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Neurochemical characteristics of calbindin-like immunoreactive coeliac-cranial mesenteric ganglion complex (CCMG) neurons supplying the prepyloric region of the porcine stomach

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ABSTRACT

The present study was designed to determine the distribution, morphology and co-localization of calbindin-D28k (CB) with other neuroactive substances in the coeliac-cranial mesenteric ganglion complex (CCMG) neurons supplying the prepyloric region of the porcine stomach. In all animals, a median laparotomy was performed and the fluorescent retrograde neuronal tracer Fast Blue was injected into the wall of the stomach prepyloric area. On the 28th day, all animals were euthanized and the CCMG complexes were then collected and processed for double-labelling immunofluorescence for CB and tyrosine hydroxylase (TH), galanin (GAL), somatostatin (SOM), leu 5-enkephalin (LENK), vasoactive intestinal peptide (VIP), substance P (SP) and cocaine- and amphetamine-regulated transcript peptide (CART), Immunohistochemistry revealed that 8.27 ± 0.51% of FB-positive neurons expressed CB-like immunoreactivity. Furthermore, CB co-localized with TH, GAL and SOM in retrogradely labelled cell bodies, whereas CART, LENK, VIP and SP were detected only in nerve terminals surrounding FB + /CB + neurons. The presence of CB in the stomach-projecting neurons may indicate the contribution of CB in the sympathetic regulation of the stomach function. Furthermore, CB-LI neurons had a catecholaminergic character and co-localized with TH, GAL and SOM, which suggests multiple functions of this neuroactive substance in the CCMG neurons supplying the porcine prepyloric area.

1. Introduction

Calbindin-D28k (CB) is an active substance, which belongs to the calcium-binding protein family. It was first detected in chicken duodenal mucosa (Wasserman et al., 1966) and since then it has been described in the central, autonomic and enteric nervous systems of various species, including humans (Baimbridge et al., 1992; Celio, 1990; Flace et al., 2014; Furness, 2000; Hermanowicz-Sobieraj and Robak, 2017; Resibois et al., 1988; Walters et al., 1993; Wojtkiewicz et al., 2012). Although its function is poorly understood, literature data suggest the participation of CB in the regulation of the calcium level in the cell (Masliukov et al., 2012a). Calcium acts as an important signal for a variety of neuronal processes, including neurotransmitter release, neuronal excitability, synaptic plasticity and gene expression (Ahmadian et al., 2015; Katz, 1996; Marty, 1989). For this reason, CB and other calcium-binding proteins (CBPs) are valuable markers for specific neuronal subpopulations in both the central and peripheral nervous systems (Baimbridge et al., 1992; Alexianu et al., 1994).

In sympathetic ganglia, CB was localized in the superior cervical ganglion (SCG), stellate ganglion (SG) and celiac ganglion (CG) in rats (Grkovic and Anderson, 1997; Richardson et al., 2006) and cats (Masliukov et al., 2012a, 2012b), as well as in the porcine caudal mesenteric ganglion (CaMG) (Lepiarczyk et al., 2016; Wojtkiewicz et al., 2012). In addition, Masliukov et al. (2012a, 2012b) described an alteration in CB immunoreactivity during ontogenesis of sympathetic ganglia in rats and cats. A similar observation was reported in the rat hippocampus and cerebellum, where the number of CB-positive sympathetic neurons decreased during the postnatal life span (Amenta et al., 1994; Villa et al., 1994). Previous studies based on retrograde tracing have shown that sympathetic neurons supplying the pineal gland, interscapular brown fat, heart, urinary bladder and descending colon displayed immunoreactivity to CB (Anderson et al., 2002;

Abbreviations: CaMG, caudal mesenteric ganglion; CB, calbindin-D28k; CART, cocaine- and amphetamine- regulated transcript peptide; CBPs, calcium-binding proteins; CG, celiac ganglion; CCMG, the coeliac-cranial mesenteric ganglion complex; DRG, dorsal root ganglia; ENS, enteric nervous system; FB, Fast Blue; GAL, galanin; GI, gastrointestinal; LENK, leu 5-enkephalin; LI, like immunoreactive; PGP 9.5, protein gene-product 9.5; PBS, phosphate-buffered saline; RTX, resiniferatoxin; SCG, superior cervical ganglion; SG, stellate ganglion; SOM, somatostatin; SP, substance P; StG, sympathetic trunk ganglia; TH, tyrosine hydroxylase; VIP, vasoactive intestinal peptide

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Lepiarczyk et al., 2016; Richardson et al., 2006; Wojtkiewicz et al., 2012). However, to date, knowledge of the distribution of CB in the porcine sympathetic neurons is very limited. It is worth mentioning that the pig is now recognized as a commonly accepted animal model for biomedical research because it shows a high degree of anatomical, histological and physiological similarity to humans, including digestive, urinary and cardiovascular systems (Verma et al., 2011; Brown and Timmermans, 2004).

The gastrointestinal (GI) tract is characterized by a unique arrangement of innervation. Besides the enteric nervous system (ENS) located in the wall of digestive tract, the GI tract also possesses extrinsic innervation originating in sympathetic, parasympathetic and sensory ganglia (Gonkowski et al., 2015; Palus and Calka, 2015; Rytel et al., 2015). The coeliac-cranial mesenteric ganglion complex (CCMG) (also known as the coeliac-superior mesenteric ganglion complex [CSMG]) is the main source of sympathetic innervation of the porcine GI tract (Palus and Calka, 2015; Kaleczyc et al., 2004). Among the plenty of neuroactive substances expressed in the CCMG neurons, it plays a regulatory role in gastric motility, acid secretion, hormone release, local blood flow and mucosal defence mechanisms (Ekblad et al., 2000). Although many of them have been investigated, knowledge concerning the distribution, morphology and co-localization of CB with other active substances, especially in neurons supplying the porcine stomach, is rather scarce. Recent data suggest that tyrosine hydroxylase (TH), galanin (GAL), somatostatin (SOM), leu 5-enkephalin (LENK), vasoactive intestinal peptide (VIP), substance P (SP) and cocaine- and amphetamine- regulated transcript peptide (CART) have a crucial regulatory function in the mammalian sympathetic neurons supplying the gastrointestinal tract (Kaleczyc et al., 2004; Palus and Całka, 2015).

Thus, the present study was designed to determine the distribution, morphology and co-localization of CB with other active substances in the CCMG neurons supplying the prepyloric region of the porcine stomach.

2. Materials and methods

2.1. Laboratory animals and surgical procedures

The experimental procedure including animal euthanasia was approved by the Local Ethical Commission for Experiments on Animals at the University of Warmia and Mazury in Olsztyn (Permit Numbers 05/2010). The study was performed on five juvenile (eight to 12 weeks old, 15–20 kg body weight) female pigs of the Large White Polish race. The animals were kept under standard laboratory conditions and were fed standard fodder and had free access to water. Before performing any surgical procedures, all of the pigs were pre-treated with azaperone (Stresnil, Jansen Pharmaceutica N.V., Belgium, 4 mg/kg of body weight, i.m.), and after 15 min the main anaesthetic drug, sodium pentobarbital (Thiopental, Sandoz, Kundl-Rakusko, Austria; 10 mg/kg of body weight), was given intravenously in a slow, fractionated infusion. In order to localize the sympathetic cell bodies supplying the stomach, the pigs were subjected to median laparotomy and received injections of the fluorescent retrograde neuronal tracer Fast Blue (FB, EMS-CHEMIE, GmbH, Germany) into the diamond-shaped part (ca. 4 cm x 4 cm) of the stomach anterior prepyloric wall in a total volume of 50 µl of a 5% aqueous solution of the Fast Blue tracer (1 µl per 1 injection). To minimize leakage of the tracer into surrounding tissues, the needle of the Hamilton syringe was left in place for 20 s after each injection, and thereafter the injection area was subsequently rinsed with isotonic saline and gently wiped with gauze. After 28 days (the required time for the marker to reach the extrinsic sources of innervation of the porcine prepyloric area), all animals were re-anaesthetized and euthanized by an overdose of sodium thiopental and were transcardially perfused with 4% buffered paraformaldehyde (pH 7.4). The coeliac-cranial mesenteric ganglion complexes (CCMG) were then collected and post-fixed by immersion in the same fixative for 20 min, rinsed in phosphate buffer (pH 7.4) over three days and finally stored in a 30% buffered sucrose solution until sectioning.

2.2. Immunohistochemical procedure

14-µm-thick serial cryostat sections of the CCMG complexes were analysed with an Olympus BX 51 fluorescent microscope (Olympus, Poland), equipped with a filter set suitable for observation of FB to localize and count neurons containing the tracer. The slices were then subjected to standard double-labelling immunofluorescence technique, which was described previously by Palus and Całka, 2015. Briefly, after air-drying at room temperature for 45 min. and rising in 0.1 M phosphate-buffered saline (PBS, pH 7.4; 3 × 10 min), the sections were blocked with a mixture containing 10% horse serum and 0.1% bovine serum albumin in 0.1 M PBS, 1% Triton X-100, 0.05% Thimerosal and 0.01% sodium azide for 1 h at room temperature to reduce non-specific background staining. After rinsing in PBS (3 × 10 min), the samples were incubated overnight with a combination of antisera directed towards protein gene-product 9.5 (PGP 9.5) (used here as a pan-neuronal marker) and calbindin (CB) as well as CB and tyrosine hydroxylase (TH), galanin (GAL), somatostatin (SOM), leu 5-enkephalin (LENK), vasoactive intestinal peptide (VIP), substance P (SP), cocaine- and amphetamine- regulated transcript peptide (CART) (Table 1). Following this, the sections were rinsed in PBS (3 × 10 min) and incubated with a mixture of secondary antibodies (Table 1) to visualize the antibody combinations: PGP 9.5/CB and CB/TH, CB/GAL, CB/SOM, CB/LENK, CB/VIP, CB/SP, CB/CART. After staining, the sections were mounted with carbonate-buffered glycerol (pH 8.6) and cover-slipped.

2.3. Negative control

Standard controls, i.e. pre-absorption for the neuropeptide antisera with appropriate antigen (20 µg of antigen/ml diluted antiserum) and the omission as well as the replacement of all primary antisera by non-immune sera, were performed to test immunohistochemical labelling. There was no fluorescence observed in any of these control stainings, which confirms the specificity of the methodology and antibody applied.

2.4. Counting and statistics

Immunostained tissue slices were evaluated using an Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets.

Table 1
List of primary antisera and secondary reagents used in the study.

Antigen	Host species	Code	Dilution	Manufacturer/ Supplier
Primary antibodies				
PGP 9.5	Mouse	7863 – 2004	1:1000	Biogenesis, UK
CB	Rabbit	CB-38	1:10000	Swant, Switzerland
TH	Mouse	MAB 318	1:200	Millipore, USA
GAL	Guinea Pig	T-5036	1:500	Penninsula, USA
SOM	Rat	8330 – 0009	1:50	AbD Serotec, UK
LENK	Mouse	4140 – 0355	1:500	AbD Serotec, UK
VIP	Mouse	9535 – 0504	1:1000	Biogenesis, UK
SP	Rat	450 – 0505	1:200	AbD Serotec, UK
CART	Mouse	MAB 163	1:1000	R&D System, USA
Secondary antibodies				
Alexa Fluor 546 nm goat anti-rabbit IgG		A11010	1:1000	Invitrogen, USA
Alexa Fluor 488 nm donkey anti-mouse IgG		A21202	1:1000	Invitrogen, USA
Alexa Fluor 488 nm donkey anti-rat IgG		A21208	1:1000	Invitrogen, USA
Alexa Fluor 488 nm goat anti-guinea pig IgG		1:1000	A11073	Invitrogen, USA

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