Glucagon-like peptide-1 modulates neurally evoked mucosal chloride secretion in guinea pig small intestine in vitro

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Baldassano S, Wang G, Mulè F, Wood JD. Glucagon-like peptide-1 modulates neurally evoked mucosal chloride secretion in guinea pig small intestine in vitro. Am J Physiol Gastrointest Liver Physiol 302: G352–G358, 2012. First published November 10, 2011; doi:10.1152/ajpgi.00333.2011.—Glucagon-like peptide-1 (GLP-1) acts at the G protein-coupled receptor, GLP-1R, to stimulate secretion of insulin and to inhibit secretion of glucagon and gastric acid. Involvement in mucosal secretory physiology has received negligible attention. We aimed to study involvement of GLP-1 in mucosal chloride secretion in the small intestine. Ussing chamber methods, in concert with transmural electrical field stimulation (EFS), were used to study actions on neurogenic chloride secretion. ELISA was used to study GLP-1R effects on neural release of acetylcholine (ACh). Intramural localization of GLP-1R was assessed with immunohistochemistry. Application of GLP-1 to serosal or mucosal sides of flat-sheet preparations in Ussing chambers did not change baseline short-circuit current (∼Isc), which served as a marker for chloride secretion. Transmural EFS evoked neurally mediated biphasic increases in ∼Isc that had an initial spike-like rising phase followed by a sustained plateau-like phase. Blockade of the EFS-evoked responses by tetrodotoxin indicated that the responses were neurally mediated. Application of GLP-1 reduced the EFS-evoked biphasic responses in a concentration-dependent manner. The GLP-1 receptor antagonist exendin-(9–39) suppressed this action of GLP-1. The GLP-1 inhibitory action on EFS-evoked responses persisted in the presence of nicotinic or vasoactive intestinal peptide receptor antagonists but not in the presence of a muscarinic receptor antagonist. GLP-1 significantly reduced EFS-evoked ACh release. In the submucosal plexus, GLP-1R immunoreactivity (IR) was expressed by choline acetyltransferase-IR neurons, neuropeptide Y-IR neurons, somatostatin-IR neurons, and vasoactive intestinal peptide-IR neurons. Our results suggest that GLP-1R is expressed in guinea pig submucosal neurons and that its activation leads to a decrease in neurally evoked chloride secretion by suppressing release of ACh at neuroepithelial junctions in the enteric neural networks that control secretomotor functions.

Gastrointestinal hormones; neurogenic chloride secretion; enteric nervous system

GLUCAGON-LIKE PEPTIDE-1 (GLP-1) is a gut-derived hormone released from L-type enteroendocrine cells after a meal (28). It attracts interest because of its incretin effect, which results in enhanced insulin secretion even before blood sugar levels become elevated (4, 26). This suggests that it might be an efficacious therapeutic agent in the treatment of diabetes mellitus (4, 28, 47). Moreover, GLP-1 has significant gastrointestinal regulatory functions (26). It suppresses motility in general (1, 32, 39, 44, 48) and retards gastric emptying (3, 32, 48) by action in the antral pump (43) and gastric reservoir (2, 16).

GLP-1 inhibits gastric and pancreatic secretory functions in humans (50) and inhibits gastric acid secretion evoked by either pentagastrin or sham feeding (41, 45, 51). GLP-1 effectively inhibits vagally evoked acid secretion but is ineffective for hormonal stimulation (45, 51). Inhibition of acid secretion by GLP-1 is lost after vagotomy, which would be consistent with suppression acetylcholine (ACh) release from vagal eff erent innervation of gastric parietal glands.

GLP-1 acts at a specific transmembrane G protein receptor, the GLP-1 receptor (GLP-1R). Messenger RNA for GLP-1R is expressed in nodose ganglia and in the gastrointestinal tract (8, 9, 27, 43). It is expressed by nitricergic neurons in the myenteric plexus of mouse intestine (1). Nevertheless, detailed evaluation of GLP-1R expression in the enteric nervous system (ENS) is lacking. We aimed to identify the classes of neurons in the submucosal plexus of guinea pig small intestine that express GLP-1R.

Interest in the biological actions of GLP-1 has focused on pancreatic endocrine and gastric secretion with little attention given to a possible role for GLP-1 in neurogenic control of the intestinal secretory glands. Our second aim was to study actions of GLP-1 on neurally evoked mucosal secretion of NaCl and H2O in guinea pig small intestine.

Neurogenic control of movement of NaCl, H2O and HCO3 into the intestinal lumen is basic for maintenance of physiological osmolarity and pH and for understanding mechanisms involved in diarrhea and constipation. The ENS, in concert with the sympathetic division of the autonomic nervous system, is responsible for neurogenic moment-to-moment control of H2O and electrolyte transport across the mucosal epithelium (15, 52, 54). Secretomotor neurons in the submucosal plexus, which release ACh and vasoactive intestinal polypeptide (VIP) at the neuroepithelial junctions, are the final motor pathways from the ENS for control of secretion (14, 33, 42).

We investigated a putative role for GLP-1 in neurogenic secretion. GLP-1 action on neurally evoked chloride secretion, immunohistochemical localization of the GLP-1R in the submucosal plexus, and its influence on stimulus-evoked release of ACh from secretomotor neurons were studied.

MATERIALS AND METHODS

Methods used in the present study are unchanged from those we reported earlier for a closely similar study of GLP-2 (5). Consequently, the presentation of methods summarizes and quotes from the earlier article (5).

Tissue preparation. Male albino Hartley guinea pigs (300–400 g) were killed instantly by stunning, followed by exsanguination from the cervical vessels according to procedures reviewed and approved by the Ohio State University Laboratory Animal Care and Use Committee and United State Department of Agriculture inspectors. Ileal segments were removed and maintained in ice-cold Krebs solu-
tion containing (in mM) 120.9 NaCl, 5.9 KCl, 1.2 MgCl₂, 1.2 NaH₂PO₄, 14.4 NaHCO₃, 2.5 CaCl₂, and 11.5 glucose.

**Immunohistochemistry.** Immunohistochemical methods were standardized and essentially the same as reported in detail for our earlier study of GLP-2 (5). Table 1 lists sources for primary and secondary antibodies and the optimal dilutions used. Whole mounts of the submucosal plexus were placed in PBS containing 10% normal donkey serum and 0.3% Triton X-100 for 30 min at room temperature to minimize nonspecific binding and increase tissue permeability and were then incubated overnight at 4°C in primary antibodies for GLP-1R or a mixture of primary antibodies for double labeling. Competition with the control peptide (catalog no. LS-P1205; Lifespan Biosciences) established antibody specificity for GLP-1R. The GLP-1R antibody was preabsorbed with the control peptide for 2 h and then used for immunohistochemical localization of the receptor. After being washed with PBS, the tissues were incubated with a single secondary antibody or a mixture of appropriate secondary antibodies conjugated with fluorescein isothiocyanate (FITC) or indocarbocyanin (Cy3) at room temperature for 1 h, followed by washing in PBS and mounting with Vectashield (Vector, Burlingame, CA). Fluorescence labeling was examined with a Nikon Eclipse 90i fluorescence microscope (Nikon Instruments, Melville, NY). Photomicrographs were acquired with a CoolSnap HQ2 monochrome digital camera and MetaMorph software (Molecular Devices, Sunnyvale, CA), stored on disk, and analyzed with MetaMorph. Images were minimally adjusted for brightness and contrast with MetaMorph. Immunoreactivity (IR) for the GLP-1R and enteric neurochemical codes and numbers of double-labeled cells were assessed in randomly chosen ganglia situated throughout the preparations.

**Ussing chamber methods.** The muscularis externa, including the myenteric plexus, was removed by microdissection to obtain flat-sheet preparations for the Ussing chamber studies. The flat-sheet preparations of submucosa/mucosa and submucosal plexus were mounted between halves of Ussing flux chambers, which had a total cross-sectional area of 0.64 cm². The tissues were bathed on both sides, with the submucosa/mucosa and submucosal plexus being mounted at the intersection between the two halves of the Ussing chamber was used to fire all submucosal neurons, including secretomotor neurons (13). Grass SD 88 stimulators (Grass Instruments, Quincy, MA) supplied current to the electrodes. The stimulus parameters were stimulus strength, 1–5 mA; frequency, 10 Hz; pulse duration, 0.5 ms; and total stimulation duration, 90 s. Changes in Iₑ during EFS were quantified as the difference between the peak response and baseline Iₑ before stimulation. Blockade of the EFS-evoked responses by tetrodotoxin (1 μM) confirmed earlier findings and was evidence that the responses were neurally mediated (13, 22, 30, 31).

**Experimental protocol.** The preparations equilibrated for 30 min at 37°C before application of EFS. Stimulation was then begun at 5-min intervals and continued until reproducible responses were recorded. Stimulation was continued to obtain control data and was then repeated in the presence of progressively increasing concentrations of GLP-1 (0.1 nM–1 μM). Incubation time for each concentration was 5 min. Pharmacological analysis consisted of application of GLP-1 in the presence of one of four different receptor antagonists: 1) the GLP-1 receptor antagonist exendin-(9–39) (10 nM); 2) the muscarinic receptor antagonist scopolamine (1 μM); 3) the nicotinic receptor antagonist hexamethonium (100 μM), or 4) a selective VIP type I antagonist, VPAC (1 μM). Each agent was placed into the chamber 30 min before addition of GLP-1.

**ACh release.** Methods for measurement of EFS-evoked release of ACh in Ussing chambers were similar to those of Yau et al. (55) and Javed and Cooke (33), with the exception that these workers measured release of [³H]ACh, whereas we determined ACh concentrations using ELISA. Flat-sheet preparations were placed in Ussing chambers with each half-chamber filled with agar to reduce the volume to 300 μl. Electrode and fluid ports were blocked with agar. The preparations were incubated with GLP-1 or GLP-1 followed by exendin-(9–39) applied in combination for 30 min. Transmural EFS was applied in each protocol. Contents of the chambers were collected immediately at the end of stimulation and stored at −20°C for subsequent analysis.

ACh concentration in each sample was determined by fluorescence immunoassay (Amplex red acetylcholine/aceteylcholinesterase assay kit, catalog no. A12217; Invitrogen, Carlsbad, CA) and a fluorescence microplate reader (SpectraMax M2; Molecular Devices). Fluorescence was measured with excitation at 560 nm and detection at 590 ± 10 nm. ACh release was expressed as micrograms per square centimeter of tissue.

**Chemicals.** The following reagents were used. Carbachol, scopolamine, and hexamethonium were purchased from Sigma (St Louis, MO). GLP-1 and exendin-(9–39) were obtained from Tocris (Ellisville, MO). The VPAC₁ receptor antagonist [Ac-His₁, β-Phε, Lys₁₅, Arg₁₆, Leu₂₇]-VIP(3–7)-GRF(8–27) was obtained from Phoenix Pharmaceuticals (Burlingame, CA).

### Table 1. Codes and sources of primary and secondary antibodies used in the study

<table>
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<tr>
<th>Antigen</th>
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<th>Source</th>
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<td>Molecular Probes</td>
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Anti-Hu, anti-human neuronal protein; GLP-1R, glucagon-like peptide-1 receptor; ChAT, choline acetyltransferase; NPY, neuropeptide Y; VIP, vasoactive inhibitory peptide.
Data analysis and statistics. Preparations from the same animal were studied at the same time in four Ussing chamber setups. Data are means ± SE; n values refer to the number of animals. Statistical significance was determined with paired Student’s t-test between control and experimental populations or ANOVA followed by Bonferroni post hoc test, when appropriate. Differences were considered significant at \( P < 0.05 \).

Labeled neurons in 30 submucosal ganglia were counted in the immunohistochemical studies. Total number of neurons, labeled with a specific neuronal marker, and the percent overlap of those markers with GLP-1R were determined.

RESULTS

Baseline \( I_{sc} \) was 150 ± 1.9 µA/cm² (n = 18), and the corresponding conductance was 37.2 ± 1.2 mS/cm². Application of GLP-1 (0.1 nM–1 nM) to the serosal side of the preparations evoked no change in the baseline \( I_{sc} \) (137.2 ± 2.5 µA/cm², n = 9, \( P > 0.05 \)) and did not alter the total tissue conductance (34.7 ± 2.2 mS/cm², n = 9, \( P > 0.05 \)).

Exposure to the muscarinic receptor agonist carbachol (10 µM) evoked a maximal increase in \( I_{sc} \) of 52.2 ± 5.0 µA/cm² (n = 3) within 3 min. Responses to carbachol were unaffected by application of 10 nM GLP-1 (50.9 ± 6.9, n = 3, \( P > 0.05 \)).

Transmural EFS. Transmural EFS evoked biphasic increases in \( I_{sc} \), which consisted of a spike-like rapid rise (phase 1) followed by a second, sustained phase 2 plateau-like response (Fig. 1A). No significant “rundown” of the stimulus-evoked responses appeared during 30 min following equilibration of the responses (Fig. 1B). GLP-1 (0.1 nM–1 µM), placed in the serosal side of the chamber, evoked concentration-dependent reductions in both phase 1 and phase 2 of the EFS-evoked responses (Figs. 1 and 2). This action of GLP-1 was suppressed following placement of the GLP-1R antagonist exendin-(9–39) (10 nM) in the chambers (Fig. 2).

Exposure to 1 µM scopolamine, a muscarinic receptor antagonist, alone abolished phase 1 and significantly reduced...
phase 2 of the EFS-evoked responses. In the presence of scopolamine, GLP-1 (0.1 nM–1 μM) failed to modify further the EFS-evoked secretory responses (Fig. 3A).

Application of the nicotinic receptor antagonist hexamethonium (100 μM) in the bathing medium on the serosal side of the preparation reduced both phase 1 and phase 2 of the EFS-evoked responses. In the presence of hexamethonium, GLP-1, in a concentration range from 0.1 nM to 1 μM, continued to inhibit the first and second phases of the EFS-evoked secretory responses (Fig. 3B).

The presence of the VIP receptor antagonist VPAC1 (1 μM) in the bathing medium on the serosal side of the preparation did not modify phase 1 but reduced significantly phase 2 of the EFS-evoked responses. In the presence of VPAC1, GLP-1 (0.1 nM–1 μM) continued to suppress both the first and second phases of the secretory responses (Fig. 3C). Suppression of phase 2 of EFS-evoked \( I_{sc} \) by VIP receptor antagonists is accepted as evidence that phase 2 is mediated in part by release of ACh from secretomotor neurons (14, 42). We confirmed that incubation with VPAC1, before placement of VIP in the Ussing chambers, suppressed VIP-evoked increases in \( I_{sc} \) (data not shown).

**ACh release.** Because functional data suggest that GLP-1 acts to suppress release of ACh from secretomotor neurons at their junctions with the epithelium, we measured the amount of ACh released from the submucosal/mucosal preparations evoked by EFS in the absence or presence of GLP-1 to test the hypothesis further. GLP-1 (10 nM) significantly reduced the amount of EFS-evoked ACh release (Fig. 3D). Pretreatment with 10 nM exendin-(9–39) reversed the action of GLP-1 to suppress stimulus-evoked release of ACh (Fig. 3D).

**Immunofluorescence.** IR for the GLP-1R in the submucosal plexus was localized to the cytoplast of the neurons (Fig. 4). GLP-1R-IR was colocalized with IR for the protein, Hu, in over 50% of the neurons (Fig. 4, A1–A3). The nuclear protein Hu is part of a family of conserved RNA-binding proteins that includes HuC, HuD, and HuR (49). It is a useful immunohistochmical tool because it labels all ENS neurons (36). No IR fluorescence was seen when the GLP-1R antibody was preadsorbed with its control peptide (not shown).

Application of double-labeling immunofluorescence methods showed that GLP-1R colocalized with 66.91 ± 4.3% \((n = 4)\) of neurons that expressed choline acetyltransferase-IR (ChAT-IR) (Fig. 4, B1–B3) and with 60.60 ± 6.2% \((n = 3)\) of neuropeptide Y-IR (NPY-IR) neurons (Fig. 4, C1–C3). GLP-1R-IR was coexpressed with somatostatin-IR in 54.8 ± 6.6% \((n = 4)\) of the neurons (Fig. 4, D1–D3). GLP-1R-IR colocalized VIP-IR in 42.0 ± 7.8% \((n = 3)\) of the neurons (Fig. 4, E1–E3).

**DISCUSSION**

GLP-1 influences gastric, insulin, and glucagon secretion (29, 40, 41, 45, 50, 51). Our results suggest, for the first time, that GLP-1 might also be involved in intrinsic neuroendocrine signaling that regulates mucosal secretion of electrolytes, H₂O, and mucus and, therefore, luminal liquidity, pH, and protection in the small intestine.

**Transmucosal EFS.** Finding of no effect of GLP-1 on baseline \( I_{sc} \) and conductance indicates a lack of direct action on epithelial ion transport, per se. Instead, GLP-1 suppression of secretion and evoked release of acetylcholine (ACh).

**3.** Pharmacological analysis of GLP-1 action on phases 1 and 2 of short-circuit current \( (I_{sc}) \) responses to electrical field stimulation and evoked release of acetylcholine (ACh). A: the muscarinic receptor antagonist scopolamine suppressed phase 1 to a greater extent than phase 2. GLP-1 suppressed phase 1 to a greater extent than phase 2. No suppression of phase 2 by GLP-1 occurred in the presence of scopolamine. B: the nicotinic receptor antagonist hexamethonium \((C_6)\) suppressed both phase 1 and phase 2. Application of GLP-1 in the presence of \( C_6 \) decreased further the amplitude of phase 1 and phase 2 responses. C: the vasoactive intestinal peptide (VIP) receptor antagonist VPAC1 suppressed phase 2 but not phase 1 responses. Suppression by GLP-1 was unaffected by the presence of VPAC1. D: the GLP-1 receptor antagonist exendin-(9–39) suppressed inhibition of stimulus-evoked ACh by GLP-1. Data are means ± SE for preparations from 6 animals. Concentrations: GLP-1, 10 nM; scopolamine, 1 μM; \( C_6 \), 100 μM; VPAC1, 1 μM; exendin-(9–39), 10 nM. * \( P < 0.05 \) for GLP-1 relative to control. # \( P < 0.05 \) for GLP-1 relative to \( C_6 \) or VPAC1. § \( P < 0.05 \) for GLP-1 relative to exendin-(9–39).
The EFS evoked biphasic increases in $I_{sc}$ in the current study were typical of earlier reports (13, 20). They mimicked observations in a variety of other species, including mice (11), rabbits (30), and humans (31). The biphasic responses in guinea pig flat-sheet preparations reflect release of multiple neurotransmitters from secretomotor neurons and release of neurotransmitters from interneurons that supply excitatory synaptic input to the secretomotor neurons. ACh and VIP are the main neurotransmitters released by the secretomotor neurons. The action of ACh is at epithelial muscarinic M3 receptors and signal transduction involves elevation of cytosolic Ca$^{2+}$ in the enterocytes and activation of protein kinase C (12, 17, 18, 38). Signal transduction for VIP differs from ACh. It involves stimulation of adenylyl cyclase, elevation of cAMP, and stimulation of PKA in the enterocytes (19, 46). Phase 1 of the EFS-evoked response in the guinea pig is mediated mainly, if not solely, by the release of ACh. Phase 2 has a peptidergic component that includes VIP as a major contributor (14, 15).

Our protocol was to evoke the biphasic increases in $I_{sc}$, add GLP-1 in the serosal compartment of the chamber, and reapply EFS. Results obtained with this protocol support a hypothesis that phase 1 of the EFS-evoked responses is mediated primarily by secretomotor release of ACh and that phase 2 includes a component of VIP release in parallel with ACh. Our data suggest that GLP-1 acts to suppress the cholinergic component in both phases 1 and 2 with marginal, if any, effect on VIP release. Consistent with this was the finding that GLP-1 did not further suppress phase 2 after blockade of the cholinergic component by scopolamine. Moreover, when the VIPergic component of phase 2 was suppressed by the selective VIP antagonist VPAC1, exposure to GLP-1 still reduced the phase 2 response. When considered in view of the results showing no effect of GLP-1 on $I_{sc}$ after neural blockade by tetrodotoxin, these results suggest that the action of GLP-1, to suppress EFS-evoked secretion, reflects inhibition of presynaptic/prejunctional release of ACh by ENS neurons.

EFS, when applied to mucosal preparations in Ussing chambers, fires most all of the cell bodies of all classes of ENS neurons in the preparations and therefore their axonal output either to synapses on cell bodies of neighboring neurons or at neuroeffector junctions (10). Blockade by hexamethonium (i.e., $C_6$) of nicotinic synaptic connections among neighboring neurons in the ENS microcircuitry partially suppressed both phases 1 and 2 of the EFS-evoked responses (see Fig. 3). The responses, remaining after nicotinic blockade, reflected direct stimulation of secretomotor neurons and release of ACh at the neuroepithelial junctions. Suppression of the residual responses by GLP-1 suggests inhibition of ACh release at the neuroepithelial junctions. Together, these results support the overall conclusion that GLP-1 acts to inhibit ACh release at synaptic connections between ENS neurons and at the junctions of secretomotor neurons with the epithelium.

**Immunofluorescence.** Our immunofluorescence results for localization of GLP-1R in the submucosal plexus are consistent with a conclusion that GLP-1R suppresses neurogenic secretion by inhibiting neuronal ACh release. Neuronal cell bodies, which expressed ChAT-IR, NPY-IR, somatostatin-IR, or VIP-IR, all expressed GLP-1R-IR. ChAT-IR and NPY-IR, in the same neuron, is a neurochemical code for the class of secretomotor neurons that do not send collaterals to innervate periglandular arterioles (25, 54). ChAT-IR neurons that contain...
calretinin are cholinergic secretomotor/vasodilator neurons (7, 25). VIP-IR is a marker for noncholinergic secretomotor/vasodilator neurons that receive sympathetic noradrenergic inhibitory input. Somatostatin-IR identifies interneurons in the ENS that supply inhibitory synaptic input to secretomotor neurons (23, 37). Expression of GLP-1R by cholinergic secretomotor/nonvasodilator neurons and cholinergic secretomotor/vasodilator neurons can account for suppression of the cholinergic component of EFS-evoked phases 1 and 2 of the secretory responses when GLP-1 was present in the tissue chamber.

Expression of GLP-1R by noncholinergic secretomotor/vasodilator neurons is inconsistent with our finding that GLP-1 had little or no effect on the VIPergic component of the stimulus-evoked phase 2 response. The discrepancy might be explained by the existence of VIPergic secretomotor neurons known to evoke mucosal HCO₃⁻ secretion (34). Expression of GLP-1R by the neurons that contained VIP might be a reflection of differences in parallel neural pathways, one for Cl⁻ secretion and the other for HCO₃⁻.

ACH release. Cholinergic neurons make up about 50% of the submucosal neurons in the guinea pig small intestine (6, 24). Finding that GLP-1R was expressed by cholinergic secretomotor neurons suggests a neuronal action for GLP-1. This and finding that suppression of EFS-evoked biphasic increases in Iₑ by GLP-1 or a muscarinic receptor antagonist lead us to suggest that the action of GLP-1 on neurogenic Cl⁻ secretion is suppression of neuronal release of ACh. Finding that GLP-1 suppressed stimulus-evoked ACh release and suppression of this action by a selective GLP-1R antagonist supports the suggestion.

Suppression of EFS-evoked ACh release could result from an inhibitory action of GLP-1 on the excitability of cholinergic secretomotor neurons, or it could result from binding at inhibitory presynaptic/prejunctional GLP-1Rs at axonal ACh release sites. Intracellular electrophysiological recording of GLP-1 action on electrical and synaptic behavior in submucosal neurons will be necessary to clarify the specific mechanism by which GLP-1 suppresses ACh release in the ENS.

Physiological significance of the action of GLP-1 in the small intestine is suggested to be a mechanism involved in minute-to-minute adjustment of the output of cholinergic secretomotor/vasodilator neurons. By adjusting secretomotor/vasodilator output, the negative feedback control networks in the ENS can regulate the osmolarity of the intraluminal milieu and thereby contribute to maintenance of an optimal environment for pancreatic and brush border digestive enzymes to work as digestion proceeds.

Conclusion. Cholinergic neurons, including secretomotor neurons, in the submucosal plexus of guinea pig small intestine express receptors for GLP-1. Stimulation of the GLP-1 receptors suppresses mucosal Cl⁻ secretion evoked by electrical stimulation of neurons in the submucosal plexus. Suppression of mucosal Cl⁻ secretion results from GLP-1 action to attenuate release of ACh from secretomotor neurons.

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

AUTHOR CONTRIBUTIONS
Author contributions: S.B., F.M., and J.D.W. conception and design of research; S.B., G.-D.W., and J.D.W. performed experiments; S.B., G.-D.W., and F.M. analyzed data; S.B., G.-D.W., F.M., and J.D.W. interpreted results of experiments; S.B., G.-D.W., and J.D.W. prepared figures; S.B. and J.D.W. drafted manuscript; S.B., G.-D.W., F.M., and J.D.W. edited and revised manuscript; S.B., G.-D.W., F.M., and J.D.W. approved final version of manuscript.

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