time of exposure. In contrast, the high affinity natural hormone TRH induces the static state that limits rapid signaling and decreases its efficacy. This may represent a more rapid mechanism than desensitization to inhibit TRH-R overstimulation. In addition, the static conformation might be associated with a distinct functionality of the agonist-receptor complex, which was not revealed in this study.

We have found no previous reports describing a similar inverse relationship between efficacies and affinities among GPCR agonists. The question whether this phenomenon is unique for TRH receptors remains open and needs further evaluation. The conditions of spare receptors common for most cellular systems using overexpressed receptors may make this phenomenon difficult to detect. According to the multiple active states theory of GPCR activation, different agonists can employ alternative mechanisms to activate a receptor, each with a distinct subset of the amino acids involved. In each case, the signal output measured (efficacy) might be different. In our case, the functional properties tested in TRH receptors stimulated by low and high affinity agonists, including regulation and G-protein specificity, appear to be similar. We suggest that all TRH analogs used in this study share a similar molecular mechanism of receptor activation, thus revealing other unappreciated aspects of the structure-activity relationships present in this agonist-GPCR system.

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TRH Analogs Are Super-agonists at TRH-R

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