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Evidence for a glutamatergic modulation of the cholinergic function in the human enteric nervous system via NMDA receptors

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Abstract

Several reports suggest that enteric cholinergic neurons are subject to a tonic inhibitory modulation, whereas few studies are available concerning the role of facilitatory pathways. Glutamate, the main excitatory neurotransmitter in the central nervous system (CNS), has recently been described as an excitatory neurotransmitter also in the guinea-pig enteric nervous system (ENS). The present study aimed at investigating the presence of glutamatergic neurons in the ENS of the human colon. At this level, the presence of ionotropic glutamate receptors of the NMDA type, and their possible interaction with the enteric cholinergic function was also studied. In the human colon, L-glutamate and NMDA concentration dependently enhance spontaneous endogenous acetylcholine overflow in Mg^{2+}-free buffer, both effects being significantly reduced by the antagonists, (±)-2-amino-5-phosphonopentanoic acid (±AP5) and 5,7-diCl-kynurenic acid. In the presence of Mg^{2+}, the facilitatory effect of L-glutamate changes to inhibition, while the effect of NMDA is significantly reduced. In addition, morphological investigations reveal that glutamate- and NR1-immunoreactivities are present in enteric cholinergic neurons and glial cells in both myenteric and submucosal plexus. These findings suggest that, as described for the guinea-pig ileum, glutamatergic neurons are present in enteric plexuses of the human colon. Modulation of the cholinergic function can be accomplished through NMDA receptors.

Keywords: Glutamate; NMDA receptor; Acetylcholine; Enteric nervous system; Colon; (Human)

1. Introduction

Different inhibitory pathways, involving both auto- and heteroreceptors, participate in the modulation of the excitatory transmission to intestinal smooth muscle, which is predominantly cholinergic in nature (Starke et al., 1989; De Luca and Coupar, 1996; De Ponti et al., 1996). Such inhibitory inputs undergo a number of adaptive changes after different experimental manipulations, indicating that they have a physiological relevance in maintaining a tonic modulation on enteric cholinergic neurons (Giaroni et al., 1999). Such restraint is also operative in the human large bowel where inhibitory responses are needed to maintain the organ in a relatively quiescent state (Karaus and Wienbeck, 1991). On opposite, there is relatively little evidence available regarding the presence of facilitatory pathways modulating the cholinergic transmission, at this level. There are reports from studies carried out on animal models suggesting that glutamate, the major excitatory neurotransmitter in the central nervous system (CNS), is likely to play a role as an excitatory neurotransmitter also in the enteric nervous system (ENS) (Kirchgessner, 2001). Glutamate immunoreactivity has been detected in subsets of submucosal and myenteric neurons in the guinea-pig ileum. At this level, glutamate is selectively concentrated in terminal axonal vesicles and can be released after application of an appropriate stimulus (Wiley et al., 1991; Liu et al., 1997; Sinsky and Donnerer, 1998; Reis et al., 2000). Immunoreactivities for both ionotropic and metabotropic glutamate receptors, and mRNAs encoding for NMDA and metabotropic receptors, have been demonstrated at different levels in the rat and guinea-pig gut (Kirchgessner, 2001). In addition, enteric neurons are endowed both with the neuronal transporter excitatory amino acid carrier 1 (EAAC1) and the vesicular glutamate transporter (VGLUT2) (Liu et al., 1997; Tong et al., 2001).

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Functional studies are consistent with a role of glutamate in the modulation of motor and secretory functions in the gut (Wiley et al., 1991; Sinsky and Donnerer, 1998; Cosentino et al., 1995; Rhoads et al., 1995). Such influence seems to primarily involve the enteric cholinergic function. Glutamate ionotropic receptors are abundantly expressed on enteric cholinergic neurons (Liu et al., 1997). In addition, glutamate may participate in the modulation of the enteric cholinergic function, since activation of NMDA receptors enhances acetylcholine release from myenteric neurons in the guinea-pig ileum and colon (Wiley et al., 1991; Cosentino et al., 1995). This latter effect has been proposed as a possible mechanism for glutamate-induced contractions of the guinea-pig ileum (Wiley et al., 1991; Sinsky and Donnerer, 1998).

Although the hypothesis that glutamate may represent an enteric excitatory neurotransmitter is gaining increased acceptance, to date few studies have been carried out to elucidate the role of glutamate, and its possible interaction with the cholinergic function, in the human ENS. In particular, it might be of significance to evaluate whether excitatory glutamatergic pathways are operative in regions of the human gut with a dominant inhibitory tone, such as the large intestine Thus, in the present study, we used morphological approaches to investigate the presence of glutamatergic neurons and of NMDA receptors in the ENS of the human colon. At this level, NMDA receptor-mediated modulation of the cholinergic function was also studied.

2. Materials and methods

Microscopically normal segments of the human ascending and sigmoid colon were obtained from colonic specimens resected from 26 patients (12 men and 14 women; mean age 68 ± 4.8) for carcinoma of the large intestine. After excision, a segment of colon from the intertaenial region was dissected along the direction of the circular muscle layer and immediately placed in a cold physiological Tyrode's solution. This study was approved by the ethical committee of the University Hospital of Varese, Italy.

2.1. Acetylcholine overflow

Acetylcholine overflow was accomplished by using 1–2-cm-long full-thickness colonic specimens deprived of the mucosal layer. Colonic segments were suspended isotonically (load 1 g) in 3 ml organ baths and perfused at a rate of 0.5 ml min^{-1} with Mg^{2+}-free Tyrode's solution (composition [mM]: 137 NaCl; 2.68 KCl; 1.8 $CaCl_2.2H_2O$; 0.47 NaH_2PO_4; 11.9 $NaHCO_3$; 5.6 glucose) containing 0.3 μM physostigmine (E8375, Sigma), gassed with O_2–CO_2 (95%–5%) and maintained at 36.5 °C. A 3-h equilibration period was always allowed, then three samples of perfusate were collected each over a period of 10 min and considered as controls. Then either L-glutamate (G1251, Sigma) or NMDA (M-102 Research Biochemical, RBI Natick, MA, USA) were added to the medium and, after allowing a contact time of 10 min with the agonist, two samples were collected over successive 10-min periods. To study the pharmacological modulation of L-glutamate and NMDA effect, the antagonists, (±)-2-amino-5-phosphonopentanoic acid (±AP5, 10 μM, A-110 RBI) and 5,7 diCl-kynurenic acid (10 μM, D-138 RBI) were added 20 min before and during the agonist treatment period, whereas Mg^{2+} (1.2 mM) and glycine (10 μM) (G7403, Sigma) were added to the Tyrode's solution at the beginning of the experiment. Some experiments were carried out to evaluate the effect of Ca^{2+} omission from the perfusing medium and of tetrodotoxin (Alomone Labs, Jerusalem, Israel) on spontaneous acetylcholine overflow. After a 60-min equilibration period, three samples of perfusate were collected each over a period of 10 min, and considered as controls. Then the perfusing medium was either changed to a Ca^{2+}-free 2 mM EGTA containing medium, or to one contained 3 μM tetrodotoxin, and after 30 min, samples were collected over two consecutive 10-min periods. The effect of test drugs on overflow was expressed as percentage variation with respect to control values. A single drug concentration was tested on each intestinal preparation. Acetylcholine in the sample was concentrated by freeze-drying 1 ml of the collected fluid (freeze dryer, Minifast 680, Edwards, Crawley, UK). The residue was reconstituted in ultra pure deionized water (200 μl), filtered (pore size 0.45 μm; Schleicher and Schuell) and assayed for acetylcholine content by high performance liquid chromatography employing electrochemical detection (HPLC-ED) with an amperometric cell as previously described (Giaroni et al., 1997).

2.2. Immunohistochemistry

Full-thickness human colonic samples were fixed with buffered formalin (4% w/v formaldehyde and acetate buffer 0.05 M) for 24 h or glutaraldehyde (2.5%) for 2 h at 4 °C and routinely embedded in paraffin.

Three-micrometer-thick sections were mounted on poly-L-lysine-coated slides and immunohistochemically stained with the avidin–biotin–peroxidase method (Hsu et al., 1981). Endogenous peroxidase activity was blocked by immersing sections for 10 min in a solution of 3% hydrogen peroxide in water. Primary antibodies (Table 1) were incu-

Table 1
Primary antisera used

Antiserum anti-	Host species	Working dilution	Clone/ code	Source
Glutamate	Mouse	1:2000	NB03L	Oncogene Res., Cambridge (MA)
NR1	Rabbit	1:1000	06–314	U.B.I., Lake Placid (NY)
NR1	Mouse	1:20	05–432	U.B.I.
ChAT	Goat	1:100 glutaraldehyde 1:50 formalin	AB144P	Chemicon Int., Temecula (CA)

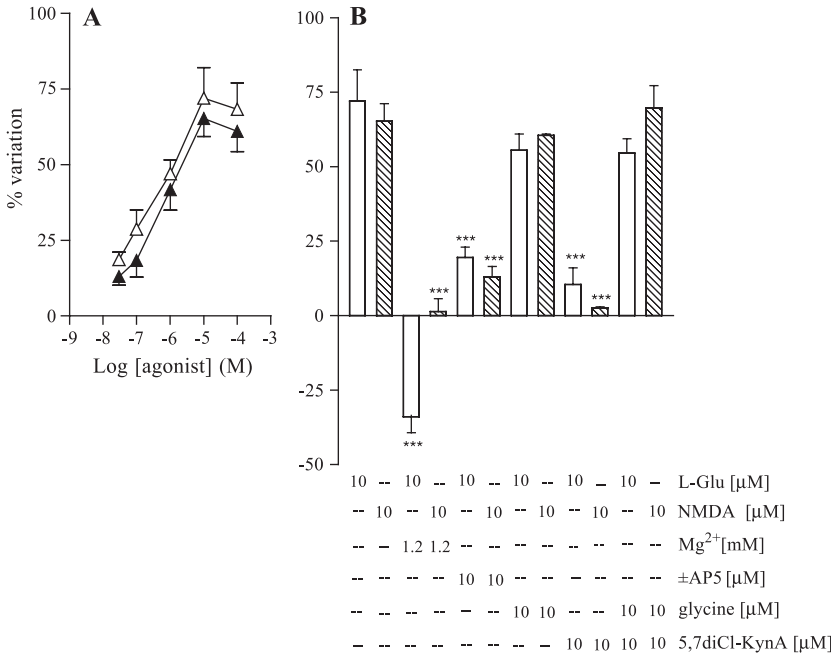

Fig. 1. (A) Stimulatory effect of L-glutamate (empty symbol) and NMDA (full symbol) on spontaneous endogenous ACh overflow from the human colon. Percentage increase in ACh overflow is plotted against log molar concentration. Each point is the mean of five experiments. Vertical bars indicate S.E.M. (B) Influence of Mg^{2+}, ± AP5, 5,7diCl-KynA, glycine and glycine in the presence of 5,7diCl-KynA on the stimulatory effect of L-glutamate (empty column) and NMDA (hatch column) on spontaneous endogenous ACh overflow from the human colon. Each column is the mean of five to six experiments. Vertical bars indicate S.E.M. In the respective experiments, NMDA, Mg^{2+}, ± AP5, 5,7diCl-KynA and glycine were added to the organ bath at the concentrations indicated at the bottom. ***$P<0.001$ by Student's t test.

bated overnight at 4 °C. Specific biotinylated secondary antibodies and avidin–biotin–peroxidase complex were consecutively applied, each for 1 h at room temperature. The immunohistochemical reaction was developed with diaminobenzidine–hydrogen peroxide reaction (Van Noorden, 1986). The sections were counterstained with haematoxylin. Microwave antigen retrieval technique with citrate buffer pH 6 ($2 \times 5'$ or $4 \times 5'$) was performed for sections incubated with anti-NR1, anti-glutamate and anti-choline acetyltransferase antibodies.

The immunoreactive structures displayed an intense brown staining.

Neurons and glial cells were identified on the basis of their morphological aspect. Neurons were evident and characterized by an eccentrically located pale staining nucleus with small chromatin clumps and a prominent central nucleolus. The cytoplasm was polygonal in shape and moderate to abundant.

Glial cells were smaller and more numerous than ganglion neurons and were molded so as to "fill in" the space remaining between neuronal processes.

The colocalization study of NR1 and choline acetyltransferase was performed immunostaining pair of "mirror-image" serial sections for each different antigen.

Specificity controls consisted of absorption of antibodies and antisera with their related antigen, omission of primary antibodies and use of control tissues with or without pertinent antigen. The absorptions of glutamate and choline acetyltransferase antibodies were carried out adding 10–100 nmol of glutamate conjugated to keyhole limpet hemocyanin with glutaraldehyde (kind gift of Dr. A. Passi, Department of Experimental and Clinical Biomedical Sciences, University of Insubria) and choline acetyltransferase (C2898, Sigma, Milan, Italy) per ml of optimally diluted antibody, respectively.

2.3. Statistical analysis

Data are presented as mean ± S.E.M., with n indicating the number of experiments. Nonlinear regression analysis of the concentration–response curves was performed in order

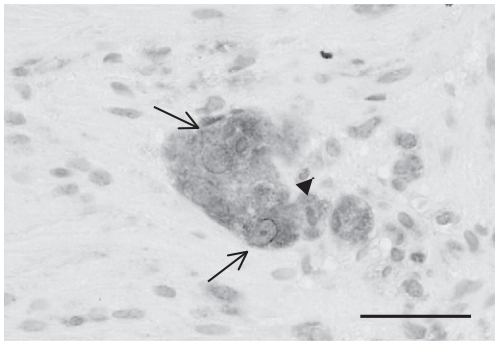

Fig. 2. Human colonic myenteric ganglia. Glutamate immunoreactivity is present in neurons (arrow) and in glial cells (arrowhead). Calibration bar: 50 μm.

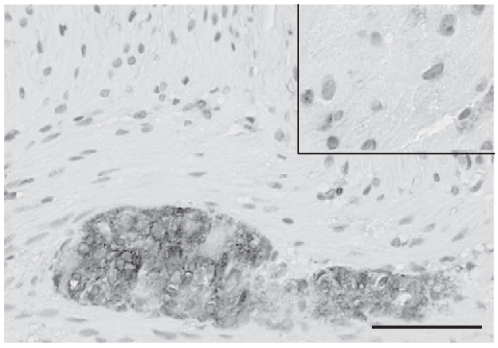

Fig. 3. Immunostaining for glutamate in the myenteric plexus of the human colon. Immunoreactivity is present in ganglionic structures and nerve endings (insert). Calibration bar: 100 μm.

to calculate the EC_{50} value (the agonist concentration that evoked 50% of the maximum response with 95% confidence limits) by use of a commercial software (Prism 3.0, GraphPad Software, San Diego CA). Significant differences were determined by Student's *t*-test (Prism 3.0, GraphPad). A probability level of $P<0.05$ was taken as significant for statistical analysis.

3. Results

3.1. Endogenous acetylcholine overflow

Endogenous acetylcholine overflow was 0.26 ± 0.02 ng g^{-1} min^{-1} ($n=190$), was stable over at least 250 min (0.25 ± 0.06 ng g^{-1} min^{-1}, $n=12$) and did not significantly differ in the presence of 1.2 mM Mg^{2+}, (0.22 ± 0.01 ng g^{-1} min^{-1}, $P>0.05$; $n=45$). Ca^{2+} omission from the superfusion medium significantly reduced acetylcholine overflow to $38 \pm 6.03\%$ ($n=6$; $P<0.05$) of control values, whereas addition of 3 μM tetrodotoxin to the superfusion medium caused a small, but not significant reduction of this parameter

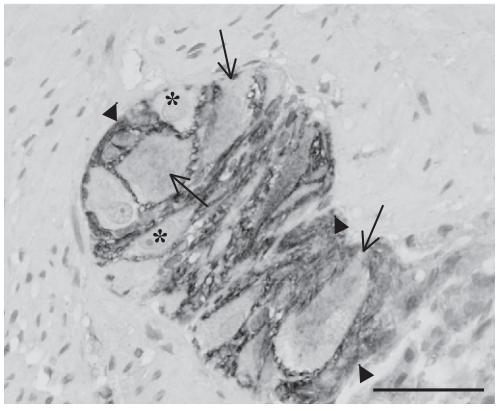

Fig. 4. NR1 immunoreactivity in human colonic myenteric plexus using the monoclonal antibody. The staining is intense in glial cells (arrowhead) and weaker in neurons (arrow). Some neurons are negative (*). Calibration bar: 50 μm.

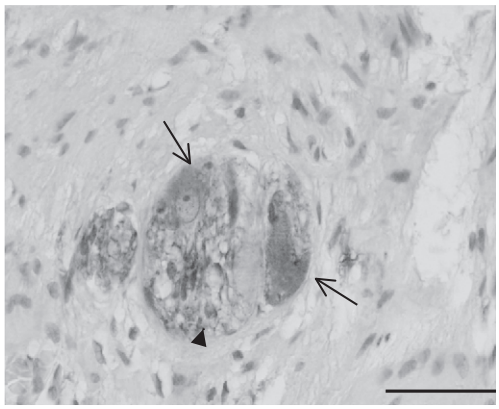

Fig. 5. Choline acetyltransferase (ChAT) immunoreactivity is present in neurons (arrow) and in glial cells (arrowhead) of human colonic myenteric ganglion. Calibration bar: 50 μm.

($-7.00 \pm 8.65\%$ with respect to control values, $n=5$, $P>0.05$).

L-Glutamate and NMDA concentration dependently enhanced acetylcholine overflow with EC_{50} values of 0.75 (0.09–6.50) μM and 0.68 (0.13–3.65) μM, respectively (Fig. 1A). In the presence of 1.2 mM Mg^{2+}, L-glutamate (10 μM) inhibited acetylcholine overflow to $34 \pm 9.24\%$ ($n=5$) of control values, whereas the facilitatory effect of NMDA (10 μM) was significantly reduced to $1.33 \pm 4.37\%$ of control values ($n=6$; $P<0.0001$) (Fig. 1B). The facilitatory effects of 10 μM L-glutamate and NMDA were significantly reduced by ± AP5 (10 μM) to $19.5 \pm 3.50\%$ ($n=6$; $P<0.001$) and to $13.00 \pm 3.51\%$ ($n=6$; $P<0.0001$), respectively, of control values. 5,7-diCl-kynurenic acid (10 μM) significantly reduced the effect of both L-glutamate ($10.50 \pm 5.50\%$ of control values, $n=6$; $P<0.001$) and NMDA ($2.50 \pm 0.50\%$ of control values, $n=6$; $P<0.0001$) (Fig. 1B). Glycine of 10 μM abolished 5,7-diCl-kynurenic acid antagonism on both L-glutamate and NMDA effects ($54.50 \pm 4.91\%$, $n=6$ and $69.67 \pm 7.59\%$, $n=6$, respectively, of control values; $P>0.05$ for both agonists). Glycine (10 μM) per se was ineffective on acetylcholine overflow ($3.54 \pm 1.40\%$ of control values, $n=6$) and failed to potentiate both L-glutamate and NMDA-induced acetylcholine overflow ($55.50 \pm 5.48\%$, $n=5$, and $60.50 \pm 0.50\%$, $n=5$,

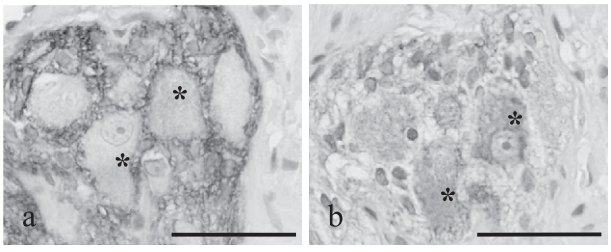

Fig. 6. Coexpression of NR1(monoclonal antibody) (A) and ChAT (B) in serial sections of human colonic myenteric ganglion. Glial cells and two neurons (*) showed the presence of both the antigens. Calibration bar: 50 μm.

respectively, of control values, $P>0.05$ for both agonists). Neither ± AP5 nor 5,7-diCl-kynurenic acid 10 μM per se had any effect on acetylcholine overflow (2.50 ± 0.50%, $n=6$ and 4.67 ± 2.76%, $n=6$, respectively, of control values).

3.2. Immunohistochemistry

Glutamate immunoreactivity was observed in myenteric and submucosal plexuses of the human colon. Glial cells and the majority of neurons within ganglia (Fig. 2) and nerve endings in the circular muscle layer (Fig. 3) showed an intense stain. No immunoreactivity was observed when glutamate antibody was absorbed with 10 nmol of the specific antigen.

The presence of NMDAR1 receptor was investigated using two antibodies. The anti-NMDA receptor NR1 monoclonal antibody (#05-432) recognizes the NMDAR1 residues 834–864, which is common to all NR1 isoforms. Using this antibody, the immunoreativity was prevalently localized in glial cells. The intensity of immunostain was strong in these cells, whereas it was weaker and variable in neurons, some of which were negative (Fig. 4).

The polyclonal antibody anti-NR1 (# 06-314) recognizes residues 864–886 splice variant which is present in the short isoforms (NR1-1). Using this antibody, we observed an intense immunostain in both the glial cells and in the majority of neuronal cells. The stain was cytoplasmic, and varied from intense, in some neurons, to weak in others. A few neurons were negative.

Choline acetyltransferase immunoreactivity was observed in both enteric plexuses: it was intense in the myenteric and weaker in the submucosal one. The immunoreactivity was localized in the cytoplasm of the majority of ganglion neurons. The intensity of immunostain, lower than that observed in the endocrine cells of the overlying mucosa, varied considerably among different cell bodies. Few neurons were negative. Choline acetyltransferase was also expressed by enteric glial cells (Fig. 5). No immunoreactivity was observed when choline acetyltransferase antibody was absorbed with 20 nmol of the specific antigen. Endocrine cell immunoreactivity was completely blocked by soluble adsorption with 50 nmol/ml.

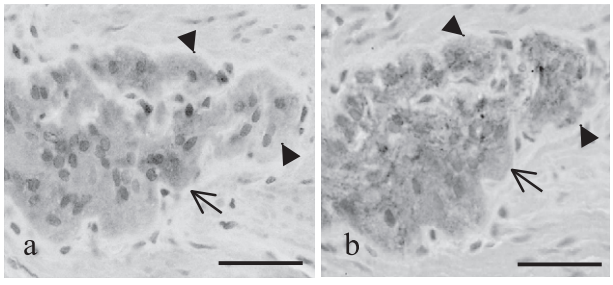

Fig. 7. Coexpression of NR1 (polyclonal antibody) (A) and ChAT (B) in glial cells (arrowhead) and neurons (arrow) on serial sections of human colonic myenteric ganglion. Calibration bar: 50 μm.

On reverse serial sections Choline acetyltransferase immunoreactivity was observed in some neurons immunoreactive for glutamate and for both anti-NR1 (Figs. 6A,B and 7A,B) antibodies.

4. Discussion

The present study shows that glutamatergic neurons are present in the human ENS. In agreement with the previous studies carried out in the guinea-pig and rat small intestine (Liu et al., 1997), glutamate immunoreactivity was found in neurons both in myenteric and submucosal ganglia as well as in axons innervating the circular muscle layer of the human large intestine. At this level, glutamate immunoreactivity was also found in enteric glial cells. This finding opens the question whether, as already described in the central nervous system (CNS) (Araque et al., 2000), a glutamate-mediated bidirectional communication between neurons and glial cells takes place also in the human ENS. This hypothesis is of particular importance because the cross-talk between neurons and glial cells might have a role in processes of potentiation as well as of neurotoxicity (Bezzi et al., 1998).

In the guinea-pig and rat bowel, immunocytochemistry and in situ hybridization studies have been carried out in order to localize different subunits for ionotropic and metabotropic glutamate receptors (Kirchgessner, 2001). As regards NR1, the ubiquitous and functional subunit of NMDA receptors (Ozawa et al., 1998), there are reports suggesting its presence in the ENS of the guinea-pig ileum (Liu et al., 1997) and rat colon (McRoberts et al., 2001). In the human colon, NR1 immunoreactivity was localized both on neurons and glial cells. In particular, in the present study, immunohistochemical distribution of NR1 has been investigated by means of two antibodies, each recognizing a specific sequence of different isoforms of the NR1 subunit. NR1 subunits exist in at least eight alternatively spliced forms, which generate NMDA receptors that differ in their physiological and pharmacological properties and in their anatomical distribution in the CNS (Zukin and Bennett, 1995; Holmes et al., 2002).

In particular, four major C-terminal splice variants (NR1-1, NR1-2, NR1-3 and NR1-4) can exist in two forms, depending either on the presence (b form) or absence (a form) of an exon cassette in the N-terminal extracellular domain (Zukin and Bennett, 1995; Holmes et al., 2002). Using the polyclonal antibody, which is specific for the short isoform NR1-1, a vast majority of neurons resulted immunoreactive. Similarly, in the guinea-pig ileum, after application of a polyclonal antibody recognizing four of the eight isoforms of the NR1 subunit, virtually all enteric neurons resulted immunoreactive (Liu et al., 1997). In the human colon, immunoreactivity to the anti-NR1-1 antibody was similar in neurons and glial cells. On opposite, a stronger immunoreactivity has been observed in glial cells than in neurons using the monoclonal antibody, which recognizes all

the NR1 isoforms. The different distribution pattern of NR1 immunoreactivity for the two antibodies in the human gut might suggest that diverse populations of NMDA receptors are present also in the human ENS. In particular, our results might suggest the presence of NR1-1 splice variant both in enteric neurons and glial cells, and of at least one of the other isoforms (NR1-2, NR1-3 and NR1-4) in glial cells.

Co-localization of NR1 and choline acetyltransferase immunoreactivities in enteric neurons suggests that NMDA receptors are present on enteric cholinergic neurons. As can be inferred from distribution of choline acetyltransferase immunoreactivity, our data suggest that, in accordance with the previous studies (Porter et al., 1996a,b), in the human colon, a high proportion of myenteric and submucosal neurons are cholinergic. However, in our model, choline acetyltransferase immunoreactivity was found also in enteric glial cells. Accordingly, in the CNS, glial cells have been shown to contain acetylcholine and choline acetyltransferase, and to synthetize acetylcholine (Lan et al., 1996; Wessler et al., 1997).

From a functional point of view, our results seem to indicate that L-glutamate and NMDA are able to modulate spontaneous endogenous acetylcholine overflow in the human colon, as described to occur in the myenteric plexus of the guinea-pig ileum and colon (Wiley et al., 1991; Cosentino et al., 1995). This evidence well correlates with the presence of NR1 immunoreactivity on enteric cholinergic neurons. L-Glutamate and NMDA concentration dependently enhanced spontaneous endogenous acetylcholine overflow from the human colon in Mg^{2+}-free buffer. Responses to the agonists were superimposable and inhibited by the antagonists, ± AP5 and 5,7-diCl-kynurenic acid. In particular, the ability of the latter compound, which is known to bind to the NMDA receptor-associated glycine modulatory site (Baron et al., 1990), suggests that NMDA receptors are under control of endogenous glycine in the human colon, as observed in the guinea-pig colon (Cosentino et al., 1995). In the present study, addition of Mg^{2+} blocked the facilitatory effect of NMDA, and changed L-glutamate-mediated facilitation to inhibition. On the whole, the present data suggest that glutamate can enhance acetylcholine overflow in the human ENS by activating NMDA receptors. However, the final effect of glutamate could depend on the responsiveness of different glutamate receptors and on the neuronal pathways involved. With this regard, it could be interesting to note that activation of AMPA receptors has been shown to inhibit spontaneous acetylcholine overflow from the guinea-pig distal colon indirectly, probably by activation of inhibitory pathways (Giaroni et al., 2000).

In conclusion, the present findings provide structural and functional basis for a glutamatergic modulation of enteric cholinergic neurons in the ENS of the human colon. At this level, glutamate receptors of the NMDA type may participate to the facilitatory modulation of excitatory cholinergic pathways, which retain a primary role in the control of motility. Secondarily, glutamate might also have a role in the modulation of sensory pathways, as already suggested for the guinea-pig ileum and rat colon (Kirchgessner, 2001; McRoberts et al., 2001). However, further investigations aimed at demonstrating the presence of neuronal storage and inactivation mechanisms for glutamate, as well as the release of endogenous glutamate from enteric nerves and its action at synaptic sites, are needed to provide definitive evidence that glutamate has a neurotransmitter role also in the human ENS.

The consequences of glutamate-mediated effects in the human ENS remain in large part to be unravelled. Hypothetically, in the complex neuronal circuitries, which constitute the ENS, glutamate might also participate to processes of activity-dependent plasticity (Giaroni et al., 1999). Secondarily, glutamate receptors might be involved in alterations of the gastrointestinal functions consequent to an alimentary intoxication, as observed after ingestion of food contaminated with domoic acid, a ionotropic non-NMDA glutamate receptor agonist (Teitelbaum et al., 1990). Finally, we cannot exclude that the present findings might represent an underlying support for successive studies concerning glutamate-mediated excitotoxicity in the human ENS. Indeed, in the guinea-pig ileum, prolonged stimulation of enteric ganglia by glutamate caused necrosis and apoptosis in the enteric neurons, which seemed primarily mediated via NMDA receptors (Kirchgessner et al., 1997), Excitotoxicity induced by glutamate may be a critical factor in the pathogenesis of some diseases of the gastrointestinal tract, as it has already been suggested for some neurological disorders in the CNS (Obrenovitch and Urenjak, 1997).

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References

Araque, A., Li, N., Doyle, R.T., Sanzgiri, R.P., Haydon, P.G., 2000. Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci. 22, 208–215.

Baron, B.M., Harrison, B.L., Miller, F.P., McDonald, I.A., Salituro, F.G., Schmidt, C.J., Sorensen, S.M., White, H.S., Palfreyman, M.G., 1990. Activity of 5,7-dichlorokynurenic acid, a potent antagonist at the *N*-methyl-D-aspartate receptor-associated glycine binding site. Mol. Pharmacol. 38, 554–561.

Bezzi, P., Carmignoto, G., Pasti, L., Vesce, S., Rossi, D., Rizzini, B.L., Pozzan, T., Volterra, A., 1998. Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. Nature 391, 281–285.

Cosentino, M., De Ponti, F., Marino, F., Giaroni, C., Leoni, O., Lecchini, S., Frigo, G.M., 1995. *N*-Methyl-D-aspartate receptors modulate neurotransmitter release and peristalsis in the guinea pig isolated colon. Neurosci. Lett. 183, 39–42.

De Luca, A., Coupar, I.M., 1996. Insights into opioid action in the intestinal tract. Pharmacol. Ther. 69, 103–115.

De Ponti, F., Giaroni, C., Cosentino, M., Lecchini, S., Frigo, G.M., 1996. Adrenergic mechanisms in the control of gastrointestinal motility: from basic science to clinical applications. Pharmacol. Ther. 69, 59–78.

Giaroni, C., Somaini, L., Marino, F., Cosentino, M., Leoni, O., De Ponti, F., Lecchini, S., Frigo, G.M., 1997. Acetylcholine detection by a modified HPLC-ED method improves the assessment of cholinergic function in the myenteric plexus of the guinea-pig colon. Neurosci. Lett. 232, 9–12.

Giaroni, C., De Ponti, F., Cosentino, M., Lecchini, S., Frigo, G.M., 1999. Plasticity in the enteric nervous system. Gastroenterology 117, 1438–1458.

Giaroni, C., Zanetti, E., Marino, F., Cosentino, M., Senaldi, A., Somaini, L., Lecchini, S., Frigo, G.M., 2000. Glutamate receptors of the AMPA type modulate neurotransmitter release and peristalsis in the guinea-pig isolated colon. Life Sci. 67, 1747–1757.

Holmes, K.D., Mattar, P., Marsh, D.R., Jordan, V., Weaver, L.C., Dekaban, G.A., 2002. The C-terminal C1 cassette of the *N*-methyl-D-aspartate receptor 1 subunit contains a bi-partite nuclear localization sequence. J. Neurochem. 81, 1152–1165.

Hsu, S.M., Raine, L., Fanger, H., 1981. Use of avidin–biotin–peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J. Histochem. Cytochem. 29, 577–580.

Karaus, M., Wienbeck, M., 1991. Colonic motility in humans—a growing understanding. Bailliere's Clin. Gastroenterol. 5, 453–478.

Kirchgessner, A.L., 2001. Glutamate in the enteric nervous system. Curr. Opin. Pharmacol. 1, 591–596.

Kirchgessner, A.L., Liu, M.T., Alcantara, F., 1997. Excitotoxicity in the enteric nervous system. J. Neurosci. 17, 8804–8816.

Lan, C.T., Shieh, J.Y., Wen, C.Y., Tan, C.K., Ling, E.A., 1996. Ultrastructural localization of acetylcholinesterase and choline acetyltransferase in oligodendrocytes, glioblasts and vascular endothelial cells in the external cuneate nucleus of the gerbil. Anat. Embryol. 194, 177–185.

Liu, M.T., Rothstein, J.D., Gershon, M.D., Kirchgessner, A., 1997. Glutamatergic enteric neurons. J. Neurosci. 17, 4764–4784.

McRoberts, J.A., Coutinho, S.V., Marvizon, J.C., Grady, E.F., Tognetto, M., Sengupta, J.N., Ennes, H.S., Chaban, V.V., Amadesi, S., Creminon, C., Lanthorn, T., Geppetti, P., Bunnett, N.W., Mayer, E.A., 2001. Role of peripheral *N*-methyl-D-aspartate (NMDA) receptors in visceral nociception in rats. Gastroenterology 120, 1737–1748.

Obrenovitch, T.P., Urenjak, J., 1997. Alterated glutamatergic transmission in neurological disorders: from high extracellular glutamate to excessive synaptic efficacy. Prog. Neurobiol. 51, 39–87.

Ozawa, S., Kamiya, H., Tsuzuki, K., 1998. Glutamate receptors in the mammalian central nervous system. Prog. Neurobiol. 54, 581–618.

Porter, A.J., Wattchow, D.A., Brookes, S.J.H., Schemann, M., Costa, M., 1996a. Choline acetyltransferase immunoreactivity in the human small and large intestine. Gastroenterology 111, 401–408.

Porter, A.J., Wattchow, D.A., Brookes, S.J.H., Schemann, M., Costa, M., 1996b. The neurochemical coding and projections of circular muscle motor neurons in the human colon. Gastroenterology 113, 1916–1923.

Reis, H.J., Massensini, A.R., Prado, M.A.M., Gomez, R.S., Gomez, M.V., Romano-Silva, M.A., 2000. Calcium channels coupled to depolarization-evoked glutamate release in the myenteric plexus of the guinea-pig ileum. Neuroscience 101, 237–242.

Rhoads, J.M., Argenzio, R.A., Chen, W., Gomez, G.G., 1995. Asparagine stimulates piglet intestinal Cl-secretion by a mechanism requiring a submucosal glutamate receptor and nitric oxide. J. Pharmacol. Exp. Ther. 274, 404–412.

Sinsky, M., Donnerer, J., 1998. Evidence for a neurotransmitter role of glutamate in guinea pig myenteric neurons. Neurosci. Lett. 258, 109–112.

Starke, K., Göthert, M., Kilbinger, H., 1989. Modulation of neurotransmitter release by presynaptic autoreceptors. Physiol. Rev. 69, 864–989.

Teitelbaum, J.S., Zatorre, R.J., Carpenter, S., Gendron, D., Evans, A.C., Gjedde, A., Cashman, N.R., 1990. Neurologic sequelae of domoic acid intoxication due to the ingestion of contaminated mussels. N. Eng. J. Med. 322, 1781–1787.

Tong, Q., Ma, J., Kirchgessner, A.L., 2001. Vesicular glutamate transporter 2 in the brain–gut axis. NeuroReport 12, 3929–3934.

Van Noorden, S., 1986. Tissue preparation and immunostaining techniques for light microscopy. In: Polak, J.M., Van Noorden, S. (Eds.), Immunocytochemistry, 2nd ed. Wright, Bristol, pp. 26–53.

Wessler, I., Reinheimer, T., Klapproth, H., Schneider, F.J., Racké, K., Hammer, R., 1997. Mammalian glial cells in culture synthetize acetylcholine. Naunyn-Schmiedeberg's Arch. Pharmacol. 356, 694–697.

Wiley, J.W., Lu, Y., Owyang, C., 1991. Evidence for a glutamatergic neural pathway in the myenteric plexus. Am. J. Physiol. 261, G693–G700.

Zukin, R.S., Bennett, M.V.L., 1995. Alternatively spliced isoforms of the NMDAR1 receptor subunit. Trends Neurosci. 18, 306–313.