A Conserved Aromatic Lock for the Tryptophan Rotameric Switch in TM-VI of Seven-transmembrane Receptors*" 

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The conserved tryptophan in position 13 of TM-VI (Trp-VI:13 or Trp-6.48) of the CWXP motif located at the bottom of the main ligand-binding pocket in TM-VI is believed to function as a rotameric microswitch in the activation process of seven-transmembrane (7TM) receptors. Molecular dynamics simulations in rhodopsin demonstrated that rotation around the chi torsion angle of Trp-VI:13 brings its side chain close to the equally highly conserved Phe-V:13 (Phe-5.47) in TM-V. In the ghrelin receptor, engineering of high affinity metal-ion sites between these positions confirmed their close spatial proximity. Mutational analysis was performed in the ghrelin receptor with multiple substitutions and with Ala substitutions in GPR119, GPR39, and the β2-adrenergic receptor as well as the NK1 receptor. In all of these cases, it was found that mutation of the Trp-VI:13 rotameric switch itself eliminated the constitutive signaling and strongly impaired agonist-induced signaling without affecting agonist affinity and potency. Ala substitution of Phe-V:13, the presumed interaction partner for Trp-VI:13, also in all cases impaired both the constitutive and the agonist-induced receptor signaling, but not to the same degree as observed in the constructs where Trp-VI:13 itself was mutated, but again without affecting agonist potency. In a proposed active receptor conformation generated by molecular simulations, where the extracellular segment of TM-VI is tilted inwards in the main ligand-binding pocket, Trp-VI:13 could rotate into a position where it obtained an ideal aromatic-aromatic interaction with Phe-V:13. It is concluded that Phe-V:13 can serve as an aromatic lock for the proposed active conformation of the Trp-VI:13 rotameric switch, being involved in the global movement of TM-V and TM-VI in 7TM receptor activation.

Despite the fact that the large superfamily of 7TM or G protein-coupled receptors are activated by agonists of incredibly different chemical nature, it is believed that they nevertheless all share a common molecular activation mechanism (1–3). A series of biochemical and biophysical studies indicate that receptor activation is associated with relatively large overall changes in the arrangement of the seven-helical bundle of transmembrane segments (4, 5). This notion has been gathered in a unifying “global toggle switch” activation model describing how in particular TM-VI performs a “vertical” see-saw movement around a pivot in the middle of the membrane during activation (2). Thus, the extracellular segment of TM-VI is supposed to tilt into the main ligand-binding pocket, whereas the intracellular segment tilts outward, away from the receptor center and thereby allows binding of the active form of the G protein. However, changes in the relative conformation of TM-V and -VII are also supposed to be important parts of the activation process in which the conserved proline residues in the middle of the transmembrane segments are involved, as in TM-VI (2, 6). Recently, the x-ray structure of opsin in complex with a peptide fragment of the corresponding G protein has confirmed such large rearrangements of at least the intracellular parts of the transmembrane helices upon G protein binding (7, 8).

It has been known for decades that certain residues or motifs in the transmembrane segments are highly conserved among rhodopsin-like 7TM receptors (e.g. the so-called DRY motif at the intracellular end of TM-III and the CWXP motif in the middle of TM-VI). It was expected that these conserved residues would have functionally important roles in the activation process. The new x-ray structures of 7TM receptors indicate that some of these residues do function as microswitches (i.e. residues that swap between significantly different conformations in the active versus the inactive state of the receptor) (9). For example, Arg-III:26 (Arg-3.50) of the DRY motif alternates between an inactive conformation, where it makes a salt bridge to the neighboring Asp-III:25, and an active conformation, where it makes a hydrogen bond to the previously rather unnoticed but highly conserved Tyr-V:24. In the active state, Arg-III:26 also makes important contacts with the C-terminal part of the G protein peptide (6–8). Tyr-VII:20 of the NPXXY motif at the intracellular end of TM-VII also appears to function as a microswitch, which apparently alternates between not two but...
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three different states (i.e. an inactive state, where the phenol side-chain of Tyr-VII:20 makes a close aromatic stacking with Phe-VIII:04 of the intracellular helix VIII; an intermediate state, where it makes a hydrogen bond to a structural water molecule of the hydrogen bond network between TM-I, -II, -VI, and -VII; and an active conformation, where Tyr-VII:20 is involved in a hydrophobic cluster stabilizing the active, outward tilted conformation of TM-VI (7–9).

The x-ray structures have, however, far from revealed all secrets of the presumed microswitches in 7TM receptors. Thus, Trp-VI:13 (Trp-6.48) of the CWXP motif, which is located in the middle of the receptor at the bottom of the main ligand-binding pocket, is generally expected to function as a key microswitch in the 7TM activation mechanism (2, 10–16). Surprisingly (and in contrast to the other microswitches), Trp-VI:13 is found in an almost identical position and conformation in all x-ray structures of 7TM receptors as yet published (i.e. including the supposedly active structure of opsin in complex with the C-terminal Ga peptide) (7, 8, 17–20). However, spectroscopic studies, including solid phase NMR analysis, strongly indicate that the side chain of Trp-VI:13 must change position and interaction partners during receptor activation (10, 12–14, 21). Thus, Trp-VI:13 is supposed to exchange between its two preferred rotamer states, g+ and trans; and, because the proline-induced bend or kink in the backbone of TM-VI is located at the position of Trp-VI:13, it is presumed that rotation of the indole side chain of Trp-VI:13 around its chi1 angle would somehow be associated with the global conformational change that occurs in TM-VI during receptor activation (9, 10, 13). The side chain of Trp-VI:13 is expected to swing away from its presumed inactive, “vertical” position between TM-III, TM-VI, and TM-VII, corresponding to the g+ conformation of its chi1 angle, to a presumed active “horizontal” position, where it instead is facing TM-V, corresponding to the trans conformation of the chi1 angle. We suggest that this presumed active conformation of Trp-VI:13 is stabilized by an aromatic interaction with Phe-V:13, which has been equally well conserved during evolution as Trp-VI:13 (Fig. 1).

In the present study, we probe the importance of Trp-VI:13 in particular the role played by Phe-V:13 as a presumed aromatic “lock” for the active rotamer form of Trp-VI:13 both in relation to constitutive and agonist-induced signaling. We use the highly constitutively active and robust ghrelin receptor as the main 7TM model system. We supplement this with the related, also constitutively active GPR39 receptor, the highly constitutively active GPR119 receptor, the native and constitutively active mutant (CAM) variants of the β2-adrenergic receptor (B2AR), and the NK1 receptor, which in respect to ligand-independent signaling is silent. In the absence of an x-ray structure showing an active 7TM receptor conformation, we use molecular modeling and molecular simulations in combination with metal ion site engineering to study the proposed proximity of the involved residues.

4 The chi1 rotamer is defined as being in the trans (t) position, when the heavy atom at the γ-position is opposite the backbone nitrogen (viewed from β-carbon to α-carbon). Similarly, the chi1 rotamer is designated to be in the gauche minus (g−) position when the γ-atom is opposite the backbone carbon and gauche plus (g+) when opposite the α-hydrogen.

EXPERIMENTAL PROCEDURES

Material—The ghrelin and substance P peptides were purchased from Bachem (Bubendorf, Switzerland). Ar231453 was kindly provided by Lisbeth Elster (7TM Pharma).

Molecular Expression—Receptor cDNAs were cloned into the eukaryotic expression vector pCMV-Tag(2B) (Stratagene, La Jolla, CA) for epitope tagging of the proteins with a FLAG tag. Mutations were constructed by PCR using the overlap extension method as previously described (22). The PCR products were digested with the appropriate restriction endonucleases (BamHI and EcoRI), purified, and cloned into the pCMV-Tag(2B) vector. All PCR experiments were performed using Pfu polymerase (Stratagene, La Jolla, CA) according to the instructions of the manufacturer. All mutations were verified by restriction endonuclease mapping and subsequent DNA sequence analysis using an ABI 310 automated sequencer. The CAM mutation of the β2-adrenergic receptor carries the following substitutions in the C-terminal part of the third intracellular loop: L266S, K267R, H269K, and L272A.

Transfections and Tissue Culture—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium 1885 supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/ml gentamicin. Cells were transfected using the calcium phosphate precipitation method with chloroquine addition as previously described (23). The amount of cDNA (20 μg/75 cm2) resulting in maximal basal signaling was used for the dose-response curves.

Phosphatidylinositol Turnover Assay—One day after transfection, COS-7 cells were incubated for 24 h with 5 μCi of [3H]myo-inositol (catalog number PT6–271, Amersham Biosciences) in 1 ml of medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/ml gentamicin per well. Cells were washed twice in 20 mM HEPES buffer, pH 7.4, supplemented with 140 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM CaCl2, 10 mM glucose, 0.05% (w/v) fetal bovine serum, and were incubated in 0.5 ml of buffer supplemented with 10 mM LiCl at 37 °C for 30 min. After stimulation with various concentrations of ligands at 37 °C, cells were extracted with 10 mM formic acid followed by incubation on ice for 30 min. The resulting supernatant was purified on Bio-Rad AG 1-X8 anion exchange resin to isolate the negatively charged inositol phosphates. After application of the cell extract to the column, the columns were washed twice with GPI buffer (60 mM sodium formate and 100 mM formic acid) to remove glycerophosphoinositol. Inositol phosphates were eluted by the addition of elution buffer (1 mM ammonium formate, 100 mM formic acid). Determinations were made in duplicates. The columns containing AG 1-X8 anion exchange resin were regenerated by the addition of 3 ml of regeneration buffer (3 mM ammonium formate, 100 mM formic acid) and 5 ml of water.

cAMP Assay—One day after transfection, COS-7 cells (2.5 × 103 cells/well) were incubated for 24 h with 2 μCi of [3H]adenine (catalog number TRK 311, Amersham Biosciences) in 1 ml of Dulbecco’s 1885 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/ml gentamicin. Cells were washed twice and incubated for 15 min at 37 °C in 1 ml of freshly prepared binding buffer supplemented with 1 mM isobutylmethylxanthine (catalog number 15879, Sigma) and...
after incubation, cells were placed on ice, medium was removed, and cells were lysed with 1 ml of 5% (w/v) trichloroacetic acid, supplemented with 0.1 mM cAMP and 0.1 mM ATP, for 30 min. The lysis mixtures were loaded onto a Dowex 50W-X4 resin (catalog number 142-1351, Bio-Rad) column (poly-prep columns, catalog number 731-1550, Bio-Rad), which was washed with 2 ml of water and placed on top of alumina columns (catalog number A9003, Sigma) and washed again with 10 ml of water. The columns were eluted with 6 ml of 0.1 M imidazole (catalog number 10125, Sigma) into 15 ml of Optiphase Highsafe scintillator (Wallac, Boston, MA). Columns were reused up to 10 times. Dowex columns were regenerated by adding 10 ml of 2 N HCl followed by 10 ml of water; the alumina columns were regenerated by adding 2 ml of 1 M imidazole, 10 ml of 0.1 M imidazole, and finally 5 ml of water. Determinations were made in duplicates or triplicates.

**Competition Binding Assays**—Transfected COS-7 cells were transferred to culture plates 1 day after transfection at a density of ~5,000 cells per well, aiming at 5–8% binding of the radioactive ligand. Two days after transfection competition, binding experiments were performed for 3 h at 4°C using 25 pM of 35S-MK-677 (provided by Andrew Howard (Merck)). Binding assays were performed in 0.1 ml of a 50 mM Hepes buffer, pH 7.4, supplemented with 1 mM CaCl2, 5 mM MgCl2, 0.1% (w/v) bovine serum albumin, and 40 µg/ml bacitracin. Nonspecific binding was determined as the binding in the presence of 1 µM unlabeled ghrelin. Cells were washed twice in 0.1 ml of ice-cold buffer, 50 µl of lysis buffer/scintillation fluid (30% ethoxylated alkylphenol and 70% diisopropyl naphthalene isomers) was added, and the bound radioactivity was counted. Determinations were made in triplicate. Initial experiments showed that steady state binding was reached with the radioactive ligand under these conditions.

**Cell Surface Expression Measurement (ELISA)**—Cells were transfected and seeded out in parallel with various concentrations of ligands or with 50 µM forskolin. After incubation, cells were placed on ice, medium was removed, and cells were lysed with 1 ml of 5% (w/v) trichloroacetic acid, supplemented with 0.1 mM cAMP and 0.1 mM ATP, for 30 min. The lysis mixtures were loaded onto a Dowex 50W-X4 resin (catalog number 142-1351, Bio-Rad) column (poly-prep columns, catalog number 731-1550, Bio-Rad), with those used for immunoprecipitation or cAMP accumulation assays. The cells were washed twice, fixed, and incubated in blocking solution (phosphate-buffered saline plus 0.2% dry milk) for 60 min at room temperature. Cells were kept at room temperature for all subsequent steps. Cells were incubated for 2 h with anti-FLAG (M2) antibody (Sigma) in a 1:300 dilution. After three washes, cells were incubated with anti-mouse
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horseradish peroxidase (Amersham Biosciences)-conjugated antibody at a 1:4000 dilution. After extensive washing the immunoreactivity was revealed by the addition of horseradish peroxidase substrate according to the manufacturer’s instructions.

Calculations—IC₅₀ and EC₅₀ values were determined by nonlinear regression using the Prism 3.0 software (GraphPad Software, San Diego). The basal constitutive activity is expressed as a percentage of the ghrelin-induced activation for each mutant construct of the ghrelin receptor.

Molecular Dynamics Simulations—The methods used for both NAMD and Monte Carlo simulations are described only briefly here but in detail in the supplemental material.

NAMD Simulations—NAMD simulations were performed using either the 2.4 Å x-ray structure of B2AR (Protein Data Bank code 2RH1) (18, 24) or the 2.65 Å x-ray structure of bovine rhodopsin (Protein Data Bank code 1GZM) (25) as a starting structure where the carazolol and 11-cis-retinal ligands were removed. The receptors were manually inserted into a hexagonal membrane surrounded by water, and structural water molecules from the x-ray structures were included. All calculations were carried out with the NAMD simulation package (26) using the CHARMM27 force field (27). Simulations were generally performed for 8 or 20 ns.

Monte Carlo Simulations—Monte Carlo simulations were performed on the B2AR (Protein Data Bank code 2RH1) structure in a setup similar to that used for the NAMD simulation but with Phe-VI:17 rotated away from the binding pocket to its other preferred conformation to allow for free movement of Phe-V:13 and Trp-VI:13. The model of the active structure of B2AR was generated by a Monte Carlo simulated annealing protocol using distance constraints applied by the nuclear Overhauser effect functionality of CHARMM corresponding to Cβ-Cβ nuclear Overhauser effect distance constraints between positions III:08, VI:16, and VII:06 based on the activating metal cation site engineered into B2AR (28), as described for the CXCRI3 receptor (29). The distance constraints for the intracellular segments of the transmembrane helices were based on the extensive double electron-electron resonance spectroscopy analysis of double spin-labeled rhodopsin (30) (see the supplemental material for details). Distance constraints were also applied between Phe-V:13 and Trp-VI:13 to ensure aromatic stacking of the two side chains (31). To allow for TM-V, TM-VI, and TM-VII to change helical kink conformation, ϕ, ψ backbone torsion angles of Trp-VI:13, which are responsible for the pronounced kink in TM-VI, were allowed to be perturbed to obtain energetically favorable conformations to stabilize the helix locally; similarly, the backbone ϕ, ψ torsion angles from Val-VII:11 to Phe-VII:15 were allowed to be perturbed in TM-VII, and the backbone ϕ, ψ torsion angles of Phe-V:13 were allowed to be perturbed in TM-V. The CHARMM program package and the CHARMM27 force field (27) were used to simulate the activation mechanism, and the Monte Carlo command (MOVE ADD) in CHARMM was used to construct the move sets (32). During the simulation protocol, perturbations were applied to translations, rotations, and torsional degrees of freedom for the defined move sets. The backbone of TM-I, -II, -III, and -IV were held fixed throughout the entire simulation. To allow for the TM-V, TM-VI, and TM-VII to move compared with each other, perturbation of the backbone ϕ, ψ angles of the ICL2 (residues 226–270) and ECL2 (residues 298–309) and the loop between TM-VII and helix VIII and helix VIII was allowed (residue 329–342). Perturbation of all side chains was allowed. Additional side-chain perturbation move sets were added for Trp-VI:13, Phe-V:13, and Phe-VI:17 in order for these residues to move more frequently than the other side chains in the receptor.

RESULTS

Molecular Dynamics Simulation of the Trp-VI:13 Rotamer Switch—Molecular dynamics simulations were performed with either rhodopsin (Protein Data Bank code 1GZM) or B2AR (Protein Data Bank code 2RH1) in a hexagonal simulation box with the receptors each placed in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine membrane surrounded by water on both sides, as described under “Experimental Procedures.” It should be noted that this type and length (up to 20 ns) of molecular dynamics simulations are particularly suited to studying side-chain movements but not, for example, large scale helical movements. The inverse agonist ligands, 11-cis-retinal and carazolol, were in most instances removed to allow Trp-VI:13 to move as freely as possible.

In rhodopsin with the inverse agonist, 11-cis-retinal still present in the structure, Trp-VI:13 (as expected) did not change rotamer state, as illustrated by its very stable chi1 angle observed throughout 8 ns of molecular dynamics simulation (supplemental Fig. 1). However, the removal of 11-cis-retinal allowed Trp-VI:13 to change rotamer state during the molecular dynamics simulations with fluctuations of up to 80° in its chi1 angle from a starting position of −70° to approximately −150°, a rotamer state that was only visited occasionally (Fig. 2a). In the retinal-free rhodopsin structure, Phe-V:13 also probed various rotameric states during the molecular dynamics simulations. In the simulation shown in Fig. 2, the side chains of Trp-V:13 and Phe-V:13 established a relatively close interaction after ~9 ns of molecular dynamics simulation, albeit with variable geometry (Fig. 2b). As shown in Fig. 2c, Trp-VI:13 and Phe-V:13 occasionally did form ideal aromatic stacking interactions, which only lasted for a short period in the retinal-free rhodopsin structure (Fig. 2a).

During similar molecular dynamics simulations of the B2AR, after removal of the inverse agonist carazolol, Trp-VI:13 was surprisingly stable, with fluctuations in its chi1 angle of only up to approximately ±15° around the starting position of −80°. This pattern was rather similar to the limited fluctuations in the chi1 angle of Trp-VI:13 observed in rhodopsin before removal of 11-cis-retinal (supplemental Fig. 1). The stability of the indole side chain of Trp-VI:13 in B2AR appeared to be related to its hydrogen bond interaction with a structural water molecule, which was stabilized by the side chains of Asp-II:10 and Ser-VII:13, of which the latter is not present in rhodopsin (supplemental Fig. 1b).

These molecular dynamics simulations indicate that upon removal of the inverse agonist ligand, Trp-VI:13 can (at least in rhodopsin) rotate into a conformation where its side chain is in rather close proximity to the equally highly conserved Phe-V:
However, in the overall inactive state of the receptor, this interaction appears to be far from ideal and stable.

Probing the Proximity of Trp-VI:13 to Phe-V:13 by Metal Ion Site Engineering—At the most extracellular ends of TM-V and TM-VI, we have previously constructed inhibitory, interhelical metal ion sites between positions V:01, V:05, and VI:24 in, for example, the NK1, the \( \delta \)-opioid, and the ghrelin receptors (33–35) (Fig. 1). In the present study, we performed metal ion site engineering 1½ to 2 helical turns “deeper” into the membrane, where a His residue is found at position VI:17 in the wild-type ghrelin receptor (Fig. 1). Substitution of Phe-V:13 or Trp-VI:13 individually with His residues in each case had only a minor effect on the affinity of Zn\(^{2+} \) as determined in competition binding experiments against the radiolabeled non-peptide agonist \( ^{35} \)S-MK677 (Table 1 and Fig. 3b). This indicates that a His residue in position V:13 or VI:13 can form a metal ion site with His-VI:17 only to a limited extent. Importantly, however, combination of the two substitutions in the FV:13H/WVI:13H double mutant increased Zn\(^{2+} \) affinity to 36 ± 7 \( \mu \)M (Table 1 and Fig. 3c). This affinity is similar to previous observations for sev-

FIGURE 2. Molecular dynamics simulations of the Trp-VI:13 rotameric switch and its interaction with Phe-V:13 in rhodopsin where 11-cis-retinal has been removed. a, chi1 torsion angle for Trp-VI:13, chi1 torsion angle for Phe-V:13, and distance between the indole group of Trp-VI:13 and the phenyl group of Phe-V:13, as measured from the center of the two aromatic rings, during 20 ns of molecular dynamics simulation of opsin. b, structures from various time points during the 20-ns molecular dynamics simulation showing the interaction between Trp-VI:13 and Phe-V:13. c, aromatic stacking of Trp-VI:13 and Phe-V:13 after 14-ns simulation.
eral bidentate metal ion sites in various receptors (33–35). This indicates that His-V:13 and His-VI:13 presumably can form a bidentate metal ion site with each other (Fig. 3c, right). The Zn\textsuperscript{2+} affinity was slightly further increased to 24 ± 7 μM by introducing a Cys residue at position V:13 (i.e. the FV:13C/WVI:13H double mutant) (Fig. 3d).

In none of the metal ion site engineered ghrelin receptor constructs did Zn\textsuperscript{2+} stimulate signal transduction as determined by inositol phosphate turnover (data not shown). Thus, the engineered metal ion binding sites between positions VI:13 and V:13 were not able to mimic the presumed aromatic-aromatic interaction between Trp-VI:13 and Phe-V:13 of the active receptor conformation. Consequently, it could be assumed that the high affinity Zn\textsuperscript{2+} binding instead would be associated with inverse agonism and/or antagonism. However, further functional analysis of the metal ion site engineered ghrelin receptor was hampered by the fact that the constitutive activity of the receptor was eliminated (see below) and that ghrelin could not activate signaling in these constructs (Table 2), despite the fact that both ghrelin and the non-peptide agonist MK677 bound with normal high affinity (Table 1 and Fig. 3).

These experiments support the notion that positions V:13 and VI:13 are in close spatial proximity (i.e. close enough to form high affinity metal ion sites when appropriately mutated with metal ion-binding residues).

**Functional Analysis of Trp-VI:13 in the Ghrelin Receptor—**

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**TABLE 1**

| Receptor construct | $B_{\text{max}}$ (fmol/10^5 cells) | $K_d$ (nM) | $K_i$ (nM) | $K_{Zn}$ (μM) |
|---------------------|------------------|----------|----------|-----------|
| Wild-type ghrelin   | 42 ± 7 (9)       | 0.34 ± 0.05 (9) | 0.75 ± 0.06 (9) | 340 ± 120 (4) |
| FV:13A              | 50 ± 19 (3)      | 2.5 ± 0.3 (3)  | 0.66 ± 0.27 (3) | ND        |
| FV:13H              | 58 ± 6 (3)       | 0.87 ± 0.09 (3) | 1.1 ± 0.4 (4) | 120 ± 30 (3) |
| FV:13Y              | 38 ± 15 (3)      | 0.43 ± 0.18 (3) | 0.22 ± 0.05 (3) | ND       |
| FV:13C              | 85 ± 33 (3)      | 2.7 ± 0.8 (3)  | 0.30 ± 0.01 (3) | ND       |
| WVI:13A             | 200 ± 54 (3)     | 0.77 ± 0.15 (3) | 0.71 ± 0.11 (3) | ND       |
| WVI:13H             | 155 ± 47 (4)     | 1.4 ± 0.1 (4)  | 1.4 ± 0.6 (5)  | 140 ± 30 (3) |
| FV:13H/WVI:13H      | 15 ± 5 (4)       | 0.40 ± 0.17 (4) | 0.72 ± 0.17 (3) | 37 ± 7 (3) |
| FV:13C/WVI:13H      | 65 ± 26 (3)      | 0.52 ± 0.21 (3) | 1.8 ± 0.4 (3) | 24 ± 7 (3) |

The EC₅₀ for ghrelin was increased by ~10-fold from 0.34 nM in the wild-type receptor to 3.2 nM in the WVI:13A mutant. An imidazole could not substitute for the indole side chain of Trp-VI:13 because the molecular pharmacological phenotype of the WVI:13H mutant was rather similar to that of the WVI:13A mutant form (i.e. including a totally eliminated constitutive activity) (Table 2). It is concluded that Trp-VI:13 is essential for the constitutive signaling of the ghrelin receptor and that it is also important for the agonist-induced signaling but, importantly, not for agonist binding as such.

**Functional Analysis of His-VI:17 in the Ghrelin Receptor—**

In its presumed active rotameric form, Trp-VI:13 could potentially interact with either Phe-V:13 or His-VI:17 (Fig. 1). In position VI:17 of 7TM receptors, one often finds either a His residue (29%), as in the ghrelin and many other peptide receptors, or a Phe residue (20%). Removal of the side chain of His-VI:17 by Ala substitution decreased the constitutive activity of the ghrelin receptor to 39% of that of the wild type (Fig. 5a, right). However, both the potency and the maximal efficacy of ghrelin was unaltered by the His-VI:17 → Ala substitution (Fig. 5a and Table 2). It is concluded that His-VI:17 to some extent could be involved in the activation process (i.e. in particular the ligand-independent, basal signaling activity) but that this is not a critical residue because it is not required for agonist activation of the receptor.

**Functional Analysis of Phe-V:13 in the Ghrelin Receptor—**

Phe-V:13 in the ghrelin receptor was substituted with Ala, His, Tyr, or Cys, and all mutants were well expressed at the cell surface (Fig. 5c). As was observed with substitution of the Trp-VI:13 rotameric switch itself, Ala substitution of Phe-V:13 totally eliminated the constitutive signaling of the ghrelin receptor (Fig. 5a, left). In the FV:13A construct, ghrelin stimulated signaling with normal potency but with reduced $E_{\text{max}}$. Although Tyr is found at position V:13 in 10% of 7TM receptors, the phenol could not in the ghrelin receptor substitute for the phenyl side chain of Phe-V:13 because the molecular pharmacological phenotype of the Tyr-V:13 construct was similar to the Ala-V:13 construct, albeit with a measurable but low degree of constitutive signaling (Fig. 5b, middle). Similarly, the introduction of His or Cys at position V:13 eliminated or seriously decreased the constitutive signaling and reduced the $E_{\text{max}}$ for ghrelin (Fig. 5b, right and left). The binding affinity of the endogenous peptide agonist ghrelin was not seriously affected by these substitutions at position V:13 (Table 1). It is concluded that Phe-V:13 is essential for the constitutive activity of the
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The constructs were expressed in transiently transfected COS-7 cells. The efficacy data on basal activity and maximal response ($E_{\text{max}}$) are expressed as a percentage of the basal activity on the wild-type ghrelin receptor. Values are shown $\pm$ S.E. Values for $n$ are shown in parentheses.

| Basal activity | $E_{\text{max}}$ | $E_{\text{max}}$ |
|---------------|------------------|-----------------|
| Wild-type ghrelin | 100 (57) | 250 $\pm$ 23 (57) | 0.34 $\pm$ 0.04 (57) |
| FV:13A | 2.6 $\pm$ 2.4 (6) | 170 $\pm$ 20 (6) | 1.40 $\pm$ 0.3 (6) |
| FV:13H | 4.2 $\pm$ 2.0 (4) | 87 $\pm$ 4 (3) | 2.00 $\pm$ 0.7 (4) |
| FV:13Y | 16 $\pm$ 3 (3) | 140 $\pm$ 30 (3) | 0.27 $\pm$ 0.05 (3) |
| FV:13C | 15 $\pm$ 7 (3) | 160 $\pm$ 30 (3) | 1.40 $\pm$ 0.7 (5) |
| WVI:13A | 0 (4) | 24 $\pm$ 3 (4) | 3.20 $\pm$ 0.8 (3) |
| WVI:13H | 0 (4) | 49 $\pm$ 5 (3) | 1.30 $\pm$ 0.4 (4) |
| HVI:17A | 0 (3) | 9 (3) | 290 $\pm$ 20 (3) | 0.60 $\pm$ 0.03 (3) |
| FV:13H/WVI:13H | 0 (3) | 0 (3) | $>$10.000 (3) |
| FV:13C/WVI:13H | 0 (3) | 0 (3) | $>$10.000 (3) |

In the transfected COS-7 cells of the present study, the ligand-independent signaling of the B2AR was below the detection limit. However, as shown in Fig. 6c, Ala substitution of the Trp-VI:13 rotameric switch basically eliminated the agonist-induced signaling of the B2AR, whereas Ala substitution of Phe-V:13 reduced the $E_{\text{max}}$ for pindolol\(^*\) to $\sim$25% of that observed in the wild-type receptor without affecting the agonist potency. The 15% constitutive activity of the so-called CAM variant of the B2AR was eliminated by Ala substitution of both Trp-VI:13 and Phe-V:13 (data not shown).

In the NK1 receptor, which is devoid of any constitutive signaling activity, Ala substitution of Trp-VI:13 almost eliminated substance P-induced signaling, whereas Ala substitution of Phe-V:13 impaired the $E_{\text{max}}$ but only by $\sim$40% (Fig. 6d). As in the other receptors, this impairment in signaling efficacy was not associated with impairment of agonist potency.

It is concluded that in all of the receptors addressed in the present study, Ala substitution of the Trp-VI:13 rotameric switch itself eliminates constitutive signaling and strongly

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5 Pindolol is used as an agonist to avoid confusing interference with endogenous adrenergic receptors being activated by, for example, isoproterenol.
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impairs agonist induced signaling without affecting agonist potency, where this could be measured. In the case of the proposed aromatic lock for the Trp-VI:13 microswitch, Phe-V:13, Ala substitution also in all cases impaired both the constitutive and the agonist-induced receptor signaling, but not to the same degree as observed in the constructs where Trp-VI:13 itself was mutated, but again without affecting agonist potency.

**Improved Trp-VI:13-Phe-V:13 Interaction Is Observed in a Presumed Active Receptor Conformation**—As shown in Fig. 2, although Trp-VI:13 and Phe-V:13 could reach each other during the molecular dynamics simulations, they were not able to establish a strong aromatic interaction in the inactive state of rhodopsin. However, such an interaction would occur preferentially in the active global toggle switch conformation of the receptor. Accordingly, we used Monte Carlo simulations combined with experimentally derived distance constraints to generate a model of a presumed active receptor conformation based on the B2AR high resolution x-ray structure. As described previously for rhodopsin-based models of the B2AR and the CXCR3 receptor (28, 29), distance constraints corresponding to the Cβ-Cβ distances for the activating metal ion binding sites built between positions III:08, VI:16, and VII:06 (29, 37) were used as nuclear Overhauser effect distance constraints for the proposed active conformation to encourage inward movement of the extracellular segments of TM-VI and TM-VII. For the intracellular segments, the distance constraints were based on the distance changes observed between a series of double spin-labeled rhodopsin constructs, as observed in the inactive (dark) versus the active (light) state as determined by double electron-electron resonance spectroscopy (30) (see Figs. 4 and 5).

**FIGURE 4. Mutational analysis of Trp-VI:13 in the ghrelin receptor.** a, competition binding experiment performed with 35S-MK677 as radioligand in COS-7 cells transiently transfected with either the wild-type (filled circles) or the Trp-VI:13 → Ala substituted ghrelin receptor (open circles). The inset shows cell surface expression of the receptors determined by ELISA under basal conditions. b, ghrelin-induced inositol phosphate accumulation in the wild-type ghrelin receptor and the Trp-VI:13 → Ala substituted receptor. The decrease in basal stimulation caused by Ala substitution of Trp-V:13 is indicated by a red arrow, and the decrease in ghrelin-induced maximum stimulation is indicated by a dotted red arrow.

**FIGURE 5. Mutational analysis of Phe-V:13 and His-VI:17 in the ghrelin receptor.** a, ghrelin-induced inositol phosphate accumulation of the wild-type ghrelin receptor (dashed line), the Phe-V:13 → Ala substituted receptor (black squares, left), and the His-VI:17 → Ala substituted receptor (black triangles, right) expressed as a percentage of the basal signaling in the wild-type receptor. b, ghrelin-induced inositol phosphate accumulation in three other mutant ghrelin receptors where Phe-V:13 was substituted with His (left), Tyr (middle), or Cys (right). In each of the panels, the decrease in basal stimulation caused by the mutations is indicated by a red arrow, and the decrease in ghrelin-induced maximum stimulation is indicated by a dotted red arrow. c, cell surface expression of mutant ghrelin receptors compared with wild-type receptor (open column) as measured by ELISA. All experiments were performed in transiently transfected COS-7 cells.
FIGURE 6. Mutational analysis of Trp-VI:13 and Phe-V:13 by Ala substitution in selected family A, rhodopsin-like 7TM receptors. a, the GPR119 receptor, which displays similar, high constitutive activity as the ghrelin receptor but signals through the G\(_{\alpha}\)q pathway and is activated by lipid metabolites, and the non-peptide, small molecule agonist Ar231453 (55). The Ar231453-induced cAMP response is shown in the Trp-VI:13 → Ala (filled squares, left) and in the Phe-V:13 → Ala (filled circles, right) substituted receptors as compared with wild-type GPR119 receptor (open squares and open circles). b, the GPR39 receptor displays ~25% constitutive signaling through the G\(_{\alpha}\)q signaling pathway and is activated by Zn\(^{2+}\) (56). The Zn\(^{2+}\)-induced inositol phosphate response is shown in the Phe-V:13 → Ala (filled circles, right) substituted receptor as compared with wild-type GPR39 receptor (open squares and open circles). The Trp-VI:13 → Ala mutant was poorly expressed (inset to the left), and its lack of response to Zn\(^{2+}\) is consequently not shown. c, the B2AR prototype family A 7TM receptor. The cAMP response in response to pindolol is shown in the Trp-VI:13 → Ala (filled squares, left) and in the Phe-V:13 → Ala (filled circles, right) substituted receptors as compared with wild-type B2AR (open symbols). d, the NK1 receptor, which displays undetectable constitutive activity, signals through the G\(_{\alpha}\)q signaling pathway and is activated by substance P. The substance P-induced inositol phosphate response is shown in the Trp-VI:13 → Ala (filled squares, left) and in the Phe-V:13 → Ala (filled circles, right) substituted receptors as compared with the wild-type NK1 receptor (open squares and open circles). The insets in each panel show the cell surface expression relative to wild-type receptor as determined by ELISA. All experiments were performed in transiently transfected COS-7 cells.
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In the present study, data from metal ion site engineering and molecular dynamics simulations demonstrate that Trp-VI:13 (Trp-6.48) and Phe-V:13 (Phe-5.47) are in close proximity and that rotation of Trp-VI:13 around its chi1 angle will bring the side chains in contact. Mutational analyses in the ghrelin and a number of other 7TM receptors show that Trp-VI:13 and Phe-V:13 both are important for constitutive activity and to a lesser degree for agonist-induced efficacy but not for agonist potency (i.e., the mutations apparently affect the activation mechanism and not the ligand binding). In a proposed active conformation with the extracellular segment of TM-VI tilted inward toward TM-III, the side chain of Phe-V:13 can form an ideal aromatic interaction with the side chain of Trp-VI:13 when this is rotated into its proposed active t-conformation.

**DISCUSSION**

Phe-V:13-Trp-VI:13 Interactions—Even in the inactive conformation of rhodopsin, where 11-cis-retinal was removed, the indole side chain of Trp-VI:13 can rotate around its dihedral chl1 to reach a position where it interacts with the phenyl side chain of Phe-V:13 (Fig. 2). However, the molecular dynamics simulations indicate that a stable interaction is not established in this overall inactive state of the receptor. Unfortunately, the supposedly active structure of opsin in complex with the G protein fragment cannot be used as a model for the active conformation of the extracellular part of 7TM receptors because it
is missing the agonist ligand, all-trans-retinal, and because it still has the extracellular “protein plug” arranged in an inactive-like conformation (7, 8, 10, 38–40). NMR spectroscopy indicates that the movement of the extracellular end of TM-VI is linked with a displacement of extracellular loop 2 (i.e. a change in the conformation of the protein plug) (38). In order to generate a model of an active receptor conformation, we used Monte Carlo simulations, which are particularly suited to simulate more large scale molecular dynamic excursions (32). Distance constraints from activating metal ion sites between TM-III, TM-VI, and TM-VII were applied for the extracellular segments, and constraints from Hubbell and co-workers (30) double electron-electron resonance experiments were used for the intracellular parts of the transmembrane segments. In these Monte Carlo simulations, where the extracellular segment of TM-VI is tilted into the main ligand-binding pocket, Trp-VI:13 could in its supposedly active, trans conformation make an ideal aromatic-aromatic interaction with Phe-V:13 (Fig. 7).

We suggest that such an interaction between the equally highly conserved Phe-V:13 and Trp-VI:13 is part of the structural constraint involved in stabilizing the global, active conformation in 7TM receptors. This notion is supported by the deleterious effect observed in a number of different receptors on the spontaneous, constitutive signaling upon Ala substitution of not only Trp-VI:13 (the rotamer switch itself) but also upon Ala substitution of Phe-V:13 (i.e. the proposed lock for the active conformation of Trp-VI:13) (Figs. 4 and 6). These mutations also impaired the agonist-induced maximally achieved efficacy, importantly, however, without affecting the potency of the agonist. This, indicates that mutation of Trp-VI:13 or Phe-V:13 in these receptors does not affect ligand binding but instead selectively impairs the activation mechanism as such.

In fact, even before x-ray structures of 7TM receptors were available, it was suggested by Gershengorn and co-workers (16) that Trp-VI:13 and Phe-V:13 could be part of a common aromatic cluster. Initially, they proposed that this interaction constrained the receptor in its inactive conformation because disruption of this aromatic cluster through substitutions increased the constitutive signaling of their original model receptor, TRHR1 (16). Subsequently, they found that Ala substitution of Trp-VI:13 had the opposite effect in the TRHR2 receptor, where it instead eliminated the high constitutive activity of this receptor (41), which we also observe in the present study for a number of other 7TM receptors: the ghrelin receptor, GPR39, GPR119, and the CAM form of the B2AR (Fig. 6). Similarly, the high constitutive activity of the 5HT4 receptor is eliminated by Ala substitution of Trp-VI:13 (15, 42). However, this is not the case for the CB1 receptor, in which the constitutive activity basically is unaffected by Ala substitution of Trp-VI:13 (43) and in the B2 bradykinin receptor, where Trp-VI:13 also only plays a subtle role in controlling the balance between the active and inactive state of the receptor (44). Nevertheless, it can still be concluded that in the many cases where Ala substitution of Trp-VI:13 impairs the constitutive activity of the receptor, Ala substitution of Phe-V:13 has a similar effect. This supports the notion that the conserved Phe-V:13 is an important part of the Trp-VI:13 rotamer switch function.

It was generally expected that TM-V would participate in the global 7TM activation mechanism (2, 4, 45–48), which recently was confirmed by the relatively large movement of the intracellular segment of TM-V observed in the x-ray structure of opsin in complex with the G-protein peptide (7, 8). Various biophysical studies, including fluorescence spectroscopy of the B2AR, suggest that relocation of the extracellular part of TM-V is required to facilitate binding of the agonist (45). Also, NMR studies on rhodopsin have demonstrated movements of TM-V due to steric interaction with the β-ionone ring and coupling with extracellular loop-2 displacement from the binding pocket (38, 39). The interaction of the Trp-VI:13 rotamer switch with Phe-V:13 demonstrated in the present study could very well constitute an important molecular communication between TM-VI and TM-V during the 7TM receptor activation process.

Other Interaction Partners for Trp-VI:13—Position III:12 (3.36) is found right across from Trp-VI:13 in the main ligand-binding pocket and could, depending on the side chain, consequently also influence the function of the Trp-VI:13 rotamer switch. Importantly, in contrast to position V:13, which has been conserved as either a Phe or a Tyr in 81% of 7TM receptors, no particular type of residue has been conserved at position III:12 (49) (Fig. 1). In the 5HT4 receptor, concerted rotation of the side chain of position III:12 and Trp-VI:13 has been proposed to occur to adopt the active conformation (15). Similarly, in the histamine H1 receptor, a Ser in position III:12 is important for the constitutive activity of this receptor and has been suggested to act in a similar manner (11). Although Ala substitution of Thr-III:12 impairs the constitutive activity of these receptors, the endogenous agonists (i.e. 5HT and histamine, respectively) are still able to activate the receptors with full efficacy (11, 15). The constitutive activity of the CB1 and the ghrelin receptors is also affected by removal of the side chain in position III:12 (i.e. Phe and Thr, respectively). But in these two receptors, the constitutive activity is, however, increased instead of decreased by Ala substitution (43, 50). In these cases, it could be speculated that the space-generating Ala substitution across from Trp-VI:13 in the main ligand-binding pocket could facilitate the rotation of the indole side chain into its presumed active trans conformation. Thus, it may be concluded that the residue found in position III:12 often influences the function of the Trp-VI:13 rotamer switch and receptor function but that it does so in a highly context-dependent manner.

From several of the x-ray structures, it appears that the indole side chain of Trp-VI:13 in its inactive, rotameric g+ conformation interacts with a structural water molecule, which is part of the hydrogen bond network between TM-I, TM-II, TM-VI, and TM-VII (9, 11, 18, 24, 51). Thus, polar residues in position VII: 12, which is 79% conserved as either an Asn or a Ser residue, and in particular position VII:13, which is 79% conserved as a Ser or a Cys residue, are probably indirectly involved in stabilizing the inactive state of Trp-VI:13 (11).

Blocking the Trp-VI:13 Rotamer Switch?—In rhodopsin, the inverse agonist 11-cis-retinal bends around TM-VI, interacting closely with Trp-VI:13, and thereby blocks any movement of this residue and TM-VI as such (2, 47, 52). However, in the novel x-ray structures of the β2-adrenergic, β1-adrenergic, and
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Adenosine A2a receptors, the co-crystallized antagonists/inverse agonists do not appear to have close interaction with or directly block Trp-VI:13 movement (9, 18–20, 24). It has even been argued that the fact that these compounds are not full inverse agonists could be related to their poor prevention of the Trp-VI:13 rotamer switch function (6). Importantly, however, for example, carazolol, timolol, and cyanopindolol all interact closely with the aromatic “sandwich” formed by Phe-V:13 and Phe-VI:17 (18–20, 24). This aromatic cluster blocks Trp-VI:13 from rotating into its presumed active, trans conformation, as observed in the molecular dynamics simulations of the present study.

Thus, although the antagonists/inverse agonists do not directly interact with the Trp-VI:13 rotamer switch, they apparently instead block its function indirectly by participating in an aromatic cluster between TM-V and TM-VI. Moreover, they all block the proposed inward movement of TM-VI, i.e. they block the proposed global toggle switch activation mechanism (9). It has also been proposed that partial agonism can be achieved by ligands without engaging the Trp-VI:13 rotameric switch, whereas full agonism appears to require this conformational change (6, 53).

30% of 7TM Receptors Function without Trp-VI:13—It may be somewhat surprising that Trp-VI:13, which often is considered to be an essential part of the activation mechanism for 7TM receptors, in fact only is found in 71% of these receptors (49). In 19% of the cases, position VI:13 is occupied by another aromatic residue (Phe or Tyr). However, around 10% of all 7TM receptors function with a non-aromatic residue, such as Gln, in position VI:13 (e.g. many chemokine receptors) (49).

Notably, the fact that a relatively large fraction of 7TM receptors function well without the Trp-VI:13 rotameric switch is only a problem in “sequential mode” allosteric activation models, where it would constitute a missing “domino brick” in the sequential line of intramolecular events connecting extracellular agonist binding with intracellular G protein activation (9, 11, 54). In contrast, in a Monod-Wyman-Changeux type of “concerted action” allosteric model, the Trp-VI:13 rotameric switch would just be one of several microswitches that form an extended allosteric interface between the transmembrane helices performing the global toggle switch movements that mediate the intramolecular signal transduction signal (9).

In such a model, any of the microswitches are in principle dispensable, provided that the active conformation is still stabilized by other parts of the allosteric interface. Thus, during the gradual evolutionary process, different parts of the allosteric interface could in certain receptors have been strengthened, thereby allowing other parts of the interface to be weakened. However, in the majority of receptors where a Trp residue is found in position VI:13, an experimental, “acute” substitution of this has a dramatic effect on both spontaneous and agonist-induced activation, as does substitution of its aromatic lock, Phe-V:13. Variations in the setting of the different microswitches of the allosteric interface, such as the Trp-VI:13 rotamer switch and the water hydrogen bond network, may be highly important for fine tuning the signal or be involved in controlling, for example, signal transduction pathway-biased signaling (9).

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6 We had to manually rotate Phe-VI:17 into another of its preferred conformers in order to break the Phe-V:13 to Phe-VI:17 interaction and thereby allow Trp-VI:13 to rotate into its trans conformation.
