

Interaction of the Growth Hormone-Releasing Peptides Ghrelin and Growth Hormone-Releasing Peptide-6 with the Motilin Receptor in the Rabbit Gastric Antrum

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ABSTRACT

The structural relationship between the motilin and the growth hormone secretagogue receptor (GHS-R), and between their respective ligands, motilin and ghrelin, prompted us to investigate whether ghrelin and the GHS-R agonist growth hormone-releasing peptide-6 (GHRP-6), could interact with the motilin receptor. The interaction was evaluated in the rabbit gastric antrum with binding studies on membrane preparations and with contraction studies on muscle strips in the presence of selective antagonists under conditions of electrical field stimulation (EFS) or not. Binding studies indicated that the affinity (pK_d) for the motilin receptor was in the order of ghrelin (4.23 ± 0.07) < GHRP-6 (5.54 ± 0.08) < motilin (9.13 ± 0.03). The interaction of ghrelin with the motilin receptor requires the octanoyl group. Motilin induced smooth muscle contractile responses but ghrelin and GHRP-6 were ineffective. EFS elicited

on- and off-responses that were increased by motilin already at 10^{-9} M, but not by 10^{-5} M ghrelin. In contrast, GHRP-6 also enhanced the on- and off-responses. The motilin antagonist Phe-cyclo[Lys-Tyr(3-tBu)- β Ala-] trifluoroacetate (GM-109) blocked the effect of GHRP-6 on the off-responses but not on the on-responses. Under nonadrenergic noncholinergic conditions, the effects of motilin and GHRP-6 on the on-responses were abolished; those on the off-responses were preserved. All responses were blocked by neurokinin (NK)₁ and NK₂ antagonists. In conclusion, ghrelin is unable to induce contractions via the motilin receptor. However, GHRP-6 enhances neural contractile responses, partially via interaction with the motilin receptor on noncholinergic nerves with tachykinins as mediator, and partially via another receptor that may be a GHS-R subtype on cholinergic nerves that corelease tachykinins.

Ghrelin, a 28 amino acid octanoylated peptide, was recently isolated from the stomach of rat, and was identified as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999). Before this discovery, the GHS-R was known to be activated by small synthetic peptides, growth hormone secretagogues (GHS), such as *H*-His-D-Trp-Ala-Trp-D-Phe-Lys-NH₂, also named growth hormone-releasing peptide-6 (GHRP-6). Ghrelin is now recognized as an important regulator of growth hormone (GH) secretion and energy homeostasis. Ghrelin is predominantly produced by the stomach, and the concentration of circulating ghrelin is influenced by acute and chronic changes in the

nutritional status (for review, see Muccioli et al., 2002). Thus, ghrelin probably also regulates diverse processes of the digestive system.

Ghrelin is structurally related to motilin. In fact, the peptide was also identified by another group who named it "motilin-related peptide" (Tomasetto et al., 2000). They found that motilin and motilin-related peptide had a complementary expression pattern (endocrine cells of the intestine and the stomach, respectively) and suggested that motilin-related peptide may also function as a gastric hormone. Because this group deduced the amino acid sequence from the nucleotide sequence of the precursor, they did not identify the octanoylation that is now known to be crucial for the biological activity of ghrelin. For this reason, it seems indicated to use the name ghrelin.

Recent studies show that indeed the spectrum of biological activities of both peptides shows some striking similarities.

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ABBREVIATIONS: GHS-R, growth hormone secretagogue receptor; GHS, growth hormone secretagogue; GHRP-6, growth hormone releasing peptide 6; GH, growth hormone; oct, octanoylated; EFS, electrical field stimulation; NK, neurokinin; NANC, nonadrenergic noncholinergic; RT-PCR, reverse transcription-polymerase chain reaction; PCR, polymerase chain reaction; bp, base pair(s); ANOVA, analysis of variance; MK 06777, *N*-[1(*R*)-[(1,2-dihydro-1-methanesulfonyl-spiro[3*H*-indole-3,4'-piperidin]-1'-yl)carbonyl]2-(phenyl-methoxy)ethyl]-2-amino-2-methylpropanamide methanesulfonate; EM-523, *de*(*N*-methyl)-*N*-ethyl-8,9-anhydroerythromycin A 6,9-hemiacetal.

Thus, although ghrelin was discovered via its effect on growth hormone release (Kojima et al., 1999), it was reported almost two decades ago that motilin, be it at high concentrations, stimulated growth hormone release in vitro and in vivo (Samson et al., 1982, 1984). Ghrelin and motilin may also act as an anabolic signal molecule during energy depletion because the levels of both peptides are increased by fasting, decreased by feeding and after an oral glucose load (Christofides et al., 1979; Peeters et al., 1980; Tschop et al., 2000), and both peptides stimulate insulin secretion (Suzuki et al., 1998; Date et al., 2002).

The effects of ghrelin on food intake confirm a role for ghrelin in the regulation of energy balance. Indeed, i.c.v. injection of ghrelin strongly stimulates feeding in rats and increases body weight by interacting with the pathways of neuropeptide Y and agouti-related protein in the arcuate nucleus (Nakazato et al., 2001). Also peripheral administration of this peptide alters food intake in mice and rats (Tschop et al., 2000) and intravenous administration enhances appetite and food intake in humans (Wren et al., 2001). An increase in food intake has also been observed after peripheral and central administration of motilin in rats and mice (Garthwaite, 1985; Rosenfeld and Garthwaite, 1987; Asakawa et al., 1998).

Hunger after ghrelin administration has been reported as a "side effect" in a clinical study analyzing GH release (Arvat et al., 2000). Motilin has also been called a "hunger hormone" because the increased plasma motilin levels during the fasted state trigger phase 3 gastric contractions during this period. Thus, the major physiological role of motilin has been attributed to its contractile effects on the gastrointestinal tract where it plays a key role in the regulation of the interdigestive motility pattern. Furthermore motilin and the motilin agonists erythromycin and its derivatives have gastroprokinetic activity (for review, see Peeters, 1993). Recent studies indicate that ghrelin has motor effects as well. Ghrelin has been reported to stimulate gastric motility in rats (Masuda et al., 2000), accelerate gastric emptying in mice (Asakawa et al., 2001), and resolve gastric postoperative ileus in rats (Trudel et al., 2002).

In addition to the structural similarities of the peptides themselves, 36% amino acid identity, and an identical precursor organization, their receptors, too, show a marked sequence homology with an overall identity of 52%, rising to 87% in the transmembrane regions. Therefore, at least some of the overlap in the spectrum of biological activities could be due to cross-interaction. To investigate this hypothesis, we compared the potency of motilin with that of ghrelin and some related peptides such as GHRP-6, a synthetic agonist of the GHS-R, in receptor binding and contractility studies in the rabbit gastric antrum, the classical in vitro model for motilin (Peeters and Depoortere, 1994). Furthermore, the mechanism of action of motilin and GHRP-6 and the receptor specificity of their contractile effects was studied in the presence of selective antagonists.

Materials and Methods

Chemicals

Norleucine¹³-porcine-motilin (motilin) was purchased from Eurogentec (Namur, Belgium). The motilin antagonist Phe-cyclo[Lys-Tyr(3-tBu)-βAla-] trifluoroacetate (GM-109) was a gift from Dr. N.

Takanashi (Chugai Pharmaceutical Company, Gotemba, Japan). Ghrelin (1-28)-OH octanoylated in Ser³ [ghrelin (1-28) oct] was from Phoenix (Belmont, CA). The growth hormone-releasing peptide GHRP-6 and its antagonist D-Lys³-GHRP-6 were purchased from Bachem (Bubendorf, Switzerland).

Octanoylated ghrelin (1-5) amide [ghrelin (1-5) oct], octanoylated ghrelin (1-23)-OH [ghrelin (1-23) oct], and nonoctanoylated ghrelin (1-23)-OH [ghrelin (1-23) nonoct] were custom synthesized by solid phase methodology using the 9-fluorenylmethoxycarbonyl strategy. Octanoylation was performed according to Bednarek et al. (2000). The neurokinin (NK₁) antagonist Sanofi Research 140333 (SR140333) and the NK₂ antagonist SR48968 were a gift from Dr. X. Emonds-Alt (Sanofi Research, Montpellier, France).

Tissue Preparation

Adult rabbits of either sex were sacrificed by a blow on the neck. The stomach was removed and rinsed with saline. All procedures were approved by the Ethical Committee for Animal Experiments of the University of Leuven.

Motilin Receptor Binding Studies

Membrane Preparation. The antrum was dissected free from the mucosa, minced, and homogenized in sucrose buffer (50 mM Tris-HCl buffer, pH 7.4, 250 mM sucrose, 25 mM KCl, and 10 mM MgCl₂) with inhibitors (1 mM iodoacetamide, 1 μM pepstatin, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 g/l trypsin inhibitor, and 0.25 g/l bacitracin). Homogenates were centrifuged at 1000g for 15 min, washed four times, and finally resuspended in 0.9% NaCl.

Displacement Curves. A competition binding assay was performed by incubating membranes (1 mg of protein) with ¹²⁵I-motilin (specific activity ± 1000 cpm/fmol, final concentration 50 pM) and increasing concentrations of motilin, ghrelin (1-5) oct, ghrelin (1-23) oct, ghrelin (1-23) nonoct, ghrelin (1-28) oct, GHRP-6, or D-Lys³-GHRP-6 for 60 min at 30°C. The reaction was stopped by adding cold buffer, and membrane-bound motilin was separated by centrifugation at 1000g. All data were corrected for nonspecific binding determined in the presence of an excess (10⁻⁶ M) of unlabeled motilin. The dissociation constant (K_d) was calculated from the displacement curves fitted to the equation of Akera-Cheng by computer (Akera and Cheng, 1977).

Contractility Studies. Circular strips, freed from mucosa (0.2 × 2.5 cm) were cut from the antrum and suspended along their circular axis in a tissue bath filled with Krebs' buffer (120.9 mM NaCl, 2.0 mM NaH₂PO₄, 15.5 mM NaHCO₃, 5.9 mM KCl, 1.25 mM CaCl₂, 1.2 mM MgCl₂, and 11.5 mM glucose) gassed with 95% O₂, 5% CO₂. After equilibration at optimal stretch, electrical field stimulation (EFS) was applied via two parallel platinum rod electrodes using a Grass S88 stimulator. Frequency spectra (1, 2, 4, 8, and 16 Hz) were obtained by pulse trains (pulse 1 ms, train 10 s, 5 V). Voltage was kept at 5 V using a Stimu-Splitter II (Med Lab, Loveland, CO). Each consecutive pulse train was followed by a 90-s interval. Contractions were measured using an isometric force transducer/amplifier (Harvard Apparatus, Inc., South Natick, MA), recorded on a multicorder, and sampled for digital analysis using the Windaq data acquisition system and a DI-2000 PGH card (Dataq Instruments, Akron, OH).

Electrical field stimulation generated muscle twitch responses consisting of on- and off-responses. When a stable response was obtained at all frequencies, the frequency spectrum was repeated in the presence of either motilin (10⁻⁹ M), ghrelin (1-23) oct (10⁻⁵ M), or GHRP-6 (10⁻⁵ M) after a preincubation period of 15 min. To evaluate the effect of antagonists, the frequency spectrum was first repeated in the presence of the motilin antagonist GM-109 (10⁻⁶ M; Takanashi et al., 1995), the GHRP-6 antagonist D-Lys³-GHRP-6 (10⁻⁵ M; Hansen et al., 1999), the NK₁ antagonist (SR140333) (5 × 10⁻⁷ M; Holzer et al., 1998), the NK₂ antagonist (SR48968) (5 × 10⁻⁷ M; Holzer et al., 1998), or under NANC (5 × 10⁻⁶ M atropine and 3 × 10⁻⁶ M guanethidine) conditions and then repeated in the presence

of antagonists + motilin or GHRP-6 in the same strip preparation. The response was calculated as the mean response during (on-response) and after (off-response) the stimulation period (from integrating the area under the curve) and was expressed in grams per square millimeter. Results are represented as the change in tension compared with the control frequency spectrum or to the frequency spectrum in the presence of antagonists.

RT-PCR for the Ghrelin Receptor (GHS-R)

Total RNA was prepared from rabbit antral smooth muscle strips using the TRIzol reagent (Invitrogen, Carlsbad, CA). Single-stranded cDNA was synthesized using a random hexameric primer (10 μ M) and 200 units of Superscript II RNase H⁻ reverse transcriptase (Invitrogen). The obtained cDNA served as a template for the polymerase chain reaction, consisting of 38 cycles of amplification (95°C for 1 min, 55°C for 1 min, 72°C for 2 min with a final elongation of 10 min at 72°C) using 0.5 U of *Taq*DNA polymerase (Amersham Biosciences AB, Uppsala, Sweden) and 0.5 μ M primers. The primers GHS-R.for (forward: 5'-GGA CCA GAA CCA CAA GCA RA-3') and GHS-R.rev (reverse: 5'-TGA GGT AGA AGA GGA CAA AGG A-3') were selected in conserved regions found after alignment of sequences published for human (GenBank no. U60179), rat (GenBank no. U94321), and swine (GenBank no. U60178) GHS-R mRNA. This PCR product was subjected to a nested PCR using as primers GHS-R2.for (5'-CMG TGA ARA TGC TKG CTG TG-3') and GHS-R2.rev (5'-TGG CTG ATC TGA GCY ATC TC-3') or GHS-R.for and GHS-R2.rev. This resulted in a PCR product of, respectively, 124 or 144 bp. PCR products were analyzed on a 1.8% agarose gel and visualized with ethidium bromide.

Statistical Analysis

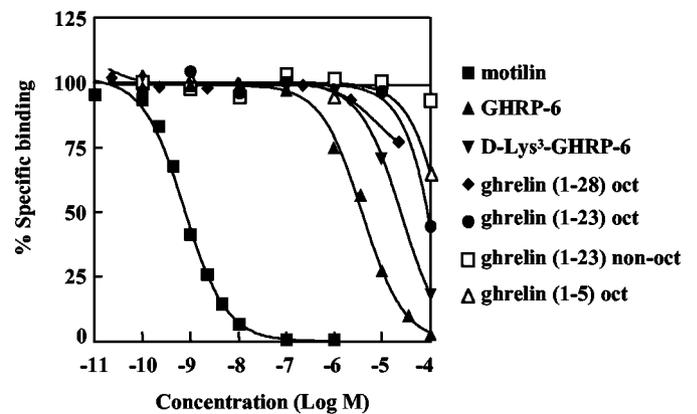
Data are represented as mean \pm S.E.M. The modulation of the response to electrical field stimulation by pharmacological agents at the individual frequencies was analyzed by Student's paired *t* test. Comparison of the response to motilin and GHRP-6 in the absence and presence of specific blockers or antagonists was analyzed by two-way ANOVA. A value of *P* < 0.05 was considered statistically significant.

Results

Interaction of Ghrelin and GHRP-6 with Motilin Receptor in Gastric Antrum of Rabbit.

Binding Studies. As shown in Fig. 1, ghrelin, GHRP-6, and related peptides weakly displaced membrane-bound ¹²⁵I-motilin. The order of affinity (pK_d) was motilin (9.13 \pm 0.03) > GHRP-6 (5.54 \pm 0.08) > D-Lys³-GHRP-6 (4.66 \pm 0.04) > ghrelin (1-28) oct (4.23 \pm 0.07) > ghrelin (1-23) oct (4.06 \pm 0.08) > ghrelin (1-5) oct (3.75 \pm 0.06). GHRP-6 had the highest affinity (5.54), which was reduced when the Ala in position 3 was replaced by D-Lys, a compound that is known to be a GHS-R antagonist. The affinity of GHRP-6 was 20.4-fold higher than that of ghrelin (1-28) oct. The weak interaction of ghrelin is due to the N terminus, because the potencies of ghrelin (1-28), ghrelin (1-23), and ghrelin (1-5) were not markedly different. The octanoyl residue, which is crucial for interaction with the ghrelin receptor, also increases affinity for the motilin receptor, because the interaction of nonoctanoylated ghrelin (1-23) was virtually zero.

Contractility Data. Nonstimulated strips. It is known that motilin contracts smooth muscle strips from the rabbit antrum, via a direct smooth muscle effect. The pEC_{50} value for this interaction is 7.48 \pm 0.11 (Van Assche et al., 1997). However, no contractile effect was observed with GHRP-6 or ghrelin (1-23) oct up to 10⁻⁵ M.



ghrelin (1-28) oct	CO-(CH ₂) ₈ CH ₃ GSSFLSPEHQVRVQQRKESKKPPAKLQPR
motilin	FVPIFTYGELQRMQEKERKNGQ
GHRP-6	HWAWFK-NH ₂

Fig. 1. Displacement of ¹²⁵I-motilin bound to a crude membrane preparation from the rabbit antrum by unlabeled motilin, GHRP-6, D-Lys³-GHRP-6, ghrelin (1-28) oct, ghrelin (1-23) oct, ghrelin (1-23) nonoct, and ghrelin (1-5) oct.

Electrical field stimulation. Electrical field stimulation evoked muscle twitch on- and off-responses that increased in amplitude with an increase in frequency from 0.55 \pm 0.14 g/mm² (1 Hz) to 8.56 \pm 0.32 g/mm² (16 Hz) for the on-responses and from 0.70 \pm 0.15 g/mm² (1 Hz) to 5.51 \pm 0.19 g/mm² (16 Hz) for the off-responses. Tetrodotoxin (3 μ M) abolished all contractions over the entire frequency spectrum, demonstrating that all responses were neurogenic (Fig. 2).

Motilin (10⁻⁹ M) significantly increased EFS-induced on-responses at 4, 8, and 16 Hz and off-responses over the entire

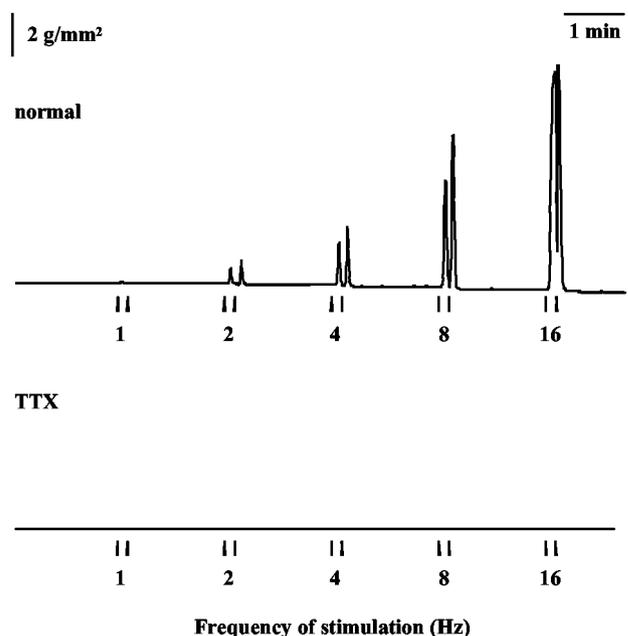


Fig. 2. Effect of tetrodotoxin (TTX) on EFS-induced responses. Muscle strips were stimulated at increasing frequencies in the absence (top) and presence of TTX (3 μ M, bottom). All responses were neurogenic because they could be abolished by TTX.

frequency spectrum. The change in tension induced by motilin was calculated at each frequency and is summarized in Fig. 3. In contrast, ghrelin (1-23) oct at concentrations up to 10^{-5} M had no effect (Fig. 3). However, GHRP-6 at a concentration of 10^{-5} M enhanced on-responses over the entire frequency spectrum to the same extent as motilin and off-responses between 2 and 16 Hz, although the effect on the off-responses was less pronounced than with motilin (Fig. 3). No significant effects were observed at 10^{-6} M.

Pharmacological analysis of neurally mediated effects. The effect of motilin on both the on- and off-responses was blocked by the motilin antagonist GM-109 (10^{-6} M), which by itself did not affect EFS-induced responses (Fig. 4). For GHRP-6 only the effect on the off-responses was blocked by GM-109, but the enhancement of the on-responses was preserved (Fig. 5).

To further explore the effects of motilin and GHRP-6, their effects were also studied under NANC conditions, and during blockade of tachykinergic receptors. The tracing in Fig. 6 illustrates that NANC conditions abolished the on-responses

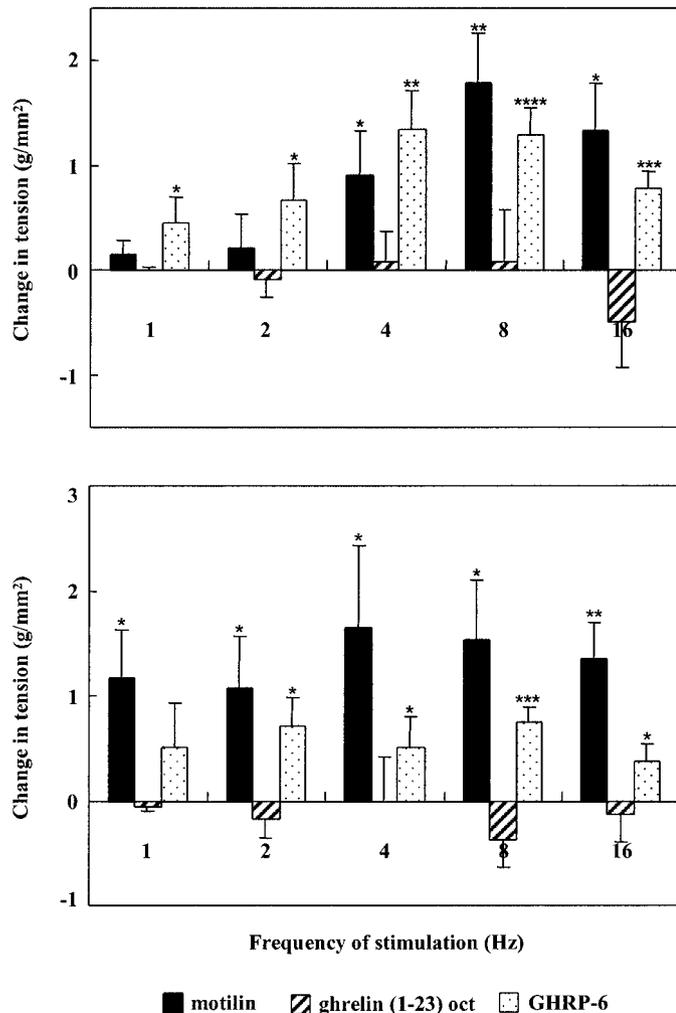


Fig. 3. Effect of ghrelin (1-23) oct, GHRP-6, and motilin on EFS-induced on-responses (top) and off-responses (bottom) in the rabbit antrum. Muscle strips were stimulated at increasing frequencies in the absence and presence of ghrelin (1-23) oct (10^{-5} M) ($n = 5$), GHRP-6 (10^{-5} M) ($n = 21$), or motilin (10^{-9} M) ($n = 14$), and the effect on the tension of the on- and off-responses was measured. Results are represented as the change in tension. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0005$; ****, $P < 0.00005$ indicate significant changes in tension compared with the response in the absence of agents in the same strip preparation.

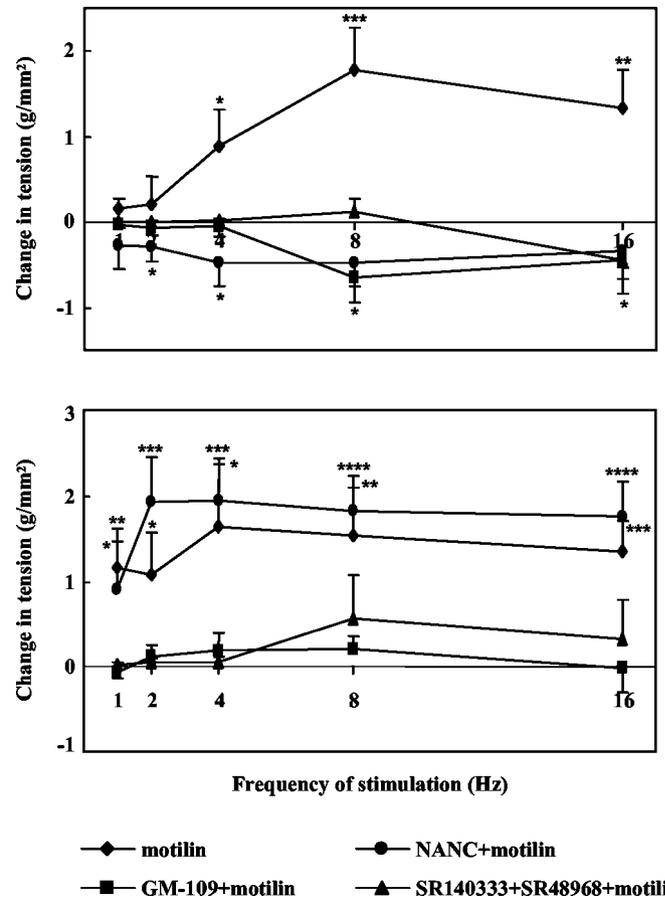


Fig. 4. Effect of a motilin antagonist, NANC conditions, and an NK_1 and NK_2 antagonist on motilin-induced neural on-responses (top) and off-responses (bottom) in the rabbit antrum. Results represent the change in tension (grams per square millimeter) induced by motilin (10^{-9} M) in the absence ($n = 14$) and presence of, respectively, GM-109 (10^{-6} M) ($n = 10$), atropine (5×10^{-6} M) + guanethidine (3×10^{-6} M) ($n = 21$), or SR140333 (5×10^{-7} M) + SR48968 (5×10^{-7} M) ($n = 8$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ indicate significant changes in tension compared with the response in the presence of antagonists without application of motilin in the same strip preparation. Two-way ANOVA revealed an overall significance for the response to motilin in the presence of GM-109 (on, $P < 0.0001$; off, $P < 0.0001$), in the presence of atropine and guanethidine (on, $P < 0.0001$; off, $P = 0.297$), and in the presence of SR140333 + SR48968 (on, $P < 0.0001$; off, $P < 0.001$) compared with the response of motilin in the absence of antagonists.

and markedly reduced the off-responses. Under these conditions, motilin markedly enhanced the off-responses over the entire frequency spectrum, but had no effect on the on-responses. The change in tension is summarized in Fig. 4. The effect of motilin on the off-responses under NANC conditions was not higher than under normal conditions.

Preincubation with the NK_1 (SR140333) and NK_2 (SR48968) antagonist also reduced EFS-induced on- and off-responses (data not shown). The remaining off-responses were abolished if the experiment was performed under NANC conditions (data not shown), indicating that tachykinins are the mediator of this noncholinergic contraction. In the presence of NK_1 and NK_2 antagonists motilin was ineffective, suggesting that tachykinergic pathways are involved in the effect of motilin on both type of responses (Fig. 4). The effect of GHRP-6 on the on-responses could not be blocked by the motilin antagonist, but as for motilin it could not be detected under NANC conditions (Fig. 5). The effect on

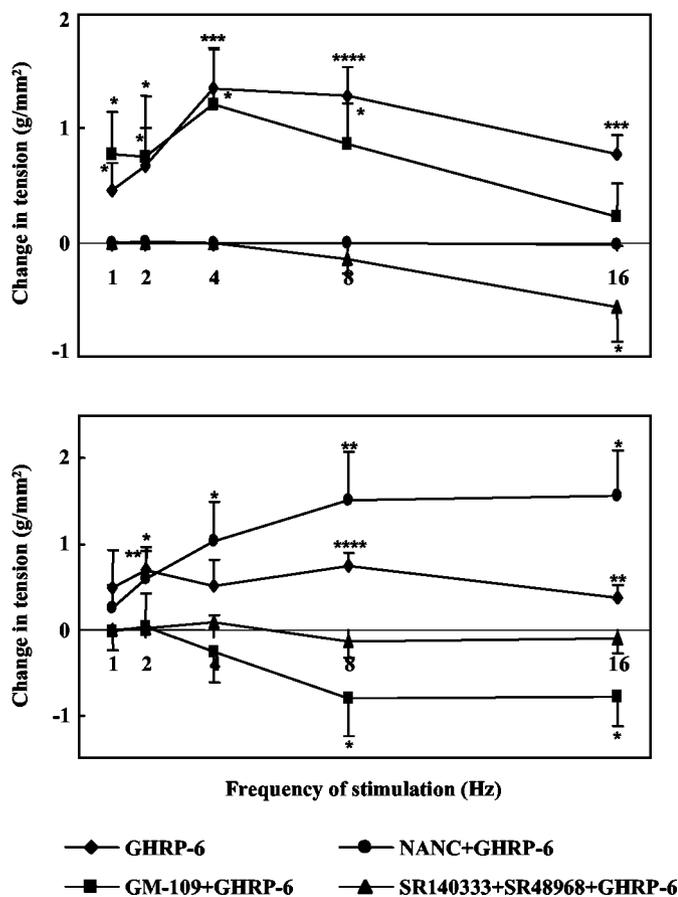


Fig. 5. Comparison of the effect of a motilin antagonist, NANC conditions, and an NK₁ and NK₂ antagonist on neural responses induced by GHRP-6. Results represent the change in tension (grams per square millimeter) induced by GHRP-6 (10^{-5} M) in the absence ($n = 21$) and presence of GM-109 (10^{-6} M) ($n = 13$), atropine (5×10^{-6} M) + guanethidine (3×10^{-6} M) ($n = 11$), or SR140333 (5×10^{-7} M) + SR48968 (5×10^{-7} M) ($n = 13$). Top, effect on the on-responses. Bottom, effect on the off-responses. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.00005$ indicate significant changes in tension compared with the response in the presence of antagonists without application of GHRP-6 in the same strip preparation. Two-way ANOVA revealed an overall significance for the response to GHRP-6 in the presence of GM-109 (on, $P = 0.163$; off, $P < 0.0001$), in the presence of atropine and guanethidine (on, $P < 0.0001$; off, $P = 0.158$), and in the presence of SR140333 + SR48968 (on, $P < 0.0001$; off, $P < 0.0001$) compared with the response of GHRP-6 in the absence of antagonists.

the off-response, which was blocked by the motilin antagonist, was under NANC conditions slightly enhanced at 8 and 16 Hz (Fig. 5). Similar to motilin, the effect of GHRP-6 on the on- and off-responses was blocked by the NK₁ and NK₂ antagonist (Fig. 5).

D-lys³-GHRP-6, known to inhibit the effect of GHRP-6 on GH release, did not antagonize the effect of GHRP-6 on the on- and off-responses (Fig. 7). In contrast, D-lys³-GHRP-6 also increased EFS-induced on-responses between 1 and 4 Hz similar to GHRP-6, but not at higher frequencies. No effect was observed on the off-responses, in agreement with its lack of effect at the concentration used in motilin receptor binding studies.

Expression of Growth Hormone Secretagogue Receptor in Rabbit Gastric Antrum

RT-PCR studies with cDNA prepared from total RNA isolated from rabbit antral smooth muscle strips revealed a

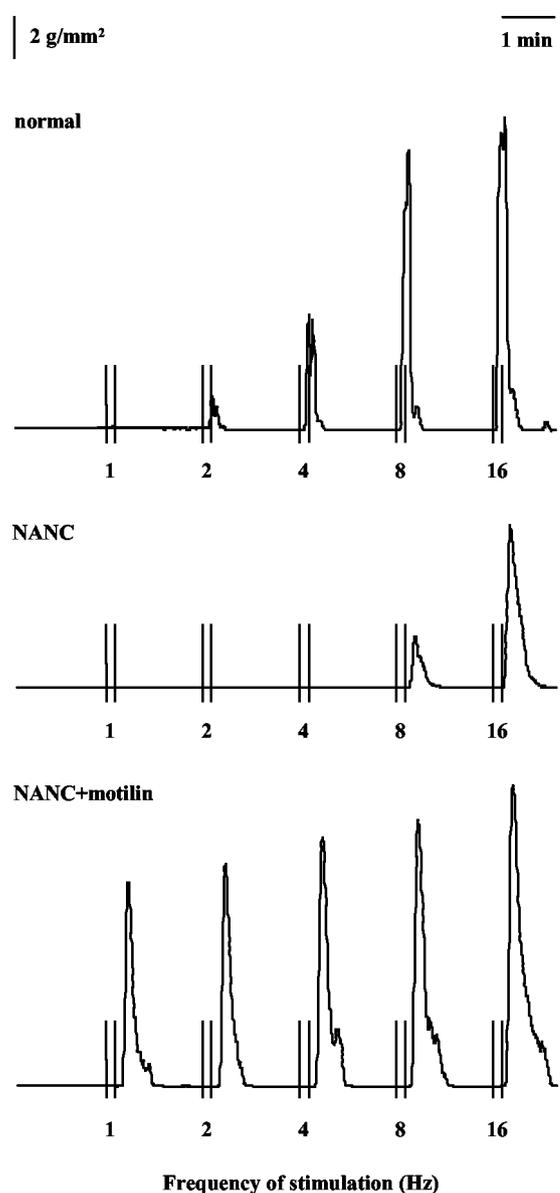


Fig. 6. Representative tracing showing the effect of motilin on EFS-induced responses at increasing frequency of stimulation under NANC conditions. The muscle strip was electrically stimulated under normal conditions and resulted in on- and off-responses (top). Under NANC conditions, only off-responses occurred at higher frequencies of stimulation (middle). Addition of motilin (10^{-9} M) under NANC conditions enhanced off-responses over the entire frequency spectrum (bottom).

GHS-R transcript product corresponding to the predicted size, based upon the set of primers used. Figure 8 shows an agarose gel electrophoresis of the nested PCR products obtained by subjecting first-round PCR products, with GHS-R.for and GHS-R.rev as primers, to a nested PCR with either GHS-R2.for and GHS-R2.rev (lane 1, expected size 124 bp) or GHS-R.for and GHS-R2.rev (lane 2, 144 bp) as primers.

Discussion

The pulsatile release of GH from the pituitary somatotrophs is regulated by two hypothalamic neuropeptides: growth hormone-releasing hormone and somatostatin that, respectively, stimulate and inhibit GH secretion. Release of GH from the pituitary somatotrophs can also be controlled by

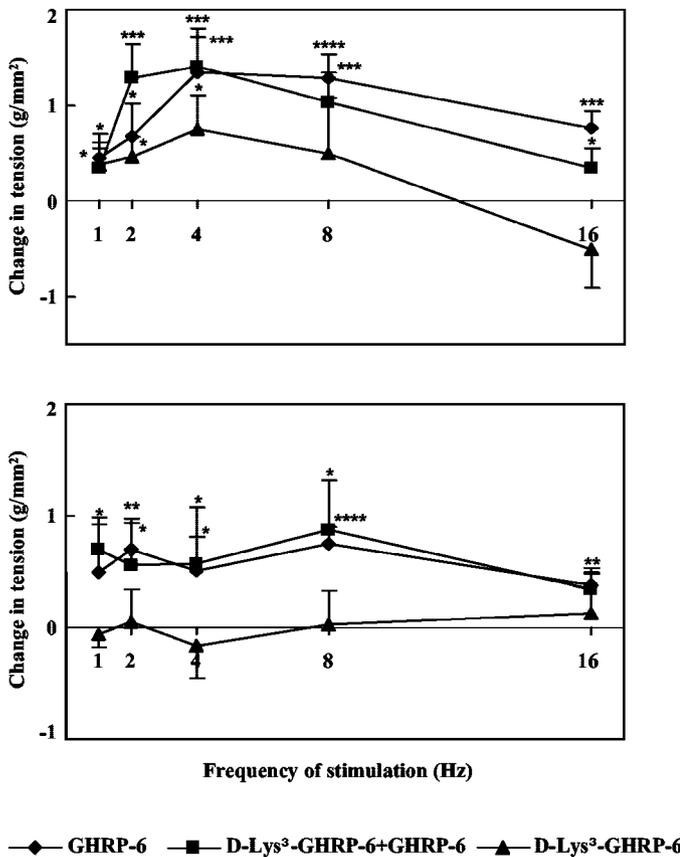


Fig. 7. Effect of the GHS-R antagonist D-Lys³-GHRP-6 on neuronal responses induced by GHRP-6. Strips were stimulated with D-Lys³-GHRP-6 (10^{-5} M) followed by stimulation with GHRP-6 (10^{-6} M). Results show the change in tension (grams per square millimeter) induced by GHRP-6 in the absence and presence of D-Lys³-GHRP-6 ($n = 16$) and of D-Lys³-GHRP-6 alone ($n = 22$). Top, effect on the on-responses. Bottom, effect on the off-responses. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; and ****, $P < 0.00005$ indicate significant changes in tension compared with the control response or the response in the presence of D-Lys³-GHRP-6 in the same preparation. Two-way ANOVA analysis revealed an overall significance for the response to GHRP-6 in the presence of D-Lys³-GHRP-6 (on, $P < 0.296$; off, $P < 0.804$) compared with the response of GHRP-6 in the absence of antagonist.

growth hormone secretagogues synthetic peptidyl (e.g., GHRP-6 and hexarelin) and nonpeptidyl molecules (e.g., MK 06777), whose actions are mediated by a specific GHS receptor (Howard et al., 1996), which is distinct from the GHRH receptor. Ghrelin was recently discovered as the natural ligand for the GHS receptor (Kojima et al., 1999). However, the principal site of ghrelin synthesis is the stomach, and the density of ghrelin binding sites is higher in several peripheral tissues than in the pituitary (Papotti et al., 2000), indicating that the role of ghrelin is not limited to the regulation of GH secretion. In this article, we explored the possibility that ghrelin could stimulate motor activity via an interaction with motilin receptors. The study was therefore performed in the rabbit gastric antrum, the classical model for the study of motor effects of motilin and motilin agonists (Peeters and Depoortere, 1994).

We found that ghrelin and ghrelin analogs interact very weakly with the motilin receptor in binding studies, whereas GHRP-6 showed some affinity ($pK_d = 5.54$). Thus, although GHRP-6 has virtually no sequence similarity with motilin, it has a stronger affinity for the motilin receptor than ghrelin.

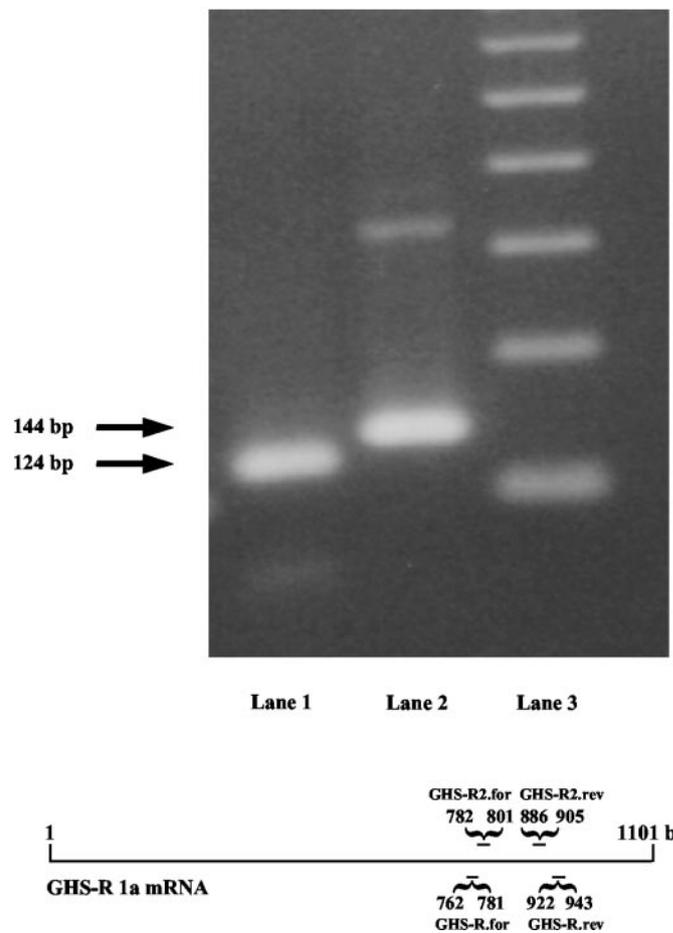


Fig. 8. GHS-R expression in the rabbit antrum. Ethidium bromide staining of agarose gel electrophoresis of the nested PCR products of cDNA prepared from the rabbit antrum. First-round PCR products obtained with GHS-R.for and GHS-R.rev as primers were subjected to a second PCR using either GHS-R2.for and GHS-R2.rev (124 bp, lane 1) or GHS-R.for and GHS-R2.rev (144 bp, lane 2) as primers. Lane 3, molecular weight marker giving bands of 100-bp spacing. A scheme indicating the primer positions in respect to human GHS-R 1a mRNA is shown.

In contrast, ghrelin does have sequence similarity (36%) with motilin but the pharmacophore of ghrelin, the [1-5] fragment and in particular the octanoyl residue on Ser³ (Bednarek et al., 2000), and the pharmacophore of motilin, the [1-7] fragment and in particular Phe¹, Val², Ile⁴, and Tyr⁷ (Macielag et al., 1992; Peeters et al., 1992), show little if any overlap. Interestingly, the weak interaction that we observed seems to be related to the octanoyl group because ghrelin (1-28) oct and ghrelin (1-5) oct have a comparable affinity, which is completely lost when the octanoyl group is removed. Ghrelin is the first peptide isolated from natural sources in which the hydroxyl group of one of its serine residues is acylated by *n*-octanoic acid, and this unique post-translational modification seems to be necessary for the GH-releasing potency of both human and rat ghrelin (Kojima et al., 1999). Apparently, it also creates a structural feature, unrelated to the amino acid sequence, which allows a weak interaction with the motilin receptor.

The weak affinity for the motilin receptor is unable to induce a contractile effect in our model. Therefore, the motor effects that have been reported for ghrelin are probably due to other pathways involving GHS receptors. In vivo, the effect of intravenous administration of ghrelin on gastric

contractions is mediated through the vagus (Masuda et al., 2000), but the concentrations of ghrelin used in this study, from 4 to 20 $\mu\text{g}/\text{kg}$, are considerably higher than for motilin, 50 ng/kg (Boivin et al., 1997; Masuda et al., 2000). In contrast, the stimulation of growth hormone secretion and the stimulation of food intake in rats are achieved at lower doses of ghrelin (growth hormone secretion 10 μg , i.v.; food intake 33 ng, i.c.v.) than for motilin (growth hormone secretion 100 μg , i.v.; food intake 1 μg , i.c.v.) (Samson et al., 1984; Rosenfeld et al., 1987; Nakazato et al., 2001; Tolle et al., 2001). Thus, although both peptides and their receptors are related to each other, the major physiological roles of motilin are its effect on intestinal motility, whereas for ghrelin, the most remarkable activities remain to be elucidated because other effects beside its effect on motility, GH secretion, and food intake have been reported. These include the cardioprotective actions of ghrelin (Nagaya et al., 2001) mediated by its antiapoptotic activity (Baldanzi et al., 2002) and the antiproliferative effects of ghrelin observed in neoplastic cell lines (Cassoni et al., 2001). In the latter two cases, the nonoctanoylated ghrelin also is effective, implicating that it might be mediated by another subtype of the ghrelin receptor. Overlapping effects at pharmacological concentrations may be due to cross-interaction but are probably physiologically not important.

GHRP-6, a ghrelin receptor agonist, had a higher affinity for the motilin receptor and did affect the response to electrical field stimulation. This effect is partially mediated through interaction with the motilin receptor, because it could be blocked by the motilin antagonist GM-109, and partially via another receptor, possibly a subtype of the GHS-R receptor with a low affinity for ghrelin. The transcripts detected by our RT-PCR studies could be transcripts of this GHS-R subtype. It is interesting to note that similarly to our study, in nonendocrine tissues, ghrelin has a much lower potency (100-fold) than hexarelin, a GHRP-6 analog, to displace bound ^{125}I -Tyr-Ala-hexarelin than in endocrine tissues (5-fold) (Papotti et al., 2000). Another indication for the involvement of a GHS-R subtype is the observation that the GHRP-6 antagonist D-Lys³-GHRP-6 was an agonist in our model. Moreover, this compound had weak excitatory effects on the on-responses but no effect on the off-responses, in agreement with its lower affinity for binding to the motilin receptor.

We have previously shown that in the rabbit antrum motilin may act on motilin receptors on both smooth muscle cells and myenteric neurons (Van Assche et al., 1997). In this study, we further explored the neurally mediated effect of motilin and obtained evidence for motilin-activated cholinergic and tachykininergic pathways. Especially, the effect of motilin on the on-response is mediated by a motilin receptor on cholinergic nerves because it is blocked under NANC conditions. This is in agreement with a previous *in vitro* study from Kitazawa et al. (1993) that indicated that motilin can induce the release of acetylcholine from enteric neurons in the rabbit duodenum. Because the effect of motilin on the on-responses was also reduced by NK₁ and NK₂ antagonists, it is plausible to assume that tachykinins synergize with acetylcholine in the transmission process. Substance P was originally characterized as an atropine-resistant stimulant of contraction of rabbit small intestine (von Euler and Gaddum, 1931). In guinea pig stomach, human ileum, and rat colon

both NK₁ and NK₂ receptors mediate NANC neuromuscular transmission (Zagorodnyuk and Maggi, 1997; Zagorodnyuk et al., 1997; Serio et al., 1998). We provided evidence that also in the rabbit antrum, the effect of motilin on the off-responses is mediated by a tachykininergic pathway that acts independent of ACh release.

In vivo studies in humans indicated that the effect of motilin and the motilin agonist erythromycin on antral motor activity is mediated through the activation of cholinergic neurons (Boivin et al., 1997; Coulie et al., 1998). Coulie et al. (1998) noted that atropine failed to block the prolonged rhythmic antral contractile activity induced by high doses of erythromycin and suggested that it might be due to activation of smooth muscle motilin receptors, which have a lower affinity (Van Assche et al., 1997). However, as the present study indicates, it might also reflect the activation of a noncholinergic neural pathway. This is in agreement with *in vivo* studies in dogs with the erythromycin derivative EM-523, which found that the contraction-stimulating activity of EM-523 in the stomach in the postprandial period is partially mediated through the cholinergic pathway; and partially through a noncholinergic pathway involving 5-hydroxytryptamine₃ and NK₁ receptors (Shiba et al., 1995).

In conclusion, our study indicates that in the rabbit antrum ghrelin is not able to have a meaningful interaction with its most related family member, the motilin receptor. Because we found no evidence that ghrelin stimulates contractile activity via local pathways. Our study, therefore, supports previous observations that ghrelin affects gastric motility via the vagal nerve. On the contrary, at high concentrations the ghrelin receptor agonist GHRP-6 acts on motilin receptors located on tachykininergic nerves that act independent of acetylcholine release to stimulate neural contractile responses. Moreover, GHRP-6 also activates another receptor that is probably a GHS-R subtype located on cholinergic neurons that corelease tachykinins. Our data therefore suggest that both the GHS-R and motilin receptor, and their ligands ghrelin and motilin, may be part of a larger family with yet-to-be-discovered peptides and receptors. The divergence in response of these peptides opens the road for the development of new gastroprokinetic drugs.

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