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Low Affinity Analogs of Thyrotropin-Releasing Hormone Are Super-Agonists

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Running title: TRH analogs are super-agonists at TRH-R

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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 re-cycling. 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 complexed to the low-affinity agonists to directly 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 $G_{q/11}$ subfamily of G-proteins and mediate the intracellular release of Ca^{2+} 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 subtle 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 $G_{q/11}$ (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.

Materials and Methods

Materials

DMEM and fetal bovine serum were purchased from Biosource (Rockville, MD). TRH (pGlu-His-ProNH$_2$), MeTRH (pGlu-His(1(τ)-methyl)-ProNH$_2$), luciferin, pertussis toxin and ammonium chloride were purchased from Sigma (St. Louis, MO). Folimycin (concanamycin A), okadaic acid, Ro-31-8425 (2-[8-(Aminomethyl)-6,7,8,9-tetrahydropyrido[1,2-a]indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide, HCl), HA-1077 (1-(5-isoquinolinesulfonyl)-homopiperazine) were purchased from (Calbiochem, San Diego, CA). TRH-Gly (pGlu-His-Pro-GlyOH) and Phe^2-TRH (pGlu-Phe-ProNH$_2$) were obtained from Bachem (Torrance, CA). TRH analogs NP 654 (pGlu-His(1-isopropyl)-ProNH$_2$) (Kaur, N.; Vikramdeep; Josan, J. S.; Jain, R. unpublished data), *R*-Desaza-TRH ((1*R*)-(3-Oxocyclopentyl)-His-ProNH$_2$) and *S*-Desaza-TRH ((1*S*)-(3-Oxocyclopentyl)-His-ProNH$_2$) (Figure 1) were synthesized as described previously (5). [^{3}H] MeTRH and [^{35}S] GTPγS were obtained from NEN Life Science Products (Boston, MA). *Myo*-[^{3}H]inositol was obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). The cDNA clone for human $G_{\alpha q}$ was obtained from the UMR cDNA Resource Center (www.cdna.org).

Cell culture and transfection

HEK293 (human embryonic kidney) cells were grown in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 100 units/ml penicillin, and 10 µg/ml streptomycin (Life Technologies Inc.) at 37 ºC in a humidified 5% CO_2 incubator. The cells were seeded in 24-well plates (75 x 10^3 cells per well) one day before transfection. For luciferase assays HEK293 cells stably expressing mouse TRH-R1 (NS R1-17 clone) were transfected with 0.8 µg/ml of pAP(Activator Protein)-1Luc vector (PathDetect *In Vivo* Signal Transduction Pathway *trans*- and *cis*-Reporting System; Stratagene, La Jolla, CA), using FuGENE^{TM}6 reagent (Roche, Basel, Switzerland).

Ligand binding assays

All binding experiments were performed in intact cell monolayer in 24 well plates. Competition binding assays at equilibrium to measure IC_{50} of inhibition were performed at 37°C for 1 h with 1 nM [^{3}H]MeTRH and various concentrations of unlabeled TRH or TRH analogs as described (6). Saturation experiments at equilibrium to determine apparent binding affinity for [^{3}H]MeTRH and TRH-R1/R2 expression level were performed at 37°C for 1 h using 0.1 to 10 nM [^{3}H]MeTRH as described (7).

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)/10mM 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 NaCI, pH 2.5). This treatment removed 96% of the [^{3}H]TRH specifically bound at the cell surface (8). The cells were washed twice with 2 ml of ice-cold HBSS/10mM Hepes and incubated at 4°C for 2h with 10 nM [H^3]MeTRH. After washing three times with 2 ml of ice-cold HBSS/10mM 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 additional 15 h in the absence or presence of 100 ng/ml PTX, and then stimulated for 5 h with or without ligand. The cells were washed with phosphate-buffered saline (PBS) and incubated at ice for 15 min with 0.5 ml lysis buffer (25 mM Gly-Gly, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol, 1% Triton X-100, pH 7.8). One hundred microliters of lysate were mixed automatically with 125 µl of reaction buffer (25 mM Gly-Gly, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol, 15 mM KH_2PO4, 2 mM ATP, pH 7.8) and 25 µl of 0.4 mM luciferin solution in reaction buffer, and the luminescence was measured for 3 s in a Victor^{TM}3 Multilabel Counter 1420 (PerkinElmer, Boston, MA). Data are expressed as relative light units (RLU).

Measurement of Phosphoinositide Hydrolysis

The cells were labeled for 24 h by incubation with 2 µCi/ml of *myo*-[^{3}H]inositol (Perkin Elmer). The cells were washed with HBSS/10mM Hepes, pH 7.4, and incubated at 37ºC in 10 mM LiCl HBSS solution in the absence or presence of agonist. The cells were lysed and accumulated inositol phosphates (IPs) were measured using ion-exchange chromatography as described (9). IPs conversion was calculated as [^{3}H]IP% = 100 x [^{3}H]IP/([^{3}H]lipids + [^{3}H]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 PBS at room temperature and incubated at 4°C for 15 min in 10 ml of 10 mM Hepes solution, pH 7.4, in the presence of 1 mM EGTA, fortified with protease inhibitor cocktail (Complete, EDTA-free, Roche, 1 tablet/25 ml). The swollen cells 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 BALBc fibroblasts expressing bombesin receptors (10), frozen and stored at -80 ºC.

Purification of G-protein Subunits and GDP/GTPγS Exchange Assay

$G_{\alpha q}$ was isolated from cuttlefish (*Sepia officnales*) retinas essentially as described by Hartman and Northup for squid $G_{\alpha q}$ (11). Cholate extracts from microvillus membrane fractions of *Sepia* retina were sequentially chromatographed over DEAE-Sephacel and Ultrogel AcA44. The peak of $G_{\alpha q}$ was exchanged into a solution containing 4 mM CHAPS by chromatography over Sephadex G-50 prior to storage at -80°C. Recombinant human $\beta_1\gamma_2$ expressed in Sf9 cells was purified as described previously (12), except that the viral infections were carried out at moi 2 for β_1 and 10 for γ_2. The purified $\beta_1\gamma_2$ 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 G_α 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 nonlinear regression using the Prism software version 4 (GraphPad, Inc., San Diego, CA).

Results

Functional characterization of a number of TRH analogs revealed that the IC_{50}/EC_{50} ratio, the ratio of the concentration of an analog that causes 50% inhibition of [^{3}H]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, Scheme 1). Although the absolute values of IC_{50}/EC_{50} ratios in cells expressing TRH-R2 were higher than in cells expressing TRH-R1, the relative differences in the ratios between TRH and TRH analogs in both cell populations were similar. The higher IC_{50}/EC_{50} ratios in cells expressing TRH-R2 may, in part, be caused by the higher level of TRH-R2 surface expression compared to TRH-R1 (~900×10^3 TRH-R2/cell versus ~300×10^3 TRH-R1/cell). The higher IC_{50}/EC_{50} ratios for certain TRH analogs compared to TRH in the same cell type is consistent with higher intrinsic efficacies for these agonists.

As differences in intrinsic efficacies of agonists are more readily apparent in cells expressing low levels of receptors because the effect of "spare receptors" is diminished, we measured the activities of various TRH analogs (Figure 1 and 2) in a cell line with a level of TRH-R1 expression (SN-R1-17 clone, ~70×10^3 receptors/cell) similar to that found in pituitary cells (13). Figure 2A illustrates a representative comparison of TRH and *R*-Desaza-TRH in activation of TRH-R1. Although *R*-Desaza-TRH exhibits a lower potency than TRH, it exhibits a higher efficacy; that is, *R*-Desaza-TRH stimulates a higher level of IP production than TRH at similar occupancy of the receptor (based on the almost parallel dose-response curves for the two agonists). In Figure 2B, the relative intrinsic efficacies of a series of TRH analogs, defined as the ratio of the maximal level of IP formation stimulated by a TRH analog divided by IP formation stimulated by TRH, were plotted versus pEC_{50} values for the agonists. 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 pEC_{50}s and intrinsic efficacies shown in figure 2B is limited because there are no TRH-analogs with potencies between Phe^2-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 (Figure 3). In this experiment, the apparent inhibitory constant for MeTRH at TRH-R1 (0.32±0.16 nM) estimated from the IC_{50} of inhibition, is similar to the dissociation constant determined in saturation binding experiments (0.61±0.096 nM), consistent with a competition between the two analogs for the same site on TRH-R1.

We measured the efficacies of TRH analogs using luciferase gene transcription as a reporter of TRH-R signaling also. 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 (PTX) (Neumann, in prep.), indicating the ability of TRH-R to couple to PTX-sensitive G-proteins, most likely of the G_i and/or G_o family, in addition to $G_{q/11}$. As shown in Figure 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 PTX sensitivity, indicating a similar ability to activate G_i and/or G_o. 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 G_s protein. 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 efficacy differences between the TRH analogs. As shown in Figure 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 of 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 re-cycling) 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 Figure 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 (Figure 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 Figure 6, the overexpression of human $G_{\alpha q}$ 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 over-expressed 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 clone, 4×10^6 receptors/cell) for the membrane preparation. The isolated membranes were extracted with 7M urea, which has been shown to eliminate the majority of GTPγS binding to endogenous proteins (23). We suppose that most of the peripheral and cytoplasmatic proteins that can affect receptor-G-protein interactions are also removed upon this treatment, making it possible to study the direct activation of exogenous G-protein by a receptor. $G_{\alpha q}$ purified from *Sepia* retina (11;23) and recombinant human $G_{\beta1\gamma2}$ (12;23) were used for these experiments. It was shown previously that this combination of subunits is most efficiently activated by TRH-R2 (not shown). The dependence of the initial rates of GDP/GTPγS exchange catalyzed by TRH-R2 on the concentrations of MeTRH and *R*-Desaza-TRH is shown in Figure 7A. At saturation, *R*-Desaza-TRH was about twice as effective in the stimulation of GTPγS binding as MeTRH. This is consistent with the results obtained in the intact cells experiments, supporting the idea that the observed differences in the efficacies of TRH analogs originate in the varied abilities of the agonist-TRH-R complexes to directly activate G-protein. The EC_{50} for stimulating GTPγS binding for both MeTRH and *R*-Desaza-TRH is one order of magnitude higher than the corresponding dissociation constant measured in the binding experiments in intact cells. This discrepancy may be attributed to the differences in the properties of the agonist-TRH-R-G-protein complex naturally occurring in the intact cells and that being formed upon reconstitution of the isolated membranes with the exogenous G heterotrimer. However, this difference had no effect on the agonist specificity of signaling efficacy. Figure 7B shows the time course of MeTRH and *R*-Desaza-TRH-stimulated GTPγS binding mediated by TRH-R2. The concentration of GTPγS was limiting in this reaction and its association with G-protein follows a pseudo-first order kinetics with half-lives of 8.6 and 4.1 min for MeTRH and *R*-Desaza-TRH, respectively.

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 $G_{\alpha q}$ saturation analysis of TRH-R2-mediated GDP/GTPγS exchange in the presence of MeTRH and *R*-Desaza-TRH (Figure 7C). The similar K_m of activation for both compounds, as well as inability of the saturating concentrations of $G_{\alpha q}$ 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 observed for TRH analogs. This is consistent with the fact that $G_{\alpha q}$ 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 (Figure 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 analog's ability 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 G_α 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 G_α subunit is not dependent on the nature of agonists. However, it has been shown by Clark et al. (25) that $G_{\beta\gamma}$ subunits can interact with an agonist-receptor complex independently of G_α, providing a scaffold facilitating the subsequent interaction of the complex with G_α, and . thereby affecting the fraction of activated complexes. Thus, it is possible that altered interactions of the receptor with $G_{\beta\gamma}$ are responsible for the efficacy differences between TRH analogs. Although the $G_{\beta1\gamma2}$ dimer 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 that 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 amino acids, Figure 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 non-specific 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 k_{on} of TRH association with TRH-R1 (28) and the dissociation constants for TRH analogs, the half-lives of receptor residency ($t_{1/2}$) for these agonists can be estimated, making an assumption that similar compounds have similar k_{on}. The first-order dissociation kinetics gives $t_{1/2}$ of ~50 min, ~20 min, ~2 sec and <1 sec 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 (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 EC_{50} of TRH-stimulated IP_3 formation in GH_4C_1 cells (~100×10^3 receptors/cell) over a short period of time (5 sec), is about two 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 et al. indicating that the dissociation rate of TRH from TRH-R decreases from a $t_{1/2}$ of less than 1 min to 18 min with increasing time of exposure (29). 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 β_2-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 Perlman et al. (28;34) 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 over-stimulation. In addition, the "static" conformation might be associated with a distinct functionality of the against-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 over-expressed 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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Figure legends

Figure 1. Structure of TRH and TRH analogs.

Figure 2. Stimulation of IP formation by TRH and TRH analogs. IP formation was measured in HEK293 cells stably expressing TRH-R1 (SN-R1-17 clone, ~70×10^3 receptors/cell) as described in *Material and Methods*. **A.** Dependence of IP formation on the concentration of TRH (■) or *R*-Desaza-TRH (○). The curves represent the non-linear regression analyses of the data using a sigmoidal dose-response function. Results are expressed as mean ± SD of assays performed in triplicate in a representative experiment. **B**. Relationship between potencies ($-\log EC_{50}$) and relative efficacies of TRH analogs. Relative efficacy is defined as the ratio of the maximal level of IP formation stimulated by a TRH analog divided by that stimulated by TRH. Results are expressed as mean ± SE of three independent experiments. The linear regressional analysis of the data gives $r^2 = 0.89$.

Figure 3. MeTRH inhibition of IP formation stimulated by *R*-Desaza-TRH. IP formation was measured in HEK293 cells stably expressing TRH-R1 (SN-R1-17 clone) in the presence of 20μM *R*-Desaza-TRH and increasing concentrations of MeTRH, as described in *Material and Methods*. The data are presented as percentage of the receptor activation by *R*-Desaza-TRH alone. The curve represents the non-linear regression analysis of the data using one-site competition function. Results are expressed as mean ± SD of assays performed in triplicate in a representative experiment.

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 33h, followed by 15h of incubation without (control) or with 100 ng/ml pertussis toxin. The cells were stimulated by 50 nM MeTRH, 50 nM TRH, 50 μM NP-654 or 50 μM *R*-Desaza-TRH for 5h, and luciferase activity was measured as described in *Material and Methods*. Results are expressed as mean ± SD of assays performed in triplicate in a representative experiment.

Figure 5. Desensitization and recycling of TRH-R are independent of the nature of agonist. HEK293 cells stably expressing TRH-R1 (SN-R1-17 clone) were used in these experiments. **A**. Effects of protein kinase inhibitors on the ability of MeTRH and *R*-Desaza-TRH to stimulate IP formation was determined as described in *Materials and Methods*. The cells were pre-incubated for 20 min without (control) or with 2 μM Ro-31-8425 or 50 μM HA-1077 and stimulated for 45 min with 50 nM MeTRH or 50 μM *R*-Desaza-TRH in the presence of inhibitor; **B**. Loss of cell surface TRH-R1 was determined in the presence of 50 nM TRH (■) or 50 μM *R*-Desaza-TRH (○) as described in *Materials and Methods*. **C**. Effects of endosomal phosphatase inhibitors on MeTRH and *R*-Desaza-TRH stimulation of IP formation were determined after 1h stimulation with 50 nM MeTRH or 50 μM *R*-Desaza-TRH in the absence (control) or presence of 0.1 μM okadaic acid, 0.1 μM folimycin or 20 mM ammonium chloride. The cells were pre-incubated with inhibitors for 30 min prior the stimulation**.** Results are expressed as mean ± SD of assays performed in triplicate in a representative experiment.

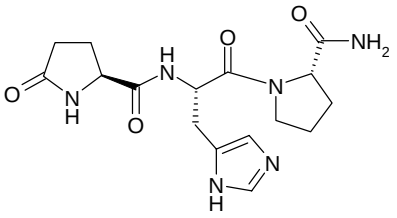
Figure 6. G_q overexpression does not affect the differences in apparent efficacies of TRH analogs. HEK293 cells stably expressing TRH-R1 (SN-R1-17 clone) were used in this experiment. The effect of overexpression of $G_{\alpha q}$ on the ability of MeTRH and *R*-Desaza-TRH to stimulate IP formation was determined after 48 h of transfection with 0.8 µg/ml DNA encoding human $G_{\alpha q}$. The cells were stimulated for 45 min with 50 nM MeTRH or 50 µM *R*-Desaza-TRH.
IP formation was determined as described in *Materials and Methods*. Results are expressed as mean ± SD of assays performed in triplicate in a representative experiment.

Figure 7. TRH-R2-catalysed [^{35}S]GTPγS binding to purified G-protein. A. Dependence of GTPγS binding on the concentration of MeTRH and *R*-Desaza-TRH was determined in urea-extracted TRH-R2-containing membranes from SN-R2-114 clone (3.5 µg protein corresponding to ~5 pmol of receptor binding sites), reconstituted at 4°C for 10 min with 5 pmol of *Sepia* retinal $G_{\alpha q}$ (assay concentration 0.1 µM) and 50 pmol of human $G_{\beta 1}\gamma_2$ (assay concentration 1 µM), in 30 µl of buffer solution containing the indicated concentrations of MeTRH (■) or *R*-Desaza-TRH (○). The binding was started by addition of 20 µl of the reaction mixture containing 2.5 nM [^{35}S]GTPγS (assay concentration 1 nM). The GTPγS binding was determined after 5 min reaction at 30°C, as described in *Materials and Methods.* The curves represent the non-linear regression analyses of the data using a sigmoidal dose-response function. **B**. Time course of GTPγS binding to the reconstituted membranes (21 µg-protein) was measured in the absence (×) or presence of 50 nM MeTRH (■) or 50 µM *R*-Desaza-TRH (○), in the reaction volume scaled up to 150 µl. Aliquots of 10µl were removed at the indicated times and GTPγS binding was determined. The lines represent the non-linear regression analysis of the data using monophase exponential association function. **C**. $G_{\alpha q}$ saturation of GTPγS binding was performed in reconstituted membranes as described in *A*, in the presence of 50 nM MeTRH (■) or 50 µM *R*-Desaza-TRH (○), and indicated concentrations of $G_{\alpha q}$. The binding of GTPγS to $G_{\alpha q}$ in the absence of membranes was also determined (×). The curves represent the non-linear regression analysis of the data using single-site saturation function. All results are expressed as mean ± SD of assays performed in duplicate in a representative experiment.

Table 1. Pharmacological characteristics of TRH analogs in TRH-R1(R1)- and TRH-R2(R2)-expressing cells.

Agonist	R1 IC_{50}, nM	R1 EC_{50}, nM	R1 IC_{50}/EC_{50}	R2 IC_{50}, nM	R2 EC_{50}, nM	R2 IC_{50}/EC_{50}
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

IC_{50}, Half maximally effective concentration for displacement of 1 nM [^{3}H]MeTRH binding. EC_{50}, Half maximally effective concentration for stimulation of IP formation. Assays were performed in HEK293 cells stably expressing TRH-R1 (SN R1-31 clone, ~300×10^3 receptors/cell) or TRH-R2 (SN R2-95 clone, ~900×10^3 receptors/cell), as described in *Material and Methods*. Results are expressed as mean ± SE of three independent experiments.



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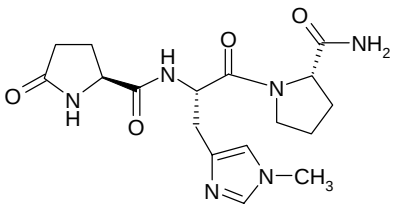

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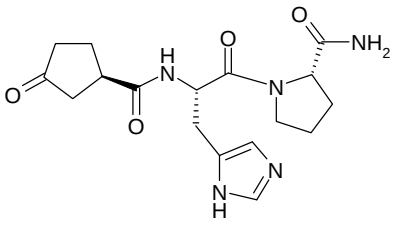

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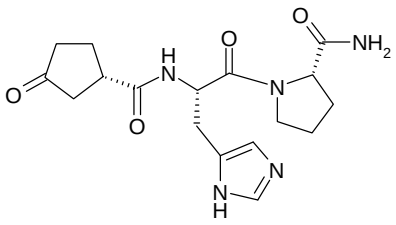

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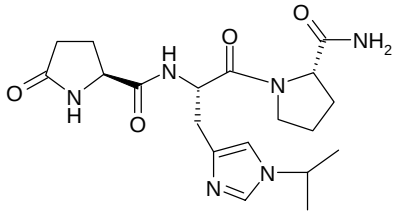

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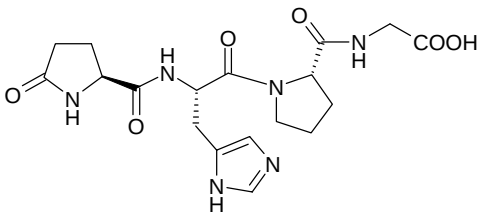

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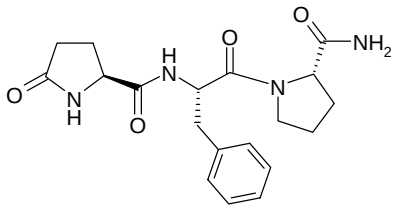
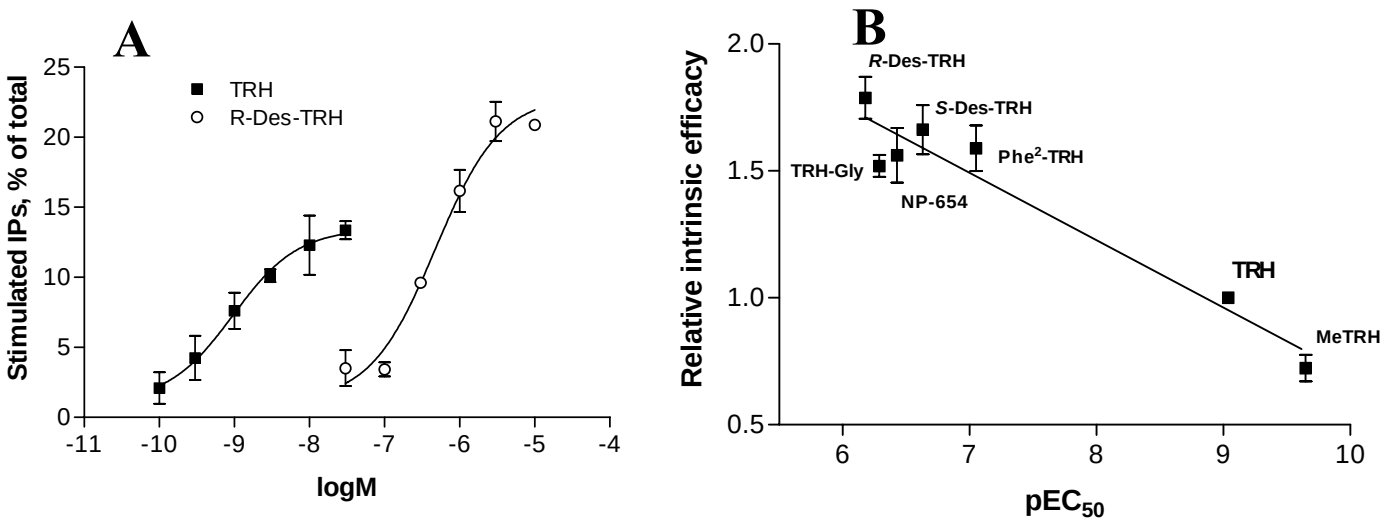


Phe^2-TRH

Figure 1



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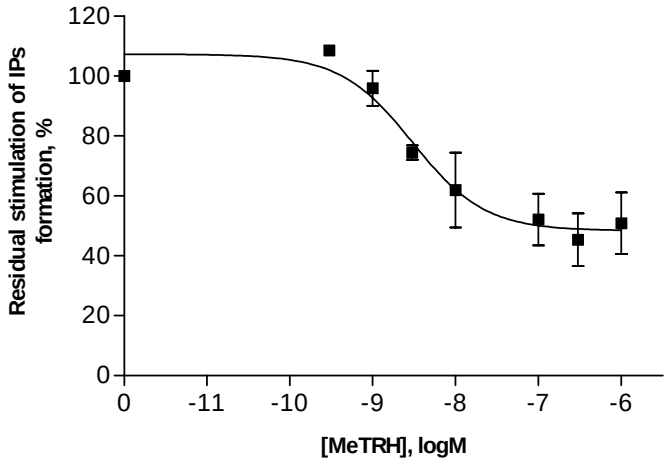

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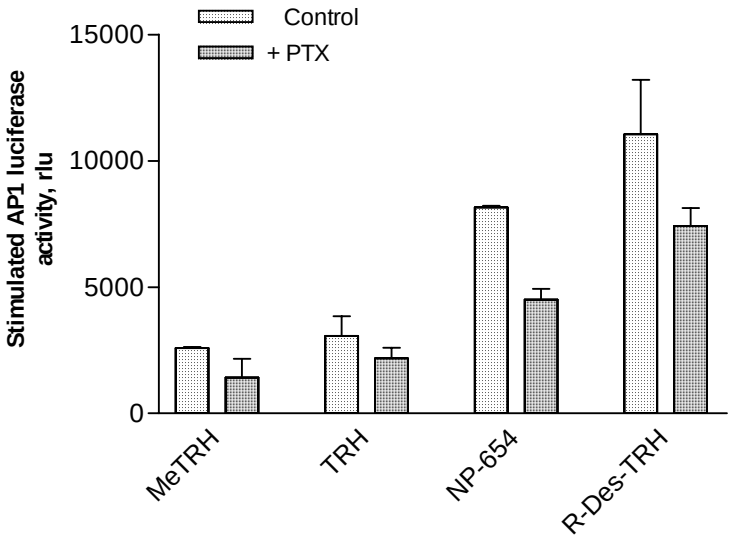

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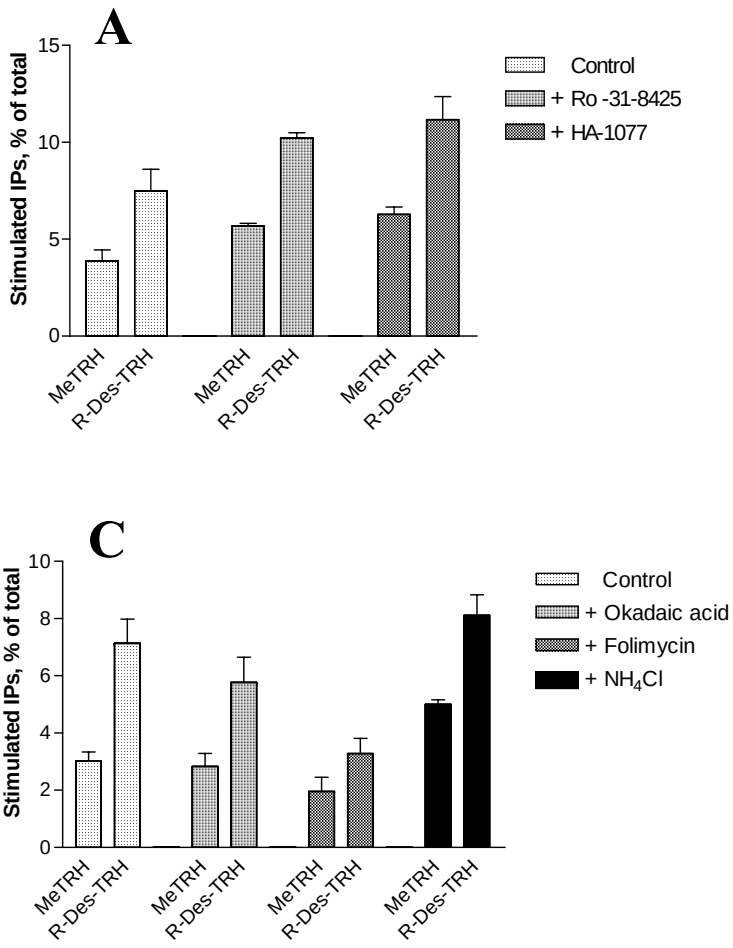


Figure 5

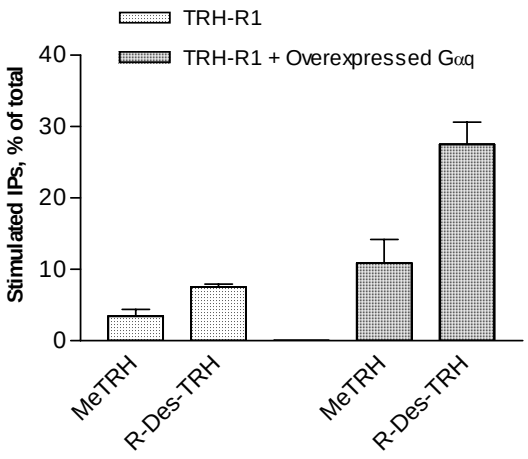
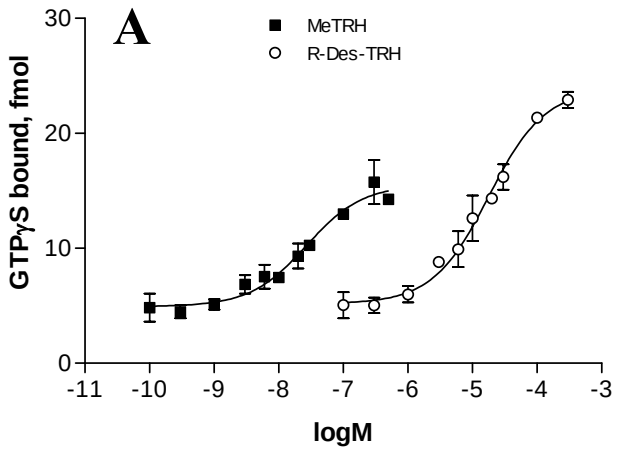
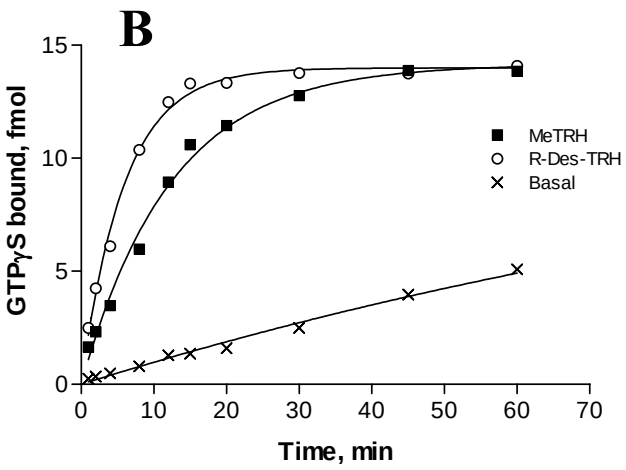


Figure 6





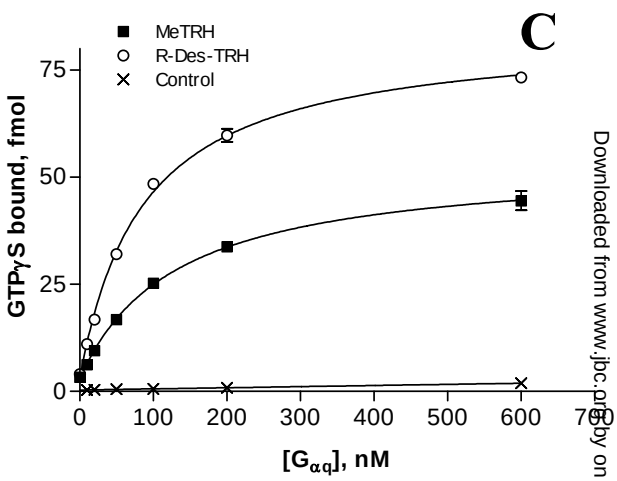


Figure 7