Functional Microdomains in G-protein-coupled Receptors

因为在第三转膜区的G蛋白偶联受体中，所有视蛋白-视黄酸结合的视蛋白受体是必需的，以确保有效的信号转导。这种Arg在促性腺激素释放激素-催乳素受体中与GnRH，Hls，或Lys失活，或严重抑制考虑刺激性不溶性磷酸盐生成，一致

Arg对活性状态的贡献。为了调查活性状态的介导和互变的结构域在作用于保守Arg的整合结构域模型和突变的

Arg侧链在活性状态被认为是必须的。在无活性的野生型受体中，Arg侧链被提议形成与Asp 

"The conservedarginine-cage motif in the gonadotropin-releasing hormone receptor*

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An Arg present in the third transmembrane domain of all rhodopsin-like G-protein-coupled receptors is required for efficient signal transduction. Mutation of this Arg in the gonadotropin-releasing hormone receptor to Gln, His, or Lys abolished or severely impaired agonist-stimulated inositol phosphate generation, consistent with Arg having a role in receptor activation. To investigate the contribution of the surrounding structural domain in the actions of the conserved Arg, an integrated microdomain modeling and mutagenesis approach has been utilized. Two conserved residues that constrain the Arg side chain to a limited number of conformations have been identified. In the inactive wild-type receptor, the Arg side chain is proposed to form an ionic interaction with Asp 

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To overcome the limitations inherent in both site-directed mutagenesis and computational modeling, we have integrated mutational studies and the application of computational techniques to the study of structural motifs in the receptor that may constitute functional microdomains. The inferences from studies of these microdomains, whose proposed structure can be substantiated by experimental data, are then evaluated in the context of a whole receptor model. This approach facilitates the elucidation of a structural basis for the phenotypes induced by site-directed mutagenesis. The effect of mutations is tested first in the microdomain models and correlated with the functional effects of site-directed mutant receptors expressed in mammalian cells. Using this approach, we have recently mapped precise interactions in segments of the binding pocket of the serotonin 5HT2A receptor (19).

In the present report, we have applied this approach to study the interaction pattern of the conserved Arg in TMD 3 in the GnRH receptor. The conserved arginine Arg3.50 (see “Experimental Procedures” for focus numbering scheme) has been implicated in the activation of various GPCRs by mutagenesis studies (20, 21) and by computational modeling (21, 22). An understanding of the molecular basis for the functional role of this Arg requires identification of those residues whose specific interactions determine its orientation within the structure of the receptor. Given the great conformational flexibility of the Arg side chain, it is likely that such orienting residues would form a three-dimensional motif to which we refer as the arginine cage.

Specific partners for Arg3.50 have been proposed, such as the conserved Asp in TMD 2 (Asp3.49) (21, 22), based on the rationale of a similar conservation pattern and the need to neutralize a positive charge in a low dielectric environment. However, a complete exploration of the conformational space of Arg3.50 in the context of a full molecular model of the receptor is not attainable with present computational techniques, and several other candidate interacting residues can be proposed. In particular, analysis of the conservation pattern centered on Arg3.50 identifies highly conserved residues that could influence the conformation of Arg3.50. These residues form the consensus sequence (I/L)XXDRYXx(I/V) (Fig. 1). Arg3.50, AspGlu3.49, and IleVal3.54 are present in all cloned GPCRs belonging to the rhodopsin family, with the exception of the platelet-activating factor receptor, which has an asparagine residue at position 3.49 (23). In an orthosteric environment, the conservation pattern described above forms an envelope of conserved residues surrounding Arg3.50, consisting in the GnRH receptor of Ile3.46, Asp3.49, and Ile3.54 (13) (Fig. 2).

To evaluate the role of these various conserved residues in caging the Arg3.50 side chain in the inactive and active forms of the receptor, we have performed a complete conformational exploration of TMD 3 using Monte Carlo simulations (15). The helical structure and the helix ends of TMD 3 have been experimentally substantiated by Cys scanning of the D2 receptor (24) and by spin-labeling studies of rhodopsin, in which the membrane/aqueous interface has been located between residues 3.52 and 3.53 (25). For the Monte Carlo simulations, a novel biphasic solvent model has been developed that reproduces the interface between the interior of a protein and a water environment. Using this Monte Carlo approach, the inferences about the possible caging interactions involving the conserved Arg have been tested by evaluating computationally the structural effects of mutations in this TMD 3 domain model and correlating these results with the functional effects of site-directed mutagenesis. These studies provide insight into the role of Arg3.50(139) in sustaining a pattern of interactions that may occur in the active and inactive forms of the receptor.

**EXPERIMENTAL PROCEDURES**

**Residue Numbering Scheme**—The residues in TMDs are numbered relative to the most conserved residue contained in the helix, as explained previously (14). On the basis of this scheme, the most conserved residue in TMD 3, Arg-139, is designated with the index number 3.50 and is hence referred to throughout as Arg3.50(139); the identification of the preceding Asp is Asp3.49(138).

**Computational Methods**—The residue of TMD 3 from Cys3.25 to Thr3.44 was modeled as an a-helix. These helix boundaries, predicted from homology studies described elsewhere (14), are consistent with recent experimental results for other GPCRs (24, 25). The environment surrounding the Arg residue in TMD 3 has been investigated in rhodopsin and found to consist of two distinct phases: a water phase and a membrane-embedded helix (25). The environment of Arg3.50(139) in the helix was assumed to be similar to the environment of residues buried inside the protein interior, consant with studies on the known structure of the photosynthetic reaction center (26), and has been modeled by a distance-dependent dielectric. To simulate the biphasic environment in our calculations, the novel mixed solvent model includes a water phase and a distance-dependent dielectric phase. The boundary between the two phases consists of a plane parallel to the membrane. TMD 3 was initially positioned perpendicular to the membrane plane at the midpoint between residues 3.52 and 3.53 following experimental observations (23) but was allowed to move ±2 Å vertically in the direction normal to the membrane plane to prevent arbitrary effects arising from the initial positioning.

To explore the conformational space available for the cytoplasmic portion of helix 3, Monte Carlo simulations were performed by varying the dihedral angles for residues Val3.44 (133) to Thr3.54 (144). The variation of backbone dihedral angles for ψ and ϕ was restrained to ±20° from their initial values. Side chain dihedral angles were rotated freely. Extensive simulations are necessary to reach convergence of the resulting conformations described by the rotamers we analyzed (see below for a description of the four consecutive dihedral angles defining the rotamers). Thus, between 100 and 400 rounds of independent random simulations were performed for each TMD 3 construct. In each round, repeated runs of Monte Carlo-simulated annealing were performed from a starting temperature of T = 2070 K, with a cooling schedule of T → 0.9 × T0 and 10,000 steps per temperature to reach 310 K. Between 19 and 76 million conformations were thus sampled for each wild-type and mutant construct. Analysis of the resulting conformations was performed at T = 310 K and restricted to backbone conformations within ±10° from their initial values to maintain an a-helical conformation. The conformations of the side chains, in particular the Arg3.50(139) side chain, are defined by the corresponding dihedral angles χ1, χ2, χ3, χ4. These dihedral angles are classified according to three main rotamers: gauche plus (g+) centered on −60° (encompassing angle values between −120° and 0°), gauche minus (g−) centered on +60° (between 0° and 120°), and trans (t) centered on 180° (between 120° and −120°). Evaluation of the preferred conformations was performed by analyzing the populations of each side chain rotamer. The rotamer state of the Arg side chain is defined by the state of each one of its four dihedral angles, e.g. the propensity of each dihedral angle (χ1, χ2, χ3, χ4) to adopt the (g+, g−, t) configuration. There are 51 possible Arg side chain rotamers, which were grouped according to their spatial orientation toward specific residues in TMD 3. The spatial orientation of each Arg3.50(139) rotamer was inferred from the average values of each of the four
The Arg-cage microdomain resulting from this conformational analysis was positioned in the context of a complete model of the transmembrane helix bundle of the GnRH receptor (9), constructed to follow the electron microscopy projection map of rhodopsin (10) using methodological steps and approaches described in detail elsewhere (14).

DNA Constructs and Transfection—Procedures for site-directed mutagenesis of the GnRH receptor, subcloning of the receptor coding region into pcDNA1/Amp and transient receptor expression have been described previously (27). COS-1 cells transfected with plasmid DNA were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. One day after transfection, COS-1 cells were split from 100-mm plates into two 12-well plates for the functional assay or into two 24-well plates for the whole-cell binding assay. Mutations of Asp3.49(138) and Arg3.50(139) were done in the mouse GnRH receptor, whereas mutations of Ile3.46(135) and Ile3.54(143) were generated in the human GnRH receptor.

Binding and Functional Assays—Binding of GnRH to the wild-type and mutant receptors was measured at 4 °C in a whole-cell agonist competition binding assay 72 h after transfection (13).125I-GnRH-A and mutant receptors was measured at 4 °C in a whole-cell agonist competition binding assay or into two 24-well plates for the whole-cell binding assay. Mutations of Asp3.49(138) and Arg3.50(139) were done in the mouse GnRH receptor, whereas mutations of Ile3.46(135) and Ile3.54(143) were generated in the human GnRH receptor.

Receptor Efficiency—We have developed an empirical representation for receptor efficiency (Q) using operational models of occupancy and response (see Equations 1 and 2),

\[ Q = \frac{[AR]/B_{\text{max}}}{1 + K_e/A} \]

\[ E/E_{\text{max}} = 1 + EC_{50}/[A] \]

where [AR] represents the concentration of ligand-receptor complex, [A] is the concentration of free ligand, and \( E_{\text{max}} \) and \( B_{\text{max}} \) represent the maximal response and maximal binding, respectively. Receptor efficiency (Q) represents the quanta functional response achieved per agonist-occupied receptor. To allow comparison of the various mutant receptors and to accommodate the influence of spare receptors on EC_{50} values (27), we define receptor efficiency as (see Equation 3),

\[ Q = E/[AR] \]

In calculating Q, \( K_e \) has been used as an estimate of \( K_p \). The receptor efficiency values obtained for the various receptor constructs are expressed relative to the wild-type receptor value.

RESULTS

Computational Simulations of TMD 3 Segment Surrounding Arg^{3.50(139)}

The GPCRs demonstrate a pattern of conservation among several residues that are in spatial proximity to Arg^{3.50(139)} when the cytoplasmic side of TMD 3 is modeled as a regular \( \alpha \)-helix (see Fig. 2). These potential local sites of interaction for Arg^{3.50} include the large hydrophobic residue (Ile/VaI/Leu) at position 3.46, the acidic residue at position 3.49, the Tyr (Ser in the GnRH receptor) at position 3.51, and the \( \beta \)-branched large hydrophobic residue (Ile/Val) at position 3.54.

The interaction patterns and rotamer positioning of Arg^{3.50(139)} with respect to these neighboring TMD 3 residues were explored with Monte Carlo simulations for the wild-type helix and for various mutant receptors. Many conformations of the flexible Arg side chain were not attainable due to steric clashes with the helix backbone. For example, all Arg rotamers whose \( \chi_1 = g \) are unpopulated because of a clash between the Arg \( \gamma \)-methyl and the backbone carbonyl of the preceding turn of the helix (30).

The most striking observation to emerge from the simulations is the tendency for Arg^{3.50(139)} to form an ionic bond with Asp^{3.49(138)} as illustrated in Fig. 2B. Nearly half of the Arg^{3.50(139)} rotamers observed were bound to this aspartic acid (Table I). As shown in Fig. 3, a variety of Arg conformations were identified that form the Arg-Asp interaction. Most other side chains remained in their original orientations throughout the simulations, consistent with their preferred rotamer populations in known \( \alpha \)-helical structures. Ser and Thr residues were overwhelmingly (92–99% of rotamers) in the \( \gamma \)-helical structures. Ser and Thr residues were overwhelmingly (92–99% of rotamers) in the \( \gamma \)-helical structures. Ser and Thr residues were overwhelmingly (92–99% of rotamers) in the \( \gamma \)-helical structures. Ser and Thr residues were overwhelmingly (92–99% of rotamers) in the \( \gamma \)-helical structures.
in different receptors. For the simulations, we substituted Ile \(^{3.46(135)}\) with Ala, Val, and Leu to test 1) the functional relevance of the wild-type side chain (by substituting Ala), 2) the role of the β-branched character of the residue (by substituting Val), and 3) the effect of the large hydrophobic side chain (by substituting Leu). Monte Carlo simulations of all three mutant constructs showed that the conformational preferences of the Arg side chain were similar to those found in the wild-type receptor. Therefore, according to our results, Ile \(^{3.46(135)}\) does not modulate the orientation of Arg \(^{3.50(139)}\).

**Asp\(^{3.49(138)}\)—**Monte Carlo simulations were performed for a mutation of the conserved acidic residue to Asn. By neutralizing the charge at this locus, this Asp \(^{3.49(138)}\) → Asn mutant would weaken the ionic bond between Arg \(^{3.50(139)}\) and Asp \(^{3.49(138)}\) and Asp \(^{3.49(138)}\) observed in simulations of the wild-type receptor. This mutation was found to significantly affect the conformational preferences of the Arg side chain (see Table I). In the Asp \(^{3.49(138)}\) → Asn mutant, the Arg side chain rarely interacts with the 3.49 locus (3% of rotamers). Two new orientations appear populated, as shown in Fig. 4. 1) Arg \(^{3.50(139)}\) is oriented toward positions 3.47–3.51 where it can H-bond to Ser \(^{3.47(136)}\) and Ser \(^{3.51(140)}\) (37% of rotamers). 2) Arg \(^{3.50(139)}\) is oriented toward positions 3.53–3.54 where it can be solvated by water at the cytoplasmic boundaries. Because activation of rhodopsin has been shown to involve a proton uptake by Glu \(^{3.49(138)}\) (32, 33), simulations were also performed for the protonated form of the aspartic acid, termed Asp \(^{3.49(138)-H}\) → Asp-H. The results yielded a pattern of preferred conformations very similar to that of Asp \(^{3.49(138)}\) observed in simulations of the wild-type receptor.

**Ser\(^{3.51(140)}\)—**A Tyr residue most commonly occurs at this position in other GPCRs. We tested the functional role of this residue with the mutant construct Ser \(^{3.51(140)}\) → Ala. Hydrogen-bonding between Arg \(^{3.50(139)}\) and Ser \(^{3.51(140)}\) was observed in the Asp \(^{3.49(138)}\) → Asn mutant receptor, but the mutation Ser \(^{3.51(140)}\) → Ala did not change significantly the orientation of the Arg side chain relative to the wild-type construct (data not shown). Evidently, this H-bond is not energetically competitive with the ionic bond Arg \(^{3.50(139)}\)-Asp \(^{3.49(138)}\). Ile \(^{3.54(143)}\)—Only Ile or Val residues appear in GPCR sequences at this position. Therefore, this locus always contains a bulky β-branched, hydrophobic side chain. To test the structural implications of these properties, we substituted this residue by Val, Leu, and Ala. The Val side chain displays similar structural features as isoleucine, being hydrophobic, bulky, and β-branched. Leu is hydrophobic and bulky but has a γ-branched side chain. Ala is hydrophobic but neither bulky nor branched. Analysis of all rotamers populated over 5%, shown in Table II, indicates that the prevailing interaction for the wild-type receptor and the three mutants is still the ionic bond between Arg \(^{3.50(139)}\) and Asp \(^{3.49(138)}\) (65–80% of rotamers). Although maintaining the same interaction, the individual rotamer conformations preferred for this interaction vary among the mutants and with respect to the wild-type receptor (Table II). Of note, a novel orientation for the Arg side chain toward residues 3.53–3.54 appears significantly populated in the Ile \(^{3.54(143)}\) → Ala mutant (14.3%) but not in the wild-type, Ile \(^{3.54(143)}\) → Val, or Ile \(^{3.54(143)}\) → Leu mutants. In the Ile \(^{3.54(143)}\) → Ala mutant, the Arg side chain can be positioned toward the aqueous cytoplasm, as illustrated in Fig. 5.

**Radioligand Binding and Agonist-stimulated Inositol Phosphate Accumulation by the Wild-type and Mutant Receptors**—To correlate the predicted local structural roles of the conserved TMD 3 residues with their effect on receptor function, constructs obtained from the mutation of Arg \(^{3.50(139)}\) and its surrounding conserved residues (see Fig. 2) were tested for their effects on ligand binding and inositol phosphate accumulation (Table III). The mutation of the Asp \(^{3.49(138)}\), Arg \(^{3.50(139)}\), and Ser \(^{3.51(140)}\) loci were carried out on the mouse GnRH receptor, whereas the Ile \(^{3.54(143)}\) and Ile \(^{3.54(143)}\) mutants were generated in the human GnRH receptor. Both human and mouse GnRH receptors have identical sequences in the TMD 3 segment studied. The amino acid substitutions were designed to test the side chain property conserved at each locus and/or a specific functional hypothesis derived from the modeling studies. The results of the radioligand binding and phosphatidylinositol assay and the relative coupling efficiencies of the various receptor constructs are summarized in Table III.

**Ile \(^{3.46(135)}\)—**Removal of the Ile side chain by the Ile \(^{3.46(135)}\) → Ala mutation abolished binding and activation. Substitution by another β-branched residue Ile \(^{3.46(135)}\) → Val also eliminated detectable ligand binding and signal transduction. In contrast, the Ile \(^{3.46(135)}\) → Leu receptor manifested coupling comparable...
to that of the wild-type receptor. However, due to poor expression of the Ile3.46(135) → Leu receptor (13% of wild-type receptor  
B_{\text{max}}), the calculated value for receptor efficiency for this mutant reveals a 5-fold increase above the value obtained for the wild-type receptor (Table III). The affinity of this mutant construct for GnRH is comparable to the wild-type receptor. The restricted pattern of amino acid substitutions that are functionally tolerated at the 3.46(135) position is most consistent with this site being involved in helix-helix packing (see “Discussion”).

Asp3.49(138)—The Asp3.49(138) → Ala mutant had no detectable agonist binding or activation and could not be evaluated. The more conservative mutation Asp3.49(138) → Asn behaved like wild type in terms of its  
K_{d}, E_{\text{C50}}, and E_{\text{max}} values (Table III). However, the lower  
B_{\text{max}} (56% relative to wild type) suggests that this construct has a modestly enhanced signaling efficiency.

Arg3.50(139)—Mutations of Arg3.50(139) to His and Lys yielded constructs with no detectable binding or activation. The Arg3.50(139) → Gln mutant expressed well and had wild-type affinity for GnRH but was very poorly coupled (Table III).

Ser3.51(140)—Consistent with the lack of structural effects observed with mutation of this locus in the computational simulations, the phenotype of the expressed Ser3.51(140) → Ala mutant receptor was similar to that of the wild-type receptor (data not shown). This finding is consistent with results reported previously (34).

DISCUSSION

Arg3.50 is absolutely conserved in all GPCRs, and its substitution in various GPCRs severely affects signal transduction (20, 21). The pattern of conservation and the effects of mutations make it likely that this side chain serves a key role in conformational changes and interactions underlying receptor activation. The results obtained with mutation of this locus in the GnRH receptor support a crucial role for this Arg in receptor function. Among the mutant receptors studied, only the construct with a Gln substitution at this site showed any detectable signal transduction. Similar results obtained by mutating the Arg3.50 in the mouse GnRH receptor have been recently reported (34).

 Oliveira et al. (22) proposed a mechanism for receptor activation in which the change in orientation of this Arg3.50 constitutes an Arg-switch leading to activation of GPCRs, a hypothesis recently expanded by a combination of mutagenesis and computational simulations on adrenergic receptors (21, 35). The present study has focused on the molecular details of the microdomains surrounding Arg3.50 by delineating the neighboring residues that interact with or restrict the position-
TABLE II
Preferred conformations of the Arg\textsuperscript{3.50(139)} side chain based on rotamer populations for the wild-type and Ile\textsuperscript{3.54(143)} mutants to Ala, Leu, and Val

| Orientation of Arg\textsuperscript{3.50} side chain | Mode of stabilization | Dihedral angles of Arg\textsuperscript{3.50} side chain | Rotamer populations |
|--------------------------------------------------|-----------------------|----------------------------------------------------------|---------------------|
| Locus 3.49 | salt bridge | \(g^+\) \(g^+\) \(g^+\) t | 2.9 | 6.8 | 4.1 | 6.3 |
| Locus 3.49 | salt bridge | g\textsuperscript{+} g\textsuperscript{+} t g\textsuperscript{+} | 3.6 | 18.9 | 7.3 | 3.3 |
| Locus 3.49 | salt bridge | g\textsuperscript{+} g\textsuperscript{+} g\textsuperscript{+} t | 3.5 | 9.9 | 7.6 | 5.6 |
| Locus 3.49 | salt bridge | g\textsuperscript{+} g\textsuperscript{+} g\textsuperscript{+} t | 11.9 | 2.8 | 4.7 | 3.2 |
| Locus 3.49 | salt bridge | g\textsuperscript{+} g\textsuperscript{+} g\textsuperscript{+} t | 1.4 | 0.0 | 6.9 | 0.5 |
| Locus 3.49 | salt bridge | g\textsuperscript{+} g\textsuperscript{+} g\textsuperscript{+} t | 11.1 | 5.8 | 3.3 | 5.5 |
| Locus 3.49 | salt bridge | g\textsuperscript{+} g\textsuperscript{+} g\textsuperscript{+} t | 0.2 | 0.5 | 5.2 | 1.2 |
| Locus 3.49 | salt bridge | t g\textsuperscript{+} g\textsuperscript{+} g\textsuperscript{+} t | 3.8 | 2.3 | 4.2 | 6.7 |
| Locus 3.49 | salt bridge | t g\textsuperscript{+} g\textsuperscript{+} g\textsuperscript{+} t | 4.3 | 0.9 | 8.4 | 6.5 |
| Locus 3.49 | salt bridge | t g\textsuperscript{+} g\textsuperscript{+} g\textsuperscript{+} t | 6.3 | 3.1 | 5.5 | 4.5 |
| Locus 3.49 | salt bridge | t g\textsuperscript{+} g\textsuperscript{+} g\textsuperscript{+} t | 7.9 | 12.5 | 19.5 | 15.5 |
| Locus 3.49 | salt bridge | t g\textsuperscript{+} g\textsuperscript{+} g\textsuperscript{+} t | 8.3 | 3.0 | 3.7 | 16.4 |
| Total rotamer populations with side chain of residue Arg\textsuperscript{3.50} oriented toward locus 3.49 | | | 65.2 | 66.5 | 80.4 | 75.2 |

| Locus 3.54 | H\textsubscript{2}O solvation | t g\textsuperscript{+} t | 0.0 | 12.7 | 0.0 | 0.0 |
| Total rotamer populations with side chain of residue Arg\textsuperscript{3.50} oriented toward locus 3.54 | | | 3.2 | 14.3 | 3.1 | 5.3 |

Fig. 5. \(R^{3.50(139)}\) orientations in the \(I^{3.54(143)}\)A construct. Three-dimensional model of TMD 3 of the GnRH receptor illustrating the preferred conformations of the Arg side chain (purple) in the Ile\textsuperscript{3.54(143)} construct. Conserved residues are highlighted by thicker bonds. Most of the populated rotamer conformations are oriented toward Asp\textsuperscript{3.49(138)}, as observed for the wild-type (Fig. 4), driven by an ionic bond between Arg\textsuperscript{3.50(139)} and Asp\textsuperscript{3.49(138)}. However, in this construct, Arg\textsuperscript{3.50(139)} is also significantly oriented toward Ala\textsuperscript{3.54(143)} (12.7%), where it would be solvated in the aqueous cytoplasm.

TABLE III
Binding and activation of wild-type and mutant GnRH receptors expressed in COS-1 cells

| Constructs | Competition binding | Phosphatidylinositol assay | Relative receptor efficiency |
|------------|---------------------|---------------------------|----------------------------|
|            | \(B_{\text{max}}\) | \(K_{\text{GnRH}}\) | \(E_{\text{max}}\) | \(E_{\text{IC}_{50}}\) |                      |
| h wild type | 100 | 1.4±0.2 | 100 | 0.3±0.1 | 1 |
| Ile\textsuperscript{3.54(143)}Ala | 109±9 | 6.8±1.5 | 69±10 | 9.5±1.3 | 0.2 |
| Ile\textsuperscript{3.54(143)}Leu | 25±4 | 3.5±1.8 | 66±10 | 0.8±0.3 | 2.5 |
| Ile\textsuperscript{3.54(143)}Val | 64±8 | 1.9±1.2 | 70±7 | 0.3±0.1 | 1.4 |
| Ile\textsuperscript{3.50(143)}Ala | u | u | u | u |                      |
| Ile\textsuperscript{3.50(143)}Leu | 13±1 | 3.2±1.5 | 96±22 | 1.2±0.1 | 4.8 |
| Ile\textsuperscript{3.50(143)}Val | u | u | u | u |                      |
| m wild type | 100 | 1.1±0.4 | 100 | 0.4±0.2 | 1 |
| Asp\textsuperscript{3.49(138)}Ala | u | u | u | u |                      |
| Asp\textsuperscript{3.49(138)}Asn | 56±5 | 1.4±0.4 | 98±6 | 0.3±0.1 | 2.9 |
| Arg\textsuperscript{3.50(139)}His | u | u | u | u |                      |
| Arg\textsuperscript{3.50(143)}Lys | u | u | u | u |                      |
| Arg\textsuperscript{3.54(143)}Gln | 559±112 | 8.9±1.0 | 68±7 | 65.2±7 | 0.04 |

The \(K_{\text{GnRH}}\) and \(B_{\text{max}}\) values are obtained from competition binding assays (mean ± S.E. from 3–5 experiments). The \(B_{\text{max}}\) and \(E_{\text{IC}_{50}}\) values are obtained from the phosphatidylinositol hydrolysis experiments (mean ± S.E. from 3–5 experiments). Basal activity was unchanged for all constructs. The mutations of Asp\textsuperscript{3.49(138)} and Arg\textsuperscript{3.50(139)} were generated in the mouse (m) GnRH receptor, whereas the mutations of Ile\textsuperscript{3.54(143)} and Ile\textsuperscript{3.46(135)} were made in the human (h) GnRH receptor. u, undetectable.

The role of all potential Arg-cage side chains studied will be discussed separately based on the results from the computational simulations and the measured properties of the mutant constructs. Asp\textsuperscript{3.49(138)}—Computational experiments indicate that the propensity to form an ionic bond between Arg\textsuperscript{3.50(139)} and Asp\textsuperscript{3.49(138)} constrains the orientation of the Arg side chain. This constraint is relieved when the simulations are carried out for the Asp\textsuperscript{3.49(138)} → Asn mutant or for a protonated Asp\textsuperscript{3.49(138)}. The results of mutagenesis in the GnRH receptor and in other receptors suggest that the interaction between Arg\textsuperscript{3.50} and Asp\textsuperscript{3.49} stabilizes the inactive receptor state. In the

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α-adrenergic receptor, the mutation Asp3.49 -> Asn leads to constitutive activation of the receptor (21, 35). We find that mutation of Asp3.49 -> Asn leads to a modest increase in the efficiency of GnRH receptor activation similar to the reported result of the Glu3.49 -> Gln mutation in rhodopsin (33).

In rhodopsin, receptor activation is accompanied by the uptake of two protons at cytoplasmic sites (32). One site of uptake has been identified as Glu3.49, based on the lack of proton uptake by the Glu3.49 -> Gln mutant (32). Thus neutralization of the 3.49 locus by either protonation or mutation favors the activated form of the receptor. To rationalize these results in the structural context of the proposed Arg3.50 - Asp3.49 interaction, we performed the relevant computational experiment of protonating Asp3.49(138) in the GnRH receptor. In the Monte Carlo simulations, neutralization of the acidic group at position 3.49 either abolished (Asp3.49 -> Asp) or dramatically decreased (Asp3.49 -> Asp-H) the orientation of the Arg side chain toward the 3.49 locus (Table I). Based on these experimental and computational findings, we hypothesize that the conserved Arg side chain is held by Asp3.49(138) through an ionic bond in the inactive state of the receptor and that receptor activation involves the release of this constraint on the Arg3.50(139) side chain conformation, most likely by protonation of Asp3.49.

Ile3.54(143) - This locus shows a 100% conservation profile as a β-branched, bulky hydrophobic residue (Ile or Val). Experimentally, the Ile3.54(143) -> Val and the Ile3.54(143) -> Leu mutants were similar or more efficient than the wild-type receptor. The Ile3.54(143) -> Ala mutant was inefficient in mediating signal transduction and displayed a much lower receptor efficiency than the wild-type receptor. In simulations, the preferred orientations of the Arg side chain in the Ile3.54(143) -> Val and the Ile3.54(143) -> Leu mutants were similar to those observed in the wild-type receptor. However, in the Ile3.54(143) -> Ala mutant, a new orientation of the Arg side chain was significantly populated (14.3%, Table II). Analysis of the new rotamer conformations that are populated after mutation of Ile3.54(143) -> Ala provides a rationale for the observed phenotype. The bulky side chain of an Ile, Leu, or Val residue at this position would clash with the Arg side chain when this adopts the (t, g, t) rotamer configuration oriented toward the C terminus or cytoplasmic boundary of TMD 3 but clashes with Ile3.54(143). Note that in this conformation Arg3.50 reaches the membrane-cytoplasm interface (white line), as determined for rhodopsin (25), and could thus become solvated.

Fig. 6. Proposed role of Ile3.54(143) in caging Arg3.50(139) through a steric clash. Three-dimensional model of TMD 3 of the GnRH receptor where R3.50(139) attempts to adopt the (t, g, t, g) rotamer configuration oriented toward the C terminus or cytoplasmic boundary of TMD 3 but clashes with Ile3.54(143). This locus shows a 100% conservation profile as a β-branched, bulky hydrophobic residue (Ile or Val). Experiments show that mutation of Ile3.54(143) to the Ala or Val residue at this position would clash with the Arg side chain when this adopts the (t, g, t, g) rotamer, as shown in Fig. 6. In contrast, an Ala at this position lacking the bulky side chain would allow this unfavorable conformation of the Arg residue, as can be seen in Fig. 5. According to the membrane-water boundary determined experimentally for rhodopsin and located between residues 3.52 and 3.53, the (t, g, t, g) rotamer of the Arg would orient the charged guanidinium group toward the aqueous cytoplasm. The strong solvation of the charged Arg side chain would inhibit it from further participation in any intramolecular interactions. Consequently, the results suggest that the structural role of Ile3.54 is to restrict the positioning of Arg3.50 during receptor activation. In the absence of a bulky side chain at this position, the solvation of Arg3.50 in the cytoplasm may prevent it from forming the interactions most conducive to establishing an active receptor state.

Ile3.46(135) - In the simulations, the wild-type receptor and Ile3.46(135) mutant constructs showed similar orientations of the Arg side chain and the surrounding residues. Mutagenesis experiments show that mutation of Ile3.46(135) to either Ala or Val is not tolerated, whereas substitution by Leu shows increased receptor efficiency as compared with the wild-type receptor. Both the lack of a local helix 3 effect of mutation of this locus in computational experiments and the highly restricted pattern of functionally tolerated mutations suggest

![Fig. 7. Schematic representation of the human GnRH receptor showing the position of Asp or Glu residues within the TMD (black circles), relative to Arg3.50 (black circle) and the Arg-cage residues studied (shaded). Modeling in three dimensions indicates that Arg3.50 cannot interact with Asp2.61 or Glu2.53 in TMD 2, based on inferences from an engineered zinc-binding site between TMD 2 and TMD 3 (positions 2.64 and 3.28) (34). Aspartic acids Asp7.49 and Asp3.49 are in position to interact with Arg3.50. Asn2.50 is emphasized because it is a conserved Asp residue in most GPCRs and is proposed to interact with Arg3.50 when Asp7.49 is replaced by an Asn in these receptors.](image-url)
that Ile3.46(135) does not form a part of the Arg-cage motif and may have a role in interhelical interactions not considered in this study.

Ser3.51(140)—Although there is a possible H-bonding interaction between Arg3.50(139) and Ser3.51(140), the interaction energy is not competitive with the strong Arg3.49(138)-Asp3.49(138) interaction that predominates in the wild-type receptor. Removal of the H-bonding group of Ser3.51(140) by substitution to Ala produced no detectable alteration of the functional properties of the GnRH receptor (32), suggesting that Arg3.50(139) does not interact with this site or that the energetic contribution of such an interaction to the receptor activation mechanism is not significant.

Mechanistic Hypothesis for the Transition from an Inactive to an Active State of the Receptor—The implications of the patterns of interactions (e.g. Arg3.50(139)-Asp2.50(87)) and preferred conformations of the Arg3.50(139) side chain derived from conformational analysis on TMD 3 alone were further analyzed in the context of a seven TMD model of the receptor. Because the strongest interaction of Arg3.50 is an ionic bond, we explored whether an alternative charge counterpart (Asp/Glu) could be found within other TMDs. The Asp or Glu residues present within the transmembrane domains of the GnRH receptor are shown schematically in Fig. 7. An acidic counterpart for the Arg would be expected to share its high degree of conservation and to reside at the cytoplasmic side of a TMD. However, the only conserved acidic group in the TMDs of the GnRH receptor is the Asp2.50(87) in the TMD 3 studied here. There are three nonconserved acidic residues present in the TMDs of the GnRH receptor at positions Glu2.53(90) and Asp2.61(98) in TMD 2 and Asp7.49(319) in TMD 7 (Fig. 7). An interaction of Arg3.50(139) with Glu2.53(90) or Asp2.61(98) is inconsistent with the geometrical constraints of an engineered Zn2+ binding site reported recently between TMD 2 and TMD 3 for the NK-1 receptor (36). Therefore these interaction possibilities were excluded. An interaction of Arg3.50(139) with the nonconserved Asp7.49(319) is possible in the complete model of the transmembrane portion of the GnRH receptor.

Notably, Asp7.49(319) forms part of a pair of conserved residues for which modeling and double mutant studies suggest an involvement in GPCR activation (8, 9). An unusual feature of the GnRH receptor is the presence of an Asn at position 2.50 in TMD 2 (Fig. 7) where nearly all other rhodopsin family GPCRs have an Asp. On the other hand, Asp7.49(319) in the GnRH receptor is an Asn in most GPCRs. This apparent interexchange of conserved residues suggests that these residues may interact, a hypothesis supported by molecular modeling and double mutation studies of both the GnRH receptor (9) and the serotonin 5-HT2A receptor (8). These results imply that Asp7.49(319) in the GnRH receptor substitutes functionally for the conserved Asp normally found at the 2.50 locus in other GPCRs and is therefore a suitable charged counterpart for Arg3.50 in terms of their high conservation profile. We suggest that one function of Asp7.49 in the GnRH receptor is to interact with Arg3.50 in the active state of the receptor. Exploring this possibility in the context of a model of the GnRH receptor supports an interchangeable role of Asp7.49(319) and Asn2.50(87) in the interaction with the Arg residue in TMD 3, as shown in Fig. 8A. The Arg side chain is capable of extending from TMD 3 toward TMD 2 and TMD 7, where it can interact simultaneously with Asp7.49(319) and Asn2.50(87). We hypothesize that during receptor activation, Asp7.49 becomes protonated, and Asp2.50 substitutes for Asp7.49 in forming an ionic interaction with Arg3.50.

In most GPCRs, the active state interaction would occur with the Arg3.50-Asp2.50 bond as shown in Fig. 8B. Several models of GPCRs have proposed an interaction of Arg2.50 with the Asp2.50-Asn2.50 locus (33, 20) but associate this interaction with the inactive state of the receptor. However, our data and results from other GPCRs described below support the role of this interaction with the conserved Asp/Asn pair at the 2.50–7.49 loci in stabilizing the active, not the inactive, receptor conformation. Thus, such a hypothesis is consistent with the finding in many GPCRs that mutations eliminating the charged character of Asp2.50, which would be expected to destabilize the proposed active-state Arg-Asp interaction, either abolish or significantly decrease receptor activity (37). Furthermore, spectroscopic experiments in rhodopsin showing that Asp2.50 is more strongly H-bonded upon activation (38), are also consistent with the proposed interaction between Arg3.50 and Asp2.50 in the active state.

In summary, the experimental and computational results suggest that the orientation of the highly conserved Arg3.50(139) side chain is constrained in the inactive receptor state by an ionic interaction with the neighboring conserved residue Asp7.49(319). During activation, Asp7.49 becomes protonated, and the Arg side chain is released. The conserved bulky side chain of Ile3.54(143) modulates the positioning of the Arg side chain by keeping it away from the cytoplasmic aqueous medium and thereby promotes the interaction with Asp7.49 (Asp2.50 in other GPCRs) that characterizes the active state of the receptor. Further studies of other structural motifs that have the role of functional microdomains will allow refinement of these proposed molecular events underlying receptor activation.
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