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Evidence for the presence of motilin, ghrelin, and the motilin and ghrelin receptor in neurons of the myenteric plexus

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Abstract

Motilin, a 22-amino acid gastrointestinal peptide, and ghrelin, the natural ligand of the growth hormone secretagogue receptor, form a new group of structurally related peptides. Several lines of evidence suggest that motilin and ghrelin are involved in the control of gastrointestinal motility by the activation of receptors on enteric neurons. The aim of this study was to look for the existence of motilin, ghrelin, and their respective receptors in the myenteric plexus of the guinea pig. We used longitudinal muscle/myenteric plexus (LMMP) preparations and cultures of myenteric neurons of the guinea pig ileum, immunohistochemistry, and reverse transcriptase-polymerase chain reaction (RT-PCR). Most of the motilin-immunoreactive (IR; 72.8%) and motilin receptor-IR (68.9%) neurons were also positive for neuronal nitric oxide synthase (nNOS), 72.8% and 68.9%, few for choline acetyl transferase (ChAT), 11.4% and 11.9%, respectively. In contrast, ghrelin was mainly colocalized with ChAT (72.2%), and only 3.6% of ghrelin-positive cells showed nNOS-IR in the LMMP. Neither motilin nor the motilin receptor or ghrelin colocalized with calbindin. RT-PCR studies revealed motilin, ghrelin, and ghrelin receptor mRNA transcripts in LMMP preparations and in cultured myenteric neurons. In conclusion, this study, for the first time, provides direct evidence for the existence of motilin and ghrelin in myenteric neurons and suggests that both peptides may play a role in the activation of the enteric nervous system and hence in the regulation of gastrointestinal motility.

Keywords: Motilin; Neuronal nitric oxide synthase; Choline acetyl transferase

1. Introduction

Motilin, a 22-amino acid gastrointestinal peptide, is released periodically from endocrine cells located in the mucosa of the upper part of the small intestine. It is generally accepted that at least in dog and in man, endogenous motilin participates in the control of the interdigestive migrating motor complex (MMC; [1,2]). The mechanism of action of motilin has not been completely elucidated yet and may implicate the vagus, the myenteric plexus, and the smooth muscle tissue. Indeed, in vivo studies in dogs led to the conclusion that low doses of motilin stimulate motilin receptors on vagal afferent neurons, whereas higher doses activate a nonvagal cholinergic pathway in the enteric nervous system. Activation of the vagal pathway induces phase 3 activity, while activation of the enteric nervous system results in strong gastrointestinal contractions [3]. Similar studies in man with the motilin agonist, erythromycin A (EM-A), showed that low EM-A doses induce a premature activity front by acting on motilin receptors on cholinergic neurons, while high doses evoke prolonged rhythmic antral contractions by activation

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of a noncholinergic neural pathway or by acting on a muscular motilin receptor [4]. In vitro studies with rabbit gastric antral strips confirmed that motilin at low doses activates motilin receptors on cholinergic and tachykinergic nerves, while at high doses, it interacts with smooth muscle motilin receptors [5,6].

Because the motilin receptor has only recently been identified [7], direct evidence for the presence of the motilin receptor or its messenger RNA in the enteric nervous system could not be obtained yet. The aim of this paper was therefore to fill this gap. We also decided to study ghrelin, the recently discovered endogenous ligand of the growth hormone secretagogue receptor [8], because this peptide shows a sequence homology (27%) with motilin, which prompted another group to propose the name "motilin-related peptide" [9]. Moreover, it is now established that the receptors of both peptide are related as well (44% overall homology, increasing to 87% in the transmembrane regions). Ghrelin is synthesized predominantly in the stomach and not only promotes growth hormone secretion but also stimulates food intake and decreases fat utilization [10–12]. In addition, ghrelin, like motilin, is capable of inducing phase 3 of the MMC and of accelerating gastric emptying in man and in rodents [13–17]. In rats, the motor effects in vivo are blocked by vagotomy [13,18], but following vagotomy, a local mechanism becomes operational [13]. In vitro studies also provided evidence for the presence of ghrelin receptors in the wall of the rat stomach [17,19].

The aim of this study was therefore to provide morphological evidence for the presence of motilin, ghrelin, and their receptors in longitudinal muscle/myenteric plexus (LMMP) preparations and cultured myenteric neurons of the guinea pig ileum by immunohistochemical and reverse transcriptase-polymerase chain reaction (RT-PCR) studies. In addition, the chemical code of the immunoreactive (IR)-positive neurons was determined by costaining for excitatory neurotransmitters [choline acetyl transferase (ChAT), a key enzyme in the synthesis of ACh], inhibitory neurotransmitters [nitric oxide synthase (NOS), the NO producing enzyme], and intrinsic primary afferent neurons (calbindin). The identity of the stained cells was checked by staining for neuron-specific enolase (NSE), a general marker for neurons.

2. Materials and methods

2.1. Longitudinal muscle/myenteric plexus (LMMP) preparations

Guinea pigs of either sex (300–400 g) were killed by cervical dislocation and exsanguinated by severing the carotid arteries. A 5-cm segment of the ileum was removed, flushed with cold sterile Krebs' solution, and pinned flat in a Sylgard bottom dissection dish. The intestine was opened along the mesenteric border, and the mucosa, submucosa, and circular muscle layer were removed with fine forceps. During the dissection, the preparation was continuously gassed with 5%/95% CO_2/O_2. The procedures were approved by the Ethical Committee for Animal Experiments of the University of Leuven.

2.2. Culturing of myenteric neurons

The LMMP was enzymatically digested in a Krebs' solution containing protease (1 mg/ml; Sigma, St. Louis, MO, USA), collagenase (1.25 mg/ml, Sigma), and bovine serum albumin (0.4% w/v; Sigma). After 30 min of incubation at 37 °C, the suspension was placed on ice and was centrifuged (1600 rpm). The pellets were washed, resuspended, and the ganglia were selected under a binocular microscope and plated in culture dishes. The growth medium was neuron-specific Medium 199 (Invitrogen, Carlsbad, CA, USA), enriched with 10% (v/v) fetal bovine serum (FBS) (Biochrom, Berlin, Germany), 50 ng/ml nerve growth factor (7sNGF; Alomone Labs, Jerusalem, Israel), and several antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin, and 50 μg/ml gentamycin; Invitrogen). In order to prevent the proliferation of dividing cells (glial cells, fibroblasts), 10 μM arabinose C-furanoside (Sigma) was added from the fourth day on. Cultured neurons (around day 8) were used for immunocytochemistry.

2.3. Immunohistochemical procedures

The LMMPs were pinned flat in a Sylgard bottom dissection dish and were fixed in freshly prepared 4%

Table 1
Characteristics of the antisera used in the immunohistochemical staining

Primary Ab (host)	Dilution	MC/PC	Supplier
Motilin (rabbit)	1:400	PC	Gut Hormone Lab, KULeuven, Belgium
Motilin receptor (rabbit)	1:500	PC	Dr. Howard, Merck, NY, USA
Ghrelin (Rb)	1:500	PC	Dr. Tomasetto, Strasbourg, France
ChAT (goat)	1:100	MC	Chemicon International, CA, USA
nNOS (mouse)	1:200	MC	Chemicon International, CA, USA
NSE (mouse)	1:10000	MC	Polysciences, USA
CALB (mouse)	1:100	MC	Sigma Immunochemicals, MO, USA

Secondary Ab
FITC-conjugated goat antirabbit IgG (1:50)
Cy3™-conjugated goat antimouse IgG (1:500)
FITC-conjugated donkey antirabbit IgG (1:50)
Cy3™-conjugated donkey antigoat IgG (1:500)
All secondary antibodies were from Jackson ImmunoResearch Lab, PA, USA

Ab: antibody; MC: monoclonal; PC: polyclonal.
FITC: fluorescein isothyocyanate; Cy3™: indocarbocyanin.