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High Constitutive Signaling of the Ghrelin Receptor—Identification of a Potent Inverse Agonist

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Ghrelin is a GH-releasing peptide that also has an important role as an orexigenic hormone-stimulating food intake. By measuring inositol phosphate turnover or by using a reporter assay for transcriptional activity controlled by cAMP-responsive elements, the ghrelin receptor showed strong, ligand-independent signaling in transfected COS-7 or human embryonic kidney 293 cells. Ghrelin and a number of the known nonpeptide GH secretagogues acted as agonists stimulating inositol phosphate turnover further. In contrast, the low potency ghrelin antagonist, [D-Arg^{1},D-Phe^{5},D-Trp^{7,9},Leu^{11}]-substance P was surprisingly found to be a high potency (EC_{50} = 5.2 nM) full inverse agonist as it decreased the constitutive signaling of the ghrelin receptor down to that observed in untransfected cells. The homologous motilin receptor functioned as a negative control as it did not display any sign of constitutive activity; however, upon agonist stimulation the motilin receptor signaled as strongly as the unstimulated ghrelin receptor. It is concluded that the ghrelin receptor is highly constitutively active and that this activity could be of physiological importance in its role as a regulator of both GH secretion and appetite control. It is suggested that inverse agonists for the ghrelin receptor could be particularly interesting for the treatment of obesity. (*Molecular Endocrinology* 17: 2201–2210, 2003)

THE STORY OF ghrelin, its receptor and synthetic compounds acting through this receptor unraveled in a unique "reverse" order. In the eighties, a synthetic hexa-peptide from a series of opioid-like peptides was found to be able to release GH (1) from isolated pituitary cells (1). Because this action was independent of the GHRH receptor, several pharmaceutical companies embarked upon drug discovery projects based on this hexa-peptide GH secretagogue (GHS) and its putative receptor. Several series of potent and efficient peptide as well as nonpeptide GHS were consequently described in the mid nineties (2–4). However, first several years later was the receptor through which these artificial GHS acted eventually cloned and shown to be a member of the 7TM G protein-coupled receptor family (5, 6). But, first in 1999 was the endogenous ligand for this receptor the hormone ghrelin finally discovered and surprisingly found to be produced in large amounts in endocrine cells in the stomach and only to a small extent centrally as originally expected (7).

Abbreviations: ADA, Adenosine deaminase; AGRP, agouti-related peptide; CRE, cAMP-responsive element; CREB, CRE binding protein; GHS, GH secretagogue; HEK, human embryonic kidney; IP, inositol phosphate; K_d, dissociation constant; K_i, affinity constant; MC, melanocortin; NPY, neuropeptide Y; ORF-74, open reading frame 74; SP-analog/SP-a, [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-substance P; 7TM, seven transmembrane.

Ghrelin is a 28-amino-acid peptide, which has a unique structure among peptide hormones as it is acylated at Ser^{3} usually with an n-octanyl moiety (7, 8). This posttranslational modification is essential for the activity of the hormone both *in vitro* and *in vivo* (7, 9). The ghrelin receptor was initially—as expected—found on GH producing cells in the pituitary and like the GHRH receptor it stimulates pulsatile secretion of GH (5). However, ghrelin receptors were also discovered in other parts of the central nervous system especially in the hypothalamus (10). Accordingly, both intracerebroventricular and iv injection of ghrelin strongly stimulates feeding and increases body weight in rodents—independent of its effect on GH secretion (11, 12). It appears that ghrelin is involved in the initiation of food intake as the plasma level of the peptide increases significantly just before meals (13, 14). Thus, ghrelin functions not only as a GH-releasing substance but also as an orexigenic hormone and the physiological role of ghrelin appears to be a link or messenger between the stomach and the hypothalamus and the pituitary. Centrally, ghrelin acts mainly on receptors expressed on neuropeptide Y (NPY)/agouti-related peptide (AGRP) producing cells in the arcuate nucleus of the hypothalamus because antibodies and antagonists of NPY and AGRP abolish the ghrelin-induced feeding response (12, 15–17). Interestingly, the ghrelin receptor was recently found to be expressed in large amounts also on afferent vagal neurons (18, 19). In

accordance with this, the effect of peripheral administration of ghrelin on c-*fos* expression in NPY/AGRP neurons and the effect on feeding in rats is totally dependent on an intact vagal nerve, whereas the effect on GH secretion was only partially mediated through the proposed vagal afferent pathway (18). These findings indicate that gastric vagal afferents may be the major pathway conveying ghrelins signaling from the stomach to the central nervous system. Interestingly, the closest homolog to the ghrelin receptor is the receptor for motilin (Fig. 1), which like ghrelin is a hormone secreted from the upper part of the gastrointestinal tract and which also interacts with the autonomic nervous system (19, 20).

For many years GH release was used to monitor activity in the—at that time still elusive—secretagogue receptor to search for agonists. Subsequently, when the receptor was eventually cloned (5), increases in intracellular calcium became the functional read-out of choice because this assay, meanwhile, was established as the dominating industrial standard for monitoring receptor signaling—especially for Gq-coupled receptors such as the ghrelin receptor. However, it is very hard to detect constitutive, ligand-independent signaling in a receptor using measurements of intracellular calcium as a read-out, due to the fact that intracellular calcium is kept within very stringent margins. In the present study, we have instead used inositol phosphate turnover as a measure of Gq signaling through the phospholipase C pathway, and we have tested the ability of the ghrelin receptor to activate cAMP-responsive element (CRE)-driven gene transcription. Surprisingly, we found that the ghrelin receptor in fact is highly constitutively active in both of these assays and among the different previously employed ligands, we identified [D-Arg^{1},D-Phe^{5},D-Trp^{7,9},Leu^{11}]-substance P as being a high efficacy and importantly a high potency inverse agonists for the ghrelin receptor.

RESULTS

Ligand-Independent Signaling of the Ghrelin Receptor through the Phospholipase C Pathway

Determinations of inositol phosphate (IP) accumulation was used as a measure of signaling through the Gq, phospholipase C pathway in COS-7 cells transiently transfected with the human ghrelin receptor. Gene-dosing experiments demonstrated a dose-dependent but ligand-independent increase in IP accumulation in cells expressing the ghrelin receptor as opposed to cells transfected with the empty pcDNA3

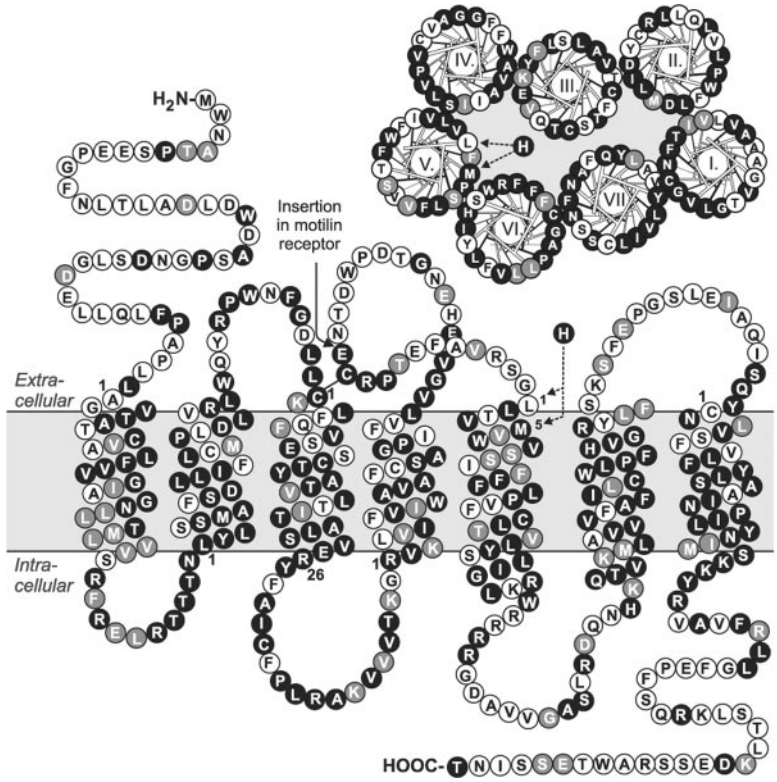

Fig. 1. Serpentine and Helical Wheel Diagram of the Ghrelin Receptor

Residues that are identical (*white on black*) or structurally conserved (*white on gray*) between the ghrelin and its closest homologue, the motilin receptor, are highlighted. The position in the extracellular loop 2 of an unusually long insertion of 39 amino acids in the motilin receptor, which is not found in the ghrelin receptor, is shown by an *arrow*. The histidine residues introduced as a bis-His metal ion site in the extracellular part of the fifth transmembrane segment are indicated with a *dotted arrow*.

vector as a negative control (Fig. 2A). Because it previously has been shown that adenosine possibly could act as an agonist on the ghrelin receptor and because adenosine perhaps could be produced by the cells used for transfection, we pretreated the cells with adenosine deaminase (ADA). However, ADA did not affect the observed ligand-independent signaling of the ghrelin receptor (Fig. 2A) and pretreatment with the same concentration of ADA totally blocked the cAMP inhibition observed upon stimulation of the cells transfected with the adenosine receptor (data not shown). An increased production of IP was observed in cells transfected with the ghrelin receptor upon stimulation with 10^{-6} M ghrelin, which was most clearly observed at the higher levels of receptor expression (Fig. 2A).

That the ghrelin receptor signals with an unusually high degree of constitutive activity, was most clearly demonstrated by comparing its activity to that displayed by its closest homolog, the motilin receptor. In cells transfected with the motilin receptor, the ligand independent production of IP was similar to that observed in cells transfected with the empty expression vector, *i.e.* being 19 and 21%, respectively, of that observed in cells transfected with the ghrelin receptor (Fig. 2B). Upon stimulation with the motilin peptide ligand, IP accumulation reached a level comparable to that observed in cells transfected with the ghrelin receptor after stimulation with the ghrelin agonist (Fig. 2B). In fact, the constitutive, ligand-independent signaling of the ghrelin receptor was comparable to that observed with one of the most well-established highly constitutively active 7TM receptors, the virally encoded ORF74 receptor (Fig. 2B) (21, 22).

Ligand Binding Analysis

A number of peptide and nonpeptides, most of which are known to be agonists on the ghrelin receptor, were selected and initially studied in binding assays (for structures see Fig. 3). The data in the literature concerning affinities of these ligands vary considerable depending on choice of tracer and assay conditions (23, 24). As shown in Table 1, ghrelin itself and the peptide agonist, ipamorelin competed against ^{125}I-ghrelin with subnanomolar affinity. Interestingly, the two nonpeptide compounds L-692,400 and L-692,429, which previously have been shown to compete with nanomolar affinity against a structurally similar radiolabeled compound, MK677 (24, 25), were unable to compete for binding against the peptide agonist ^{125}I-ghrelin in concentrations up to 1 uM (Table 1). In other 7TM receptor systems, differences in affinities of 100- to 10,000-fold have been observed dependent on whether it was determined in homologous or heterologous competition assays (21, 26–28). However, [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P, which has been described to act as an antagonist with micromolar potency on the ghrelin receptor (23), was found to compete against ^{125}I-ghrelin with a surprisingly high affinity, *i.e.* K_i = 45 nM.

Peptide and Nonpeptide Agonist Modulation of the Constitutive Signaling of the Ghrelin Receptor

Ghrelin stimulated IP accumulation with an EC_{50} of 0.19 nM up to approximately the double level of that observed without ligand (Fig. 3). As expected based on previous reports using calcium mobilization assays most of the peptide and nonpeptide ligands behaved as agonist with variable potencies and efficacies also in the functional assay measuring IP turnover. L-692,429 displayed an efficacy like ghrelin and a potency only 20-fold less than the endogenous agonist, EC_{50} = 3.9 nM (Fig. 3). The hexa-peptide analog ipamorelin had a potency of 13 nM and an efficacy of 65% of that of ghrelin. NN703, which in respect of GH secretion is known as a full agonist (23), surprisingly

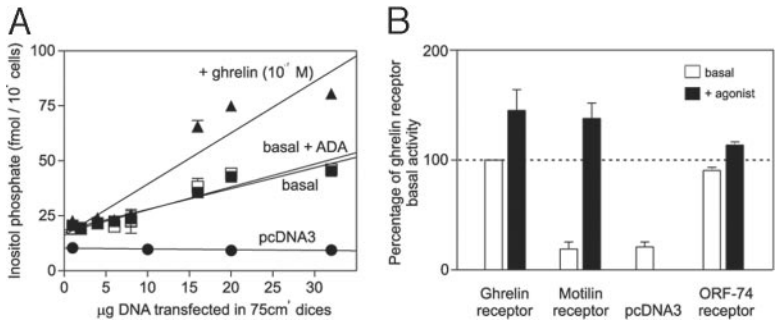

Fig. 2. Constitutive Signaling of the Ghrelin Receptor as Determined by Analysis of IP Turnover

A, Gene-dosing experiments with the ghrelin receptor in transiently transfected COS-7 cells: basal constitutive activity (*full square*), constitutive activity after incubation for 30 min with adenosine deaminase (ADA) (*open square*) compared with the ghrelin agonist-stimulated activity (*full triangle*) and the activity in cells transfected with the empty vector pcDNA3 (*full circles*). Transfection with 20 μg DNA gives a receptor expression of 12.6 fmol/10^5 cells measured by homologous competition binding curves. Data are mean ± SE of three independent experiments made in triplicate. B, Comparison of the basal, constitutive activity (*open bars*) and the agonist-stimulated activity (*closed bars*) of the ghrelin receptor (ghrelin 10^{-6} M), the motilin receptor (motilin 10^{-6} M), and the ORF-74 receptor from human herpes virus 8 (GRO-α 10^{-7} M) all transfected with 20 μg DNA, as described in *Materials and Methods*. Data are mean ± SE of three independent experiments made in triplicate.

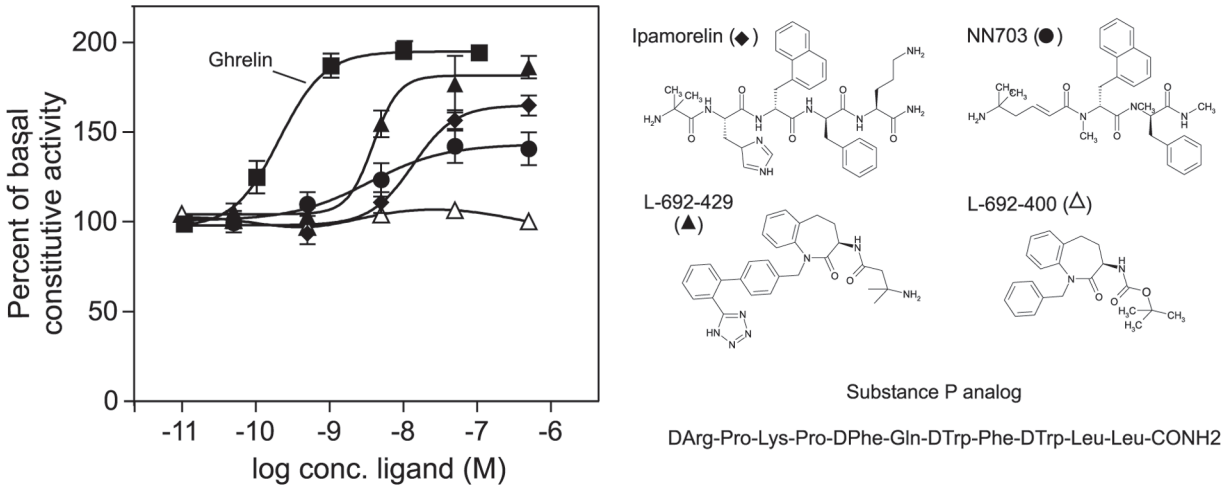

Fig. 3. Dose Response Curves for a Selection of GHS for Stimulation of IP Turnover

The EC_{50} for ghrelin (*full square*) was 0.19 ± 0.06 nM, for ipamorelin (*full diamond*) 13 ± 4 nM, for NN0703 (*full circles*) 3.4 ± 0.7 nM and for L-692–429 (*full triangle*) 3.9 ± 1.2 nM. L-692–400 (*open triangle*) did not increase the basal activity of the receptor through the phospholipase C pathway. Experiments were performed in transiently transfected COS-7 cells (20 μg DNA in 75-cm^2 discs) and mean ± SE of three to five independent experiments made in duplicate are shown.

Table 1. Competition Binding Experiments Using ^{125}I-Ghrelin as Tracer

	$K_{d/I}$	SEM n
	(nM)	
Ghrelin	0.36 ± 0.3	4
Ipamorelin	2.2 ± 1.3	3
NN703	35 ± 3	3
L-692,429	[a]	4
L-692,400	[a]	4
[D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}] (Substance P)	45 ± 8	3

The ghrelin receptor was transiently transfected into COS-7 cells as described in *Materials and Methods*. B_{max} is 12.6 fmol/10^{-5} cells.

[a] The compounds were unable to displace the radioligand.

also functioned only as a partial agonist (43%) in respect of IP turnover (Fig. 3). L-692,400, a compound structurally similar to L-692,429, which has been shown to be an antagonist on the ghrelin receptor (29), was here found to be a neutral ligand as it did not change IP turnover in the ghrelin receptor-transfected cells (Fig. 3).

Inverse Agonist Modulation of the Constitutive Signaling of the Ghrelin Receptor

[D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P has previously been described as being a low potency ghrelin receptor antagonist (23). We could confirm that, as the substance P analog inhibited the ghrelin-stimulated IP accumulation with an EC_{50} value of 630 nM. Importantly, when [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P was applied to the ghrelin receptor in the absence of ghrelin it was—in contrast to the hexapeptide analogs and nonpeptide ligands—found to function as a high efficacy, full inverse agonist as it inhibited the spontaneous, ligand-independent signaling in cells transfected with the ghrelin receptor down to the level observed in cells transfected with the empty expression vector (Fig. 4A). [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P had no effect on the constitutive activity of the ORF-74 receptor (data no shown). Unexpectedly, the potency of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P as an inverse agonist on the ghrelin receptor was observed to be 5.2 nM, which is approximately 50- to 100-fold higher than the potency of the same peptide when studied as an antagonist against submaximally, ghrelin stimulated IP accumulation (Fig. 4A). Schild-type analysis also demonstrated that the ligand independent, basal signaling of the ghrelin receptor was inhibited by low doses of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P, which did not shift the dose-response curve for ghrelin to the right (Fig. 4B). Thus [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P is a high potency, high efficacy inverse agonist for the constitutive, ligand-independent signaling of the human ghrelin receptor, which functions as a relative low potency antagonist for ghrelin-induced signaling.

Metal-ion site engineering has previously been used as a molecular probe for both antagonism, agonism and inverse agonism (21, 30, 31). Here we built a metal-ion binding site into the ghrelin receptor by substituting residues V:01 and V:05 with His residues (Fig. 1). As shown in Fig. 5, Zn(II) functioned as a full inverse agonist on the metal-ion site engineered receptor with a potency of 4.3 μM through binding to the two His residues located in an *i* and *i+4* position at the extracellular end of TM-V. This demonstrates that the li-

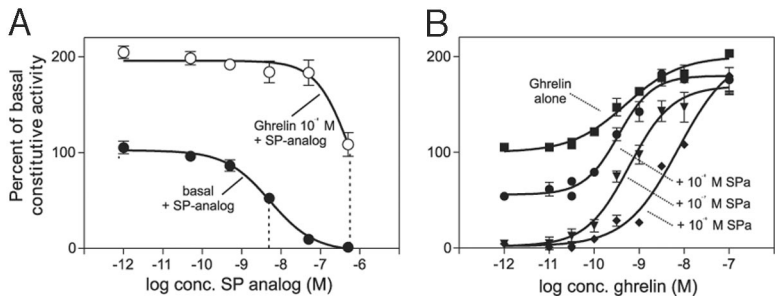

Fig. 4. Effect of [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]-Substance P on the Constitutive Activity (*Full Circle*) and on the Ghrelin-Stimulated IP Turnover (*Open Circle*)

A, The IC_{50} for [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]-Substance P acting as an antagonist against ghrelin stimulated signaling was 630 ± 20 nM, whereas its IC_{50} as an inverse agonist was 5.2 ± 0.7 nM. The stimulatory dose-response curve for ghrelin is indicated as a *dotted curve* for comparison (see Fig. 3). B, Dose-response curves for ghrelin in the presence and absence of D-Arg[1], D-Phe[5], D-Trp[7,9],Leu[11]]-Substance P (SP-analog) in three different concentrations; 10^{-6} M (*diamonds*), 10^{-7} M (*triangles*), and 10^{-8} M (*squares*). Experiments were performed in transiently transfected COS-7 cells (20 μg DNA in 75-cm^2 discs) and mean ± SE of three to five independent experiments made in duplicate are shown.

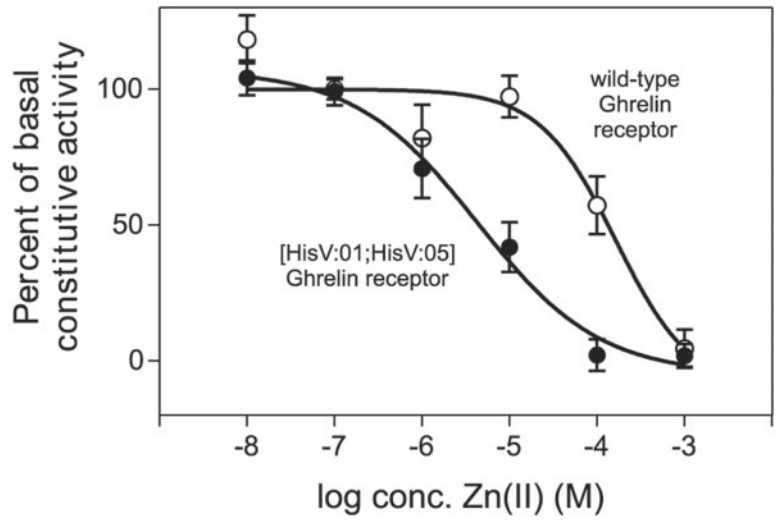

Fig. 5. Inverse Agonism of Zn(II) through Binding to a Metal-Ion Site Engineered into the Extracellular End of TM-V of the Ghrelin Receptor

The IC_{50} for Zn(II) on the wild-type ghrelin receptor (*open circles*) and on the metal ion site engineered receptor (*closed circles*) was 160 ± 70 μM and 4.3 ± 0.2 μM, respectively. The EC_{50} for ghrelin is 1.9 as shown in Fig. 3 and the EC_{50} for the bis-His modified receptor is 12 ± 2 nM. Data are mean ± SE of three independent experiments performed in duplicate in transiently transfected COS-7 cells (20 μg DNA in 75-cm^2 discs).

gand-independent activity of the ghrelin receptor can be blocked through binding and stabilization of the extracellular end of TM-V as in the ORF-74 receptor (21).

Signaling through the CRE Pathway

Activity in Gq coupled receptors can often be determined in CRE reporter assays through phosphorylation of the CRE binding protein (CREB) by downstream kinases of the Gq pathway especially the Ca^{++}/calmodulin-dependent kinase IV (32, 33). Thus, gene-dosing experiments with the ghrelin receptor performed in transiently transfected human embryonic kidney (HEK)-293 cells resulted in a dose-dependent but ligand-independent stimulation of the CRE-luciferase reporter assay (Fig. 6A). Ghrelin stimulated this activity further whereas [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]-substance P again acted as an inverse agonist, here by decreasing the constitutive CREB-dependent transcriptional activity of the ghrelin receptor (Fig. 6A and *inset*). The substance P analog did not appear to be a full inverse in this assay, conceivably due the technical, timing difficulties in inhibiting a constitutive activity determined in a far downstream reporter system.

The homologous motilin receptor displayed no detectable constitutive activity in the CRE-luciferase reporter assay, but upon stimulation with motilin a

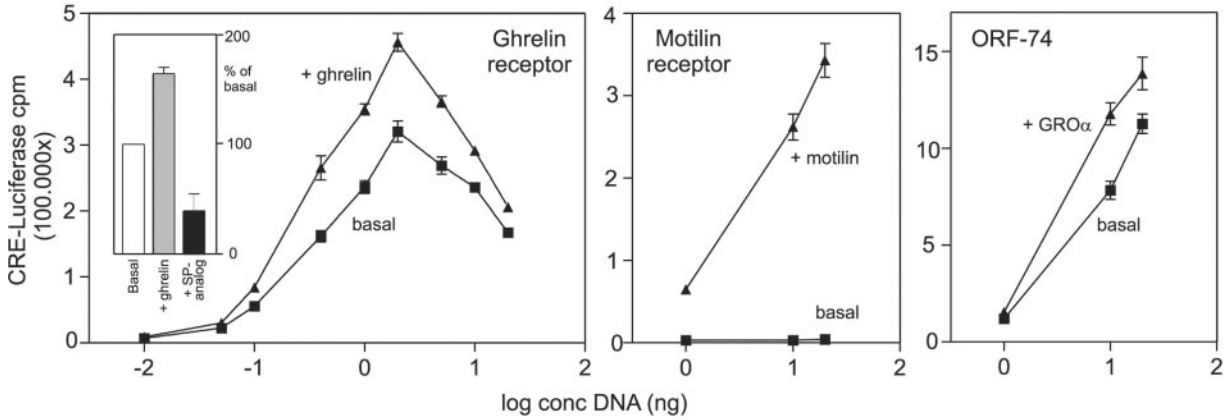

Fig. 6. Induction of cAMP-Responsive Element (CRE) Dependent Gene-Transcriptional Activity by the Ghrelin Receptor (A), the Motilin Receptor (B), and by ORF-74 (C)

The ligand-independent, basal signaling activities of the three receptors (*filled squares*) and the signaling in the presence of a maximal dose of the relevant full agonist: ghrelin (10^{-6} M), motilin (10^{-6} M), and GROα (10^{-7} M) (*filled triangles*) was measured by CREB-luciferase reporter assay in transiently transfected HEK-293 cells (for details see *Materials and Methods*). Shown are representative experiments out of at least four independent gene dosage experiments performed in quadruplicates in 96-well plates. *Inset* in panel A is shown the effect of ghrelin (10^{-6} M) and of [D-Arg^1, D-Phe^5,D-Trp^{7,9},Leu^{11}]-Substance P (10^{-6} M) in cells transfected with 2 ng ghrelin receptor DNA. RLU, Relative light units, as measured in a Packard TopCounter (5 sec/well).

strong signal was observed of a magnitude similar to that observed with the ghrelin receptor (Fig. 6B). Like the ghrelin receptor, the virally encoded ORF-74 receptor also signaled with high ligand-independent activity through the CRE pathway with an efficacy, which was even somewhat higher than the maximal efficacy observed for the ghrelin receptor (Fig. 6C). However, as compared with both the motilin receptor (agonist-stimulated response) and the ORF-74 receptor (ligand-independent response) the gene-dose required for ghrelin receptor to stimulate CREB transcriptional activity was surprisingly almost two orders of magnitude lower. In fact, a bell-shaped stimulation was observed with the ghrelin receptor. Thus, the ghrelin receptor in a highly efficient, ligand-independent manner stimulates transcriptional activity though the CRE pathway.

DISCUSSION

In the present study, we have discovered that the human ghrelin receptor is characterized by a surprisingly high degree of constitutive signaling activity and that it very efficiently signals also through the CREB pathway. The ligand-independent signaling of the ghrelin receptor has been overlooked until present conceivably due to the fact, that the receptor previously was studied almost exclusively in calcium mobilization assays. In a single preceding publication, IP turnover was also employed (23); however, in that study an ultra-short incubation period of only 1 min was employed due to the high noise level. Further downstream signaling pathways such as CREB-dependent gene transcription have to our knowledge not previously been reported for the ghrelin receptor.

The fact that the ghrelin receptor is highly constitutively active raises a series of questions concerning the physiological importance of this activity and whether the receptor is regulated not only by the ghrelin agonist but also by a yet unknown endogenous inverse agonist. Moreover, knowledge of the high constitutive activity opens for novel pharmaco-therapeutic opportunities in developing inverse agonist compounds for the ghrelin receptor for the treatment of, for example obesity.

Possible Physiological Importance of the High Constitutive Activity of the Ghrelin Receptor

First, it should be emphasized that both in general and in the specific case of the ghrelin receptor, it is very difficult to determine the *in vivo* physiological importance of high ligand-independent signaling activity. However, in the *in vitro* setting the ghrelin receptor is massively constitutively active and with the homologous motilin receptor as a conveniently silent control receptor for effect of *in vitro* expression. In fact, the ligand-independent signaling of the ghrelin receptor is similar to that displayed by one of the most vigorously constitutively active receptors yet reported, the ORF-74 oncogene, encoded by human herpes virus 8 (21, 22). Thus, we expect that the constitutive activity of the ghrelin receptor is a physiologically important phenomenon.

It is interesting to note that GH is secreted from isolated pituitary cells in culture in a manner, which not only can be stimulated by GHRH and ghrelin, but which can be inhibited by somatostatin (34). Thus, there is a high level of GH secretion even from isolated cells, where no hormonal tonus should be present. It could be speculated that this ligand-independent GH

secretion may be a consequence of the high level of constitutive signaling from the ghrelin receptor, which is known to be found on such pituitary cells. The substance P analog [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P described in the present study as an inverse agonist for the ghrelin receptor could be a useful tool to probe this hypothesis.

As presented in the introduction, ghrelin has been strongly implicated in the control of appetite and food intake acting both centrally via receptors found in the arcuate nucleus of the hypothalamus and peripherally through receptors on afferent vagal neurones (11, 12, 18). Centrally the ghrelin receptor is expressed mainly on the NPY and AGRP containing cells and antibodies and antagonists against NPY and AGRP have been shown to abolish the ghrelin induced effect on feeding (12, 15, 16). It has been hypothesized that ghrelin should increase the expression of NPY, however this has not been tested directly yet (35). Fasting induces an increase in the NPY level which appears to be mediated through an increase in CREB-dependent gene transcription as shown in transgenic mice expressing a CRE-lacZ construct (36). Both the CRE-activation and the NPY up-regulation in response to fasting were clearly attenuated by leptin. However, in view of the strong effect of the ghrelin receptor on CREB-dependent transcription observed in the present paper (Fig. 6) and the fact that ghrelin is the major chemical messenger of fasting and appetite signals could suggest that the CRE-mediated up-regulation of NPY is regulated through the ghrelin receptor. To what degree the constitutive activity of the ghrelin receptor is involved in this mechanism remains to be shown. Also in this case, inverse agonists such as the [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P could be valuable pharmacological tools.

Is There an Endogenous Inverse Agonist for the Ghrelin Receptor?

Food intake is regulated by a very intricate and redundant system of chemical messengers acting through a complicated neuronal network. The system is characterized by several layers of parallel, stimulating, and inhibitory mechanisms. However, in this system ghrelin is the only known peripherally acting orexigenic hormone. The fact that the ghrelin receptor is characterized by a high degree of constitutive activity raises the question of whether this receptor is regulated by an endogenous inverse agonist, which would act by decreasing the constitutive activity of the receptor. In this connection it should be noted that the MC-4 melanocortin receptor, which on the inhibitory side is crucially involved in the control of food intake, also is characterized by a high degree of constitutive activity (37). Importantly, the activity of the MC-4 receptor is know to be controlled not only by an endogenous agonist—α-MSH—but also by an endogenous inverse agonist—AGRP (37). Apparently, for certain systems it must be an advantage to modulate or fine-tune a high level of constitutive activity through the action of two ligands having opposite effects on the same receptor in stead of having two ligands acting as agonists on two receptors, which have opposite signaling effects. The latter arrangement is clearly a regulatory set-up used in many other systems in the organism. One interesting observation is, that the effect of the inverse agonist AGRP on the MC-4 receptor both *in vitro* and *in vivo* can be surprisingly long lasting (38, 39). The molecular/cell biological mechanism behind this phenomenon is not yet clear.

Ghrelin Receptor Inverse Agonists for the Treatment of Obesity?

Whether or not an endogenous inverse agonist exists for the ghrelin receptor, the high constitutive signaling activity of the receptor indicates that a compound acting as an efficient inverse agonist could be an interesting antiobesity agent. Today, the strong orexigenic effect of ghrelin as such clearly suggests that an antagonist of the ghrelin receptor could be a powerful regulator of appetite (15, 16). In view of the ligand-independent signaling of the ghrelin receptor, it is tempting to suggest that a neutral antagonist would in fact not be efficient in an *in vivo* setting and that an antagonist, which also is an inverse agonist, would be required for a ghrelin receptor ligand to be a truly efficacious antiobesity agent. As shown in the present study, [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P is a potent and highly efficacious inverse agonist for the ghrelin receptor; however, it is probably not very optimal as a general pharmacological tool because it also has effects on the tachykinin NK1, *i.e.* the substance P receptor and at higher concentrations even affects a number of other receptors including the bombesin receptor (40). Nevertheless, dipeptide libraries based on this and similar substance P antagonists have proven to be useful starting points for the development of nonpeptide antagonists for several types of peptide receptors (41, 42). [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P in fact has a very interesting molecular pharmacological phenotype as it is a rather clean, high affinity inverse agonist with a low potency as an antagonist (Fig. 4). The *in vivo* efficacy of an inverse agonist for the ghrelin receptor as an antiobesity agent remains to be demonstrated. However, if the high constitutive signaling activity of the receptor observed in the present study occurs for example in the NPY/AGRP neurons in the arcuate nucleus, then this could very likely constitute the signaling "set point" against which the receptors for the many different appetite suppressing hormones and transmitters, such as leptin, insulin, melanocortin, PYY3–36, *etc*. (43). Thus, it could be envisioned that an inverse agonist for the ghrelin receptor would be useful to suppress appetite especially in between meals and thus would be efficient for example to prevent the craving for second order meals, desserts and snacks.

Interestingly, the ghrelin receptor belongs to a small subset of 7TM receptors for which almost exclusively agonist ligands have as yet been discovered through chemical file screening. Some other members of this subset are the motilin receptor, the C5a, the 5HT2c and to a certain degree the somatostatin and opioid receptors.

Molecular Mechanism of the Constitutive Activity in Ghrelin Receptor

Why is the ghrelin receptor so strongly constitutively active and the highly homologous motilin receptor apparently totally silent without stimulation by agonist? There is at present no clear answer to that, since mutations and substitutions at multiple places in a 7TM receptor can lead to pronounced constitutive activity. Nevertheless, one possible molecular mechanism for the constitutive activity of the ghrelin receptor could be that TM-VI is pulled toward TM-III by a salt-bridge formed between ArgVI:20 (Arg^{281}) and GluIII:09 (Glu^{124}) (Fig. 1). This would fit well with our observations on the apparent requirement for an inwards movement of TM-VI and -VII at the extracellular end in receptors with engineered activating metal-ion sites (31) (our unpublished observations). Moreover, both Arg^{281} and Glu^{124} have through mutagenesis previously been implicated to be involved in agonist ligand binding (44). However, ArgVI:20 and GluIII:09 are both conserved in the nonconstitutively active motilin receptor (Fig. 1). An interesting speculative possibility for the silences of the motilin receptor could be that the conspicuous, long, and Pro-rich insertion in extracellular loop 2 of this receptor (Fig. 1) could function as a tethered inverse agonist. The neurotensin 2 receptor is another 7TM receptor characterized in respect of ligand binding and functionality which has the constellation; ArgVI:20 together with GluIII:09. Importantly, the neurotensin 2 receptor was recently shown to be as highly constitutively active as the ghrelin receptor (45).

MATERIALS AND METHODS

Materials

Ghrelin, [D-Arg^1,D-Phe^5,D-$Trp^{7,9}$,Leu^{11}], and substance P were purchased from Bachem (Bubendorf, Switzerland). Ipamoreline, NN703, L-692-629 and L-692-400 were kindly provided by Michael Ankersen, Novo Nordisk (Måløv, Denmark).

Molecular Biology

The cDNA for the motilin receptor was provided by Bruce Conklin, The Gladstone Institute (San Francisco, CA) and the cDNA for the human herpes virus 8 encoded ORF74 receptor by Mette Rosenkilde, Laboratory for Molecular Pharmacology. The human ghrelin/GHS receptor (GHS-R) cDNA was cloned by PCR from a human brain cDNA library and sequenced using an ABI 310 automated sequencer and found to be identical to the GHS receptor having accession no. NP004113. The cDNA was cloned into the eukaryotic expression vector pcDNA3 (Invitrogen, Carlsbad, CA). Mutations were constructed by PCR using the overlap expression method (46). The PCR products were digested with appropriate restriction endonucleases, purified and cloned into pcDNA3. All PCR experiments were performed using *pfu* polymerase (Stratagene, La Jolla, CA) according to the instructions of the manufacturer. All mutations were verified by restriction endonuclease mapping and subsequent DNA sequence analysis using an ABI 310 automated sequencer.

Transfections and Tissue Culture

COS-7 cells were grown in DMEM 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 previously described (47). For Fig. 2A various amounts of DNA were used, whereas for Fig. 2B-5 20 μg DNA were used per 75-cm^2 plates. In Fig. 2A tranfection with 20 μg DNA per 75-cm^2 plates gave a receptor expression of 126 fmol/10^5 cells measured by homologous competition binding curves. Assuming a linear correlation between DNA amount and protein expression, an estimation of the expression level for the plates transfected with 1, 2, 4, 6, 8, 16, and 32 μg DNA are made. HEK-293 cells were grown in DMEM 31966 with high glucose supplemented with 10% fetal calf serum, 2 mM glutamine and 0.01 mg/ml gentamicin. Cells were transfected with Lipofectamine 2000 (Life Technologies, Gaithersburg, MD).

Competition Binding Assays

Transfected COS-7 cells were transferred to culture plates 1 d after transfection at a density of 1×10^5 cells per well aiming at 5–8% binding of the radioactive ligand. Two days after transfection, competition binding experiments were performed for 3 h at 4 C using 25 pM of ^{125}I-ghrelin (Amersham, Little Chalfont, UK). Binding assays were performed in 0.5 ml of a 50 mM HEPES buffer (pH 7.4), supplemented with 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.1% (wt/vol) BSA, 40 g/ml bacitracin. Nonspecific binding was determined as the binding in the presence of 1 M of unlabeled ghrelin. Cells were washed twice in 0.5 ml of ice-cold buffer and 0.5–1 ml of lysis buffer (8 M urea, 2% Nonidet P-40 in 3 M acetic acid) was added and the bound radioactivity was counted. Determinations were made in duplicate. Initial experiments showed that steady-state binding was reached with the radioactive ligand under these conditions.

Phosphatidylinositol Turnover

One day after transfection, COS-7 cells were incubated for 24 h with 5 Ci [^{3}H]-*myo*-inositol (Amersham, PT6–271) in 1 ml 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, 0.05% (wt/vol) bovine serum; and were incubated in 0.5 ml buffer supplemented with 10 mM LiCl at 37 C for 30 min. The indicated curves were furthermore incubated with adenosine deaminase ADA (200 U/mg, Roche Molecular Biochemicals, Mannheim, Germany) for 30 min in a concentration of 1 U/ml.

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 potassium hydroxide in HEPES buffer, and the generated [^{3}H]-inositol

phosphate was purified on Bio-Rad (Hercules, CA) AG 1-X8 anion-exchange resin as described. Determinations were made in duplicates.

CRE Reporter Assay

HEK-293 cells (30,000 cells/well) seeded in 96-well plates were transiently transfected with a mixture of pFA2-CREB and pFR-Luc reporter plasmid (PathDetect CREB trans-Reporting System, Stratagene) and the indicated amounts of receptor DNA. One day after transfection, cells were treated with the respective ligands in an assay volume of 100 μl medium for 5 h. When treated with the ligands cells were maintained in low serum (2.5%) throughout the experiments. The assay was terminated by washing the cells twice with PBS and addition of 100 μl luciferase assay reagent (LucLite, Packard, Meriden, CT). Luminescence was measured in a TopCounter (Top Count NXT, Packard) for 5 sec. Luminescence values are given as relative light units.

Calculations

IC_{50} and EC_{50} values were determined by nonlinear regression using the Prism 3.0 software (GraphPad Software, San Diego, CA). Values of the dissociation and inhibition constants (K_d and K_i) were estimated from competition binding experiments using the equations $K_d = IC_{50}$-L and $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of radioactive ligand. Maximal binding capacity (B_{max}) values were estimated from competition binding experiments using the equation $B_{max} = B_0$ IC_{50}/[ligand], where B_0 is the specifically bound radioligand.

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REFERENCES

1. Bowers CY, Momany F, Reynolds GA, Chang D, Hong A, Chang K 1980 Structure-activity relationships of a synthetic pentapeptide that specifically releases growth hormone *in vitro*. Endocrinology 106:663–667
2. Bowers CY, Momany FA, Reynolds GA, Hong A 1984 On the *in vitro* and *in vivo* activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. Endocrinology 114:1537–1545
3. Patchett AA, Nargund RP, Tata JR, Chen MH, Barakat KJ, Johnston DB, Cheng K, Chan WW, Butler B, Hickey G, 1995 Design and biological activities of L-163,191 (MK-0677): a potent, orally active growth hormone secretagogue. Proc Natl Acad Sci USA 92:7001–7005
4. Smith RG, Cheng K, Schoen WR, Pong SS, Hickey G, Jacks T, Butler B, Chan WW, Chaung LY, Judith F, 1993 A nonpeptidyl growth hormone secretagogue. Science 260:1640–1643
5. Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJ, Dean DC, Melillo DG, Van Der Ploeg LH, 1996 A receptor in pituitary and hypothalamus that functions in growth hormone release. Science 273:974–977
6. Smith RG, Van Der Ploeg LH, Howard AD, Feighner SD, Cheng K, Hickey GJ, Wyvratt Jr MJ, Fisher MH, Nargund RP, Patchett AA 1997 Peptidomimetic regulation of growth hormone secretion. Endocr Rev 18:621–645
7. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656–660
8. Hosoda H, Kojima M, Mizushima T, Shimizu S, Kangawa K 2002 Structural divergence of human ghrelin; identification of multiple ghrelin-derived molecules produced by post-translational processing. J Biol Chem 278:64–70
9. Bednarek MA, Feighner SD, Pong SS, McKee KK, Hreniuk DL, Silva MV, Warren VA, Howard AD, Van Der Ploeg LH, Heck JV 2000 Structure-function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. J Med Chem 43: 4370–4376
10. Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, Bhattacharya S, Carpenter R, Grossman AB, Korbonits M 2002 The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. J Clin Endocrinol Metab 87:2988
11. Tschop M, Smiley DL, Heiman ML 2000 Ghrelin induces adiposity in rodents. Nature 407:908–913
12. Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S 2001 A role for ghrelin in the central regulation of feeding. Nature 409:194–198
13. Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS 2001 A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 50:1714–1719
14. Cummings DE, Weigle DS, Frayo RS, Breen PA, Ma MK, Dellinger EP, Purnell JQ 2002 Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. N Engl J Med 346:1623–1630
15. Horvath TL, Diano S, Sotonyi P, Heiman M, Tschop M 2001 Minireview: ghrelin and the regulation of energy balance—a hypothalamic perspective. Endocrinology 142:4163–4169
16. Kojima M, Hosoda H, Matsuo H, Kangawa K 2001 Ghrelin: discovery of the natural endogenous ligand for the growth hormone secretagogue receptor. Trends Endocrinol Metab 12:118–122
17. Schwartz MW, Woods SC, Porte Jr D, Seeley RJ, Baskin DG 2000 Central nervous system control of food intake. Nature 404:661–671
18. Date Y, Murakami N, Toshinai K, Matsukura S, Niijima A, Matsuo H, Kangawa K, Nakazato M 2002 The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. Gastroenterology 123:1120–1128
19. Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, Makino S, Fujimiya M, Niijima A, Fujino MA, Kasuga M 2001 Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. Gastroenterology 120:337–345
20. Itoh Z 1997 Motilin and clinical application. Peptides 18:593–608
21. Rosenkilde MM, Kledal TN, Brauner-Osborne H, Schwartz TW 1999 Agonists and inverse agonists for the

herpesvirus 8-encoded constitutively active seven-transmembrane oncogene product, ORF-74. J Biol Chem 274:956–961
22. Bais C, Santomasso B, Coso O, Arvanitakis L, Raaka EG, Gutkind JS, Asch AS, Cesarman E, Gershengorn MC, Mesri EA, Gerhengorn MC 1998 G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator. Nature 391: 86–89
23. Hansen BS, Raun K, Nielsen KK, Johansen PB, Hansen TK, Peschke B, Lau J, Andersen PH, Ankersen M 1999 Pharmacological characterisation of a new oral GH secretagogue, NN703. Eur J Endocrinol 141:180–189
24. McKee KK, Palyha OC, Feighner SD, Hreniuk DL, Tan CP, Phillips MS, Smith RG, Van Der Ploeg LH, Howard AD 1997 Molecular analysis of rat pituitary and hypothalamic growth hormone secretagogue receptors. Mol Endocrinol 11:415–423
25. Pomes A, Pong SS, Schaeffer JM 1996 Solubilization and characterization of a growth hormone secretagogue receptor from porcine anterior pituitary membranes. Biochem Biophys Res Commun 225:939–945
26. Holst B, Hastrup H, Raffetseder U, Martini L, Schwartz TW 2001 Two active molecular phenotypes of the tachykinin NK1 receptor revealed by G-protein fusions and mutagenesis. J Biol Chem 276:19793–19799
27. Rosenkilde MM, Cahir M, Gether U, Hjorth SA, Schwartz TW 1994 Mutations along transmembrane segment II of the NK-1 receptor affect substance P competition with non-peptide antagonists but not substance P binding. J Biol Chem 269:28160–28164
28. Ahuja SK, Murphy PM 1996 The CXC chemokines growth-regulated oncogene (GRO) α, GROβ, GROγ, neutrophil-activating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 are potent agonists for the type B, but not the type A, human interleukin-8 receptor. J Biol Chem 271:30545–20550
29. Cheng K, Chan WW, Butler B, Wei L, Smith RG 1993 A novel non-peptidyl growth hormone secretagogue. Horm Res 40:109–115
30. Elling CE, Nielsen SM, Schwartz TW 1995 Conversion of antagonist-binding site to metal-ion site in the tachykinin Nk-1 receptor. Nature 374:74–77
31. Elling CE, Thirstrup K, Holst B, Schwartz TW 1999 Conversion of agonist site to metal-ion chelator site in the β(2)-adrenergic receptor. Proc Natl Acad Sci USA 96: 12322–12327
32. Chen W, Shields TS, Stork PJ, Cone RD 1995 A colorimetric assay for measuring activation of Gs- and Gq-coupled signaling pathways. Anal Biochem 226:349–354
33. Matthews RP, Guthrie CR, Wailes LM, Zhao X, Means AR, McKnight GS 1994 Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. Mol Cell Biol 14: 6107–6116
34. Cervia D, Petrucci C, Bluet-Pajot MT, Epelbaum J, Bagnoli P 2002 Inhibitory control of growth hormone secretion by somatostatin in rat pituitary GC cells: sst(2) but not sst(1) receptors are coupled to inhibition of single-cell intracellular free calcium concentrations. Neuroendocrinology 76:99–110
35. Shintani M, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, Hayashi T, Inoue G, Hosoda K, Kojima M, Kangawa K, Nakao K 2001 Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. Diabetes 50:227–232
36. Shimizu-Albergine M, Ippolito DL, Beavo JA 2001 Down-regulation of fasting-induced cAMP response element-mediated gene induction by leptin in neuropeptide Y neurons of the arcuate nucleus. J Neurosci 21: 1238–1246
37. Nijenhuis WA, Oosterom J, Adan RA 2001 AgRP(83–132) acts as an inverse agonist on the human-melanocortin-4 receptor. Mol Endocrinol 15:164–171
38. Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, Barsh GS 1997 Antagonism of central melanocortin receptors *in vitro* and *in vivo* by agouti-related protein. Science 278:135–138
39. Ollmann MM, Barsh GS 1999 Down-regulation of melanocortin receptor signaling mediated by the amino terminus of Agouti protein in *Xenopus* melanophores. J Biol Chem 274:15837–15846
40. Woll PJ, Rozengurt E 1988 [D-Arg1,D-Phe5,D-Trp7,9,Leu11] substance P, a potent bombesin antagonist in murine Swiss 3T3 cells, inhibits the growth of human small cell lung cancer cells *in vitro*. Proc Natl Acad Sci USA 85:1859–1863
41. Fincham CI, Higginbottom M, Hill DR, Horwell DC, O'Toole JC, Ratcliffe GS, Rees DC, Roberts E 1992 Amide bond replacements incorporated into CCK-B selective "dipeptoids." J Med Chem 35:1472–1484
42. Horwell DC 1996 Use of the chemical structure of peptides as the starting point to design nonpeptide agonists and antagonists at peptide receptors: examples with cholecystokinin and tachykinins. Bioorg Med Chem 4:1573–1576
43. Schwartz MW, Morton GJ 2002 Obesity: keeping hunger at bay. Nature 418:595–597
44. Feighner SD, Howard AD, Prendergast K, Palyha OC, Hreniuk DL, Nargund R, Underwood D, Tata JR, Dean DC, Tan CP, McKee KK, Woods JW, Patchett AA, Smith RG, Van Der Ploeg LH 1998 Structural requirements for the activation of the human growth hormone secretagogue receptor by peptide and nonpeptide secretagogues. Mol Endocrinol 12:137–145
45. Richard F, Barroso S, Martinez J, Labbe-Jullie C, Kitabgi P 2001 Agonism, inverse agonism, and neutral antagonism at the constitutively active human neurotensin receptor 2. Mol Pharmacol 60:1392–1398
46. Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR 1989 Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77: 61–68
47. Gether U, Johansen TE, Schwartz TW 1993 Chimeric NK1 (substance P) NK3 (neurokinin B) Receptors. J Biol Chem 268:7893–7898