Three members of the ghrelin receptor family were characterized in parallel: the ghrelin receptor, the neurotensin receptor 2 and the orphan receptor GPR39. In transiently transfected COS-7 and human embryonic kidney 293 cells, all three receptors displayed a high degree of ligand-independent signaling activity. The structurally homologous motilin receptor served as a constitutively silent control; upon agonist stimulation, however, it signaled with a similar efficacy to the three related receptors. The constitutive activity of the ghrelin receptor and of neurotensin receptor 2 through the $G_{o\alpha}$ phospholipase C pathway was $50\%$ of their maximal capacity as determined through inositol phosphate accumulation. These two receptors also showed very high constitutive activity in activation of cAMP response element-driven transcription. GPR39 displayed a clear but lower degree of constitutive activity through the inositol phosphate and cAMP response element pathways. In contrast, GPR39 signaled with the highest constitutive activity in respect of activation of serum response element-dependent transcription, in part, possibly, through $G_{12/13}$ and Rho kinase. Antibody feeding experiments demonstrated that the epitope-tagged ghrelin receptor was constitutively internalized but could be trapped at the cell surface by an inverse agonist, whereas GPR39 remained at the cell surface. Mutational analysis showed that the constitutive activity of both the ghrelin receptor and GPR39 could systematically be tuned up and down depending on the size and hydrophobicity of the side chain in position VI:16 in the context of an aromatic residue at VII:09 and a large hydrophobic residue at VII:06. It is concluded that the three ghrelin-like receptors display an unusually high degree of constitutive activity, the structural basis for which is determined by an aromatic cluster on the inner face of the extracellular ends of TMs VI and VII.

In principle, all 7TM$^1$ G protein-coupled receptors are to some degree constitutively active, in that they are able to change into an active conformation without the presence of the agonist ligand (1). For the majority of receptors, the constitutive activity is so low that it is not picked up by the various signaling assays employed. Some receptors, however, such as the $\beta_2$-adrenergic receptors show a low but significant degree of constitutive activity of perhaps 3–10% of the maximal signaling capacity depending on the system in which it is studied (2). A few endogenous receptors have been described to display very high constitutive activity (for example, the CB1 cannabinoid receptor (3) and certain sphingolipid receptors (4)). However, it is still debated whether these receptors are in fact truly constitutively active, because it is difficult to rule out the possible presence of lipid ligands in the cellular system in which they are studied. Nevertheless, high constitutive activity is indisputably a hallmark of many virally encoded 7TM receptors such as ORF74 from human herpes virus 8 and US28 from human cytomegalovirus (5). The virus has optimized, for example, ORF74 to signal constitutively with $50\%$ efficacy and to be modulated positively by endogenous angiogenic chemokines and negatively by endogenous angiostatic or modulatory chemokines (6, 7).

Recently, we discovered that the receptor for the hormone ghrelin also signals with $50\%$ of maximal activity in the absence of its peptide ligand (8). This receptor property remained unnoticed for many years because mobilization of intracellular calcium had been used almost exclusively to monitor the signal transduction activity of this receptor (9). When inositol (1,4,5)-triphosphate (IP) turnover was measured instead, it became clear that the ghrelin receptor in fact was highly constitutively active (8). Ghrelin is an acylated 28-amino acid peptide hormone that is released from endocrine cells of the stomach in the premeal situation and functions as an important orexigenic from the periphery that stimulates especially acute food intake. Neuropeptide Y/agouti-related peptide neurons of the arcuate nucleus are a major target of ghrelin in the hypothalamus. Ghrelin increases the firing rate and induces an increased expression and release of neuropeptide Y and agouti-related peptide (10, 11). Furthermore, ghrelin also seems to exert important actions on energy expenditure, both through receptors on adipocytes and through modulation of thermogenesis (12, 13). Thus, it has been suggested that the high constitutive signaling activity of the ghrelin receptor could serve as a signaling set-point, for example, in the control of appetite and energy expenditure, where it would counterbalance; PBS, phosphate-buffered saline; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; ELISA, enzyme-linked immunosorbent assay; CRE, CAMP-responsive element; PTK, pertussis toxin; PI3K, phosphoinositide 3-kinase; TM, transmembrane domain.

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$^2$ The abbreviations used are: 7TM, seven transmembrane; IP, inositol (1,4,5)-triphosphate; NMU, neuropeptide U; NT-R2, neurotensin receptor 1; HEK, human embryonic kidney; CRE, serum-responsive element; CRE, cAMP-responsive element; PTX, pertussis toxin; PI3K, phosphoinositide 3-kinase; TM, transmembrane domain.
Constitutive Activity of the Ghrelin Receptor-like Family

Fig. 1. The ghrelin receptor family. A, schematic phylogenetic tree of the ghrelin receptor family indicating the relative relationship of the receptor. The black dots indicate the three receptors that either previously (the ghrelin receptor and NT-R2 (8, 17)) or in the present study (GPR39) have been demonstrated to display a high degree of constitutive signaling activity. B, serpentine model of GPR39. Residues that are identical among GPR39, the ghrelin receptor, and NT-R2 are highlighted in white on black. The generic numbering system for 7TM receptor residues described by Schwartz (59) is used throughout the article, and the proposed first residues of each transmembrane helix are indicated by 1.

ance a large number of inhibitory hormones and neurotransmitters, such as leptin, insulin, and PYY3–36 (14).

The ghrelin receptor belongs to a small family of receptors for peptide hormones and neuropeptides; besides ghrelin, the family includes motilin, neurotensin, and neuromedin U (NMU) (Fig. 1A). Like ghrelin, motilin is expressed in neuroendocrine cells of the gastrointestinal tract but acts mainly locally on neurons in the gut, where it stimulates meal-related secretions and motility of the intestine (15). The motilin receptor is completely silent with respect to constitutive signaling and consequently serves as a good control receptor in this study, as in previous studies (8). Neurotensin is both a gut hormone and a neuropeptide located in various parts of the central nervous system (16). The receptor that mediates most of the functions of neurotensin is neurotensin receptor 1. However, a structurally related receptor NT-R2 was recently cloned and was shown to bind neurotensin with lower affinity; it is interesting that neurotensin apparently has only minimal effects on the signaling of this receptor (17). It is noteworthy that in the context of the present study, NT-R2, like the ghrelin receptor, signals constitutively with ~50% of its maximal efficacy as determined by measurements of IP accumulation (17). NMU is a neuropeptide that acts through two structurally related receptors, NMU receptors 1 and 2 (18) (Fig. 1A). There are no indications in the literature or in our preliminary studies that these receptors are constitutively active.2

Among the members of the ghrelin receptor family is an orphan receptor, GPR39, that, together with GPR38 (later de-orphanized as the motilin receptor), were initially cloned as structural homologues to the ghrelin receptor (19) (Fig. 1, A and B). Very little information is available concerning GPR39 except that Northern blot analysis indicates that its peripheral expression is restricted to the stomach and the small intestine, whereas it is much more widely expressed in the central nervous system (19). Inspired by the knowledge of the high constitutive signaling of the ghrelin receptor and the NT-R2 receptor, we cloned the human GPR39 and discovered that it is also was highly constitutively active. In the present study, the signaling properties of the three constitutively active members of the ghrelin receptor family are characterized in parallel, and the internalization property of GPR39 is compared with that of

2 B. Holst and T. W. Schwartz, unpublished observations.
the ghrelin receptor. Furthermore, through a mutational analysis of the opposing faces of TM3, VI, and VII of the ghrelin receptor in particular, an aromatic cluster is identified that seems to be structurally important for the constitutive activity of this family of receptors. This was verified through a series of corresponding substitutions in both the ghrelin receptor and GPR39 at position VI:16 with residues of variable aromaticity and size through which the constitutively active molecule could systematically be tuned up and down.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ghrelin, [Δ-Arg⁶, N-pentapeptide (Δ-Tyr)⁸]Leu¹-substance P, motilin, and neurotensin were purchased from Bachem (Bubendorf, Switzerland). LY294062 and Y27632 were from Calbiochem. Pertussis toxin was purchased from Sigma Chemical Co.

**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 (University of Copenhagen, Copenhagen, Denmark). The human ghrelin/growth hormone secretagogue receptor cDNA and the GPR39 were cloned by PCR from a human brain cDNA library. The cDNAs were subcloned into pCDNA4-IRE (Invitrogen, Carlsbad, CA). Mutations were constructed by PCR using the overlap extension method (20). 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 (Applied Biosystems, Foster City, CA).

**Transfections and Tissue Culture—**COS-7 cells were grown in Dulbecco’s modified Eagle’s medium 1885 supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/ml gentamicin. Cells were transfected using calcium phosphate precipitation method with chloroquine (20 μg/ml; Sigma Chemical Co.) and transferrin-Texas Red (1:200; Molecular Probes, Paisley, UK) for 30 min at 4° or 37 °C in serum-free Dulbecco’s modified Eagle’s medium/1% bovine serum albumin (final volume 200 μl). Subsequent washes (15 min, 37 °C with vehicle (5 μl of Dulbecco’s modified Eagle’s medium), ghrelin (final concentration, 100 nm), and [Δ-Arg⁶, N-pentapeptide (Δ-Tyr)⁸]Leu¹-substance P (100 nm) were terminated by washes with ice-cold PBS. Processing for immunofluorescence microscopy was performed at room temperature. Cells were fixed (2% paraformaldehyde in PBS, 15 min; quenched by PBS and 25 mM glycine; 2 x 5 min) and permeabilized (0.075% Triton X-100 in PBS, 5 min), and the primary antibody was then detected with goat anti-mouse IgG-Alexa 488 (1:200 in PBS/1% bovine serum albumin for 30 min; Molecular Probes). After removal of non-specifically bound secondary antibody (six washes), cells were postfixed in 2% paraformaldehyde, nuclear DNA stained with 4’,6-diamidino-2-phenylindole (Sigma), and coverslips mounted in Mowiol 48-88 (Calbiochem). Immunofluorescence analysis was performed as previously described in detail (21). In brief, a vertical stack of 25–30 fluorescent images was acquired digitally on a Zeiss Axiovert 100 microscope (63 x objective; Omega Optical excitation and emission filter sets), using Openlab 2.0 (Improvision, Coventry, UK) to direct a piezo z-axis drive in 0.2-μm steps. The central 15 images for each fluorophore were then deconvolved to remove out-of-focus light (Openlab) and reconstructed in three dimensions (3.0 μm z-section) in Volocity (Improvision). Fluctuation analysis—CRE and SRE reporter assay—COS-7 cells transfected and seeded out in parallel with those used for IP accumulation assay. The cells were lysed in sample buffer and separated on SDS/10% PAGE according to the method of Laemmli (23). Proteins were transferred to nitrocellulose, and Western blot analysis was performed using a 1:5000 dilution of mouse monoclonal anti-erbB1/erbB2 antibody (Santa Cruz Biotechnology). Blots were probed anti-mouse horseradish peroxidase-conjugated secondary antibodies, visualized using enhanced chemiluminescence reagent (Amerham Biosciences) and quantified by densitometric analysis. ERK1/2 phosphorylation was normalized against the loading of protein by expressing the data as a ratio of phospho-ERK1/2 over total ERK1/2. Results were expressed as percentage of the value obtained in nonstimulated, mock-transfected cells.

**Cell Surface Expression Measurement (ELISA)**—Cells were transfected and seeded out in parallel with those used for IP accumulation assay. The cells were washed twice, fixed, and incubated in blocking buffer (0.2% dry milk in PBS) for 60 min at room temperature. Cells were kept at room temperature for all subsequent steps. Cells were incubated 2 h with anti-FLAG (M2) antibody (Sigma Chemical Co) in 1:300 dilution. After three washes, cells were incubated with anti-mouse horseradish peroxidase-conjugated antibody (Amerham Biosciences) at a 1:4000 dilution. After extensive washing, the immunoreactive activity was revealed by the addition of horseradish peroxidase substrate according to manufacturer’s instruction.

**RESULTS**

**Signaling through the Phospholipase C Pathway—**Determination of IP accumulation was used as a measure of signaling through the Gαs-phospholipase C pathway in transiently transfected COS-7 cells. As described previously (8), the ghrelin receptor displays a high degree of constitutive signaling activity through the phospholipase C pathway, demonstrated by the gene dose-dependent but ligand-independent increase in IP production (Fig. 2A). Gene-dosing experiments with GPR39 also demonstrated a dose-dependent increase in IP accumulation in cells expressing the GPR 39 receptor as opposed to cells transfected with the empty vector (Fig. 2B). Surface ELISA of the FLAG-tagged GPR39 receptor confirmed that the gene-dosing experiments in fact did result in a dose-dependent surface expression of the orphan receptor (Fig. 2B, inset). The lack of an endogenous ligand for GPR39 prevented us from evaluating the degree of constitutive activity compared with the maximal stimulation achieved by the agonist. However, as a result of our general probing of receptors with meal-ions (24, 25) we discovered that Zn(II) administered as ZnCl₂ acted as a...
low potency agonist with an EC50 value of 34 μM in GPR39-transfected cells as opposed to non-transfected cells (Fig. 2C). The zinc ion increased the receptor-mediated signaling from 22 fmol/10^5 cells to 46 fmol/10^5 cells at the highest expression level (Fig. 2C). In parallel transfections of COS-7 cells, it was found that the ghrelin receptor displayed a higher level of constitutive activity compared with GPR39, although a slightly lower degree of surface expression was observed by ELISA. The highest achievable level of constitutive signaling activity observed for the ghrelin receptor was 45 fmol/10^5 cells (Fig. 2, A and C). Higher receptor gene doses resulted in lower responses in IP accumulation, and a similar phenomenon was observed in the in the downstream signaling pathways described below (data not shown). Addition of ghrelin further increased the IP signaling by ~2-fold, to 80 fmol/10^5 cells.

The NT-R2 receptor has previously been described to signal through the phospholipase C pathway with ~50% constitutive activity (17), which was in accordance with the observation that ligand-independent IP accumulation in COS-7 cells transfected with this receptor was similar to the levels observed in cells transfected with the ghrelin receptor (Fig. 2C). It is interesting that neurotensin did not act as an agonist, in that it did not further increase the signaling of the receptor above the basal level (Fig. 2C). In the study in which this phenomenon was initially described, the non-peptide ligand SR48692 was used as a high potency agonist, which further increased the IP accumulation by ~2-fold (17). The SR48692 ligand was unfortunately not available for the present study. Thus, the ghrelin receptor and NT-R2 signaled with a similar high constitutive activity corresponding to ~50% of their maximal efficacy through the phospholipase C pathway. GPR39 also displayed constitutively active members of the ghrelin receptor family showed clear ligand-independent signaling through the ERK1/2 signaling pathway.

**Signalining through the CRE Pathway**—Although the activity of the important transcription factor CRE binding protein is generally believed to be controlled by cAMP-dependent kinases, it can also be activated by downstream kinases of the Gq pathway, such as Ca^2+/-calmodulin kinase IV or protein kinase C (29, 30). As demonstrated previously (31), gene-dosing experiments with the ghrelin receptor performed in transiently transfected HEK293 cells result in a dose-dependent but ligand-independent stimulation of the CRE pathway as monitored by a reporter assay using CRE-driven luciferase activity (Fig. 4A). The CRE-dependent transcriptional activity could be further increased by ghrelin and could be decreased by the inverse agonist [D-Arg^1,D-Phe^3,D-Trp^7,Nle^11]-substance P. However, the effects of the agonist and inverse agonist were smaller in the CRE reporter assay than in the IP assay, probably because of the high degree of amplification in the reporter assay. The NT-R2 also showed gene dose-dependent signaling through the CRE pathway as a ~5-fold increase in the CRE-mediated luciferase activity was observed in cells transiently transfected with this receptor (Fig. 4B). Similar to what was observed when measuring inositol phosphate accumulation, neurotensin was also a neutral ligand in the CRE assay (17). Transfecting with increasing amounts of GPR39 cDNA induced only ~2-fold increase in CRE-mediated luciferase activity (Fig. 4C), and the addition of Zn(II) induced only a very limited stimulation of the CRE-mediated luciferase activity. The moti-
Constitutive Activity of the Ghrelin Receptor-like Family

To determine whether the SRE activation was caused by a functional coupling of the receptors to Go_{12/13} proteins, we treated the cells with pertussis toxin (PTX; 100 ng/ml for 24 h). This treatment did not affect the constitutive activity of SRE-mediated transcription for the ghrelin receptor or for GPR39, but PTX reduced the constitutive SRE activity mediated by NT-R2 by ~50% (Fig. 5, insets). Because Go_{12/13} are often functionally coupled to the Rho signaling pathway, we used a specific Rho kinase inhibitor (Y-27632) to probe this pathway. Y-27632 decreased the basal GPR39-mediated SRE activity by ~60%, whereas the ghrelin receptor and NT-R2 mediated SRE activity was inhibited by only ~20%. A similar inhibition of the GPR39-induced SRE activity was observed by treatment of the cells with the PI3K inhibitor LY-294002. It is noteworthy that in cells transfected with either the ghrelin receptor or with NT-R2, a slight increase in the basal SRE signaling was observed by addition of the PI3K inhibitor (Fig. 5, A and B, insets). Thus, GPR39 signals with an extraordinarily high constitutive activity through the SRE pathway compared with NT-R2 and the ghrelin receptor; the latter did display a strong ligand-mediated signal in this pathway.

Constitutive Internalization of Receptors—The ghrelin receptor and GPR39 were FLAG-tagged at their extracellular N termini, and the ability of the receptors to internalize was studied in stably transfected HEK293 clones. To specifically examine the fate of receptors expressed at the cell surface, antibody feeding experiments were performed with the M2 antibody (recognizing the FLAG tag), which was added to the cell medium before ligand stimulation, fixation, and detection. When cells were incubated with the M2 antibody at 4 °C (to prevent internalization) the immunoreactivity was confined to the plasma membrane (data not shown). However, when cells expressing the FLAG-tagged ghrelin receptor were incubated at 37 °C for 45 min with the M2 antibody, but no ligand added, the majority of the immunoreactivity was at the end of the incubation period found intracellularly in a vesicular pattern (Fig. 6, top row). Thus, in HEK293 cells, the ghrelin receptor is constitutively internalized. Numerous intracellular puncta contained both FLAG immunoreactivity and transferrin-Texas Red, a marker for clathrin-coated internalization and recycling endosomes. Treatment with the ghrelin agonist (100 nM for the last 15 min before fixation) did not alter this pattern of presumably endosomal receptor localization (Fig. 6, second row). Addition of the inverse agonist [D-Arg^1,D-Phe^5,D-Trp^7,9,Leu^11]-substance P, however, induced a substantial redistribution of the ghrelin receptors to the plasma membrane (Fig. 6, third row). Ghrelin receptors were also trapped at the cell surface by treatment with concanavalin A (0.3 mg/ml), a nonspecific inhibitor of internalization, which also prevented transferrin-endocytosis (data not shown). In parallel experiments, internalization of FLAG-tagged β_2-adrenergic receptors to transferrin-positive endosomes was observed only in the presence of the agonist isoproterenol (data not shown).

In contrast to the ghrelin receptor, GPR39 was almost exclusively located at the plasma membrane even when the incubation with the M2 antibody was performed at 37 °C for 45 min (Fig. 6). This was also the case in HEK293 cells stably transfected with a FLAG-tagged version of the [Asn^VI:16Phe]GPR39 mutant receptor, which signals with the same high basal activity as the ghrelin receptor through the G_{q/11}, phospholipase C pathway (see below).

Thus the ghrelin receptor is constitutively internalized and this can be prevented by the inverse agonist [D-Arg^1,D-Phe^5,D-Trp^7,9,Leu^11]-substance P, whereas the homologous GPR39 receptor, which also is highly constitutively active, does not undergo constitutive internalization.
Mutational Analysis of the Structural Basis for the Constitutive Activity of the Ghrelin Family of Receptors—Previous studies using metal-ion site engineering have indicated that activation of 7TM receptors involves an inward movement of TMs VI and VII toward TM III in the main ligand-binding pocket at the extracellular ends of these helices (25). Based on the
Constitutive Activity of the Ghrelin Receptor-like Family

![FIG. 6. Internalization properties of the ghrelin receptor and of GPR39.](image)

assumption that the high constitutive activity of the ghrelin, NT-R2, and GPR39 receptors could be a result of an increased tendency of TM6 and VII to “dock” with each other and with the inner face of TM III, we performed a mutational analysis, mainly of residues located at the inner faces of these transmembrane segments initially in the ghrelin receptor (Fig. 7A). Residues were substituted either with Ala or with the corresponding residues of the silent motilin receptor (31) (Figs. 4 and 5). IP accumulation was chosen as the signaling read out because this is most closely associated with the receptor and the G protein. As shown in Fig. 7B, the constitutive activity of the ghrelin receptor was somewhat reduced in several of these mutants, and it was totally eliminated in four of the mutants: GlnIII:05 to Leu; PheVI:16 to Ala; ArgVI:20 to Ala; and PheVII:06 to Ala. However, in three of these mutants, the ability of the agonist to stimulate the receptor was also eliminated, which made interpretations of the results difficult. For example, the lack of signaling could be the result, in principle, of a lack of sufficient surface expression of the mutant receptor. But in the case of the PheVI:16-to-Ala substitution, a selective effect on the constitutive activity of the receptor was observed, in that the ghrelin stimulation of receptor signaling was intact (Fig. 7B). This is a particularly interesting position, because an activating metal-ion site has been built in the β2-adrenergic receptor from this position to two positions in TMs III and VI, respectively.

As shown in Fig. 8A, when a large aromatic Tyr residue (as found in the neurotensin 2 receptor) was introduced at position VI:16 of the ghrelin receptor, the mutant receptor displayed constitutive activity similar to that of the wild-type ghrelin receptor, which has a structurally similar Phe at this position. However, when a small polar residue such as Asn (as found in the GPR39 receptor) was introduced at position VI:16, this resulted in an intermediate but clear constitutive activity, which corresponded well with the constitutive activity observed in the GPR39 receptor (Fig. 8A). The surface expression of the mutant receptors was similar to that of the wild-type receptor as reflected in the similar maximal signaling efficacy measured during stimulation with the ghrelin agonist (Table I). Only the FVI:16A mutation displayed a somewhat lower $E_{\text{max}}$ compared with the wild-type receptor. In contrast, the other mutant receptor with a very low basal signaling (FVII:09A) was in fact highly expressed on the surface, with a higher $E_{\text{max}}$ in response to ghrelin than that observed for the wild-type receptor. When the corresponding substitutions were performed in the GPR39 receptor, a very similar picture was found; i.e. introduction of either a Phe or a Tyr residue at position VI:16 instead of the endogenous Asn resulted in increased constitutive activity of a magnitude similar to that observed in the wild-type ghrelin and neurotensin receptors (Fig. 8). In GPR39, Ala substitution at this position decreased the observed constitutive activity but did not totally eliminate it as observed in the ghrelin receptor. Thus, it is possible to tune the constitutive activity of both the ghrelin receptor and the GPR39 receptor up and down in a relatively systematic manner by changing the physicochemical property of the inner face of TM VI in the main ligand binding pocket at position VI:16. To probe whether the mutations affected the surface expression, ELISA assay was performed with the FLAG-tagged GPR39 mutations, which showed a very similar surface expression for all the mutations (Fig. 8B).

The mutations of the hydrophobic pocket between TMs VI and VII modulated the signaling through CRE- and SRE-mediated transcription in a manner similar to that of the affected phospholipase C-mediated signaling. It is interesting that in CRE-mediated signaling, ghrelin was able to stimulate FVI:16A equally efficiently as the wild-type ghrelin receptor (Fig. 9A). Because of the close proximity and, therefore, likely structural interaction between PheVI:16 and PheVII:06 and PheVII:09 at the inner face of TM VII (Fig. 10), these two residues were also subjected to further mutational analysis in the ghrelin receptor. A similarly high constitutive activity was observed when PheVII:06 was substituted with other large residues such as Tyr, His, or Leu. However, the constitutive activity was eliminated if Ala was introduced in position VII:06. In contrast, only substitution with Tyr for PheVII:09 was allowed, giving normal high constitutive activity, whereas substitution with His, Leu, or Ala in all cases impaired the constitutive activity of the ghrelin receptor.

**DISCUSSION**

In the present study, the orphan receptor GPR39 was identified as a constitutively active member of the ghrelin receptor
family. A parallel analysis of the signaling properties of GPR39, NT-R2, and the ghrelin receptor itself demonstrates considerable differences in the relative efficacy by which these structurally related, constitutively active receptors signal through different pathways. Thus, the ghrelin receptor and NT-R2 are more efficacious than GPR39 in Gq/phospholipase C and CRE signaling, whereas GPR39 clearly was the most constitutively active through the SRE pathway. The receptors also displayed major differences in their internalization properties. The ghrelin receptor internalized in a ligand-independent fashion and accumulated in intracellular vesicles in contrast to GPR39, which despite its high constitutive signaling activity remained at the cell surface. Despite their differences in signaling and internalization properties, a common structural “volume knob” or lever could be identified in this family of receptors in the form of an aromatic cluster of residues on the inner face of TMs VI and VII. Thus, both in the ghrelin receptor and in GPR39, the level of constitutive activity could, in a similar manner, gradually be turned up or down depending on the size and hydrophobic properties of the side chain of the residue in position VI:16.

Different and Similar Signaling Properties among Three Structurally Related, Constitutively Active Receptors—First, it should be emphasized that constitutive activity of a receptor is a difficult property to measure quantitatively because it is dependent on the expression level of the receptor as emphasized by the gene dosing experiments. Therefore, the structurally homologous motilin receptor serves as a highly important
“negative” control in both the present and the previous studies of the ghrelin receptor (14). Not only does the motilin receptor in parallel gene dosing experiments display no sign of constitutive activity, but also administration of the agonist ligand (the peptide motilin) results in signaling through the various pathways of a magnitude comparable to that observed in the basal state with the three constitutively active receptors (Figs. 4 and 5). This strongly indicates that the expression levels of the receptors are similar and that the constitutive signaling observed with GPR39, NT-R2, and the ghrelin receptor is a physiologically important phenomenon and not an experimental artifact caused, for example, by overexpression of these receptors.

In the signaling assay, which measures activity near the receptor i.e. determination of inositol phosphate accumulation as a reflection of Gq-mediated phospholipase activity, the ghrelin receptor and NT-R2 both signal with a similar constitutive efficacy of -50% of the maximal capacity (8, 17). GPR39 (although clearly being constitutively active) signaled through this pathway with a somewhat lower efficacy (Fig. 2). However, when more downstream signaling pathways were studied, the relative efficacy and even the rank order of constitutive signaling efficacy varied among the three related receptors. With respect to CRE-mediated transcription, the ghrelin receptor showed very strong ligand-independent activity, reaching a level 2-fold higher than the neurotensin receptor, whereas GPR39 showed only a very limited degree of constitutive activity in this signaling pathway. Because there is no indication that these receptors couple through Gs and cAMP, it is likely that the CRE activation is mediated through a Gq pathway and various down-stream kinases (29, 30). When SRE mediated transcriptional activity was measured instead, a different rank order of potency was observed among the three related receptors. GPR39, in contrast, which clearly signaled with the lowest

| Activity of ghrelin receptor mutations | Efficacy | Basal activity as percentage of the ghrelin-induced maximum activity | Basal activity as percentage of the ghrelin-induced maximum activity |
|----------------------------------------|----------|---------------------------------------------------------------|---------------------------------------------------------------|
| Mock                                   | 15 ± 1   | 13                                                           | 15                                                           |
| Ghrelin-R WT                          | 100      | 200 ± 9                                                      | 15                                                           |
| D99N                                   | 124 ± 36 | 221 ± 69                                                     | 5                                                            |
| FIII:04S                               | 102 ± 25 | 191 ± 45                                                     | 5                                                            |
| QIII:05L                               | 13 ± 1   | 11 ± 3                                                       | 3                                                            |
| SIII:08A                               | 95 ± 13  | 213 ± 19                                                     | 4                                                            |
| EIII:09Q                               | 89 ± 14  | 189 ± 28                                                     | 3                                                            |
| E196Q                                  | 141 ± 14 | 240 ± 4                                                      | 4                                                            |
| R198L                                  | 58 ± 9   | 189 ± 31                                                     | 5                                                            |
| MV:05A                                 | 66 ± 9   | 198 ± 19                                                     | 5                                                            |
| FVI:16A                                | 15 ± 1   | 145 ± 23                                                     | 4                                                            |
| FVI:16N                                | 33 ± 4   | 173 ± 13                                                     | 4                                                            |
| FVI:16Y                                | 82 ± 8   | 181 ± 18                                                     | 4                                                            |
| FVII:20A                               | 14 ± 1   | 16 ± 1                                                       | 4                                                            |
| QVII:02A                               | 79 ± 13  | 186 ± 39                                                     | 3                                                            |
| NVII:02A                               | 67 ± 15  | 191 ± 19                                                     | 5                                                            |
| FVII:06A                               | 17 ± 3   | 16 ± 4                                                       | 3                                                            |
| FVII:06L                               | 134 ± 19 | 345 ± 49                                                     | 3                                                            |
| FVII:06H                               | 84 ± 15  | 185 ± 51                                                     | 3                                                            |
| FVII:06Y                               | 83 ± 5   | 210 ± 18                                                     | 3                                                            |
| FVII:09A                               | 34 ± 1   | 211 ± 19                                                     | 3                                                            |
| FVII:09L                               | 37 ± 2   | 169 ± 15                                                     | 3                                                            |
| FVII:09H                               | 50 ± 4   | 203 ± 20                                                     | 3                                                            |
| FVII:09Y                               | 115 ± 8  | 237 ± 13                                                     | 3                                                            |

**a** WT, wild type.

![Figure 9](image_url) Effect of substitutions of PheVI:16 in the ghrelin receptor in SRE- and CRE-dependent gene transcription. A, CRE-dependent and B, SRE-dependent gene transcription were performed in transiently transfected HEK293 cells in which the basal, ligand-independent signaling activities (black bars) and the signaling in the presence ghrelin (10⁻⁷ M) (white bars) were measured for wild-type (WT) ghrelin receptor and ghrelin receptor with PheVI:16 substituted with Ala, Asn, or Tyr.
activity in GPR39-transfected cells by specific Rho kinase blocker Y-27632 decreased the basal SRE sensitive to SRE activation through Rho kinase (32). The previously been suggested to mediate the coupling of several other 7TM receptors to SRE activation, including Gβγ, Go, and G13. SRE activation can also be mediated through Go (33), but the lack of effect of PTX would tend to exclude this pathway for at least GPR39 and the ghrelin receptor, whereas the NT-R2 coupling to SRE seemed to be at least partly dependent on Go due to the effect of PTX.

We would have expected that the constitutive activity of the ghrelin-like receptors could be detected in MAP kinase activation as well, as observed for many virally encoded receptors (37). However, all three ghrelin-like receptors, otherwise constitutively active, were almost silent in respect of ligand-independent signaling through at least ERK1/2 phosphorylation. This is surprising, especially because the ghrelin receptor itself showed a clear ligand-mediated activation of this MAP kinase (Fig. 5). We cannot at present explain this discrepancy. However, it illustrates the notion that there is more than one active conformation of a 7TM receptor; although constitutive activity may to a certain degree reflect the repertoire of the agonist induced signaling, it may not cover all the signaling pathways (38). This could be an important point, because we are here dealing not with virally encoded “exogenous receptors” but with endogenous receptors, which probably have evolved a high degree of constitutive activity for a particular physiological purpose and in which a continuous high signaling through other pathways could be problematic in leading, for example, to cell proliferation and cancer development.

NT-R2 is an atypical neurotensin receptor because the peptide only binds with relatively low potency to this receptor and because neurotensin has almost no effect on receptor signaling, as observed both in this study and by Richard et al. (17). It is interesting that a small non-peptide compound, SR48.692, which is a high-affinity antagonist for the normal neurotensin receptor NT-R1, in fact functions as a high-potency, high-efficiency agonist for NT-R2 (17).

**Different Internalization Pattern for the Ghrelin Receptor and GPR39—**7TM receptors are generally targeted to the cell surface; upon agonist activation, they are internalized as part of a desensitization process. Thereafter, they are either recycled to the membrane or, in certain cases, sorted to the lysosomal pathway for degradation. It is generally assumed that constitutive activity would lead to constitutive phosphorylation and constitutive internalization. In the present study, we find by antibody feeding experiments that the ghrelin receptor is constitutively internalized, whereas GPR39, which in certain signaling assays is even more constitutively active, remains at the cell surface (Fig. 6). However, GPR39 was clearly not as constitutively active as the ghrelin receptor in the Gq-mediated IP signaling. However, even the mutant form of GPR39 ([QVI: 16F]GPR39), which through the IP pathway signaled with a degree of constitutive efficacy similar to that of the ghrelin receptor, showed no sign of constitutive internalization. This is in complete agreement with observations in both constitutively active, virally encoded receptors and in constitutively active mutant forms of endogenous receptors, such as the C5a receptor (38, 39). There simply is no direct correlation between constitutive signaling and constitutive internalization. In the case of the virally encoded US28 receptor, it could even be demonstrated that the structural basis for the constitutive internalization was located in the C-terminal tail and was totally independent upon the constitutive signaling (40).

The observation that a relatively short treatment with the inverse agonist for the ghrelin receptor is able to trap the vast

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**Fig. 10.** Molecular model of the residues proposed to be part of the structural basis for the high constitutive activity in the ghrelin receptor. A. molecular model of the ghrelin receptor built over the inactive structure of rhodopsin (60, 61). The seven helical bundle is displayed without the loops as viewed from the extra-cellular side. Only the residues on the inner faces of TMs III, VI, and VII, which in the mutational analysis were identified to potentially be involved in the constitutive activity, are shown. The two arrows indicate the proposed inward movement of TM VI and VII toward TM III that occur during activation of 7TM receptors based on metal-ion site engineering between residues: III:08, VI:16, and VII:06. B. the extracellular ends of TMs VI and VII as viewed from between TMs IV and V, showing the close proximity of the three aromatic residues: PheVI:16, PheVII:06, and PheVII:09. These three residues were subject to further mutational analysis, as shown in Fig. 8.

The degree of constitutive activity in the possibly Gq-mediated IP and CRE pathways, showed the highest degree of constitutive signaling in the SRE pathway. This suggests that a G-protein other than Gq might be responsible for at least the majority of the observed constitutive activation of SRE. G12/13 has previously been suggested to mediate the coupling of several other 7TM receptors to SRE activation through Rho kinase (32). The specific Rho kinase blocker Y-27632 decreased the basal SRE activity in GPR39-transfected cells by about 50%, indicating that G12/13 is involved in the GPR39-induced, ligand-independent stimulation of SRE activation. The constitutive activation of SRE observed in cells transfected with the ghrelin receptor or with NT-R2 was much lower than that observed with GPR39; this SRE activity was also inhibited only marginally by the Rho kinase blocker (Fig. 4). A close interaction between PI3K- and SRE-mediated transcription has been described previously (36). Through the use of a selective blocker of PI3K (LY-294002), we found for GPR39 that this pathway was responsible for a fraction of SRE activation similar to that of Rho. However, many different signal transduction pathways have been suggested as links between 7TM receptors and PI3K activation, including Gβγ, Go, and G13. SRE activation can also be mediated through Go (33), but the lack of effect of PTX would tend to exclude this pathway for at least GPR39 and the ghrelin receptor, whereas the NT-R2 coupling to SRE seemed to be at least partly dependent on Go due to the effect of PTX.

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The observation that a relatively short treatment with the inverse agonist for the ghrelin receptor is able to trap the vast
majority of the receptors at the cell surface indicates that the internalization of this receptor is associated with signaling and postsignaling events and that the receptor is probably recycled relatively rapidly to the cell surface. More detailed cell biological studies are required to determine this; recently, however, this phenomenon was characterized in detail in the constitutively active CB1 receptor, where an inverse agonist also traps the constitutively internalized receptor (41). Trapping of constitutively active receptor mutants at the cell surface by inverse agonists has been shown in several systems (42–44).

It should be noted that a different group has recently reported that a green fluorescent protein-tagged ghrelin receptor is surface expressed in Chinese hamster ovary cells and internalizes upon ligand stimulation (45). The discrepancy may be explained by the large GFP tag, by the cell type, or by the fact that only antibody feeding experiments are more sensitive in detecting internalization of cell surface receptors (45).

**A Molecular Switch Modulating the Level of Constitutive Activity**—In the present study, we identified an aromatic cluster on the inner face of TMs VI and VII that seems to be at least part of the structural basis for the constitutive activity of the ghrelin-like receptors.

The molecular activation mechanism for 7TM receptors is not understood in details. However, a series of biophysical studies, including EPR studies with site-directed spin labeling and studies with various fluorescent probes, all indicate that activation of rhodopsin and β-adrenergic receptors is associated with a movement of especially TM VI out and away from TM III at the intracellular face of the receptor (46–48). The corresponding movements occurring at the extracellular, agonist-binding part of the receptor have as yet not been characterized through similar biophysics methods. Nevertheless, construction of activating metal-ion sites in the main ligand-binding pocket indicates that activation of 7TM receptors involves an inward movement of TMs VI and VII toward each other and toward TM III within the main ligand binding pocket (25).2 Agonists are according to this model believed to act through binding to and stabilization of an active conformation in which the extracellular ends of TMs III, VI, and VII are “docked” onto each other. However, the receptor is assumed to be in a dynamic equilibrium between inactive and active conformations even in the absence of the agonist, which is responsible for the constitutive activity of these receptors.

Through a systematic mutational approach, we identified three positions on the inner face of the ghrelin receptor that seemed to be responsible for stabilizing the active conformation of the receptor in the absence of agonist ligand: PheVI:16, PheVII:06, and PheVII:09 (Figs. 7 and 8). Most convincingly, the constitutive signaling activity of both the ghrelin receptor and GPR39 could be raised or lowered through variation of the size and hydrophobicity or aromaticity of the residue located in position VI:16. In the model described above for 7TM receptor activation, the interpretation would be that a Phe or Tyr in position VI:16 in the context of a Phe in position VII:09 and a large hydrophobic residue in position VII:06, will ensure a favorable “docking” of the extracellular end of TM VI on TM VII through the formation of a hydrophobic core between these helices.

Although Phe and Tyr are very common residues at position VI:16 (found in 66% of 7TM receptors) the residue at position VII:09 is usually a small residue such as Ala, Gly, or Ser (found in 73% of receptors) (49). It should be noted that our mutational analysis in the ghrelin receptor, with a Phe at position VI:16, showed that the high constitutive activity was observed only with a Tyr or a Phe in position VII:09 (Fig. 8). Not even a His in position VII:09 would give the high constitutive signaling.

Mutations at two other positions in the ghrelin receptor also eliminated the constitutive activity (i.e. GlnIII:05 and ArgVI:20), which are both located just above the described aromatic cluster (Fig. 7). It is likely that these two residues may also be involved in creating the structural basis for the high constitutive activity of this receptor through a polar interaction across the main ligand binding pocket for example hydrogen bond formation with or without water molecules. However, because ghrelin activation was also eliminated by these mutations, we cannot at present confirm this notion.

**Biological Significance of High Constitutive Receptor Activity**—The level of constitutive activity displayed by the three ghrelin-like receptors (~50% or more of their maximal capacity) is highly unusual among 7TM receptors. We would suggest that this property of the receptors is an important part of their physiological repertoire in vivo. Cells in which such receptors are expressed will be provided with a signaling tone against which other receptors may act. Thus, it has been suggested that the ghrelin receptor, which is the only stimulatory receptor expressed on the important sensory neuropeptide Y/agouti-related peptide neurons in the arcuate nucleus of the hypothalamus, provides a positive signaling tone (perhaps most significantly exemplified in the CRE activity) against which the receptors for all the appetite inhibitory hormones and transmitters act, such as leptin, insulin, PYY3–36, etc. (14). A high constitutive signaling also means that regulation of the expression of the receptor as such has direct consequences on the signaling activity independent of the hormone. It is interesting to note, therefore, that the ghrelin receptor has been described as being up-regulated by 8-fold in the hypothalamus during fasting (50). According to our model, this would provide a ligand-independent increase in ghrelin receptor signaling (i.e. stimulation of appetite) (14). The cannabionoid CB1 receptor is another highly constitutively active receptor that stimulates food intake. The CB1 receptor is expressed, for example, in vagal afferent neurons, where its expression is also highly up-regulated during fasting, and the expression returns rapidly to normal when the animals are fed again (51).

Ligands binding to a receptor that displays high constitutive signaling activity can either regulate its signaling up (agonists) or down (inverse agonists). Although only a couple of endogenous inverse agonists have been described so far (i.e. agouti and agouti gene related peptide for the melanocortin receptors), it is likely that others will be identified in the future, especially because the only reason that agouti was discovered was the skin color produced by a natural genetic variant of the protein (52). Thus, it could be interesting to search for endogenous inverse agonists for the ghrelin-like receptors. It has been suggested that inverse agonists against the ghrelin receptor could be particularly attractive antiobesity agents (14).

The orphan receptor GPR39 is widely expressed in the central nervous system, and it could be of great importance that this receptor signals constitutively with very high efficacy through SRE, possibly mediated to a large degree through Gq12/13 and RhoA. The activity of Gq12/13 and RhoA has recently been associated with cellular plasticity in neurons, particularly with cytoskeletal contractions that prevent neural outgrowth (53). Hence, the expression of GPR39 and the following constitutive signaling through Gq12/13 may decrease the sensitivity of neurons for factors that induce sprouting (53, 54). Similar effects and signaling pathways could perhaps be involved in the recently demonstrated rapid rewiring of the arcuate nucleus feeding circuits in response to leptin and ghrelin, which have opposite effects in this case (55). Thus, it seems that the ghrelin receptor, and perhaps also GPR39, regulates neuronal activity not only through altering transmitter expression and release...
but also through altering neuronal plasticity (56). It has also recently been shown that SRE-induced transcription is an important factor for cell survival, including neuronal survival through inhibition of the complex apoptosis cascade (57). The strong increase in SRE activity induced by ghrelin through its receptor (Fig. 5) could perhaps be involved in the antiapoptotic effects observed for ghrelin on for example adipocytes (58).

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