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A Model of Inverse Agonist Action at Thyrotropin-Releasing Hormone Receptor Type 1: Role of a Conserved Tryptophan in Helix 6

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ABSTRACT

A binding pocket for thyrotropin-releasing hormone (TRH) within the transmembrane helices of the TRH receptor type 1 (TRH-R1) has been identified based on experimental evidence and computer simulations. To determine the binding site for a competitive inverse agonist, midazolam, three of the four residues that directly contact TRH and other residues that restrain TRH-R1 in an inactive conformation were screened by mutagenesis and binding assays. We found that two residues that directly contact TRH, Asn-110 in transmembrane helix 3 (3.37) and Arg-306 in transmembrane helix 7 (7.39), were important for midazolam binding but another, Tyr-282 in transmembrane helix 6 (6.51), was not. A highly conserved residue, Trp-279 in transmembrane helix 6 (6.48), which was reported to be critical in stabilizing TRH-R1 in an inactive state but not for TRH binding, was critical for midazolam binding. We used our previous model of the unoccupied TRH-R1 to generate a model of the TRH-R1/midazolam complex. The experimental results and the molecular model of the complex suggest that midazolam binds to TRH-R1 within a transmembrane helical pocket that partially overlaps the TRH binding pocket. This result is consistent with the competitive antagonism of midazolam binding. We suggest that the mechanism of inverse agonism effected by midazolam involves its direct interaction with Trp-279, which contributes to the stabilization of the inactive conformation of TRH-R1.

Seven transmembrane-spanning receptors (7TMRs) comprise one of the largest protein families in mammalian genomes that transduce a variety of signals across cell-surface membranes to activate numerous cellular responses. Because of the involvement of the 7TMRs in many physiological processes, they have been selected as targets for many therapeutic drugs. All 7TMRs share a putative topological structure, but the molecular details of receptor conformational change upon agonist and inverse agonist binding that lead to activation and inactivation, respectively, have not yet been fully understood. For the last decade, intensive research has been focused on identifying receptor agonist binding sites and the dynamic conformational changes induced upon agonist binding (Osman et al., 1999). However, data about the binding of inverse agonists and the inactivation mechanism of 7TMRs are more limited. Interpretations of the results of these studies have been made more complex because the three-dimensional structure of only a single 7TMR, bovine rhodopsin, has been solved by X-ray crystallography (Palczewski et al., 2000).

Thyrotropin-releasing hormone (TRH) is a tripeptide pyro-Glu-His-Pro-amide that is synthesized and released from the hypothalamus to regulate pituitary hormone levels. TRH functions as a neurotransmitter/neuromodulator in the central and peripheral nervous systems also. TRH binds to specific 7TMRs, TRH receptors (TRH-Rs), and induces a cellular response through G-protein-related signaling pathways. The three-dimensional structure of TRH receptor subtype-1 (TRH-R1) and its TRH binding pocket have been studied by

[1] In addition to numbering residues according to their position within the TRH-R1 sequence, the general numbering schema of 7TMRs proposed by Ballesteros and Weinstein is provided in parentheses.

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ABBREVIATIONS: 7TMR, seven transmembrane-spanning receptor; TRH, thyrotropin-releasing hormone; TRH-R, thyrotropin-releasing hormone receptor; MeTRH, [3-methyl-His]-TRH; HEK, human embryonic kidney; DOPC, dioleoylphosphatidylcholine; ABNR, Adopted Basis Newton-Raphson; Ach, acetylcholine; NMS, *N*-methylscopolamine; NPY, neuropeptide Y; BIBP3226, (*R*)-*N*2-(diphenylacetyl)-*N*-[(4-hydroxy-phenyl)methyl]-D-argininamide; CREB, cAMP response element-binding protein.

a combination of molecular mutagenesis, analog synthesis, and computer simulation modeling (Gershengorn and Osman, 1996; Laakkonen et al., 1996; Perlman et al., 1996). Direct interactions between residues within TRH-R1 and aspects of TRH were identified experimentally when complementary changes could be made within the receptor and the ligand. These interactions were supported by a molecular model that was constructed on the basis of the properties of the transmembrane helices and the nature of the extracellular loops. The model led to the proposition that TRH binds to residues located within the extracellular third of the transmembrane helical bundle of TRH-R1. Tyr-106 (3.34) and Asn-110 (3.37) in transmembrane helix 3 interact with the pyro-Glu, Tyr-282 (6.51) in transmembrane helix 6 with His, and Arg-306 (7.39) in transmembrane helix 7 contacts the Pro-amide. These residues, through their direct contact with TRH, demarcate the putative transmembrane helical TRH binding pocket (Gershengorn and Osman, 1996). In addition, Tyr-181 in extracellular loop 2 (Perlman et al., 1997) and Asn-289 at the junction of extracellular loop 3 and transmembrane helix 6 (Han and Tashjian, 1995) also seem to interact directly with TRH. Because our computer model indicated that TRH could not bind to these two extracellular loop residues and the residues in the transmembrane helices simultaneously, we proposed that Tyr-181 and Asn-289 are part of an intermediate TRH binding site (Perlman et al., 1997; Colson et al., 1998b; Gershengorn and Osman, 2001). Because extracellular loop 2 projects into the transmembrane helical bundle in the crystal structure of rhodopsin (Palczewski et al., 2000), it is possible that TRH could bind to extracellular loop 2 and transmembrane helical residues simultaneously under certain conditions. Further studies of the structure and mobility of extracellular loop 2 of TRH-R1 are needed to distinguish between these possibilities. The conformational changes induced in the receptor after TRH binding have not yet been fully delineated.

Several nonpeptide benzodiazepine drugs exhibit antagonistic activity by competing with TRH for binding to TRH-R1 and inhibiting its stimulation. In addition, benzodiazepine drugs have been shown to act as inverse agonists on TRH-R1, in that they inhibit constitutive (or agonist-independent) signaling activity (Jinsi-Parimoo and Gershengorn, 1997). Based on the different effects of TRH and benzodiazepine drugs on receptor function, delineating the structures of TRH-R1/TRH and TRH-R1/midazolam complexes may elucidate the mechanisms of TRH-R1 activation and inactivation.

In these studies, we attempted to delineate the binding pocket for benzodiazepine drugs within TRH-R1. We screened residues that comprise the transmembrane TRH binding pocket and found that only two of the residues that directly contact TRH are also involved in midazolam binding. In contrast, different residues in the extracellular loops were found to be involved in either TRH or midazolam binding. Furthermore, a highly conserved Trp residue in 7TMRs, which is Trp-279 (6.48) in transmembrane helix 6 in TRH-R1, was found to play an important role in benzodiazepine drug binding even though it is not involved in TRH binding. The model that we generated of the TRH-R1/midazolam complex suggests a mechanism of TRH-R1 inactivation by benzodiazepine drug inverse agonists.

Materials and Methods

Materials. [^{3}H][3-methyl-His]-TRH ([^{3}H]MeTRH) was purchased from DuPont Pharmaceuticals (Wilmington, DE). TRH was from Calbiochem (La Jolla, CA). Midazolam, triazolam, and alprazolam were purchased from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium was from Mediatech (Herndon, VA), and fetal bovine serum was from BioSource Technologies (Camarillo, CA). The cDNA encoding mouse TRH-R1 was in the mammalian cell expression vector pcDNA3.1 (+). Single amino acid mutations were engineered by overlapping polymerase chain reaction, products of which were digested by EcoR1 and NotI and inserted into pcDNA3.1 (+) vector. All constructs were verified by dideoxy sequencing.

Cell Culture and Transfection. HEK293EM cells (Robbins and Horlick, 1998) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. On the day before transfection, the cells were seeded into 24-well plates (1.5×10^5/well). After 16 h, the media were aspirated, and the cells (~50% confluent) were transfected with 2 μg/ml of receptor-encoding plasmid DNA using calcium phosphate. Binding assays were performed 24 h after transfection. For signaling assays, cells were cotransfected with 100 ng/ml of receptor-encoding plasmid DNA and plasmids encoding CREB and CREB-activated luciferase gene (PathDetect CREB *trans*-Reporting System; Stratagene, La Jolla, CA) as described previously (Wang and Gershengorn, 1999). In brief, 6 h after transfection, medium containing 10% FBS was changed to medium containing 1% FBS, and TRH 1, 10, or 100 nM and/or midazolam 50 μM were added to the medium. Luciferase activity was measured 24 h after transfection.

Ligand Binding Assays. Apparent binding affinity constants (K_d) were measured at equilibrium using 0.1 to 10 nM [^{3}H]MeTRH, an analog of TRH with 5- to 10-fold higher affinity to TRH receptors than native TRH. Equilibrium was achieved at 4°C for 4-h incubation as described previously (Perlman et al., 1992). Competition binding assays at equilibrium to measure apparent inhibitory constants (K_i) were performed at 4°C for 4 h with 2 nM [^{3}H]MeTRH for cells expressing TRH-R1 and mutant receptors with high affinity for TRH or 5 nM [^{3}H]MeTRH for cells expressing mutant receptors with low affinity for TRH and various concentrations of unlabeled TRH or antagonist as described previously (Perlman et al., 1996). Equilibrium binding constants were derived from competition binding experiments using the formula $K_i = (IC_{50})/(1 + ([L]/K_d))$, where IC_{50} is the concentration of unlabeled ligand that half-competes with specifically bound [^{3}H]MeTRH, and K_d is the equilibrium dissociation constant for [^{3}H]MeTRH. Curves were fitted by nonlinear regression analysis and drawn with the Prism software version 3 (GraphPad, Inc., San Diego, CA).

Computer Modeling. The unoccupied and TRH-occupied TRH-R1 models were constructed previously by Colson and colleagues (Colson et al., 1998a,b). The models in the present studies were built with explicit lipid bilayers and water placed in periodic boxes. For the construction, we adapted the multistep protocol developed by Woolf and Roux (1994, 1996). We used a pre-equilibrated conformer library of dioleoylphosphatidylcholine (DOPC) provided by Dr. Richard Venable (Dolan et al., 2002). The system contains 56 DOPC and about 4700 water molecules in a tetragonal unit cell. The unit cell dimensions are $55 \times 55 \times 88$ Å. The details of construction were described elsewhere (Huang, 2003).

The midazolam-occupied TRH-R1 model was constructed by manually docking midazolam into the unoccupied TRH-R1 receptor in the crevice formed by the extracellular portions of transmembrane helices 3, 5, 6, and 7, as suggested by experimental results. The force field for midazolam was constructed by quantum mechanical calculations of the electrostatic potential of midazolam for an optimized structure. The partial charges were fitted to reproduce the electrostatic potential. The initial structure of the complex was equilibrated by a 1000-step Adopted Basis Newton-Raphson (ABNR) minimization and a subsequent 2-ns molecular dynamics simulation. Midazo-

lam remained in the same crevice during the simulation. The midazolam molecule was then rotated about its three principle axes, producing more than 1700 rotational combinations. Each docked configuration was followed by a 100-step Steepest Descents minimization, a 250-step ABNR minimization, a 5-ps molecular dynamics simulation, and a 250-step ABNR minimization. All molecular dynamics simulations were run at 310 K. The simulations were carried out with the all-atom PARAM 27 force field of CHARMM 29 (Brooks et al., 1983; Mackerell et al., 1998).

Data Analysis. All binding data were analyzed using GraphPad Prism. Statistical significance was determined using Student's t test with a probability criterion of $p < 0.05$.

Results

Residues Important for TRH and Midazolam Binding. Several benzodiazepine drugs have been reported to function as competitive inverse agonists of TRH-R1. The structure of midazolam, one of the benzodiazepine drugs with the highest affinity for TRH-R1, is shown in Fig. 1. To begin to determine the binding site for midazolam in TRH-R1, three of the four residues that have been shown to directly contact TRH within the transmembrane binding pocket were screened for binding of midazolam. Residues Asn-110, Tyr-282, and Arg-306 were substituted with Ala, Phe, and Lys, respectively. Binding assays were performed in cells expressing the mutant receptors with N110A, Y282F or R306K as described ubder *Materials and Methods*. As illustrated in Fig. 2 and Table 1, the affinities of N110A and R306K for TRH were decreased only 3.9- and 1.9-fold, respectively, but midazolam affinities were reduced 120- and 9-fold, respectively. In contrast, the affinity of Y282F for TRH was 10-fold lower than TRH-R1 but was not different from TRH-R1 for midazolam (Table 1). These results suggest that residues Asn-110 and Arg-306 are important for midazolam binding, whereas the hydroxyl group of Tyr-282 is only important for TRH binding. Tyr-106 in transmembrane helix 3 was reported previously to interact with the pGlu moiety of TRH (Perlman et al., 1994b), but substitution of Tyr-106 with Phe, Ala, Asn, or Ser disturbs MeTRH binding so severely that it was not possible to test whether Tyr-106 is involved in midazolam binding.

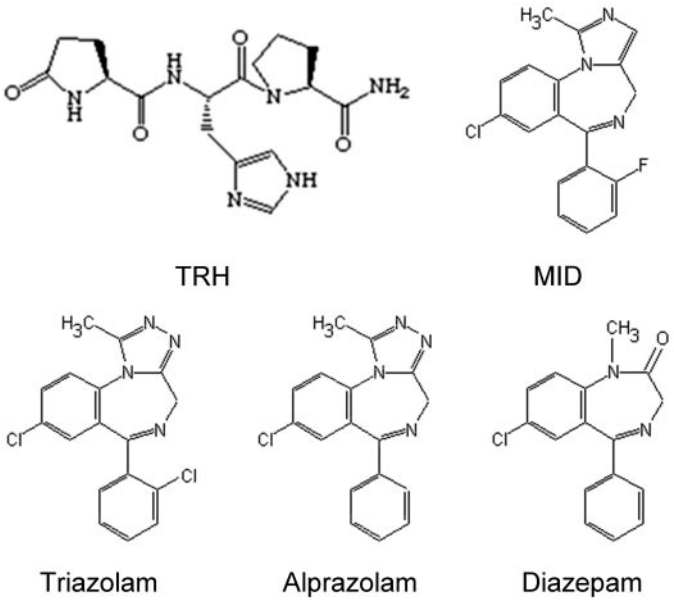

Fig. 1. Chemical structures of TRH, midazolam, triazolam, alprazolam, and diazepam.

TRH and Midazolam Binding to Extracellular Loop Residues. Three residues in the extracellular loops of TRH-R1, Tyr-181 (Perlman et al., 1997) and Arg-185 (X. Lu and M. C. Gershengorn, unpublished data) in extracellular loop 2 and Asn-289 (Han and Tashjian, 1995; Colson et al., 1998b) in extracellular loop 3, were found previously to contact TRH directly. To determine whether these residues participate in midazolam binding, Arg-185 and Asn-289 were replaced with His and Ala, respectively. As shown in Table 2, the affinities of R185H and N289A for TRH were reduced 11- and 17-fold, respectively, whereas the affinities of R185H and N289A for midazolam were indistinguishable from that of TRH-R1. Mutant receptors in which Tyr-181 in extracellular loop 2 was substituted by Ala or Phe exhibited very low affinities for MeTRH; therefore, no binding assay could be performed for testing whether Tyr-181 might be involved in the binding of midazolam.

We screened several residues in the extracellular loops with hydrophobic or charged side chains in an attempt to discover whether they affect midazolam binding. We selected Trp91 and Tyr93 in extracellular loop 1, and Asp-165, Leu-166, Tyr-171, Lys-172, and Lys-182 in extracellular loop 2, and mutated them by Ala substitution. As illustrated in Table 2, the affinity of mutant Y93A for TRH was similar to that of TRH-R1, but the affinity of Y93A for midazolam was 6.5-fold lower than that of TRH-R1. On the other hand, the affinities of D165A, L166A, Y171A, K172A, and K182A for TRH and midazolam were similar to those measured in TRH-R1. W91A, however, exhibited a 44-fold reduction in affinity for TRH compared with TRH-R1 (Table 2). To evaluate the cause of the reduction in affinity of W91A for TRH, the affinities and potencies of three TRH analogs, [desaza^1]-TRH, [Phe^2]-TRH, and [Pyr^3]-TRH, for W91A were measured. W91A displayed the same proportional reduction as TRH-R1 in affinities for TRH analogs [Phe^2]-TRH and [Pyr^3]-TRH, but the affinity of W91A for [desaza^1]-TRH decreased 5- to 10-fold more than to TRH-R1 (data not shown). Because changes at all three positions of TRH caused similar decreases in affinities to TRHR-R1 and W279A, these findings suggest that Trp-91 is probably not involved in direct TRH binding but may be important for maintaining the native conformation of the extracellular loops and thereby indirectly affect TRH binding. It is noteworthy that the affinity of W91A for midazolam was 640-fold lower than that of TRH-R1 (Table 2). The much greater reduction in affinity for midazolam of W91A may indicate not only that Ala substitution at position 91 may lower midazolam binding by disturbing the conformation of the receptor but also that Trp-91 may directly interact with midazolam. Taken together, these results suggest that residues Trp-91 and Tyr-93 in extracellular loop 1 may be involved in the binding of midazolam to TRH-R1.

Trp-279 in Transmembrane Helix 6 (6.48) Is Critical for Midazolam Binding. Trp-279 (6.48) in transmembrane helix 6 of TRH-R1 is a highly conserved residue in subfamily A 7TMRs. Trp-279 was identified as important for restraining TRH-R1 in an inactive state by forming a hydrophobic interaction with Phe-199 (5.46) in transmembrane helix 5 (Colson et al., 1998a). Therefore, binding of midazolam to mutant receptors W279A and F199A was measured. As shown in Table 1, the affinity of mutant receptor W279A for

TRH was minimally increased, but the affinity for midazolam was decreased 21-fold compared with TRH-R1, indicating that Trp-279 is important for midazolam binding. In contrast, the affinity of F199A for TRH was 18-fold lower, whereas for midazolam it was 14-fold lower than that of TRH-R1 (Table 1). The reduction in TRH affinity to F199A was shown previously to be an indirect effect (Perlman et al., 1994a) and a similar lowered affinity of F199A for midazolam binding may also be caused by a conformational change rather than loss of a direct interaction between midazolam and Phe-199.

To further investigate the role of the side chain of Trp-279 in midazolam binding, we substituted Trp-279 with Phe and His. The affinities of W279F and W279H for TRH were *increased* approximately 6-fold above that of TRH-R1 (Table 1). By contrast, the affinities for midazolam of both mutant receptors were 4-fold decreased compared with TRH-R1. The differences in the affinities of W279A, W279F, and W279H to midazolam suggest that Trp-279 may contact midazolam directly via hydrophobic interactions.

Midazolam Inhibition of Basal and TRH-Stimulated Signaling. To determine whether midazolam maintains its antagonist property with mutant receptors studied, we measured the effect of midazolam on basal signaling and on TRH-stimulated signaling using a CREB reporter system. We used midazolam at 50 μM because the affinity of TRH-R1 for midazolam at 37C (K_i ~3 μM) is much lower than at 4C. As shown in Fig. 3, W279A, W279F, and W279H displayed higher basal signaling activities than TRH-R1; W279F and W279H were significantly more active than W279A. Basal signaling was reduced by adding 50 μM Midazolam to cells expressing TRH-R1, W279A, W279F, or W279H. midazolam inhibited basal signaling of TRH-R1, W279A, W279F and W279H receptors by 41 ± 4.3, 32 ± 11, 20 ± 2.5, and 19 ± 4.3%, respectively. Midazolam also inhibited TRH-stimulated signaling by TRH-R1, W279A, W279F, and W279H (Fig. 3) and by Y282F, Y282S, and Y282N also (data not shown). These results indicate that midazolam behaves as an inverse agonist and TRH antagonist at the mutant receptors tested.

Other Benzodiazepines Binding in TRH-R1. To gain further insight into how midazolam binds to TRH-R1, we studied three other benzodiazepine drugs, triazolam, alprazolam, and diazepam, which are structurally similar to midazolam (Fig. 1). We measured the binding affinities of TRH-R1, N110A, W279A, R306K, N289A, and Y282F for these three compounds. As shown in Table 3, all receptors exhibited lower affinities for triazolam, alprazolam, and diazepam, as they did for midazolam. These results are consistent with the idea that other benzodiazepine drugs interact with TRH-R1 in the same binding pocket as midazolam. However, the hierarchies of binding affinities could not be used to predict direct contacts with specific residues within TRH-R1. Nevertheless, the results point to certain possible contacts between midazolam and TRH-R1. For instance, diazepam, which does not have the imidazole moiety of midazolam, does not discriminate N110A mutant receptor from TRH-R1 (15-fold lower affinity) as clearly as midazolam does (120-fold). This suggested that the imidazole moiety of midazolam might interact with Asn-110. With computer modeling of midazolam-bound TRH-R1 and proper midazolam analogs available, we would be able to identify the direct interactions between them.

A Model of the TRH-R1/Midazolam Complex. The TRH-R1/midazolam model was built using the previously constructed unoccupied TRH-R1 model (Huang, 2003), because we reasoned that the structure of midazolam-bound TRH-R1 should be more similar to the inactive, unoccupied TRH-R1 than to the activated TRH-occupied TRH-R1. Midazolam was first manually docked near Trp-279 of transmembrane helix 6 and Asn-110 of transmembrane helix 3 in the crevice formed by transmembrane helices 3, 5, 6, and 7 guided by the experimental evidence of possible direct interactions between midazolam and Asn-110 and midazolam and Trp-279. Energy minimization and molecular dynamics simulations were performed to relax and equilibrate the complex. Midazolam was stable in the same crevice during the length of the simulation. At the end of the equilibration simulation, 1700 additional dockings were performed by rotating midazolam about *x*-, *y*-, and *z*-axes. Each orientational configuration was followed by minimization and a short molecular dynamics simulation. These resulted in many docking configurations that significantly deviated from each other in terms of location and orientation. Therefore, we used three interaction energy terms to evaluate them. The interaction energies of midazolam to Asn-110 and midazolam to Trp-279 provided information concerning the location of midazolam in the binding pocket and its proximity to the two residues. More specific interaction energy between the imidazole ring of midazolam and Asn-110 provided additional orientational information. The configurations that fit this set of criteria were selected. Within the 1700 docked configurations, the

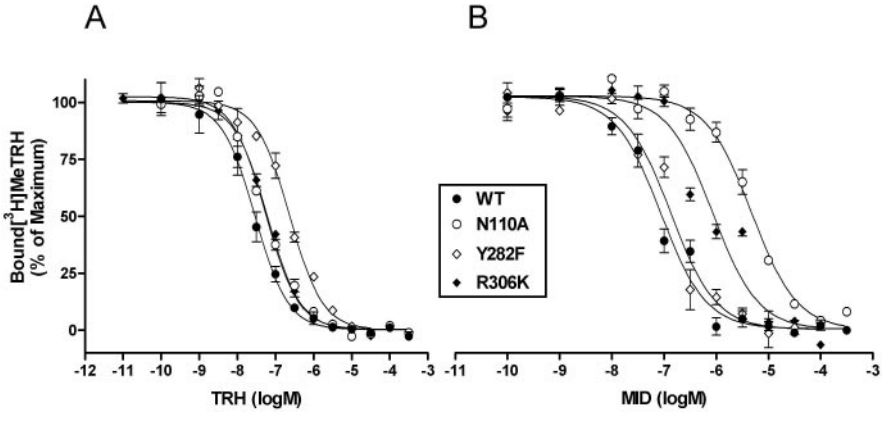

Fig. 2. Binding affinities of TRH-R1, N110A, Y282F, and R306K for TRH and midazolam. HEK293EM cells expressing WT or mutant receptors were incubated in HBSS buffer with 2 or 5 nM [^{3}H]MeTRH in the presence of various doses of TRH (A) or midazolam (MID) (B) at 4°C for 4 h. Each point represents duplicate determinations from at least two independent experiments. Data are expressed as percentages of total specific binding.

values were -0.75 ± 0.85 kj/mol/Å^2, -3.80 ± 1.46 kj/mol/Å^2, and -0.08 ± 0.20 kj/mol/Å^2 for the three interaction energies, respectively, in which the majority only exhibited strong interactions in one or two terms. We identified 38 configurations that had low values in all three interaction energy terms, with average values of -1.69, -4.51, and -0.78, respectively, and percentiles of 85th, 72nd, and 98th, respectively. Twenty of the 38 configurations fell into the same group of structures as characterized by a pair-wise root-mean-square deviation of 1.5 ± 0.4 Å between the midazolam molecules. The rest of the configurations were divided into five minor groups. The root-mean-square deviations between the members of different groups were 5.4 ± 0.7 Å. The members of the major group also exhibited closer proximity of midazolam to both Asn-110 and Trp-279 than members of the other groups. Therefore, this group of structures is most consistent with low-energy configuration and with the experimental results. A representative docked configuration is shown in Fig. 4. The phenyl ring in the 5-position (see Fig. 1 for numbering) is interacting with Trp-279 of transmembrane helix 6, possibly through ring stacking. The imidazole moiety is interacting with Asn-110 of transmembrane helix 3. The other residues that interact with midazolam in this model are not included in the figure for simplicity.

TABLE 1
Comparison of affinities of TRH-R1 and transmembrane residue mutant receptors for TRH and midazolam
The data are presented as the mean (95% confidence interval).

TRH-R1	K_i	
	TRH	Midazolam
	μM	
Wild type	0.009 (0.007–0.012)	0.026 (0.020–0.036)
N110A	0.035 (0.03–0.04)	3.07 (2.30–4.08)
Y282F	0.09 (0.07–0.11)	0.05 (0.03–0.08)
R306K	0.017 (0.013–0.021)	0.23 (0.15–0.34)
F199A	0.16 (0.13–0.18)	0.37 (0.27–0.50)
W279A	0.007 (0.006–0.008)	0.55 (0.41–0.75)
W279F	0.0016 (0.0014–0.0018)	0.11 (0.09–0.14)
W279H	0.0015 (0.0013–0.0018)	0.11 (0.09–0.12)

TABLE 2
Comparison of affinities of receptors with mutations in the extracellular loops for TRH and midazolam
The data are presented as the mean (95% confidence interval).

TRHR	K_i	
	TRH	Midazolam
	μM	
TRHR	0.009 (0.007–0.012)	0.026 (0.020–0.036)
R185H	0.10 (0.03–0.4)	0.04 (0.01–0.14)
N289A	0.15 (0.09–0.27)	0.04 (0.025–0.07)
W91A	0.40 (0.33–0.49)	16.67 (11.68–23.78)
Y93A	0.02 (0.014–0.04)	0.17 (0.10–0.28)
D165A	0.017 (0.001–0.002)	0.023 (0.017–0.032)
L166A	0.008 (0.007–009)	0.03 (0.02–0.04)
Y171A	0.01 (0.008–0.012)	0.036 (0.029–0.044)
K172A	0.015 (0.012–0.018)	0.04 (0.03–0.05)
K182A	0.009 (0.007–0.01)	0.02 (0.012–0.027)

It has been proposed previously (Colson et al., 1998a) that the activation of TRH-R1 induced a movement of the cytoplasmic ends of transmembrane helices 5 and 6 that increased the distance between them. This proposal is supported by recent disulfide cross-linking studies showing that TRH binding induces a separation of transmembrane helice 5 and 6 and a counter-clockwise rotation of transmembrane helix 6 at the cytoplasmic ends (Huang 2003). The movement of helices 5 and 6 relative to each other can be estimated by the distance between the α carbons of Tyr-211 (at the cytoplasmic end of transmembrane helix 5) and Gln-265 (at the cytoplasmic end of transmembrane helix 6). The distances between these residues in the models of unoccupied, TRH-occupied, and midazolam-occupied TRH-R1 are shown in Fig. 5. For unoccupied TRH-R1, 8 structures were randomly selected from a 5-ns molecular dynamics simulation and subsequently minimized to have a quality comparison similar to that of the other structures. Similar calculations were performed with the models of midazolam-occupied and TRH-occupied TRH-R1. In the models of midazolam-occupied TRH-R1, the distances were calculated from the members of the major cluster of configurations with the low interaction energy. In the unoccupied TRH-R1 model, the average distance was 11.6 ± 0.7 Å, whereas in the TRH-occupied model, the average distance increased to 16.5 ± 0.7 Å. In contrast, in the midazolam-occupied models, the average distance decreased by 1.3 Å to 10.3 ± 0.5 Å. These differences were statistically different (***, $P < 0.001$). Thus, the distances between the cytoplasmic ends of transmembrane helix 5 and transmembrane helix 6 correlate directly with the signaling activities. The largest distance is observed in TRH-occupied receptor, which is in an activated form conducive to signaling, whereas the midazolam-occupied TRH-R1 has the smallest distance consistent with the inverse agonist properties of midazolam that inhibit the basal activity. The unoccupied receptor has an intermediate distance consistent with modest constitutive activity. These results are consistent with our previous suggestion that a separation of transmembrane helix 5 from transmembrane helix 6 may be part of the mechanism of TRH-R1 activation (Colson et al., 1998a).

Discussion

The competitive binding nature of benzodiazepine drugs to TRH-R1 led us to hypothesize that the binding site for benzodiazepine drugs in TRH-R1 may overlap with the binding pocket of TRH within the transmembrane helices. Four residues within the transmembrane helices of TRH-R1 have been identified to be involved in TRH binding: Tyr-106 (3.33), Asn-110 (3.37), Tyr-282 (6.51), and Arg-306 (7.39). Herein, we have demonstrated that mutant receptors in which Asn-110 and Arg-306 are substituted with Ala and Lys, respectively, exhibited modestly lower binding affinities for TRH

compared with TRH-R1 but lost 120- and 9-fold in affinity, respectively, for midazolam compared with TRH-R1. On the other hand, mutant receptor Y282F displayed a 10-fold reduction in affinity for TRH but exhibited a similar affinity for midazolam as TRH-R1. These results support the hypothesis that the binding site of midazolam is located within the transmembrane helices of TRH-R1 at a site partially overlapping the binding pocket of TRH. In contrast to the residues within the transmembrane helical binding crevice, residues Arg-185 and Asn-289 in the extracellular loops were not involved in midazolam binding. Rather, two other residues in the extracellular loops seemed to be involved in the binding of midazolam. Receptors in which Tyr-93 in extracellular loop 1 was mutated to Ala displayed similar affinity for TRH but a 7-fold decrease in affinity for midazolam. A mutant receptor in which Trp-91 in extracellular loop 1 was substituted by Ala exhibited reduced affinities for TRH and midazolam partly because of a conformational change in TRH-R1, but the much greater decrease in affinity for midazolam compared with TRH suggests that Trp-91 contacts midazolam directly. We postulated previously that residues within extracellular loops may participate in initial interactions with TRH but may not be part of the transmembrane helical TRH binding pocket (Perlman et al., 1997; Colson et al., 1998b). In light of the finding that a section of extracellular loop 2 between transmembrane helix 5 and the highly conserved Cys in extracellular loop 2 projects into the transmembrane helical bundle in the X-ray crystal structure of rhodopsin (Palczewski et al., 2000), this suggestion should be reconsidered. However, we must be cautious in generalizing this finding from rhodopsin to other 7TMRs. First, extracellular loop 2 may play different roles in rhodopsin versus other 7TMRs because rhodopsin is constitutively occupied by its ligand, retinal, whereas diffusible ligands for other 7TMRs must move through extracellular loop 2 to reach their binding pockets within the transmembrane helical bundles. It is likely that extracellular loop 2 adopts different conformations before and after ligand binding. Second, the corresponding section of extracellular loop 2 of TRH-R1 is shorter than that of rhodopsin by four residues, which reduces the possibility that extracellular loop 2 projects into the transmembrane helical bundle of TRH-R1.

Trp-279 (6.48) is a highly conserved residue in subfamily A 7TMRs that has been proposed in TRH-R1 to form a hydrophobic interaction with Phe-199 (5.46) between transmembrane helix 5 and 6 and thereby restrain TRH-R1 in an inactive conformation (Colson et al., 1998a). Substitution of Trp-279 by Ala resulted in a mutant receptor with a minimally *increased* affinity for TRH but a 21-fold decrease in affinity for midazolam compared with TRH-R1. This finding

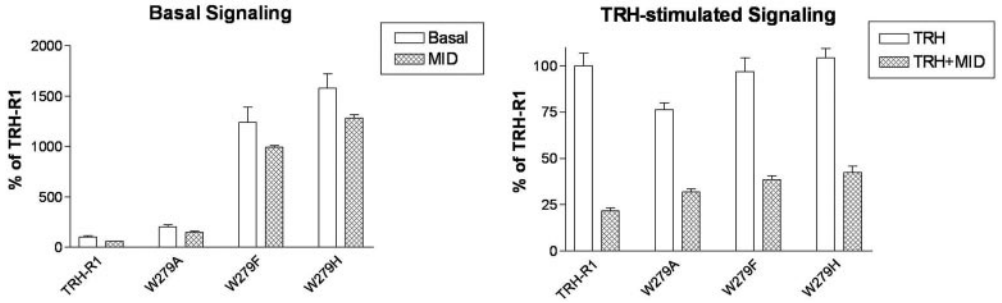

Fig. 3. The effects of midazolam on basal signaling and TRH-stimulated signaling of TRH-R1 and W279 mutant receptors. HEK293EM cells expressing TRH-R1, W279A, W279F, or W279H were treated with or without TRH, midazolam (MID), or TRH plus midazolam for 18 h. Cells were washed and CREB-mediated luciferase activities were measured as described under *Materials and Methods*. To measure basal signaling, luciferase activity was measured in cells transfected with various amounts of receptor-encoding plasmids and basal signaling was estimated as the slope of the line in a plot of signaling activities versus receptor expression levels. In five experiments, the slopes of the lines with TRH-R1, W279A, W279F, and W279H were 0.89 ± 0.20, 1.8 ± 0.32, 11 ± 2.3, and 14 ± 2.2, respectively. Results are expressed as percentage of TRH-R1 as the mean ± S.E. of assays performed in triplicate in five experiments. The results for TRH-stimulated signaling are from a representative experiment performed in triplicate and expressed as percentage of TRH stimulation of TRH-R1 as the mean ± S.D. RLU, relative light units.

TABLE 3
Affinities of mutant receptors for different benzodiazepines
The data are presented as the mean (95% confidence interval).

Receptor	K_i				
	TRH	Midazolam	Tria	Alp	Dia
			μM		
TRH-R1	0.009 (0.007–0.012)	0.026 (0.020–0.036)	0.14 (0.11–0.18)	0.21 0.09–0.48)	1.6 (0.9–2.7)
N110A	0.035 (0.03–0.04)	3.07 (2.30–4.08)			23.2 (15.5–34.9)
W279A	0.007 (0.006–0.008)	0.55 (0.41–0.75)	4.50 (2.80–7.25)	0.84 0.51–1.40)	15.2 (11.5–20.0)
R306K	0.017 (0.013–0.021)	0.23 (0.15–0.34)	0.32 0.15–0.68)	0.39 0.24–0.62)	2.1 (1.5–3.0)
Y282F	0.09 (0.07–0.11)	0.05 (0.03–0.08)	0.79 (0.49–1.27)	0.54 (0.36–0.81)	
N289A	0.15 (0.09–0.27)	0.04 (0.025–0.07)	0.53 (0.21–1.30)	0.60 (0.29–1.25)	

suggests that the side chain of Trp-279 is important for midazolam binding but is not involved in TRH binding. To further analyze the structural feature of W279 that is important in the binding of midazolam, we substituted Trp-279 with Phe and His, which also have hydrophobic side chains. The mutant receptors W279F and W279H displayed *higher* affinities for TRH than TRH-R1. Unlike W279A, which showed a 21-fold reduction in affinity for midazolam, W279F and W279H displayed smaller reductions (4-fold) in affinities for midazolam compared with TRH-R1. These findings are consistent with the idea that the bulky hydrophobic side chain of Trp is directly contacting midazolam and that smaller hydrophobic side chains can partially substitute the effect of Trp. However, because we were not able to make complementary substitutions in TRH-R1 and midazolam, as was done for TRH-R1 and TRH (Gershengorn and Osman, 1996; Perlman et al., 1996), we cannot determine the specific details of the interaction between midazolam and TRH-R1.

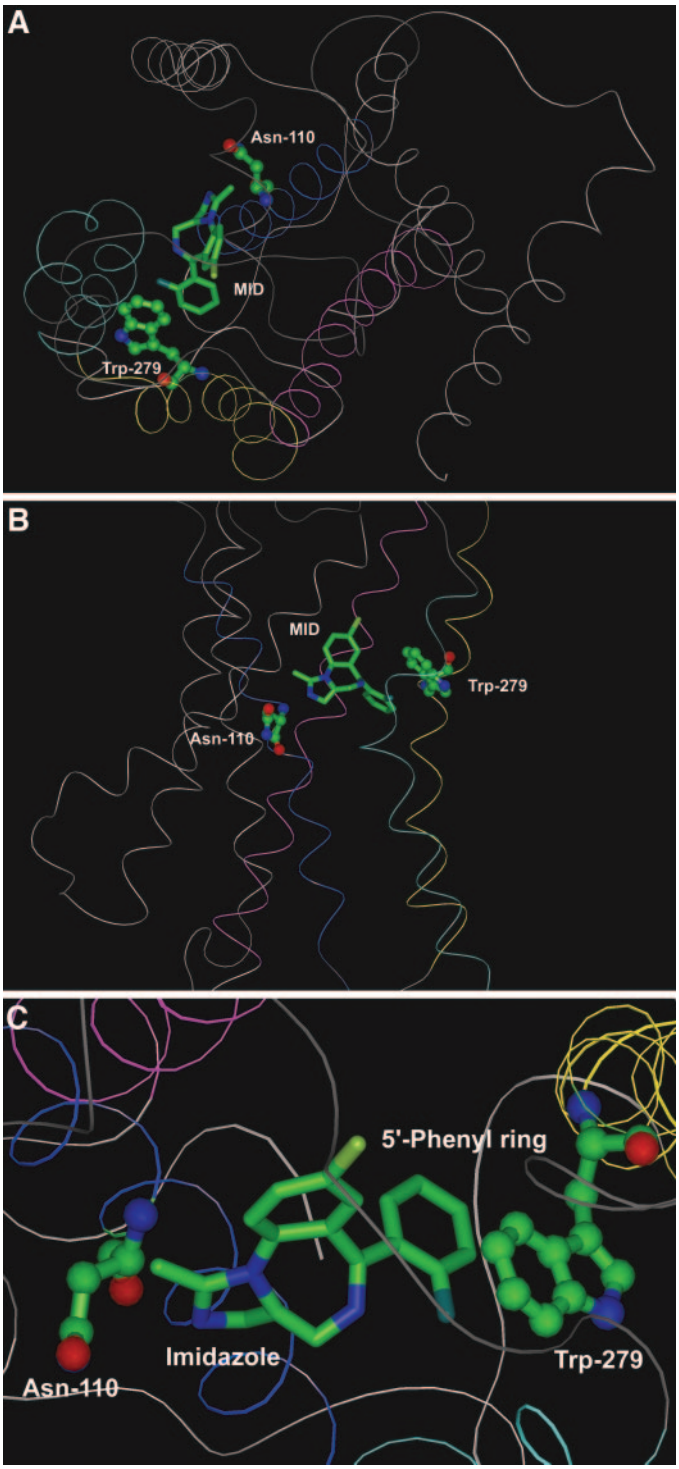

Fig. 4. The model of midazolam-bound TRH-R1. A, a view from extracellular space. B, a side view of the complex. C, a close-up view. Only Asn-110 (transmembrane helix 3), Trp-279 (transmembrane helix 6), midazolam, and the backbone of the receptor (ribbon representation) are shown. Transmembrane helix 3 (transmembrane helix 3) is blue, transmembrane helix 5 is cyan, transmembrane helix 6 is yellow, and transmembrane helix 7 is purple. The rest of the receptor is gray. Lipid bilayer and water molecules are not shown.

Several other receptors of subfamily A of the 7TMR superfamily have displayed apparently overlapping binding sites for agonists and antagonists. In the muscarinic 1 receptor agonist acetylcholine (Ach) and the atropine-like muscarinic receptor antagonist *N*-methylscopolamine (NMS) exhibit contact points within the receptor that are highly homologous but the side chain interactions are different. The NMS and Ach binding sites overlap in that the cationic headgroups of NMS and Ach bind within the same charge-stabilized aromatic cage of the receptor, but Ach has a compact acetoxy side chain and cannot replicate the stabilizing interhelical interactions provided by the bulky side-chains of NMS (for review, see Lu et al., 2002). In the neuropeptide Y (NPY) receptor, the 36-amino acid C-terminal amidated neuropeptide NPY and a small nonpeptide NPY1 antagonist, BIBP3226, seem to share residues for interactions within the transmembrane regions of the receptor, including Gln-120 (3.32), Asn-283 (6.55), and His-306 (7.39), which are in the same position of key residues for ligand binding of other peptide and biogenic amine receptors (Du et al., 1997).

Trp-279 (6.48) in TRH-R1 is a highly conserved residue in subfamily A receptors and plays an important role in several 7TMRs. In rhodopsin, 11-*cis*-retinal acts as a potent inverse agonist, suppressing the activity of the receptor to an undetectable level (ground state). Trp (6.48) within rhodopsin interacts with the C13 methyl group of retinal in the ground state, and this contact is modified by isomerization of the 11-cis linkage to trans in the active form of the retinal/rhodopsin complex (Lin and Sakmar, 1996). Deletion of the

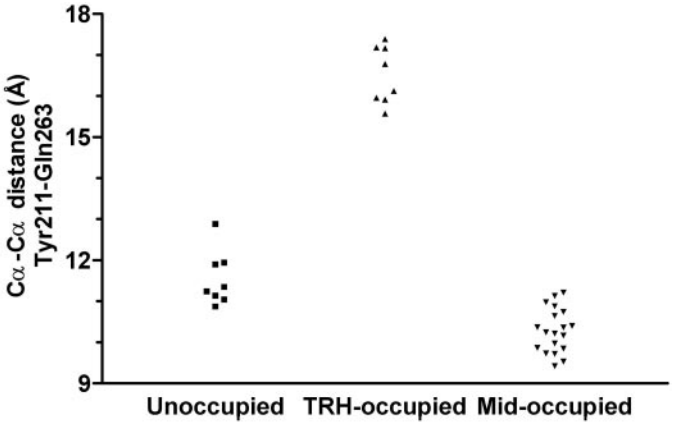

Fig. 5. Distances between the Cα atoms of Tyr-211 in transmembrane helix 5 and Gln-263 in M6 in unoccupied, TRH-occupied, and midazolam-occupied TRH-R1 models. The averages are 11.5, 16.5, and 10.3 Å for the three models, respectively.

C13 methyl group on retinal results in increased constitutive activity (Ebrey et al., 1980). Moreover, in the ground state, a retinal analog with a photoreactive moiety in the β-ionone ring labeled Trp (6.48). At a later stage in the activation process, the retinal analog identified a more superficial residue Ala (4.58), consistent with the idea that this highly conserved Trp (6.48) plays a critical role in maintaining rhodopsin in an inactive state (Borhan et al., 2000). In 5-HT_4 receptor, substitution of Trp (6.48) by Ala blocked antagonist inhibition, but this mutant receptor still retained the ability to be stimulated by agonist (Joubert et al., 2002). In B2 bradykinin mutant receptors in which Trp (6.48) was substituted to either Ala, Phe, or Gln, two inverse agonists to wild-type receptor acted as agonists for the mutant receptors (Marie et al., 2001). The latter two mutant receptors also exhibited constitutive activities. In the case for A_3 adenosine receptor, substitution of Trp (6.48) by either Ala or Phe reduced affinities for inverse agonists and maintained the same affinities for agonists. Meanwhile, Cl-IB-MECA, an agonist, could not activate these two Trp (6.48) mutants (Gao et al., 2002). Indeed, this conserved Trp is involved in receptor inactivation/activation in a growing number of 7TMRs. However, the mechanism is probably more complicated than a simple "switch". The complexity is perhaps best exemplified by the different roles played by Trp (6.48) in TRH receptors subtype 1 and 2 (TRH-R2). In TRH-R1, substitution of this Trp to Ala resulted in a more constitutively active receptor. In contrast, the same substitution inactivated the highly basally active TRH-R2 (Sun and Gershengorn, 2002).

In conclusion, the results of our experiments and computer simulations provide strong evidence that the inverse agonist midazolam binds within the transmembrane bundle of TRH-R1 in a pocket that overlaps the TRH binding pocket and that midazolam seems to bind directly to Trp-279. Furthermore, the computer simulations suggest a mechanism for inactivation of TRH-R1 signaling by midazolam. Midazolam binding enhances the interactions between residue(s) in transmembrane helices 5 and 6, thus strengthening the constraints holding transmembrane helices 5 and 6 together and leading to a decrease in the distance between the cytoplasmic ends of these helices. In that movement of transmembrane helix 5 away from transmembrane helix 6 seems to be a component of the process of TRH-R1 activation (Colson et al., 1998a), increases in the contacts between transmembrane helices 5 and 6 would decrease the distance between the cytoplasmic ends of transmembrane helices 5 and 6 that prevents activation. This hypothesis is consistent with the general mechanism proposed for 7TMR activation based on structural studies of rhodopsin (Cai et al., 1999; Okada et al., 2001) in which movement (or rotation) of transmembrane helix 6 away from transmembrane helix 2, 3, and 5 "opens" the site on the rhodopsin cytoplasmic surface that interacts with transducin.

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References

Borhan B, Souto ML, Imai H, Shichida Y, and Nakanishi K (2000) Movement of retinal along the visual transduction path. *Science (Wash DC)* **288:**2209–2212.

Brooks BR, Bruccoleri RE, Olafson BD, Statea DJ, Swaminathan S, and Karplus M (1983) CHARMM: a program for macromolecular energy, minimization and dynamics calculations. *J Comp Chem* **4:**187–217.

Cai K, Klein-Seetharaman J, Hwa J, Hubbell WL, and Khorana HG (1999) Structure and function in rhodopsin: effects of disulfide cross-links in the cytoplasmic face of rhodopsin on transducin activation and phosphorylation by rhodopsin kinase. *Biochemistry* **38:**12893–12898.

Colson AO, Perlman JH, Jinsi-Parimoo A, Nussenzveig DR, Osman R, and Gershengorn MC (1998a) A hydrophobic cluster between transmembrane helices 5 and 6 constrains the thyrotropin-releasing hormone receptor in an inactive conformation. *Mol Pharmacol* **54:**968–978.

Colson AO, Perlman JH, Smolyar A, Gershengorn MC, and Osman R (1998b) Static and dynamic roles of extracellular loops in G-protein-coupled receptors: a mechanism for sequential binding of thyrotropin-releasing hormone to its receptor. *Biophys J* **74:**1087–1100.

Dolan EA, Venable RM, Pastor RW, and Brooks BR (2002) Simulations of membranes and other interfacial systems using P2(1) and Pc periodic boundary conditions. *Biophys J* **82:**2317–2325.

Du P, Salon JA, Tamm JA, Hou C, Cui W, Walker MW, Adham N, Dhanoa DS, Islam I, Vaysse PJ, et al. (1997) Modeling the G-protein-coupled neuropeptide Y Y1 receptor agonist and antagonist binding sites. *Protein Eng* **10:**109–117.

Ebrey T, Tsuda M, Sassenrath G, West JL, and Waddell WH (1980) Light activation of bovine rod phosphodiesterase by non-physiological visual pigments. *FEBS Lett* **116:**217–219.

Gao ZG, Chen A, Barak D, Kim SK, Muller CE, and Jacobson KA (2002) Identification by site-directed mutagenesis of residues involved in ligand recognition and activation of the human A3 adenosine receptor. *J Biol Chem* **277:**19056–19063.

Gershengorn MC and Osman R (1996) Molecular and cellular biology of thyrotropin-releasing hormone receptors. *Physiol Rev* **76:**175–191.

Gershengorn MC and Osman R (2001) Minireview: insights into G protein-coupled receptor function using molecular models. *Endocrinology* **142:**2–10.

Han B and Tashjian AH Jr (1995) Identification of Asn289 as a ligand binding site in the rat thyrotropin-releasing hormone (THR) receptor as determined by complementary modifications in the ligand and receptor: a new model for THR binding. *Biochemistry* **34:**13412–13422.

Huang W (2003) Agonist-induced conformational changes in TRH receptor: A disulfide cross-linking and molecular modeling approach. Doctoral thesis, Cornell University, New York.

Jinsi-Parimoo A and Gershengorn MC (1997) Constitutive activity of native thyrotropin-releasing hormone receptors revealed using a protein *kinase C-responsive* reporter gene. *Endocrinology* **138:**1471–1475.

Joubert L, Claeysen S, Sebben M, Bessis AS, Clark RD, Martin RS, Bockaert J, and Dumuis A (2002) A 5-HT4 receptor transmembrane network implicated in the activity of inverse agonists but not agonists. *J Biol Chem* **277:**25502–25511.

Laakkonen LJ, Guarnieri F, Perlman JH, Gershengorn MC, and Osman R (1996) A refined model of the thyrotropin-releasing hormone (TRH) receptor binding pocket. Novel mixed mode Monte Carlo/stochastic dynamics simulations of the complex between TRH and TRH receptor. *Biochemistry* **35:**7651–7663.

Lin SW and Sakmar TP (1996) Specific tryptophan UV-absorbance changes are probes of the transition of rhodopsin to its active state. *Biochemistry* **35:**11149–11159.

Lu ZL, Saldanha JW, and Hulme EC (2002) Seven-transmembrane receptors: crystals clarify. *Trends Pharmacol Sci* **23:**140–146.

Mackerell AD Jr, Bashford D, Bellot M, Dunbrack RL, Evanseck JD, Field MJ, Fischer S, Gao J, Guo H, Ha S, et al. (1998) All-atom emperical potential for molecular modeling and dynamics studies of proteins. *J Phys Chem B* **102:**3586–3616.

Marie J, Richard E, Pruneau D, Paquet JL, Siatka C, Larguier R, Ponce C, Vassault P, Groblewski T, Maigret B, et al. (2001) Control of conformational equilibria in the human B2 bradykinin receptor. Modeling of nonpeptidic ligand action and comparison to the rhodopsin structure. *J Biol Chem* **276:**41100–41111.

Okada T, Ernst OP, Palczewski K, and Hofmann KP (2001) Activation of rhodopsin: new insights from structural and biochemical studies. *Trends Biochem Sci* **26:**318–324.

Osman R, Colson A-O, Perlman JH, Laakkonen LJ, and Gershengorn MC (1999) Mapping binding sites for peptide G protein-coupled receptors: The receptor for Thyrotropin-releasing hormone, in *Structure-Function Analysis of G Protein-Coupled Receptors* (Wess J ed) pp 59–84, John Wiley & Sons, New York.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, et al. (2000) Crystal structure of rhodopsin: a G protein-coupled receptor. *Science (Wash DC)* **289:**739–745.

Perlman JH, Colson AO, Jain R, Czyzewski B, Cohen LA, Osman R, and Gershengorn MC (1997) Role of the extracellular loops of the thyrotropin-releasing hormone receptor: evidence for an initial interaction with thyrotropin-releasing hormone. *Biochemistry* **36:**15670–15676.

Perlman JH, Laakkonen L, Osman R, and Gershengorn MC (1994a) A model of the thyrotropin-releasing hormone (TRH) receptor binding pocket. Evidence for a second direct interaction between transmembrane helix 3 and TRH. *J Biol Chem* **269:**23383–23386.

Perlman JH, Laakkonen LJ, Guarnieri F, Osman R, and Gershengorn MC (1996) A refined model of the thyrotropin-releasing hormone (TRH) receptor binding pocket. Experimental analysis and energy minimization of the complex between TRH and TRH receptor. *Biochemistry* **35:**7643–7650.

Perlman JH, Nussenzveig DR, Osman R, and Gershengorn MC (1992) Thyrotropin-releasing hormone binding to the mouse pituitary receptor does not involve ionic interactions. A model for neutral peptide binding to G protein-coupled receptors. *J Biol Chem* **267:**24413–24417.

Perlman JH, Thaw CN, Laakkonen L, Bowers CY, Osman R, and Gershengorn MC (1994b) Hydrogen bonding interaction of thyrotropin-releasing hormone (TRH) with transmembrane tyrosine 106 of the TRH receptor. *J Biol Chem* **269:**1610–1613.

Robbins AK and Horlick RA (1998) Macrophage scavenger receptor confers an adherent phenotype to cells in culture. *Biotechniques* **25:**240–244.

Sun Y and Gershengorn MC (2002) Correlation between basal signaling and internalization of thyrotropin-releasing hormone receptors: evidence for involvement of similar receptor conformations. *Endocrinology* **143:**2886–2892.

Wang W and Gershengorn MC (1999) Rat TRH receptor type 2 exhibits higher basal signaling activity than TRH receptor type 1. *Endocrinology* **140:**4916–4919.

Woolf TB and Roux B (1994) Molecular dynamics simulation of the gramicidin channel in a phospholipid bilayer. *Proc Natl Acad Sci USA* **91:**11631–11635.

Woolf TB and Roux B (1996) Structure, energetics and dynamics of lipid-protein interactions: A molecular dynamics study of the gramicidin A channel in a DMPC bilayer. *Proteins* **24:**92–114.

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