Expression of substance P, vasoactive intestinal peptide and galanin in cultured myenteric neurons from the ovine abomasum

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ABSTRACT: Enteric neurons are able to alter their neurotransmitter content during adaptation to new artificial conditions. The aim of the present study was to investigate changes in vasoactive intestinal peptide (VIP), substance P (SP) and galanin expression during culture of myenteric neurons from the ovine abomasum. In order to accurately reflect the *in vivo* situation cryostat sections from the ovine abomasum were used. Cultured and non-cultured myenteric neurons were immunohistochemically stained with a mixture of antibodies raised against Hu C/D (neuronal marker) and VIP, SP or galanin. Double labeling revealed that Hu C/D-IR/VIP-IR myenteric neurons were very rare in cryostat sections (1.4 ± 0.2%) but significantly increased to 21.3 ± 1.7% when cultured for three days. A significant increase in Hu C/D-positive/VIP-positive myenteric neurons were also found in 6- and 9-days cultures (23.9 ± 1.9 and 24.5 ± 2.0%, respectively). *In vivo*, the expression of SP was found in 9.7 ± 1.0% of myenteric perikarya. After 3, 6 and 9 days of incubation the proportion of Hu C/D-IR/SP-IR myenteric perikarya significantly increased to 19.3 ± 1.3%, 22.3 ± 1.2% and 24.1 ± 1.7% (respectively). When compared to the *in vivo* situation the proportion of galanin-expressing myenteric neurons was unchanged after 3, 6 and 9 days of culturing. In conclusion, alterations in VIP and SP (but not galanin) expression occur during neuronal culturing. Our results supports the idea that both VIP and SP may act as factors which increase neuronal survival.

Keywords: enteric nervous system; myenteric neurons; neuronal plasticity; neuropeptides; abomasum; sheep

The enteric nervous system coordinates the regulation of many gastric functions including acid production, hormone secretion as well as stomach contraction and emptying. Over the last years, the morphology of the gastric myenteric plexus and the neurochemical coding of its neurons have been extensively studied using many different techniques (for a review see Ekblad et al., 2000). Both radioimmunoassay (Ferrante et al., 1991; Hannibal et al., 1998; El-Salhy, 1999), and immunocytochemistry (recently Jarvinen et al., 1999; Reiche and Schemann, 1999; Vanden Berghe et al., 1999; Hu et al., 2002; Pfannkuche et al., 2003; Pimont et al., 2003) have revealed that neuronal elements of the stomach express a wide spectrum of various neurotransmitters including neuropeptides. Some of the latter,

besides their regulatory function in the gastrointestinal tract, also play a crucial role in the intestinal neuronal response to inflammation (Neunlist et al., 2003), lipopolysaccharide exposition (Arciszewski et al., 2005) and axonal transport blockade or nerve injury (Ekblad et al., 1996). So far, there has been very little data published concerning the plasticity of gastric myenteric neurons. Hydrochloric acid administrated intragastrally selectively activates a subpopulation of myenteric neurons from the rat stomach also exhibiting immunoreactivity to nitric oxide synthase (NOS), vasoactive intestinal peptide (VIP) and neuropeptude Y (NPY), but not choline acetyltransferase (Schicho et al., 2004). In the stomach corpus of streptozotocin-induced diabetic rats an increased content of calcitonin

gene-related peptide (CGRP), but not substance P (SP) has been reported (Miyamoto et al., 2001). Moreover, in hibernating hamsters up-regulated expression of CGRP as well as SP and a constant level of VIP- and NOS-immunoreactivity has been found in myenteric neurons of the proventriculus and proximal stomach (but not of the distal stomach) as compared with normal non-hibernating animals (Shochina et al., 1997).

The aim of the present study was to evaluate whether culturing may provoke changes in the chemical content of myenteric neurons from the ovine abomasum. Therefore, the presence of VIP, SP and galanin was immunohistochemically assessed in cultured myenteric neurons from the ovine abomasum. To reflect the *in vivo* situation immunoreactivities to VIP, SP and galanin were studied in abomasal cryostat sections from the healthy animals. The choice of the sheep as an experimental model is justified as in this species, to our best knowledge, there is a paucity of reports concerning the plasticity of the enteric neurons.

MATERIAL AND METHODS

Animals and anesthesia

Handling of the animals and experimental procedures were conducted according to guidelines of the local Ethical Committee and were in agreement with Principles of Laboratory Animal Care, NIH publications No. 86–23, revised 1985. Ten adult male sheep (weighing ca. 35 kg) were used. The animals were deeply anesthetized with xylazine (Rometar, Spofa Prague, Czech Republic, 0.4 mg/kg b.w.) and ketamine (Narkamon, Spofa Prague, Czech Republic, 10 mg/kg b.w.). In five animals (n = 5) cultures of stomach myenteric neurons were made. In another group of five animals (n = 5) serial frozen sections of the abomasum wall were cut in order to reflect an *in vivo* situation.

Neuronal culturing

The abdomen was opened by a midline incision and the abomasum was visualized. Using a dissecting microscope and a pair of sharp scissors strips of the muscular layer of the abomasum (approx. 2 cm long) were gently removed and washed thrice

(2 min each) in cold Ca^{+2} and Mg^{+2} free Hank's balanced salt solution (HBSS, Gibco BRL, Life Technologies, CA, USA). After the final washing, the material was cut into smaller pieces and placed into a vial containing a mixture of HBSS, trypsin (1.3 mg/ml; Gibco BRL) and collagenase type II (1.5 mg/ml; Gibco BRL). For the mechanical dissociation the material was 15 times triturated with a 5 ml glass pipette and placed in a waterbath for 20 min (37°C, 60 IU/min). Next, 1.5 mg/ml of protease (Sigma-Aldrich, St Louis, MO, USA) was added. The trituration step was repeated and the material was once again placed in a waterbath for another 20 min. In order to stop the digestion 12 ml of fetal calf serum (Gibco BRL) was added, and the specimen was three times centrifuged (1 000 rpg, 37°C, 10 min). After every centrifugation the supernatant was gently removed and the cell pellet was supplemented with HBSS. After the final centrifugation medium containing Neurobasal A supplemented with 10% fetal calf serum, 0.5nM L-glutamine, 100 μl/ml streptomycin sulphate and 100 units/ml penicilin G sodium (Gibco BRL) was added to the cell pellet. At the bottom of each of a four-well multidish (Nunclon™Surface, Nunc, Denmark) a cover slip coated with laminin (Sigma-Aldrich) was placed. Wells were filled with 950 μl of medium and after that seeded with 50 μl of cell suspension. Finally the multidishes were placed in an incubator (37°C, 5% CO_2) and incubated for 3, 6 or 9 days. On every third day of incubation the medium was changed.

Cryostat sections (CS)

The pieces of the ovine stomach (n = 5, approx. 4 cm^2) were dissected out, immediately rinsed with cold 0.9 natrium chloride and pinned serosa-side up onto a piece of balsa wood. The material was fixed (four days) with Stefanini solution containing paraformaldehyde and picric acid and four times washed (once per day) for cryoprotection in a Tyrode solution containing sucrose. Next, the abomasum was cut into smaller segments. The specimens were grouped, embedded in O.C.T compound and frozen in dry ice. Using a cryostat serial transverse as well as longitudinal sections of 10 μm thickness were made. Every fifth section was placed on a glass slide (SuperFrost® Plus, Mezel, Germany) and stored at −20°C for further double immunohistochemical staining.

Immunocytochemistry

Both multidishes and cryostat sections were first washed with three changes of 0.1M phosphate buffered saline (PBS, pH 7.4) containing 0.25% Triton X-100 (Sigma-Aldrich). After final washing a combination of primary anisera diluted in PBS, 0.25% triton X-100 and 0.25% bovine serum albumin (BSA, Sigma-Aldrich) were dropped into the slides. Mouse antibodies raised against Hu C/D (1 : 400; Molecular Probes, Eugene, OR, USA, code A-21271) used as pan-neuronal markers were mixed with antibodies raised against VIP (1 : 100; Biogenesis, Poole, UK, code 9535-0504), SP (1 : 200; Biogenesis, code 8450-0505) or galanin (1 : 1 200; Biogenesis, code 4600-5004). Following overnight incubation (4°C) in a humid chamber the slides were several times rinsed with PBS and a combination of secondary antibodies conjugated to FITC (1 : 200; MP Biomedicals, Solon, OH, USA) and Texas Red (1 : 100; MP Biomedicals) were applied for one hour incubation at room temperature. After the final washing in PBS (three times for 10 min) the slides were covered with a cover slip in buffered glycerol and viewed under an epifluorescent microscope equipped with a set of filters appropriate for FITC and Texas Red.

In order to assess the specificity of antibodies an inactivation with an excess amount of antigen (10–100 µg of synthetic substance per ml of diluted antiserum) were performed. The omission of primary antibodies was also used as a negative control. Control stainings did not exhibit any immunostaining.

Cell counting and statistical analysis

Both in cryostat sections and cover slips with cultured neurons the percentage of perikarya immunoreactive to Hu C/D and VIP, SP or galanin were estimated by cell counting. All data were presented as mean ± S.E.M. In each animal ($n = 5$) at least two cryostat sections from different parts of the abomasum were examined. On every slide at least 150 myenteric neurons were assessed. In each animal from the second group ($n = 5$) the mean from two cultures of gastric myenteric neurons run simultaneously were calculated. All neurons found in the area of a cover slip (but a minimum of 100 neuronal cell bodies) were studied. Statistical difference between multiple groups of data were compared using the one-way ANOVA test followed by Bonfernoni's post-hoc test. The level for significance was $P < 0.05$.

RESULTS

After 3, 6 and 9 days of culturing a flat layer of cell bodies immunoreactive to Hu C/D (a neuronal marker) was found in all glass-slides. The number and density of Hu C/D-positive myenteric neurons found in 3-, 6- and 9-days cultures are summarized in Table 1. The neuronal perikarya had a tendency to group in larger clusters (up to 20 neurons) but numerous individual neuronal cells were also observed. In general, most of the clusters were situated in the central part of the glass-slide but single

Table 1. The number and density of Hu C/D-positive myenteric neurons found after 3-, 6- and 9-days of culturing

Days of culturing	Number of neurons (min–max)	Density (neuron/mm^2) (min–max)
3	248–302	1.9–2.3
6	147–221	1.1–1.7
9	105–139	0.8–1.0

colonies of myenteric neurons located peripherally were also observed.

Immunocytochemistry revealed that the myenteric neurons cultured for 3, 6 and 9 days frequently sent VIP-, galanin-, but no SP-containing processes which usually contacted other perikarya or nerve fi-
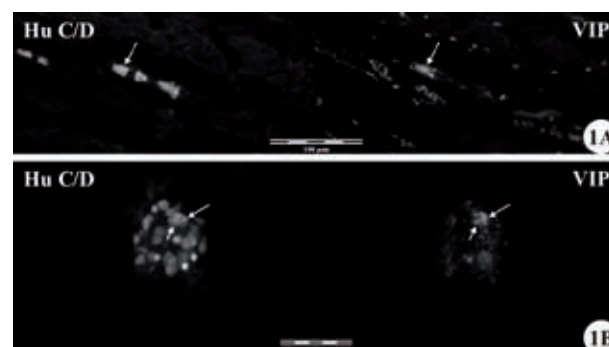

Figure 1. Paired micrographs of cryostat sections of the ovine abomasum (1A) and abomasal myenteric neurons cultured for three days (1B). In (1A) and (1B) VIP-immunoreactive neurons are marked with arrows; bars represent 100 µm
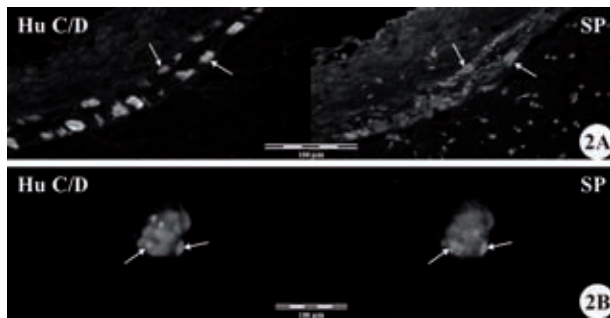
Figure 2. Arrows in (2A) indicate SP-positive myenteric neurons found in cryostat transverse sections of the ovine abomasum. Micrographs (2B) show a subpopulation of myenteric neurons from the ovine abomasum cultured for six days and double stained for Hu C/D and SP (arrows); bars represent 100 μm

bres and thus created multiconnected dense meshes. Double labeling revealed that Hu C/D-IR/VIP-IR myenteric neurons were very rare in CS (1.4 ± 0.2%; n = 5, Figure 1) but significantly increased to 21.3 ± 1.7% (n = 5; P < 0.05) when cultured for three days. A significant increase in VIP-expression was also found in myenteric neurons cultured for six (23.9 ± 1.9%; n = 5; P < 0.05) and nine days (24.5 ± 2.0%; n = 5; P < 0.05). The proportions of VIP-IR myenteric neurons were statistically similar (P < 0.05) regardless of if the cultures were incubated for 3, 6 or 9 days. The expression of SP *in vivo* was found in 9.7 ± 1.0% of Hu C/D-positive myenteric perikarya (n = 5; Figure 2), whereas after three days of culturing *in vitro*, the proportion of Hu C/D-IR/SP-IR myenteric perikarya significantly increased to 19.3 ± 1.3% (n = 5, P < 0.05). As compared to the
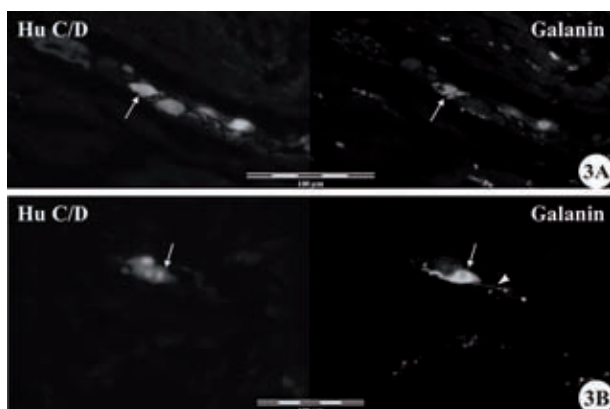

Figure 3. Galanin-positive myenteric neurons are seen both *in vitro* (arrow in 3A) and after nine days of culturing (arrow in 3B). Note that the galanin-IR neurons presented in the lower panel send galanin-containing process (arrowhead); bars represent 100 μm

in vivo situation, increased percentages of cultured myenteric neurons immunoreactive to SP were also found in 6- and 9-days cultures (22.3 ± 1.2% and 24.1 ± 1.7%, respectively; n = 5). The incubation time did not significantly change (P < 0.05) the proportions of Hu C/D-IR/SP-IR myneteric neurons. The percentage of Hu C/D-expressing non-cultured abomasal myenteric neurons displaying IR to galanin was statistically similar when compared to the proportions of galanin-expressing neurons found after 3, 6 and 9 days of culturing (25.0 ± 1.1% vs. 23.1 ± 1.0%, 22.4 ± 1.9% and 21.4 ± 1.3%, respectively; n = 5, P < 0.05; Figure 3).

DISCUSSION

The results of present study demonstrated that culturing substantially increased the proportions of VIP- and SP-expressing ovine abomasal myenteric neurons, whereas the percentage of galanin-containing myenteric neurons was unchanged when compared to the situation found *in vivo*. Because previous reports have clearly demonstrated that up-regulation of VIP occurred in cultured myenteric neurons from the small intestine of the rat (Lin et al., 2003) and pig (Arciszewski et al., 2005), such a result was to be expected in the present study. In fact, in the cultured gastric perikarya we noted a nearly 20% increase in VIP expression which supports the previously formulated hypothesis and indicates that VIP is an important element participating in enteric neuronal plasticity (reviewed by Ekblad and Bauer, 2004). In the rat, VIP has been found to promote survival of cultured enteric neurons (Sandgren et al., 2003), also when exposed to lipopolysaccharide (Arciszewski et al., 2008). The mechanism underlying the neuroprotective action of VIP is related to a direct stimulation of VIP receptor-expressing glial cells to secrete neurotrophic factors, particularly interleukin-1α and 1β (Brenneman et al., 1995), interleukin-3 and interleukin-6 (Brenneman et al., 2003); however, another mode resulting in the inhibition of the production of microglia-derived pro-inflammatory agents has also been described (Delgado and Ganea, 2003).

A substantial increase in SP content has previously been described in neuronal cultures of the porcine myenteric ganglia (Grider and Bonilla, 1994), which is consistent with the results presented herein. Since the participation of SP in neu-

roimmune processes is well known it is possible that one of the first protective reactions of gastric myenteric neurons in response to culturing may be *de novo* synthesis of SP. Different lines of evidence indicate that SP has neuroprotective properties. In cultured striatal neurons SP was found to reverse the toxicity induced by quinolinic acid (Calvo et al., 1996). Additionally, SP as well as SP-agonists reduced trophic factor deprivation-induced cell death of spiral ganglion neurons (Lallemend et al., 2003). Tachykinin family peptides showed highly selective neuroprotective effects for cultured mesencephalic dopaminergic neurons and this action was correlated with an increase in neuronal excitability (Salthun-Lassalle et al., 2005). However, another mechanism may also underlie the neuronal survival-promoting effect of SP. SP-preferring G protein-coupled NK1 receptors have been found in numerous immune cells including monocytes, macrophages (Ho et al., 1997), lymphocytes (McCormack et al., 1996) and mast cells (Okada et al., 1999). In monocytes as well as mast cells stimulation with SP induced the increased levels of pro-inflammatory but also neurotrophic cytokines such as IL-1, IL-6 and TNFα (Lotz et al., 1988; Azzolina et al., 2003) which suggests similarities between the neuroprotective mechanisms of SP and VIP.

In our previous experiment we discovered that culturing *per se* markedly increased the number of galanin-expressing myenteric neurons from the porcine small intestine (Arciszewski and Ekblad, 2005). Moreover, the addition of exogenous galanin to the cultures slightly reduced neuronal survival whereas VIP-supplementation increased the proportion of galanin-IR perikarya (Arciszewski and Ekblad, 2005). To our surprise, in the present study we observed no significant changes between the percentage of non-cultured and cultured subpopulations of gastric myenteric neurons exhibiting galanin-IR. This suggests that in the mammalian stomach galanin may have no or only a minor influence on neuronal survival and adaptation. However this hypothesis needs to be further explored experimentally, especially since galanin is overexpressed not only in, as mentioned above, cultured enteric neurons but also in mechanically lesioned sensory (Landry et al., 2000) and sympathetic nervous cells (Klimaschewski et al., 1996). Moreover, in afferent neurons the up-regulated galanin, due to its inhibitory action, is believed to exert neurotrophic effects (for a recent review see Wiesenfeld-Hallin and Xu, 2001).

In summary, the present study has reported the increased expression of VIP and/or SP (but not galanin) in cultured myenteric neurons from the ovine abomasum. This study provides anatomical evidence in support of a significant role of VIP and SP in enteric neuronal plasticity (a consequence of neuronal adaptation to new artificial conditions).

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