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Expression of β_2 adrenoceptors within enteric neurons of the horse ileum

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ABSTRACT

The activity of the gastrointestinal tract is regulated through the activation of adrenergic receptors (ARs). Since data concerning the distribution of ARs in the horse intestine is virtually absent, we investigated the distribution of β_2-AR in the horse ileum using double-immunofluorescence. The β_2-AR-immunoreactivity (IR) was observed in most (95%) neurons located in submucosal plexus (SMP) and in few (8%) neurons of the myenteric plexus (MP). Tyrosine hydroxylase (TH)-IR fibers were observed close to neurons expressing β2-AR-IR. Since β_2-AR is virtually expressed in most neurons located in the horse SMP and in a lower percentage of neurons in the MP, it is reasonable to retain that this adrenergic receptor could regulate the activity of both secretomotor neurons and motor neurons innervating muscle layers and blood vessels. The high density of TH-IR fibers near β_2-AR-IR enteric neurons indicates that the excitability of these cells could be directly modulated by the sympathetic system.

1. Introduction

Sympathetic noradrenergic neurons supplying the gastrointestinal tract (GIT) have three main functions: (1) reduce oral to anal propulsion of the contents (Quinson et al., 2001), (2) reduce fluid and electrolyte secretion (Quinson et al., 2001), and (3) constrict the arteries that supply the digestive organs (Quinson et al., 2001). It has also been shown that adrenergic innervation is able to modulate mucosal IgA secretion (Schmidt et al., 2007) and the cells of the immune system (Straub et al., 2006). Furthermore, gut sensations seem also to be modulated by the sympathetic system, through the activation of intestinal monocytes (Viramontes et al., 2001; Blandizzi, 2007).

Given the considerable interaction between the sympathetic extrinsic innervation and the enteric nervous system (ENS), a more thorough knowledge of sympathetic innervation is thus pertinent to understand enteric control. The organization of the sympathetic pathways and the ways in which the endings of sympathetic postganglionic motor neurons are related to ENS neurons and other intramural targets, such as smooth muscle cells of muscular layers and vessels as well as other different GIT elements, are all well established especially in laboratories species (Furness, 2006).

The sympathetic motor neurons supplying horse GIT are located in the ganglia of the thoracic sympathetic chain (TSC), in the splanchnic ganglia (when these exist), and in pre-vertebral celiac ganglia (CG), cranial mesenteric ganglia (CranMG), and caudal mesenteric ganglia (CaudMG) (Russo et al., 2010). TSC ganglia should harbor the neurons involved in vascular blood flow control whereas the neurons residing in the pre-vertebral ganglia should mainly control motility, secretion, and blood flow as well (Furness, 2006).

The sympathetic system activates the G-protein-coupled adrenergic receptors (ARs) found in both nervous and non-nervous tissues (Civantos Calzada and Aleixandre de Artiñano, 2001), which are the cellular membrane binding sites through which natural catecholamines and sympathomimetic drugs exert their physiological and pharmacological effects. ARs types and subtypes and their distribution along the GIT have been the object of investigation mainly in laboratory species (Paton and Vizi, 1969; Hirst and McKirdy, 1974; Surprenant and North, 1988; Dobreva et al., 1998; Nasser et al., 2006) but also in humans (Cellek et al., 2007; Schemann et al., 2010). At present, α1 and α2 are considered as

Abbreviations: AR, adrenoceptor; CaudMG, caudal mesenteric ganglia; CCK, cholecystokinin; CG, celiac ganglia; CGRP, calcitonin gene-related peptide; CML, circular muscular layer; CranMG, cranial mesenteric ganglia; DBH, dopamine β-hydroxylase; ENS, enteric nervous system; FITC, fluorescein isothiocyanate; GIT, gastrointestinal tract; ICJ, ileocaecal junction; IFANs, intestinofugal neurons; IML, intermediolateral cell column; IPANs, intrinsic primary afferent neurons; IR, immunoreactivity, immunoreactive; LML, longitudinal muscle layer; mm, muscularis mucosae; MP, myenteric plexus; PVG, prevertebral ganglia; SMP, submucosal plexus; TH, tyrosine hydroxylase; WB, Western blot.

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two distinct subfamilies, and consequently ARs are subdivided into three subfamilies: α1-ARs (consisting of three subtypes: α_{1A}, α_{1B}, and α_{1D}), α2-ARs (consisting of α_{2A}, α_{2B}, and α_{2C} subtypes) (Docherty, 1998), and β-ARs [consisting of β_1, β_2, and β_3 subtypes] (Boss et al.,1999; Dzimiri, 1999; Zhong and Minneman, 1999; Kobilka, 2011).

In the gastro-intestinal tract (GIT), β-adrenoceptors are subdivided into three types, β_1, β_2, and β_3-adrenoceptors (Manara et al., 1995). In the different species, the distribution of the β_2-ARs differs among different tracts of the GIT (Meylan et al., 2004; Nasser et al., 2006). In the cattle, a detailed research on mRNA codifying for ARs showed that mRNA for α_{2AD}- and β_2-ARs were more expressed in the intestine than in the abomasum (Meylan et al., 2004) while in the mouse, the distribution of β_2-ARs did not show meaningful differences in its different intestinal tracts. In the mouse, in addition, β_2-ARs were equally distributed in both the MP and SMP, whereas in the rat and guinea-pig these receptors were expressed only in the SMP (Nasser et al., 2006). Nasser et al. (2006) indicated that the effect of the β_2-AR act via the ENS. In rabbit ileum, β_2-AR appears to be involved in physiologic adrenergic inhibition (Wagner et al., 1981). More recently, Seiler et al. (2005) suggested that β_2-AR mechanisms are involved in the relaxation of rat ileal longitudinal muscle. The relaxation of the guinea-pig ileum seems to be mediated only *via* the β_1-adrenoceptors (Grassby and Broadley, 1984) whereas the distension of the taenia caecum is mediated *via* β_2-ARs.

The data concerning the activity of the β_2ARs in the horse intestine are instead limited. The sympathetic relaxation of equine ileal smooth muscle seems to depend mainly on β_3-AR subtype activation, with a minor involvement of the β_2 subtype (Re et al., 1997).

In recent studies we investigated the enteric and sympathetic neurons innervating the horse ICJ (Chiocchetti et al., 2009a; Russo et al., 2010). The knowledge of ARs subtypes involved in the regulation of equine gastrointestinal functions provides important opportunities from a pharmacological point of view and needs to be further investigated clinically.

The main goal of the present research was to demonstrate the presence of β_2-AR in the ileal ENS. In addition, we also studied the relationship between the β_2-AR-immunoreactive (IR) cells and cathecolaminergic fibers, showing immunoreactivity for the enzyme tyrosine hydroxylase (TH).

2. Materials and methods

Four Italian trotter horses, two young females aged 2.5 and 3.5 years, and two males age 8 months and 5 years were utilized. These animals were destined to be euthanized because of musculoskeletal problems. None had gastrointestinal disorders. In addition, four ileal horse tissues were collected at the public slaughterhouse; among these specimens, two tissues showed slight mucosal inflammation of unknown reason. All procedures on trotter horses were carried out in accordance with Italian legislation regarding experimental animals, after approval by the Ethic Scientific Committee for Experiments on Animals of the University of Bologna (Prot Rif. BQ/gf PROT 13825–X/10 – All. 67). The animals were deeply anesthetized and killed by means of embutramide, mebenzonium iodide and tetracaine hydrochloride (Tanax) administration. The ileum of all subjects utilized was immediately removed and then cut open along the mesenteric border, flushed out with phosphate-buffered saline (PBS: 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) and fixed in 2% paraformaldehyde containing 0.2% picric acid in 0.1 M sodium phosphate buffer (pH 7.0) at 4 °C overnight. After rinsing in PBS, the tissue was stored in PBS containing 30% sucrose and sodium azide (0.1%) (pH 7.4) at +4 °C. Subsequently, the tissue was cut into small pieces (2 × 0.5 cm), transferred to a mixture of PBS–sucrose–azide and OCT compound (Tissue Tek) at a ratio of 1:1 and then embedded in 100% OCT. The tissue was frozen in isopentane cooled in liquid nitrogen, mounted in Tissue Tek® (Sakura Finetek Europe, NL) mounting medium and sectioned at 14–16 μm on a cryostat. The sections were collected on gelatin-coated slides and left to dry for 1 h at room temperature (RT).

2.1. Double-immunofluorescence

Cryostat sections were washed in PBS and then processed for immunostaining. To block non-specific binding, the sections were incubated in a solution containing 10% normal goat serum (Colorado Serum Co., Denver, CO, #CS 0922) and 0.5% Triton X-100 (Merck, Darmstadt) in PBS for 1 h at RT. Thereafter, the sections were incubated in a cocktail of primary antibodies diluted in antibody diluent (1.8% NaCl in 0.01 M sodium phosphate buffer containing 0.1% sodium azide) (Tables 1 and 2) for 24 h at room temperature (RT). After washing in PBS (3 × 10 min) the sections were incubated in a cocktail of secondary antibodies diluted in PBS (Table 3) for 1 h at RT. After washing in PBS (3 × 10 min), the slides were coverslipped with buffered glycerol (pH 8.6). The antibody cocktails were added with 10% normal goat serum and 0.5% Triton X-100. All the incubations were performed in a humid chamber.

2.2. Specificity of the primary antibodies

The specificity of the antibody mouse anti-TH has been already tested by Western blot (Wb) analysis on equine tissues (Russo et al., 2010). The anti-β_2AR antibody utilized in this study was tested for its specificity by (WB) analysis, which indicated that it was specific for the targeted molecules (Fig. 1).

The enteric neuronal cell bodies were identified with the anti-human neuronal protein (HuC/HuD) antiserum, which recognizes an RNA–binding protein (RBP) of the embryonic lethal abnormal visual (Elav) family. Hu family proteins are conserved

Table 1
Details of the primary antibodies used (suppliers: Biogenex, San Ramon, California, USA; Novocastra, Leica Microsystems – Biosystems Division, Newcastle, UK; Oncogene, CA, USA; Santa Cruz Biotechnology, CA, USA. β2-AR, β2-adrenoceptor; NSE, neuron specific enolase; S100, calcium binding protein; TH, tyrosine hydroxylase.

Tissue antigens	Immunogen	Host species	Dilution	Code	Sources and references
β2-AR	Peptide mapping at the C-terminus of β2-AR of mouse origin	Rabbit	1:100	Sc-570	Santa Cruz
HuC/D	Human neuronal protein HuC/HuD	Mouse	1:200	A21271	Molecular Probes
NSE	Purified human gamma enolase	Mouse	1:6	AM055-5M	Biogenex
S100	S100 protein isolated from bovine brain	Rabbit	1:80	PC157	Oncogene
TH	Recombinant protein corresponding to the carboxyl terminal end (120 amino acids) of mouse tyrosine hydroxylase (Leu 379–Ser 498)	Mouse	1:40	NCL-TH Clone 1B5	Novocastra