Role of cholinergic neurons in the motor effects of glucagon-like peptide-2 in mouse colon

Antonella Amato,1 Alessandra Rotondo,1 Lorenzo Cinci,2 Sara Baldassano,1 Maria Giuliana Vannucchi,2* and Flavia Mulè1

1Dipartimento di Biologia cellulare e dello Sviluppo, Università di Palermo, Palermo; and 2Dipartimento di Anatomia, Istito e Medicina legale, Università di Firenze, Firenze, Italy

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Amato A, Rotondo A, Cinci L, Baldassano S, Vannucchi MG, Mulè F. Role of cholinergic neurons in the motor effects of glucagon-like peptide-2 in mouse colon. Am J Physiol Gastrointest Liver Physiol 299: G1038–G1044, 2010. First published August 12, 2010; doi:10.1152/ajpgi.00282.2010.—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 1) 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; 2) to characterize GLP-2 mechanism of action; and 3) to determine the distribution of GLP-2 receptor (GLP-2R) in the mouse colonic muscular wall. Exogenous GLP-2 (0.1–300 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 Nω-nitro-L-arginine methyl ester (a nitric oxide synthase inhibitor), apamin (a blocker of small conductance Ca2+-dependent K+ channels), or [Lys1,Pro2,5,Arg3,4,Tyr6]VIP 7–28 (a VIP receptor antagonist), but it was prevented by atropine or pertussis toxin (PTX), a Gαi/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 muscular wall. 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 G, protein.

enteric nervous system; gastrointestinal hormones; colonic motility; acetylcholine

GLUCAGON-LIKE PEPTIDE-2 (GLP-2) is a 33-amino acid peptide derived from proglucagon that is secreted by enteroneodocrine 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 monophosphate (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 rodent and human intestinal subepithelial myofibroblasts (21), in human and pig enteroneodocrine 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 authorized 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 ± 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 cecum. The content of the excised segment was cleaned with Krebs solution and segment of ~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% O₂ and 5% CO₂) and heated (37°C) Krebs solution with the following composition (mM): 119 NaCl, 4.5 KCl, 2.5 MgSO₄, 25 NaHCO₃, 1.2 KH₂PO₄, 2.5 CaCl₂, 11.1 glucose.

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) 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, Quincy, MA).

To provide electrical field stimulation (EFS), we used an S88 square-wave pulse generator (Grass Medical Instruments) 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 ~60 min before the start of the experiment.

Functional Experiments

In a first set of experiments, after the equilibration period, the response of the preparation to noncumulative concentrations of GLP-2 (0.1–300 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 submaximal 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 pretreatment of intestinal preparations with N°-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 (Tocris-Bioscience, Bristol, UK), a VIP receptor antagonist; atropine (1 μM), a muscarinic receptor antagonist; or pertussis toxin (PTX) (300 ng/ml). 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, ~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, NaVO₄) for 1 h. Subsequently, it was centrifuged for 15 min at 12,000 rpm and supernatant was isolated. Protein concentration was measured by 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 by using a coelectrophoresed prestained molecular weight protein ladder (Invitrogen, Paisley, UK). The membranes were sequentially incubated overnight with antibodies to GLP-2R (Santa Cruz Biotechnology, Santa Cruz, CA) raised against the NH₂-terminal extracellular domain of rodent GLP-2R (diluted 1:200), or mouse β-tubulin (diluted 1:5,000) (Sigma Aldrich) applied as a loading control, and proteins were visualized by using an anti-goat IgG secondary antibody conjugated to horseradish peroxidase (diluted 1:10,000) (Santa Cruz Biotechnology). The target proteins were detected by enhanced chemiluminescence (Pierce, Rockford, IL).

For immunohistochemistry, proximal colon specimens (n = 3 mice) were fixed in 4% paraformaldehyde for 4 h at 4°C. The specimens were cryoprotected in 30% sucrose in phosphate-buffered saline (PBS) for ~12 h at 4°C, frozen in Killik cryostat embedding
isolated mouse proximal colon. GLP-2 produced a maximal effects induced by GLP-2 on the spontaneous contractions of preparations were pretreated for 40 min with GLP-2 (10 nM) 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-h washout.

The response to GLP-2 (0.1–300 nM) was abolished by TTX (1 μM), which per se reduced the spontaneous contraction amplitude (~4 cmH₂O), 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 Ca²⁺-dependent K⁺ channels; or [Lys1,Pro2,5,Arg3,4,Tyr6]VIP7–28 (300 nM), a VIP receptor antagonist (Fig. 2). In contrast, the GLP-2 inhibitory effect was significantly reduced by atropine (1 μM), a muscarinic receptor blocker, that per se decreased the amplitude of the spontaneous contractions (~7 cmH₂O), 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 nitricergic small relaxation (0.4 ± 0.2 cmH₂O; n = 12) (abolished by L-NAME) which was always followed by cholinergic contraction (14.1 ± 2.3 cmH₂O; n = 12) (abolished by atropine). GLP-2 (0.1–300 nM) caused a concentration-dependent reduction of the electrically evoked cholinergic contractions, without affecting the neural inhibitory response (Fig. 4, A and B). GLP-2 (100 nM) produced ~60% of reduction of the evoked contractile response amplitude and this effect was reversible after washing out. The

Quantitative Analysis

By using a ×40 objective, GLP-2R-immunoreactivity (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 mesentery. 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 via a ×40 objective and transformed into TIFF files by use of Scion Image. Field edges were defined on the basis of structural details within the tissue section to ensure that the fields did not overlap. Comparison between pictures taken from GLP-2R- and 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’s results on a total of 12 slices for each antibody. The results were expressed as means ± 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 cmH₂O (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 washout 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 ~75% of reduction of amplitude of spontaneous contractions with an EC₅₀ = 4.0 nM (CI = 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) 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-h washout.
inhibitory action of GLP-2 was significantly reduced by GLP-2R desensitization or by PTX (300 ng/ml) (Fig. 4B). GLP-2R desensitization caused a transient reduction of the evoked contractions, which was followed by recovery to the initial amplitude, whereas PTX did not affect the evoked responses.

In the tissue, GLP-2 (30 nM) did not affect the concentration-dependent contractile response induced by CCh (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 were observed given that GLP-2R levels were relatively higher in gastric fundus and colon than in the small bowel (Fig. 6).

**Immunohistochemistry.** GLP-2R-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 labeling was detected in the perikaryon and in numerous nerve varicosities inside the myenteric ganglia. The labeling had a granular aspect. IR fibers 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-IR neurons were present in both plexuses (myenteric and submucous plexuses). They had a round or oval perikaryon and the labeling was homogenously distributed in the cytoplasm. Some IR nerve fibers 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 per slice in the myenteric plexus is reported in Table 1. The mean number of neurons per 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 (e.g., epithelial integrity, secretion, local blood flow, nutrient uptake, and utilization) (8, 28). However, little is known about its role in the control of gastrointestinal motility. In anesthetized pigs, GLP-2 acts as an inhibitor of gastric motility, because it abolishes the motor response induced by hypoglycemia (30). In humans, results about the ability of GLP-2 to suppress gastric motility are conflicting and 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 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 to date this has not been inves-

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**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>
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<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>
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</tbody>
</table>

Data are means ± SE (n = 3). GLP-2R, glucagon-like peptide-2 receptor; ChAT, choline acetyltransferase.
GLP-2 AND CHOLINERGIC NEURONS

The inhibitory effects induced by GLP-2 were not mediated by the release of any inhibitory neurotransmitters because the NO synthase inhibitor L-NAME, the VIP receptor antagonist [Lys1,Pro2,5,Arg3,4,Tyr6]VIP7–28, or the blocker of small conductance Ca\(^{2+}\)-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 both in the soma of myenteric and submucosal neurons and in the nerve fibers, especially at the border between the circular muscle layer and the submucosa.

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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 were tonically activated, as reported in a previous investigation (18). Atropine significantly attenuated GLP-2 inhibitory effects, indicating that muscarinic receptors were involved in 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 nitric 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 CCh 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 acetylcholine release (3). Consistent with our functional and morphological results, recent findings have pointed out the importance of the enteric excitatory motoneurons in the downstream signaling 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 represents an explanation for the GLP-2 inability to inhibit motility in these animals.

Because studies characterizing GLP-2R-regulated intracellular signaling 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 pretreatment with PTX, a G\(_{1043}\) 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 moder-
ate 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.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**REFERENCES**


