ROLE OF CHOLINERGIC NEURONS IN THE MOTOR EFFECTS OF GLUCAGON-LIKE PEPTIDE-2 IN MOUSE COLON

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Running title: GLP-2 and cholinergic neurons

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Abstract

Glucagon-like peptide-2 (GLP-2) reduces mouse gastric tone and small intestine transit, but its action on large intestine motility is still unknown. The purposes of the present study were: i) to examine the influence of GLP-2 on spontaneous mechanical activity and on neurally-evoked responses, by recording intraluminal pressure from mouse isolated colonic segments; ii) to characterize GLP-2 mechanism of action; iii) to determine the distribution of GLP-2 receptor (GLP-2R) in the mouse colonic muscle coat by immunohistochemistry. Exogenous GLP-2 (0.1 nM - 300 nM) induced a concentration-dependent reduction of the spontaneous mechanical activity, which was abolished by the desensitization of GLP-2 receptor or by tetrodotoxin, a voltage-dependent Na⁺-channel blocker. GLP-2 inhibitory effect was not affected by L-NAME, a nitric oxide-synthase inhibitor, apamin, a blocker of small conductance Ca²⁺-dependent K⁺ channels, or [Lys1,Pro2,5,Arg3,4,Tyr6]VIP⁷⁻²⁸, a VIP receptor antagonist, but it was prevented by atropine or pertuxis toxin (PTX), a Gi/o protein inhibitor. Proximal colon responses to electrical field stimulation were characterized by nitrergic relaxation which was followed by cholinergic contraction. GLP-2 reduced only the cholinergic evoked contractions. This effect was almost abolished by GLP-2 receptor desensitization or PTX. GLP-2 failed to affect the contractile responses to exogenous carbachol. GLP-2R-immunoreactivity (IR) was detected only in the neuronal cells of both plexuses of the colonic muscle coat. More than 50% of myenteric GLP-2R-IR neurons shared the choline-acetyltransferase-IR. In conclusion, the activation of GLP-2R located on cholinergic neurons may modulate negatively the colonic spontaneous and electrically evoked contractions through inhibition of acetylcholine release. The effect is mediated by Gi protein.

Key words: enteric nervous system; GI hormones; colonic motility; acetylcholine.
**Introduction**

Glucagon-like peptide-2 (GLP-2) is a 33-amino acid peptide derived from proglucagon that is secreted by enteroendocrine L cells of the small and large intestine following nutrient intake, especially carbohydrate and fats (7). The gastrointestinal tract is the principal target for GLP-2 action where it affects multiple facets of physiology, including growth, absorption and motility (28). In particular, GLP-2 has been shown to be an important intestinotrophic factor that stimulates epithelial cell proliferation and inhibits apoptosis, increases crypts and villi and enhances intestinal digestive and absorptive capacity (8, 28). Accordingly, GLP-2 is regarded with interest also in relation to its therapeutic potential in several intestinal syndromes (9, 12).

Furthermore, several studies in human and animal models have demonstrated that GLP-2 is able to affect gut motor activity. Specifically, GLP-2 reduces the vagally induced antral motility in pigs (30), it slows human gastric emptying (13, 20) and it decreases the mouse gastric fundic tone leading to an increase of the stomach capacity (1). GLP-2 seems to act in concert with glucagon-like peptide-1 (GLP-1) to inhibit rat small bowel myoelectric activity in the fasted state (6). The peptide modulation on the gastrointestinal motility appears to be due mainly to central nervous mechanisms (30), but involvement of the enteric nervous system has been also shown (1). In addition, GLP-2 has been reported as an inhibitor of intestinal transit in the mouse (16), but the action of GLP-2 on large intestine motility has not been explored yet.

The GLP-2 effects are mediated by the interaction with a specific GLP-2 receptor (GLP-2R), belonging to the class of seven transmembrane-spanning G-protein coupled receptors. After GLP-2 binding to the transfected receptor, adenosine 3',5'-cyclic monophoshate (cAMP) levels are increased (19, 32), consistent with findings from studies of related members of the glucagon/GLP-1R family. However, GLP-2R ability to couple to different G-protein subunits and to activate multiple signaling pathways has been also demonstrated (14, 22). The presence of GLP-2R-mRNA transcripts has been demonstrated by Northern blot analysis along the murine gastrointestinal tract with high levels of expression in bowel (31) and, by in situ hybridization, in the murine enteric neurons (5). Furthermore, GLP-2R protein has been detected in the rodents and human intestinal subepithelial myofibroblasts (21), in human and pig enteroendocrine cells and in enteric neurons, suggesting that many of the GLP-2 actions may be indirect through release of not yet identified secondary mediators (11, 31).

The present study was undertaken to examine potential peripheral motor effects of GLP-2 on the spontaneous mechanical activity and on neurally-evoked responses in mouse proximal colon and to determine the mechanism responsible for the observed effects. In addition, we determined
the protein expression of GLP-2R in different regions of mouse gastrointestinal tract by Western blotting and the distribution of GLP-2R in the colonic mouse muscle coat by immunohistochemistry.

**Materials and methods**

**General.** The experiments were authorised by Ministero della Sanità (Rome, Italy), following the guidelines of the European Communities Council Directive of 24 November 1986. Adult male mice (C57BL/10SnJ; weighing $25.5 \pm 1.5\; g$) were killed by cervical dislocation. The abdomen was immediately opened and the proximal colon was removed from a position just distal to the caecum. The content of the excised segment was cleaned with Krebs solution and segment of about 2 cm in length was cut. Preparation was mounted in a custom designed horizontal organ bath (volume=5 ml), which was continuously perfused with oxygenated (95% $\text{O}_2$ and 5% $\text{CO}_2$) and heated (37 °C) Krebs solution with the following composition (mM): NaCl 119; KCl 4.5; MgSO$_4$ 2.5; NaHCO$_3$ 25; KH$_2$PO$_4$ 1.2; CaCl$_2$ 2.5; glucose 11.1.

The mechanical activity was recorded as previously described (18). In brief, the distal end of each segment was tied around the mouth of a J-tube, which was connected *via* a T catheter to a pressure transducer (Statham Mod. P23XL; Grass Medical Instruments, Quincy, MA, USA) and to a syringe for filling the preparation with Krebs solution. Each preparation was filled with 0.1 ml Krebs solution. The ligated proximal end was secured with a silk thread just to preload the preparations of 0.5 g. The mechanical signals were detected as changes in endoluminal pressure, which are mainly generated by circular muscle (1), and recorded on an ink writer polygraph (Grass model 7D; Grass Instruments Co., Quincy, MA).

To provide electrical field stimulation (EFS), was used an S88 square-wave pulse generator (Grass Medical Instruments, Quincy, MA, USA) coupled *via* a stimulus isolation unit (Grass SIU5) to a pair of platinum plates, which were placed in parallel on either sides of the intestinal segment. Preparations were allowed to equilibrate for about 60 min before starting the experiment.

**Functional experiments.** In a first set of experiments, after the equilibration time, the response of the preparation to non-cumulative concentrations of GLP-2 (0.1 nM - 300 nM) was examined. The peptide was added into the bath at increasing concentrations in volumes of 50 μl and each concentration was left in contact with the tissue for 7 min. The interval between single concentrations was 40 min to avoid tachyphylaxis. In the absence of well-characterized GLP-2R antagonists, to confirm the specificity of the observed effect, the peptide-induced response was tested after 40 min of tissue pretreatment with GLP-2 (10 nM) to induce desensitization of the
receptors. Moreover, to investigate the neuronal nature of inhibitory responses, a sub-maximal concentration of GLP-2 (30 nM) was tested in presence of tetrodotoxin (TTX) (1 µM), a voltage-dependent Na⁺-channel blocker. Thus, the effects of increasing concentrations of GLP-2 were evaluated after pre-treatment of intestinal preparations with Nω-nitro-L-arginine methyl ester (L-NAME) (300 µM), an inhibitor of nitric oxide (NO)-synthase, apamin (0.1 µM), a blocker of small conductance Ca²⁺-dependent K⁺ channels, [Lys1,Pro2,5,Arg3,4,Tyr6]VIP⁷⁻²⁸, a VIP receptor antagonist, atropine (1 µM), a muscarinic receptor antagonist, or pertussis toxin (PTX) (300 ng/ml), a Gi/o protein inhibitor. These agents were added to the perfusing solution at least 30 min before testing the peptide, except PTX which was left in contact with the tissue for 3 h. The concentrations of the inhibitors used were determined from previous experiments, where they have been shown to be effective in mouse colon or from literature (1, 15, 18, 26).

The influence of GLP-2 (0.1 nM – 300 nM) on the electrically-evoked responses was evaluated in separate set of experiments, being the spontaneous mechanical activity abolished by the repetitive application of electrical field stimulation (EFS). Trains of stimuli (duration 5 s, supramaximal voltage, 8 Hz and 0.5 ms pulse duration) were applied to colonic preparation at intervals of 70 s and stable and reproducible responses for a time-period of 3 h were observed. EFS induced a biphasic response, characterized by muscular relaxation followed by contraction, which was abolished by TTX (1 µM), suggesting its neural origin. The responses evoked by EFS were analysed in the presence of non-cumulative increasing concentrations of GLP-2 (0.1 nM – 300 nM).

The contact time for each concentration was 7 min. The effect of GLP-2 was evaluated after GLP-2R desensitization with GLP-2 (10 nM for 40 min) or (PTX) (300 ng/ml). In separate experiments, tissues were exposed to cumulative increasing concentrations of carbachol (CCh) (10 nM - 30 µM) or to KCl (30 mM), and the myogenic contractions produced were evaluated in the absence or in the presence of GLP-2 (30 nM).

Data analysis and statistical tests. For data analysis, the mean amplitude of the spontaneous pressure waves was determined for 7 min before and after GLP-2 administration. The inhibitory response of the colonic circular muscle to GLP-2 was taken as the percent change from the resting spontaneous activity (e.g. 100% corresponds to the abolition of spontaneous activity). The inhibitory effect of GLP-2 on evoked cholinergic contractions was expressed as a percentage of the response produced by EFS in control conditions. Contractile effects induced by CCh were expressed as a percentage of the maximal response. All data are mean values ± SE. The letter n indicates the number of experimental animals. The concentration (EC₅₀) with 95% confidence intervals (Cls) producing half maximum response was calculated using Prism 4.0, GraphPad (San
Diego, CA, USA). Statistical analysis was performed by means of ANOVA followed by Bonferroni post hoc test. A probability value of less than 0.05 was regarded as significant.

Drugs. The following drugs were used: apamin, atropine sulphate, carbachol (CCh), Nω-nitro-L-arginine methyl ester (L-NAME) (Sigma Aldrich, Milan, Italy), tetrodotoxin citrate (TTX) (Ascent scientific LtD., Bristol, UK), rat glucagon-like-peptide 2 (GLP-2), pertuxis toxin (PTX) [Lys1,Pro2,5,Arg3,4,Tyr6]VIP7-28 (Tocris-Bioscience, Bristol, UK). Each compound was prepared as a stock solution in distilled water. The working solutions were prepared fresh the day of the experiments by diluting the stock solutions in Krebs.

Western blotting and immunohistochemistry. For Western blotting, about 30 mg of tissue from fundus, antrum, duodenum, jejunum and colon was incubated on ice in RIPA Buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl, 1% Nonidet P-40) containing protease inhibitors (2 mM PMSF, NaVO3) for 1 h. Subsequently, it was centrifuged for 15 minutes at 12000 rpm and supernatant isolated. Protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Milan, Italy). Proteins (50 μg) were separated by 10% SDS-PAGE containing 0.1% SDS and transferred to Hybond-C nitrocellulose membranes (Amersham Life Science, Munich, Germany) by electroblotting. Loading and transfer conditions were assessed by staining of the gel with Ponceau-Red. The relative migration position of target protein was detected using a coelectrophoresed pre-stained molecular-weight protein ladder (Invitrogen, Paisley, UK). The membranes were sequentially incubated overnight with antibodies to GLP-2R (Santa Cruz Biotechnology, Inc., CA, USA) raised against the N-terminal extracellular domain of rodent GLP-2R (diluted 1:200), or mouse β-tubulin (diluted 1:5000) (Sigma Aldrich, Milan, Italy) applied as a loading control, and proteins were visualized by using an anti-goat IgG secondary antibody conjugated to horseradish peroxidase (diluted 1:10000) (Santa Cruz Biotechnology, Inc., CA, USA). The target proteins were detected by enhanced chemiluminescence (Pierce, Rockford, USA).

For immunohistochemistry, proximal colon specimens (mice=3) were fixed in 4% paraformaldehyde for 4 hours at 4°C. The specimens were cryoprotected in 30% sucrose in phosphate-buffered saline (PBS) for approximately 12 hours at 4°C, frozen in Killik cryostat embedding medium (Bio-Optica, Milan, Italy). Transverse sections, 12 μm thick, were cut, collected on polylysine-coated slides and pre-incubation in 0.5% Triton (Sigma Aldrich, Milan, Italy) and 1.5% bovine serum albumin (BSA) (Sigma Aldrich, Milan, Italy) in PBS for 15m at room temperature. Then, they were incubated with GLP-2R polyclonal antibody (Santa Cruz Biotechnology, Inc., CA, USA) at final dilution of 1:50 overnight at 4°C. The immunoreaction was revealed by using the secondary antibody Alexa Fluor 488 Donkey anti Goat (Invitrogen, Paisley,
UK) 1:333 for 2h at room temperature. To identify the potential localization of GLP-2R on cholinergic neurons, transverse sequential sections, 4 μm thick, were cryocut from each specimens and collected on slides (4 sections/slide, 2 slides/specimens) in two separate areas, one area containing the first and the third section, the other area containing the second and the fourth section. Each area was bordered with a pap pen and the two sections of one area were incubated with GLP-2R antibody as described above while the two sections of the neighbour area were incubated with Choline Acetyltransferase (ChAT) polyclonal antibody (a generous gift of Dr M. Schemann) (23), at final dilution of 1:500 overnight at 4°C. The two immunoreactions were revealed by using, the Alexa Fluor 488 secondary donkey anti goat 1:333 and the Alexa Fluor 568 secondary goat anti rabbit 1:333 respectively, both incubated for 2h, at room temperature and then observed under an epifluorescence Zeiss Axioskop microscope. Negative controls were performed by omitting the primary antibodies and all of them had no labelling (data not shown).

Quantitative analysis. By using a x40 objective, GLP-2R-IR neurons were counted along the entire perimeter of each section (4 sections each animal) taking as starting and ending point the insertion of the mesentere. Quantification of the neurons sharing GLP-2R- and ChAT-IR was done on the sequential sections collected as above described. Digitized images of the entire perimeter of the muscle wall were acquired using a x40 objective and transformed into TIFF files using Scion Image. Field edges were defined based on structural details within the tissue section to ensure that the fields did not overlap. Comparison between pictures took from GLP-2R-IR sections and from ChAT-IR sections at the same level was done to identify and quantify those neurons that shared the two markers. Only the labeled neuronal bodies were considered for quantification. The count was done by two of us blind to each other results on a total of 12 slices for each antibody. The results were expressed as mean ± SE.

Results

Functional studies

Influence of GLP-2 on spontaneous mechanical activity. Mouse proximal colon exhibited spontaneous mechanical activity consisting of phasic contractions at a frequency of 1.8 ± 0.3 (n=15) and an amplitude of 10.6 ± 2.5 cm H2O (n=15). GLP-2 (0.1 nM- 300 nM) produced inhibitory effects on the pressure waves, characterized by a decrease in the mean amplitude of spontaneous contractions, without affecting the frequency and the resting tone (Fig.1A). The effect occurred within 1 min after addition of the peptide and was completely reversible after washing out.
with normal Krebs solution. Figure 1B shows the concentration-response curve for the inhibitory effects induced by GLP-2 on the spontaneous contractions of isolated mouse proximal colon. GLP-2 produced a maximal effect corresponding to about 75% of reduction of amplitude of spontaneous contractions with an EC$_{50}$ = 4.0 nM (Cls=2.3 - 7 nM; n=6). To assess the specificity of the effect, the preparations were pretreated for 40 min with GLP-2 (10 nM) in order to desensitize the receptors. This treatment produced an early and transient reduction of the spontaneous contractions, which was followed by recovery to the initial amplitude. In these conditions, the inhibition of mechanical activity induced by the peptide was significantly reduced over the full concentration used (Fig.1B). The desensitization effect was not reversible after 2 hour wash out.

The response to GLP-2 (0.1-300 nM) was abolished by TTX (1 µM), which per se reduced the spontaneous contraction amplitude (about 4 cm H$_2$O), suggesting its neural origin. However, it was not affected by L-NAME (300 µM), a blocker of the NO synthase, that per se increased amplitude of spontaneous activity, apamin (100 nM), a blocker of small conductance Ca$^{2+}$-dependent K$^+$ channels, or [Lys1,Pro2,5,Arg3,4,Tyr6]VIP$_{7-28}$ (300 nM), a VIP receptor antagonist (Fig. 2). In contrast, the GLP-2 inhibitory effect was significantly reduced by atropine (1 µM), muscarinic receptor blocker, that per se decreased the amplitude of the spontaneous contractions (about 7 cm H$_2$O), or by PTX (300 ng/ml), which per se was without any effect on spontaneous mechanical activity (Fig. 3).

Influence of GLP-2 on the neurally-evoked responses. EFS (trains of 8 Hz for 5 s) induced a nitrergic small relaxation (0.4 ± 0.2 cm H$_2$O; n=12) (abolished by L-NAME) which was always followed by cholinergic contraction (14.1 ± 2.3 cm H$_2$O; n=12) (abolished by atropine). GLP-2 (0.1 nM - 300 nM) caused a concentration-dependent reduction of the electrically-evoked cholinergic contractions, without affecting the neural inhibitory response (Fig.4 A and Fig.4B). GLP-2 (100 nM) produced about 60% of reduction of the evoked contractile response amplitude and this effect was reversible after washing out. The inhibitory action of GLP-2 was significantly reduced by GLP-2R desensitization or by PTX (300 ng/ml) (Fig.4B). GLP-2R desensitization caused an transient reduction of the evoked contractions, which was followed by recovery to the initial amplitude, while PTX did not affect the evoked responses.

In the tissue, GLP-2 (30 nM) did not affect the concentration-dependent contractile response induced by carbachol (10 nM - 30 µM), a muscarinic agonist, (Fig.5) or the contraction induced by KCl (30 mM) (data not shown).
Expression analysis

Western blotting. Western blot analyses of mouse fundus, antrum, duodenum, jejunum and colon yielded a single 72-kDa band corresponding to the molecular weight of the GLP-2R. Tissue-specific differences in protein expression was observed as GLP-2R levels were relatively higher in gastric fundus and colon than in the small bowel (Fig. 6).

Immunohistochemistry. GLP-2R-immunoreactivity (IR) in the proximal colon was detected only in the neuronal cells of myenteric and submucous plexuses. The IR neurons were round or oval in shape; the labelling was detected in the perikarion and in numerous nerve varicosities inside the myenteric ganglia. The labelling had a granular aspect. IR fibres were seen outside the ganglia, few in the thickness of the circular muscle coat, many at the submucosal border (Fig. 7) in the region containing the so-called submucosal interstitial cells of Cajal. Some of the IR myenteric neurons were also ChAT-IR (Fig. 8).

ChAT-immunoreactivity (IR) neurons were present in both plexuses (myenteric and submucous plexuses). They had a round or oval perikaryon and the labelling was homogenously distributed in the cytoplasm. Some IR nerve fibres were detected in the muscle coat and numerous at the submucosal border of the circular muscle layer (data not shown). Some of the IR neurons were also GLP-2R-IR (Fig. 8).

Quantitative analysis. The mean number of GLP-2R-IR and ChAT-IR neurons/slice in the myenteric plexus is reported in Table 1. The mean number of neurons/slice that, by comparison, shared the two markers was 18.44±1.32 at the myenteric plexus.

Discussion

The present study demonstrates that, in mouse proximal colon, GLP-2 is able to modulate negatively the spontaneous mechanical activity and the electrically-evoked cholinergic contractions through inhibition of acetylcholine release from enteric neurons. These conclusions are also supported by immunohistochemistry, showing the presence of the GLP-2R on myenteric neurons, half of which shared ChAT-IR.

It is well accepted that GLP-2 affects multiple facets of gastrointestinal physiology, concerning mainly the control of mucosal growth and function (eg, epithelial integrity, secretion; local blood flow; nutrient uptake and utilization) (8, 28). However little is known about its role in the control of the gastrointestinal motility. In anesthetized pigs, GLP-2 acts as an inhibitor of gastric motility, because it abolishes the motor response induced by hypoglycaemia (30). In humans results
about the ability of GLP-2 to suppress gastric motility are conflicting, and it could depend on the methodology used to assess antral emptying or on the type of administered test meal (low-calorie liquid meal or high-calorie solid meal) (13, 17, 20, 24). In mouse, GLP-2 decreases the gastric tone leading to an increase of the stomach capacity (1) and it inhibits the intestinal transit (16).

We initially used Western blotting to examine the distribution and relative abundance of GLP-2R along the mouse gastrointestinal tract, because up to date this has not been investigated and the only available information is about GLP-2R gene expression (31). GLP-2R was observed in all gastrointestinal regions examined, with relatively higher levels of expression in gastric fundus and colon than in small intestine. The GLP-2R high level in gastric fundus supports our recent evidence for GLP-2 ability to induce gastric relaxation (1).

Therefore, our working hypothesis was that GLP-2R activation may induce motor effects also in mouse colon. Actually, our data show that exogenous GLP-2 reduced in a concentration-dependent manner spontaneous mechanical activity suggesting a potential inhibitory role of the peptide on mouse colonic circular muscle. The effect is mediated by GLP-2R because the agonist-induced desensitization almost abolished the GLP-2 response. In fact, in the absence of GLP-2R well-characterized antagonists, desensitization can represent a useful pharmacological tool in the study of receptors and GLP-2R has been shown to undergo rapid and sustained homologous desensitization induced by the agonist (10, 29). Indeed, GLP-2R antagonist (3, 25) has been used as a GLP-2R antagonist (3, 25), but it works as well as a weak partial agonist (27). The potency (EC50) of GLP-2 in inducing colonic inhibitory effects was 4.0 nM, which is in agreement with that reported for the human and/or rat GLP-2 ability to stimulate GLP-2 receptor in various cell types (EC50 ranging from 0.04 to 14 nM) (10, 19, 27, 29) or for inducing mouse gastric relaxation (1). Preliminary analyses in our laboratory have indicated that the GLP-2 plasma concentration is about 0.7 ng/ml in ad libitum feeding mice in well accord with the range of effective concentrations used in this study. Moreover, a dipeptidyl peptidase IV inhibitor (DPPI 1c) caused reduction of evoked contractions suggesting the occurrence of a system for metabolizing GLP-2 in the colon (F. Mulè, unpublished observations). Therefore, we are encouraged to consider the GLP-2 effect as a physiological action. We can only speculate about hormonal role on colonic motility because it could be released in the blood stream from L-type enteroendocrine cells of the small and large intestine after nutrient ingestion or it could act with paracrine mechanism near its site of production (colonic enteroendocrine L-cells). Reduced colonic propulsion caused by GLP-2 could lead to greater absorption of water and electrolytes, in agreement with its nutrient absorptive function (8,28).

Moreover, we investigated whether GLP-2 induces inhibition of colonic motility via a direct action on the smooth muscle cells and/or via an indirect action, mediated by enteric nervous system,
because expression in enteric neurons of both submucosal and myenteric plexus has been reported 
(3, 5, 11). The observation that TTX, a blocker of neuronal voltage-dependent Na⁺ channels, 
abolished the GLP-2 effects suggests that neurons within the intramural plexuses are responsible for 
the action of the peptide in mouse colonic circular muscle. Indeed, our immunohistochemical study 
showed that in the proximal colon, the GLP-2R was expressed only by the neuronal cells. IR was 
detected either in the soma of myenteric and submucosal neurons and in the nerve fibres, especially 
at the border between the circular muscle layer and the submucosa.

The inhibitory effects induced by GLP-2 were not mediated by the release of any inhibitory 
neurotransmitters because L-NAME, NO synthase inhibitor, [Lys1,Pro2,5,Arg3,4,Tyr6]VIP7-28, VIP 
receptor antagonist, or apamin, a blocker of small conductance Ca²⁺-dependent K⁺ channels, which 
antagonizes the inhibitory purinergic transmission in this preparation (26), failed to affect the 
mechanical response to GLP-2.

Therefore, we addressed the possibility that GLP-2 inhibitory effects could be due to a 
reduction of ongoing release of acetylcholine. Cholinergic motor neurons have been reported to be 
spontaneously active in mouse ileum (4) and we observed a reduction of the amplitude of 
spontaneous contractions by atropine suggesting that, in our preparation, muscarinic receptors are 
tonically activated, as reported in a previous investigation (18). Atropine significantly attenuated 
GLP-2 inhibitory effects indicating that muscarinic receptors were involved on the hormone 
mechanism of action. Therefore, our data can be interpreted as suggesting that circular smooth 
muscle of mouse proximal colon is under a tonic influence by neural acetylcholine and GLP-2R 
activation would reduce the release of the excitatory transmitter from the cholinergic nerves. To 
further support our hypothesis we tested GLP-2 on neurally-mediated responses evoked by EFS. 
The observations that the peptide reduced, in a concentration-dependent manner, the electrically 
evoked cholinergic responses, without affecting the nitrergic relaxation, and this effect was almost 
completely abolished after GLP-2R desensitization, provide further evidence that GLP-2 receptor 
activation is able to modulate the release of acetylcholine. On the other hand, GLP-2 failed to affect 
the contractions induced by carbachol or KCl, confirming that the peptide does not directly interfere 
with smooth muscle muscarinic receptors and the inhibitory action is achieved primarily by acting 
on prejunctional receptors. Once more, immunohistochemical data support our hypothesis because 
more than 50% of myenteric GLP-2R-IR neurons shared ChAT-IR. Our proposed mechanism is in 
agreement with a previous report in guinea-pig ileum, showing that activation of GLP-2R, present 
on cholinergic secretomotor neurons, decreases epithelial chloride secretion by suppressing 
aeetylcholine release (3). Consistent with our functional and morphological results, recent findings 
have pointed out the importance of the enteric excitatory motoneurons in the downstream signalling
of the glucagon-like peptides to inhibit mouse intestinal motility (16). In fact, they showed that in a murine animal model with a partial enteric nervous system deficit, characterized by a dramatic decrease of cholinergic neurons number (GFRα2-deficient animals), GLP-2 was not able to induce reduction of intestinal transit, as did in wild-type animals (16). The reduction of acetylcholine release could represent an explanation for the GLP-2 inability to inhibit motility in these animals.

Because studies characterizing GLP-2R-regulated intracellular signalling pathways in transfected cell lines and in intestinal mucosa have reported increases in cAMP (19, 29, 32), GLP-2 should be thought as facilitating synaptic transmission. The observation that, in our experimental model, the inhibitory effects of GLP-2 on spontaneous mechanical activity and on evoked cholinergic contractions were significantly inhibited by pre-treatment with pertuxis toxin, a Gi/o protein inhibitor, supports the hypothesis that in mouse colonic cholinergic myenteric neurons, GLP-2R may be coupled to inhibitory G protein, leading in turn to reduction of the cAMP level. GLP-2R ability to couple to different G-protein subunits and activate multiple signaling pathways has been demonstrated (14, 22). In particular, GLP-2R may be coupled in a dose-dependent manner to alternate G protein and cAMP accumulation occurs at moderate concentrations (0.1 – 1 nM), but there is reduction with higher levels of GLP-2 (25, 29).

In conclusion, in mouse proximal colon, GLP-2 is able to inhibit the spontaneous and electrically evoked contractions acting peripherally through inhibition of prejunctional acetylcholine release. The slowing of colonic motility could lead indirectly to an increase of water and electrolyte absorption, in accordance with GLP-2 role as nutrient absorption stimulator.

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References


Figure Legends

Figure 1. A: Typical recordings showing the inhibitory effects of increasing concentrations of GLP-2 on spontaneous contractions in mouse proximal colon. B: Concentration-response curves for the inhibitory effects induced by GLP-2 before and after GLP-2R desensitization (10 nM for 40 min). Data are means ± SE (n=5) and are expressed as percentage inhibition of the spontaneous contraction. SE is reported only if it exceeds the dimension of the symbol. * P<0.05 compared with the respective control conditions.

Figure 2. Concentration-response curves for the inhibitory effects induced by GLP-2 in control conditions (n=14) and in the presence of TTX (1 µM; n=3), L-NAME (300 µM; n=4), apamin (0.1 µM, n=3) or [Lys1,Pro2,5,Arg3,4,Tyr6]VIP7-28 (300 nM, n=4). Data are means ± SE and are expressed as percentage inhibition of the spontaneous contraction. The control curves are the means of the control data obtained before each treatment. SE is reported only if it exceeds the dimension of the symbol.

Figure 3. Concentration-response curves for the inhibitory effects induced by GLP-2 in control conditions (n=10) and in the presence of atropine (1 µM; n=5), or PTX (300 ng/ml; n=5). Data are means ± SE and are expressed as percentage inhibition of the spontaneous contraction. The control curves are the means of the control data obtained before each treatment. * P<0.05 compared with the respective control conditions.

Figure 4. A: Typical recordings showing the inhibitory effects of increasing concentrations of GLP-2 on cholinergic contractile response to EFS (train of 5 s, 0.5 ms, supramaximal voltage, 8 Hz) in mouse proximal colon. B: Concentration-response curves for the inhibitory effects induced by GLP-2 before (n=10) and after GLP-2R desensitization (10 nM for 40 min; n=5) or after PTX (300 ng/ml; n=5) on the neurally evoked cholinergic contraction. Data are means ± SE and are expressed as a percentage of the response obtained in the absence of GLP-2. SE is reported only if it exceeds the dimension of the symbol. The control curves are the means of the control data obtained before each treatment. * P<0.05 compared with the respective control conditions.

Figure 5. Concentration-response curves for the contractile effects induced by carbachol in mouse proximal colon before and after pre-treatment with GLP-2 (10 nM). Contractile response is...
expressed as percent of the maximal response. Data are means ± SE (n= 4). SE is reported only if it exceeds the dimension of the symbol.

**Figure 6.** Immunoblots of whole homogenates from mouse gastric fundus, antrum, duodenum, jejunum and colon showing the expression of GLP-2R and β-tubulin (loading control). Negative control was obtained by omitting the primary antibody. Shown are representative blots from three independent experiments.

**Figure 7.** GLP-2R-IR in the mouse colon. The labelling is present only in neuronal cells. The neuronal bodies show a diffuse IR with granular aspect. Several IR fibres are present in the muscle coat especially at the border with the submucosa. LML=longitudinal muscle layer; CML=circular muscle layer; SM=submucosa. Bar=75 μm.

**Figure 8.** One myenteric ganglion. **A:** Three GLP-2R-IR neurons. The labelling is detected in the perikarion and along the cell processes and had a granular aspect. **B:** Six ChAT-IR neurons. The labelling is homogenously distributed in the perikarion and has a granular aspect in the cell processes. * indicates the neurons that express both labelings. Bar=30 μm.
Fig. 3
**Fig. 4**

A. Graph showing the effects of various concentrations of GLP-2 on EFS-evoked contractions. The graph displays the percentage inhibition of contractions at different concentrations of GLP-2.

B. Graph showing the comparison of control, GLP-2R desensitization, and PTX treatments. The x-axis represents the logarithm of [GLP-2] (M), while the y-axis represents the cholinergic-evoked contraction (% inhibition).
Fig. 5

CCh-evoked contraction (%)

- Log [CCh] (M)

(control) (GLP-2)
GLP-2R 70 KDa

β-tubulin 45 KDa

Fig. 6
Table 1. Quantitative analysis of immunoreactive neurons/slice in myenteric plexus of mouse proximal colon.

<table>
<thead>
<tr>
<th>Chemical coding</th>
<th>Myenteric plexus</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-2R</td>
<td>32.88±2.14</td>
</tr>
<tr>
<td>ChAT</td>
<td>44.33±1.94</td>
</tr>
<tr>
<td>GLP-2R/ChAT</td>
<td>18.44±1.32</td>
</tr>
<tr>
<td>% colocalization vs GLP-2R</td>
<td>55.10±3.17</td>
</tr>
<tr>
<td>% colocalization vs ChAT</td>
<td>41.67±6.16</td>
</tr>
</tbody>
</table>

Data are means ± SE (n=3).