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Identification of Expression and Function of the Glucagon-like Peptide-1 Receptor in Gastrointestinal Smooth Muscle

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IDENTIFICATION OF EXPRESSION AND FUNCTION OF THE GLUCAGON-LIKE PEPTIDE 1 RECEPTOR IN GASTROINTESTINAL SMOOTH MUSCLE

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Table of Contents

Page

List of Figures

Page

Abstract

Identification of Expression and Function of the Glucagon-like Peptide-1 Receptor in Gastrointestinal Smooth Muscle.

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In response to ingestion of nutrients enteroendocrine L cells secrete a potent incretin hormone, glucagon-like peptide-1 (GLP-1) to enhance glucose-dependent release of insulin and regulate glucose metabolism. Therapies related to GLP-1 (e.g., exendin-4) are currently approved for type 2 diabetes. The GLP-1 receptor (GLP-1R) is expressed in cells of the gastrointestinal tract (enteric neurons, enterocytes) and elsewhere (pancreatic cells, heart, and vascular smooth muscle). In pancreatic cells, GLP-1R are coupled to stimulatory G protein, Gs, generation of cAMP and activation of cAMP-dependent protein kinase. Although a decrease in the motility of stomach and colon is commonly associated with diabetes, the expression and function of GLP-1R in gastrointestinal smooth muscle are not known. **Aim.** To test the hypothesis that GLP-1 regulates smooth muscle function by acting on GLP-1R expressed on smooth muscle. **Methods.** Intestine and colon were removed from mice, and smooth muscle cells were isolated by collagenase digestion, and cultured in DMEM-10. Expression of GLP-1R mRNA was measured by RT-PCR using specific primers and the expression of GLP-1R protein was measured by western blot using GLP-1R specific antibody (sc-66911 and 1:200 dilution). The effect of GLP-1 (7-36) amide on Gαs activation (by western blot using Gαs-GTP

specific antibody), cAMP formation (by ELISA), and PKA activity (by a kinase assay using [^{32}P]ATP) was examined in cultured smooth muscle cells. The effect of GLP-1 on basal activity and on acetylcholine (ACh)-induced contraction was measured in muscle strips and intact colon in organ bath experiments. **Results.** Amplification of GLP-1R mRNA was obtained in mRNA isolated from mucosa derived from colon and intestine and also in mRNA obtained from tissue preparation devoid of mucosa suggesting expression of GLP-1R mRNA in mucosal as well as non-mucosal cells (enteric neurons or muscle cells) of the colon. The specificity of GLP-1R primers was corroborated using mRNA from pancreatic □ cells line (MIN-6) shown to express GLP-1R in our previous studies. A similar pattern of GLP-1R protein expression was obtained with western blot. To identify the GLP-1R expression specifically in muscle cells devoid of other cells such as enteric neurons, expression was examined in cultured smooth muscle. Expression of GLP-1R mRNA was confirmed in these pure smooth muscle cell cultures of colon and intestine. The role of GLP-1R in the regulation of smooth muscle function was analyzed in organ bath studies. The addition of GLP-1 (1 nM-1 □M) caused dose-dependent relaxation of basal tone in colonic muscle strips and relaxation of acetylcholine (ACh, 1 □M)-induced phasic activity and tonic contractions in both muscle strips and intact colon. The effect of GLP-1 on ACh-induced contraction suggests a role of smooth muscle Gs-coupled GLP-1R in mediating relaxation. In smooth muscle cells, GLP-1 (7-36) amide caused activation of Gαs, increased cAMP levels, and stimulated PKA activity. **Conclusion**. Colonic smooth muscle cells express GLP-1R, and GLP-1 inhibits both basal and acetylcholine-induced contraction. The GLP1-R is coupled to the heterotrimeric G protein, Gαs.

CHAPTER 1: INTRODUCTION

Anatomy and Function of the Gastrointestinal Tract

The gastrointestinal (GI) tract is a tubular series of organs arranged in a linear fashion spanning from the oral cavity to the anus. The physiological processes of digestion begin when the body is presented with olfactory or visual stimuli signaling an impending meal. Food begins to be broken down as it is chewed and swallowed, from where the bolus travels down the esophagus and into the stomach. Once mixed with hydrochloric acid in the stomach, chyme is systematically deposited into the proximal small intestine (duodenum), followed by migration distally into the jejunum and then the ileum. Chyme then leaves the small intestine and travels through the cecum, ascending colon, transverse colon, descending colon, and sigmoid colon, culminating in the rectum before it has amassed sufficiently to be excreted from the anus as feces.

Figure 1: Diagram of the Gastrointestinal Tract

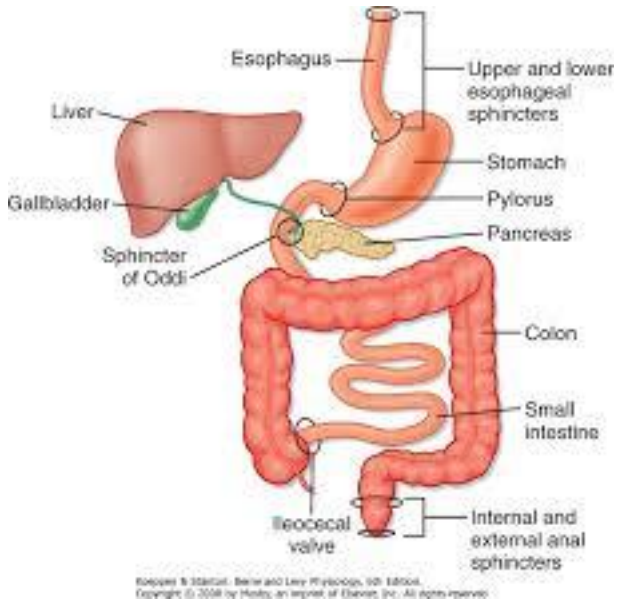


Figure 1. Diagram of the Gastrointestinal Tract. This figure provides a schematic of gastrointestinal anatomy. Food is swallowed and passes through the esophagus into the stomach, from where it is emptied into the small intestine. It passes into the colon and is then excreted. (Berne, 2008)

Description of the Mucosa and Circular and Longitudinal Muscle Layers

The GI tract is arranged into layers with distinct structures and functions. Adjacent to the luminal space is the mucosa layer, containing an innermost epithelium, a middle lamina propria comprised of connective tissue, and an outer muscularis mucosa, a thin layer of smooth muscle. Surrounding the mucosa is the submucosa, a layer of loose collagenous tissue containing vasculature, neurons, and lymphatic vessels. The submucosa is surrounded by the muscularis propria, comprised of two smooth muscle layers. The muscularis propria contains an inner circular layer and outer longitudinal layer that mediate peristalsis in conjunction. The adventitia layer is the most superficial,

composed of supporting tissues, blood vessels, nerves, and adipose tissue. Within the peritoneal cavity, a serosa is also present, lined with mesothelium composed of simple squamous cells.

Figure 2: Layers of the Gastrointestinal Tract

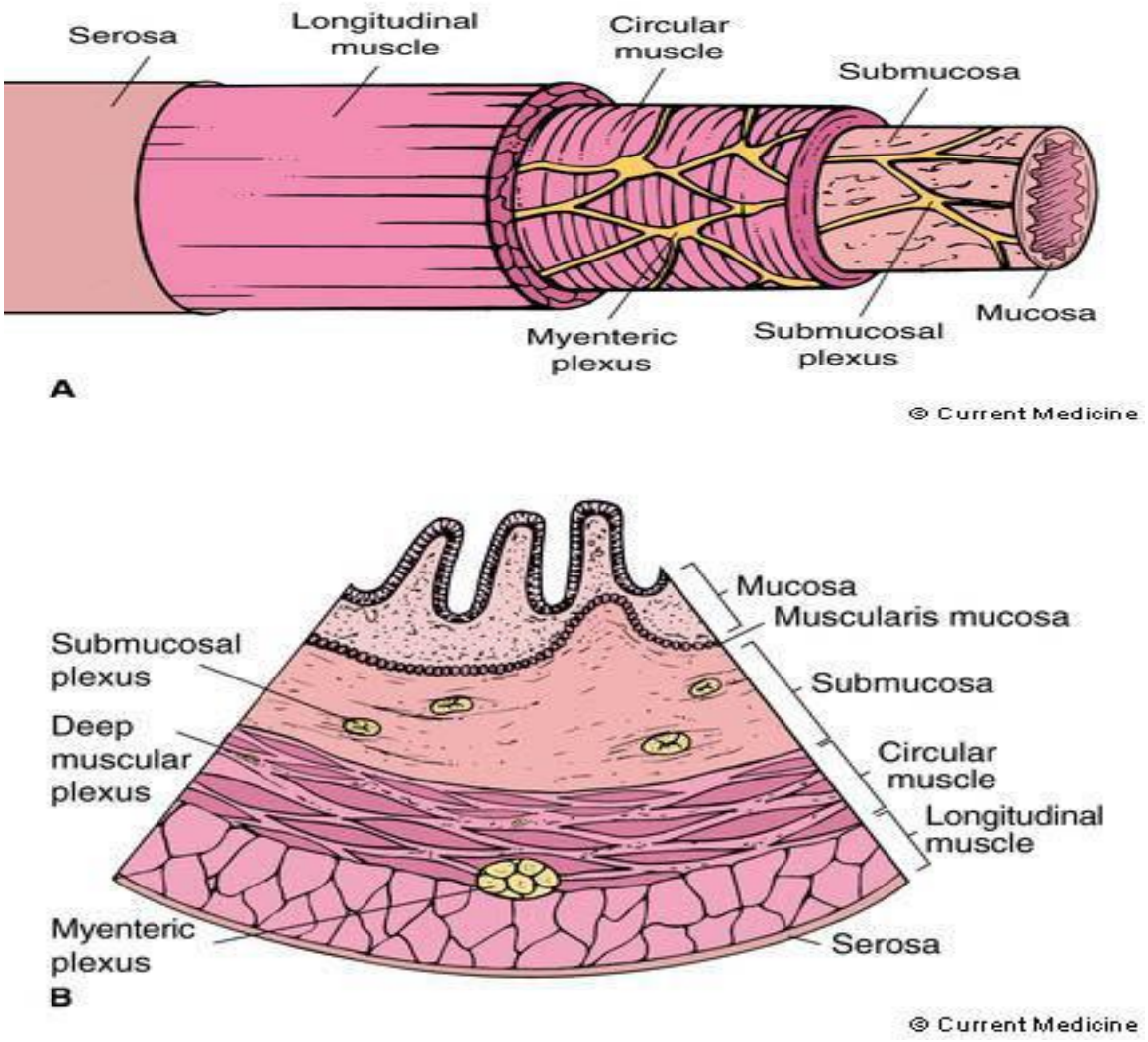


Figure 2. Layers of the Gastrointestinal Tract. Panel A represents a longitudinal view of the gastrointestinal tract with layers exposed. Panel B displays a schematic of a slice of the tract from a cross-sectional view. (Berne, 2008).

Hormonal Regulation of Motility and Food Intake

The gastrointestinal tract is the largest endocrine system in the human body with respect to quantity of cells. Enteroendocrine cells (EECs) lie throughout the GI mucosa in crypts and villi and represent approximately 1% of gut epithelial cells. There are differentiated subgroups of EECs that secrete distinct combinations of hormones. In addition, EECs can be classified by morphology and position in mucosa. Open type EECs possess a bottle-neck shape with an apical elongation, allowing microvilli to face the intestine. Closed type EECs are located to the basal membrane and do not have luminal projections or microvilli.

A cells are open-type EECs located in the stomach that secrete ghrelin. G cells are closed-type EECs in the duodenum and antrum of the stomach that secrete gastrin. D cells are open-type with long or short basal cytoplasmic processes and secrete somatostatin from the small intestine and gastric corpus and antrum regions of the stomach. L cells, which contain glucagon-like peptide-1 (GLP-1), GLP-2, and PYY, are open type and release their contents in response to ingested ingredients (Latorre, 2016).

Much is known about the specific hormones that mediate gastrointestinal motility, and their effects are well documented. Once released from A cells in the stomach, ghrelin increases food intake and feelings of hunger. Administration of ghrelin agonists has also been observed to increase gastric emptying in diabetic patients with gastroparesis. Peptide YY (PYY) is a gut hormone with structural similarities to neuropeptide Y (NPY) and reduces food intake in both humans and rodents once released from L cells. PYY is localized at its highest concentration along the distal aspects of the gastrointestinal tract but is also present in the proximal sections (Murphy, 2006). Cholecystokinin (CCK) is

secreted from I cells, a type of EEC (Latorre, 2016), in the small intestine and attenuates food intake, likely acting through CCK1 receptors localized to the vagus nerve. Pancreatic peptide (PP) is synthesized and secreted from the endocrine pancreas after meals, resulting in decreased food intake and delayed gastric emptying. Amylin is released simultaneously with insulin from pancreatic β-cells after meals, and reduces food intake when administered peripherally at supraphysiological doses.

GLP-2 in peripheral circulation increases GI motility, but does not have an apparent effect on appetite. Oxyntomodulin is released post-prandially as a product of preproglucagon and reduces food intake. (Murphy, 2006). 5-hydroxytryptamine/serotonin (5-HT) is released from a variety of cells, including I cells, K cells, L cells, and enterochromaffin (EC) cells, and is thought to stimulate motility through both neuronal and mucosal mechanisms. Somatostatin is produced by D cells and stomach and small intestine and inhibits gastric acid secretion (Latorre , 2016). Glucose-dependent insulinotropic polypeptide (GIP) is released from duodenal K cells after meals. GIP has not been observed to influence food intake acutely, but it has been shown that GIP-receptor knockout (KO) mice are resistant to obesity while subjected to high-fat diets. (Murphy, 2006) (Suzuki, 2017).

Figure 3: Schematic of a Typical Enteroendocrine Cell

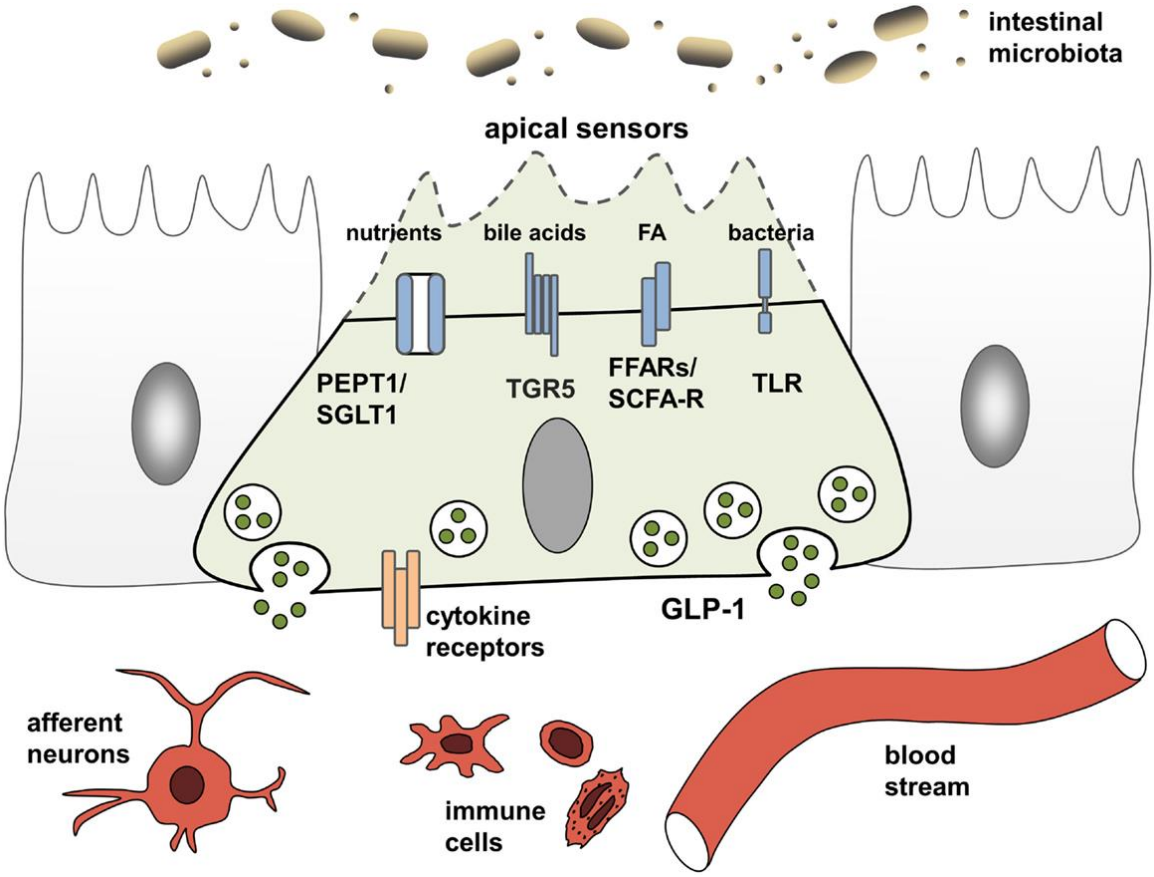


Figure 3. Schematic of a Typical Enteroendocrine Cell. This figure provides a conceptual schematic of an EEC. Cell polarity is indicated by the distinct apical and basolateral aspects, with the apex adjacent to the gut lumen and the basolateral side adjacent to highly vascularized and innervated layers. Nutrients are sensed at the apical membrane, intracellular signaling occurs, and hormones are exocytosed from the basolateral membrane (Zietek, 2016).

Description of Incretins

The term ‘incretin’ originated in the early 1900s when clinicians first isolated glucose-lowering intestinal factors from gut extracts. The name references the ability of incretins to increase the insulinemic peak following orally administered glucose, compared with intravenous glucose infusion. ‘The incretin effect’ refers to this disparity in insulinotropic capabilities.

Food intake is the physiological stimulus for incretin release from enteroendocrine cells, and the primary effect of the incretin family is reduction of blood glucose levels. This effect is mediated by the regulation of hormonal pancreatic secretions, leading to inhibition of gastric emptying and reduction of appetite and food intake. GIP was the first incretin identified, and the failure of its inactivation to remove insulinotropic effects suggested the presence of other peptides with similar actions. GLP-1 was identified years later after the preproglucagon gene was cloned and characterized on the long arm of human chromosome 2 (Cantini, 2016).

Biochemistry, Production, and Secretion of GLP-1

Food intake is the primary stimulus for the release of GLP-1 from L cells, localized mostly in the ileum of the small intestine. Plasma GLP-1 levels increase minutes after a meal before digested food reaches the L cells, suggesting that GLP-1 production is mediated by neural and endocrine stimulation instead of direct nutrient sensing by the intestinal walls. In fasted humans, plasma levels of active GLP-1 (7-36) amide tend to remain in the range of 5-10 pmol/liter. Plasma levels peak in the range of

15-50 pmol/liter after 30-120 minutes (Cantini, 2016).

Enteroendocrine L cells synthesize a large precursor protein, preproglucagon, which is processed to produce GLP-1 as well as other biologically active peptides. The most common form of GLP-1 in circulation is the GLP-1 (7-36) amide (Murphy, 2006). GLP-1 (7-36) amide is the biologically active metabolite in circulation that causes the increase of glucose-dependent insulin secretion after binding to GLP-1R (Barale 2017). GLP-1 (7-36) amide is rapidly degraded by the enzyme dipeptidyl peptidase (DPP)-4 into GLP-1 (9-36) amide, the primary GLP-1 metabolite without the ability to interact with GLP-1R, and has a short half-life (Barale 2017), (Cantini 2016). The half-life of intravenously injected GLP-1 in humans is only 2-3 minutes.

GLP-1 (7-36) amide is a peptide consisting of 30 amino acids after the alternative processing of preproglucagon. The preproglucagon gene is located in human chromosome 2q36-q37 and contains six exons and five introns. Exon 4 contains the entire coding sequence for GLP-1. In mammals, the preproglucagon gene is transcribed into an mRNA that yields a single 180-amino acid precursor. The post-translational processing undergone by preproglucagon is tissue-specific, with different prohormone convertase (PC) enzymes located in the pancreas, intestine, and brain.

The proglucagon protein contains active peptides other than GLP-1, including glucagon, GLP-2, oxyntomodulin, and other fragments with unclear roles. PC1/3 is expressed primarily in the central nervous system and GI tract, yielding the products GLP-1, GLP-2, and oxyntomodulin. Pancreatic α cells contain high levels of a different convertase isoform, PC2, which is responsible for significant glucagon production in the pancreas, in addition to modest glucaon production in the brain. Pancreatic α cells also

express low levels of PC1/3, implying modest production of GLP-1 from pancreatic islets.

The various isoforms of GLP-1 are produced by additional post-translational modification, specifically by cleavage of six amino acids from the N-terminus of GLP-1 (1-37). The products exhibit varying degrees of activity and include GLP-1 (7-37) and GLP-1 (7-36) in addition to the C-terminal-amidated forms, GLP-1 (7-37) amide and GLP-1 (7-36) amide. The amide isoforms require further post-translational modification by a peptide-amidating monooxygenase. All isoforms mentioned are able to recognize and exert equipotent effects on pancreatic GLP-1R (Cantini, 2016) (**Figure 4**).

Figure 4. Diagram of GLP-1 Processing: From DNA to the Amino Acid Sequence

(adapted from Cantini, 2016)

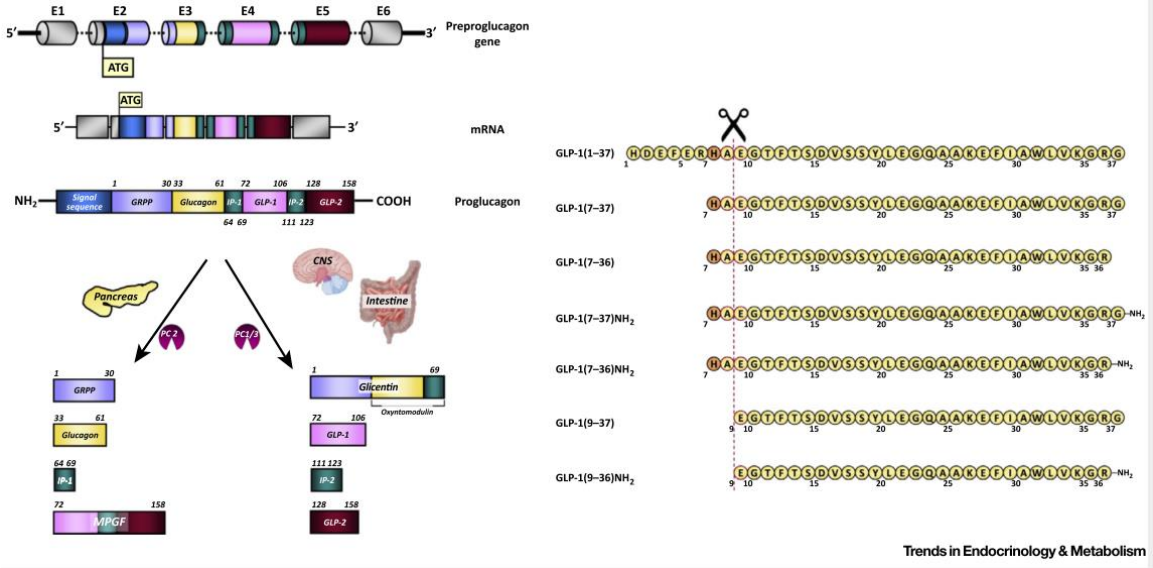


Figure 4. GLP-1 Processing. The preproglucagon gene contains six exons (E1, E2, E3, E4, E5, E6) and five introns. It is transcribed into mRNA and translated into the proglucagon precursor. The prohormone convertases, PC1/3 and PC2 perform tissue-specific post-translational processing to generate different active peptides, including GLP-1, GLP-2, glucagon, oxyntomodulin, glicentin, glicentin-related polypeptide, major glucagon fragment, intervening peptide-1, and intervening peptide-2. Further post-translational cleavage of six amino acids from the N-terminus of GLP-1 (1-37) produce the active forms, GLP-1 (7-37) and GLP-1 (7-36). The forms amidated at the C-terminus are produced by additional post-translational modifications from a peptide-amidating monooxygenase. Sites of cleavage by DPP-4 are noted by the dotted red line.

Characteristics of the Glucagon-like Peptide-1 Receptor

GLP-1R is a G-protein coupled receptor with seven transmembrane domains and 463 amino acids. It contains a large hydrophilic extracellular domain at the N-terminus, which also contains a 23 amino acid leader sequence essential for the biosynthesis of the receptor. There are six cysteine residues at the N-terminus which are essential for ligand binding, in conjunction with other cysteine residues in the extracellular loop between transmembrane regions 1 and 3. Distinct domains within the third intracellular loop at the C-terminus are essential for selective binding of specific G proteins. The receptor enters the endoplasmic reticulum (ER) during maturation, where it undergoes N-linked glycosylation in specific amino acid residues. This process is associated with proper expression of GLP-1R at the plasma membrane (Donnelly, 2012) (Cantini, 2016).

Figure 5: Schematic of Typical G-protein Coupled Receptor

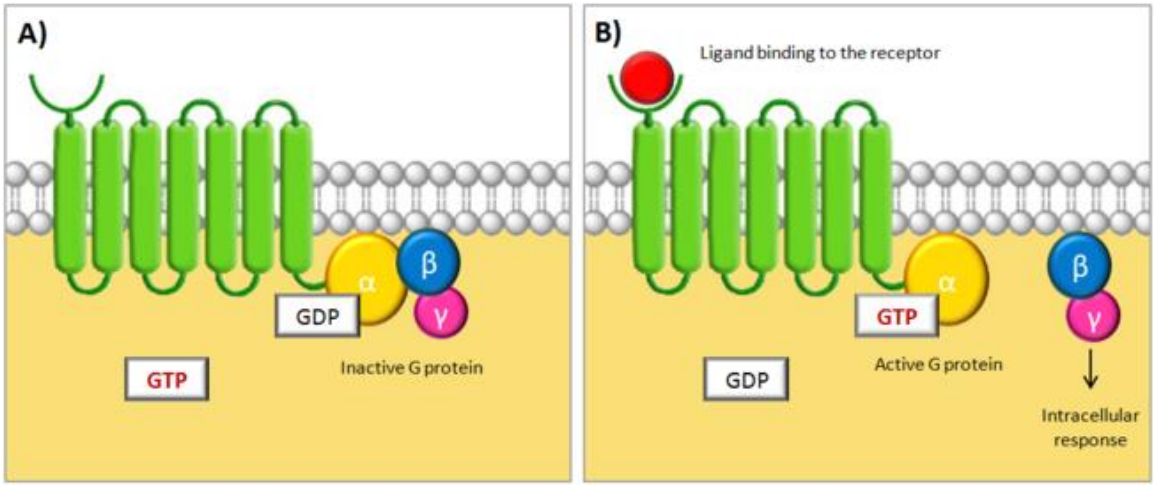


Figure 5: Schematic of a Typical G-protein Coupled Receptor. This figure provides a representation of a typical GPCR, such as GLP1-R, in both inactivated (A) and activated (B) states (Alberts, 2008). There is an extracellular N-terminus, 7 transmembrane domains, and an intrcellular C-terminus. The βγ complex dissociates from the Gα subunit after activation by ligand binding.

Known GLP-1R Expression

GLP-1R mRNA has been identified in the lung, pancreatic islets, stomach, kidney, hypothalamus, and heart. Studies failed to identify expression in adipose tissue, liver, or skeletal muscle. Expression of mRNA in these regions suggests the presence of translated GLP-1R protein. Interestingly, GLP-1R isoforms identified in the kidney and heart are suggested to be structural variants to the pancreatic classical receptor. In the pancreas, GLP-1R is expressed primarily in β cells and to a lesser degree in δ cells. The expression of the receptor in α cells is debated and unclear. Within the heart, GLP-1R is

expressed at the atrial level and sinoatrial myocytes, but not in the ventricular myocardium (Richards, 2014) (Kim, 2013).

Physiological Effects of GLP-1

After secretion, GLP-1 (7-36) amide binds to a specific GLP-1R and causes tissue-specific responses (Barale 2017). The effects of GLP-1 in pancreatic β cells are well documented, although the underlying mechanisms are not well understood. GLP-1 in pancreatic β cells has an insulinotropic effect, leading to instantaneous insulin secretion and lowered blood glucose levels. Some evidence suggests that GLP-1 induces insulin and zinc secretion from pancreatic β and δ cells, which would act on ATP-dependent potassium channels to block glucagon secretion from α cells. It is unclear if GLP-1 acts directly on α cells, since GLP-1R expression has not been confirmed there (Franklin, 2005) (Tuduri, 2016).

In cardiovascular tissue, activation of GLP-1R by GLP-1 is thought to induce cardioprotective effects via activation of anti-apoptotic mechanisms in cardiomyocytes (Noyan-Ashraf, 2009) (Richards, 2014). Liraglutide, a GLP-1R agonist, was shown in mice to improve cardiomyocyte survival after induced ischemia, and it also resulted in sustained improvement of cardiac function after myocardial infarction. These effects are thought to be due to modulation of the expression of genes with known cardioprotective effects, including Nrf2, peroxisome proliferator-activated receptor, and heme oxygenase 1, via activation of the second messengers Akt and glycogen synthase kinase. This would suggest a mechanism of cardiovascular GLP-1R distinct from pancreatic GLP-1R (Noyan-Ashraf, 2009).

In the human and mouse CNS, GLP-1R have been identified in the arcuate and

paraventricular nuclei in the hypothalamus, which are involved with regulation of appetite and satiety.

G Protein Signaling

G proteins are membrane-associated proteins that are usually classified as either 'small' or 'large/heterotrimeric.' They are coupled with membrane-bound proteins known as G-protein coupled receptors (GPCRs), and the various relationships between G proteins and GPCRs mediate various signal transduction pathways. This study will focus on heterotrimeric G proteins.

Heterotrimeric G proteins contain 3 subunits, classified as α, β, and γ. In the inactive conformation, the α subunit is bound to guanosine diphosphate (GDP) and forms a stable complex with the β and γ subunits. Upon GPCR activation by an agonist, the receptor couples with its respective G protein, and the GDP bound to the α subunit is exchanged for guanosine triphosphate (GTP). Now bound to GTP and activated, the Gα-GTP complex dissociates both from the GPCR and the Gβγ complex. The Gα-GTP or Gβγ complexes may then induce signal transduction by activating intracellular downstream effectors. The signal is ultimately terminated by the intrinsic GTPase activity of the α subunit, which hydrolyzes GTP to GDP and enables the re-association of the α, β, and γ subunits, yielding the inactive heterotrimer.

Specificity and selectivity of G protein signaling systems arise from the distinct heterotrimers formed by different isoforms of the subunits. In humans, there are 21 known isoforms encoded by 16 Gα subunit genes, 6 known Gβ subunits encoded by 5 genes, and 12 known Gγ isoforms, resulting in over 700 combinations of Gαβγ heterotrimers. These heterotrimers are further classified into 4 main classes: Gαs, Gαi/o,

Gαq/11, and Gα12/13, all with distinct downstream effects. (Ross and Gilman, 1980) (Moreira, 2014) (Yu, 2017) (Duc, 2017)

Rationale

Glucagon-like peptide-1 is an incretin peptide hormone secreted in response to nutrient ingestion. It increases insulin secretion, decreases glucagon secretion, and reduces gastrointestinal motility. The GLP1-R is coupled to Gαs proteins in pancreatic β cells and activates the adenylyl cyclase/cAMP/PKA pathway. However, the presence of the GLP1-R is gastrointestinal smooth muscle is not known, nor is the mechanism of action of GLP-1 in gastrointestinal smooth muscle. The aim of this study is to test the hypothesis that GLP-1 regulates smooth muscle function by acting on GLP-1R expressed on smooth muscle.

CHAPTER 2: MATERIALS AND METHODS

Reagents

Antibodies for GLP-1R, GLP-1R (D6) and GLP-1R (H-55) were obtained from Santa Cruz Biotechnologies, Inc. (Dallas, TX); [^{35}P]ATP was obtained from NEN Life Sciences (Boston, MA); NF449 (4,4',4",4"'-[Carbonylbis(imino-5,1,3-benzenetriyl-bis(carbonylimino))]tetrakis-1,3-benzenedisulfonic acid), GLP-1(7-37), and GLP-1(7-36) amide were obtained from Tocris Bioscience (Bristol, United Kingdom); (RNAqueous™ kit and TRIzol® Reagent were obtained from Ambion (Austin, TX). High Capacity cDNA Reverse Transcription Kit was obtained from Applied Biosystems (Foster City, CA); Q5® Reaction Buffer, Q5® High GC Enhancer, Deoxynucleotide (dNTP) Solution Mix, Q5® High-Fidelity DNA Polymerase were obtained from New England Biolabs (Ipswitch, MA); Western blotting materials, 4x Laemmli Sample Buffer, and DC™ Protein Assay Reagents were obtained from Bio-Rad Laboratories (Hercules, CA); SuperSignal® West Pico Chemiluminescent Substrate, Dulbecco's Modified Eagle Medium, and Trypsin-EDTA (0.25%) with phenol red were obtained from ThermoFisher Scientific (Waltham, MA); Collagenase CLS type II and soybean trypsin inhibitor were obtained from Worthington (Freehold, NJ); T-PER® Tissue Protein Extraction Reagent was obtained from Thermo Scientific (Rockford, IL); Monoclonal Anti-cAMP Antibody Based Direct cAMP ELISA Kit was obtained from NewEast Biosciences (Malvern, PA); All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Animals

Wild-type mice (C57BL/6) were used purchased from Jackson Laboratories (Sacramento, California). The mice were housed in an animal facility directed by the Division of Animal Resources at Virginia Commonwealth University. Mice were subjected to 12-hour light and 12-hour dark cycles with food and water provided. Mice were euthanized by asphyxiation by carbon dioxide (CO_2) under approval by the Institutional Animal Care and Use committee of Virginia Commonwealth University.

Preparation of Mouse Intestine and Colon

Mice were euthanized by CO_2 inhalation, and they were carefully observed to ensure cessation of life. Abdominal incisions were made vertically, and the small intestine and colon were removed and placed into beakers filled with a smooth muscle buffer (SMB) consisting of NaCl 120 mM, KCl 4 mM, KH_2PO_4 2.6 mM, CaCl2 2.0 mM, MgCl2 0.6 mM, HEPES (N-2-hydroxyethylpiperazine-N' 2-ethanesulfonic acid) 25 mM, glucose 14 mM, and Basic Eagle Medium (essential amino mixture) 2.1% (pH 7.4). Luminal contents of the intestinal and colonic tubes were flushed with a blunt syringe filled with SMB. The tubes were opened along the mesenteric border with scissors. The mucosal layer, containing EEC, was carefully separated from the smooth muscle layer, containing smooth muscle cells, ICC, and the myenteric plexus, using the rounded edge of forceps. For protein and RNA extraction, tissues were homogenized on ice in a phosphate-buffered saline (PBS) solution supplemented with protease inhibitors. For preparation of cell culture, smooth muscle layer tissues were placed in SMB. All tissues were used for experiments immediately.

Preparation of Dispersed Small Intestine and Colon Smooth Muscle Cells

Smooth muscle cells were isolated from colonic and small intestinal smooth muscle by sequential enzymatic digestion of muscle strips, filtration, and centrifugation. Tissues were chopped with scissors and incubated at 35°C for 20-30 minutes in SMB containing 0.1% collagenase (300 U/ml) and 0.01% soybean trypsin inhibitor (w/v). The SMB containing the tissue received 100% oxygen during the entire procedure. Partially digested tissues were washed twice with collagenase-free SMB and were then permitted to disperse spontaneously in collagenase-free DMEM. Smooth muscle cells were then harvested via filtration using 500 μm Nitex and centrifuged at 2000 rpm for 10 minutes. To wash the cells, the supernatant was aspirated, cells were resuspended in fresh DMEM, and the mixture was centrifuged again at 2000 rpm for 10 minutes. Cells were washed twice before being prepared for culture.

Preparation of Cultured Small Intestine and Colon Smooth Muscle Cells

Dispersed smooth muscle cells isolated from mouse small intestine and colon were resuspended in DMEM-10 containing penicillin (200 U/ml), streptomycin (200 μg/ml), gentamycin (100 μg/ml), amphotericin B (2.5 μg/ml) and 10% fetal bovine serum. Using proper sterile protocol, cells were plated at a concentration of approximately 5 X 10^5 cells/mL and stored at 37°C in an incubator with 10% CO_2. DMEM-10 was replaced every 1-3 days as needed until 80-90% confluence was reached. Primary culture smooth muscle cells were trypsinized (Trypsin-EDTA, 0.25%) and observed via microscopy for detachment. The mixture was collected via autopipette and DMEM-10 was added to cease trypsinization. The supernatant was aspirated, cells were

resuspended in DMEM-10, and the cells were either used immediately for experiments or replated at a 2:1 dilution to continue culture under the same conditions as previously described. Experiments were performed on cells in the first and second passage.

Protein and mRNA Extraction from Smooth Muscle Cells

To obtain protein, cells or tissue were isolated and mixed into T-PER containing a phosphatase inhibitor cocktail (cantharidin, bromotetramisole, and microcytin-LR) (1:200 dilution) and a protease inhibitor cocktail (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, leupeptin, bestatin, pepstatin, and E-64) (1:200). 4 mL of T-PER were used per gram of tissue, and the mixture was allowed to incubate at room temperature for 15 minutes. Tissues or cells were homogenized and then allowed to incubate for 15 minutes at room temperature. The mixture was homogenized a second time and then separated by centrifugation for 20 minutes at 12,000 rpm for 20 minutes. The supernatant containing extracted protein was then transferred to a new tube, and protein concentration was determined via Bio-Rad DC Protein Assay Kit against a bovine serum albumin standard curve.

To obtain mRNA from tissue, 0.5 mL of TRIzol was added to 50-100 mg of tissue. The mixture was permitted to incubate at room temperature for 5 minutes, and 0.2 mL chloroform was added. After violent mixing, the solution was incubated at room temperature for 2-3 minutes and then centrifuged at 12000 g at 4°C for 15 minutes. The upper aqueous phase was transferred to a new tube, and the 0.2 mL of chloroform was added. The mixture was incubated and centrifuged a second time as previously described. Next, 0.5 mL of 2-propanol was added, the solution was mixed, and the mixture was

incubated at room temperature for 10 minutes. The tube was centrifuged at 12000 g for 15 minutes at 4°C. The supernatant was discarded and 1 mL of cold ethanol (75%) was used to resuspend the pellet. The mixture was again centrifuged at 12000 g for 15 minutes at 4°C. The supernatant was aspirated with care taken to leave the pellet intact, which was allowed to air dry at room temperature for 15 minutes. Total RNA was then dissolved into 0.1% diethylpyrocarbonate (DEPC)-treated water, and concentration was determined via spectrophotometry.

RNA was obtained from cultured smooth muscle cells using guanidinium-based lysis and glass fiber filter separation with the RNAqueous Total RNA isolation kit as described by the included protocol.

RT-PCR Analysis of Mouse Colon and Small Intestine Mucosa and Smooth Muscle

Specific primers for GLP-1R were designed to span across exons of sequences homologous in mice and humans. The primer sequences for mouse GLP-1R were as follows: Forward 5'-CCC GCC CTC AGG GTA CCA CGG T-3' and Reverse 5'-TCA GGA AAG TTT CTC TCC CCT C-3', yielding a fragment of 346 bp (obtained from Invitrogen, Carlsbad, CA). Sequences for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene with known expression in smooth muscle cells (Perrotta, 2017), primers were as follows: Forward 5'-AGA AAC CTG CCA AGT ATG ATG-3' and Reverse 5'-GGA GTT GCT GTT GAA GTC G-3'. The High Capacity cDNA Reverse Transcription Kit was used to synthesize cDNA. The reaction mixture was prepared as described by the given protocol using 1 μg of RNA. Negative controls were created by substituting the reverse transcriptase enzyme with an equal volume of

DNase/RNase-free water. An Eppendorf Vapo Protect Mastercycler was used for thermal cycling with the following conditions: 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C, and a hold at 4°C as needed.

PCR products were generated using primers specific for GLP-1R and GAPDH as previously described. The PCR reaction mixture was prepared as follows: 5 μL of 5x Q5 Reaction buffer, 0.5 μL of 10 mM dNTP, 1.25 μL of 10 μM forward primer, 1.25 μL of 10 μM reverse primer, 0.25 μL of Q5 DNA polymerase, 2 μL of cDNA, and 9.75 μL of DNase/RNase-free water. Due to the GC-rich content of the forward and reverse GLP-1R primers, 5 μL of 5x Q5 GC Enhancer was also added for a total reaction volume of 25 μL. Thermal cycling conditions were determined empirically, with the following conditions used to generate GLP-1R PCR products: initial denaturation step at 95°C for 3 minutes; 37 cycles of 95°C for 30 seconds, 55°C for 45 seconds, and 72°C for 1 minute; final extension step at 72°C for 10 minutes; hold at 4°C as needed. The following conditions were used to generate GAPDH PCR products: initial denaturation step at 95°C for 3 minutes; 30 cycles of 95°C for 30 seconds, 55°C for 45 seconds, and 72°C for 1 minute; final extension step at 72°C for 10 minutes; hold at 4°C as needed. The PCR products were separated by electrophoresis in a 1.2% agarose gel at 100 volts in the presence of ethidium bromide. Bands were visualized by ultraviolet fluorescence and recorded with a Bio-Rad Gel Doc EZ Imager.

Western Blot Analysis

Protein was extracted from colon and intestine tissues, with concentration determined by protein assay. Tris/Glycine sodium dodecyl sulfate-polyacrylamide gels

for electrophoresis (SDS-PAGE) were made with the following components: 2.0 mL of ddH_20, 1.3 mL of 30% acrylamide mix, 1.3 mL of 1.5 M Tris (pH=8.8), 0.05 mL of 10% SDS, 0.05 mL of 10% ammonium persulfate solution (APS), and 0.002 mL of TEMED. After polymerization, a stack buffer (0.68 mL of ddH_20, 0.17 mL of 30% acrylamide mix, 0.13 mL of 1.0 M Tris (pH=6.8), 0.01 mL of 10% SDS, 0.01 mL of 10% (APS), and 0.001 mL of TEMED) was added and allowed to polymerize. 40 μg of protein were used per sample, which was diluted 3:1 with 4x Laemmli Sample Buffer (with 10% 2-mercaptoethanol). β-actin was used as a positive control. Samples were heated at 95°C for 5 minutes, loaded into wells, and separated via SDS-PAGE for 30-60 minutes at 75 V through the stack buffer and then for 30-90 minutes at 100 V through the resolving buffer. Protein bands were transferred onto a nitrocellulose membrane via Bio-Rad Trans-Blot Turbo Transfer system. The membrane was then blocked with 5% (w/v) non-fat dried milk/TBS-T (Tris-buffered saline [pH = 7.6] and 0.1% Tween-20) for 1 hour at room temperature.

The membrane was incubated overnight at 4°C with a primary antibody specific for GLP-1R (Santa Cruz Biotechnology, sc-390774, 1:200 in TBS-T/1% non-fat dried milk) and then washed thrice with TBS-T. It was then incubated for 1 hour at room temperature with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:2000 in TBS-T/1% non-fat dried milk). The membrane was washed thrice with TBS-T, and immunoreactive proteins were visualized using the SuperSignal West Pico Chemiluminescent substrate. Membranes were covered in clear plastic wrap, exposed to film, and developed.

Gs Activation Assay.

Activation of Gs in response to GLP-1 was analyzed by western blot using an antibody that specifically recognizes the activated (GTP-bound) Gαs subunit (NewEast Biosciences; catalogue # 80801). Muscle cells in culture were treated with GLP-1 (7-36) amide (1 μM) for 5 minutes in the presence or absence of Gs activation inhibitor, NF449 (10 μM) (Hohenegger, 1997). In some experiments, muscle cells were treated with 1 μM vasoactive intestinal peptide (VIP), a known ligand for Gs-coupled $VPAC_2$ receptor, for 5 minutes. At the end of 5-minute incubation, medium was removed and muscle cells were solubilized on ice for one hour in medium containing 20 mM Tri-HCl (pH 8.0), 1 mM DTT, 100 mM NaCl, 0.5% sodium dodecyl sulfate, 0.75% deoxycholate, 1 mM PMSF, 10 μg/ml of leupeptin and 100 μg/ml of aprotinin. The proteins in the lysate were resolved by SDS/PAGE and electrophoretically transferred onto nitrocellulose membranes. The membranes were incubated for 12 h with anti-Gαs-GTP antibody (1:1500) and then for 1 h with horseradish peroxidase-conjugated secondary antibody (1:5000). The protein bands were identified by enhanced chemiluminescence reagent.

Generation of cAMP in Smooth Muscle

Cyclic AMP was measured by a competitive ELISA using a monoclonal anti-cAMP antibody-based direct cAMP assay kit following the protocol provide by NewEast Biosciences (catalogue # 80203). Muscle cells in culture were treated with GLP-1 (7-36) amide (1 μM) for 5 minutes in the presence or absence of Gs activation inhibitor, NF449 (10 μM). In some experiments, muscle cells were treated with 1 μM VIP for 5 minutes. 100 μM of isobutyl methyl xanthine was used in the incubation to prevent cAMP

hydrolysis. Briefly, a 96-well plate was coated with goat-anti-mouse serum. cAMP from colonic smooth muscle extracts were competitively bound to the monoclonal anti-cAMP antibody in the presence of known amounts of cAMP-conjugated horseradish peroxidase, which were used to generate a standard curve. The plate was read on a Victor2 1420 Multi-Level microplate reader at 450 nm. Optical density readings were used to calculate the concentration of cAMP in samples based on the standard curve. Results were expressed as cAMP in pmol/mg protein.

Activation of PKA in Colonic Smooth Muscle

Cyclic AMP-dependent protein kinase activity was measured in an in vitro kinase assay using PKA-selective substrate peptide kemptide and [^{32}P]ATP. Muscle cells in culture were treated with GLP-1 (7-36) amide (1 μM) for 5 minutes in the presence or absence of Gs activation inhibitor, NF449 (10 μM). In some experiments, muscle cells were treated with 1 μM VIP for 5 minutes. 100 μM of isobutyl methyl xanthine was used in the incubation to prevent cAMP hydrolysis. After a 5-minute incubation period, cells in the plate were rinsed with a medium containing 50 mM Tris•HCl (pH 7.4), 10 mM EDTA, 0.5 mM IBMX, 10 mM 2-mercaptoethanol, and 100 mM NaCl, and were then homogenized in 0.5 mL of ice-cold medium. The mixture was separated by centrifugation at 48,000 g for 15 minutes. The supernatant was transferred to a new tube and used as a source for protein kinase. 20 μL of supernatant, containing 50 μg of protein, was used to initiate an assay in a medium containing 50 mM Tris, 20 mM magnesium acetate, 200 μM [^{35}P]ATP, 100 μM IBMX, 150 μM kemptide [LRRASG], a PKA substrate, and 0.25 mg/mL BSA in the presence and absence of 5 μM cAMP. The reaction mixtures were

incubated at 35-37°C for 30 minutes and the reaction was stopped by spotting the reaction mixture onto a phosphocellulose filter discs. The filters were washed 3-4 times with 75 mM H_2PO_4 and then dried before transferring to vials for radioactive counting using a beta counter. Results were expressed as a ratio activity in the presence or absence of cAMP.

Smooth Muscle Strip Preparation

Mice were euthanized as previously described, and colons were removed and immediately washed and flushed Krebs solution containing 118 mM NaCl, 4.8 mM KCl, 1mM $MgSO_4$, 1.15 mM NaH_2PO_4, 15 mM $NaHCO_3$, 10.5 mM Glucose and 2.5 mM $CaCl_2$ (95% O_2/5%CO_2, pH 7.4). Colons were cut into segments approximately 1 cm in length, leaving some tubes intact while cutting others along the longitudinal axis. Intact segments were tied at each end with silk thread using care not to obstruct the lumen. The tissues were mounted between a glass rod and FT-03C isometric transducer (Grass Technologies, East Warwick, RI), oriented near the bottom of the 5 mL well (Radnoti, Monrovia, CA) to ensure constant submersion in Krebs buffer. Water was pumped constantly through the jackets surrounding the wells at 37°C to maintain constant temperature in the bath. Signals from the transducer were amplified by an Octal Bridge Amplifier (AD Instruments, Colorado Springs, CO), transmitted to a Powerlab 8/35 with Grass Adaptor unit, and recorded with ADInstruments LabChart Pro7 software. Force was recorded in grams (transduced from mV), and the transducer was calibrated with a 2-point curve.

Effects of GLP-1 on Spontaneous Phasic and Acetylcholine-induced Contractions

Intact colon segments were prepared as previously described. All tubes were raised to 1 gram of tension and were then allowed to equilibrate in Krebs buffer for 45 minutes. Tubes were washed out with fresh Krebs every 15 minutes during equilibration. Contraction was induced with acetylcholine (ACh) at a concentration of 1 μM. GLP-1 (7-37) and GLP-1 (7-36) amide were added at target concentrations between 1 nM and 1 μM. Measurements were taken prior to administration of ACh, during the plateau after ACh-induced contraction, and during the plateau after GLP-1 administration, and they were averaged to account for naturally occurring variations in smooth muscle. Samples were washed to encourage return to baseline tension.

Statistical Analysis

Results were expressed as means ± standard error of the mean of n experiments. Experiments were designed to compare treatment to control conditions, with each individual strip serving as its own control. Paired t-tests were conducted in GraphPad Software Prism 6 (La Jolla, CA) with significance set at $P<0.05$ and denoted with "*". Each experiment was performed on tissues obtained from different animals.

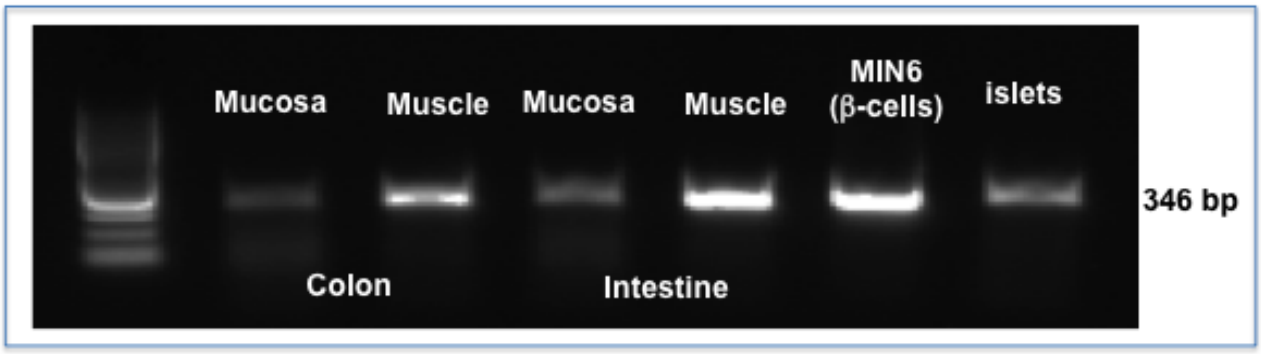
CHAPTER 3: RESULTS

Expression of GLP-1R mRNA

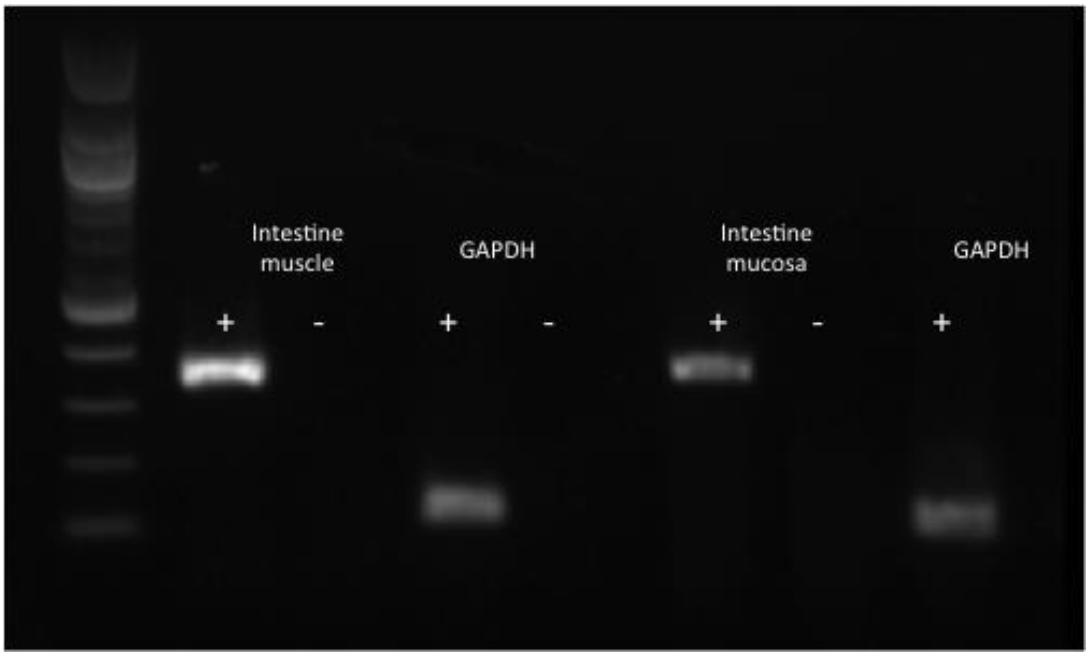
Muscle and mucosal layers were isolated from the colon and small intestine of mice. RNA was extracted and reversibly transcribed to synthesize cDNA, which was then amplified via PCR. Specific primers for GLP-1R were used to yield fragments spanning 346 bp in length. Pancreatic β cells (MIN6 cell line) and islets of Langerhans have known expression of GLP-1R and were used as positive controls in tissue. GAPDH was used as a housekeeping gene. Negative controls were made by substituting the reverse transcriptase enzyme for an equal volume of water to check for genomic DNA contamination. Expression of GLP-1 mRNA was observed in both mucosa and muscle layers of both small intestine and colon. Results are demonstrated in Figure 6.

Figure 6: Expression of GLP-1R mRNA in Small Intestine and Colon Tissue

A)



B)



C)

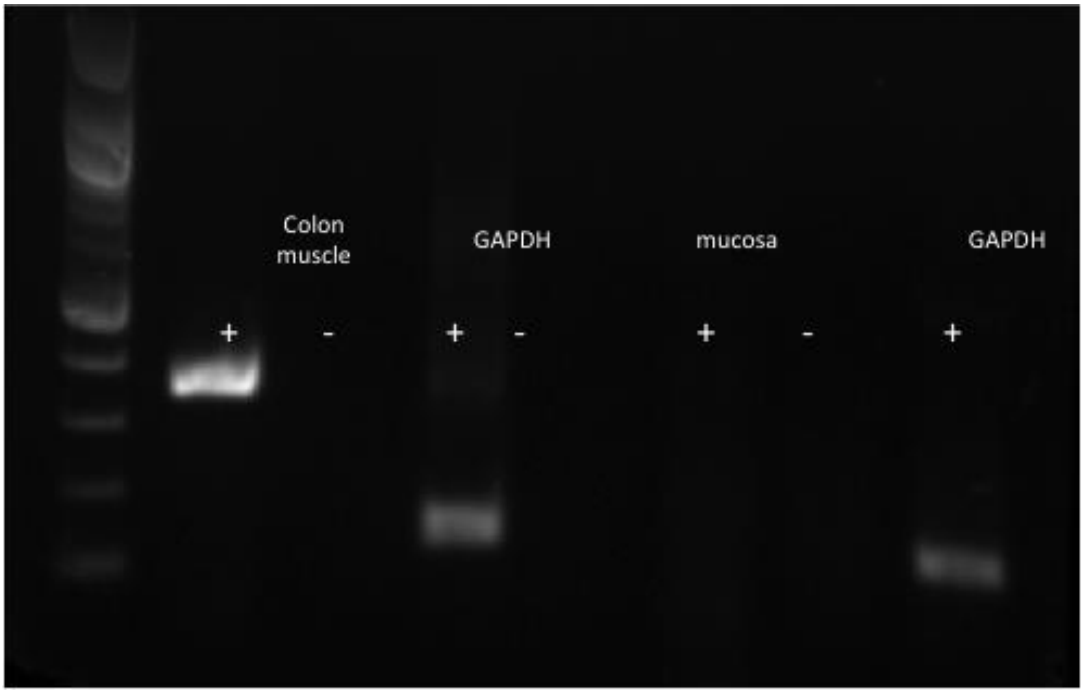


Figure 6. Expression of GLP-1R mRNA. A) This figure demonstrates expression of GLP-1R mRNA in mouse colon muscle and mucosa layers as well as small intestine muscle and mucosa layers. Bands are present in all lanes, including MIN6 and islets, the positive controls. ***B)*** This shows an example of RT-PCR experiments conducted in the small intestine. Bands are present in the intestinal muscle and mucosa lanes as well as the GAPDH lanes. No bands are present in the negative control lanes (absence of reverse transcriptase enzyme), suggesting lack of genomic DNA contamination. ***C)*** This shows an example of RT-PCR experiments conducted in the colon. Bands are present in the intestinal muscle lane as well as the GAPDH lanes. Expression was not observed in colonic mucosa in the experiment. No bands are present in the negative control lanes (absence of reverse transcriptase enzyme), suggesting lack of genomic DNA contamination.

Expression of GLP-1R Protein

Muscle and mucosal layers were isolated from the colon and small intestine of mice. Protein was extracted and quantified against a bovine serum albumin standard curve. Specific antibodies for GLP-1R were used to detect the protein target protein, sized 56 KDa. The pancreas has known expression of GLP-1R and was used as a positive control in tissue. β-actin was used as a loading control. Expression of GLP-1 protein was observed consistently in the muscle layers of both small intestine and colon. Results are demonstrated in Figure 7.

Figure 7: Expression of GLP-1R Protein

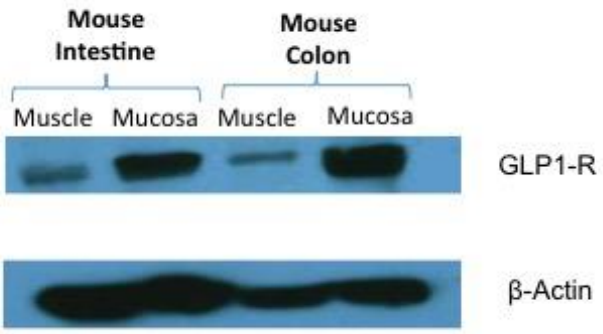


Figure 7. Expression of GLP-1R Protein. The figure demonstrates expression of GLP-1R in small intestine and colon muscle and mucosa. The top panel demonstrates expression of GLP-1R protein at 56 kDa in the muscle and mucosa layers of both colon and intestine. The bottom panel shows expression of the loading control, β-actin, in all four samples.

Expression of GLP-1R in Smooth Muscle Cells

The aim of this study was to determine presence of GLP-1R on smooth muscle cells. The muscle layer of the gastrointestinal tract contains various other cell types, including enteric neurons and interstitial cells of Cajal. To address this, smooth muscle cells devoid of other cell types were isolated, cultured, and used to identify GLP-1R expression as well as the signaling pathway activated by GLP-1R in smooth muscle cells. RNA was extracted, reversibly transcribed, and amplified via PCR using specific primers. mRNA expression was consistent with results in tissue as previously described. Smooth muscle cells in the colon and small intestine were shown to express GLP-1R mRNA. Results are demonstrated in Figure 8.

Figure 8: Expression of GLP-1R in Smooth Muscle Cells

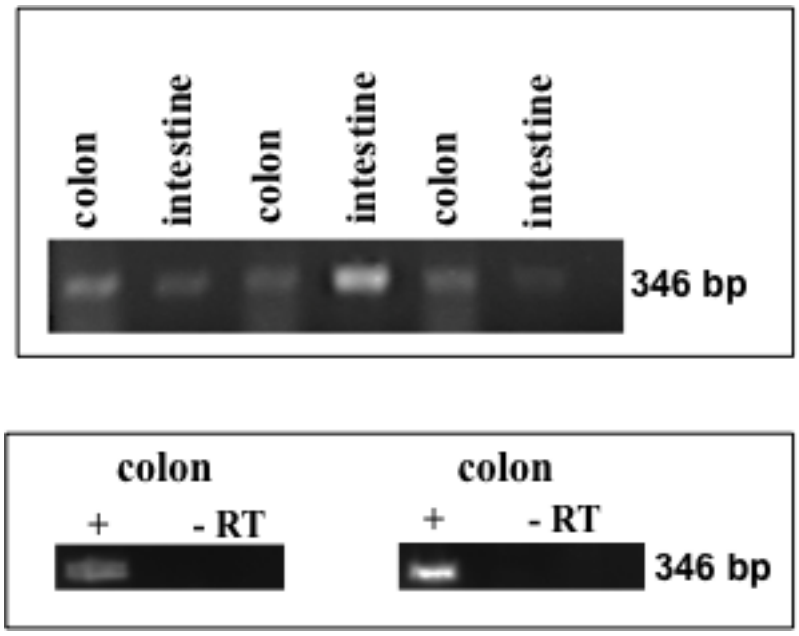


Figure 8. Expression of GLP-1R in Smooth Muscle Cells. The figure shows intestinal expression from 3 different experiments and colonic expression from 5 different experiments. The bottom panel displays an example of a negative control, where water was substituted for the reverse transcriptase enzyme. Bands appear at the expected base pair length.

Activation of Ga_s by GLP-1 in Smooth Muscle Cells

Previous studies have shown that GLP-1 receptors are coupled to activation of Gs proteins in pancreatic beta cells. We examined whether Gs proteins are activated in response to GLP-1(7-36) amide in western blot using an antibody that specifically bind activatde (GTP-bound) Gαs subunit. Addition of 1 μM GLP-1(7-36) amide caused a 3 fold increase in the activation of Gs suggesting that GLP-1 receptors in smooth muscle are coupled to activation of Gs. This was further examined using a selective inhibitor (NF449). The effect of GLP-1 in Gs activation was blocked in the presence of NF449. Control studied showed that VIP, a ligand known to activate Gs-coupled VPAC2 receptors, also stimulated Gs. The extent Gs stimulation y GLP-1 and VIP appears similar. These results suggest that in smooth muscle cells, GLP-1R are coupled to G_s proteins similar to pancreatic β cells. Results are demonstrated in Figure 9.

Figure 9: Activation of Ga_s by GLP-1 in Smooth Muscle Cells

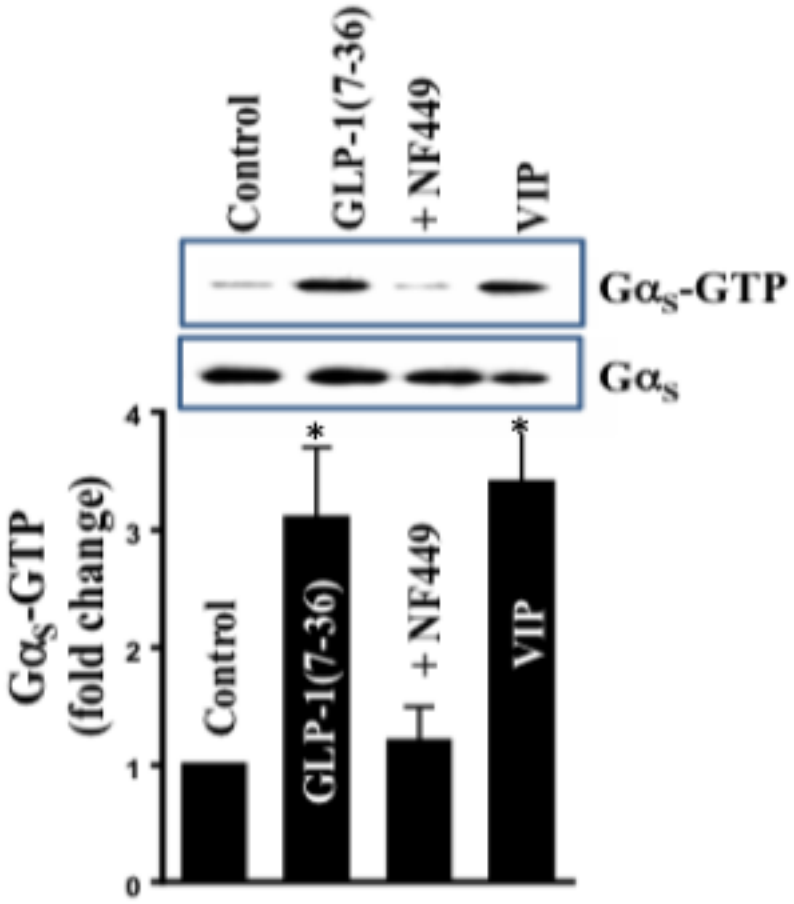


Figure 9. Gs activation by GLP-1. Muscle cells were treated with 1 μM GLP-1 (7-36) amide in the presence or absence of Gs activator, NF449 (10 μM). In some experiments, cells were treated with 1 μM of VIP. Activation of Gs was measured in a western blot analysis using an antibody selective for GTP-bound Gαs. The density of bands was calculated by image analysis. A representative image of 3 separate experiments is shown in the figure.

Increase in cAMP Levels by GLP-1 in Smooth Muscle Cells

Consistent with activation of Gs, treatment of cells with GLP-1(7-36) amide significantly increased cAMP levels compared to basal levels. The effect of GLP-1 on cAMP levels was significantly inhibited by treatment of cells with GLP-1 in the presence of 10 μM NF449, an inhibitor of Gs activity. The effect of NF449 on GLP-1-induced cAMP levels is consistent with its inhibitory effect on GLP-induced Gs activity. VIP also caused a stimulation in cAMP levels and the results are consistent with its effect on Gs-coupled VPAC2 receptors in smooth muscle cells. Results are demonstrated in Figure 10.

Figure 10: Increase in cAMP Levels by GLP-1 in Smooth Muscle Cells

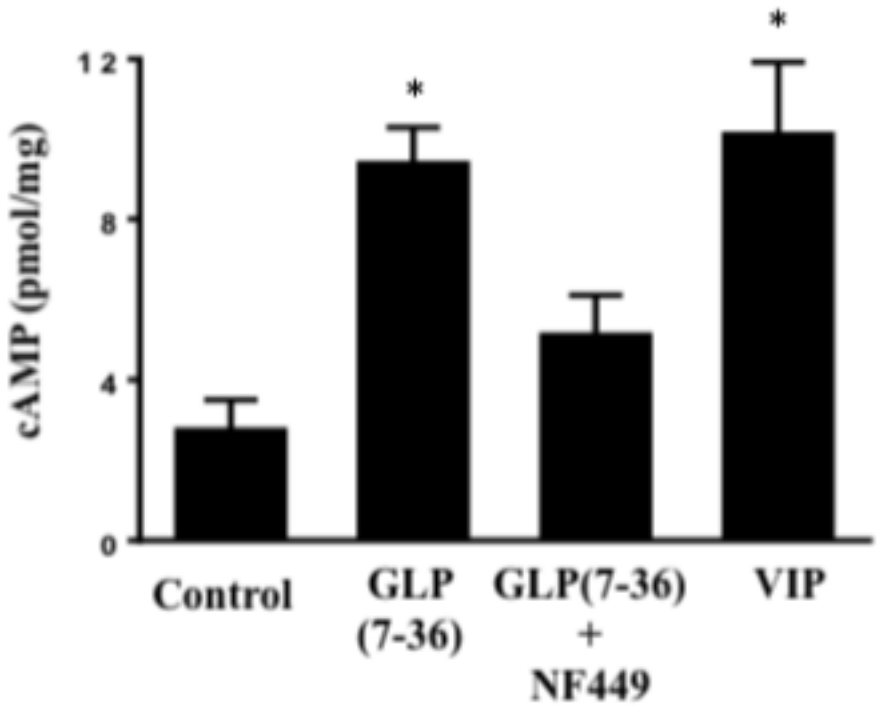


Figure 10. GLP-1 induced increase in cAMP levels. Muscle cells in culture were treated with GLP-1(7-36) amide in the presence or absence of 10 μM NF449. In some experiments cells were treated with 1 μM VIP. cAMP was measured by ELISA as described in the Methods. Results are expressed as pmol of cAMP/mg protein. Values are mean ± SEM of 4 separate experiments

Ga_s-dependent Activation of PKA by GLP-1 in Smooth Muscle Cells

Stimulation of Gs and increase in cAMP leads to stimulation PKA activity. Treatment of cells with GLP-1 caused a significant increase in PKA activity and the stimulation was attenuated in the presence of Gs inhibitor, NF449. The effect of NF449 is consistent with its inhibitory effect on Gs activation and cAMP generation. VIP also caused am increase in PKA activity. These results suggest that in smooth muscle cells, GLP-1R are coupled to the G_s/cAMP/PKA pathway, similar to that of VIP. Results are demonstrated in Figure 11.

Figure 11: Ga_s-dependent Activation of PKA by GLP-1 in Smooth Muscle Cells

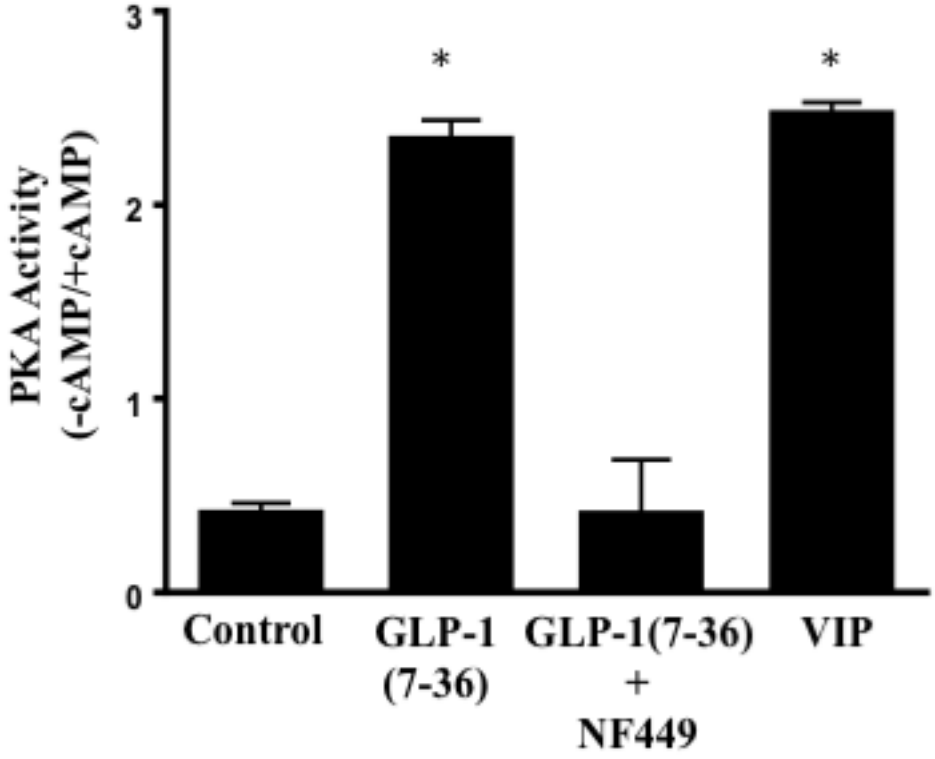


Figure 11. GLP-1 induced increase in cAMP-Dependent protein kinase (PKA) activity. Muscle cells in culture were treated with GLP-1(7-36) amide in the presence or absence of 10 μM NF449. In some experiments cells were treated with 1 μM VIP. PKA activity was measured in the lysates using kemptide and [^{32}P]ATP in the presence or absence of cAMP as described in the Methods. Results are expressed as ratio of PKA activity in the absence or presence of cAMP (-cAMP/+ cAMP). Values are mean ± SEM of 3 separate experiments

Inhibition of Spontaneous Phasic Activity by GLP-1 in Colon

The effects of GLP-1 (7-36) amide on spontaneous phasic activity in the mouse colon were observed using intact colon segments in an organ bath equipped with a force transducer. The effects of GLP-1 on both basal and agonist-induced colonic muscle contraction were examined. GLP-1 (7-36) amide was shown to inhibit spontaneous phasic activity at a concentration of 1 μM. Results are demonstrated in Figure 12.

Figure 12: Inhibition of Spontaneous Phasic Activity by GLP-1 in Colon

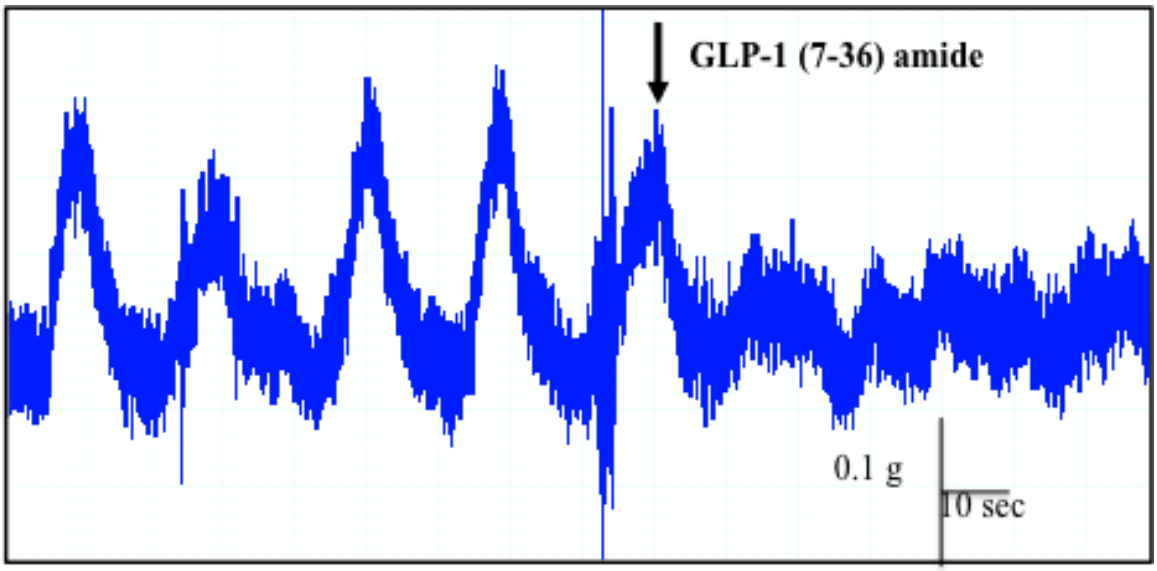


Figure 12. Inhibition of Spontaneous Phasic Activity by GLP-1 in Colon. The figure displays a tracing from an intact colon segment in an organ bath. The left side of the panel shows baseline spontaneous phasic activity with consistent frequency and amplitude. Addition of GLP-1 (7-36) amide at a concentration of 1 μM immediately causes an inhibition of the amplitude of spontaneous contractions. The vertical bar represents 0.1 grams of force, and the horizontal bar represents 10 seconds.

Inhibition and Relaxation of Agonist-induced Tone by GLP-1 in Colon

The effects of GLP-1 (7-36) amide on agonist-induced tone in the mouse colon were observed using intact colon segments in an organ bath equipped with a force transducer. The effects of GLP-1 on both basal and agonist-induced colonic muscle contraction were examined. Contraction was induced by ACh at 1 μM, and GLP-1 (7-36) amide was added to the organ bath at 1 μM after tension plateaued. Relaxation was observed immediately after addition of GLP-1, suggesting a relaxant effect on smooth muscle. Results are demonstrated in Figure 13.

Figure 13: Inhibition and Relaxation of Agonist-induced Tone by GLP-1 in Colon

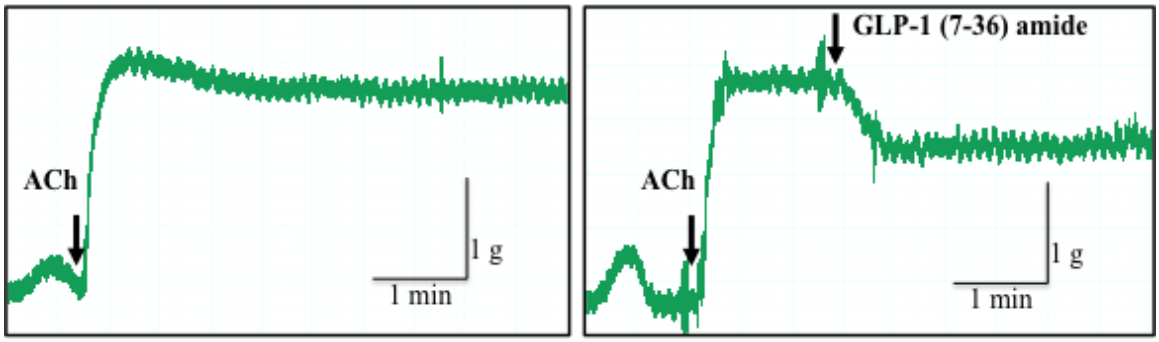


Figure 13. Inhibition and Relaxation of Agonist-induced Tone by GLP-1 in Colon. Contraction was induced by ACh at 1 μM, and GLP-1 (7-36) amide was added to the organ bath at 1 μM after tension plateaued. Relaxation was observed immediately after addition of GLP-1, suggesting a relaxant effect on smooth muscle, and decrease in tone by approximately 40% was observed. The horizontal line represents 1 minute, and the vertical line represents 1 gram of tension. GLP-1 exerts a clear relaxant effect on smooth muscle on tissues contracted by agonists. Mean relaxation in terms of percentage of peak contracted tension = 22.6%±4.6, n=4.

CHAPTER 4: DISCUSSION

The aim of this study was to examine the presence and function of the glucagon-like peptide-1 receptor in gastrointestinal smooth muscle. GLP-1 is released after meals in response to stimuli such as glucose, lipid digestion products, amino acids, bile salts, and free fatty acids. Enteroendocrine cells then secrete hormones such as GLP-1 into the bloodstream with a host of effects on factors such as gastrointestinal motility, secretion, and energy metabolism. Expression of the GLP-1R has been identified throughout the human body, both centrally and peripherally, and many of its effects are well documented. GLP-1 is a potent insulinotropic hormone, assisting with the regulation of postprandial glucose homeostasis. Activation of GLP-1R results in increased insulin secretion and decreased glucagon secretion, decreased gastric emptying and motility, and increased satiety (Latorre, 2016) (Cantini, 2016).

Clinicians have identified the therapeutic potential of GLP-1 and its analogues for the treatment of type 2 diabetes mellitus due its insulinotropic effects. Patients with type 2 diabetes have a significantly decreased incretin secretory response after eating, resulting in hyperglycemic conditions in postprandial and fasted states. While the effects of GLP-1 secretion seem promising, the efficacy of GLP-1 as a long-term treatment option is limited by its rapid life; the peptide is degraded by DPP-4 within minutes of entering circulation. Analogues such as exendin-based therapies, DPP-4 resistant analogues, and human GLP-1 analogues are being explored due to the potential of a prolonged therapeutic effect. Currently, commercially available analogues include exanatide, exanatide once weekly, and liraglutide.

Interestingly, augmentation of the incretin system results in downregulation of glucagon secretion from pancreatic α cells, possibly resulting in more stability in blood glucose levels. While these properties are promising, very little is known about the distribution of GLP-1R throughout GI smooth muscle. In addition, the mechanisms by which the known properties of GLP-1 act are not understand. By identifying enzymes, substrates, and signaling molecules involved with the response, researchers improve their chances of identifying specific targets that might be useful for inducing a longer lasting insulinotropic response.

This study has identified $G\alpha_s$ as the G protein coupled with the GLP-1R, which can now be associated with insulin secretion, inhibition of glucagon secretion, slowed gastric emptying, increased fullness, and relaxation of gastrointestinal smooth muscle. Perhaps pharmacologically addressing the signal cascade initiated by $G\alpha_s$ activation could elucidate methods to increase the practical efficacy of incretin therapy.

In addition, it is known that agonist-bound GPCRs undergo multiple conformational changes while transitioning between their inactive and active forms. It has been suggested that allosteric interactions at the cytoplasmic aspect of GPCRs induce conformational changes that alter ligand-binding affinity. The potential to form active complexes of G protein, agonist, and receptor with enhanced stability and duration has great potential for therapeutic use of incretins, especially considering the short half-life of peptide hormones like GLP-1 (Duc, 2017).

This study identified expression in the colon and small intestine, but further research should be done to quantify relative levels of GLP-1R in mucosa and muscle of each organ and across different regions of the GI tract. The current research did not test

differences in response between distal and proximal regions of the colon and small intestine, nor did it quantify expression. Spatiotemporal mapping could be done to observe luminal behavior during GLP-1 induced relaxation. In addition, more focus could be allocated to comparing different isoforms of the bioactive GLP-1 peptide in different tissue types to compare responses. The minimal concentration of GLP-1 (7-36) amide at which a response was observed (threshold dose) was 10 nM with more dramatic responses occurring at 1 μM, but little else is known about dose-dependent responses to GLP-1. Propulsion studies could also be done with GLP-1 administered to different GI regions to observe effects on the peristaltic response.

When more is understood about the direct and indirect effects of GLP-1, expression in all tissue types has been determined, and the characteristics of its analogues and GLP1-R agonists have been identified, clinicians will have a valuable pathway to manipulate for therapeutic benefit. A drug that induces a constant, longer-lasting GLP-1R response will significantly impact treatment of diabetic and obese patients. Route of administration and dosage plans need to be determined. Finally, long-term studies on safety and desensitization to treatment should be explored.

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Vita

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