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Distinct Roles for Arginines in Transmembrane Helices 6 and 7 of the Thyrotropin-Releasing Hormone Receptor

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SUMMARY

The thyrotropin-releasing hormone (TRH) receptor (TRH-R) is a member of the seven-transmembrane region, G protein-coupled receptor family. Arg-283 and Arg-306, in transmembrane helices 6 and 7, respectively, are putatively in positions homologous to those of residues that are important for agonist and antagonist binding in receptors for neurotransmitters. These arginines were mutated and the mutant receptors were transiently expressed in COS-1 cells. The affinity of the R306K TRH-R was similar to that of the wild-type (WT) TRH-R, whereas no specific binding was detected in cells expressing R306A, R306E, or R306L TRH-Rs. Because TRH stimulated inositol phosphate (IP) formation to similar maximal extents in cells expressing WT and Arg-306 mutant TRH-Rs, relative potencies were used to estimate the relative affinities of the receptors. The EC_{50} values for stimulation of R306A, R306E, and R306L TRH-Rs were 1500-, 1200-, and 3000-fold higher than that for the WT TRH-R. No specific binding was measurable in COS-1 cells expressing R283K, R283H, or R283A TRH-Rs, whereas maximal TRH stimulation of IP formation was to levels 64%, 42%, or <1%, respectively, of that in cells expressing WT TRH-Rs; for R283K and R283H TRH-Rs, EC_{50} values were 6300- and 50,000-fold higher, respectively, than that for the WT TRH-R. In AtT-20 cells stably expressing R283A TRH-Rs, the binding affinity was 39,000-fold lower than that of the WT TRH-R and the number of receptors was estimated to be 0.88 $\times 10^6$/cell, but TRH did not stimulate IP formation. Thus, in the TRH-R, Arg-306 appears to be important for binding but not for activation, whereas Arg-283 appears to be important for binding and activation.

The TRH-R (1) is a member of the seven-TM GPCR family (2). To understand the biology of GPCRs, computer-generated three-dimensional models of the TMs of several of these receptors have been developed based on the crystal structure of bacteriorhodopsin or *de novo* constructions. In the first approach, the helical bundle of bacteriorhodopsin (3) served as a template on which the other receptor models were constructed, in spite of their low sequence homology with bacteriorhodopsin (4, 5). In the second approach, the helices were packed into a seven-TM bundle based on hydrophobicity (6) or on complementarity of conserved patches in adjacent helices (7). We developed a three-dimensional model of the unoccupied TRH-R[1] based on a method of sequence analysis of GPCRs (8) that predicted a structure of rhodopsin in agreement with the recently obtained rhodopsin projection map (9). A model of the TRH-R binding pocket was then constructed (10) based on previous experimental observations (11, 12) and preliminary data that suggested that Arg-283 in TM-6 and Arg-306 in TM-7 might be involved in binding TRH. We therefore performed a detailed analysis of the roles of these TM arginine residues in the binding and activation of TRH-R.

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[1]Submitted for publication.

Experimental Procedures

Materials. TRH was purchased from Calbiochem, MeTRH from Sigma, and [^{3}H]MeTRH from DuPont-NEN. The cloning vector pBluescript was from Stratagene. The expression vectors pCDM8 and pcDNAINeo were from Invitrogen. Dulbecco's modified Eagle's medium, fetal calf serum, and Geneticin were from Life Technologies. NuSerum was from Collaborative Research. Restriction endonucleases were from New England Biolabs.

Mutagenesis. The full length mouse TRH-R cDNA in pBluescript (1) was used for mutation and transformation. Mutants were prepared by the uridine method template (R283L, R283E, R306L, and R306E TRH-Rs) (R283L is, for example, a receptor in which arginine at position 283 is substituted by leucine) or the polymerase chain reaction, and plasmid sequences were confirmed by the dideoxy chain termination method. All mutant receptors in pBluescript were digested with *Xho*I and *Not*I, and 3.2-kilobase *Xho*I/*Not*I restriction

ABBREVIATIONS: TRH-R, thyrotropin-releasing hormone receptor; TRH, thyrotropin-releasing hormone; GPCR, G protein-coupled receptor; pyro-Glu, pyroglutamyl; TM, transmembrane helix; MeTRH, thyrotropin-releasing hormone in which *N*-γ-methylhistidine is substituted for histidine; IP, inositol phosphate; WT, wild-type.

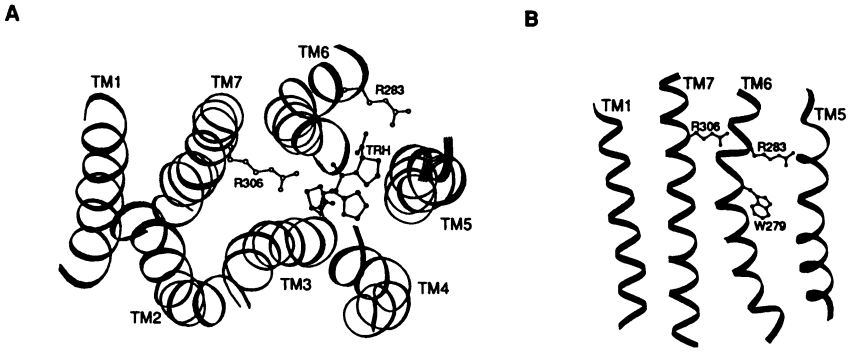

Fig. 1. Model of part of the binding pocket of the TRH/TRH-R complex. *Ribbons*, α-helical arrangements of putative TM backbone structures. A, Top view (from the extracellular side) showing the positions of the TMs, the side chains of Arg-283 (TM-6) and Arg-306 (TM-7), and the docked TRH. B, Side view of selected helices. TM-2, -3, and -4 are omitted to show the relative positioning of Trp-279, Arg-283, and Arg-306 with respect to each other and to the proposed helical boundaries.

fragments were subcloned into the full length, mouse TRH-R cDNA in pCDM8 (pCDM8mTRHR) (13), which was used for transfection.

Cell culture and transfection. COS-1 cells were maintained and transfected as described (11). AtT-20 cells, maintained in Dulbecco's modified Eagle's medium supplemented with 10% NuSerum, were co-transfected with pCDM8 containing cDNA for WT or Arg-283 mutant TRH-Rs and with pcDNAINeo by electroporation. Stable cell lines were selected for resistance to Geneticin, which was added at 400 μg/ml 72 hr after transfection. Geneticin-resistant clones (11–20 clones of each receptor) were picked and assayed for binding and activation.

IP formation. Assays were performed as described (11).

Receptor binding studies. Assays were performed as described (11).

Statistical analyses. Curves were fitted by nonlinear regression and drawn with the Inplot program (GraphPAD Software, San Diego, CA). Significance levels were assessed by *t* test.

Results

The putative positions of Arg-283 in TM-6 and Arg-306 in TM-7 of the TRH-R are shown in Fig. 1, illustrating that the residues are proposed to lie within the transmembrane bundle. Arg-306, Arg-283, and Trp-279, which is predicted to reside on the same face of TM-6 as Arg-283 (Fig. 1B), were each mutated to alanine and the mutant receptors were transiently expressed in COS-1 cells. As a measure of receptor activation, the cells were stimulated with TRH and formation of IP second messengers was assayed (Fig. 2; Table 1). Relative potencies can be used to assess relative affinities in receptor-ligand systems of equal efficacies (14). For all Arg-306 mutant receptors, the maximal extents of TRH stimulation of IP formation were the same as in cells expressing WT TRH-Rs, suggesting that these mutant receptors and WT receptors have similar efficacies. Mutation of Arg-306 to alanine increased the EC_{50} of the mutant TRH-R by a factor of 1500, compared with the WT TRH-R. In contrast, the maximal level of stimulation of IP formation by R283A TRH-R was <1% of that of the WT TRH-R. Thus, Arg-306 appeared to be important for binding, because the EC_{50} of R306A TRH-R was higher than that of the WT TRH-R, and Arg-283 appeared to be important for activation; it was not possible to assess the role of Arg-283 in binding from these experiments. The EC_{50} and maximal extent of TRH stimulation of IP formation in cells expressing W279A TRH-Rs were the same as those in cells expressing WT TRH-Rs. Thus, Trp-279 does not appear to be involved in the binding of TRH or in the activation of TRH-Rs.

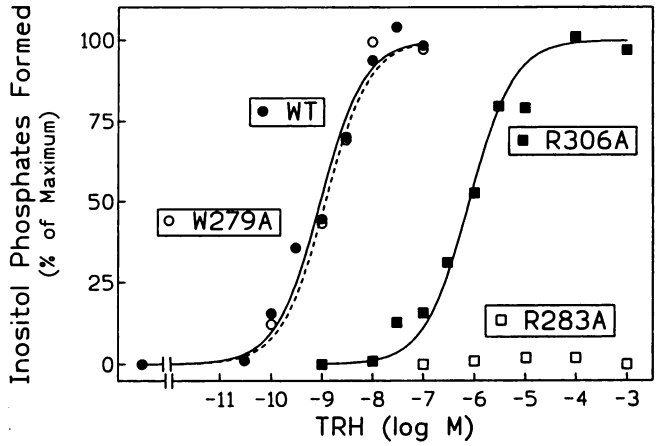

Fig. 2. TRH stimulation of IP formation in COS-1 cells expressing WT, W279A, R306A, or R283A TRH-Rs. Data points represent mean values of duplicate or triplicate determinations in two to seven experiments. There was no TRH stimulation of IP formation in AtT-20 cells stably expressing approximately 0.88×10^6 R283A TRH-Rs/cell (see text).

TABLE 1

Binding and activation of WT and mutant TRH-Rs

The values presented are the mean ± standard error of duplicate or triplicate measurements in two to seven individual experiments.

TRH-R	K_d, MeTRH[a]	EC_{50}, TRH
	μM	μM
WT	0.0012 ± 0.00015	0.0011 ± 0.00030
R306K	0.0029 ± 0.00087	0.011 ± 0.0045
R306A		1.6 ± 0.53
R306E		3.3 ± 1.6
R306L		1.3 ± 0.38
R283K		6.9 ± 1.7
R283H		55 ± 18
R283A	39 ± 14	ND[b]

[a] For binding assays, the concentration of [^{3}H]MeTRH was 2 nM for WT TRH-Rs, 4 nM for R306K TRH-Rs, and 100 nM for R283A TRH-Rs. We could not consistently measure specific binding of Arg-283 mutant TRH-Rs in COS-1 cells. For R283A TRH-Rs in two different stably transfected AtT-20 cell lines, the specifically bound [^{3}H]MeTRH was 2560 ± 1680 dpm/1 × 10^6 cells (three experiments) and 2250 ± 260 dpm/1 × 10^6 cells (four experiments).

[b] ND, No stimulation of IP formation was detected.

Fig. 3 and Table 1 show the results of mutating Arg-306 to residues other than alanine. Compared with WT TRH-R, the

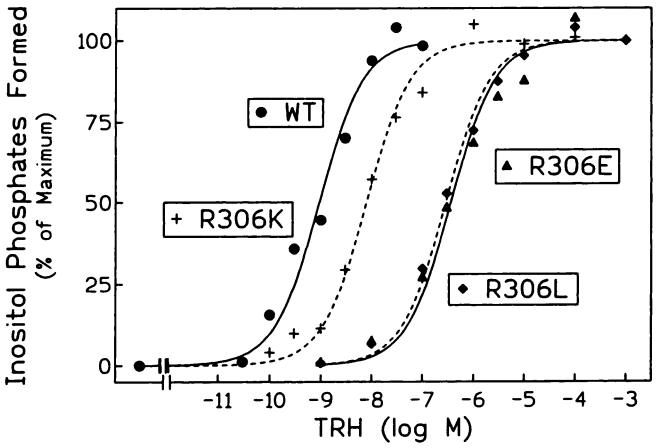

Fig. 3. TRH stimulation of IP formation in COS-1 cells expressing WT or Arg-306 mutant TRH-Rs. Data points represent mean values of duplicate or triplicate determinations in two to five experiments. The data obtained with WT TRH-Rs are reproduced here for comparison.

EC_{50} values for TRH stimulation of IP formation in COS-1 cells expressing R306L and R306E TRH-Rs were 1200- and 3000-fold higher, respectively, whereas that in cells expressing R306K TRH-Rs was only 10-fold higher. These data are consistent with the idea that Arg-306 is critical for binding and indicate that the side-chain ammonium group of lysine mimics the guanidinium moiety of Arg-306. Arg-306 is not important for receptor activation.

Fig. 4 shows the effects of mutating Arg-283 to residues other than alanine. Like R283A TRH-R, there was minimal stimulation by TRH of IP formation in COS-1 cells expressing R283S, R283L, or R283E TRH-Rs. In contrast, COS-1 cells expressing R283K or R283H TRH-Rs exhibited stimulation of IP formation by maximally effective doses of TRH that were 64 ± 16% (mean ± standard error) or 42 ± 7.3%, respectively, of that observed in cells expressing WT TRH-Rs. The EC_{50} values for stimulation of IP formation in cells expressing R283K and R283H TRH-Rs were 6300- and 50,000-fold higher, respectively, than that of the WT TRH-R (Fig. 4; Table 1). These findings are consistent with the idea that Arg-283 is critical for activation and suggest that TRH-Rs with a residue containing an N-H group at position

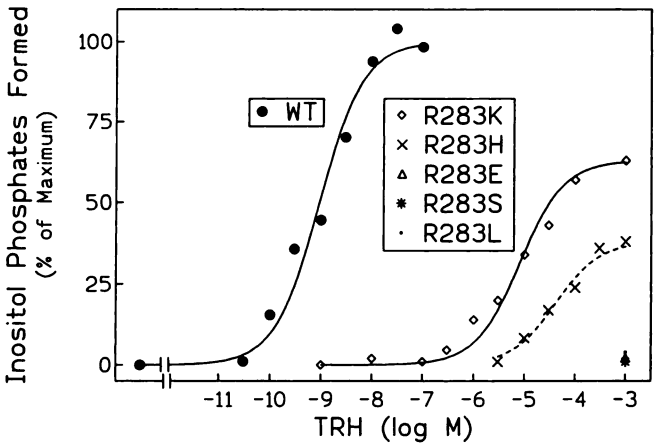

Fig. 4. TRH stimulation of IP formation in COS-1 cells expressing WT or Arg-283 mutant TRH-Rs. Data points represent mean values of duplicate or triplicate determinations in three to five experiments. The data obtained with WT TRH-Rs are reproduced here for comparison.

283 retain the capacity to stimulate IP formation. To confirm this finding, AtT-20 cells were stably transfected with plasmids containing cDNA for R283K, R283A, or R283S TRH-Rs. Stimulation of AtT-20 cells expressing R283A or R283S TRH-Rs by maximally effective doses of TRH caused negligible IP formation. In two cell lines stably expressing R283A TRH-Rs, TRH stimulated IP formation 1.08 ± 0.06-fold (five experiments) and 1.15 ± 0.06-fold (seven experiments), compared with 22 ± 2.2-fold (nine experiments) in AtT-20 cells stably expressing WT TRH-Rs. Stimulation of cells expressing R283K TRH-Rs led to IP formation that was 16 ± 1.9% of that in cells expressing WT TRH-Rs. Thus, in AtT-20 cells, as in COS-1 cells, only a receptor with an N-H group-containing side chain at position 283 was capable of activation. These data suggest that Arg-283 is important for TRH-R activation and for high affinity binding and that, in contrast to activation, other N-H group-containing side chains cannot substitute for arginine in producing high affinity binding.

Because maximal stimulation of IP formation by TRH is directly related to TRH-R number (15, 16), it was possible that the lower activities observed with Arg-283 mutants were due to lower receptor expression rather than lower intrinsic efficacies of these TRH-R mutants. For high affinity TRH-Rs, MeTRH binding allows accurate measurements of receptor number. WT and R306K TRH-Rs exhibited high affinity for MeTRH (Table 1). In COS-1 cells, no specific binding was detected for Arg-283 or Arg-306 mutant receptors other than R306K TRH-R using 20 nM [^{3}H]MeTRH in the binding assay, and the level of nonspecific binding using 100 nM [^{3}H]MeTRH was too high to allow detection of specific binding. An immunological approach would allow estimation of the number of low affinity receptors, but TRH-R-specific antibodies were not available. Therefore, we measured binding of [^{3}H]MeTRH to R283A TRH-Rs stably expressed in AtT-20 cells, in which the level of nonspecific binding (30 ± 9.5% of total binding, three experiments) was lower than in COS-1 cells, to estimate receptor number in cells expressing these low affinity receptors. The K_d of [^{3}H]MeTRH binding was estimated to be 39 ± 14 μM (Table 1). In two different R283A TRH-R-expressing AtT-20 cell lines, the receptor number was estimated to be at least as high as that in WT cells (880,000 ± 320,000 receptors/cell). These estimates of receptor number should be viewed as approximate, because of the low affinity of R283A TRH-Rs. It is possible that there may have been an underestimation of the binding affinity due to dissociation of radiolabeled ligand from low affinity receptors during separation of cell-bound and free radioligand. This would result in an overestimation of receptor number. We attempted to minimize dissociation by rapid separation of cells from the incubation buffer by centrifugation of the cells through an oil/sucrose interface (14). We think, however, that it is unlikely that any overestimation of receptor number would be large enough to affect the interpretation of our results, because we can detect TRH stimulation of IP formation in AtT-20 cells expressing as few as 10,000 WT TRH-Rs/cell.[2] Thus, the decreased efficacy observed with the R283A TRH-R cannot be attributed to a decreased number of cell surface receptors and must, therefore, be due to decreased intrinsic efficacy of this mutant receptor.

[2] J. H. Perlman, M. C. Gershengorn, unpublished observations.

Discussion

Our results indicate that Arg-283 and Arg-306, two residues that appear to reside within the transmembrane core of TRH-R, are involved in the binding of TRH. It is important to note that these data do not show that either of these residues makes a direct contact with TRH. Without direct structural information regarding the receptor-ligand complex, only indirect approaches are available to infer the roles of these residues. One such strategy has been applied to TRH-R (11) and other members of the GPCR superfamily (17–20). In this method, a site-specific mutation in the receptor that deletes a potential interacting moiety is constructed and a ligand analog that lacks the specific moiety that is proposed to bond with the substituted receptor residue is synthesized. It is predicted that the decrease in affinity observed when the native ligand is bound to the mutated receptor would be similar to the decrease found when the analog is bound to the native receptor and, furthermore, that the decrease measured when the analog is bound to the mutated receptor would be less than the sum of the individual decrements. For example, mutation of Tyr-106 to phenylalanine in TRH-R, which removes only the hydroxyl group, caused the affinity for TRH to be decreased by 100,000-fold. The affinity of WT TRH-R for Pro-His-Pro-NH_2, an analog in which the C=O group of pyro-Glu is deleted, was 110,000-fold lower than that for TRH. However, the affinity of interaction between Y106F TRH-R and Pro-His-Pro-NH_2 was only 16-fold lower than either of the aforementioned binding affinities (11). From these data, we concluded that the hydroxyl group of Tyr-106 forms a hydrogen bond with the C=O of pyro-Glu. Similar data using analogs of TRH and TRH-Rs mutated at the arginine residues in TM-6 and TM-7 have not yet been obtained.

We have recently proposed a model of unoccupied TRH-R,[1] portions of which were verified by our experimental observations, and a model for a complex of TRH with the receptor that was based on our experimental findings (10). The model of the receptor-ligand complex incorporates our earlier findings that Tyr-106 (11) and Asn-110 (10) of TM-3 form hydrogen bonds with the ring carbonyl and N-H groups, respectively, of pyro-Glu of TRH, and it predicts roles, based on preliminary findings, for Arg-283 and Arg-306 in forming the binding pocket. The results of the experiments reported herein are consistent with those predictions and expand them by suggesting a role for Arg-283 in receptor activation. The model proposes that the binding pocket of TRH-R resides completely within the TM bundle, and it predicts that Arg-306 forms a hydrogen bond with the hydroxyl group of Tyr-106 and the side-chain carbonyl group of Gln-105 in TM-3. The model also predicts an interaction between Arg-283 and the terminal carbonyl group of Pro-NH_2 of TRH. We plan to test these hypotheses using TRH analogs and these mutant receptors. Thus, in addition to incorporating experimental data, the model provides hypotheses that can be tested.

The hydrogen bonding network of which Arg-306 is a part, which is predicted by the model to surround Tyr-106, helps explain the larger than expected decrease in affinity upon mutation of Tyr-106 (11), because several hydrogen bonds would be broken as a result of this mutation. Lysine mimics a functional group of Arg-306 that appears to be responsible for maintaining high affinity binding. The model proposes that the role of this group is to stabilize the bond between Tyr-106 and the ring C=O of pyro-Glu of TRH. Another role of Arg-306, which is not mimicked by lysine, appears to be responsible for a small decrease in binding affinity. The model proposes that this group interacts with the side-chain C=O of Gln-105. This is consistent with our finding that the affinity of Q105A TRH-R is only 3-fold less than that of WT TRH-R (12). Furthermore, our results indicate that a group in Arg-283 not mimicked by either lysine or histidine is responsible for high affinity binding.

Residues in other GPCRs in positions homologous to these arginine residues have been shown to be important in binding. Asparagine and phenylalanine in homologous positions of TM-7 in the β-adrenergic and α-adrenergic receptors, respectively, have been demonstrated to be important for antagonist subtype selectivity (21, 22), and an homologous tyrosine in TM-7 of the muscarinic M3 receptor has been proposed to bind an ester group of the ligand (20). A threonine or asparagine in an analogous position in serotonin receptors is responsible for the differential selectivity of human and rodent receptors (23–25). Aromatic residues in homologous positions in TM-6 have been implicated in neurotransmitter (26, 27), adenosine A_1 (28), and neurokinin-1 (19) GPCR binding. A side-chain N-H group is strongly conserved at this position in GPCRs for peptides and, in the case of the neurokinin-1 receptor, histidine at this site has been demonstrated to directly contact a nonpeptide antagonist (19). Whereas asparagine, glutamine, or histidine is found in GPCRs for other peptides, TRH-R is unusual in that an arginine is located at this position. The importance of nonconserved residues at these two GPCR positions for the binding of ligands of various structures (for example, neurotransmitters and peptides) argues for evolutionary conservation of interactions involving these sites. That is, an interaction is conserved at these sites although the properties of the residues needed for these interactions are not conserved.

An interesting difference between these two arginine residues is exhibited by the effects on efficacies for stimulation of IP formation with the mutant TRH-Rs. At position 306, substitution for arginine by all amino acids tested led to mutant TRH-Rs that were capable of stimulating IP formation to the same maximal level as WT TRH-Rs. In contrast, in cells expressing mutant TRH-Rs in which Arg-283 was substituted by alanine, serine, leucine, or glutamate, TRH caused virtually no stimulation of IP formation, whereas substitution by lysine or histidine led to receptors with decreased, but measurable, IP-stimulating activities. It was possible that the decreases in apparent stimulating activities of these mutant TRH-Rs were caused by lower levels of expression, rather than decreases in their intrinsic efficacies. We were able to show that this was not the case in AtT-20 cells stably expressing R283A TRH-Rs. In these cells, we found that the binding affinity of the R283A TRH-R was 39,000-fold lower than that of the WT TRH-R, its level of expression was similar to that of the WT TRH-R, and activation of R283A TRH-Rs caused no measurable increase in IP formation.

We were able to estimate the affinities of R283K and R283H TRH-Rs from the dose-response curves for stimulation of IP formation. Theoretically, decreases in efficacy would result in decreases in potency until the EC_{50} of stimulation is equal to the K_d of binding. Further decreases in

efficacy would result in decreases in the maximal extent of stimulation but not a further increase in EC_{50} (14). Therefore, we may assume that the EC_{50} values of R283K and R283H TRH-Rs for TRH equal the K_d values of TRH binding. We estimate that the affinities of R283K and R283H TRH-Rs are 1300- and 10,000-fold lower, respectively, than the K_d of WT TRH-Rs (5 nM) (1). These estimates are consistent with our inability to measure specific binding in AtT-20 cells expressing R283K TRH-Rs, using 20 nM [^{3}H]MeTRH. Although R283S, R283L, and R283E TRH-Rs appeared incapable of stimulating IP formation, we cannot exclude the possibility, as we did for R283A TRH-Rs in AtT-20 cells, that this was due to markedly reduced receptor expression. We attempted to do so with radioligand binding assays in COS-1 cells expressing these mutant receptors, but we could not consistently measure specific binding.

Although the molecular details of activation of GPCRs are not known, it has been postulated that, upon the binding of agonist, conformational changes are induced in the receptor that cause alterations in intracellular domains (2), such as intracellular loop 3 (29), to enhance coupling to G proteins. A corollary to this hypothesis is that new intramolecular interactions would form to constrain the agonist-occupied receptor in the "activated" conformation. This idea is supported by the findings that some unliganded mutant receptors appear able to attain an active conformation (30). It is of note, therefore, that Arg-283 is in TM-6, which is contiguous with intracellular loop 3, and that only receptors with residues containing an N-H group at position 283 were capable of stimulating IP formation. Based on these findings, it is tempting to speculate that the N-H group is involved in formation of an intramolecular hydrogen bond with a residue in another region of the receptor to effect activation.

In conclusion, Arg-283 in TM-6 and Arg-306 in TM-7 of the TRH-R are both important residues but in different ways. Arg-283 is involved in activation and binding, whereas Arg-306 is necessary for binding but not activation. The dual roles of Arg-283 are dissociable through mutagenesis. A model of the binding pocket of TRH-R has been proposed that is consistent with these findings.

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