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# Overlapping Binding Site for the Endogenous Agonist, Small-Molecule Agonists, and Ago-allosteric Modulators on the Ghrelin Receptor

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**ABSTRACT**

A library of robust ghrelin receptor mutants with single substitutions at 22 positions in the main ligand-binding pocket was employed to map binding sites for six different agonists: two peptides (the 28-amino-acid octanoylated endogenous ligand ghrelin and the hexapeptide growth hormone secretagogue GHRP-6) plus four nonpeptide agonists—the original benzolactam L-692,429 [3-amino-3-methyl-*N*-(2,3,4,5-tetrahydro-2-oxo-1-([2′-(1*H*-tetrazol-5-yl) (1,1′-biphenyl)-4-yl]methyl)-1*H*-1-benzazepin-3(*R*)-yl)-butanamide], the spiroindoline sulfonamide MK-677 [*N*-[1(*R*)-1,2-dihydro-1-ethanesulfonylspiro-3*H*-indole-3,4′-piperidin)-1′-yl]carbonyl-2-(phenylmethoxy)-ethyl-2-amino-2-methylpropanamide], and two novel oxindole derivatives, SM-130686 [(+)-6-carbamoyl-3-(2-chlorophenyl)-(2-diethylaminoethyl)-4-trifluoromethyloxindole] and SM-157740 [(±)-6-carbamoyl-3-(2,4-dichlorophenyl)-(2-diethylaminoethyl)-4-trifluoromethyloxindole)]. The strongest mutational effect with respect to decrease in potency for stimulation of inositol phosphate turnover was for all six agonists the GluIII:09-to-Gln substitution in the extracellular segment of TM-III. Likewise, all six agonists were affected by substitutions of PheVI:16, ArgVI:20, and PheVI:23 on the opposing face of transmembrane domain (TM) VI. Each of the agonists was also affected selectively by specific mutations. The mutational map of the ability of L-692,429 and GHRP-6 to act as allosteric modulators by increasing ghrelin's maximal efficacy overlapped with the common mutational map for agonism but it was not identical with the map for the agonist property of these small-molecule ligands. In molecular models, built over the inactive conformation of rhodopsin, low energy conformations of the nonpeptide agonists could be docked to satisfy many of their mutational hits. It is concluded that although each of the ligands in addition exploits other parts of the receptor, a large, common binding site for both small-molecule agonists—including ago-allosteric modulators—and the endogenous agonist is found on the opposing faces of TM-III and -VI of the ghrelin receptor.

The gastrointestinal peptide hormone ghrelin is an important regulator of appetite, energy expenditure, and acute growth hormone secretion through interaction with the ghrelin receptors located mainly in the central nervous system (Tschöp et al., 2000). From a drug discovery point of view, these functions of ghrelin qualify ghrelin receptor agonists as anabolic compounds with potentials for treatment of, for example, cachexia.

The initial agonists synthesized for the ghrelin receptor were peptides, sharing common structural features, including a central hydrophobic motif and a positive charge in the amidated C-terminal end. Despite relatively poor bioavailability and low therapeutic index/window, these peptides efficiently induced GH secretion in vitro as well as in vivo both in animal and human models (Bowers et al., 1984). GHRP-6 is a prototype for these peptides (Fig. 1). In an attempt to increase oral bioavailability, a series of nonpeptide compounds was subsequently developed. They were

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**ABBREVIATIONS:** GH, growth hormone; GHS, growth hormone secretagogue; GHRP, growth hormone releasing peptide; CGP7930, 2,6-di-tert-butyl-4-(3-hydroxy-2,2-dimethyl propyl)-phenol; MK-677, *N*-[1(*R*)-1,2-dihydro-1-ethanesulfonylspiro-3*H*-indole-3,4′-piperidin)-1′-yl]carbonyl-2-(phenylmethoxy)-ethyl-2-amino-2-methylpropanamide (L-163.191); L-692,429, 3-amino-3-methyl-*N*-(2,3,4,5-tetrahydro-2-oxo-1-([2′-(1*H*-tetrazol-5-yl) (1,1′-biphenyl)-4-yl]methyl)-1*H*-1-benzazepin-3(*R*)-yl)-butanamide (MK-751); SM-130686, (+)-6-carbamoyl-3-(2-chlorophenyl)-(2-diethylaminoethyl)-4-trifluoromethyloxindole; SM-157740, (±)-6-carbamoyl-3-(2,4-dichlorophenyl)-(2-diethylaminoethyl)-4-trifluoromethyloxindole; ELISA, enzyme-linked immunosorbent assay; TM, transmembrane; wFw, the tri-peptide D-Trp-Phe-D-Trp.

termed growth hormone secretagogues (GHS), an abbreviation used for the entire group of compounds that act through the ghrelin receptor and increases GH secretion. The initially reported nonpeptide GHS compounds were based on a central benzolactam moiety, with L-692,429 as a prototype having a potency of approximately 10 nM but an oral bioavailability of only 1 to 8% (Smith et al., 1997). However, subsequent series based on the spiropiperidine privileged structure, MK-677 being the most advanced prototype compound, exhibited favorable in vivo properties and have been investigated in several human clinical studies, including a phase II study for bone healing. A number of different series of nonpeptide GHS compounds, inspired by this pioneering work, were subsequently developed at different companies, including the highly conformationally constrained oxindole compounds from Tokunaga et al. (2001, 2005) (Fig. 1).

The ghrelin receptor is special in that it is highly constitutively active (Holst et al., 2003), and a large number of chemically very different agonists are available. We compared a selection of prototype ghrelin receptor agonists in receptor binding and signaling and discovered that the small-molecule compounds acted not only as high-potency, efficacious agonists but also—surprisingly—as allosteric modulators of ghrelin signaling (Holst et al., 2005). Thus, for example, coadministration of nanomolar concentrations of L-692,429 shifted the dose-response curve for ghrelin up to 10-fold to the left. In contrast, the GHRP-6 peptide acted as a negative allosteric modulator by decreasing the potency of the endogenous agonist 5-fold, whereas MK-677 did not affect the potency of ghrelin. It is noteworthy that all the tested small-molecule agonists acted as coagonists with ghrelin (i.e., they improved the maximal efficacy of the endogenous agonist). Although in general this dual function had not been specifically appreciated, it became clear that several partial agonists for other receptors had very similar properties, because they were allosteric modulators and coagonists on top of being agonists on their own (for example, $Zn^{2+}$ on the melanocortin melanocortin-1 and melanocortin-4 receptors and CGP7930 on the GABAb receptor) (Holst et al., 2002; Binet et al., 2004). Based on these observations, the term ago-allosteric modulators was suggested for such compounds (Schwartz and Holst, 2006; Kenakin, 2007; Schwartz and Holst, 2007). According to the basic concept and according to the official International Union of Pharmacology definition, allosteric ligands are supposed to bind to a site that is dis-

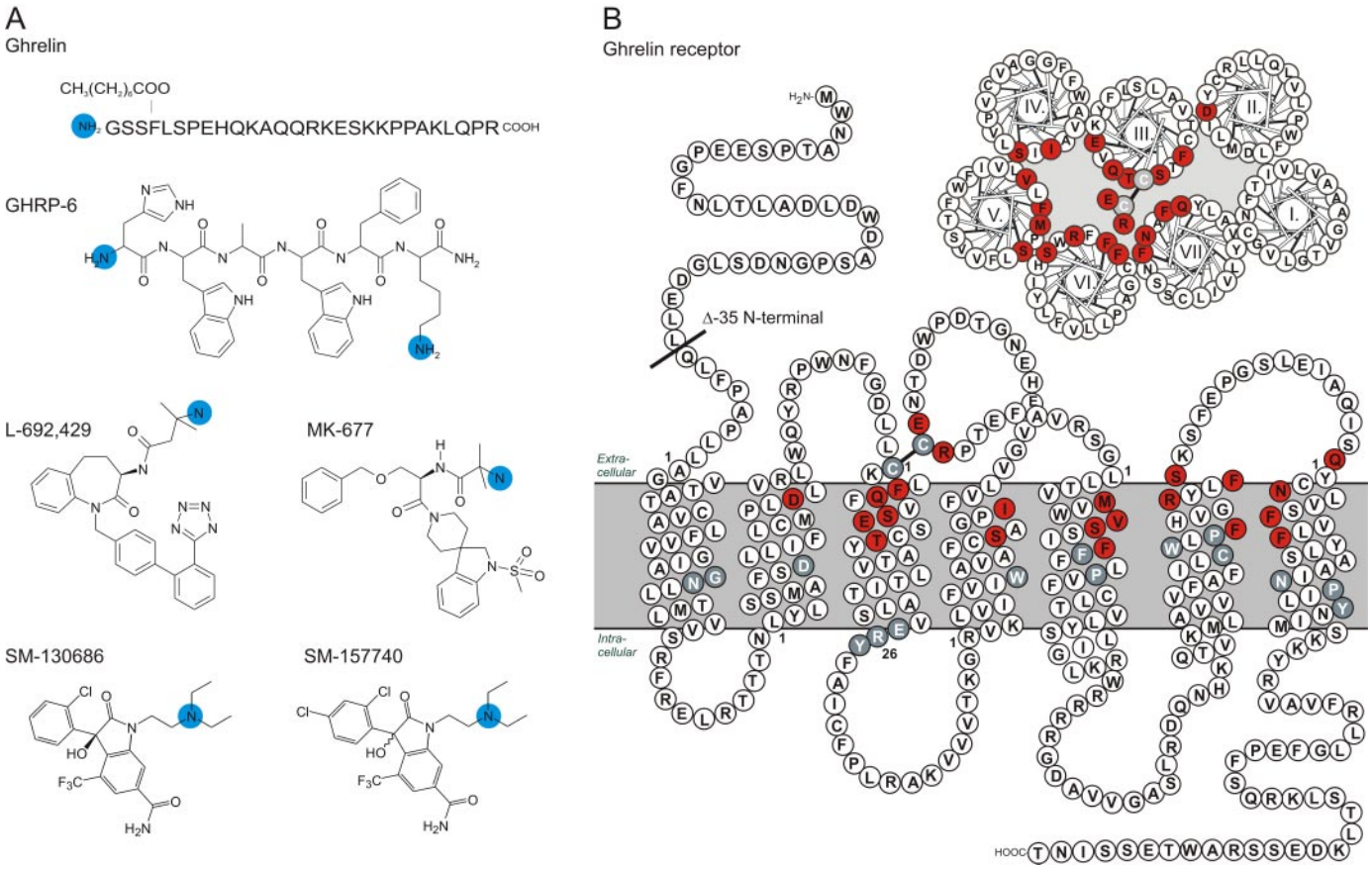


**Fig. 1.** Structure of the ghrelin receptor agonist and schematic figure of ghrelin receptor. A, the structure of the small-molecule synthetic ghrelin receptor agonist investigated in the present study: the endogenous agonist ghrelin (Kojima et al., 1999), which requires octanoylation of Ser3 for most biological effects; the original hexapeptide GH secretagogue GHRP-6 of Bowers; the initially identified nonpeptide GH secretagogue, the benzolactam L-692,429; the subsequent spiroindanylpiperidine nonpeptide GH secretagogue, MK-677, which has been in several clinical trials; and the novel oxindole derivatives SM-130686 and SM-157740. The blue shadow indicates the positive charge. B, helical wheel and serpentine diagram of the ghrelin receptor. The residues mutated in the present study are highlighted in red. Schwartz/Baldwin's generic numbering system for 7TM receptor, which is based on the actual location of the residues in each transmembrane helix, is used throughout the article. For reference, the numbering of a highly conserved residues in each helix is marked (highlighted in white on gray): AsnI:18, AspII:10, ArgIII:26, TrpIV:10, ProV:16, ProVI:15, and ProVII:17, and the proposed first residue of each transmembrane helix is indicated by "1." The position of the deletion of the amino terminal is indicated by a line, Δ-35 N-terminal.

tinct from the binding site of the endogenous agonist (Neubig et al., 2003). In the present article, we use a large library of receptor mutants combined with computational chemistry to characterize the binding site for the small-molecule agonists shown in Fig. 1. We were surprised to find a large overlap in the binding site for the endogenous ligand, ghrelin, and the binding site for the ago-allosteric modulators.

## Materials and Methods

**Material.** Ghrelin and GHRP-6 were purchased from Bachem (Bubendorf, Swicherland). The nonpeptide compounds MK-677 (L-163,191) (Patchett et al., 1995) and L-692,429 (MK-751) (Smith et al., 1993) were kindly provided by Andrew Howard (Merck Research Laboratories, Rahway, NJ). SM-130686 and SM-157740 (Tokunaga et al., 2005) were kindly provided by Ryu Nagata (Dainippon Sumitomo Pharma Co., Osaka, Japan).

**Molecular Biology.** The human ghrelin/GHS receptor cDNA was cloned by PCR from a human brain cDNA library. The cDNA was cloned into the eukaryotic expression vector pCMV-Tag(2B) made by Stratagene (La Jolla, CA) for epitope-tagging of proteins. Mutations were constructed by PCR using the overlap expression method (Holst et al., 2004). The PCR products were digested with appropriate restriction endonucleases (BamHI and EcoRI), purified, and cloned into the vector pCMV-Tag (2B). 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 automated sequencer (ABI PRISM 310; Applied Biosystems, Foster City, CA).

**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 calcium phosphate precipitation method with chloroquine addition. The amount of cDNA (20 $\mu$g/75 cm$^2$) resulting in maximal basal signaling was used for the dose-response curves.

**Phosphatidylinositol Turnover.** One day after transfection, COS-7 cells were incubated for 24 h with 5 $\mu$Ci of [*myo*-$^3$H]inositol (Amersham, Chalfont St. Giles, Buckinghamshire, UK) 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 buffer [20 mM HEPES, pH 7.4, supplemented with 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, and 0.05% (w/v) 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 peptide and/or non peptides for 45 min 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 anion exchange resin (AG 1-X8; Bio-Rad Laboratories, Hercules, CA) to isolate the negatively charged inositol phosphates. After application of the cell extract to the column, the content was washed twice with washing buffer (60 mM sodium formate and 5 mM sodium tetraborate decahydrate) to remove glycerophosphoinositol. Inositol phosphates were eluded by addition of elution buffer (1 M ammonium formate, 100 mM formic acid). Determinations were made in duplicates. The columns containing AG 1-X8 anion exchange resin were regenerated by addition of 3 ml of regeneration buffer (3 M ammonium formate and 100 mM formic acid) and 10 ml of water.

**Cell Surface Expression Measurement (ELISA).** Cells were transfected and seeded out in parallel with those used for inositol phosphate accumulation assay. The cells were washed twice, fixed, and incubated in blocking solution (phosphate-buffered saline/3% 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 Chemical Co., St. Louis, MO) in 1:300 dilution. After three washes, cells were incubated with antimouse horseradish peroxidase (Amersham) conjugated antibody in dilution 1:4000. After extensive washing, the immunoreactivity was revealed by the addition of horseradish peroxidase substrate according to manufacturer's instructions.

**Calculations.** $EC_{50}$ values were determined by nonlinear regression using the Prism 3.0 software (GraphPad Software, San Diego, CA). The basal constitutive activity is expressed as percentage of the ghrelin-induced activation for each mutant construct of the ghrelin receptor. In Tables 1 and 2, Fmut indicates the -fold shift in potency induced by the structural change in the mutated receptor compared with the wild-type receptor. Student's *t* test was used to evaluate the allosteric effect of GHRP-6 and L-692,429 in the wild-type ghrelin receptor compared with the mutated constructs.

**Computational Chemistry.** Construction of ghrelin receptor model—the primary sequence of the human ghrelin receptor GHS-R1a was obtained from the Swiss-Prot database (accession number Q92847). Homology models were constructed by MODELLER using the crystal structure of bovine rhodopsin Protein Data Bank ID 1gzm (solved to 2.65 Å resolution), obtained from the Brookhaven Protein Data Bank, as structural template (Edwards et al., 2004). Pairwise sequence alignment between GHS-R1a and bovine rhodopsin was obtained by use of the biopolymer modules within SYBYL7.3 (Tripos, St. Louis, MO), using environment-dependent substitutions tables and generic residues finger-print motifs in the 7TMs (Schwartz et al., 2006). The N-terminal domain, the extra- and intracellular loops, and the C-terminal domain were excluded. The software packaged MODELLER was configured to produce an ensemble of 10 three-dimensional comparative homology models of GHS-R1a (Marti-Renom et al., 2000). The individual receptor models were evaluated based on MODELLERs fitness score. Further analysis of accessible side-chain rotamer states for residues at the inner faces of the extracellular segments of TM-III, -IV, -V, -VI, and -VII were conducted within SYBYL1 and the Biopolymer module using the Lovel rotamer library to optimize local interactions between residue side chains. The resulting GHS-R-1a models were relaxed by subsequent minimization (in vacuo) using 1000 steepest descent minimization steps, in which backbone C$\alpha$ atoms were harmonically restrained with a force constant of 50 kcal/mol, followed by 2000 minimization steps without backbone C restrains.

Conformational search for low energy conformations of the nonpeptide compounds L-692,429, MK-677, SM-130686, and SM-157740 were performed in SYBYL using simulated annealing and clustering of minimized conformations obtained form the trajectory (data not shown). Analysis of the resulting clusters identified two possible low-energy conformations for the highly rigid SM compounds. The slightly larger L-692,429 can accommodate several conformations. However, the analysis of associated conformational energies identified only few distinct conformations that were comparable with the global minimum conformation and therefore relevant for docking. Finally, MK-677 was the most flexible compound that could adopt several possible conformations. However, the central methyleneoxy derivative adopts only one clearly favored coplanar conformation, where the oxygen seems intramolecularly hydrogen-bounded to the amide, and the compound is also stabilized by a strong dipole moment. This structural arrangement was in fact found in seven of eight phenoxyacetamide hits in a search for stereospecific methyleneoxy-based ligand fragments in Relibase, a database of experimentally determined high-resolution protein-ligand complexes (http://relibase.rutgers.edu). Consequently, the favored planar arrangement of the methyleneoxy derivative effectively decreased the conformational flexibility and reduced the number of relevant bioactive conformations for MK-677.

The identified low-energy conformations of the nonpeptide agonists were manually docked to the constructed ghrelin receptor model to maximize complementary ligand receptor interactions using the presumed major anchor point GluIII:09 as an initial interaction partner for the positively charged nitrogen moiety found in each of the compounds. The receptor ligand complex was subjected to 300 minimization steps using steepest descent and the MMFF force field.

During the minimization, only atoms within 8 Å from any agonist atom were allowed to move. The resulting root-mean-square deviation was less than 0.15 Å.

## Results

Single amino acid substitutions at 22 positions, located in and above the supposed main ligand-binding pocket between TM-II, -III, -IV, -V, -VI, and -VII in the ghrelin receptor, were used to map the binding sites of six different ghrelin receptor agonists (Fig. 1). The substitutions were selected from a large library of mutants based on their properties in both introducing significant structural change and still being expressed at a reasonable level at the cell surface compared with the wild-type receptor. Thus, as judged by cell surface ELISA, the expression levels of the mutants were between 0.37- and 1.3-fold of the expression level of the wild-type receptor (Table 1). A truncated version in which the 35 amino-terminal residues of the receptor were deleted and a methionine residue was introduced in front of $Gln^{35}$ was included in the analysis (Fig. 1). The mutants were transiently transfected into COS-7 cells, and the signaling property of the agonists was evaluated by full dose-response curves of stimulation of inositol phosphate accumulation. For most of the mutants, the high constitutive signaling activity of the ghrelin receptor was preserved as another certification of the robustness of this receptor. Because of its central location in the binding pocket the PheVI:16 to Ala mutant was included in the analysis although its constitutive signaling—as reported previously—was eliminated (Table 1). It is noteworthy that the mutant had a relatively good expression level as judged from the ELISA (Table 1).

### Residues Important for the Ghrelin Function

Sixteen of the mutations have previously been described in terms of cell surface expression and effects on constitutive activity and ghrelin-induced activation (Holst et al., 2006). In the present study, the mutational map for Ghrelin, which previously included GlnIII:05 and GluIII:09 in TM-III, ArgVI:13 and PheVI:16 in TM-VI, and AsnVII:02 in TM-VII, was expanded (Table 1). It is noteworthy that the strongest novel hit, PheVI:23, which decreased the signaling of ghrelin 25-fold, is located one and two helical turns, respectively, above two previously identified hits, ArgVI:20 and PheVI:16. Two additional new hits, $Glu^{197}$ and GlnVII:-02 ($Gln^{302}$), also located more superficially in the receptor (i.e., at the interface between the transmembrane domain and the extracellular parts of the receptor), were weaker in that they decreased the potency of ghrelin less than 10-fold. Thus, alanine substitution of GlnVII:-02 shifted the dose-response curve for ghrelin 5.6-fold to the right (-fold changes in $EC_{50}$ value between wild-type and mutated receptor are expressed as Fmut) (Table 1). This residue is positioned at the C-terminal end of extracellular loop 3 or one helical turn above AsnVII:02 in TM-VII, which previously was suggested to be a hit for ghrelin (8.5-fold in previous study and 3.5-fold in the present study) (Holst et al., 2007). Substitution of $Glu^{197}$ in extracellular loop 2 located next to $Cys^{198}$, which is part of the conserved disulfide bridge down to CysIII:01 in TM-III, decreased the potency of ghrelin 6.9-fold (Fig. 1). In contrast

TABLE 1

Characterization of a library of 23 mutant versions of the ghrelin receptor with substitutions systematically placed throughout the main ligand-binding crevice and in the extracellular part of the receptor

The constructs were expressed in transiently transfected COS-7 cells. In the first column, expression of each mutation is assessed by cell surface ELISA stated as fraction of wild-type receptor expression. In the second column is shown the constitutive activity of the mutant receptors expressed as percentage of basal signaling activity compared to the maximal Ghrelin-stimulated activity The potency ($EC_{50}$) of the compounds with respect to stimulating inositol phosphate accumulation was determined in cells expressing either the wild-type or the mutant forms of the ghrelin receptor. Fmut indicates the -fold shift in potency induced by the structural change in the receptor compared with the wild-type receptor.

| | B&W | Expression Level | *n* | Constitutive Activity | *n* | Ghrelin | *n* | Fmut |
|---|---|---|---|---|---|---|---|---|
| | | | | % | | *nM* | | |
| WT-Ghrelin R1a | | 1[a] | 3 | 46 ± 1 | 52 | 0.34 ± 0.02 | 52 | 1.0 |
| AspII:20Asn ($Asp^{99}$) | 2.60 | 0.62 ± 0.15 | 3 | 69 ± 2 | 8 | 1.10 ± 0.2 | 8 | 3.4 |
| PheIII:04Ser($Phe^{119}$) | 3.28 | 1.20 ± 0.21 | 3 | 39 ± 2 | 21 | 0.44 ± 0.04 | 17 | 1.3 |
| GlnIII:05Ala($Gln^{120}$) | 3.29 | 0.37 ± 0.11 | 3 | 49 ± 3 | 11 | 2.30 ± 0.6 | 11 | 6.8 |
| SerIII:08Ala ($Ser^{123}$) | 3.32 | 1.20 ± 0.08 | 3 | 36 ± 2 | 16 | 0.77 ± 0.30 | 16 | 2.3 |
| GluIII:09Gln ($Glu^{124}$) | 3.33 | 0.75 ± 0.18 | 3 | 44 ± 2 | 10 | 52.00 ± 11 | 10 | 150 |
| ThrIII:12Ala ($Thr^{127}$)[b] | 3.36 | 0,73 ± 0.17 | 3 | 64 ± 5 | 4 | 0.61 ± 0.22 | 4 | 3.2 |
| SerIV:16Ala ($Ser^{174}$) | 4.56 | 0.78 ± 0.12 | 3 | 41 ± 3 | 10 | 0.65 ± 0.10 | 10 | 2.1 |
| IleIV:20Ala ($Ile^{178}$) | 4.60 | 1.00 ± 0.3 | 3 | 46 ± 2 | 10 | 0.82 ± 0.15 | 10 | 2.4 |
| Arg199Leu[b] | | 0.94 ± 0.08 | 3 | 31 ± 3 | 7 | 0.76 ± 0.34 | 7 | 2.2 |
| Glu197Gln[b] | | 0.99 ± 0.04 | 3 | 49 ± 2 | 8 | 2.30 ± 0.9 | 8 | 6.9 |
| MetV:05Ala ($Met^{213}$) | 5.39 | 1.30 ± 0.4 | 3 | 49 ± 2 | 13 | 0.44 ± 0.07 | 13 | 1.3 |
| ValV:08Ala ($Val^{216}$) | 5.42 | 0.53 ± 0.06 | 3 | 53 ± 1 | 11 | 0.91 ± 0.24 | 6 | 2.7 |
| SerV:09Ala ($Ser^{217}$) | 5.43 | 0.73 ± 0.12 | 3 | 41 ± 2 | 7 | 0.37 ± 0.09 | 7 | 1.1 |
| PheV:12Ala ($Phe^{220}$) | 5.46 | 0.68 ± 0.07 | 3 | 22 ± 2 | 12 | 0.48 ± 0.08 | 13 | 1.4 |
| PheVI:16Ala ($Phe^{279}$) | 6.51 | 0.72 ± 0.23 | 3 | 0 ± 1 | 13 | 12.00 ± 3 | 12 | 36 |
| ArgVI20:Gln ($Arg^{283}$) | 6.55 | 0.67 ± 0.26 | 3 | 20 ± 2 | 12 | 19.00 ± 4 | 10 | 55 |
| PheVI:23Ala ($Phe^{286}$)[b] | 6.58 | 1.00 ± 0.20 | | 45 ± 2 | 7 | 8.50 ± 2.6 | 7 | 25 |
| SerVI:24Ala ($Ser^{287}$)[b] | 6.59 | 1.10 ± 0.20 | 3 | 50 ± 2 | 7 | 0.57 ± 0.16 | 7 | 1.7 |
| GlnVII:-02Ala ($Gln^{302}$)[b] | 7.32 | 0.49 ± 0.06 | 3 | 43 ± 4 | 6 | 1.90 ± 0.7 | 6 | 5.6 |
| AsnVII:02Ala ($Asn^{305}$) | 7.35 | 1.10 ± 0.2 | 3 | 26 ± 2 | 14 | 1.20 ± 0.3 | 11 | 3.5 |
| PheVII:06Leu ($Phe^{309}$) | 7.39 | 1.20 ± 0.5 | 3 | 38 ± 3 | 8 | 0.32 ± 0.07 | 8 | 0.9 |
| PheVII:09Ala ($Phe^{312}$) | 7.42 | 0.64 ± 0.14 | 3 | 24 ± 5 | 8 | 0.88 ± 0.13 | 6 | 2.6 |
| Ξ-35 N-terminal | | 0.28 ± 0.18 | 3 | 41 ± 4 | 5 | 0.67 ± 0.05 | 5 | 2.0 |

B&W, the nomenclature as described by Ballesteros and Weinstein

[a] $B_{max}$ value is 55 ± 12 fmol/$10^5$ cells.

[b] Novel mutations with respect to effect on ghrelin.

TABLE 2

Mutational mapping of the binding site for the ghrelin receptor agonists, GHRP-6, L-692,429, MK-677, SM-157740, and SM-130686, using a library of 23 mutant versions of the ghrelin receptor with substitutions systematically placed through the main ligand-binding crevice and in the extracellular part of the receptor

The potency ($EC_{50}$) of the compounds with respect to stimulating inositol phosphate accumulation was determined in COS-7 cells transiently transfected with either the wild-type or the mutant forms of the ghrelin receptor. Fmut indicates the -fold shift in potency induced by the structural change in the receptor as compared to the wild-type receptor. In the first column is shown the Fmut value of ghrelin in the respective ghrelin receptor mutation.

| | B&W | Ghrelin Fmut | GHRP-6 | | | L-692,429 | | | MK-677 | | | SM-157740 | | | SM-130686 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $EC_{50}$ | $n$ | Fmut | $EC_{50}$ | $n$ | Fmut | $EC_{50}$ | $n$ | Fmut | $EC_{50}$ | $n$ | Fmut | $EC_{50}$ | $n$ | Fmut |
| | | | *nM* | | | *nM* | | | *nM* | | | *nM* | | | *nM* | | |
| WT-Ghrelin R1a | | 1.0 | 1.8 ± 0.21 | 18 | 1.0 | 39.0 ± 7 | 11 | 1.0 | 0.30 ± 0.03 | 21 | 1.0 | 0.49 ± 0.08 | 18 | 1.0 | 0.31 ± 0.04 | 20 | 1.0 |
| AspII:20Asn ($Asp^{99}$) | 2.60 | 3.4 | 11.0 ± 2.0 | 3 | 5.9 | 6.3 ± 1.7 | 4 | 0.2 | 400.00 ± 300 | 3 | 1300.0 | 9.80 ± 0.7 | 3 | 20.0 | 130.0 ± 90 | 4 | 410.0 |
| PheIII:04Ser($Phe^{119}$) | 3.28 | 1.3 | 2.7 ± 0.6 | 3 | 1.5 | 24.0 ± 8 | 3 | 0.6 | 1.30 ± 0.2 | 5 | 4.4 | 2.00 ± 0.7 | 3 | 4.1 | 2.50 ± 0.5 | 3 | 8.2 |
| GlnIII:05Ala($Gln^{120}$) | 3.29 | 6.8 | 51.0 ± 2.0 | 3 | 28.0 | 91.0 ± 21 | 4 | 2.3 | 3.60 ± 1.5 | 4 | 12.0 | 2.10 ± 0.7 | 3 | 4.2 | 2.00 ± 0.8 | 3 | 6.5 |
| SerIII:08Ala ($Ser^{123}$) | 3.32 | 2.3 | 1.5 ± 0.4 | 6 | 0.8 | 89.0 ± 26 | 4 | 2.3 | 2.90 ± 1.1 | 6 | 9.7 | 0.57 ± 0.14 | 4 | 1.2 | 0.87 ± 0.20 | 3 | 2.8 |
| GluIII:09Gln ($Glu^{124}$) | 3.33 | 150.0 | >10.000[a] | 4 | >5400.0 | >10.000[a] | 4 | >250.0 | >1000[a] | 4 | >3300.0 | 140.00 ± 40 | 5 | 290.0 | 320.0 ± 90 | 4 | 1100.0 |
| ThrIII:12Ala ($Thr^{127}$) | 3.36 | 3.2 | 6.0 ± 3.0 | 3 | 3.2 | 930.0 ± 250 | 4 | 23.0 | 1.20 ± 0.2 | 3 | 4.0 | 0.53 ± 0.26 | 3 | 1.1 | 1.10 ± 0.1 | 3 | 3.6 |
| SerIV:16Ala ($Ser^{174}$) | 4.56 | 2.1 | 3.9 ± 2.7 | 4 | 2.1 | 100.0 ± 30 | 6 | 2.7 | 0.38 ± 0.13 | 4 | 1.3 | 0.61 ± 0.10 | 3 | 1.3 | 1.30 ± 0.1 | 3 | 4.3 |
| IleIV:20Ala ($Ile^{178}$) | 4.60 | 2.4 | 230.0 ± 20.0 | 3 | 130.0 | >1000[a] | 4 | >25.0 | 1.60 ± 0.6 | 4 | 5.3 | 4.40 ± 1.2 | 3 | 8.9 | 23.00 ± 15 | 3 | 76.0 |
| Arg199Leu | | 2.2 | 5.6 ± 0.8 | 4 | 3.0 | 180.0 ± 60 | 3 | 4.6 | 0.22 ± 0.02 | 3 | 0.7 | 0.34 ± 0.15 | 3 | 0.7 | 0.36 ± 0.01 | 3 | 1.1 |
| Glu197Gln | | 6.9 | 2.7 ± 0.9 | 3 | 1.5 | 43.0 ± 7 | 3 | 1.1 | 1.30 ± 0.8 | 3 | 4.4 | 0.57 ± 0.15 | 3 | 1.1 | 0.55 ± 0.20 | 3 | 1.8 |
| MetV:05Ala ($Met^{213}$) | 5.39 | 1.3 | 4.1 ± 1.0 | 6 | 2.2 | 77.0 ± 18 | 5 | 2.0 | 0.18 ± 0.05 | 3 | 0.6 | 0.89 ± 0.22 | 3 | 1.8 | 0.64 ± 0.21 | 3 | 2.4 |
| ValV:08Ala ($Val^{216}$) | 5.42 | 2.7 | 5.3 ± 2.3 | 3 | 2.9 | 130.0 ± 4 | 3 | 3.2 | 0.67 ± 0.17 | 4 | 2.2 | 0.48 ± 0.20 | 3 | 1.0 | 0.38 ± 0.19 | 3 | 1.2 |
| SerV:09Ala ($Ser^{217}$) | 5.43 | 1.1 | 4.4 ± 1.2 | 3 | 2.4 | 38.0 ± 10 | 3 | 1.0 | 0.30 ± 0.07 | 4 | 1.0 | 0.55 ± 0.12 | 3 | 1.1 | 1.60 ± 0.4 | 3 | 5.2 |
| PheV:12Ala ($Phe^{220}$) | 5.46 | 1.4 | 7.8 ± 1.4 | 3 | 4.2 | 310.0 ± 170 | 3 | 8.0 | 0.27 ± 0.10 | 6 | 0.9 | 0.68 ± 0.13 | 3 | 1.4 | 0.33 ± 0.05 | 3 | 1.1 |
| PheVI:16Ala ($Phe^{279}$) | 6.51 | 36.0 | >10,000[a] | 3 | >5400.0 | >10,000[a] | 4 | >250.0 | 36.00 ± 6 | 3 | 120.0 | 13.00 ± 2 | 3 | 27.0 | 58.00 ± 12 | 3 | 190.0 |
| ArgVI20:Gln ($Arg^{283}$) | 6.55 | 55.0 | >10,000[a] | 3 | >540.0 | >10,000[a] | 4 | >250.0 | 57.00 ± 23 | 3 | 190.0 | 110.00 ± 20 | 3 | 230.0 | 340.00 ± 90 | 3 | 1100.0 |
| PheVI:23Ala ($Phe^{286}$) | 6.58 | 25.0 | 27.0 ± 7.0 | 4 | 14.0 | >1000[a] | 4 | >25.0 | 4.30 ± 0.1 | 3 | 14.0 | 6.50 ± 4.2 | 3 | 13.0 | 59.50 ± 21 | 3 | 190.0 |
| SerVI:24Ala ($Ser^{287}$) | 6.59 | 1.7 | 3.1 ± 1.0 | 4 | 1.7 | 22.0 ± 9 | 3 | 0.6 | 0.60 ± 0.21 | 4 | 2.0 | 1.30 ± 0.5 | 3 | 2.5 | 1.70 ± 0.9 | 3 | 5.2 |
| GlnVII:-02Ala ($Gln^{302}$) | 7.32 | 5.6 | 8.9 ± 1.1 | 3 | 4.8 | 45.0 ± 18 | 3 | 1.1 | 0.95 ± 0.22 | 3 | 3.2 | 0.94 ± 0.22 | 3 | 1.9 | 0.72 ± 0.14 | 3 | 2.3 |
| AsnVII:02Ala ($Asn^{305}$) | 7.35 | 3.5 | 125.0 ± 10 | 6 | 68.0 | >10.000[a] | 4 | >250.0 | 3.50 ± 1.1 | 3 | 12.0 | 0.67 ± 0.10 | 4 | 1.4 | 0.69 ± 0.35 | 3 | 2.2 |
| PheVII:06Leu ($Phe^{309}$) | 7.39 | 0.9 | 28.0 ± 14.0 | 5 | 15.0 | 260.0 ± 50 | 3 | 6.7 | 0.40 ± 0.08 | 5 | 1.3 | 1.10 ± 0.2 | 3 | 2.2 | 1.00 ± 0.3 | 3 | 3.3 |
| PheVII:09Ala ($Phe^{312}$) | 7.42 | 2.6 | 32.0 ± 10.0 | 3 | 18.0 | 85.0 ± 16 | 3 | 2.2 | 1.20 ± 0.3 | 3 | 4.1 | >1000[a] | 3 | >2000.0 | >1000[a] | 4 | >3200.0 |
| Ξ-35 N-terminal | | 2.0 | 3.0 ± 0.9 | 3 | 1.6 | 22.0 ± 7 | 3 | 0.6 | 0.36 ± 0.05 | 4 | 1.2 | 0.69 ± 0.12 | 3 | 1.4 | 0.52 ± 0.16 | 4 | 1.7 |

B&W, the nomenclature as described by Ballesteros and Weinstein

[a] The $EC_{50}$ values have been estimated based on graphic illustration of the dose-response curve. $EC_{50}$ > 10,000 nM means that only the highest concentration applied ($10^{-5}$ M) increases slightly above basal.

to many other peptide hormones acting through 7TM receptors, truncation of the extracellular amino-terminal segment of the receptor had no effect on ghrelin function (Table 1). Thus, as reported previously, ghrelin is dependent on residues located on the opposing faces of TM-III, -VI, and -VII, which now includes residues also at the interface between the helices and the extracellular domains.

### The Strongest Hits for Ghrelin in TM-III and VI Are Shared by the Small-Molecule Agonists

**Residues in TM-III.** The single strongest interaction partner for ghrelin, GluIII:09 (Fmut = 150), was found to be shared by all other agonists tested in the present study (Table 1 and Fig. 2): L-692,429 (Fmut >250), SM-157740 (Fmut = 290), SM-130686 (Fmut = 1100), MK-677 (Fmut = 3300), and GHRP-6 (Fmut = >5400). In all cases, GluIII:09-to-Gln was the largest or equal to the largest mutational hit observed. It is noteworthy that the GluIII:09 substitution had no effect on either the constitutive activity or on the cell surface expression, which suggests that the observed effect on the potency of the ligands is caused by disruption of a direct interaction and that GluIII:09 serves as a key anchor point for all these agonists.

Alanine substitution of GlnIII:05 located one helical turn closer to the extracellular surface compared with GluIII:09, decreased the ghrelin potency only 6.8-fold. Among the small-molecule agonists, MK-677 and GHRP-6 were affected 12- and 28-fold (Fig. 2 B and D), respectively, and L-692,429 and SM-157740 less than 5-fold (Fig. 2C). It should be noted that the GlnIII:05-to-Ala mutation almost totally eliminated the function of the wFw-containing oligopeptides—i.e., both inverse agonists and agonists (Holst et al., 2007). The surface expression of this mutant receptor was apparently decreased to approximately 40% compared with that observed for the wild type receptor; but—importantly—the level of constitutive activity was not affected by the GlnIII:05 mutation (Table 1). We were surprised to find that mutations of the two other residues in TM-III, PheIII:04 and SerIII:08, which function as a crucial efficacy switch-region for the wFw-containing peptides and have major effects on the potencies of these receptor ligands, and even swap their efficacies (Holst et al., 2007), had very little if any effect on the potencies of the small-molecule agonists and did not swap their efficacy either, just as they had little effect on ghrelin action (Table 2).

**Residues in TM-VI.** Three different residues in TM-VI—located approximately one helical turn apart from each other (Fig. 1)—were identified as major mutational hits for ghrelin (Fig. 2). The most deeply located of these, PheVI:16, was—as previously shown—essential also for the ligand-independent, constitutive signaling (Holst et al., 2004). The small synthetic agonists were affected between 27- and more than 5400-fold by this substitution (Table 2). Gln substitution of ArgVI:20—located one helical turn closer to the extracellular surface—also strongly affected the potency of all agonists analyzed in the present study (i.e., ghrelin 55-fold and the synthetic agonist between 190- and 1100-fold) (Table 2 and Fig. 2, E–H). However, in analogy with substitution of PheVI:16, this mutation also decreased the constitutive activity of the receptor from 46 ± 1% to 20 ± 2% of the maximal ghrelin induced stimulation. Because both the PheVI:16-to-Ala and the ArgVI:20-to-Gln mutations also affected the constitutive activity of the receptor (Table 1), it is not possible to conclude whether these substitutions in fact are directly involved in the binding of the various agonists or by impairing the general conformational movements required to obtain the active conformation of the receptor indirectly affect the potency of the agonists.

It is noteworthy that this was not at all an issue for the most superficially located mutational hit for Ghrelin in TM-VI (i.e., Ala substitution of PheVI:23 facing toward TM-VII). This substitution did not affect cell surface expression, which was identical to that measured for the wild-type receptor, or affect the constitutive activity, which was 45 ± 2% of maximal ghrelin-induced stimulation compared with 46 ± 1% for wild-type receptor (Table 1). This mutation impaired ghrelin potency 25-fold and was a solid hit for all the synthetic agonists as well: L-692,429, > 25-fold; GHRP-6, 14-fold, MK-677, 14-fold, and SM-157740, 13-fold (Table 2 and Fig. 2, E–H). It should be noted that mutation of the neighboring residue at the extracellular end of TM-VI, SerVI:24 facing TM-V, affected neither ghrelin nor any of the small-molecule agonists (Table 2).

The two relatively weak mutational hits for ghrelin, which are located in the extracellular part of the transmembrane region in position GlnVII:-02 and $Glu^{197}$, did not affect the potency of any of the small ghrelin receptor agonists more than 5-fold (Table 2). In conclusion, all four major mutational hits for the endogenous agonist ghrelin are shared with all the small-molecule agonists (i.e., the key anchor point in TM-III, GluIII:09, as well as the three residues on the opposing face of TM-VI: PheVI:16, ArgVI:20, and PheVI:23).

### Selective Mutational Hits for the Synthetic Agonist Are Located in TM-II, -IV, and -VII

**Residues in TM-II.** In contrast to Ghrelin, all small-molecule agonists were affected by substitution of AspII:20 to Asn located at the extracellular end of TM-II; however, the effect varied significantly from compound to compound. Most interestingly, a 6-fold *improved* potency was observed for L-692,429 in response to this substitution (i.e., the $EC_{50}$ was shifted from 39 ± 7 to 6.3 ± 1.7 nM) (Fig. 3 A). In contrast, the two other nonpeptide agonists displayed 20-fold (SM-157740) and >1300 fold (MK-677) *decreased* potency (Fig. 3, D and G). For the hexa-peptide GHRP-6, this was only a 5.9-fold hit.

**Residues in TM-IV.** Substitutions of IleIV:20 with Ala at the extracellular end of TM-IV, which is an important anchor point position [for example, for certain CXCR4 antagonists (Gerlach et al., 2001)] and a position that has been used for metal-site engineering to tether small chelator agonists (Rosenkilde et al., 2007), did not affect ghrelin but was a mutational hit for all the synthetic agonists. Also in this position, the magnitude of the hit varied from 130-fold for GHRP-6 and 25-fold for L-692,429 to 5.3-fold for MK-677 (Table 2, Fig. 6). Ala substitution of SerIV:16 one helical turn below IleIV:20, which improved the potency of wFw-containing inverse agonist peptides up to 20-fold or more (Holst et al., 2007), did not affect any of the tested agonist compounds (Table 2).

**Residues in TM-V.** It is noteworthy that none of the four substitutions located on the three most extracellular helical turns in TM-V facing into the main ligand-binding pocket affected ghrelin or any of the small-molecule agonists (Table 2).

**Residues in TM-VII.** Two different residues located in TM-VII (AsnVII:02 and PheVII:09) pointing inward and to-

ward TM-VI displayed significant selectivity within the group of small-molecule agonist. That is, substitution of the most extracellularly located residue AsnVII:02 totally destroyed the function of L-692,429 (Fig. 3C) whereas SM-130686 (Fig. 3I) was not affected at all.

The opposite pattern was observed by Ala substitution of PheVII:09 located two helical turns below AsnVII:02. Thus, in this mutant, the potency of L-692,429 was unchanged, whereas SM-130686, even at 100 nM, was unable to stimulate the receptor (Fig. 3, B and H). MK-677 was somewhat

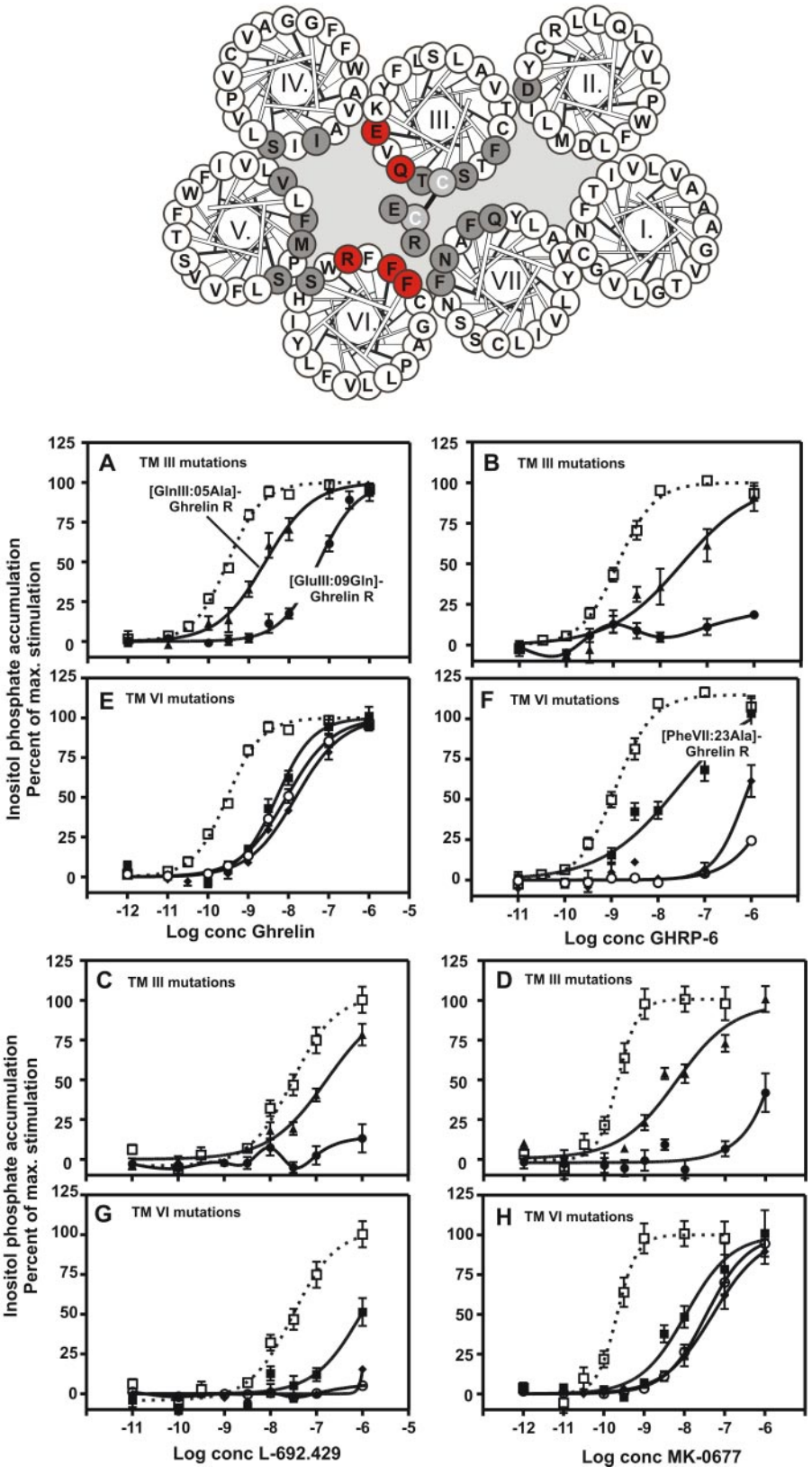


**Fig. 2.** Substitutions in the ghrelin receptor that affect the potency of all agonists tested. In the helical wheel diagram at the top are indicated in red the positions in TM-III and TM-VI of the residues, which upon mutagenesis affects the signaling property of all tested agonists (i.e., both the endogenous agonist ghrelin and synthetic agonists some of which also display allosteric properties). The graph depicts dose-response curves for ghrelin (A and E), GHRP-6 (B and F), L-692,429 (C and G), and MK-677 (D and H) on the wild-type ghrelin receptor (□ and dotted line), GlnIII:05Ala (▲ in A–D), GluIII:09Gln (● in A–D), PheVI:23Ala (■ in E–H), ArgVI:20Gln (♦ in E–H), and PheVI:16Ala (○ in E–H). Data are mean ± S.E. of 3 to 20 independent experiments performed in triplicate.

affected by both of these substitutions, although only the AsnVII:02 would be considered a hit (Fig. 3, E and F). In conclusion, on top of the mutational hits shared with ghrelin, the small-molecule agonists each have a number of mutational selective hits, which vary between the compounds with the most clear ones being IleIV:20, AsnVI:02, and PheVII:09.

### Mutational Mapping of Residues Involved in the Allosteric Effect of L-692,429 and GHRP-6 on Ghrelin Signaling

For the ago-allosteric modulators, the residues involved in allosteric modulation are not necessarily identical to the residues required for agonism. The ago allosteric compounds

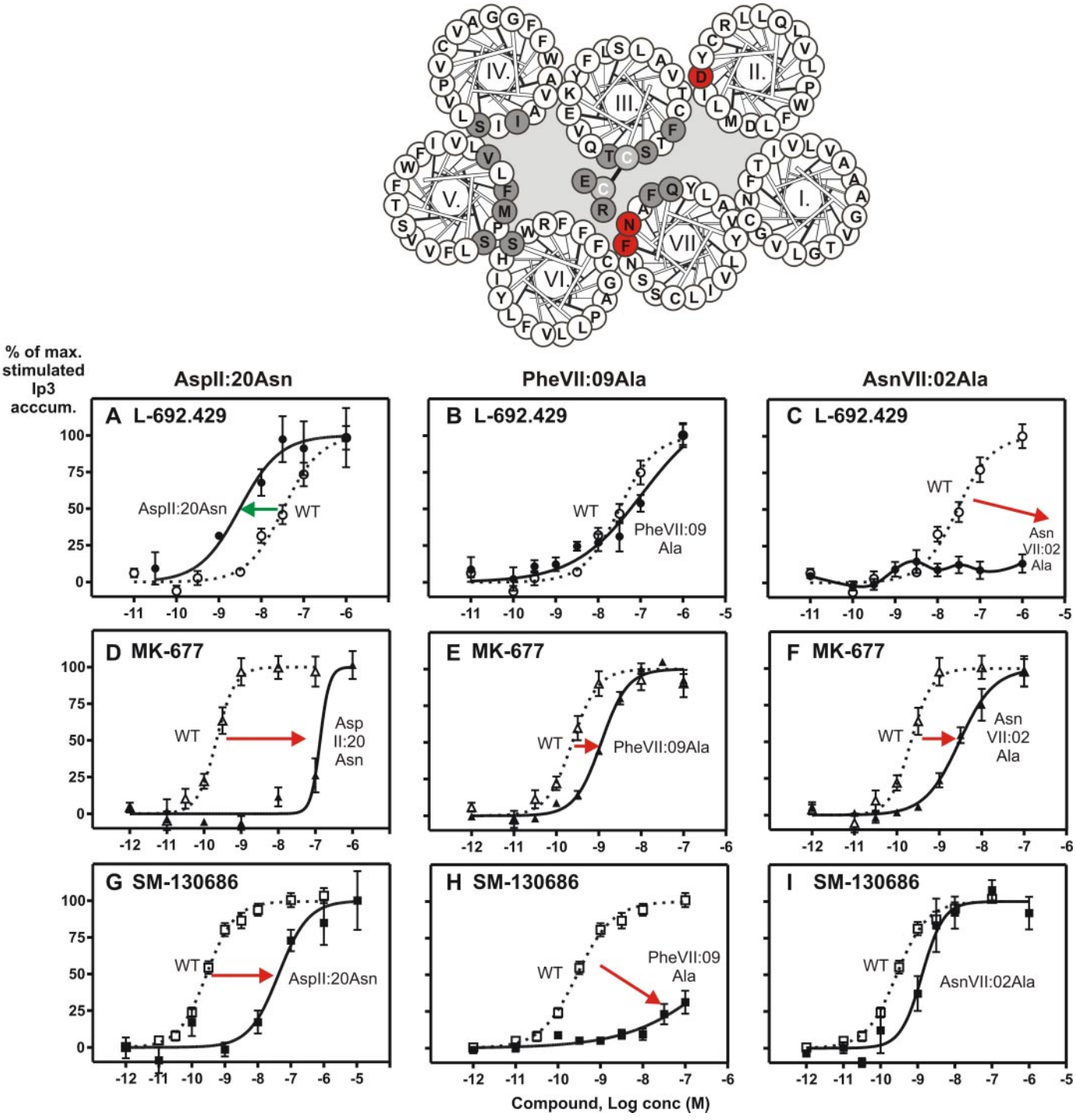


**Fig. 3.** Some substitutions in the ghrelin receptor that selectively affect the potency of certain nonpeptide agonists. In the helical wheel diagram at the top are indicated in red three positions in TM-II and TM-VII of the residues, which upon substitution selectively affect one or more nonpeptide agonists as opposed to the endogenous ligand, ghrelin. Dose-response curves for L-692,429 (●) in AspII:20Asn (A), PheVII:09Ala (B) and AsnVII:02Ala (C) compared with WT-ghrelin receptor (○ and dotted line). Dose-response curve for MK-677 (▲) on AspII:20Asn (D), PheVII:09Ala (E), and AsnVII:02Ala (F) compared with WT-ghrelin receptor. Dose-response curve for SM-130686 (■) on AspII:20Asn (G), PheVII:09Ala (H), and AsnVII:02Ala (I) compared with WT-ghrelin receptor. See Table 2 for full data set. Data are mean ± S.E. of 3 to 20 independent experiments performed in triplicate.

L-692,429 or GHPR-6 are characterized by modulating the dose-response curve of ghrelin both in terms of potency and efficacy (Holst et al., 2005). However, as measurement for the allosteric modulation in various mutated ghrelin receptor constructs, we here used the increase in efficacy for ghrelin stimulation of inositol phosphate accumulation in transfected COS-7 cells. The window of more than 20% increase in efficacy caused by coadministration of ghrelin with L-692,429 or GHPR-6 (Holst et al., 2005) (Fig. 4C) was sufficient to allow for evaluation of changes induced by mutations, similar to previous studies in the A3 receptor (Gao et al., 2003). Nine mutations were selected for the mapping of the allosteric modulation, eight of which were mutational hits (>20-fold reduction in potency) for the agonism of either L-692,429 or GHPR-6. The last mutation, Arg199Leu, was located next to the conserved Cys residue in extracellular loop 2 (ECL-2) at a position described previously to be an interaction site for allosteric modulators (May et al., 2007a).

An efficacy level of 75 to 100% of maximal ghrelin-induced stimulation (dashed line) was modulated by coadministration of 100 nM L-692,429 (Fig. 4A) or 10 nM GHPR-6 (Fig. 4B) on wild-type ghrelin receptor and the indicated nine mutant constructs. The concentration of ghrelin required to obtain 75 to 100% of maximal stimulation varied dependent on the importance of the respective residues for ghrelin-induced stimulation (Table 1). For the GluIII:09 (Fig. 4D), PheVI:16, ArgVI:20, and PheVI:23 mutations, 100 nM ghrelin was used, whereas for the rest of the mutants, as well as for the wild-type (Fig. 4C), 10 nM ghrelin was sufficient to obtain 75 to 100% of maximal stimulation.

The GluIII:09Gln substitution, which also was the strongest mutational hit in respect of agonism for all compounds tested, completely abolished the allosteric effect of both L-692,429 (Fig. 4A) and GHPR-6 (Fig. 4B). We were surprised to find that this was the only mutation that significantly reduced the allosteric effect of GHRP-6 on ghrelin signaling (Fig. 4B). In Fig. 4D are shown full-dose response curves demonstrating the lack of allosteric effect of 10 nM GHRP-6 in the GluIII:09Gln mutant ghrelin receptor. The same was observed with 100 nM GHRP-6 (data not shown). For L-692,429 four of the seven mutational hits for its agonistic property were also hits for its allosteric effect, that is IleIV:20Ala, PheVI:23Ala, and AsnVII:02Ala besides GluIII:09Gln (Fig. 4A). Thus, two of the mutations that most strongly impaired their agonist property, PheVI:16Ala and ArgVI:20Gln, had no effect on the ability of L-692,429 and GHPR-6 to increase the efficacy of ghrelin upon coadministration. It is noteworthy that the allosteric effect of L-692,429 was diminished significantly by the $Arg^{199}$-to-Leu substitution in ECL-2, which had no effect on its agonist property (Table 2).

In conclusion, the mutational map for the allosteric property of both L-692,429 and GHPR-6 overlaps with the mutational map for agonism for both these compounds and for ghrelin. However, the mutational map for the allosteric effect is also clearly different and includes fewer residues in the main ligand binding pocket—especially for GHRP-6.

### Molecular Modeling of Receptor-Ligand Complexes

The low-energy conformations of the four nonpeptide agonists were manually docked into the pocket shared among TM-III, -IV, -V, -VI, and -VII in the ghrelin receptor model to maximize complementary ligand receptor interactions using the presumed major anchor point GluIII:09 as an initial interaction partner for the positively charged nitrogen moiety found in each of the compounds. Initially, we made a number of alternative docking attempts with the compounds, considering, for example, AspII:20 as an ionic interaction partner. However, the pocket shared among TM-II, -III, and -VII could not accommodate either of the agonists without steric clashes to other parts of the receptor.

**Docking of SM-157740 and SM-130686.** These two compounds—different by only a single Cl (Fig. 1)—were docked in a similar overall mode as shown for SM-157740 in Fig. 5A. The terminal *N*-diethyl functionality of the SM-compounds can—with the basic ammonium group engaged in a charge-charge interaction with GluIII:09—interact with the side chain of IleIV:20 on the opposing face of TM-IV (Fig. 5A, left), which upon Ala substitution were 8.9- and 76-fold hits for the two compounds, respectively (Table 2). The chlorophenyl of SM-130686 or the dichlorophenyl group of SM-157740 was docked into the deeply located hydrophobic pocket between residues TyrIII:13, PheV:13, TrpVI:13, and HisVI:17. The oxindole scaffold seems to make aromatic interactions with the aromatic cluster on the interface between TM-VI and VII: PheVI:16; PheVII:06 and PheVII:09. In this docking mode, ArgVI:20 can make a close interaction with the hydroxyl group of the oxindole scaffold. Moreover, the amide attached to the oxindole can make hydrogen bond interactions with SerIII:08 and potentially also to GlnIII:05. However, although some of the indicated interaction residues are mutational hits for the SM compounds, many of them are not (Table 2). For example, a couple of residues that were clear hits in the mutational mapping, AspII:20 and PheVI:23, are not able to interact with the SM-compounds in this docking mode. In fact AspII:20 is located so far away, 12 to 13 Å from GluIII:09, even in a straight line going through TM-III, that it is impossible for a single SM molecule to interact with both of these residues in any docking mode (Fig. 5B). In the case of PheVI:23, which is a hit for all the small-molecule agonists, the distance between this reside and PheVII:09 is too long (15–16 Å) for the SM compounds to interact simultaneously with both residues—i.e., while being anchored to GluIII:09 at the other end of the pocket (Fig. 5B). Because Ala substitution of PheVII:09 is the largest mutational hit for both SM-157740 and SM-130686 (>2000- and >3200-fold, respectively) we favor the relatively deep binding mode for these two ligands. It is noteworthy that analogs containing aliphatic or aromatic derivatives attached to the oxindole amide are recognized to have high affinity to the ghrelin receptor as well (Holst et al., 2006). It is noteworthy that the proposed docking modes of SM-157740 and SM-130686 are consistent with the active analogs, which in addition has the possibility to probe an aliphatic, aromatic extracellular pocket formed between TM-II, -VI, and -VII.

**Docking of** L-692,429. When the basic amine group of L-692,429 is engaged in a charge-charge interaction with the GluIII:09 anchor-residue, the terminal dimethyl functionality can interact with IleIV:20—which is a 25-fold mutational hit—in analogy with the interactions observed for the SM-compounds (Fig. 5A, middle). The benzamide derivative of L-692,429 was docked into the aromatic, hydrophobic pocket between TM-III, -VI, and -VII. This enables the biphenyl tetrazol ring system of L-692,429 to form aromatic interac-

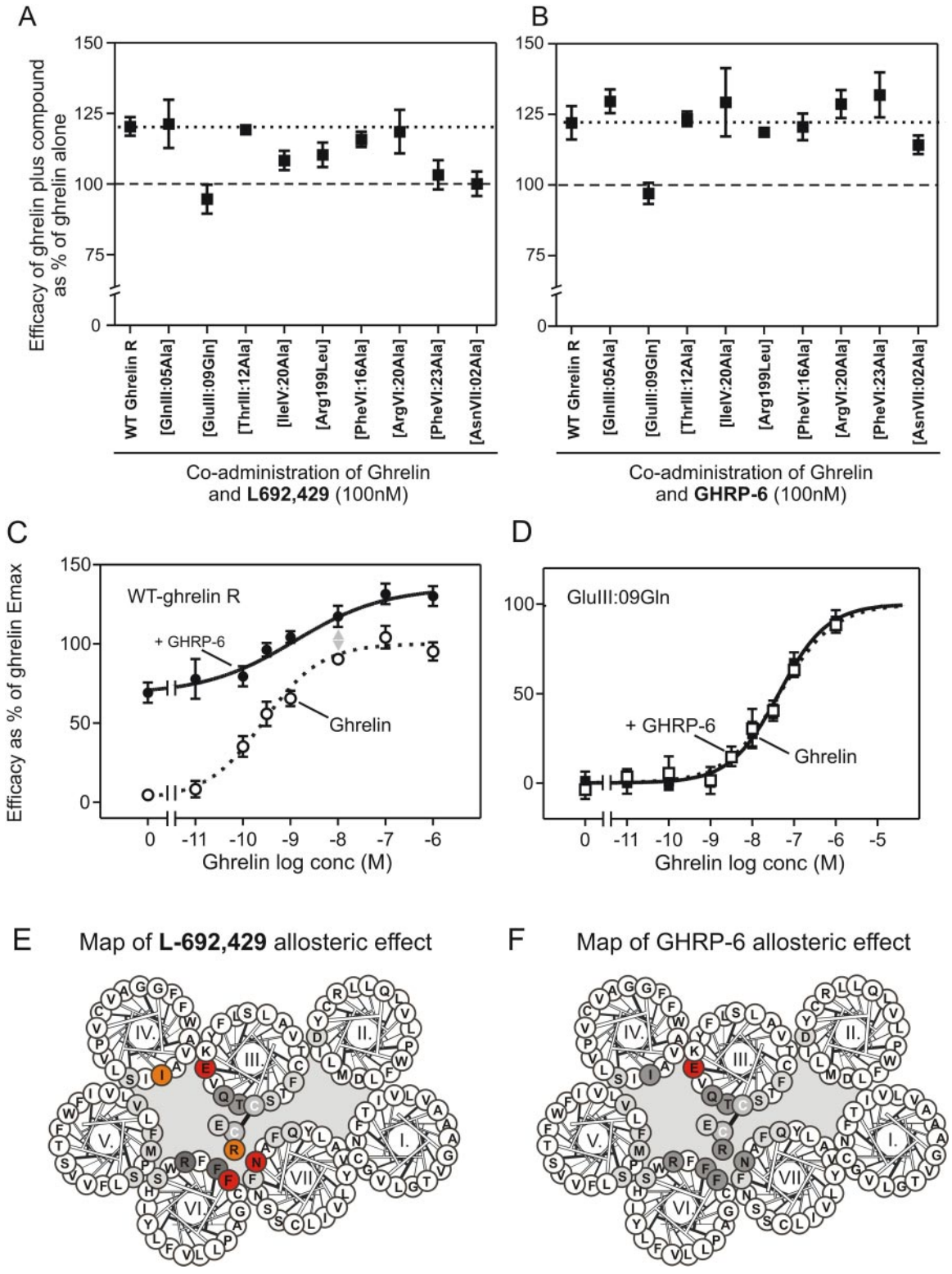


**Fig. 4.** Mapping of the allosteric effect of L-692,429 and GHRP-6. The increase in efficacy obtained by coadministration of ago-allosteric modulators with ghrelin as measured by inositol phosphate accumulation in COS-7 cells transiently transfected with wild-type ghrelin receptors or mutated versions as indicated. Ghrelin was administrated in a concentrations of 100 nM for GluIII:09, PheVI:16, ArgVI:20, and PheVI:23 and 10 nM for the rest, which induced 75 to 100% of the maximal efficacy. Subsequently the ago-allosteric modulator L-692,429 (A) and GHRP-6 (B) was administered at 100 and 10 nM, respectively, which are the concentrations that have previously been demonstrated to induce the most potent allosteric effect (Holst et al., 2005). The dashed line indicates the efficacy obtained by ghrelin alone normalized to 100% to compare the wild-type receptor with the mutated receptor constructs. The dotted line indicates the efficacy induced by coadministration of ghrelin with the ago-allosteric modulator in the wild-type ghrelin receptor. Data are mean ± S.E. of three to five independent experiments performed in triplicate. Full dose-response curve of ghrelin in the presence and absence of 10 nM GHRP-6 is shown for the wild-type ghrelin receptor in C and for the GluIII:09Gln substitution in D. Helical wheel diagram with the mutational map for of the allosteric effect observed for L-692,429 (E) and GHRP-6 (F). The residues that upon mutational substitution completely abolished the allosteric effect are marked in red, those that partially eliminated the allosteric effect are marked with orange, and the residues that had no effect on the allosteric modulation are marked in dark gray. The residues included in the agonist mapping but not in the allosteric mapping are marked in light gray.

tion with PheIII:04, PheVI:16, and PheVII:09 and PheVII:06, whereas the benzodiazepine moiety is positioned in a lower, aromatic hydrophobic pocket, where it apparently interacts with, for example, residues ThrIII:12, TyrIII:13, PheV:12, TrpVI:13, PheVI:16, and PheVII:09. Several of these residues are however not recorded as mutational hits. In this docking mode, the tetrazol group is in close interaction with ArgVI:20. However, the distance to AsnVII:02, which is a mutational hit and a potential hydrogen bond interaction partner at the extracellular end of TM-VII, and the distance to PheVI:23, which also is a mutational hit and a potential aromatic interaction partner, is relatively long: approximately 6 Å (Fig. 5B). As for the SM compounds, AspII:20, which upon substitution to Asn is a 5-fold gain-of-function hit for L-692,429, is located far away from the docked compound. However, it is likely that much of the effects of the AspII:20 substitution is indirect (see *Discussion*).

**Docking of MK-677.** The primary basic ammonium group of MK-677 is docked to engaged in a charge-charge interaction with the anchor-residue GluIII:09, which enables the dimethyl functionality of the 2-amino-2-methylproprionamide group to make hydrophobic interactions with IleIV:20 located in the subpocket between TM-III, -IV, and -V opposite GluIII:09—in analogy with the interactions observed for the SM compounds and L-692,429 (Fig. 5A, right). The two carbonyl atoms of 2-amino-2-methylproprionamide and the central piperidin-1 amide of MK-677 can make hydrogen bond interactions with ArgVI:20—a 190-fold mutational hit in TM-VI. In the opposite side of the pocket, the oxygen atom of the phenyl side chain linker can, in this docking mode, make hydrogen bond interac-

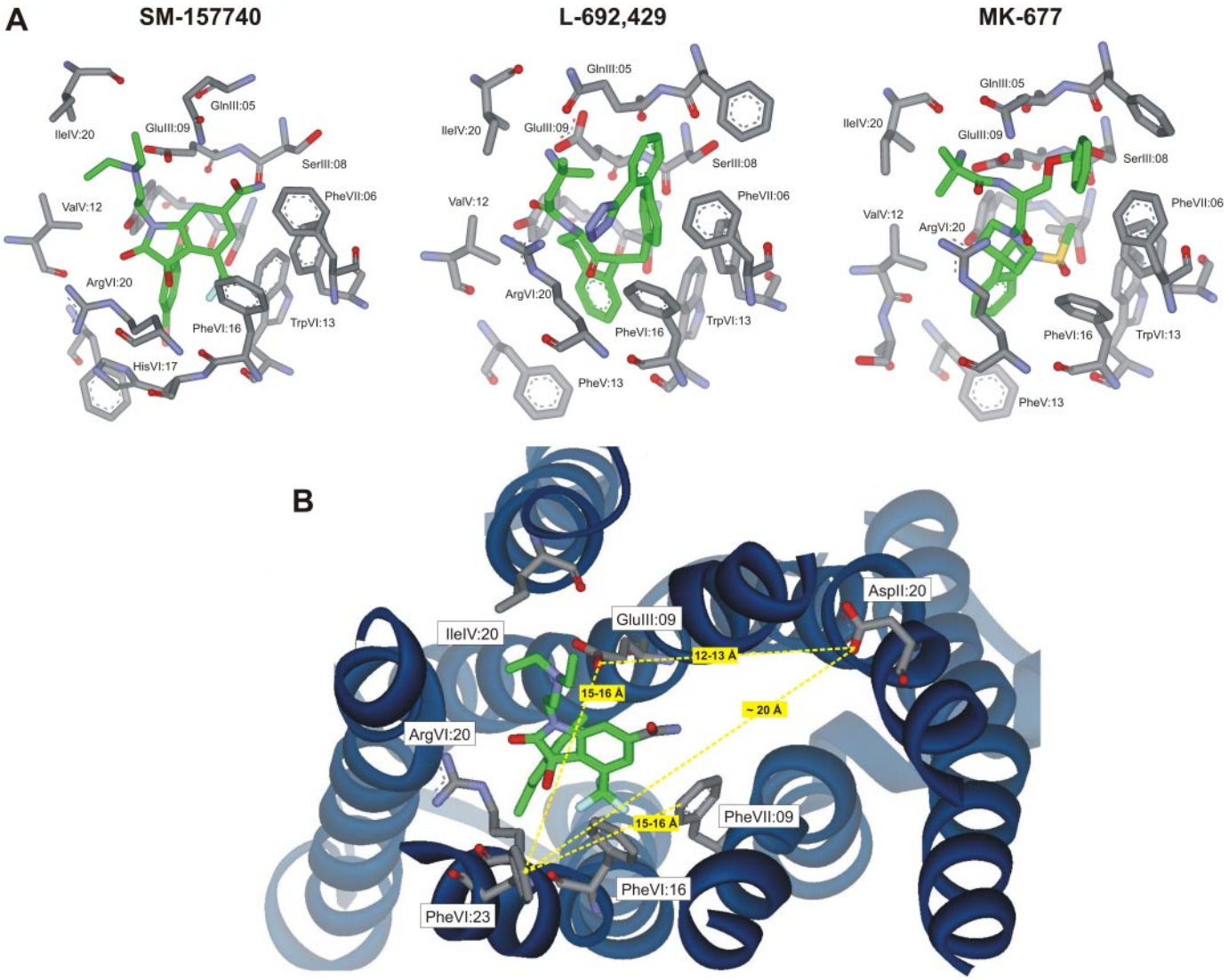


**Fig. 5.** Molecular models of the nonpeptide agonists in complex with the ghrelin receptor. Low-energy conformations of SM-157740 (A, left structure and B), L-692,429 (A, middle structure), and MK-677 (A, right structure) docked into the ghrelin receptor modeled over the inactive, dark state of rhodopsin to optimize for structural complementarities and using the proposed charge-charge interaction of the positively charged nitrogen of each of the ligands with GluIII:09 as an initial anchoring site (see *Molecular Modeling of Receptor-Ligand Complexes* for details). The nonpeptide ligands are shown with carbon atoms in green. A, the receptor residues that in the molecular model were found to be near the nonpeptide agonists are shown with carbon atoms in gray. B, the main mutational hit residues for SM-157740 are highlighted in the ghrelin receptor model with the seven helical bundle shown in dark blue, solid ribbon format and with which SM-157740 docked as shown in A, left. It should be noted that the AspII:20 and PheVI:23 mutational hits were not depicted in the corresponding structure in A (left structure) because these residues are located too far away from the ligand in the chosen docking mode (see *Results* and *Discussion*). Certain distances are indicated by yellow dotted lines.

tions with SerIII:08, which is a 9.7-fold selective hit for MK-677 as opposed to the other nonpeptide agonists. In addition, GlnIII:05, positioned one helical turn above SerIII:08, which is a 12-fold hit for MK-677, seems to make hydrogen bond interactions to the same oxygen atom as well as the amide carbonyl in the 2-amino-2-methylproprionamide group. The phenyl itself is able to make aromatic interactions both with PheIII:04 (a 4.4-fold hit) and with PheVII:06, which upon Leu substitution is not a mutational hit for MK-677 (Table 2). In this docking mode, the spiroindane derivative is positioned in the lower aromatic hydrophobic pocket surrounded by TrpVI:13, PheVI:16, and HisVI:17 in TM-VI; ValV:08 and PheV:12 from TM-V; and ThrIII:12 and TyrIII:13 from TM-III as well as PheVII:09. Several of these residues, however, were not recorded as mutational hits. Nevertheless, the oxygen of sulfonyl spiroindane moiety would seem to be involved in a hydrogen bond interaction with the hydroxyl of ThrIII:12, which is a weak 4-fold hit, together with PheVII:09, which is a 4.1-fold hit. In contrast, substitution of ValV:08, SerV:09, or PheV:12 does not affect MK-677 despite the apparent close proximity to MK-677. Conversely, two clear mutational hits in this pocket of the ghrelin receptor are, in the present docking mode, too far away to be able to make meaningful interactions with the ligand: PheVI:23, a 14-fold hit located ~10 Å away, and AsnVII:02, a 12-fold hit located 5 to 6 Å away. Moreover, for MK-677, AspII:20 is located way outside its presumed binding site despite that this is one of the strongest mutational hits for this compound (Fmut = 1300).

Thus, it is possible to dock each of the nonpeptide agonists in low-energy conformations into the main ligand-binding pocket of the ghrelin receptor in a mode that is in agreement with many of the major mutational hits. Nevertheless, the resulting ligand-receptor complexes are far from perfect because it is not possible to account for all the mutational hits with respect to close interactions in the receptor ligand complex. Conversely, several apparent interactions observed in the molecular models are not reflected in the mutational analysis of the different compounds. This is most clearly illustrated by the relatively small and conformational constrained SM-compounds.

No attempt was made to dock the ghrelin peptide in the receptor, mainly due to problems in identifying the presumed conformation of this peptide when bound to the receptor. Moreover, it is very likely that the peptide also has major—as yet unidentified—interactions with the external domains, which in the case of the ghrelin receptor would be highly difficult to model. Nevertheless, in the favored docking mode for all three small-molecule ligands, each of these basically fills up the main ligand binding pocket and totally prevent any meaningful docking of even, for example, the N-terminal oligopeptide of ghrelin in this pocket, where the mutational hits for ghrelin are located.

## Discussion

In the present study, we found that the small-molecule synthetic agonists—both peptide and nonpeptides—share major interaction points with the endogenous agonist ghrelin confined to the interface between the extracellular ends of TM-III and TM-VI (Holst et al., 2006). In addition, the agonist property of the small-molecule compounds was affected by a number of receptor substitutions in TM-II, -IV, and -VII not involved in ghrelin binding. It is noteworthy that the mutational map of the ability of L-692,429 and GHRP-6 to act as allosteric modulators overlapped with the common mutational map for agonism, but it was not identical with the map for the agonist property of the small-molecule ligands. Thus an important observation of the present study is that ago-allosteric modulators may have binding sites that overlap with those of the endogenous agonist. This fits well with the agonist property of these compounds—but such an overlap in binding site does not fit with their property as allosteric modulators (Schwartz and Holst, 2007)

**Mutational Mapping of a Common Agonist Binding Pocket in the Ghrelin Receptor.** We obviously cannot rule out the possibility that the effects of the mutants on both the agonistic and the allosteric properties of the compounds, as in basically all mutational studies, could be indirect because of a generally disturbed receptor structure. However, the ghrelin receptor is particularly suited for mutational mapping of ligand binding sites, both because it inherently is relatively robust and because the high constitutive signaling activity of the receptor provides an extra and very relevant quality control parameter on top of the ELISA measurement of cell surface expression. Moreover, the mutational hits for the ligands seem to cluster in meaningful "footprints" or maps, which clearly stand out from a large number of surrounding "negative" substitutions (Fig. 6).

The ghrelin receptor agonists all seem to use GluIII:09 ($Glu^{124}$) as a key—supposedly charge-charge—anchor point in TM-III located next to the classic monoamine binding site AspIII:08. The interaction with GluIII:09 was described very early on for MK-677 and later also for ghrelin (Feighner et al., 1998; Holst et al., 2006) just as the corresponding GluIII:09 ($Glu^{119}$) in the closely related motilin receptor has been shown to be a key charge-charge interaction point for small-molecule agonists—such as erythromycin (Xu et al., 2005).

Besides GluIII:09, all the ghrelin agonist of the present study seem to interact also with PheVI:16, ArgVI:20, and PheVI:23 on the opposing face of TM-VI. In addition, they are also all dependent upon residues located on the inner face of TM-VII. However, here the agonists differ with respect to which residues they interact with. For example, MK-677 and L-692,429 are affected by substitutions at the most extracellular end of TM-VII—i.e., AsnVII:02 and PheVII:06—whereas the smaller SM compounds seem to interact exclusively and rather strongly with the deeply located PheVII:09 (Fig. 6). In this area, ghrelin is affected only by the substitution of GlnVII:-02, which in fact is located in ECL-3—as indicated by the generic numbering. This fits well with the general concept that larger, peptide agonists for binding tend to exploit mainly residues at the most extracellular end of the helices and the extracellular domains (Schwartz et al., 2006)—as demonstrated for example also for the motilin receptor (Matsuura et al., 2002, 2006).

At the other end of the binding pocket (i.e., at the interface between TM-III and TM-IV), all the small-molecule ghrelin agonists—but not ghrelin—seem to be dependent upon IleIV:20, which is located spatially close to the important GluIII:09 (Fig. 5). It is noteworthy that, together with position III:05, located right above III:09, position IV:20 was recently used for building an anchoring metal-ion site in the CXCR3 receptor for small-molecule agonists (Rosenkilde et al., 2007).

Thus, aromatic chelators—such as bipyridine and phenanthrolene—could be tethered through a bridging $Zn^{2+}$ or $Cu^{2+}$ ion in the resulting HisIII:05-AspIV:20 bidentate metal-ion site, to act as highly efficacious and potent agonists for the CXCR3 receptor. The receptor activation was achieved through a second-site aromatic-aromatic interaction between the chelator and TyrVI:16 requiring an inward movement of TM-VI in accordance with the Global Toggle Switch Model for 7TM receptor activation (Rosenkilde et al., 2007). Thus, we suggest that the small-molecule ghrelin receptor ligands may function as agonists in a manner rather similar to that shown in Fig. 7. That is, they bind to GluIII:09 and IleIV:20 at the interface between TM-III and -IV anchored through a charge-charge interaction instead of a metal-ion site and make second-site interactions with residues on the inner face of TM-VI and -VII, especially the aromatic cluster including PheVI:16 corresponding to TyrVI:16 of CXCR3. The aromatic cluster located between TM-VI and VII has previously been demonstrated to be essential for the high constitutive signaling of the ghrelin receptor (Holst et al., 2004).

**AspII:20—An Indirect Mutational Hit?** AspII:20, which upon substitution to Asn apparently is a strong hit for MK-677 and surprisingly increases the potency of L-692,429, is located far from the other mutational hits, which all cluster at the other end of the main ligand-binding pocket (Fig. 4B). Thus, most likely, the AspII:20 substitution affects the small-molecule agonists indirectly. It should be noted that substitutions of inward-facing residues in TM-II may have special effects in 7TM receptors. Thus, mutations in TM-II of the NK-1 receptor apparently affected the conformational interchange between agonist and antagonist preferring conformations, which could be mistaken for direct effects on ligand binding (Rosenkilde et al., 1994). It is likely, therefore, that the interface between TM-II and VII and perhaps III constitutes an important allosteric transition interface in the activation mechanism for the receptors and consequently that mutations here will be able to affect ligand binding and action indirectly in both positive and negative ways

**Overlapping Binding Site for Allosteric Modulators and the Endogenous Agonist.** Three of the small-molecule compounds—MK-677, L-692,429, and GHRP-6—have previously been shown to act not only as agonists but also as allosteric modulators. That is, they all shift the dose-response curve for ghrelin upward and thereby increase its maximal efficacy. In addition, L-692,429 acts as positive allosteric modulator and GHRP-6 as a negative modulator in respect of either increasing or decreasing the potency of ghrelin (Holst et al., 2005). Moreover, in radioligand binding experiments, neither L-692,429 nor GHRP-6 is able to inhibit the binding of the orthosteric ligand ghrelin—even at concentrations 100-fold above their observed $EC_{50}$. Thus, as judged through three different pharmacological approaches—as suggested by Christopoulos and Kenakin—these compounds behave as allosteric ligands (Christopoulos and Kenakin, 2002; May et al., 2007b). In the present study, we have mapped the residues important both for simple agonism and for allosteric modulation. It is noteworthy that the negatively charged glutamic acid in position III:09, which is totally essential for all tested agonists, including ghrelin, was also required both for the ability of L-692,429 and GHRP-6 to act as allosteric modulators. It should be emphasized that the GluIII:09-to-Gln mutation otherwise behaves totally normally with respect to both cell surface expression and ligand independent signaling. In addition, substitution of PheVI:23, which is another key interaction site for ghrelin, abolished L-692,429's allosteric modulation of ghrelin. Thus, two of the

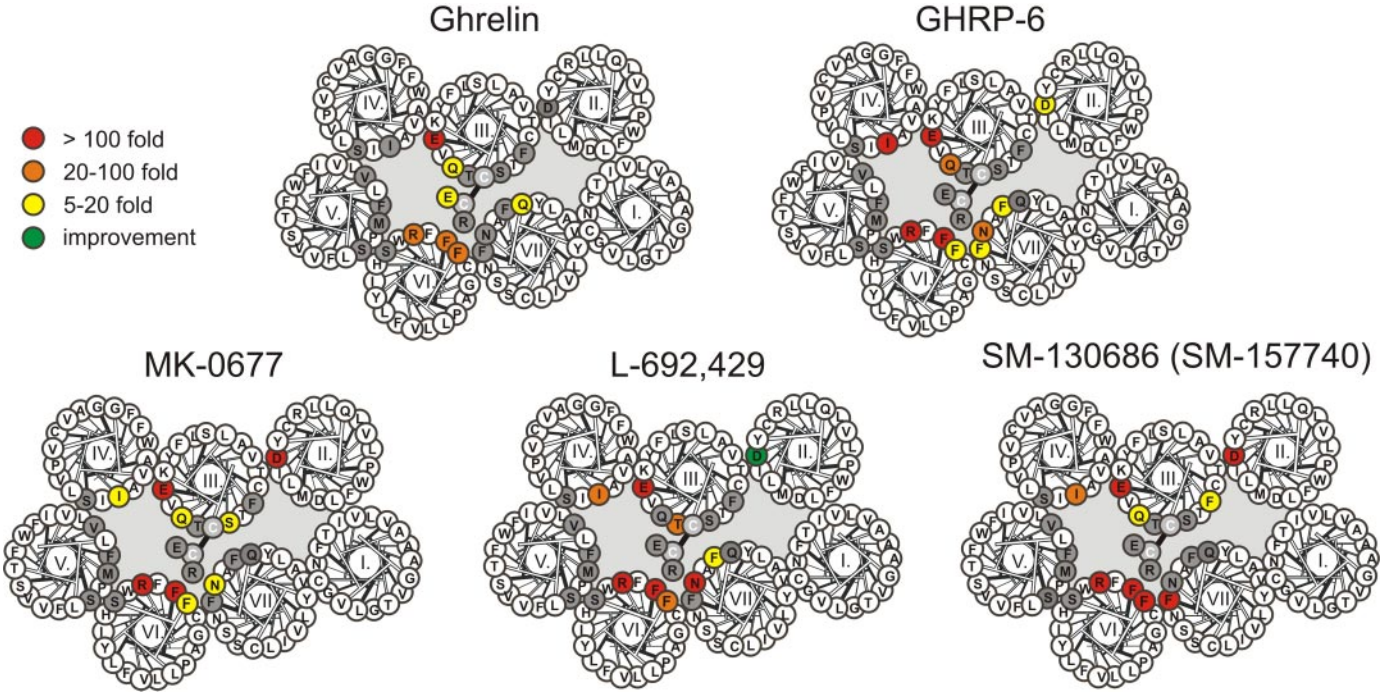


**Fig. 6.** Helical wheel diagram with the mutational map for each of the ghrelin receptor agonists analyzed. Residues that upon mutational substitution as indicated in Tables 1 and 2 affect the potency of the ligand 5- to 20-fold are marked in yellow, those that affect the potency of the ligand 20- to 100-fold are marked in orange, and those that decrease the potency more than 100-fold are indicated in red. The residue AspII:20, which upon mutational substitution increases the potency of the L-692,429, is indicated in green. Only the interaction pattern of SM-130686 is shown on this helical wheel diagram; however, SM-157740 is affected by the same mutations but approximately 10-fold less for each of the substitutions (see Table 2).

important interaction sites identified for ghrelin are shared with L-692,429 when it acts as an allosteric modulator. The allosteric properties of GHRP-6 were affected only by substitution of GluIII:09 and not by any other of the residues identified as agonist interaction hits.

Three different explanations have been suggested for how allosteric ligands could have overlapping binding sites with the endogenous agonist (Schwartz and Holst, 2007):

1. The ago-allosteric modulator could interchange between two—perhaps partly overlapping—but different binding modes. That is, one binding mode when it binds alone acting as an agonist, which is the binding site being mapped by mutations, and another binding mode when it acts as an allosteric modulator. The ago-allosteric modulator would then adopt the latter binding mode when the orthosteric binding site is occupied by the endogenous agonist. This type of dual interaction mode has been demonstrated for allosteric modulators acting on the A3 adenosine receptor (Gao et al., 2003; Gao and Jacobson, 2006).
2. The ago-allosteric ligand could influence the efficacy and potency of the endogenous ligand not by binding to the receptor at the same time but instead by biasing the dynamic interchange between inactive and active receptor conformations toward the active conformations, for which the endogenous agonist binds with high affinity.
3. A scenario in which the ligands act in different protomers of a dimeric receptor complex. The ago-allosteric modulator could then act through the orthosteric binding pocket of an "allosteric protomer," whereas the endogenous agonist bind to the corresponding orthosteric binding pocket of the "orthosteric protomer." There is very strong evidence that allosteric modulators acting on heterodimers of family C 7TM receptors function in this way (Pin et al., 2005; Schwartz and Holst, 2006, 2007). Although the occurrence and especially the physiological significance of dimers is still being debated for family A and B receptors, there is increasing evidence that small-molecule ligands may affect the binding and function of endogenous agonists through binding in "the other" protomer of a dimeric receptor complex, for example in chemokine receptors (Sohy et al., 2007).

**Differences in Mutational Map for Agonist versus Allosteric Property of the Same Compounds.** Although there was a clear overlap between the residues being important for the agonist effect and those being important for the allosteric effect of the two ago-allosteric modulators, it was also apparent that these mutational maps differed considerably. Besides GluIII:09 and the neighboring IleIV:20, the only identified mutational hits for the allosteric property of L-692,429 were the two most extracellularly located residues also being important for its agonism (i.e., PheVI:23 and AsnVII:02). In addition, the allosteric effect of L-692,429 was partly dependent upon $Arg^{199}$ in ECL-2, which was not a mutational hit for its agonism (Fig. 6). Thus, it seems that when acting as an allosteric modulator, L-692,429 binds more superficially in the binding pocket than when acting as an agonist. For GHRP-6, only a single residue down in the actual ligand-binding pocket was a hit for its allosteric effect, GluIII:09.

**Molecular Modeling of Agonist Receptor Complexes.** When the inactive, dark state of rhodopsin is used as a template for 7TM receptor modeling, a relatively large empty space is found corresponding to where the inverse agonist, 11-cis retinal was bound. As shown in Fig. 5 for the ghrelin receptor, low energy conformations of the nonpeptide agonists can be docked relatively well into this tempting, deep pocket to satisfy many of their respective mutational hits. In previous molecular modeling studies, which were based on

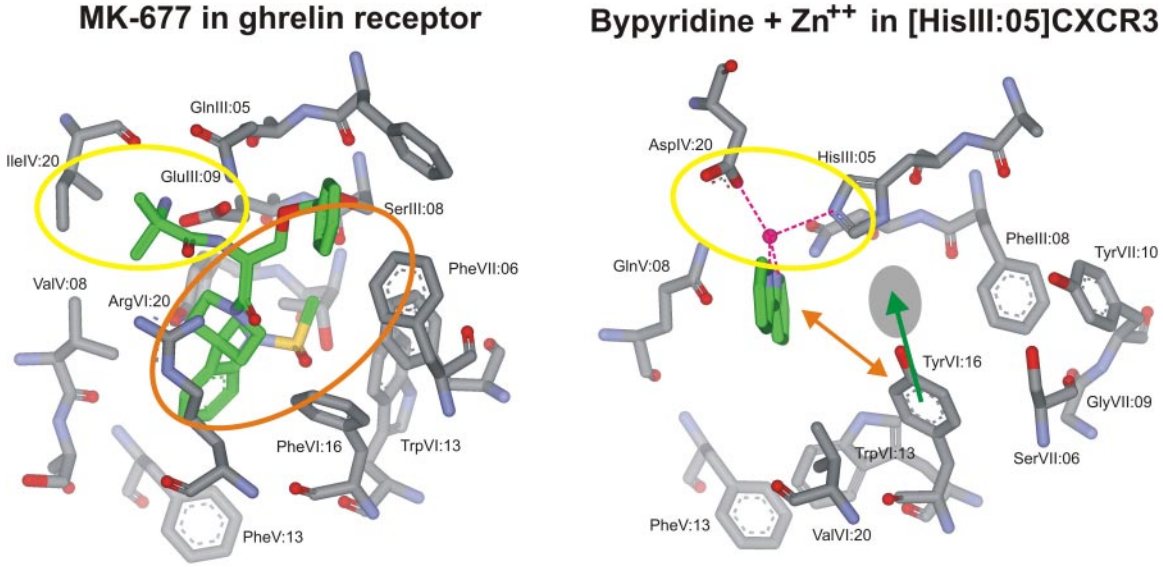


**Fig. 7.** Comparison of the proposed molecular interactions of a selected nonpeptide agonist—MK-677—and bipyridine in the metal-ion site engineered CXCR3 receptor. Yellow circles indicate the proposed initial anchoring sites of the nonpeptide compounds between TM-III and IV [i.e., by either a bridging metal-ion site (HisIII:05-AspIV:20) for bipyridine or by a charge-charge-interaction to GluIII:09 (stabilized by hydrophobic, aliphatic interactions with IleIV:20)]. In orange is indicated the "second site" interaction to key residues in TM-VI (i.e., an orange double arrow, showing for bipyridine the aromatic-aromatic interaction with TyrVI:16 in the CXCR3 receptor, and an orange circle, indicating the larger interaction pattern of MK-677 with residues on the corresponding face of TM-VI and VII in the ghrelin receptor). A green arrow indicates the direction of the inward movement of TM-VI, which is required for establishing the second site interaction in the CXCR3 receptor (see *Mutational Mapping of a Common Agonist Binding Pocket in the Ghrelin Receptor* for discussion of issues related to ligand docking and receptor activation).

very limited mutational data—i.e., basically only GluIII:09—compounds such as MK-677 were proposed to bind in relatively similar manners (Ye et al., 2000). However, although such ligand-receptor complex models may show nice complementary structural fits they are nevertheless somewhat problematic. Most importantly, these ligands are agonists but the presence of a large bulky, hydrophobic part of the ligands between TrpVI:13 and TM-V will most likely block the proposed TrpVI:13 rotamer-switch; i.e., it will in analogy with 11-cis retinal prevent the indole side chain of TrpVI:13 from rotating from its inactive conformer (shown in Fig. 7A) into what is believed to be its active rotamer state (as shown in Fig. 7B) (Shi et al., 2002; Schwartz et al., 2006). In this context, it is interesting that MK-677, for example, has been used as the basis for the development of structurally related ghrelin receptor antagonists, which very likely could bind in a mode similar to what is shown in Fig. 5A.

In contrast, for the agonists a well controlled mutational hit—PheVI:23 located at the extracellular end of TM-VI—is in the models not close enough to make a direct interaction with any of the compounds (Fig. 5). We propose that the limitation of the molecular models is due mainly to the fact that it is the inactive form of rhodopsin, which is used as template for the receptor model. The high resolution X-ray structure of the $\beta$2-adrenergic receptor in complex with an inverse agonist has been published; however, the helices surrounding the main ligand-binding pocket were in a configuration rather similar to that of rhodopsin (Cherezov et al., 2007; Rosenbaum et al., 2007). Unfortunately, no active form of any 7TM receptor is yet available. We have used Monte Carlo molecular simulations—guided by distance constraints derived form EPR and metal-ion site engineering—to generate models of presumed active 7TM receptor conformations (Hubbell et al., 2003; Schwartz et al., 2006; Rosenkilde et al., 2007). However, this was done in relatively simple cases [i.e., where the agonist was either a metal ion or a small, very simple metal-ion chelator (bipyridine)].[1] Nevertheless, the proposed inward movement of TM-VI during receptor activation—in accordance with the Global Toggle Switch Model—will bring PheVI:23 closer to the ligand, and it is likely that rather small adjustments in the backbone structure could allow for the phenyl side chain to position itself on top of the ligand and thereby help to hold TM-VI in the inwardly bent proposed active conformation.

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