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Intrinsic neuronal control of the pyloric sphincter of the lamb

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ABSTRACT

To better understand the local neuronal network of the gastro-duodenal junction in ruminants, we identified the components of the enteric nervous system (ENS) innervating the pyloric sphincter (PS) of the lamb abomasum. The neurons were labelled after injecting the tracer Fast Blue (FB) into the wall of the PS, and the phenotype of the FB-labelled neurons was immunohistochemically investigated using antibodies against nitric oxide synthase (NOS), choline acetyltransferase (ChAT), tachykinin (substance P) and tyrosine hydroxylase (TH).

The FB-labelled abomasal myenteric plexus (MP) neurons, observed up to 14 cm from the PS, were NOS-immunoreactive (IR) (82 ± 12%), ChAT-IR (51 ± 29%), SP-IR (61 ± 33%), and also TH-IR (2%). The descending nitrergic neurons were also SP-IR (64%) and ChAT-IR (21%); the cholinergic descending neurons were SP-IR (3%).

The FB-labelled duodenal neurons were located only in the MP, up to 8 cm from the sphincter and were ChAT-IR (79 ± 16%), SP-IR (32 ± 18%), NOS-IR (from 0 to 2%), and also TH-IR (4 ± 3%). The cholinergic ascending neurons were also SP-IR (60%) whereas no ChAT-IR cells were NOS-IR.

The findings of this research indicate that the sheep PS is innervated by long-projecting neurons of the abomasal and duodenal ENS.

1. Introduction

The abomasum, the only part of the ruminant stomachs that secretes digestive juices, is anatomically divided into *fundus*, *corpus* and *pylorus*, and functionally divided into an abomasal body and antrum. The pylorus represents a short section of the gastro-duodenal junction whose lumen contains a rounded thickness of the wall termed pyloric torus. The pyloric region has been the subject of numerous investigations in man (Torgesen, 1942; Fisher and Cohen, 1973; Ramkumar and Schulze, 2005), dogs (Torgesen, 1942; Allescher et al., 1988; Daniel et al., 1989; Mochiki et al., 2001), guinea-pigs (Cai and Gabella, 1984; Iino, 2000; Yuan et al., 2001), rats (Kressel et al., 1994; Lindeström and Ekblad, 2002), ruminants (Ruckebusch and Malbert, 1985; Malbert and Ruckebusch, 1991; Plaza et al., 1996), pigs (Torgesen, 1942; Treacy et al., 1992), and several other species, such as rabbits, cats, and horses (Torgesen, 1942).

The pyloric sphincter (PS) plays a crucial role in regulating the emptying of chyme from the stomach into the duodenum, which, in a fully fed ruminant, is not a continuous process. The pylorus is densely innervated by extrinsic and intrinsic nerves; branches of the vagus nerve constitute, in general, the bulk of the extrinsic innervation and carry mostly afferent signals (Ramkumar and Schulze, 2005); this evidence was also confirmed in sheep by studies carried out after the injection of retrograde tracers into the pylorus (Cottrell and Greenhorn, 1987; Chiocchetti et al., 2003).

The PS presents, as do all sphincters of the digestive tract, a continuous myenteric plexus (MP) and is richly innervated by enteric nervous system (ENS) excitatory motoneurons whose principal transmitter is acetylcholine but which contain tachykinins, and by inhibitory motoneurons whose principal neurotransmitter is nitric oxide (NO), but which also have adenosine triphosphate, vasoactive intestinal peptide and pituitary adenylyl cyclase activating peptide as co-transmitters (Furness, 2006). Adrenergic innervation is abundant in the pylorus as well (Cottrel and Stanley, 1992).

It has been shown that antral contractions were reduced in the extrinsically isolated sheep abomasum, but they were not totally abolished (Cottrel and Stanley, 1992). In addition, following a vagotomy, there was a complete resumption of the normal reflex behaviour within 4 weeks (Bell et al., 1977), indicating the ability of intrinsic reflex pathways to control abomasal motility.

To date, enteric circuits of the abomasum which may play a crucial rule in PS motility as well, have been investigated only in cattle (Geishauser et al., 1998; Vittoria et al., 2000; Pfannkuche

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Table 1
Details of primary antibodies used (suppliers: Biogenex, San Ramon, CA, USA; Biomol Res. Lab., Plymouth Meeting, PA, USA; Chemicon International, Temecula, CA, USA; Molecular Probes, Eugene, Ore, USA; Novocastra Lab., Newcastle, UK; BD Transduction Lab., Lexington, KY 40511; Sigma, Saint Louis, Missouri, USA)

Tissue antigen	Host species	Serum code	Dilution	Source
ChAT	Rabbit	P3YEB	1:250	Prof. Michael Schemann
Human neuronal protein Hu	Mouse	A-21271	1:1000	Molecular Probes
NF 200 kDa	Rabbit	4142	1:400	Sigma
NOS	Mouse	N31020	1:40	BD Transduction Laboratories
NOS	Rabbit	SA-227	1:100	Biomol
SP	Rat	10-S15	1:500	Fitzerald
TH	Mouse	NCL-TH	1:40	Novocastra
TH	Rabbit	AB151	1:500	Chemicon

Abbreviations: ChAT, choline acetyl transferase; NF 200, neurofilament 200 kDa; NOS, nitric oxide synthase; PGP 9.5, protein gene product; S100, calcium binding protein; SP, substance P; TH, tyrosine hydroxylase.

et al., 2002); to our knowledge, no studies have been carried out regarding sheep PS intrinsic control.

There is also evidence that duodenal local nerve fibres may modulate the PS in monogastric species; neuronal feedback mechanisms triggered by the luminal contents in the duodenum seem to be very important for PS motility (Yuan et al., 2001; Lindeström and Ekblad, 2002; Treacy et al., 1992; Allescher et al., 1989).

To better understand the local neuronal network of the gastro-duodenal junction, the present study aimed at characterizing the distribution, morphology and phenotype of abomasal anally and duodenal orally projecting neurons residing within the ENS of sheep.

2. Materials and methods

All the procedures described below were carried out in agreement with the Italian legislation regarding experimental animals, after having been approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna.

2.1. Fluorescent tracer injections

The surgical procedure and details related to anesthesia, surgery and the slaughter of the animals has been published (Chiocchetti et al., 2006). Briefly, 1 month before euthanasia, five lambs 2–6 months of age, underwent a surgical procedure to inject the fluorescent retrograde tracer Fast Blue (FB) into the PS. After anesthesia and following a midline laparotomy, a ring (1 cm wide) of PS was intramurally infiltrated with 80 μl of FB (2%), injected at multiple sites by means of a Hamilton 10 μl microsyringe. Each injection was performed carefully so as to avoid any leakage of the tracer from the injected site. Any visible leakage of FB was immediately removed with a cotton bud. After a 1-month survival time, the lambs were deeply anesthetized and killed by the administration of Tanax® (0.5 ml/kg; Intervet Italia).

2.1.1. Wholemount processing

The gastro-duodenal junction (GDJ), composed of a 20 cm length of abomasum, the PS and a 12 cm length of the duodenum, was collected, cut open, flushed out and immersed in sodium phosphate buffer (PBS) containing the type-l calcium channel blocker nicardipine for 15 min. The tissues were distended and then pinned on balsa wood, mucosal surface facing down, and subsequently fixed in 2% paraformaldehyde containing 0.2% picric acid in 0.1 M PBS (pH 7.0) at 4 °C overnight; they were subsequently removed from the balsa wood and washed in dimethylsulphoxide (3 × 15 min), followed by washing in PBS (3 × 15 min). The tissues were stored at 4 °C in PBS containing sodium azide (0.1%) until they were processed to obtain wholemount preparations of abomasal and duodenal MP. The tissues (1 × 2 cm) from both the abomasum and the duodenum were pinned flat, mucosa side up, in a Sylgard-covered Petri dish containing PBS. The mucosa and submucosal layers were carefully cut off. The specimens were turned upside down to cut off the serosa and turned back again. Then the circular muscle layer was removed to expose the MP.

2.1.2. Vagal afferents

The proximal and distal vagal ganglia were collected from two lambs.

2.1.3. Cryosections

Segments of the abomasum, together with proximal and distal vagal ganglia, were removed and fixed overnight at 4 °C in 4% paraformaldehyde in PBS; details related to the preparations of 12 μm thick cryosections have been published (Chiocchetti et al., 2006).

2.2. Immunohistochemistry

Wholemount preparations, in which abomasal and duodenal FB-labelled cells were observed using epifluorescence microscopy, were incubated with primary antisera (Table 1). Single or double labelling studies using the indirect immunofluorescence method were performed. The tissues were incubated in 10% normal goat serum in PBS containing 1% TritonX-100 for 30 min at room temperature (RT) to reduce non-specific binding of the primary antibodies and to permeabilize the tissue to the antisera. The tissues were then incubated at 4 °C in a humid chamber for 3 days in a mixture of two primary antisera diluted in a suitable medium (1.8% NaCl in 0.01 M phosphate buffer containing 0.1% Na-azide). After washing in PBS (3 × 10 min), the tissues were incubated for 4 h at RT in a humid chamber in a mixture of the secondary antibodies diluted in PBS (Table 2). The tissues were then washed in PBS (3 × 10 min) and mounted in buffered glycerol pH 8.6. Wholemount preparations of abomasal MP and duodenal MP and submucosal plexus (SMP), taken from different locations in each animal, at least 2 cm from the FB injection sites, were analyzed.

To study the morphology of the FB-labelled neurons, we utilized a primary antiserum to neurofilament NF 200 kDa whereas their percentage on the total neuronal population was investigated by the use of an anti-Hu; the same anti-Hu antibody was utilized to study the proportion of the subpopulations within the MP of the abomasum and the duodenum.

The cryosections were processed for double labelling. The tissues were incubated in 10% normal goat serum in PBS containing 1% Triton X-100 for 30 min at RT. Following incubation in combined primary antibodies for one night at 4 °C in a humid chamber, preparations were given 3 × 10 min washes in PBS and then incubated for 1 h at RT with appropriate secondary antibodies. The sections were cover-slipped with buffered glycerol, pH 8.6.

2.2.1. Antibody specificity

The specificity of mouse anti-NOS, rabbit anti-ChAT and rat anti-SP had already been tested (Pfannkuche et al., 2002). The antibody to TH has been well characterised in other species and is raised against highly conserved protein, it probably recognise the appropriate antigens in sheep; it has already been localized in neurons of the sheep sympathetic system and ENS (Chiocchetti et al., 2006). However, this was not directly tested.

The specificity of the secondary antibodies was tested by applying these antisera without the use of primary antibodies. No stained neurons or fibres were seen after omitting the primary antisera.

2.2.2. Fluorescence microscopy

Preparations were examined using a Zeiss Axioplan microscope equipped with the appropriate filter cubes for discriminating between fluorescein isothiocyanate (FITC) and Alexa 594 fluorescence. Images, recorded using a Polaroid DMC digital camera (Polaroid Corporation, Cambridge, Ma, USA) and DMC 2 software, were further processed using Corel Photo Paint X3 and Corel Draw X3 (Corel Corporation, Dublin, Ireland) and Adobe Photoshop CS software programs.

Table 2
Details of secondary antisera used (suppliers: Calbiochem-Novabiochem Corporation, San Diego, CA, USA)

Antibody	Dilution	Source
Goat anti-mouse IgG Alexa 594	1:200	Molecular Probes
Goat anti-rabbit IgG FITC	1:40	Calbiochem-Novabiochem Corpor.
Donkey anti-rat IgG 594	1:50	Molecular Probes
Rabbit anti-rat IgG FITC	1:50	Chemicon

FITC: fluorescein isothiocyanate.