A Local, Exendin 9-39-Insensitive, Site of Action of GLP-1 in Canine Ileum*

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Running Title: Paracrine action of GLP-1

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Abstract: GLP-1 modulates glucose levels following a meal, including by inhibition of gastric emptying and intestinal transport. Intra-arterial injection of GLP-1 into the gastric corpus, antrum or pylorus of anesthetized dogs had no effect on the contractile activity of the resting or neurally activated stomach. GLP-1 injected i.a. inhibited intestinal segments when activated by enteric nerve stimulation, but not by acetylcholine. Isolated ileum segments were perfused intra-arterially, instrumented with strain gauges to record circular muscle activity and with sub-serosal electrodes to stimulate enteric nerves. GLP-1 caused concentration-dependent inhibition of nerve-stimulated phasic, but not tonic activity. This was absent during TTX-induced activity and partly prevented by L-NOARG. Exendin 9-39, the GLP-1 antagonist, had no intrinsic activity and did not affect the actions of GLP-1. Capsaicin mimicked the effects of GLP-1 and may have reduced the effect of subsequent GLP-1. GLP-1 may mediate paracrine action on afferent nerves in the canine ileal mucosa using an unusual receptor.

Key Words: gastric emptying, intestinal motility, capsaicin, afferent nerves, paracrine action
Introduction:

Glucagon-like peptide-1 (GLP-1) is produced in L-type endocrine cells of the ileal and colonic mucosa where it is usually co-localized with PYY and GLP-2 (8,68,53,24,42,20,51,21). It contains sequence similarities with glucagon and is derived by alternate processing of a common precursor, proglucagon (aa 78-107). It is released by glucose, fatty and peptone meals (42,21,51,3,20), which is constrained by somatostatin (41). GLP-1 acts as an incretin (59,50,62) with multiple actions on pancreatic beta cells: it raises cAMP, inhibits the K_{ATP}-sensitive channel, depolarizes, enhances Ca^{2+} entry and promotes insulin release (40,46). It also inhibits release of glucagon from pancreatic alpha cells, possibly by increasing release of somatostatin by D cells (42,21,51,20). When GLP-1’s action is inhibited, glucose tolerance is impaired (11). Receptors for GLP-1 are present in the periphery as well as the central nervous system, where it may act to modulate fluid and food intake (18,19,39,10,52,15,7,72,48). GLP-1 receptors are also present on afferent nerves and several of its actions are postulated to involve activation of vagal afferents or those from the dorsal roots (44,70,54).

Up to now most receptors have been characterized by their susceptibility to agonists and antagonists and in several cases by cloning and expression of the receptor in cell lines. Only one cloned GLP-1 receptor has been found (18,39,10,52,15,7,72), but there is one previous case in which receptors have distinctly different pharmacology, such as insensitivity to the antagonist, exendin 9-39 have been described (54).

GLP-1 has important affects on gastrointestinal motility; it inhibits gastric emptying and intestinal transit (4,7, 66,67,47,57) and is postulated to be involved in ileal braking of gastric emptying and intestinal transit following glucose (48,49,71,22,5), fat (37,38,61,60) and peptone (38) meals. The locations of receptors for these actions have not been identified, but they are know to require an intact vagus nerve (44,36,70). We demonstrated in awake instrumented dogs that GLP-1 inhibits gastric emptying of a saline meal by decreasing the number and volume of flow pulses through the pylorus. These changes were
associated with inhibition of antropyloric pressure waves, stimulation of isolated pyloric pressure waves and an increase in pyloric tone (4).

The half life of GLP-1 in the plasma is short (1-2 min.) as it is rapidly degraded by dipeptidyl peptidase IV and it is uncertain if the active peptide reaches all its receptors in an intact form (16,17,56,6,9,43,55,63). Therefore we hypothesize that it might have a local action in the ileum. The objectives of this study were to determine if GLP-1 acted on the canine ileum, a site at which it is released in the canine intestine, and to determine the site and nature of any such actions.
Methods:

Animals: Random source dogs were studied. Animals of both sexes were used if they were in good health and had been treated in our Animal Facility for parasites. They were anesthetized with 40 mg/kg of Na pentobarbital and maintained at a level of anesthesia in which they did not respond to pain and had no eye blink to touching the cornea. The were respired artificially throughout the experiment. After surgery (described below), and completion of the experiment, they were euthanized with intravenous hypertonic KCl. These procedures were approved by the McMaster University Animal Care Committee.

Preparation of isolated segments: This has previously been described in detail \(45, 31-35, 13, 14, 69\) and is summarized briefly. The abdomen was opened with a midline incision and the ileum brought to the exterior. A segment of 10-15 cm was isolated completely from the body by cannulating a terminal artery and vein, perfusing the artery at 2.7mL/min with physiological saline, collecting the venous effluent, cutting all connection by mesentery, nerves and vasculature to the body, and separating the segment from the intestine by double ties at each end. At the distal end a drainage cannula was inserted into the lumen through a stab wound and tied in place with a purse string suture. Luminal drainage was by gravity. Two strain gauges were sewed to the serosa in order to record circular muscle contraction, one 2-3 cm from the proximal and the other 2-3 cm from the distal end. Two pairs of 1cm long silver stimulating electrodes were inserted subserosally, oriented in the circumferential axis. Each pair was 0.5-1 cm distal to the strain gauges in order to initiate ascending excitation. The exposed ileal segment was wrapped in paraffin soaked gauze and warmed with a lamp. The animal was warmed with a heater in the operating table.

Close intra-arterial perfusion: The techniques for perfusing the gastric corpus, antrum, pylorus and duodenum have been previously described \(25-30, 2\). Briefly, in animals anesthetized as described above, an artery close to the organ was selected, cannulated with a small cannula containing heparinized physiological solution. The area perfused was delineated with a perfusion of physiological solution, a strain gauge was
attached to record circular muscle contractions and electrodes pairs inserted 0.5-1cm below in circumferential orientation.

**Protocols:** In all cases, a recovery period of 30 minutes was followed by perfusion of increasing concentrations of acetylcholine, until a maximum response was attained. Other contractions were evaluated in terms of the percentage maximal response to acetylcholine. Acetylcholine was also infused intermittently and after completion of the experiment to ascertain that contractility remained unimpaired. If the response to a maximal dose was decreased to <80%, the experiment was discarded. After a further rest period of 10-15 min, the tissues were stimulated with electrical field stimulation (EFS) of the electrodes distal to the strain gauges (3 pps, 40V/cm, 0.5ms duration). In isolated segments, EFS was continued for 6 minutes, with a change in the polarity of the stimulating electrodes after 3 min. The two sets of electrodes (below the proximal and distal strain gauges) were each stimulated alternately for 6 min. throughout the experiment and data from both electrode sites was combined to study the time of affects of GLP-1. EFS was carried out initially until stable responses were obtained. GLP-1 infusions in physiological solution, by way of a T-tube in the intra-arterial cannula, were found in preliminary experiments to have a delayed onset of action, partly due to the dead space in the perfusion tubing but mostly due to the inherent action of the agent. In experiments with close intra-arterial infusion, actions of GLP-1 were studied on responses to EFS as described above or on responses to repetitive infusions of a sub-maximal dose of acetylcholine (34). The physiological solution contained (in mM): NaCl 115.5; NaHCO₃ 21.9; KCl 4.6; MgSO₄.7H₂O 1.16; NaH₂PO₄.H₂O; CaCl₂ 2.5; glucose 11.1. It was maintained at 37°C and bubbled with 95%O₂-5%CO₂.

**Data Analysis:** Records of the second minute of each recording at a given polarity of stimulation were analyzed (during the initial minute, contractile activities were unstable). Tonic tension data were analyzed as area under the contraction curve (AUC) as previously described (13). Phasic tension increments were analyzed separately from tonic increments by summing each contraction during the second minute and
averaging the value. To obtain data on the duration of action of GLP-1, response from both proximal and distal electrodes and at both polarities of stimulation were combined. L-NOARG infusion increased tonic and phasic activity. The effect of L-NOARG on responses to GLP-1 was studied by evaluating the percentage decrease in the increment of activity caused by electrical field stimulation after L-NOARG to the percentage decrease in the increment before L-NOARG.

**Statistical Analysis:** Data were usually analyzed by paired comparisons with Dunnett’s correction when multiple comparisons were made (Prism 3 Software). When the effects of L-NOARG on responses to GLP-1 were assessed, the comparison was between the percentages inhibition of responses to the same concentration of GLP-1 before L-NOARG compared to responses after L-NOARG.

**Drugs and Reagents:** GLP-1 and exendin (9-39) amide were obtained from BACHEM Bioscience Inc. Philadelphia, U.S.A. C capsaicin and the capsaicin receptor antibody were from Calbiochem-Novabiochem Corporation, San Diego, California, USA. Other drugs and chemicals were from Sigma (St. Louis, MO, USA).
Results:

In vivo GLP-1 caused marked inhibition of gastric emptying in dogs fed a saline meal. This resulted from increased pyloric tone and inhibition of propulsive antral contractions (4). Therefore we initially evaluated the effects of intra-arterial infusion of GLP-1 (0.1 to 100ng) into the antral and pyloric regions of anesthetized dogs at rest and during stimulation of enteric and vagal nerves as previously described (26,2). There were no effects (data from 6 experiments, not shown). We also infused GLP-1 intravenously while activating enteric nerves, again without effect. These results shifted our focus to the ileum.

When GLP-1 (10^{-11} \text{ mol to } 10^{-9}\text{mol}) was infused intra-arterially into the ileum (n=6) during stimulation from serosal electrodes placed 0.5 to 1 cm distal to the strain gauge over the perfused site (to elicit the ascending excitatory reflex), there was persistent inhibition of the phasic activity elicited by EFS (Figure 1B at bottom). A similar GLP-1 infusion had no significant effect on responses to acetylcholine given intra-arterially every two minutes. These studies (n=4) also showed that, under resting conditions, GLP-1 perfusions were without significant contractile effect (Figure 1A at top). We observed that recovery of phasic activity in response to EFS after i.a. infusions of GLP-1 was delayed for long periods.

To understand the mechanisms involved, we used isolated arterially perfused ileal segments instrumented with two strain gauges, each with a distal pair of serosal electrodes, stimulated alternately as described in Methods. Infusion of GLP-1 inhibited responses to electrical field stimulation (EFS) of these electrodes applied alternately to the proximal and distal pair. Figure 2 shows an example of the concentration-dependent (10^{-8} \text{ M to } 3\times10^{-7}\text{M}) effects of GLP-1 infusions. Infusions were carried out for 8 minutes and studied at least for 6 minutes after infusions were stopped on responses to EFS. Maximum inhibition was obtained with 10^{-7} \text{ M GLP-1 (10^{-9}\text{M had no consistent effect). Figure 3 summarizes}}
experiments of this type, demonstrating that GLP-1 primarily inhibited phasic but not tonic responses to EFS.

In order to define the duration of action of GLP-1, experiments were carried out using a concentration of $10^{-7}$M for 8 minutes. EFS was carried out alternately at both proximal and distal strain gauges and the effects were assembled (Figures 4A and 4B). The inhibition of phasic activity extended for greater than 22 minutes (more than 15 minutes after perfusion was stopped), while there were no consistent effects on tonic contractile activity..

Exendin (9-39) amide ($3\times10^{-8}$M) infused for 14 minutes before and during infusion of either $3\times10^{-8}$ or $10^{-7}$M GLP-1 had no ability to interfere with the inhibitory action of GLP-1 on phasic activity in response to EFS (Figure 5). GLP-1 infused after exendin 9-39 had no effect on tone as usual (not shown). Exendin 9-39 itself had no effects on phasic responses to EFS (Figure 6) or tonic responses (not shown).

When TTX ($10^{-20}$Fg) was infused and responses to EFS abolished, persistent phasic and tonic activity ensued as previously described (12,34). GLP-1 had no effect on this activity in concentrations through $3\times10^{-7}$M (data not shown), indicating that it had no effect on myogenic activity, consistent with its lack of inhibitory effect on responses to intra-arterial acetylcholine. Perfusion of L-NOARG ($10^{-4}$M) also induced persistent tonic and phasic activity, but responses to excitatory stimulation of proximal enteric nerves were preserved, as previously described$^{12}$. GLP-1 had a reduced inhibitory effect on these responses, suggesting that NO release mediated its effects in part (Figure 7), and the normalized results were significantly less reduced than in the same tissues perfused with GLP-1 before L-NOARG ($p<0.03$).

Capsaicin infused at $10^{-6}$ or $3\times10^{-6}$M had no effects on responses to EFS, but at $10^{-5}$M it like GLP-1 caused significant and persistent inhibition of phasic, but not tonic responses to EFS (Figure 8). We attempted to achieve desensitization of responses to capsaicin, by subsequent infusions of capsaicin after an infusion of $10^{-5}$M, but we were unable to obtain persistent lack of effect to a subsequent infusion; i.e., when
the phasic responses recovered sufficiently to test if GLP-1 could inhibit it, capsaicin was also capable of reducing it. After capsaicin, GLP-1 still causes inhibition of the residual phasic responses to EFS (Figure 9), but the magnitude appeared to be less (non-significantly). There was no effect of GLP-1 on tone after capsaicin (not shown).
Discussion:

The main findings of this study are that GLP-1 acts locally in the ileal region of the gastrointestinal tract near its release sites to inhibit activity of the enteric nervous system initiated by local activation of the ascending excitatory peristaltic reflex. Under the conditions of our experiments, using an isolated-perfused ileal segment, no extrinsic neural or hormonal influences are involved. GLP-1 causes concentration-dependent persistent inhibition of reflex neural activity, abolished by TTX, reduced by L-NOARG and without any effect on responses to exogenous acetylcholine.

The most surprising and interesting finding was that these effects of GLP-1 were unaffected by prior infusion of exendin (9-39) amide, an antagonist of the actions of GLP-1 at the known receptor (18,39,10,52,15,7,72). This receptor mediates most of the known actions of GLP-1: e.g., actions to enhance pancreatic beta cell growth and release insulin (59,49,50,62,22,23,40,46), inhibition of gastric emptying, secretion and intestinal motility (44,37,38,66,67), central actions on the dorsal vagal nucleus and other CNS sites (58,65). In the current literature, there is only one reported action of GLP-1 which was exendin 9-39-insensitive; the activation of hepatic vagal afferent nerves by intra-portal infusion of GLP-1 or exendin 4 (54). Thus our data as well as that of Nishizawa et al., (54) suggest that a neural receptor class for the action of GLP-1 may exist. One caveat is that we did not demonstrate that our exendin 3-39 was bioactive. In anesthetized animals there is no readily available test of bioactivity. Thus, although three different batches from the supplier, Bachem, behaved the same in our experiments, it is possible that they were not bioactive. Another caveat is that we did not go to higher concentrations of exendin 3-39, because of the very high costs that would have resulted from perfusion before and during the exposure to GLP-1 in multiple experiments. However, we believe that our concentrations were sufficient, at least, to affect the actions of GLP-1 based on reports in the literature (e.g., 54).
Interestingly, the action of GLP-1, persistent inhibition of phasic activity, was mimicked by capsaicin in a concentration-dependent manner. Capsaicin may have reduced the inhibitory action of subsequent GLP-1, but it clearly did not abolish it and the change was not significant. Thus was probably because, under our conditions, we were unable to demonstrate persistent desensitization of responses to capsaicin; i.e., when the phasic responses to EFS recovered after capsaicin enough to test the inhibitory effects of GLP-1, the inhibitory response to capsaicin had also recovered. Thus the interpretation of these results must be restrained. One suggestive evidence was that some L cells containing GLP-1 were near sites of immunoreactivity to the capsaicin receptor, presumably on sensory nerves (unpublished observations).

The site of action of GLP-1 released from ileal L-cells is unclear because the peptide is rapidly degraded by a peptidase located in the intestine and on endothelial cells (16,17,43,6,9,1). Whether or not GLP-1 released at this site should be considered as having a hormone-like action under physiological conditions is unclear. It may function as a paracrine agent. Thus it is possible that a local site of action near the L cell, where GLP-1 would be present in higher concentrations before degradation, may participate in the physiological role of GLP-1 and may do so by activation of a unique GLP-1 receptor. Clearly further study is required to evaluate it importance. In this connection, the possibility that the action is on sensory nerves, since it appears that all GLP-1 actions on gastrointestinal motor function operate through neural connections and are not local to the sites of the response such as the stomach and small intestine, needs to be considered.
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References:


40. Hallbrink M, Holmqvist T, Olsson M, Ostenson CG, Efendic S, and Langel U. Different domains in the third intracellular loop of the GLP-1 receptor are responsible for Galpha(s) and Galpha(i)/Galpha(o) activation. *Biochim Biophys Acta* 1546:79-86, 2001.


Legends:

**Figure 1.** Top: representative tracing showing that intra-arterial GLP-1 $10^{-10}$mol had no consistent effect on the amplitude of responses to acetylcholine (Ach) 0.5Fg given every two minutes. Fl refers to a control flush of equal volume to the acetylcholine injection. (n=4)

Bottom: representative trace showing that intra-arterial GLP-1 $10^{-10}$mol nearly abolished the responses to electrical field stimulation (EFS at 40V/cm, 0.5ms, 3pps). Polarity was reversed to ensure that electrode polarization played no role. Fl refers to a control flush of equal volume to the GLP-1 injection. (n=5)

The vertical bar represents 100% of the maximal response to Ach in this tissue for the top trace and 25% of the maximal response to Ach in the tissue in the bottom trace.

**Figure 2.** Excerpts from a representative experiment showing the effect of increasing concentrations of GLP-1 on phasic and tonic activity from EFS in isolated ileal segments. GLP-1 was given for 8 minutes and the recordings were made 6 minutes later (14 minutes after the administration of GLP-1 was begun). Tracing marked with the baseline to show how tonic and phasic activities were determined. One minute intervals were marked as well. Arrows up indicate the onset of EFS. Arrows down indicate the reversal of polarity of stimulation and the double line indicates the end of EFS stimulation. The distance between the second and third tracings correspond to 100% of the maximal Ach response.

**Figure 3.** Top: Summary of the analysis of 4 phasic activities in experiments with increasing concentrations of GLP-1. All values were significantly different from controls and the changes at $10^{-7}$ and $3 \times 10^{-7}$ M from significantly different from that at $10^{-8}$M, but not from one another. We used $10^{-7}$M as the maximal concentration. (n=7)

Bottom: Summary of the same experiments showing that there were no significant changes in tonic activity from these concentrations of GLP-1.
**Figure 4A.** Figure showing the time course of the action of GLP-1 (10^-7M), perfused for 8 minutes. The data were assembled from recordings from the proximal and distal sites in the same segment. Note that the onset of the response was at about 7 minutes after the infusion and persisted until about 25 minutes from the onset of perfusion. (n=5-7)

**Figure 4B.** Figure with data from the same experiments as in Figure 4A showing that there was no consistent effect of GLP-1 on tonic activities.

**Figure 5.** Figure showing the effect of 3x10^{-8}M GLP-1 after and during infusion of exendin 9-39. Note that the inhibitory effects of GLP-1, at a submaximal concentration, were not reduced by exendin 9-39. As usual there were no changes in the tonic activity (not shown). (n=5)

**Figure 6.** Figure showing the lack of significant effect of 3x10^{-8}M exendin 9-39 on phasic activity in response to EFS. There was also no effect on the tonic response to EFS. (n=3)

**Figure 7.** Figure summarizing effects of GLP-1 on phasic and tonic activities in response to EFS after L-NOARG (10^{-4}M) infused for 10 minutes. L-NOARG produced ongoing phasic and tonic activity on which response to EFS were superimposed. The EFS-initiated activity and the activity to L-NOARG were not affected significantly after L-NOARG. (n=5)

**Figure 8A.** Figure showing effects of capsaicin on phasic activity in response to EFS. Note that inhibition was delayed until 19 min. after the start of infusion, but then persisted until about 37 min. after start of infusion. (n=6)

**Figure 8B.** Figure showing effects of capsaicin on tonic activity in response to EFS in the same experiments as in Figure 8A. Note that there was no significant inhibition,

**Figure 9.** Figure showing effects of 10^{-7}M GLP-1 after 10^{-5}M capsaicin. There appeared to be a reduced inhibition in response to GLP-1. However, we were unable to show that capsaicin produced persistent desensitization to its own action and were thus unsure of the significance of this result. (n=4)
Effects of Increasing GLP-1 Concentrations

All significant differences from Control p<0.01.

Effects of Increasing GLP-1 Concentrations

None significant differences from Control or one another.
Effect of GLP-1

From 7 through 22 min, sign. diff. from 100%. T₀ at onset of GLP-1 infusion.
Effect of GLP-1 on Tone

* Sign. diff. from 100%. T₀ at onset of GLP-1 infusion.
Effect of GLP-1 after EXENDIN

* Sign. diff. from 100%

GLP-1 ($3 \times 10^{-8}$ M) started after 14 min infusion of Exendin (0.3 or $1 \times 10^{-7}$ M). $T_0$ at onset of GLP-1 infusion.
Exendin Effects During EFS

Exendin infusion begun at $T_0$.
No significant change.

% CONTROL PHASIC ACTIVITY

TIME min

0 4 7 10 13
Effect of GLP-1 before L-NOARG

** p<0.01

Effect of GLP-1 after L-NOARG

p=0.01 diff. from 100%
Effect of GLP-1 before L-NOARG

No sign. diff. from 100%

% CONTROL TONIC ACTIVITY

TIME min

0 4 7 10 13 16 19

Effect of GLP-1 after L-NOARG

No sign. diff. from 100%

% CONTROL TONIC ACTIVITY

TIME min

0 4 7 10 13 16 19
Effect of Capsaicin (10^{-5}M)

Capsaicin infusion begun at T_0.

Means sign. diff., p=0.015

* p<0.05  ** p<0.01

% CONTROL PHASIC ACTIVITY

TIME min

0 4 7 10 13 16 19 22 25 28 31 34 37
Effect of Capsaicin (10^{-5} M)

Capsaicin infusion begun at T_0. No value sign. diff. from 100%.
Effect of GLP-1 after Capsaicin

% CONTROL PHASIC ACTIVITY

TIME min

* p<0.05  ** p<0.01 diff, form 100%