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Ghrelin Receptor Inverse Agonists: Identification of an Active Peptide Core and Its Interaction Epitopes on the Receptor

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ABSTRACT

[D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]Substance P functions as a low-potency antagonist but a high-potency full inverse agonist on the ghrelin receptor. Through a systematic deletion and substitution analysis of this peptide, the C-terminal carboxyamidated pentapeptide wFwLX was identified as the core structure, which itself displayed relatively low inverse agonist potency. Mutational analysis at 17 selected positions in the main ligand-binding crevice of the ghrelin receptor demonstrated that ghrelin apparently interacts only with residues in the middle part of the pocket [i.e., between transmembrane (TM)-III, TM-VI and TM-VII]. In contrast, the inverse agonist peptides bind in a pocket that extends all the way from the extracellular end of TM-II (AspII:20) across between TM-III and TM-VI/VII to TM-V and TM-IV. The potency of the main inverse agonist could be *improved* up to 20-fold by a number of space-generating mutants located relatively deep in the binding pocket at key positions in TM-III, TM-IV and TM-V. It is proposed that the inverse agonists prevent the spontaneous receptor activation by inserting relatively deeply across the main ligand-binding pocket and sterically blocking the movement of TM-VI and TM-VII into their inward-bend, active conformation. The combined structure-functional analysis of both the ligand and the receptor allowed for the design of a novel, N-terminally Lys-extended analog of wFwLL, which rescued the high-potency, selective inverse agonism that was dependent upon both AspII:20 and GluIII:09. The identified pharmacophore can possibly serve as the basis for targeted discovery of also nonpeptide inverse agonists for the ghrelin receptor.

Since the hormone ghrelin was discovered in 1999, data have accumulated suggesting that it plays an important role not only in the control of growth hormone secretion—as originally expected and as reflected by its name—but also, importantly, in appetite regulation and food intake (Tschop et al., 2000; Nakazato et al., 2001). In the period leading up to a meal, increasing amounts of ghrelin are secreted from endocrine cells, especially in the stomach, apparently as a hunger signal from the gastrointestinal tract. The rise in plasma ghrelin levels is independent of time and food-related cues but correlates well with hunger scores, supporting the notion that ghrelin is of major importance for meal initiation (Cummings et al., 2004). The surge in plasma ghrelin levels drops shortly after food reaches the upper part of the intestine. The decrease in plasma ghrelin is proportional to the energy content of the meal, and simple distension of the stomach by water does not suppress plasma ghrelin levels (Callahan et al., 2004). Although ghrelin may act through afferent vagal mechanisms on centers in the brain stem, a major target for ghrelin seems to be the arcuate nucleus of the hypothalamus (Nakazato et al., 2001; Date et al., 2002). Ghrelin stimulates expression and release of transmitters, especially from the NPY/AgRP neurons. NPY will subsequently stimulate postsynaptic Y1 and Y5 receptors, whereas AgRP inhibits the activity of MC4 receptor-expressing neurons, which are believed to convey the major orexigenic (i.e., appetite stimulating) action of ghrelin (Schwartz and Morton, 2002; Holst and Schwartz, 2004).

We have reported that the ghrelin receptor apparently plays an important role on its own, independent of the ghrelin hormone. That is, the receptor is characterized by a very

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ABBREVIATIONS: NPY, neuropeptide Y; AgRP, Agouti related peptide; 7TM, seven transmembrane; SAR, structure-activity relationship; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction; MK-677, 2-amino-*N*-((*R*)-2-(benzyloxy)-1-((1-(methylsulfonyl)spiro(indoline-3,4′-piperidin)-1′-yl)carbonyl)ethyl)-2-methyl-propionamide monomethanesulfonate.

high basal signaling activity (Holst et al., 2003). Thus, in the absence of agonist, the ghrelin receptor signals with approximately 50% of its maximal efficacy as measured by $G\alpha_q$-mediated inositol phosphate accumulation. Ghrelin as well as various synthetic agonists for the ghrelin receptor will increase the signaling activity to approximately twice the basal level (Holst et al., 2005). Constitutive, ligand-independent activation of several other signaling pathways, including cAMP responsive element-controlled transcriptional activity, has also been demonstrated for the ghrelin receptor (Matthews et al., 1994). The cAMP responsive element signaling is particularly interesting, because it has been shown that this is an important signaling pathway for the inhibitory effect of leptin on the NPY/AgRP neurons of the hypothalamus in vivo (Shimizu-Albergine et al., 2001). High constitutive activity has been demonstrated for many 7TM receptors in various in vitro settings (Seifert and Wenzel-Seifert, 2002). However, it has been very difficult to verify whether such ligand-independent receptor signaling is of physiological importance. In the case of the ghrelin receptor, this verification was recently achieved through the identification of a naturally occurring mutation that selectively eliminated the constitutive activity without affecting the affinity, potency, or efficacy of the endogenous hormone ghrelin (Pantel et al., 2006). It is noteworthy that the occurrence of the mutation, which selectively affected the ghrelin receptor constitutive signaling, segregated with the development of very short stature and obesity (Holst and Schwartz, 2006; Pantel et al., 2006). It is conceivable that the decreased height of the carriers is mediated through decreased activity of the growth hormone pathway (Pantel et al., 2006).

[D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]Substance P was originally described to act as a rather low-potency (micromolar) antagonist for a number of peptide receptors, including the neurokinin receptors, the bombesin receptors, and the ghrelin receptor (Tsou et al., 1985; Woll and Rozengurt, 1988; Tullin et al., 2000). When probing the effect of various ghrelin receptor ligands on the very high constitutive activity of the receptor, we discovered that [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P, surprisingly, had a 100-fold higher potency as an inverse agonist than as an antagonist (Holst et al., 2003). This was demonstrated also by use of Schild-type analysis. Thus, over a reasonable dose-range, [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P functions as a selective inverse agonist for the ghrelin receptor with minimal antagonist activity. In the present study, we perform a systematic analysis of the structure-function relationship (SAR) for this peptide and thereby identify the active core structure. Moreover, by use of a mutational library with systematic substitutions throughout the main ligand-binding pocket of the ghrelin receptor, most of which display normal high constitutive activity, we identify presumed interaction sites both for the endogenous agonist ghrelin and for two inverse agonist peptides including the original [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P. The SAR analysis of the inverse agonists and the identification recognition epitopes for the different types of ligands allowed for the development of a novel, high-potency inverse agonist for the ghrelin receptor.

Materials and Methods

Materials. Ghrelin peptide was purchased from Bachem (Bubendorf, Switzerland). The N^{α}-9-fluorenylmethoxycarbonyl-protected amino acids, 1-hydroxy-benzotriazole and the 4-(2′,4′-dimethoxyphenyl-9-fluorenylmethoxycarbonyl-aminomethyl)-phenoxy (Rink Amide) resin were purchased from Novabiochem (Schwalbach, Germany). The side-chain protecting groups were *tert*-butyl for Tyr; trityl for Gln and His; 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl for Arg, *tert*-butyl ester for Asp, and *t*-butoxycarbonyl for Lys and diaminopropionic acid. *N*,*N*′-Diisopropylcarbodiimide was obtained from Sigma-Aldrich (Taufkirchen, Germany). Trifluoroacetic acid (TFA), 1-methyl-2-pyrrolidone, *N*-ethyldiisopropylamine, *t*-butanol, thioanisole, piperidine, propionic acid anhydride, *O*-(7-azobenzotriazol-1-yl)-*N*,*N*,*N*,*N*-tetramethyluronium hexafluorophosphate, *p*-thiocresol, 1,2-ethanedithiol, and trimethylsilylbromide were purchased from Fluka (Taufkirchen, Germany). Acetonitrile (for HPLC) was obtained from Merck (Darmstadt, Germany). Diethyl ether, dichloromethane, and dimethyl formamide (peptide synthesis grade) were obtained from Biosolve (Valkenswaard, The Netherlands).

Peptide Synthesis. The inverse agonist peptides were synthesized by solid-phase technique on an automated multiple peptide synthesizer (Syro-MultiSynTech, Bochum, Germany) by using the Rink amide resin (30 mg; resin loading, 0.6 mmol/g) as described recently (Lang et al., 2004). All peptides were cleaved from the resin in one step with the use of TFA, precipitated from ice-cold diethyl ether, washed, and finally lyophilized. Partially oxidized Met was reduced after lyophilization by applying a mixture of TFA/ethanedithiol/trimethylbromosilane [97.2:1.6:1.2 (v/v/v)] for at least 20 min and subsequently recovered from ice-cold diethyl ether, washed, and finally lyophilized. Purification of the peptides was achieved by preparative HPLC on an RP C-18 column (250 × 25 mm, 10 μm; Grace Vydac, Hesperia, CA) with a gradient of 20 to 60% B in A (A = 0.1% TFA in water; B = 0.08% TFA in acetonitrile) over 60 min and a flow of 10 ml/min (λ = 220 nm). The peptides were analyzed by matrix-assisted laser desorption ionization mass spectrometry on an Voyager-DE RP workstation (Applied Biosystems, Darmstadt, Germany) and by analytical reversed-phase HPLC on a Grace Vydac RP18-column (4.6 × 250 mm; 5 μm, 300 Å) using linear gradients of 10 to 60% B in A over 30 min and a flow rate of 0.6 ml/min (λ = 220 nm). The observed masses were in full agreement with the calculated masses, and the purity of all peptides was >95% according to analytical HPLC.

Molecular Biology. The human ghrelin/growth hormone secretagogue receptor cDNA was cloned by PCR from a human brain cDNA library. The cDNA was cloned into the eukaryotic expression vector pCMV-Tag(2B) (Stratagene, La Jolla, CA) for epitope tagging of proteins. Mutations were constructed by PCR using the overlap extension method (Horton et al., 1989). 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) 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 (Applied Biosystems).

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 as described previously (Holst et al., 1998). The amount of cDNA (20 μg/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 μCi of [*myo*-^{3}H]inositol (GE Healthcare, Little Chalfont, 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 supple-

mented with 10 mM LiCl at 37°C for 30 min. After stimulation with various concentrations of peptide for 45 min at 37°C, cells were extracted with 10% ice-cold perchloric acid followed by incubation on ice for 30 min. The resulting supernatants were neutralized with KOH in HEPES buffer, and the generated [^{3}H]inositol phosphate was purified on AG 1-X8 anion exchange resin (Bio-Rad Laboratories, Hercules, CA). Determinations were made in duplicates.

Competition Binding Assays. Transfected COS-7 cells were transferred to culture plates one day after transfection at a density of approximately 5000 cells per well aiming at 5 to 8% binding of the radioactive ligand. Two days after transfection, competition binding experiments were performed for 3 h at 4°C using approximately 25 pM [^{35}S]MK-677 (provided by Andrew Howard, Merck Research Laboratories, NJ). Binding assays were performed in 0.1 ml of a 50 mM HEPES buffer, pH 7.4, supplemented with 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 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 and 50 μl of lysis buffer/scintillation fluid (ethoxylated alkylphenol 30% and diisopropyl naphthalene isomers 70%) 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

Calculations. IC_{50} and EC_{50} values were determined by nonlinear regression using Prism software (ver. 3.0; GraphPad Software, San Diego, CA). The basal constitutive activity is expressed as a percentage of the ghrelin-induced activation for each mutant construct of the ghrelin receptor. Fmut indicates the -fold shift in potency or affinity induced by the structural change in the ligand compared with the [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P peptide in Table 1. In Table 2 Fmut indicates the -fold shift in potency induced by the structural change in the mutated receptor compared with the wild-type receptor.

Results

Structure Activity Relationship for the Inverse Agonist [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]Substance P. A systematic series of 20 analogs of the [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P peptide, which previously was found to be a high-potency inverse agonist for the ghrelin receptor (Holst et al., 2003), were made by solid phase synthesis, purified and characterized in COS-7 cells transiently transfected with the ghrelin receptor using both signal transduction assays measuring inositol phosphate turnover and competition binding assays against the ^{35}S-radiolabeled nonpeptide agonist MK-677 (Table 1 and Fig. 1).

Determination of the Active Peptide Core. Sequential truncation of the four N-terminal residues of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P (compounds 2–5) had almost no effect on the inverse agonist property of the peptide. Thus, the C-terminal heptapeptide, fQwFwLL (compound 5) had an inverse agonist potency of 29 nM, which is only 1.6-fold less than the full-length [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P (Fig. 2A). Likewise, the affinity of fQwFwLL was 17 nM versus 3.2 nM for the full-length peptide as measured in competition binding experiments. Further truncation of the C-terminal heptapeptide fQwFwLL by deletion of the N-terminal D-Phe residue (corresponding to D-Phe^5 in the mother peptide) (compound 6) decreased the inverse agonist potency and the binding affinity by 55- and 38-fold, respectively (Table 1, Fig. 2). This indicates that D-Phe^5 of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P is important for its inverse agonist activity.

Systematic alanine-substitutions were subsequently performed in the full-length peptide [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P (compounds 8–14) to try to determine the relative importance of the side chains within the fQwFwLL heptapeptide sequence, which apparently contained all of the structural information required to obtain potent and efficacious inverse agonism. The Ala-scan clearly showed that the side chains of Gln^6 and Leu^{11} were of minor importance for function and binding (compounds 9 and 14, Table 1 and Fig. 3). In contrast, the side-chains of D-Trp^7,

TABLE 1

Analysis of the SAR for [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P on the ghrelin receptor with respect to inverse agonist potency (i.e., EC_{50}) for inhibition of the constitutive stimulation of inositol phosphate accumulation and affinity (i.e., IC_{50}) in competition binding experiments using [^{35}S]MK677 as radioactive ligand as determined in transiently transfected COS-7 cells

Fmut indicates the -fold shift in potency or affinity induced by the structural change in the ligand compared with the [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P peptide.

		EC_{50}	n	Fmut	IC_{50}	n	Fmut
		nM			*nM*		
1	rPKPfQwFwLL-NH_2	18 ± 2	11	1.0	3.2 ± 0.7	4	1.0
2	PKPfQwFwLL-NH_2	25 ± 9	5	1.4	2.2 ± 0.4	3	0.7
3	KPfQwFwLL-NH_2	44 ± 3	4	2.4	17.0 ± 3	3	5.3
4	PfQwFwLL-NH_2	61 ± 23	3	3.4	>1000.0	4	>310
5	fQwFwLL-NH_2	29 ± 9	8	1.6	17.0 ± 4	3	5.3
6	QwFwLL-NH_2	990 ± 90	4	55.0	120.0 ± 30	4	38.0
7	wFwLL-NH_2	>1000[a]		5≫55.0	530.0 ± 230	4	170.0
8	rPKPAQwFwLL-NH_2	350 ± 50	3	19.0	68.0 ± 20	3	21.0
9	rPKPfAwFwLL-NH_2	32 ± 1	3	1.8	10.0 ± 3	3	3.1
10	rPKPfQAFwLL-NH_2	>1000	3	>55.0	>1000	3	>310
11	rPKPfQwAwLL-NH_2	310 ± 40	3	17.0	64.0 ± 5	3	20.0
12	rPKPfQwFALL-NH_2	>1000	3	>55.0	>1000	3	>310
13	rPKPfQwFwAL-NH_2	>1000	3	>55.0	110.0 ± 40	3	34.0
14	rPKPfQwFwLA-NH_2	31 ± 6	3	1.7	19.0 ± 3	4	5.9
15	rPKPfQWFwLL-NH_2	210 ± 6	3	12.0	97.0 ± 25	3	30.0
16	rPKPfQwFWLL-NH_2	>1000	3	>55.0	150.0 ± 19	3	47.0
17	rPKPFQwFwLL-NH_2	23 ± 5	4	1.3	4.3 ± 0.5	3	1.3
18	rPKPyQwFwLL-NH_2	12 ± 3	4	0.7	3.9 ± 1.8	3	1.2
19	rPKPwQwFwLL-NH_2	11 ± 4	3	0.6	11.0 ± 2	3	3.4
20	rPKP QwFwLL-NH_2	52 ± 14	4	2.9	26.0 ± 3.5	3	8.1
21	KPfQwFwL -NH_2	>1000	3	>55.0	38.0 ± 13	3	12.0
22	KPfQwFw -NH_2	>1000	3	>55.0	150.0 ± 30	3	47.0

[a] The dose-responds curve is biphasic, composed of a partial agonist component at 10 nM concentration and after partial inverse agonism.

D-Trp[9], and Leu[10] were all very important for the function of [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]substance P, because the corresponding Ala-substituted peptides were totally devoid of any inverse agonist activity (compounds 10, 12, and 13) (Table 1 and Fig. 3). Ala substitution of Phe[8] (compound 11) located between the two D-Trp residues shifted the dose-response curve 17-fold to the right.

The importance of the D versus L configuration of the three aromatic D-amino acid residues was further examined. The two essential D-Trp residues differed somewhat in this respect. Substitution of D-Trp[9] with L-Trp (compound 16), like the Ala substitution (compound 12), totally eliminated the inverse agonist activity, whereas substitution of D-Trp[7] with L-Trp (compound 15) was less deleterious in that it shifted the does-response curve only 12-fold to the right (Table 1). Substitutions of D-Phe[5] presented a different picture. Whereas deletion of D-Phe[5] as part of the initial N-terminal truncation resulted in an impressive 55-fold reduction in potency (compound 6) and Ala substitution of D-Phe[5] had a 19-fold effect (compound 8), substitution of D-Phe[5] with L-Phe (compound 17) was almost without effect. Similarly substitutions with D-Tyr or D-Trp (compounds 18 and 19) had limited effect on the inverse agonist function. We were most surprised to find that D-Phe[5] could be totally deleted from the middle of the [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]substance P sequence (compound 20) with only a minimal 2.9-fold shift in the inverse agonist dose-response curve (Table 1). Thus, although D-Phe[5] in some peptide constellations clearly seems to play a role for the inverse agonist property (compounds 6 and 8), the D-configuration is not in itself important, and the structurally unrelated N-terminal tetrapeptide of D-Arg-Pro-Lys-Pro can substitute for D-Phe[5] as an N-terminal extension of the wFwLL peptide (compound 5 and 20), despite the fact that each of these four residues in the truncation analysis apparently had no effect (compounds 2–5). Thus, D-Phe[5] is not in itself a critical residue, but it seems to contribute to the inverse agonist property in a constellation-dependent manner (see *Discussion*). One interpretation of these results could be that one or more positive charges—as found in the side chains of Arg and Lys and in the α-amino group—need to be present at a certain distance from the pentapeptide core to obtain the inverse agonist property.

Ala-substitutions performed at the C-terminal end of the [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]substance P showed that the

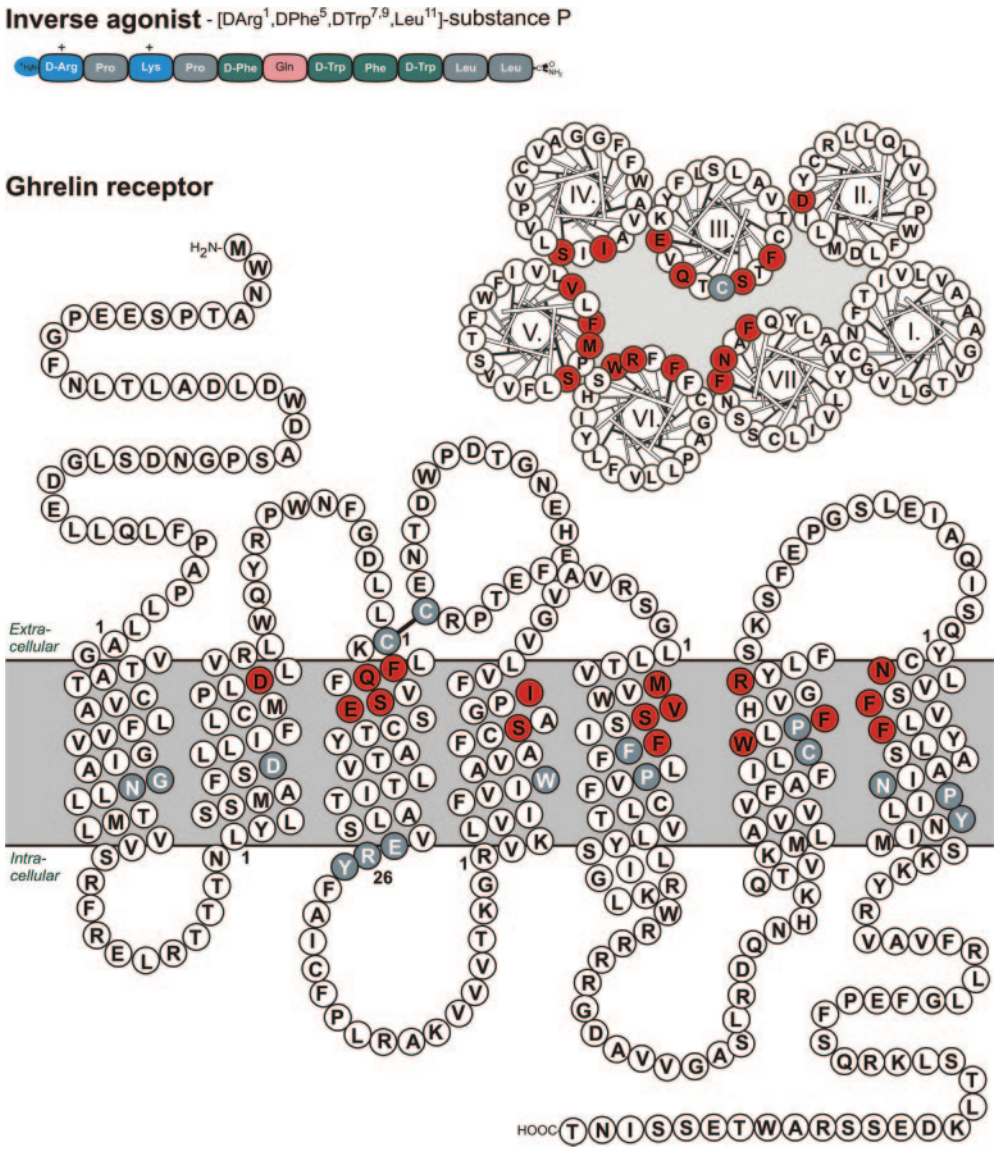

Fig. 1. Structure of the [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]substance P and the ghrelin receptor. On the schematic drawing of the amino acid sequence of [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]substance P in the upper part of the figure, the aromatic amino acids are green, the hydrophobic amino acids are gray, the hydrophilic noncharged amino acids are purple, and the positively charged amino acid is blue. In the serpentine and helical wheel diagram of the ghrelin receptor, the residues that are mutated in the present study are marked in black on red. The generic numbering system for 7TM receptor residues described by Baldwin (1993) and Schwartz (1994) is used throughout the article, and the most conserved residues of each helix, which are used as fix point for the numbering, are marked with white on gray. The numbers of the conserved residues are determined by their location in the helix and the proposed first residue of each transmembrane helix is indicated by "1".

side-chain of Leu[10] was essential whereas the side chain of Leu[11] was dispensable (compounds 13 and 14 and Fig. 3). Nevertheless, C-terminal truncation of Leu[11] totally eliminated the inverse agonism (compounds 21 and 22), whereas binding properties were preserved; the affinities were 38 and 150 nM, respectively. The combination of these observations indicates that although the side chain of Leu[11] is not important for the function of the peptide, this residue still serves an essential function, probably as a simple backbone spacer that positions the C-terminal carboxy-amide group correctly.

In conclusion, the initial truncation analysis defined the C-terminal heptapeptide as important for the inverse agonist property of [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]substance P; however, more detailed analysis showed that both D-Phe[5] and Gln[6] are in fact dispensable, which indicates that the core peptide for the inverse agonist property is the C-terminal pentapeptide. This pentapeptide, wFwLL (compound 7) in itself displayed an interesting biphasic dose-response curve (Fig. 2). At low nanomolar concentrations, wFwLL acted as a partial agonist, whereas at higher micromolar concentrations, it was a partial inverse agonist (Fig. 2A). In competition binding against [^{35}S]MK-677, the wFwLL pentapeptide competed with an apparent affinity of 530 nM (Table 1 and Fig. 2B).

Ghrelin Receptor Mutants for Mapping of Ligand Binding Sites. Mutations at 17 key positions in the main ligand-binding pocket of the ghrelin receptor were selected from a large library of ghrelin receptor mutants for analysis of their absolute and relative effect on the potency of the agonist ghrelin compared with two inverse agonist peptides: the full-length [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]substance P and the C-terminal heptapeptide fQwFwLL (Table 2). These mutations cover generally known, ligand-binding positions located in the extracellular segments of TM-II through TM-VII (Fig. 1). To be able to determine the effect of the substi-

TABLE 2

Mutational mapping of the binding site for the agonist Ghrelin and the inverse agonists [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]substance P (SP-analog) and fQwFwLL using a library of 17 mutant versions of the ghrelin receptor with substitutions systematically placed through the main ligand-binding crevice

The potency (EC_{50}) of the compounds with respect to either stimulating (agonist) or inhibiting (inverse agonist) the constitutive stimulation of 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 compared with the wild-type receptor. In the first column is shown the constitutive activity of the mutant receptors expressed as percentage of basal signaling activity compared with the maximal ghrelin-stimulated activity.

	Constitutive Activity	n	Ghrelin		Fmut	SP Analog		Fmut	fQwFwLL		Fmut
			EC_{50}	n		EC_{50}	n		EC_{50}	n	
	%		*nM*			*nM*			*nM*		
WT-Ghrelin R1a	42 ± 1	53	0.34 ± 0.04	53		18.0 ± 2	12		21 ± 5	15	
AspII:20Asn (Asp[99])	56 ± 5	4	1.10 ± 0.3	4	3.2	850.0 ± 150	3	47.00	530 ± 230	3	25.0
PheIII:04Ser(Phe[119])	38 ± 4	6	0.42 ± 0.05	6	1.3	160.0 ± 18	5	8.90	180 ± 24	5	8.5
GlnIII:05Ala(Gln[120])	47 ± 4	7	2.20 ± 0.8	7	6.5	>1000		>56.00	>1000		>47.0
SerIII:08Ala (Ser[123])	35 ± 3	8	0.32 ± 0.08	8	0.9	4.7 ± 0.2	3	0.26	39 ± 11	3	1.8
GluIII:09Gln (Glu[124])	41 ± 3	4	86.00 ± 22	8	250.0	260.0 ± 59	3	14.00	170 ± 45	3	8.0
SerIV:16Ala (Ser[174])	43 ± 2	8	0.47 ± 0.07	8	1.4	1.0 ± 0.3	4	0.06	68 ± 12	3	3.2
IleIV:20Ala (Ile[178])	46 ± 2	11	0.72 ± 0.11	11	2.5	400.0 ± 40	4	22.00	>1000	3	>47.0
MetV:05Ala (Met[213])	43 ± 6	8	0.40 ± 0.04	8	1.2	8.4 ± 1.3	3	0.47	64 ± 4	3	3.0
ValV:08Ala (Val[216])	52 ± 2	5	1.00 ± 0.3	5	2.9	2.7 ± 0.8	3	0.15	49 ± 1	3	2.3
SerV:09Ala (Ser[217])	46 ± 4	4	0.39 ± 0.13	4	1.1	11.0 ± 3	3	0.61	98 ± 11		4.7
PheV:12Ala (Phe[220])	20 ± 1	12	0.44 ± 0.07	12	1.3	1.9 ± 0.3	4	0.11	14 ± 4	4	0.67
TrpVI:13Ala[a] (Trp[276])		4	3.2 ± 0.8	3	9.4			(13)[a]			(5.0)[a]
PheVI:16Ala (Phe[279])	2 ± 2	10	14.00 ± 3	10	41.0						
ArgVI20:Gln (Arg[283])	17 ± 4	7	13.00 ± 2	7	38.0	74.0 ± 2	3	4.10	160 ± 50	4	7.6
AsnVII:02Ala (Asn[305])	16 ± 2	5	2.90 ± 0.5	5	8.5	14.0 ± 6	3	0.78	110 ± 30	3	5.2
PheVII:06Leu (Phe[309])	42 ± 2	6	0.50 ± 0.12	6	1.5	120.0 ± 32	5	6.70	>1000	3	>47.0
PheVII:09Ala (Phe[312])	15 ± 1	8	1.30 ± 0.2	8	3.8	46.0 ± 7	3	2.60	>1000		>47.0

[a] Because of the lack of constitutive activity of TrpVI:13Ala, the inverse agonists are tested in competition binding using [^{35}S]MK-677 as a radioligand.

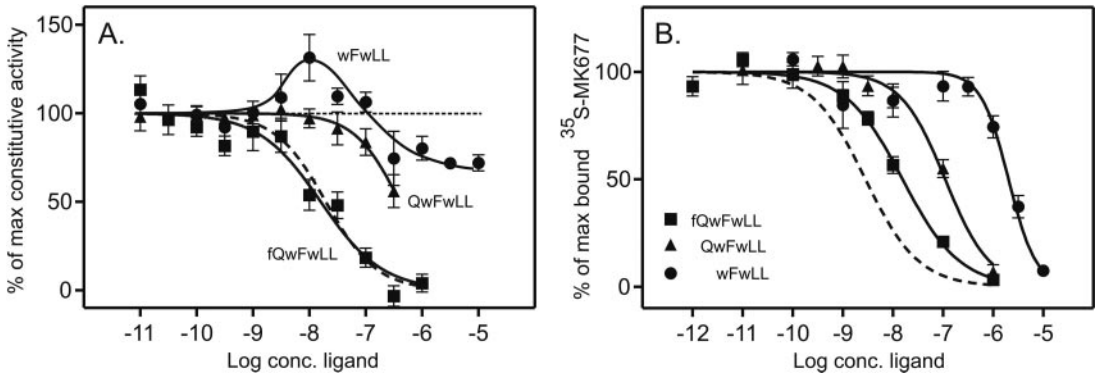

Fig. 2. Amino-terminal truncation of [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]substance P tested for inverse agonist properties and binding affinity on COS-7 cells transiently transfected with wild-type ghrelin receptor. Inhibition of basal inositol phosphate turnover (A) and competition binding study using ^{35}S-labeled MK-677 as a radioligand (B). The ligands used are [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]substance P (dashed line) and three truncated versions of this peptide: FQwFwLL (■), QwFwLL (▲) and wFwLL (●). Data are mean ± S.E. of three to five independent experiments performed in triplicate.

tutions on the potency of the inverse agonists, mutations were selected to have little or no effect on the constitutive activity and yet represent a reasonable structural change. As shown in Table 2, except for PheVI:16 to Ala and TrpVI:13 to Ala, the mutants displayed a constitutive activity of 15% or more as measured in the inositol phosphate turnover assay. This allowed a robust determination of the potency of the inverse agonist peptides. A slight increase in constitutive activity from 42% in the wild-type ghrelin receptor to 56% was observed in the AspII:20 mutant (Table 2), which is particularly interesting because this residue selectively affects inverse agonists and not the agonist ghrelin (see next section).

Mutational Mapping of the Presumed Binding Site for the Ghrelin Hormone. Three major plus three minor mutational hits were identified among the 17 mutations tested in the present study: GluIII:09 (250-fold rightward shift in the dose-response curve), PheVI:16 (41-fold), ArgVI:20 (38-fold); as well as GlnIII:05 (6.5-fold), TrpVI: 13Ala (9.4-fold), and AsnVII:02 (8.5-fold). In view of the results with the inverse agonist peptides (see next section) it should be noted that none of the substitutions in TM-II, TM-IV, and TM-V affected the potency of ghrelin; i.e., the Fmut values varied between 1.1 and 3.2 (Table 2). Thus, within the main ligand-binding pocket, the mutational map for the relatively large (28 amino acid residue) ghrelin is limited and restricted to key residues on the opposing faces of the extracellular ends of TM-III, TM-VI, and TM-VII, in accordance with the toggle-switch model for 7TM receptor activation.

Mutational Mapping of the Presumed Binding Site for the Inverse Agonist [D-Arg^1,D-Phe^5,D-Trp^{7,9}, Leu^{11}]Substance P. When comparing the mutational maps of the inverse agonist [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}] substance P and the smaller heptapeptide inverse agonists with that of ghrelin, there is clearly an overlap in the mutational hits; however, there are also noticeable differences in the quantitative effects of the common hits (Table 2 and Figs. 4 and 6). The major mutational hits, which decreased the potency for [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P were: AspII:20 (47-fold), GlnIII:05 (>58-fold), GluIII:09 (14-fold), IleIV:20 (20-fold) and TrpVI:13 (13-fold) (Fig. 4), whereas the minor hits were: PheIII:04 (8.9-fold) and PheVII:06 (6.7-fold). Thus, the mutational map for [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}] substance P overlaps with that of ghrelin at the interface between TM-III, TM-VI, and TM-VII. However, the mutational map for the inverse agonist extends both toward TM-II (PheIII:04 and AspII:20) and toward TM-IV (IleIV:20), which was not the case for the ghrelin agonist.

In addition, among the substitutions in TM-V and its interface with TM-IV, an interesting picture emerged where several mutations actually *increased* the potency of the inverse agonist

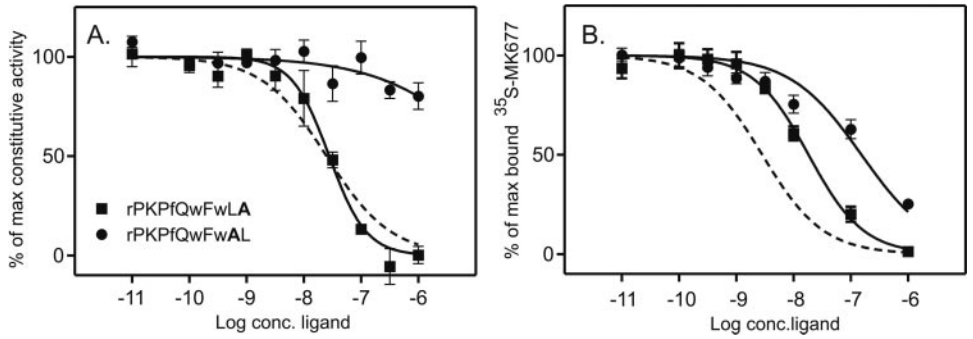

Fig. 3. Carboxyl-terminal alanine substitution of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P. The modified peptides are tested for inverse agonist properties and binding affinity on COS-7 cells transiently transfected with wild-type ghrelin receptor. Inhibition of basal inositol phosphate turnover (A) and competition binding using ^{35}S-labeled MK-677 as a radioligand (B) by [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P (dashed line), [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Ala^{11}] substance P with an alanine most C terminally (■) and [D-Arg^1,D-Phe^5,D-Trp^{7,9},Ala^{10},Leu^{11}]substance P with an alanine substitution in position 10 (●). Data are mean ± S.E. of three to five independent experiments performed in duplicate.

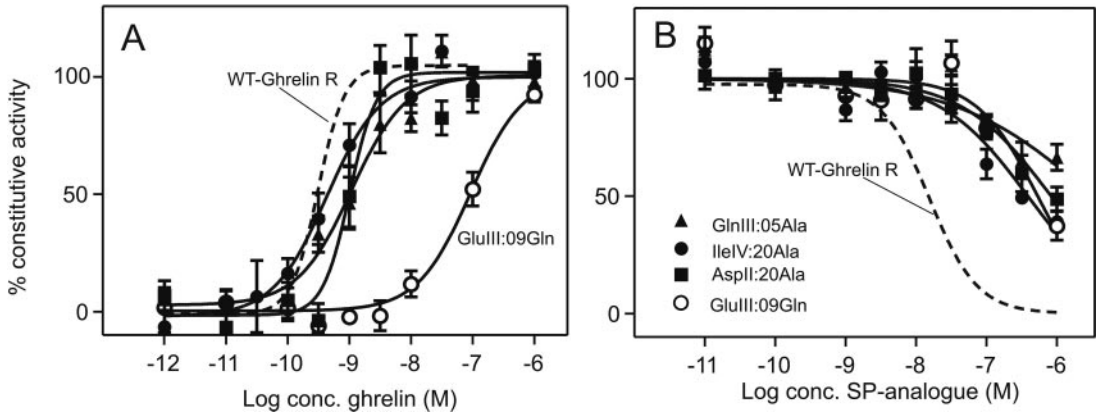

Fig. 4. Structural determinants for function of the agonist ghrelin and the inverse agonist [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P. Ghrelin (A) and [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P (B) were tested for modulation of inositol phosphate turnover in COS-7 cells transiently transfected with the wild-type ghrelin receptor (dashed line) or three different mutant versions of the ghrelin receptor: IleIV:20Ala (●), GlnIII:05Ala (▲), GluIII:09Gln (○), and AspII:20Ala (■). The localizations of the mutated residues on the receptor are shown in Fig. 7. Data are mean ± S.E. of three to five independent experiments performed in duplicate.

(Fig. 5), which is a rather unusual observation in mutational mapping of ligands. The potency for [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P was increased almost 20-fold in the SerIV:16 mutant compared with the wild-type ghrelin receptor (Fig. 5C). Similarly, in all four mutations in TM-V, the potency of the inverse agonist was increased from approximately 2- to 10-fold (Fig. 5C, Table 2). Thus, the mutational map for [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P in fact extends from AspII:20 all the way to TM-V, with a cluster of mutational hits that increased the potency of the inverse agonist.

Mutational Mapping of the Presumed Binding Site for the Heptapeptide Inverse Agonist fQwFwLL. Both the major and the minor mutational hits for the C-terminal heptapeptide fQwFwLL were very similar to those of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P itself. However, for fQwFwLL, two additional major hits were found in TM-VII: PheVII:06 (>47-fold), which was a minor hit for the full-length peptide, and PheVII:09 (>47-fold). Moreover, the mutations in TM-IV and TM-V, all of which clearly increased the potency for the longer [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P, had either no effect or a slight decreasing effect on the C-terminal heptapeptide (Table 2).

We conclude that the inverse agonist peptides bind in an extended pocket reaching from AspII:20 between TM-III and TM-VI/VII all the way to TM-IV and TM-V (Fig. 7). This is in contrast to the agonist ghrelin, which seems to interact only with residues in the center of this pocket (i.e., between TM-III, TM-VI, and TM-VII. It is noteworthy that the potency of the [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P inverse agonist could be significantly improved by space-generating mutants located relatively deep in the pocket between TM-III, TM-IV, and TM-V (Fig. 7).

Rescue of High Potency Ghrelin Receptor Inverse Agonism. As mentioned above, the interpretation of the SAR analysis of the inverse agonist peptide was that one or more positive charges need to be placed at a certain distance N-terminally to the wFwLL pentapeptide core. This fits well also with the mutational analysis identifying an elongated, presumed binding pocket for these inverse agonists in which negatively charged residues are found at each end (i.e., GluIII:09 and AspII:20). Thus, to try to rescue the high potency inverse agonism, which was lost when the [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P was truncated to the core pentapeptide wFwLL (Fig. 6B), we synthesized the carboxy-amidated KwFwLL peptide in which both an α-amino group and an ε-amino group is available at two different distances N-terminally to the core peptide. As shown in Fig. 6, KwFwLL was in fact a highly efficacious and potent inverse agonist for the ghrelin receptor displaying a potency similar to that of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P. Preliminary mutational mapping of this novel compound demonstrated that the inverse agonist activity of KwFwLL was dependent upon both AspII:20 and GluIII:09 and to a larger degree than the two other inverse agonists (Fig. 6C, Table 2). It is noteworthy that Ala-substitution of each of these acidic residues converted the high-potency dose-response curve observed for the KwFwLL inverse agonist in the wild-type ghrelin receptor into a biphasic curve similar to that observed for the core-pentapeptide in the wild-type receptor.

Discussion

Systematic structure-function analysis of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P (Fig. 1), a potent undecapeptide inverse agonist of the ghrelin receptor, revealed that the C-terminal heptapeptide fQwFwLL is sufficient to obtain full potency and full inverse agonist efficacy. C-terminal pentapeptide wFwLX (where X represent any amino acid) probably represents the core peptide. Mutational analysis of the receptor binding site for the two inverse agonists (i.e., the full-length [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P and the fQwFwLL heptapeptide) gave a rather similar picture, with mutational hits spanning across the entire main ligand-binding crevice of the ghrelin receptor (Fig. 7). This picture was strikingly different from that obtained for the ghrelin agonist peptide, which was affected only by a few centrally located substitutions in the main ligand binding pocket. It is noteworthy that a cluster of space-generating substitutions on the inner faces of TM-III, TM-IV, and TM-V *improved* rather than impaired the potency for the [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P inverse agonist without affecting the potency for ghrelin. It should be noted that [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P has the novel property of being a highly selective inverse agonist with minimal antagonist activity on the ghrelin receptor (Holst et al., 2003).

Inverse Agonist SAR. The conclusion on the SAR analysis of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P was that the core peptide, which is responsible for the inverse agonism, is

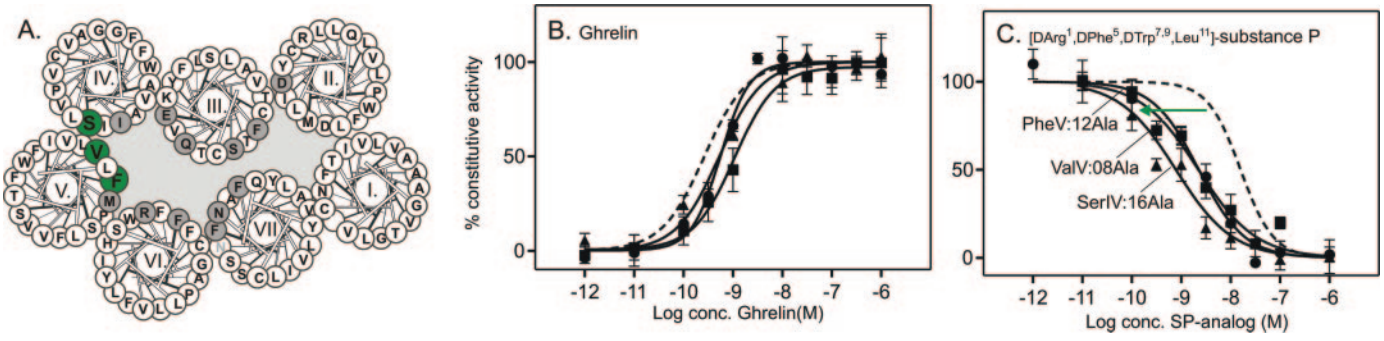

Fig. 5. Improved inverse agonism of the mutation made in TM-IV and TM-V. A, a helical wheel diagram of the most extra-cellular located residues of the wild-type ghrelin receptor where the residues that have been substituted according to Table 1 are marked with gray. The residues in TM-IV and TM-V that have been substituted are marked with green. Dose-response curve of ghrelin (B) and [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P (C) on the following ghrelin receptor mutations: SerIV:16Ala (▲), ValV:08Ala (■), and PheV:12Ala (●). The wild-type curve is represented by a dashed curve. In B and C, the experiments are made in transiently transfected COS-7 cells. Data are mean ± S.E. of three to five independent experiments performed in duplicate.

the C-terminal carboxyamidated pentapeptide wFwLX, in which the presence—but not the side chain—of the last residue is important. However, on its own, this pentapeptide did not inhibit the constitutive signaling of the ghrelin receptor. On the other hand, potent and efficient inverse agonism could be obtained by extending this peptide at the N-terminal end with different amino acid residues, which surprisingly could be varied considerably in respect to structure. For example, D-Phe-Gln- (compound 5) and D-Arg-Pro-Lys-Pro-Gln (compound 20) were rather similar in providing inverse agonist potency and efficacy to the wFwLL peptide core. The Ala-substitution analysis revealed that each of the side chains of these N-terminal amino acids was replaceable by a simple methyl group or, as observed for D-Phe^5, could be deleted entirely. The hypothesis that an N-terminal positive charge placed at a certain distance from the wFwLL core peptide, which is the key to the inverse agonism, was confirmed through the synthesis of KwFwLL. This Lys-extended pentapeptide is the smallest, high-potency full inverse agonist yet described for the ghrelin receptor.

The analogs and fragments of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P were characterized both in a functional inositol phosphate accumulation assay and in competition binding assays. It is noteworthy that the results from these two assays did not in all cases quantitatively agree. For example, the PfQwFwLL peptide (compound 4) was an inverse agonist almost equally potent and efficacious compared with the two other peptides, which were one amino acid residue shorter or longer (compounds 3 and 5, respectively). In contrast to these two peptides, the PfQwFwLL peptide was unable to displace [^{35}S]MK-677 from the ghrelin receptor even at a concentration of 1000 nM. A similar phenomenon has previously been observed where an agonist was competing at apparent low affinity against a radiolabled antagonist tracer despite its actual high affinity for the receptor (Rosenkilde et al., 1994). Conversely, the C-terminally truncated peptide KPfQwFwL (compound 21) rather selectively lost its inverse agonist property with only approximately a 10-fold decrease in binding affinity (Table 1). In principle, an apparent loss of the inverse agonist property can be the result of either a loss of the ability to bind to the receptor (i.e., it is a hit both in functional and binding assay) or a specific loss of inverse agonist activity (i.e., the compound still binds, but it cannot decrease the constitutive activity). In the latter case, the compound is then either a neutral antagonist or it may even have gained agonist efficacy and may be "balancing between" agonism and inverse agonism. It should be noted that small chemical changes in nonpeptide agonists can, in certain receptor systems, change them into antagonists (Perlman et al., 1997).

Receptor Binding Mode for Ghrelin. In the present study, we find that the mutational hits for ghrelin itself in the main ligand binding crevice of the receptor are restricted to residues located on the opposing, inner faces of TM-III, TM-VI, and TM-VII (i.e., restricted to the middle part of the main ligand-binding crevice) (Fig. 7A). It is noteworthy that

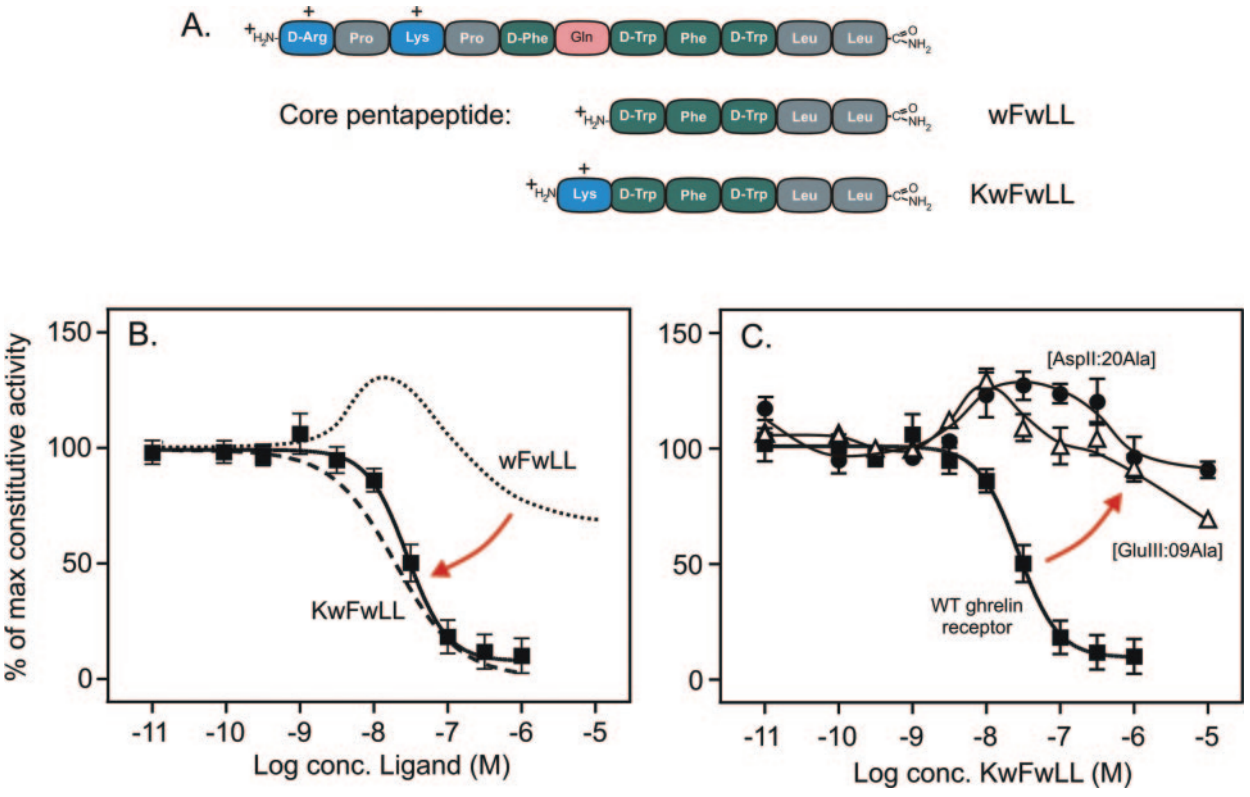

Fig. 6. Rescued inverse agonism through N-terminal extension of the core pentapeptide wFwLL with positively charged Lys, which is dependent on AspII:20 and GluIII:09 at each end of the presumed binding pocket. A, schematic drawing of the mother peptide inverse agonist [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P, the pentapeptide wFwLL (biphasic dose-response curve), and the novel lysine-modified pentapeptide KwFwLL (rescue the inverse agonist function). B, dose-response curve of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P (dashed line), the pentapeptide wFwLL (dotted line), and the lysine-modified pentapeptide KwFwLL (–■–) measured as inositol phosphate turnover. C, dose-response curve of the lysine-modified pentapeptide KwFwLL on the wild-type ghrelin receptor (■) and two mutant versions of the receptor GluIII:09Gln (Glu^{124}) (△) and AspII:20Ala Asp^{99} (●) measured as inositol phosphate turnover.

these proposed interaction sites for the ghrelin agonist coincide with the center of action in the proposed global toggle switch model for 7TM receptor activation (Schwartz et al., 2006). According to this model, receptor activation is associated with a vertical seesaw movement of mainly TM-VI and TM-VII around a pivot corresponding to the conserved Pro residue in the middle of each of these helices. It is noteworthy that the extracellular segments of TM-VI and TM-VII in particular are believed to move inward toward TM-III in the main ligand-binding crevice, whereas the intracellular ends of these helices are moving the opposite way to reveal epitopes to be recognized by, for example, G proteins (Hubbell et al., 2003; Schwartz et al., 2006). This model is supported by observations including distance constraints from activating metal ion sites and disulfide tethered ligands, both cases involving residues on the opposing faces of TM-III, TM-VI, and TM-VII (i.e., where the ghrelin hits are found in the present study) (Elling et al., 1999, 2006; Buck and Wells, 2005). In the case of the relatively large ghrelin peptide, we envision that in addition to the proposed contact residues in the middle of the main ligand-binding pocket (Fig. 7A), the 28-amino acid agonist peptide will probably have additional contact points with the receptor located, for example, in the loop regions—by analogy to many other peptide hormones, chemokines, etc. (Schwartz et al., 2006). It is noteworthy that GluIII:09, which in the present study is found to be a major

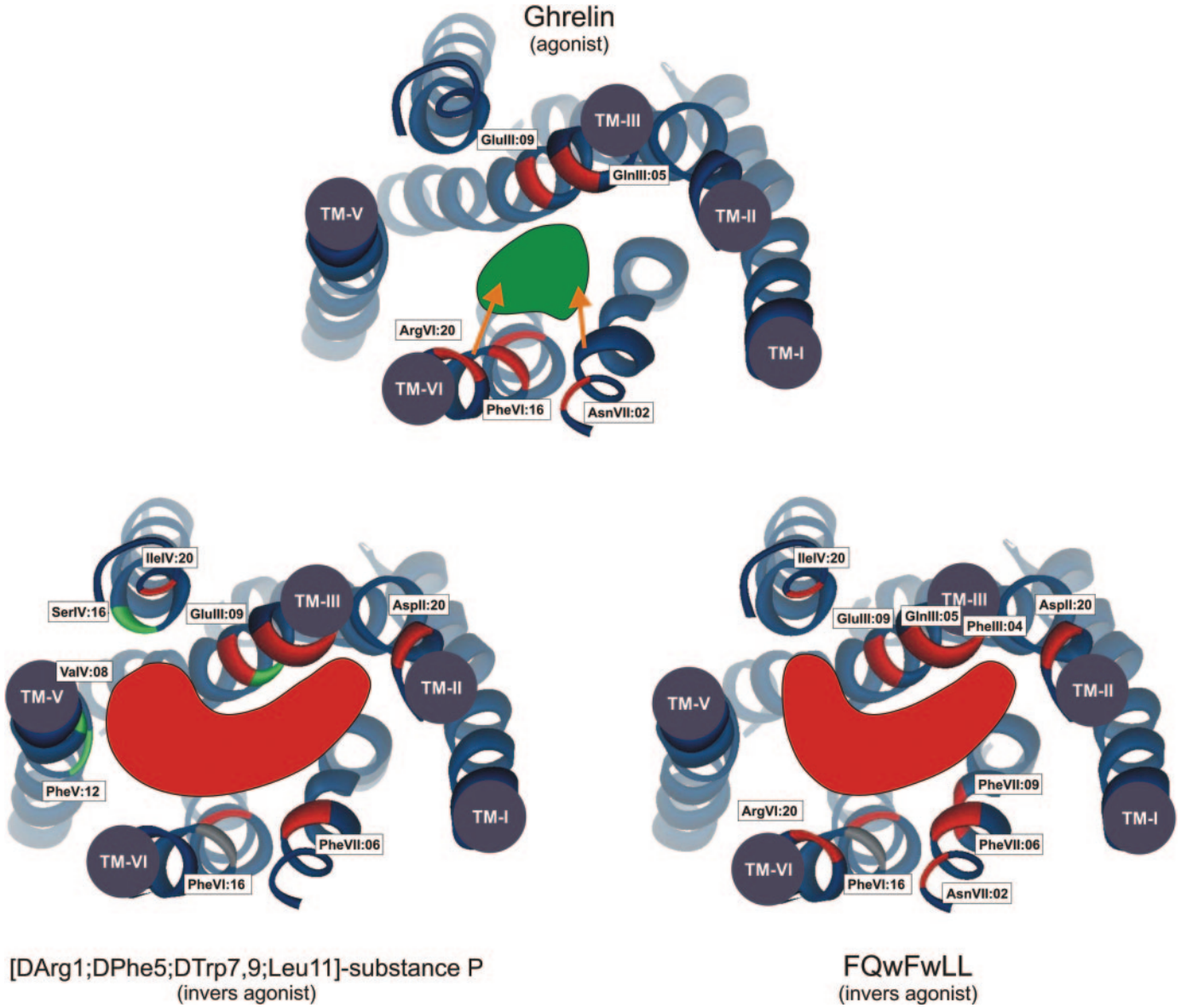

Fig. 7. Schematic model of the proposed binding mode for the inverse agonist and agonist in the main ligand-binding crevice of the ghrelin receptor. Molecular models of the ghrelin receptor—built over the X-ray structure of the inactive form of rhodopsin—as viewed from the extracellular side, where only the seven helical domains are shown in solid ribbon with the identified mutational hits for the inverse agonist peptides (lower part) and the endogenous agonist ghrelin (upper part) highlighted. Mutational hits that impair potency are shown in red, and hits that improve inverse agonist potency are shown in green. The location of PheVI:16, which is part of the aromatic cluster between the extracellular ends of TM-VI and TM-VII, is shown in gray. This residue may be part of the binding pocket for the inverse agonists; however, this could not be determined in the present study because mutation of this residue eliminates the constitutive activity. The space presumed to be occupied by the agonist ghrelin is indicated in green on the upper part of the figure; orange arrows indicate the movements occurring during activation. The much larger space presumed to be occupied by the inverse agonist to prevent activation is indicated in red on the lower part of the figure.

hit for ghrelin, was in an early report on the molecular mechanism of action of growth hormone secretagogues found also to be essential for the action of positively charged nonpeptide agonists, such as MK-677 (Feighner et al., 1998). It is noteworthy that GluIII:09 is apparently less important for the action of the long inverse agonist peptides, which are also all positively charged but instead are highly dependent on the presence of AspII:20 in TM-II (Table 2).

Receptor Interaction Mode for the Inverse Agonist Peptides. The mutational maps for the two inverse agonists that were characterized in the present study were rather similar to each other but differed from the mutational map for the agonist ghrelin by also including residues in, for example, TM-II and TM-IV (i.e., on each side of the central binding pocket between TM-III, TM-VI, and TM-VII (Fig. 7). Thus, the mutational analysis in the main ligand-binding crevice of the receptor demonstrated that the inverse agonist peptides act through binding to residues scattered throughout a pocket extending all the way from AspII:20 at the extracellular end of TM-II across the main ligand-binding crevice between TM-III and TM-VI/VII to the extracellular ends of TM-IV and TM-V (Fig. 7, B and C). A major difference between the mutational map for [D-Arg^{1},D-Phe^{5},D-Trp^{7,9},Leu^{11}]substance P and that for its C-terminal heptapeptide, fQwFwLL, was that a relatively large cluster of space-generating mutation in the pocket between TM-III, TM-IV, and TM-V increased the potency for [D-Arg^{1},D-Phe^{5},D-Trp^{7,9},Leu^{11}]substance P but not for the heptapeptide inverse agonist (Fig. 7).

It is not possible, based on the available data, to determine precisely how a large flexible peptide of 7 or even 11 amino acids is located in the proposed binding pocket of the receptor. Nevertheless, we suggest that the central aromatic tripeptide—wFw—of the inverse agonists interacts with the characteristic central aromatic cluster in TM-VI and TM-VII of the ghrelin receptor (i.e., TrpVI:13, PheVI:16, Phe,VII:06, and PheVII:090, and perhaps also PheIII:04). This aromatic cluster has previously been shown to be essential for the constitutive activity of the ghrelin receptor, and other members of this family of receptors, by systematic structure-function analysis (Holst et al., 2004). The constitutive activity of the ghrelin receptor and the homologous GPR39 receptor can, in fact, be systematically tuned up and down depending on the size and aromaticity of, in particular, the residue in position VI:16 in the presence of a large hydrophobic residue in positions VII:06 and VII:09 (Holst et al., 2004). It has been suggested that these aromatic residues may serve as covalently "tethered" ligands that are located strategically at the interface between TM-III, TM-VI, and TM-VII and thereby hold or "glue" the extracellular segments of these helices in the active conformation and thus mediate the high constitutive activity of the ghrelin receptor (Schwartz et al., 2006). In the present context, we propose that by interacting with this aromatic cluster in the receptor, the inverse agonists prevent the inward movement of TM-VI and TM-VII in particular and thereby block receptor activation (Fig. 7). It should be noted that we were not able to probe the importance of the central PheVI:16 residue for the binding and action of inverse agonists because mutations of this residue eliminate its constitutive activity (Holst et al., 2004).

In a scenario where the aromatic side chains in the middle of the peptide interact with residues in TM-VI and TM-VII, it is likely that the backbone of this peptide makes hydrogen bond interactions with the key residues in TM-III (i.e., GlnIII:05 and SerIII:08) (Table 2). In this crude picture of a hypothetical binding mode, the two ends of the inverse agonists could, in principle, extend into each of the two "side pockets" of the main ligand binding crevice (Fig. 7). In the "shallow" TM-II, TM-III, TM-VII pocket of the receptor, the inverse agonists are all highly dependent on AspII:20. Thus, it could be speculated that this negatively charged residue could make a charge-charge interaction with the positively charged amino-terminal end of the inverse agonist ligands, which needs to be spaced to a certain distance from the wFw core of the ligand to obtain high potency and efficacy (Table 1). In this scenario, the C-terminal carboxyamidated -Leu-X peptide could then extend toward GluIII:09 and IleIV:20 (Fig. 7). The opposite possibility, where the highly divergent N-terminal end of the inverse agonists instead bind in the TM-III, TM-IV, TM-V pocket is supported by the observation that the mutational maps for the two inverse agonists differ mainly in this pocket, where a number of mutations increase the potency of the larger ligand, [D-Arg^{1},D-Phe^{5},D-Trp^{7,9},Leu^{11}]substance P (Fig. 7). The relatively deeply located space-generating substitutions in the TM-III, TM-IV, TM-V pocket could allow the peptide to bind deeply in the main ligand binding pocket and thereby perhaps obtain a more optimal, specific interaction elsewhere in the receptor (for example, with the aromatic cluster on TM-VI and TM-VII). More experiments are clearly needed to determine the precise binding mode for both the inverse agonist peptides and the ghrelin agonist. Nevertheless, we propose that the bulky peptides obtain their inverse agonist property by binding relatively deeply and broadly in the main ligand binding crevice, thereby preventing the inward movement of TM-VI and TM-VII toward TM-III in accordance with the global toggle switch model for 7TM receptor activation (Fig. 7) (Schwartz et al., 2006). The high dependence upon a charge-charge interaction between the receptor and the ligand is supported by the notion that the minimal inverse agonist KwFwLL rescued the inverse agonist properties lost by N-terminal truncation of [D-Arg^{1},D-Phe^{5},D-Trp^{7,9},Leu^{11}]substance P. Furthermore, mutations in the receptor that removed the negatively charged GluIII:09 or AspII:20 induced the same molecular phenotype as observed by the core peptide wFwLL in the wild-type receptor (Fig. 6).

Development of Nonpeptide Ghrelin Receptor Inverse Agonists. The ghrelin receptor is an agonist prone receptor; i.e., it is relatively "easy" to discover small molecule ligands that act as agonists on this receptor, as exemplified by the multitude of different growth hormone secretagogue compounds (Smith et al., 1997). Nevertheless, we have previously proposed that inverse agonists for the ghrelin receptor could possibly be useful antiobesity agents, in that they would be expected to eliminate the high constitutive, ghrelin-independent signaling activity of this "hunger receptor" between meals (i.e., at a time where the ghrelin hormone levels are low) (Holst and Schwartz, 2004). Thus, in theory, ghrelin receptor inverse agonists should be able to decrease the craving for second-order meals, desserts, and snacks. The present SAR analysis of the inverse agonist peptides and their binding site in the ghrelin receptor provides important information about the key structural elements that are required to obtain inverse agonism in this receptor. The development of

the KwFwLL inverse agonist peptide indicates that the residues identified as mutation hits are in fact important for a pharmacophore model for inverse agonism in the ghrelin receptor. Several structure- or knowledge-based approaches have been applied to the discovery of nonpeptide antagonists for 7TM receptors such as site-directed drug discovery, tethered ligand discovery etc. (Buck et al., 2005; Frimurer et al., 2005). It will be interesting, based on the present knowledge about inverse agonist receptor interactions, to apply such methodd to specifically attempt to develop antagonists, and inverse agonists in particular, for the ghrelin receptor.

Acknowledgments

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