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Comparison of the gastroprokinetic effects of ghrelin, GHRP-6 and motilin in rats in vivo and in vitro

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Abstract

Ghrelin and motilin form a new family of structurally related peptides. We compared the gastroprokinetic effects of ghrelin, the ghrelin receptor agonist, growth hormone releasing peptide 6 (GHRP-6), and motilin in rats in vivo and in vitro.
Methods: Ghrelin, GHRP-6 or motilin (10–150 μg/kg) were injected i.p. and the effects on gastric emptying and transit were measured after intragastric application of Evans blue. In antral and fundic strips the effect of motilin, ghrelin or GHRP-6 was studied during electrical field stimulation (EFS) in the absence and presence of N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) (300 μM).
Results: Ghrelin and GHRP-6 but not motilin accelerated gastric emptying and transit in rats. Ghrelin was more potent than GHRP-6 and the dose–response relationship for ghrelin but not for GHRP-6 was bell-shaped. In fundic or antral strips, neural responses to EFS consisted of an on-relaxation that was reversed into a cholinergically mediated contraction by addition of the nitric oxide (NO)-synthase blocker, L-NAME. The post-stimulus off-contraction was cholinergically mediated. Under normal conditions, the ghrelin agonists reduced the on-relaxations in fundic strips and increased the cholinergic off-contractions in antral and fundic strips. The concentration response curves in muscle strips of the fundus were bell-shaped with maximal effects for ghrelin at 1.2 μM (on-responses) and 0.66 μM (off-responses) and for GHRP-6 at 0.50 μM (on-responses) and 0.26 μM (off-responses). No effects were observed with motilin between 1 nM and 0.1 μM. Studies in the presence of L-NAME confirmed the effect of the ghrelin agonists on cholinergic excitatory motor responses. No effects were observed with motilin under the different experimental conditions. The presence of growth hormone secretagogue receptor 1a transcripts in the strip preparations was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR).
Conclusion: Ghrelin and GHRP-6 but not motilin accelerate gastric emptying and transit by activating cholinergic excitatory pathways in the enteric nervous system in addition to the known vagal pathways.

Keywords: Peptide hormone; Gastric emptying; Intestinal transit; Enteric nervous system

1. Introduction

Ghrelin, a 28-amino acid peptide with an octanoyl at Ser^3, was discovered 5 years ago as the endogenous ligand of the growth hormone secretagogue receptor, now often referred to as the ghrelin receptor (Kojima et al., 1999). This receptor was first characterized, cloned and identified as the receptor for a family of synthetic ligands (e.g., GHRP-6) known as growth hormone secretagogues that stimulate the release of growth hormone (GH) (Howard et al., 1996; Bowers et al., 1977).

Soon after the discovery of ghrelin, it became clear that the functions of the peptide extend beyond the control of GH secretion, an observation consistent with the extensive presence of the ghrelin receptor in endocrine and non-endocrine peripheral organs (Papotti et al., 2000).

A clear role for ghrelin as the first systemically active orexigenic hormone has been shown in rodents as exoge-

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nous application of ghrelin induces weight gain by stimulating an acute increase in food intake as well as by decreasing fat utilization (Tschop et al., 2000; Nakazato et al., 2001). Also in humans, ghrelin may enhance appetite and food intake (Wren et al., 2001). In addition, circulating ghrelin levels are decreased in chronic (obesity) and acute (caloric intake) states of positive energy balance, whereas plasma levels of ghrelin are increased by fasting and in cachectic patients with anorexia nervosa (Cummings et al., 2001; Otto et al., 2001; Tschop et al., 2001). A variety of other functions have been ascribed to ghrelin, including cardiovascular (anti)-proliferative and slow wave sleep promoting effects (for a review, see Murray et al., 2003).

Ghrelin was identified almost simultaneously by another group who named it "motilin-related peptide" because of the similarity with the gastrointestinal peptide, motilin (Tomasetto et al., 2000). In addition, their receptors constitute a new subfamily within class A of rhodopsin-like G-protein-coupled receptor.

The physiological role of motilin is related to the induction of the interdigestive motility pattern. Motilin also accelerates gastric emptying (Peeters et al., 1992) and these effects are leading to the development of a new class of gastroprokinetics, motilides, erythromycin derivatives that interact with the motilin receptor (Janssens et al., 1990; Peeters, 1993). The recent findings that ghrelin can stimulate interdigestive motor activity (Fujino et al., 2003; Tack et al., 2004a) and gastric emptying in several species including man (Trudel et al., 2002; Asakawa et al., 2001; De Winter et al., 2004; Tack et al., 2004b) also support a physiological role for ghrelin in the regulation of gastrointestinal motility.

Although ghrelin was isolated from rat stomach extracts, attempts to isolate motilin from rat extracts have failed as were attempts to isolate cDNA encoding the motilin precursor (Vogel and Brown, 1990; Huang et al., 1999). The only species near to rats and mice in terms of evolution, from which motilin has been isolated, is the guinea pig (Xu et al., 2001).

The aim of the present study was to compare the dose-dependent effects of ghrelin and the synthetic agonist of the ghrelin receptor, growth hormone releasing peptide-6 (GHRP-6), with those of guinea pig motilin on motility: (a) in vivo by measuring their effect on gastric emptying and intestinal transit, (b) in vitro by measuring their effect on neural contractile responses in strips from rat antrum and fundus. More specifically, their effect on excitatory responses was characterized pharmacologically.

2. Materials and methods

2.1. Materials

Rat ghrelin was obtained from Tocris Cookson (Bristol, UK). Guinea pig motilin was custom synthesized by Eurogentec (Namur, Belgium). GHRP-6 was purchased at Bachem (Bubendorf, Switzerland). Atropine sulfate, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), guanethidine monosulfate and Evans blue were obtained from Sigma (St Louis, MO, USA).

2.2. In vivo experiments

All procedures for the in vivo experiments received approval from the Medical Ethical Committee of the University of Antwerp. Male Wistar rats (250–350 g) were fasted for 24 h with free access to water. They received an i.p. administration of the drug under study, ghrelin (10, 20, 50 or 100 μg/kg), GHRP-6 (15, 50, 100 or 150 μg/kg), motilin (10 or 100 μg/kg) or saline in a random order. Rats were divided in three experimental set-ups, studying the different drugs. Each group consisted of at least 7 rats in the ghrelin and GHRP-6 experiments and of at least 5 rats in the motilin experiments. Immediately after the injection of the drug, 1.5 ml Evans blue (50 mg/ml in 0.9% NaCl with 0.5% methylcellulose) was administered intragastrically with an orogastric canula. Twenty minutes later, the rats were sacrificed. The stomach was clamped with a string above the lower oesophageal sphincter and a string beneath the pylorus to prevent leakage of Evans blue. The stomach was cut just beneath the strings and was frozen at −70 °C until measurement of gastric emptying. The small intestine was also resected from the stomach to caecum and small intestinal transit was measured immediately.

Gastric emptying was determined spectrophotometrically, the method was adapted from the measurement of gastric emptying in mice previously described (De Winter et al., 2002, 2004). The stomach of each individual rat was cut just above the lower oesophageal sphincter and the pyloric sphincter. Evans blue remains largely in the lumen of the stomach, a part of the Evans blue is trapped in the mucus layer of the stomach and a very small amount of Evans blue is resorbed in the mucosa after 20 min (Lange et al., 1994). Then the stomach and its contents were put in 15 ml 0.1 N NaOH. These samples therefore contain the total amount of Evans blue present in the stomach (mostly luminal and within the mucus layer). The stomach was minced and homogenised (PRO 200, Pro Scientific Inc., CT, USA) during 30 s. The samples were further diluted to 30 ml with 0.1 N NaOH and left at room temperature for 1 h. Five milliliters of the supernatant was then centrifuged at 1356 *g* for 20 min at 4 °C. Samples were further diluted (1/50) with 0.1 N NaOH and the absorbance was read at a wavelength of 565 nm (A565) with a spectrophotometer (Varian, Victoria, Australia). The stomach and its contents obtained from a rat sacrificed immediately after orogastric administration of Evans blue served as standard (reference stomach). Percent gastric emptying was calculated as [(A565 reference−A565 sample)/A565 reference] × 100.

Evans blue colours the intestinal lumen dark blue and clearly shines through the intestinal wall. Therefore, *small intestinal transit* can be measured as the migration of Evans

blue from the pylorus to the most distal point of migration and was expressed in percent transit (compared to the total length of the small intestine) (De Winter et al., 1999, 2004). Twenty minutes after gavage, Evans blue migrated from the stomach into the small intestine, never reaching the caecum or colon within this time interval.

2.3. In vitro experiments

2.3.1. Tissue preparation

Male Wistar rats (350–400 g) were sacrificed, the stomach was removed and rinsed with saline. All procedures were approved by the Ethical Committee for Animal Experiments of the University of Leuven.

2.3.2. Contractility measurements

Circular strips (0.2 × 1.5 cm) freed from mucosa were cut from the antrum or fundus and suspended along their circular axis in a tissue bath filled with Krebs-buffer at 37 °C (NaCl: 120.9 mM; NaH_2PO_4: 2.0 mM; $NaHCO_3$: 15.5 mM; KCl: 5.9 mM; $CaCl_2$: 1.25 mM; $MgCl_2$: 1.2 mM; glucose: 11.5 mM) and gassed with 95% O_2/5% CO_2. After equilibration at optimal stretch (antrum: 2 g, fundus: 1.5 g), electrical field stimulation (EFS) was applied via two parallel platinum rod electrodes using a Grass S88 stimulator (Grass, Guincy, MA, USA). Frequency spectra (1–16 Hz) were obtained by pulse trains (pulse 1 ms, train 10 s, 6 V (fundus), 8 V (antrum)). Voltage was kept constant by using a Med Lab Stimu-Splitter II (Med Lab, Loveland, CO, USA). Each consecutive pulse train was followed by a 90-s interval. Contractions were measured using an isometric force transducer/amplifier (Harvard Appartus, South Natick, MA, USA), recorded on a multicorder and sampled for digital analysis using the Windaq data acquisition system and a DI-2000 PGH card (Dataq Instruments, Akron, Ohio, USA).

Frequency spectra were repeated every 30 min with washes in between until a stable response was obtained at all frequencies (usually after 3 times) following which strips were preincubated (15 min) with either 1 μM motilin, ghrelin or GHRP-6 before the frequency spectrum was repeated. Concentration response curves were established in a non-cumulative manner. Strips were electrically stimulated at increasing frequencies (1–16 Hz) in the absence or presence of different doses (10 nM–10 μM) of ghrelin, GHRP-6 or motilin and the change in tension of the EFS-induced on- and off-responses at 8 Hz was calculated.

The effect of the compounds on excitatory responses was studied in the presence of L-NAME (300 μM) in both antral and fundic strips. The neural response obtained in the presence of EFS was calculated as the mean response during (on-response) and after (off-response) the stimulation period and was expressed in grams and normalized for the cross-sectional area of the strip (g/mm^2) using the equation: cross-section (mm^2) = tissue wet weight (mg)/[tissue length (mm) × density (mg/mm^3)]. The density of smooth muscle was assumed to be 1.05 mg/mm^3.

2.3.3. RT-PCR for the growth hormone secretagogue receptor

Total RNA was prepared from rat fundic and antral smooth muscle strips using the TRIzol reagent (Gibco BRL, NY, USA). Single-stranded cDNA was synthesized using an oligo(dT) anchor primer (5′-GACCACGCGTATC-GATGTCGAC$(T)_{16}$-3′) (0.5 μM) and 200 units of super-script™ II RNase H$^-$ reverse transcriptase (Gibco BRL, NY, USA). 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 (Pharmacia Biotech, Uppsala, Sweden) and 0.5 μM primers (forward: 5′-GGA CCA GAA CCA CAA GCA RA-3′ and reverse: 5′-TGA GGT AGA AGA GGA CAA AGG A-3′). This PCR product was subjected to a nested PCR using as forward primer (5′-CMG TGA ARA TGC TKG CTG TG-3′) and as reverse primer (5′-TGG CTG ATC TGA GCY ATC TC-3′). All primers were selected in conserved regions found after alignment of sequences published for human (Genbank U60179), rat (Genbank U94321) and swine (Genbank U60178) growth hormone secretagogue receptor mRNA. This resulted in a PCR product of 124 bp. PCR products were analysed by electrophoresis on a 1.4% agarose gel and, after a capillary transfer of the cDNA fragments on a Biodyne A Membrane (Gibco BRL, NY, USA), hybridized with a [^{32}P]-labeled oligonucleotide probe (5′-GGTGTTTGCTTTCATCCTCT-GCTG-3′) at 54 °C overnight. The oligonucleotide probe (1 μM) was labelled using 25 μCi γ[^{32}P]-ATP (ICN, Costa Mesa, CA, USA) and 5 units of T_4 polynucleotide kinase (Promega, Southampton, UK).

2.4. Statistical analysis

Data are represented as mean ± standard error of the mean (S.E.M.). The effect of motilin, ghrelin and GHRP-6 on gastric emptying and intestinal transit was analysed by one-way analysis of variance (ANOVA) followed by Dunnett's test.

The modulation of the response to electrical field stimulation by the compounds under investigation at the individual frequencies was analysed by Student's paired *t*-test. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Contractility in vivo

3.1.1. Gastric emptying

Compared to saline, ghrelin at a dose of 20 μg/kg and 50 μg/kg significantly accelerated gastric emptying of the semi-liquid meal from 22.4 ± 4.5% to respectively 59.2 ± 6.8% ($P < 0.0001$) and 41.9 ± 6.9% ($P < 0.05$) (Fig. 1). Because higher doses (100 μg/kg) of ghrelin failed to accelerate

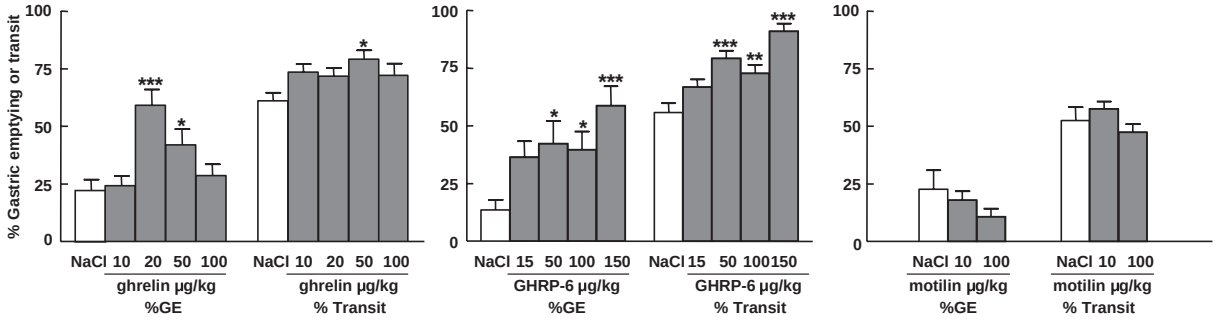

Fig. 1. Comparison of the effect of i.p. administration of increasing doses (0–150 µg/kg) of ghrelin, GHRP-6 or motilin on gastric emptying (GE) and transit in rats. Results are represented as the mean±S.E.M. and are the mean of at least 5 animals. *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus saline.

gastric emptying, the dose–response curve to ghrelin was bell-shaped. On the other hand, GHRP-6 increased gastric emptying dose-dependently between 15 and 150 µg/kg with significant effects at 50 µg/kg, 100 µg/kg and 150 µg/kg (Fig. 1). No significant effects were observed with motilin at 10 or 100 µg/kg (Fig. 1).

3.1.2. Small intestinal transit

Ghrelin significantly accelerated small intestinal transit in a bell-shaped manner with significant effects at a dose of 50 µg/kg (Fig. 1). Similar with the effects on gastric emptying, GHRP-6, dose-dependently increased small intestinal transit between 15 and 150 µg/kg with significant effects at 50 µg/kg (1.4-fold), 100 µg/kg (1.3-fold) and 150 µg/kg (1.6-fold) (Fig. 1). Application of motilin at 10 or 100 µg/kg had no effect on intestinal transit (Fig. 1).

3.2. Contractility in vitro

3.2.1. Fundic strips

EFS evoked changes in muscle contractility that were frequency-dependent and consisted of on- and off-responses. The responses were entirely neurogenic as they were blocked by tetrodotoxin (3 µM). The on-responses consisted of a relaxation, partially mediated by the release of nitric oxide because addition of L-NAME turned the relaxations into a cholinergically mediated contraction (Fig. 2A). The off-contractions were also cholinergically mediated as they disappeared under non-adrenergic, non-cholinergic (NANC) conditions (Fig. 2A).

A representative tracing of the effect of ghrelin (1 µM) on EFS-induced responses at 8 Hz under normal conditions is shown in Fig. 2B. Ghrelin reduced the EFS-induced on-relaxation and increased the EFS-induced off-contraction. The effect of ghrelin at the other frequencies is summarized in Fig. 3 and compared with the effects of GHRP-6 and motilin. Both ghrelin (1–8 Hz) and GHRP-6 (1–16 Hz) but not motilin at 1 µM reduced the on-relaxations and facilitated the off-contractions at high frequencies (8–16 Hz). A concentration response curve to ghrelin, GHRP-6 and motilin was established in a non-cumulative manner and the change in tension of the on- and off-responses induced by the three agonists at different concentrations (10 nM–10 µM) at 8 Hz was calculated and is represented in Fig. 4. The

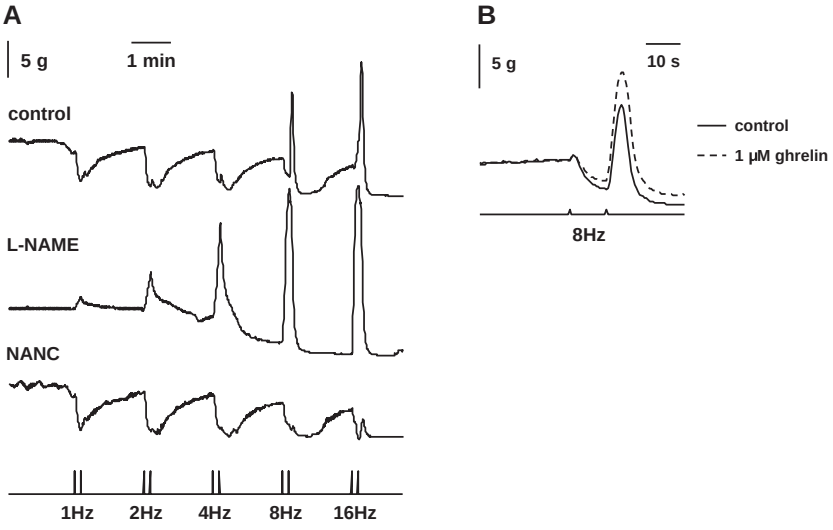

Fig. 2. (A) Representative tracing of neural responses induced by electrical field stimulation of rat fundic strips at increasing frequency of stimulation (1–16 Hz) under normal conditions, in the presence of L-NAME (300 µM) or under NANC (5 µM atropine and 3 µM guanethidine) conditions. (B) Representative tracing of the effect of ghrelin (1 µM) on EFS-induced on- and off-responses at 8 Hz in rat fundic strips.

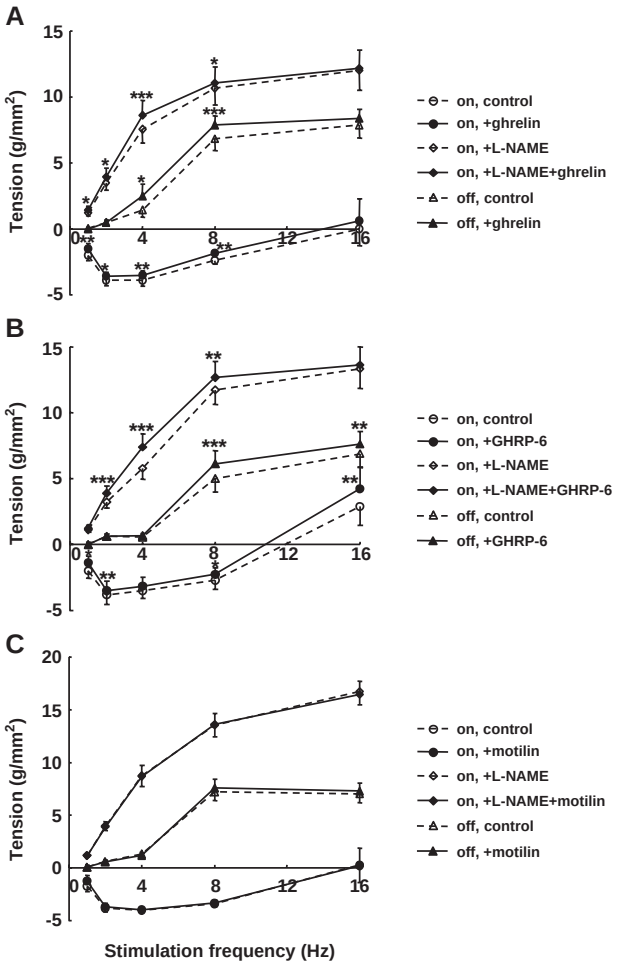

Fig. 3. Effect of ghrelin (A), GHRP-6 (B) or motilin (C) on EFS-induced responses in rat fundic strips. Muscle strips were electrically stimulated (1–16 Hz) preceding and during incubation with L-NAME in the absence (dashed lines, open symbols) or the presence (full lines, filled symbols) of ghrelin (1 μM), GHRP-6 (1 μM) or motilin (1 μM) and the tension of the on- and off-responses was measured. Results are mean±S.E.M. of 8–11 strip preparations. *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus the control frequency spectrum or the spectrum in the presence of L-NAME in the same strip preparation.

concentration response curves were bell-shaped with maximal effects for ghrelin at 1.2 μM (on-responses) and 0.66μM (off-responses) and for GHRP-6 at 0.50 μM (on-responses) and 0.26 μM (off-responses). No effects were observed with motilin between 1 nM and 0.1 μM.

The effect of the ghrelin receptor agonists on excitatory responses was studied into more detail in the presence of L-NAME (300 μM). Both ghrelin and GHRP-6 increased cholinergic motor responses in the presence of L-NAME over almost the entire frequency spectrum (1–8 Hz) (Fig. 3). Also under these conditions, motilin did not affect nerve-mediated excitatory responses.

Neither of the compounds studied affected smooth muscle responses in the absence of electrical field stimulation (data not shown).

3.2.2. Antral strips

Due to the small basal tone of the antral strips, EFS did not induce pronounced on-relaxations as in fundic strips but off-contractions were already apparent at lower frequencies of stimulation (Fig. 5). In the presence of L-NAME, EFS unmasked cholinergically mediated on-contractions. The off-contractions almost completely disappeared under NANC conditions (Fig. 5).

None of the agonists affected the on-relaxations under normal conditions but the off-contractions were enhanced by 1 μM ghrelin (4–16 Hz) or GHRP-6 (4–8 Hz) but not by motilin. The maximal change in tension induced by ghrelin amounted 0.34±0.11 g/mm^2 (4 Hz) and by GHRP-6, 0.57±0.16 g/mm^2 (8 Hz).

The effect of the compounds on excitatory cholinergic motor responses was also studied in the presence of L-NAME and revealed an enhancement of the response after application of ghrelin (4–8 Hz) and GHRP-6 (1–16 Hz) (Fig. 6). No effect was observed with motilin.

None of the compounds studied induced smooth muscle responses in the absence of electrical field stimulation (data not shown).

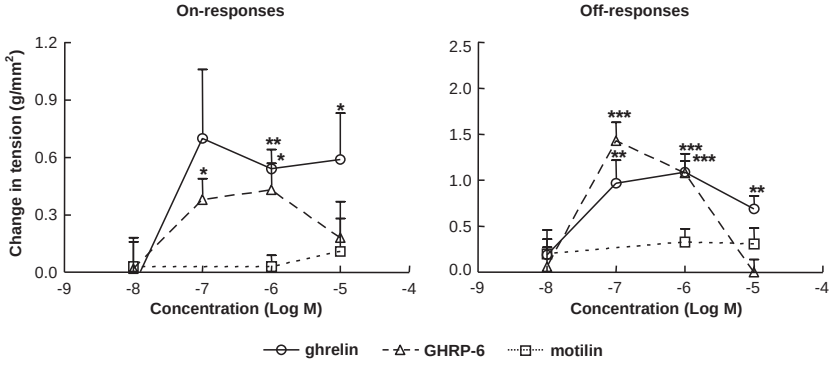

Fig. 4. Non-cumulative concentration response curves to ghrelin, GHRP-6 or motilin in rat fundic strips. Strips were electrically stimulated at increasing frequencies (1–16 Hz) in the absence or presence of different doses (10 nM–10 μM) of ghrelin, GHRP-6 or motilin and the change in tension of the EFS-induced on- and off-responses at 8 Hz was calculated. Results are mean±S.E.M. of 6–11 strip preparations. *$P<0.05$, **:$P<0.01$, ***$P<0.001$ versus the control response.

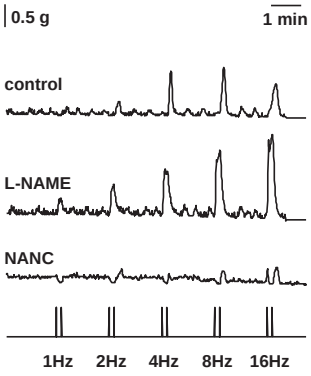

Fig. 5. Representative tracing of neural responses induced by electrical field stimulation of rat antral strips at increasing frequency of stimulation (1–16 Hz) under normal conditions, in the presence of L-NAME (300 μM) or under NANC (5 μM atropine and 3 μM guanethidine) conditions.

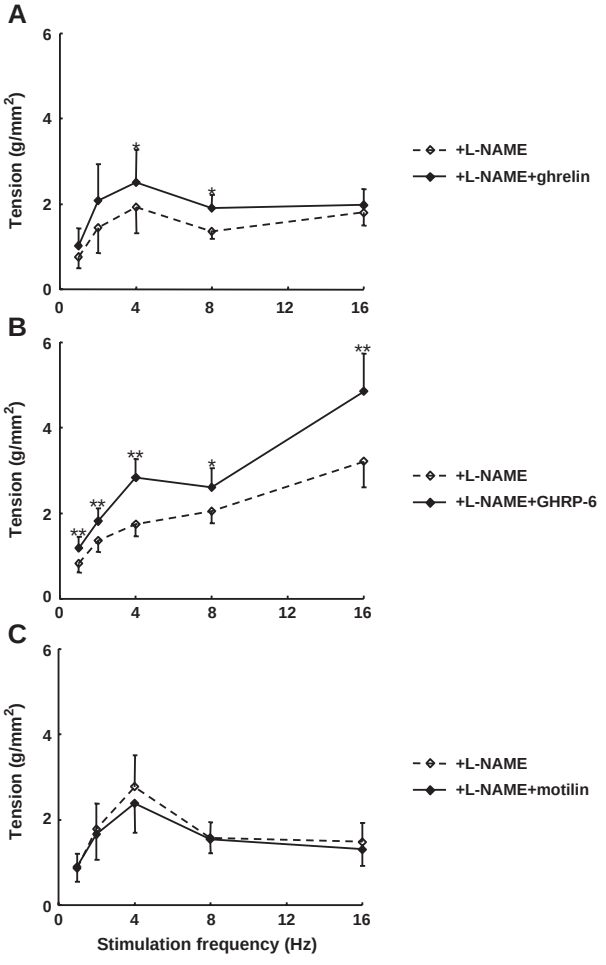

Fig. 6. Effect of ghrelin (A), GHRP-6 (B) or motilin (C) on EFS-induced responses in rat antral strips. Muscle strips were electrically stimulated (1–16 Hz) during incubation with L-NAME in the absence (dashed lines, open symbols) or the presence (full lines, filled symbols) of ghrelin (1 μM), GHRP-6 (1 μM) or motilin (1 μM) and the tension of the on-responses was measured. Results are mean ± S.E.M. of 8–11 strip preparations. *$P<0.05$, **$P<0.01$ versus the control frequency spectrum or the spectrum in the presence of L-NAME in the same strip preparation.

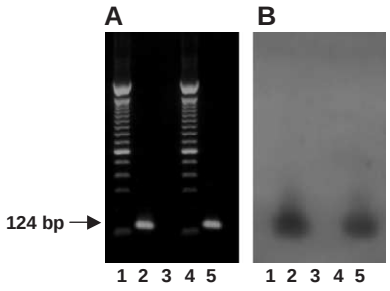

Fig. 7. Expression of ghrelin receptor mRNA in the rat fundus and antrum. (A) Ethidium bromide staining of agarose gel electrophoresis of the nested PCR products of cDNA prepared from the rat antrum (lane 2) and fundus (lane 5). The band of 124 bp corresponds to the amplified ghrelin receptor cDNA product with the expected length. Lane 1 and 4, molecular weight marker giving bands of 100-bp spacing. (B) Southern blot hybridization of the agarose gel (A) with the [^{32}P]-labeled oligonucleotide ghrelin receptor probe. The positive signal in lane 2 and 5 confirmed the existence of ghrelin receptor transcripts in respectively rat antrum and fundus.

3.3. Expression of the ghrelin receptor in rat fundic and antral strips

The presence of mRNA for the ghrelin receptor 1a variant in the rat antral and fundic smooth muscle strips was verified by RT-PCR with gene specific primers and the specificity of the amplified products was confirmed by Southern blot hybridization. Analysis of the PCR products on electrophoresis revealed in both preparations a band with the expected length of 124 bp, hybridizing with the [^{32}P] labelled ghrelin receptor oligonucleotide probe (Fig. 7).

4. Discussion

The ghrelin receptor agonists, ghrelin and GHRP-6, but not motilin stimulated gastric emptying and small intestinal transit in vivo and enhanced neurally mediated contractions in rat fundic and antral strips in vitro.

The situation is therefore opposite to the results in rabbit antral smooth muscle strips where motilin and GHRP-6 but not ghrelin enhanced neural responses and induced smooth muscle contractions (Van Assche et al., 1997; Depoortere et al., 2003; Dass et al., 2003a). However, the effect of GHRP-6 was partially mediated via cross-interaction with the motilin receptor and partially via another receptor that might be the ghrelin receptor or a subtype (Depoortere et al., 2003).

Thus, although motilin is able to induce contractile responses in vitro in rabbit, man and cat (Peeters et al., 1988; Van Assche et al., 2001; Depoortere et al., 1993), preparations of rat intestine have proven to be refractory to motilin (Segawa et al., 1976; Strunz et al., 1975; Sato et al., 1997) and the motilin agonist erythromycin A (Peeters et al., 1989) or one of its derivatives (Sato et al., 1997) in vitro. Other studies (Tani and Muto, 1985; Bertaccini and Coruzzi, 1977), except one (Nakazawa et al., 1978), also failed to

observe an effect with motilin on gastric emptying or transit in vivo in control rats or in rats with postoperative ileus (De Winter et al., 1999; Trudel et al., 2002) when administered peripherally.

In the present study, we took another approach and used guinea pig motilin, the only species near to rats and mice in terms of evolution from which motilin has been isolated (Xu et al., 2001), to test the effect of motilin in rats. In addition, the effect of motilin on neurally mediated contractions, elicited by electrical field stimulation, was for the first time addressed in rats. However, none of these attempts led to a positive result. The situation could therefore be similar to dogs where motilin is effective in vivo but not in vitro, except at high concentrations (Itoh et al., 1976; Poitras et al., 1987). For that reason, in dogs, it has been concluded that motilin affects motility via vagal efferents stimulated by motilin receptors on 5-hydroxytryptamine-containing neurons in the area postrema (Itoh, 1997). However, in the present in vivo study, guinea pig motilin did also not affect gastric emptying and transit in rats even at a dose that was 50 times higher than the dose used to accelerate gastric emptying in humans (Peeters et al., 1992).

The lack of effect of motilin in rats might be explained by the fact that motilin and its receptor are non-existent or non-functional in rats. The recent publication of the complete genome of the rat allowed to apply in silico strategies to identify the rat genes for motilin and its receptor and showed that only pseudogene sequences exist for the peptide and its receptor (Aerssens et al., 2004). This explains the negative results with guinea pig motilin in rats in the present study although it remains to be elucidated why this diversion of evolution for motilin did occur. Is it because of a rodent-specific difference in gastrointestinal regulation or because the actions of ghrelin now combine the functions of both peptides? The fact that the migrating motor complex length in rats is much shorter than in mammals suggests that it is perhaps not regulated by motilin but by other neuropeptides. The ligand activation domain of the ghrelin receptor has been evolutionary conserved from Pufferfish (*Spheroides nephelus*) to human (400 million years) thereby stressing the importance of ghrelin for human biology (Palyha et al., 2000). For the motilin receptor, the situation is less clear. Although a clone has been isolated from the genomic library of the Pufferfish that was thought to represent an ortholog of the motilin receptor, motilin failed to activate this receptor when expressed in HEX 293 cells (Palyha et al., 2000).

Our data with ghrelin in vivo are in accordance with previous studies in rats. In anesthetized rats, Masuda et al. (2000) showed excitatory effects of ghrelin on gastric contractions and in conscious rats Trudel et al. (2002) demonstrated that ghrelin accelerated gastric emptying, enhanced small bowel transit and was able to overcome postoperative ileus. However, this is the first report on the effects of GHRP-6 on gastric motility and transit in rats. In addition, we showed that, in vivo, the dose–response relationship to ghrelin was bell-shaped but not for GHRP-6 in the dose-range investigated. There are many reasons for bell-shaped dose–response curves. Desensitization is one of the possibilities especially because the ghrelin receptor is susceptible to rapid desensitization (Camina et al., 2004; Orkin et al., 2003). Another possibility is the existence of a high and a low affinity receptor binding site.

Ghrelin was more potent than GHRP-6 to accelerate gastric emptying and transit because, if expressed in nmol/kg, ghrelin had significant effects between 6 nmol/kg (20 μg/kg) and 15 nmol/kg (50 μg/kg) while GHRP-6 was effective between 57 nmol/kg (50 μg/kg) and 172 nmol/kg (150 μg/kg). However, in vitro GHRP-6 was slightly more potent than ghrelin and the concentration response curve for both agonists was bell-shaped in vitro. A different susceptibility to peptidase activity in vivo and a different ability of the agonists to reach the neurons in the myenteric plexus in vitro may account for the different potency of ghrelin and GHRP-6 in vivo and in vitro.

There is little doubt that the vago-vagal reflex pathway plays an important role in the motility effects of ghrelin in rats because the effects can be blocked by atropine or vagotomy (Masuda et al., 2000; Fujino et al., 2003). In addition, the presence of the ghrelin receptor in rat nodose ganglion was confirmed by RT-PCR, direct sequencing, in situ hybridization histochemistry and electrophysiology (Date et al., 2002).

Fujino et al. (2003) showed that following vagotomy a peripheral mechanism may become active in rats. In the present study, we provided evidence for the existence of ghrelin receptor 1a transcripts in the rat fundic and antral strips while morphological evidence for the presence of ghrelin receptors in the enteric nervous system but not in smooth muscle tissue of rat stomach was recently reported by Dass et al. (2003b). These authors reported that ghrelin could increase the EFS-evoked off-contractions in strips from rat forestomach. In the present study, we compared the effects of ghrelin with those of GHRP-6 in more clearly defined regions of the stomach, fundus and antrum and investigated their effect on the on- and off-responses into more detail. Studies in rat fundic strips in the presence and absence of L-NAME confirmed the effect of the ghrelin agonists on cholinergic excitatory responses and suggested that the reduction of the on-relaxation was due to stimulation of cholinergic nerves partially masked by nitrergic nerves. Also in rat antral strips ghrelin and GHRP-6 enhance cholinergic excitatory motor responses.

In conclusion, this study for the first time compared the effects of ghrelin with that of the growth hormone secretagogue, GHRP-6, on gastrointestinal motility in vivo and in vitro in rats. Our data show that both peptides have prokinetic properties on gastric emptying and small intestinal transit but with a different dose–response relationship. In addition to the known vagus nerve-dependent mechanisms, we provide evidence that both peptides may also have a direct effect on the enteric nervous system through

activation of cholinergic excitatory nerves. However, the rather small effects observed in vitro suggest that the major effects of ghrelin and GHRP-6 are mediated via the vagus. The lack of effect of motilin in rats is the result of a divergence in evolution and it remains to be investigated whether ghrelin could be the surrogate of motilin in rats.

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