Hydrogen Bonding Interaction of Thyrotropin-releasing Hormone (TRH) with Transmembrane Tyrosine 106 of the TRH Receptor*

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Jeffrey H. Perlman, Colette N. Thaw, Lisa Laakkonen, Cyril Y. Bowers, Roman Osman, and Marvin C. Gershengorn

From the  Divisions of Molecular Medicine, Department of Medicine, Cornell University Medical College and The New York Hospital, New York, New York 10021, the Department of Physiology and Biophysics, Mount Sinai School of Medicine, City University of New York, New York, New York 10029, and the Endocrine Section, Department of Medicine, School of Medicine, Tulane Medical Center, New Orleans, Louisiana 70112

Thyrotropin-releasing hormone (TRH, pyroglutamatic acid-histidine-proline-amide) binds to a seven-transmembrane-spanning, G protein-coupled receptor. We tested the hypothesis that Tyr

The affinities of [Pro]

The binding of endothelin type-A selective antagonists and endothelins and an approximately 16,000-fold decrease in affinity for the endothelin type B-selective agonist sarafotoxin 6c (21). The binding of endothelin type-A selective antagonists and endothelin type-B selective agonists appear to be mediated by different subdomains of their respective receptors (22). Mutation of a Lys residue in the fifth transmembrane helix of the angiotensin type 1 receptor caused an 8-fold decrease in binding of angiotensin (23). A Val residue in the sixth transmembrane helix of the cholecystokinin-B/gastrin receptor has been demonstrated to be involved in binding a nonpeptide antagonist (24).

It has been demonstrated in the GPCRs for neurotransmitters that an aspartate in the third transmembrane helix is important for binding and appears to serve as a counterion to the positively charged ligand. In contrast, we have previously demonstrated that the interaction between TRH and its receptor is non-ionic (25). We postulated that a residue in the third transmembrane helix of TRH-R might be involved in binding TRH through a nonionic interaction. The Tyr

EXPERIMENTAL PROCEDURES

Materials—TRH and chlordiazepoxide were purchased from Sigma, and [Pro]

The abbreviations used are: TRH, thyrotropin-releasing hormone; TRH-R, TRH receptor; GPCR, guanine-nucleotide-binding protein-coupled receptor; [Pro]

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were prepared by the polymerase chain reaction (28), and plasmid sequences were confirmed by the dideoxy chain termination method (29).

**Cell Culture and Transfection—**COS-1 cells were maintained and transfected as described (4). Cells were seeded 1–3 days prior to transfection at 3.0 x 10^6 to 1.2 x 10^6 cells/100-mm dish, transfected using the DEAE-dextran method, and maintained in medium with 10% Nu-Serum for 2–3 days, at which time cells were harvested and seeded into 12-well plates at 100,000 cells/well in medium with 5% Nu-Serum. Mock transfection was performed as described but without plasmid DNA.

**Results and Discussion**

To test the hypothesis that Tyr^{106} was important for binding, Tyr^{106} was mutated to Phe and this mutant TRH-R was expressed in COS-1 cells. In preliminary experiments, it became evident that [Phe^{106}]TRH-TRH-R exhibited very low affinities for TRH analogs that would be difficult to measure accurately in binding experiments. We therefore assessed relative affinities by measuring potencies of stimulation of IP formation. Relative potencies may be used to assess relative binding affinities if the efficacies of the compared systems are the same (31). The magnitude of IP stimulation by TRH correlates directly with the level of TRH-R expression (32, 33). The similar extent of maximal stimulation by TRH analogs for WT receptors and by TRH for WT and Phe^{106} receptors is consistent with the idea that the efficacies are similar. To confirm for WT TRH-Rs that relative potencies reflect relative binding affinities, activation and binding studies (Fig. 1, upper panel; Table 1) were performed with intact cells under identical conditions using a TRH analog substituted at the <Glu moiety with a prolyl moiety ([Pro’ITRH] (see Fig. 2 for structures). The EC_{50} for stimulation of IP formation by [Pro’ITRH] in cells expressing WT TRH-Rs was 110,000-fold higher than that for TRH. The K_{i} of WT TRH-Rs for [Pro’ITRH] was 43,000-fold higher than for TRH. Thus, although the absolute values of the EC_{50} were lower than their respective K_{i} values, a common finding with GPCRs (34), the relative potencies and relative affinities were similar. These data are consistent with the idea that the relative order of potencies reflects the relative order of affinities of binding under these conditions. Of note, we have found similar results for all mutant TRH-Rs in which we have been able to assay binding directly.

We measured the potencies of stimulation of IP formation by TRH analogs in cells expressing [Phe^{106}]TRH-Rs (Fig. 1, lower panel; Table 1). The EC_{50} for TRH stimulation of IP formation in cells expressing [Phe^{106}]TRH-Rs was 100,000-fold higher than in cells expressing WT TRH-Rs. These data indicate the importance of Tyr^{106} in binding TRH. Substitution of Pro for <Glu results in a TRH analog in which the carboxyl group of <Glu has been substituted by a methyl group (Fig. 2). The EC_{50} for [Pro’ITRH] in cells expressing WT or [Phe^{106}]TRH-Rs were 110,000- and 16-fold higher, respectively, than for stimulation by TRH. These data provide compelling evidence that the carboxyl group of <Glu binds to the hydroxyl group of Tyr^{106}.

**Activation and binding of wild-type (WT) or [Phe^{106}]TRH receptors by TRH analogs**

The EC_{50} values of activation correspond to the data shown in Fig. 1. Binding parameters were derived from competition for [3H]MeTRH binding by unlabeled TRH analogs in COS-1 cells expressing WT TRH-Rs as described (25). Duplicate or triplicate determinations of binding at each concentration of unlabeled ligand were obtained in two experiments.

| Ligand (log M) | EC_{50} (μM) | K_{i} (μM) |
|---------------|--------------|----------|
| TRH           | 0.001 ± 0.001| 110 ± 3.2|
| Phe^{106}     | 100 ± 0.51   | 1600 ± 22|
| [Pro’ITRH]    | 0.013 ± 0.0001| 560 ± 6.3|

First, the decrease in apparent affinity of [Phe^{106}]TRH-R, in which the hydroxyl group of Tyr^{106} was removed, for TRH is the same as the decrease in apparent affinity of WT TRH-R for [Pro’ITRH], in which the carbonyl group of <Glu was removed. Second, there were only small differences in apparent affinities of [Phe^{106}]TRH-R for TRH or [Pro’ITRH], which reflects a lack of additivity of effects of [Phe^{106}]TRH-R and [Pro’ITRH] on binding.

Substitution of an aminobutyryl residue for <Glu ([Abu’ITRH]) results in a residue with a side chain consisting of an ethyl group and lacking a carbonyl group. The K_{i} of WT TRH-Rs for [Abu’ITRH] was 15,000-fold higher than that for TRH. The EC_{50} values of stimulation of IP formation by [Abu’ITRH] in WT and Phe^{106} receptors were also similar to those found for [Pro’ITRH] (data not shown). The similar results obtained with [Pro’ITRH] and [Abu’ITRH] were predicted by our model in which the Tyr^{106} hydroxyl group binds the <Glu carbonyl group.

There was a small decrease in the potency of [Pro’ITRH] relative to TRH in cells expressing [Phe^{106}]TRH-Rs. This 16-fold decrease observed when an analog without the <Glu resi-
and millimolar range. This approach may not be possible with receptors for larger peptides or proteins. The possibility that small amounts of contaminating TRH could become significant at high concentrations of TRH analogs and bind to TRH-R is very unlikely because these analogs were synthesized de novo by solid state technique (41).

Relative potencies of activation reflect relative binding affinities for systems of comparable efficacy (31). However, variations in potencies relative to affinities of up to 30-fold have been found for some ligand-GPCR systems (42, 43). Although this may cause a problem in interlaboratory results of some ligand-receptor systems, it is unlikely that it would affect our conclusions. Therefore, when the 100,000-fold decrease in potency observed with [Phe^{106}]TRH-R is much greater than would be expected for a change in efficacy that does not affect maximal stimulation. Thus, although we have not measured the affinity of [Phe^{106}]TRH-R directly, the decreased potencies observed are almost certainly caused by decreased affinity.

We were able to use complementary site-specific mutations in the receptor and substitutions of specific groups in the ligand to test our hypothesis. A lack of additivity of similar effects of receptor mutants and ligand analogs is strong evidence that the changes in the receptor and the ligand are affecting the same bond. An appropriate choice of mutations and ligand substitutions can provide detail at an atomic level as described in this work. This approach has been used in studies of binding sites of GPCRs for neurotransmitters (44) and nonpeptide substance P antagonists (20) involving interactions of substantially lower affinity than that described here.

In summary, we have found compelling evidence, based on the results of experiments using site-specific mutagenesis and selective analogs of TRH, that the hydroxyl group of Tyr^{106} binds the carboxyl group of the <Glu moiety of TRH. To our knowledge, this represents the highest affinity, non-covalent bond yet observed between single functional groups of a GPCR and a ligand, and is the first delineation of a direct binding interaction between a residue in the transmembrane core of a GPCR and a specific moiety of a peptide agonist.

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