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Effects of vasoactive intestinal peptide and galanin on survival of cultured porcine myenteric neurons

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Abstract

Enteric neuronal plasticity is probably fundamental in order to withstand injury or changes in intestinal activity. The role of the neuropeptides in neuroprotection is still enigmatic. The expression of galanin and vasoactive intestinal peptide (VIP) and the effects of the two peptides on survival of small intestinal porcine myenteric neurons cultured for 6 days were studied. Immunocytochemistry and cell counting were used to evaluate the numbers of surviving neurons and their expression of galanin and VIP. To reflect the in vivo situation, cryostat sections of porcine mid-jejunum were used. A concentration-dependent and marked increase in neuronal survival was noted when neurons were grown in the presence of VIP (10^{-8}–10^{-6} M), whereas addition of galanin (10^{-8}–10^{-6} M) slightly decreased neuronal survival. A dramatic increase in the proportions of myenteric neurons containing VIP or galanin immunoreactivity occurred during culturing. The presence of VIP further increased the number of galanin-expressing neurons. A majority of the galanin-immunoreactive neurons lacked VIP, while all VIP-immunoreactive neurons contained galanin.

In conclusion, culturing porcine myenteric neurons in the presence of VIP increases, while the presence of galanin reduces, survival. Culturing significantly increased the proportion of neurons expressing VIP and/or galanin; the presence of VIP further increased the number of galanin-expressing neurons.
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1. Introduction

A wide array of neuropeptides has by now been discovered and thoroughly studied. Two of them, galanin and vasoactive intestinal peptide (VIP) seem to be of particular importance for optimal regulation of the alimentary tract. Both peptides are reported to play multiple roles in the gastro-intestinal tract; regulating peristalsis, secretion, blood flow and feeding, and both are widely distributed in the enteric nervous system (ENS) and found to act as classical neurotransmitters as well as neuromodulators (see Refs. [1–3] for recent reviews).

"The cell body reaction" refers to the dramatic shifts in gene expression induced in adult peripheral neurons in response to axotomy [4,5]. In particular, the expression of neuropeptides is subjected to prominent changes in the injured neurons. Induction or up-regulation of the expression of neurotransmitters promoting regeneration and survival of damaged neurons is accompanied by a down-regulation of the expression of neurotransmitters not needed in this particular situation [4,6].

The ENS has, no doubt, similar defense systems which we at present know very little of. Evidence of an altered expression of neuropeptides within the enteric neurons in response to neuronal injury, e.g., axotomy, colchicine treatment, isolation and culturing or in response to changes in intestinal activity, is steadily amounting [7–9]. Recent investigations clearly indicate that neuronal VIP is strongly

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involved in enteric neuronal protection (see [7,10] for reviews). Another putative candidate to be involved in enteric neuronal plasticity is galanin. This since galanin expression was found to be up-regulated in rat myenteric neurons after axotomy, colchicine treatment or isolation and autotransplantation [7]. Also in the hypertrophic rat small intestine, the number of galanin-expressing myenteric neurons increases [11].

Culturing myenteric neurons has provided a strong tool for studying various aspects of enteric neuronal plasticity and survival [8,9,12–14]. The goal of the present study was to evaluate, for the first time, the effects of galanin and VIP on survival of cultured porcine myenteric neurons. In addition, immunocytochemistry was used to determine whether culturing with or without the presence of galanin or VIP influenced the neuronal expression of the two peptides.

2. Material and methods

2.1. Animals

Primary cultures of myenteric neurons were prepared from nine piglets of both sexes, 3–6 weeks old (10–15 kg body weight). The animals were divided into two groups. One group (*n*=5) was used to study the possible influence of galanin on survival and expression of VIP and galanin in the myenteric neurons whereas the other (*n*=4) was used to test VIP. All experimental procedures were approved by the Animal Ethic Committee, Lund and Malmö and in agreement with NIH publication No. 86-23, revised 1985. The piglets were anaesthetised with metomidatum hydrochloridum (Hypnodil, Janssen Pharmaceutica, Belgium; 15 mg/kg b.w.) and killed by an overdose of pentobarbital (Pentobarbitalnatrium, Apoteket, Sweden; 30 mg/kg b.w.). The abdomen was opened by a midline incision and a portion of mid-jejunum, 15 cm long, was quickly removed and transferred into cold 0.9% sodium chloride.

2.2. Culture of myenteric neurons

The method for culturing porcine myenteric neurons was adapted from that previously used for rat [8,13,14], with some modifications. By using a dissecting microscope strips of longitudinal muscles with adherent myenteric ganglia were dissected out, washed (2×10 min) in Ca^{2+} and Mg^{2+} free Hanks balanced salt solution (HBSS; Gibco BRL, Life Technologies, Sweden) and cut with a pair of fine scissors into smaller pieces. The tissue was incubated (2 h, 37 °C) in HBSS supplemented with digestive enzymes (1.3 mg/ml trypsin, 1.5 mg/ml collagenase type II and 1.5 mg/ml protease; Sigma-Aldrich, Sweden). After digestion, the tissue was mechanically dissociated by trituration and then three times centrifuged at 1000 rpm in 18 °C. After final centrifugation, the pellet was suspended in 2 ml medium containing Neurobasal A (Gibco BRL, Life Technologies) with the addition of 10% fetal calf serum, 0.5 nM L-glutamine, 100 μl/ml streptomycin sulphate and 100 units/ml penicillin G sodium (Gibco BRL, Life Technologies). Equal volumes (100 μl) of the cell suspension containing myenteric neurons were seeded with 900 μl medium on laminin-coated cover glass slides placed in a four-well multidish (Nunclon™Surface, Nunc, Denmark). Cultures of the first group of animals (*n*=5) were as follows: One well contained medium only and this culture served as a parallel control, one contained medium with the addition of 10^{-6} M galanin (porcine; Sigma-Aldrich), one medium with 10^{-7} M galanin and one medium with 10^{-8} M galanin. Myenteric neurons from the piglets of the second experimental group (*n*=4) were cultured as follows: one well contained medium only (parallel control), the medium in the three remaining wells was supplemented with different concentrations of porcine VIP (10^{-6}, 10^{-7} and 10^{-8} M; Sigma-Aldrich). At least 3 four-well multidishes were seeded from each pig. In order to examine the presence of glia and smooth muscle cells within the cultures an additional four-well multidish (without the addition of VIP or galanin) was also prepared. The cultures were placed in an incubator (5% CO_2 atmosphere; 37 °C) and kept for 6 days. Medium (and peptides) was changed on the third day of incubation. All procedures were done aseptically. After 6 days of incubation, the cultures of myenteric neurons were fixed (30 min) in Stefanini solution containing 2% formaldehyde and 0.2% picric acid followed by rinsing (three times, 15 min each) with Tyrode solution containing 10% sucrose. The cultures were then frozen until being processed for immunocytochemistry.

In order to establish the numbers of neurons seeded (0 days in vitro), 100 μl of the cell suspension was placed onto glass slides. At least two slides were performed from each piglet. The slides were dried (30 min in 37 °C) followed by fixation and rinsing as described above and processed for immunocytochemistry.

2.3. Cryostat sections

To reflect the in vivo situation specimens from mid-jejunum (approximately 1 cm long) from five piglets were dissected out, opened longitudinally and pinned onto a piece of balsa wood with serosal surface up. Following fixation overnight in Stefanini solution the intestinal segments were rinsed three times (1 per day) with Tyrode solution containing 10% sucrose. After final washing, tissue samples were embedded in O.C.T. compound and frozen on dry ice. Longitudinal and transversal cryostat sections of 12 μm thickness were made. Every fifth section was placed on glass slides (SuperFrost® Plus, Menzel, Germany) and processed for double staining immunocytochemistry.

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