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Muscarinic Receptors Control Postprandial Release of Glucagon-Like Peptide-1: *In Vivo* and *in Vitro* Studies in Rats

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Plasma levels of glucagon-like peptide-1 (GLP-1) rise rapidly after nutrient ingestion through an indirect mechanism triggered from the proximal intestine and involving the vagus nerve that stimulates the L cell in the distal gut. The role of muscarinic receptors in this pathway was thus investigated using the anesthetized rat and fetal rat intestinal cells (FRIC) in culture. GLP-1 secretion from the distal gut increased 5-fold after 3 ml corn oil were placed into the proximal duodenum ($P < 0.001$). Atropine (a nonspecific muscarinic receptor antagonist) completely inhibited fat-induced GLP-1 secretion *in vivo* ($P < 0.01$). Pirenzepine (an M1 muscarinic receptor antagonist) also inhibited fat-induced GLP-1 secretion *in vivo*, by 91 ± 6% ($P < 0.01$). Gallamine (an M2 muscarinic receptor antagonist) and 4-diphenylacetoxy-*N*-methylpiperidine (an M3 muscarinic receptor antagonist) had no effect. Incubating FRIC cultures with bethanechol (a muscarinic receptor agonist) stimulated GLP-1 secretion to 200 ± 22% of control ($P < 0.01$). Pirenzepine and gallamine significantly inhibited bethanechol-stimulated GLP-1 secretion, by 96 ± 12% and 98 ± 8%, respectively ($P < 0.01$). Unexpectedly, 4-diphenylacetoxy-*N*-methylpiperidine stimulated GLP-1 secretion by FRIC cells, to 324 ± 52% of the control value ($P < 0.01$). Double immunofluorescent staining using GLP-1 and M1, M2, and M3 muscarinic receptor antibodies showed expression of the three subtypes of muscarinic receptors by the L cells in rat ileal sections and FRIC cultures. These results demonstrate the role of M1 muscarinic receptors expressed by L cells in the control of postprandial secretion of GLP-1. M2 muscarinic receptors also seem to play a role in controlling GLP-1 secretion by fetal, but not adult, L cells. (*Endocrinology* 143: 2420–2426, 2002)

THE INSULINOTROPIC hormone glucagon-like peptide-1 [GLP-1-(7–36) amide] is secreted by enteroendocrine L cells in the distal intestine and colon in response to nutrient ingestion, especially fat and carbohydrates (1, 2). GLP-1 then stimulates insulin secretion in a glucose-dependent manner (3–5). GLP-1 also inhibits glucagon secretion (6), delays gastric emptying (7), and inhibits food intake (8). Furthermore, GLP-1 has recently been shown to stimulate pancreatic β-cell proliferation and neogenesis (9). These pleiotropic actions of GLP-1 therefore offer great potential for the treatment of hyperglycemia in patients with type 2 diabetes mellitus. Indeed, numerous studies to date have demonstrated that administration of GLP-1 stimulates glucose-dependent insulin secretion in patients with type 2 diabetes, resulting in a normalization of both fasting and fed glycemia (10, 11).

Release of GLP-1 from the distal L cells is under complex regulation. Agents that can directly stimulate the secretion of GLP-1 have been analyzed in various models of the intestinal L cells, including a primary cell culture of fetal rat intestinal cells (FRIC) (12–14), murine intestinal cell lines (15), and the perfused rat ileum (16) and colon (17). The major secretagogues identified using these models include glucose-dependant insulinotropic peptide (GIP) (14–17), gastrin-releasing peptide (GRP) (14, 16, 17), calcitonin gene-related peptide (14, 16, 17), and agonists of acetylcholine (14–17). Fatty acids, when applied directly to L cells in culture, also stimulate GLP-1 secretion (13, 15).

In the *in vivo* setting, GLP-1 is rapidly released from L cells upon the ingestion of a mixed meal (1, 2) or after infusion of fat directly into the duodenum (18–20). Indeed, plasma levels of GLP-1 peak within 15–30 min of nutrient administration despite the fact that nutrients do not reach the distal ileum or colon within this time frame (21). These observations are not consistent with the idea that nutrients act directly on ileal L cells to stimulate the release of GLP-1, at least during the early phase of secretion. Several studies by our group have demonstrated that administration of nutrients into the duodenum stimulates GIP secretion by local endocrine K cells, which, in turn, stimulates the afferent vagus nerve to the central nervous system, stimulating the efferent vagus (celiac) nerve to the distal gut (18, 19). As the vagus nerve releases acetylcholine, we have hypothesized that this pathway signals through muscarinic receptors expressed at the level of the L cell. Consistent with this hypothesis, several *in vitro* studies demonstrated the involvement of cholinergic muscarinic receptors in the regulation of GLP-1 secretion (12, 14, 15, 22). Furthermore, Balks *et al.* (23) used atropine, a nonspecific muscarinic antagonist, to demonstrate that GLP-1 release in humans is under the control of muscarinic receptors. However, additional studies have shown the involvement of cholinergic nicotinic receptors in the regulation of GLP-1 secretion (1), as well as the neuropeptide, GRP (24). The relationships between these different neuromodulatory pathways remain to be determined. The goal of the present study was to investigate the role of cholinergic muscarinic

Abbreviations: AUC, Area under the curve; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine; FRIC, fetal rat intestinal cells; GIP, glucose-dependant insulinotropic peptide; GLP-1, glucagon-like peptide-1; GRP, gastrin-releasing peptide; PMA, phorbol myristate acetate.

receptors in the control of GLP-1 secretion, using both the anesthetized rat model and FRIC cultures.

Materials and Methods

Studies in anesthetized rats

After fasting for 18–24 h, male Wistar rats, weighing 250–300 g (Charles River Laboratories, Inc., St. Constant, Canada), were lightly anesthetized with halothane and given an ip injection of sodium pentobarbital (60 mg/kg) to achieve a surgical plane of anesthesia. The carotid artery was cannulated to allow for arterial blood sampling, and the jugular vein was cannulated for injections; the femoral vein was also cannulated for solution infusion. Blood samples (1.2 ml) were collected into 120 μl Trasylol (5000 kallikrein inactivating units/ml), EDTA (1.2 mg/ml), and diprotin-A (0.1 mM). Plasma was separated from red blood cells by centrifugation and was stored at −20 C until assay. Red blood cells were immediately resuspended in heparinized saline and reinjected via the jugular cannula. All animal procedures were approved by the Animal Care Committee of the University of Toronto.

After median laparotomy, a loose ligation was set up surrounding the pylorus, and 3 ml fat in the form of corn oil (56% polyunsaturated fatty acids, 32% monounsaturated fatty acids, and 12% saturated fatty acids; Sunfresh Ltd., Toronto, Canada) were then infused into the proximal part of the duodenum at time zero. Blood was collected at −10 and 0 min to ascertain basal levels of secretion, and manipulation of the gastrointestinal tract was performed in the interval between these two time points. Blood samples were also collected at 10, 20, 30, and 60 min thereafter. In a previous study (21) we showed that fat does not reach the distal ileum within 60 min after intraduodenal administration in rats, thus precluding any direct effect of the fat on the distal L cell.

Twenty minutes before corn oil administration, groups of 6 rats received one of the following primed infusions: atropine sulfate (Sigma, St. Louis, MO; 500 μg/kg and 500 μg/kg·h, iv), pirenzepine (RBI, Natick, MA; 200 μg/kg and 200 μg/kg·h, iv), gallamine (RBI; 200 μg/kg and 200 μg/kg·h, iv), or 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP; RBI; 200 μg/kg and 200 μg/kg·h, iv). Control animals received a solution of 0.9% NaCl, iv The flow rate was 3 ml/h. These doses were selected according to Steinle and Smith (25).

Plasma was stored at −20 C before RIA for bioactive GLP-1 [GLP-1-(7–36) amide]. Plasma was extracted in ethanol and assayed using an RIA kit from Linco Research, Inc. (St. Charles, MO). The assay uses an antibody specific for the N-terminal end of GLP-1 and therefore does not cross-react with the circulating degradation product, GLP-1-(9–36) amide. The detection limit of the assay is 3 pM.

FRIC cultures

FRIC cultures were prepared as described in detail previously (12–14). In brief, intestines from a litter of 19- to 21-d gestation fetal Wistar rats were pooled, and the cells were dispersed by two sequential 15-min incubations with collagenase (45 mg/dl), hyaluronidase (50 mg/dl), and deoxyribonuclease I (5 mg/dl; Sigma). The dispersed cells were washed and placed into monolayer culture on 60 × 15-mm dishes in culture medium [DMEM containing 5% (vol/vol) FBS, 4.5 g/liter glucose, and penicillin (50 IU/ml)-streptomycin (50 μg/ml)] at a final concentration of 0.6 fetal rat intestines/2 ml·dish. The cells were then incubated at 37 C with 10% (vol/vol) CO_2 and constant humidity for 16–24 h. Before each experiment, cultures were washed twice with HBSS to remove dead or floating cells, and groups of two dishes were then incubated for 2 h with experimental agents in 2 ml/dish experimental medium [DMEM containing 0.5% (vol/vol) FBS, 1 g/liter glucose, 20 μU/ml insulin, and penicillin (50 IU/ml)-streptomycin (50 μg/ml)].

Some cells were pretreated for 30 min with medium alone (control) or with pirenzepine (1 mM), gallamine (1 mM), or 4-DAMP (1 mM), followed by 2-h treatment with medium alone (control), phorbol myristate acetate (PMA; 1 μM; positive control), pirenzepine (1 mM), gallamine (1 mM), 4-DAMP (1 mM), bethanechol (1 mM; Sigma), McN-A-343 (RBI; 10–5000 μM), or oxotremorine-M (RBI; 10–5000 μM). Each treatment was tested in 6–10 independent experiments.

After the incubation period, cell viability was checked visually by phase-contrast microscopy, and then cell and medium peptides were collected as described previously (12–14). In brief, medium was centrifuged to remove any floating cells, and trifluoroacetic acid was added to the collected medium to a final concentration of 0.1% (vol/vol). Cells were collected by scraping and homogenization in 2 ml/dish 1 N HCl containing 5% (vol/vol) HCOOH, 1% (vol/vol) trifluoroacetic acid, and 1% (vol/vol) NaCl at 4 C. Peptides and small proteins in acidified cell medium or cell extracts were then collected by reverse phase adsorption to C_{18} silica (C_{18} Sep-Pak, Waters Corp., Milford, MA) as previously reported (12–14). We have demonstrated that this methodology permits greater than 88% recovery of intact proglucagon-derived peptides, including GLP-1, from tissues and cell cultures (12). Extracts were stored at −20 C until assay.

Peptides extracted from the intestinal cultures were assayed for the presence of GLP-1 using an antiserum directed against the carboxyl-terminus of GLP-1-(7–36) amide (Affinity Research Products, Nottingham, UK) as described previously (18, 25). Previous studies have demonstrated that FRIC cultures synthesize and secrete GLP-1-(7–36)NH_2 (26). Secretion of GLP-1 was calculated as the total amount of GLP-1 released into the medium over the 2-h incubation period, normalized for the total content of GLP-1 in each dish (*i.e.* medium plus cells).

Immunohistochemistry

Rats were anesthetized with halothane. Pieces of distal ileum were removed, fixed with formalin, embedded in paraffin, and 5-μm sections were prepared. After deparaffinization and hydration, sections were incubated with blocking solution (5% normal donkey serum, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 30 min. Sections were then incubated with primary antiserum for GLP-1 (rabbit polyclonal antibody, used at a 1:1250 dilution) (11) or for M1 (goat polyclonal antibody C-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; used at a1:50 dilution), M2 (goat polyclonal antibody C-18, Santa Cruz Biotechnology, Inc.; used at a 1:50 dilution), or M3 (goat polyclonal antibody C-20, Santa Cruz Biotechnology, Inc.; used at a 1:50 dilution) muscarinic receptors for 1 h at room temperature. After three serial washes with PBS, sections were incubated with secondary antibodies, Cy3-conjugated donkey antirabbit IgG (Jackson ImmunoResearch Laboratories, Inc.; used at a 1:250 dilution) or Cy2-conjugated donkey antigoat IgG (Jackson ImmunoResearch Laboratories, Inc.; used at a 1:50 dilution), for 1 h at room temperature. After rinsing with PBS, the sections were mounted and visualized using a fluorescent microscope.

Some tissue was fresh-frozen and postfixed in methanol, then acetone, at −20 C. Sections (20 μm) were double stained for GLP-1 (as described above) and for neurofilament 68 using a monoclonal antineurofilament 68 antibody (Sigma; used at a 1:100 dilution) for 1 h at room temperature, then incubated with the secondary antibody (donkey antimouse IgG, Jackson ImmunoResearch Laboratories, Inc.; used at a 1:300 dilution). These sections were visualized using 500-nm optical sections on a confocal microscope.

FRIC cultures were grown in eight-well chamber slides (Nalge Nunc International, Naperville, IL) overnight. The medium was removed, and the cells were washed with PBS and fixed in methanol at −10 C for 5 min. Slides were incubated with blocking solution (5% normal donkey serum, Jackson ImmunoResearch Laboratories, Inc.) for 30 min, then incubated with primary antiserum for GLP-1 (rabbit polyclonal antibody, used at a 1:1250 dilution) or for M1 (goat polyclonal antibody C-20, Santa Cruz Biotechnology, Inc.; used at a 1:50 dilution), M2 (goat polyclonal antibody C-18, Santa Cruz Biotechnology, Inc.; used at a 1:50 dilution), or M3 (goat polyclonal antibody C-20, Santa Cruz Biotechnology, Inc.; used at a 1:50 dilution) muscarinic receptors for 1 h at room temperature. After three serial washes with PBS, sections were incubated with secondary antibodies, Cy3-conjugated donkey antirabbit IgG (Jackson ImmunoResearch Laboratories, Inc.; used at a 1:250 dilution) or Cy2-conjugated donkey antigoat IgG (Jackson ImmunoResearch Laboratories, Inc.; used at a 1:50 dilution). After rinsing with PBS, the sections were mounted and visualized using a fluorescent microscope. For the controls, no immunostaining was observed when the primary or secondary antibodies were omitted (not shown).

Data analysis

Peptide secretion is expressed as the change from basal values (*in vivo* experiments) or as a percentage of control secretion (*in vitro* experi-

ments). The area under the curve (AUC) for changes in hormone levels was determined using the trapezoidal rule. All data are expressed as the mean ± SEM. Statistical significance between experimental groups was assessed by ANOVA using n-1 *post hoc* custom hypothesis tests. Significance was determined at the $P < 0.05$ level in these comparisons.

Results

The level of basal plasma GLP-1-(7–36) amide in the anesthetized rats was 3.6 ± 0.3 pM. Plasma GLP-1-(7–36) amide concentrations increased very quickly after the infusion of 3 ml fat in the form of corn oil into the proximal duodenum, to 8 ± 0.3 pM at 10 min (*i.e.* 2.2 times the basal value), and continued to increase to 15.4 ± 1.6 pM at 60 min (*i.e.* 5 times the basal level, $P < 0.001$) (Fig. 1). The AUC over the basal value after fat infusion was 723 ± 65 pmol/liter·h. Infusion of 3 ml saline into the proximal duodenum had no effect on plasma GLP-1-(7–36) amide during the 1 h of the experiment.

To determine the role of muscarinic receptors in postprandial release of GLP-1 *in vivo*, rats were given atropine; a nonspecific muscarinic receptor antagonist, in association with lumenal nutrients. Intravenous infusion of atropine (500 μg/kg and 500 μg/kg·h) completely inhibited corn oil-induced GLP-1-(7–36) amide secretion (Fig. 1). The AUC over the basal value during 1 h of atropine infusion was 65 ± 13 pmol/liter·h (*i.e.* 91% inhibition; $P < 0.001$), showing the involvement of muscarinic receptors in the control of postprandial GLP-1-(7–36) amide secretion in rats.

To assess which subtype of muscarinic receptor is involved in fat-induced GLP-1 secretion in rats, specific antagonists for the M1, M2, and M3 subtypes of muscarinic receptors were infused into rats receiving intraduodenal corn oil. As shown in Fig. 2, infusion of pirenzepine (200 μg/kg and 200 μg/kg·h; an M1 muscarinic receptor subtype antagonist) significantly inhibited fat-induced GLP-1 secretion by 91 ± 6% ($P < 0.01$). Infusion of the M2 receptor antagonist, gallamine (200 μg/kg and 200 μg/kg·h) decreased fat-induced GLP-1 secretion by 43 ± 8%, but this did not reach significance. Furthermore, infusion of 4-DAMP (200 μg/kg and 200 μg/kg·h), a specific M3 muscarinic subtype antagonist, had no effect on fat-induced GLP-1 secretion (Fig. 2).

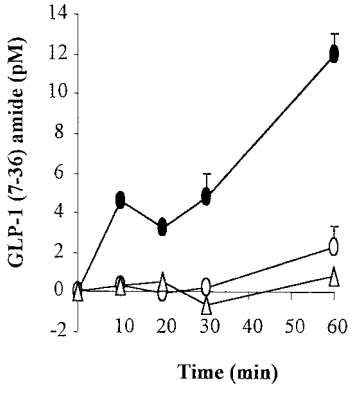

FIG. 1. Effect of atropine on corn oil-induced GLP-1 secretion *in vivo* in rats. Rats received either atropine (500 μg/kg and 500 μg/kg·h; ○) or saline (●) 20 min before 3 ml fat in the form of corn oil were infused into the proximal duodenum. A control group received 3 ml saline into the proximal duodenum (△). Bioactive GLP-1 secretion is expressed as the change from basal values (n = 6). Values are the mean ± SEM. ($P < 0.01$ for fat infusion *vs.* saline at t = 10, 30, and 60 min.)

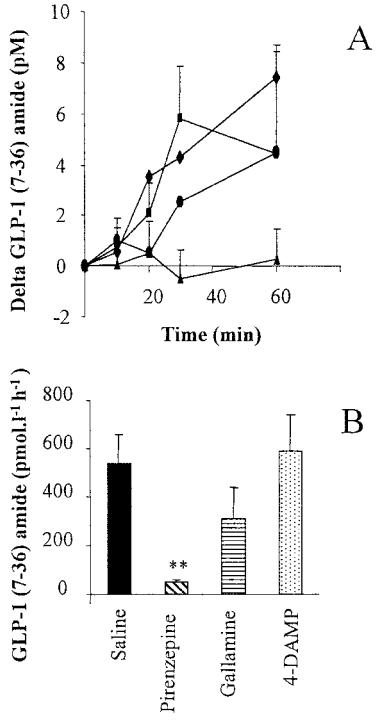

FIG. 2. Effect of specific muscarinic antagonists, M1 (pirenzepine; ▲), M2 (gallamine; ●), and M3 (4-DAMP; ■; 200 μg/kg and 200 μg/kg·h), on corn oil-induced GLP-1 secretion (♦). Bioactive GLP-1 secretion is expressed as the change from basal values (A) or as the 1 h integrated response over the basal level (B). Values are the mean ± SEM. **, $P < 0.01$ [*vs.* the control (saline) group (n = 6)].

Expression of muscarinic receptors by ileal L cells

To determine whether acetylcholine acts on muscarinic receptors localized at the level of endocrine L cells in the distal intestine, double immunostaining (GLP-1 and M1, M2, or M3 muscarinic receptors) was performed and visualized using a fluorescent secondary antiserum. L cells were mainly localized in the crypts, with a few cells detectable in the villus epithelium. Double staining for muscarinic (*green*) receptors and GLP-1 (*red*) showed the expression of M1 (Fig. 3A), M2 (Fig. 3B), and M3 (Fig. 3C) subtypes of muscarinic receptors on all of the ileal L cells. Most of the epithelial cells demonstrated muscarinic receptor positivity. Furthermore, double immunofluorescent staining for GLP-1 (*green*) and neurofilament-68 (*red*) confirmed that endocrine L cells are connected to neurons in the enteric nervous system (Fig. 3D).

Muscarinic receptors and GLP-1 secretion by FRIC cultures

To determine the involvement of muscarinic receptors in the regulation of GLP-1 secretion at the cellular level, FRIC cultures were incubated for 2 h with medium alone (negative control), PMA (1 μM, a PKC activator known to stimulate GLP-1 secretion in this model) (12, 13) or different muscarinic receptor subtype antagonists (Fig. 4). PMA stimulated GLP-1 secretion to 199 ± 24% to 299 ± 80% of the control level ($P < 0.01$ to $P < 0.001$). Bethanechol (1 mM; a nonspecific muscarinic receptor agonist) also stimulated GLP-1 secretion by FRIC cultures to 200 ± 34% to 253 ± 42 of the control level ($P < 0.05$ to $P < 0.001$).

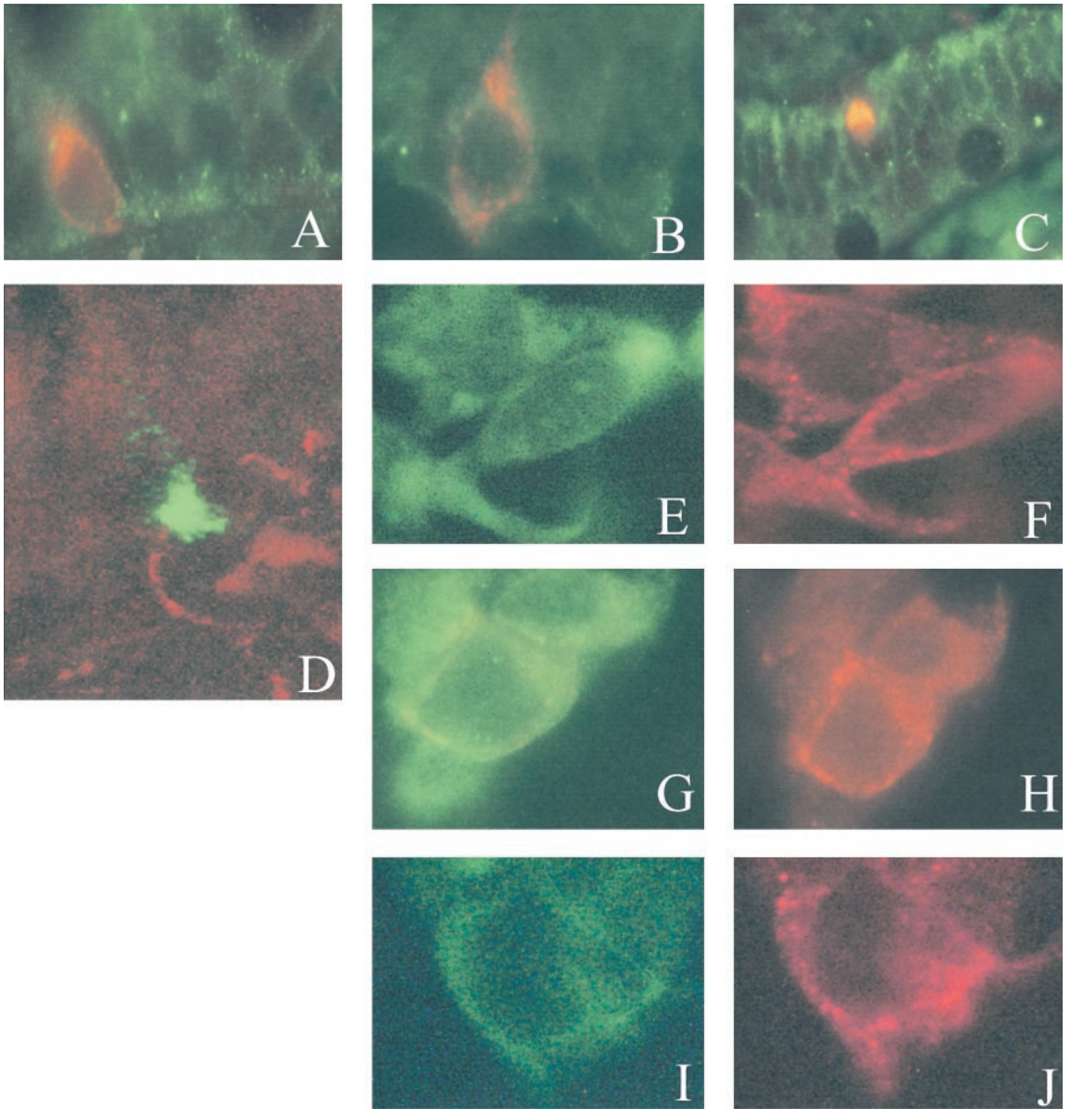

FIG. 3. Double immunofluorescent staining for GLP-1 and either muscarinic receptor subtypes or neurofilament in rat intestine. A–C, GLP-1 (*red*) overlaid on M1 (A), M2 (B), and M3 (C) receptors (*green*) in paraffin-embedded 5-μm rat ileal sections visualized by fluorescence microscopy. D, GLP-1 (*green*) and neurofilament-68 (*red*) in paraffin-embedded 20-μm rat ileal sections were visualized by confocal microscopy using 500-nm optical sections. E–J, GLP-1 (*red*; F, H, and J) and M1 (E), M2 (G), and M3 (I) receptors (*green*) in methanol-fixed FRIC cultures visualized by fluorescence microscopy. The same cells are shown in each of E–F, G–H, and I–J, and were double stained for GLP-1 and one of the muscarinic receptors.

Preincubation with pirenzepine (1 mM) completely inhibited bethanechol-stimulated GLP-1 secretion (104 ± 33% of the control; $P < 0.01$ *vs.* bethanechol alone; Fig. 4A), whereas pirenzepine alone had no effect on GLP-1 secretion by FRIC cultures (102 ± 30% of the control). Gallamine (1 mM) also completely inhibited bethanechol-stimulated GLP-1 secretion (102 ± 8% of the control; $P < 0.01$ *vs.* bethanechol alone; Fig. 4B), whereas gallamine alone had no effect on GLP-1 secretion *in vitro* (92 ± 19% of the control). Unexpectedly, 4-DAMP (1 mM) alone stimulated GLP-1 secretion to 324 ± 52% of the control level ($P < 0.01$; Fig. 4C). However, 4-DAMP had no significant effect on bethanechol-stimulated GLP-1 secretion by FRIC cultures (343 ± 92% of the control; Fig. 4C).

To further establish the role of muscarinic receptors in regulating GLP-1 secretion by FRIC cultures, cells were incubated for 2 h in the presence of graded concentrations of McN-A-343 (an M1 muscarinic receptor subtype agonist) or oxotremorine-M (an M3 muscarinic receptor subtype agonist). McN-A-343 significantly stimulated GLP-1 secretion to 197 ± 24% of the control level ($P < 0.01$) when used at a concentration of 5 mM (Fig. 5A). By contrast, oxotremorine-M had no effect on GLP-1 secretion by FRIC cultures when used at concentrations between 10 and 5000 μM (Fig. 5B).

Immunostaining of FRIC for GLP-1 showed that approximately 1% of FRIC cells are L cells. Double immunostaining for GLP-1 and the muscarinic receptor subtypes M1 (Fig. 3,

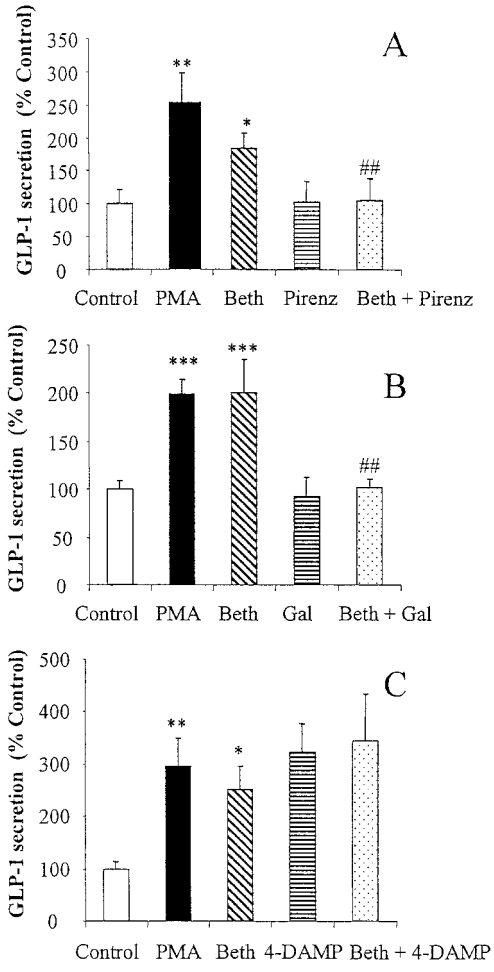

FIG. 4. Release of GLP-1 by FRIC cultures in response to 2-h treatment with medium alone (negative control), PMA (1 μM; positive control), or bethanechol (1 mM; a muscarinic agonist). Cells were pretreated for 30 min with either medium alone or 1 mM pirenzepine (A; an M1 antagonist), 1 mM gallamine (B; an M2 antagonist), or 1 mM 4-DAMP (C; an M3 antagonist) before stimulation with bethanechol. GLP-1 secretion is expressed as a percentage of the control (n = 6). Values are the mean ± SEM. **, $P < 0.01$; ***, $P < 0.001$ *vs.* the control; ##, $P < 0.01$ *vs.* bethanechol.

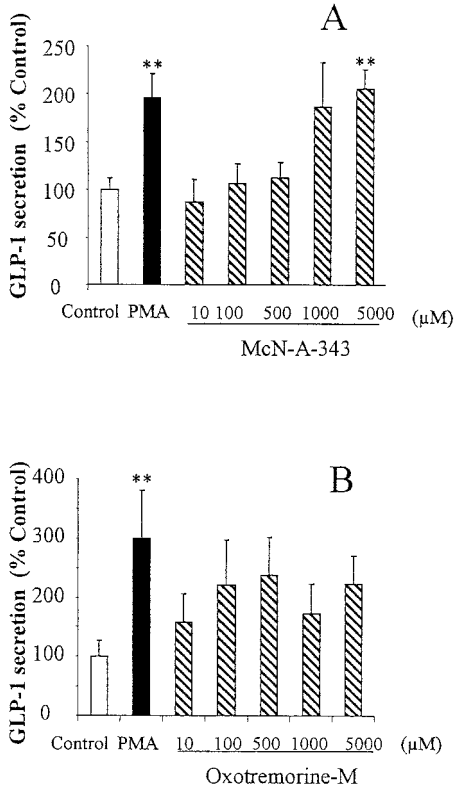

FIG. 5. Release of GLP-1 by FRIC cultures in response to 2-h treatment with medium alone (negative control), PMA (1 μM; positive control), or graded concentrations of McN-A343 (A; an M1 agonist) or oxotremorine (B; an M3 agonist). GLP-1 secretion is expressed as a percentage of the control (n = 6; mean ± SEM). **, $P < 0.01$ *vs.* the control.

E and F), M2 (Fig. 3, G and H), or M3 (Fig. 3, I and J) further showed the expression of all three subtypes of muscarinic receptors on L cells. In contrast to staining for GLP-1, which was found in only a small percentage of FRIC cells, the majority of cells were immunopositive for the muscarinic receptors.

Discussion

GLP-1 is rapidly secreted by L cells upon the ingestion of a mixed meal or after the infusion of fat directly into the duodenum (1, 2, 18–20). Indeed, plasma levels of GLP-1 peak within 15–30 min of nutrient administration despite the fact that nutrients do not reach the distal ileum within this time frame (21). These observations are not consistent with the idea that nutrients act directly on ileal L cells to stimulate the release of GLP-1, at least during the early phase of secretion. We have previously demonstrated that this secretion of GLP-1 is stimulated by nutrients in the proximal duodenum through activation of a vagal pathway (18). The results of the present study extend these finding by demonstrating that GLP-1 secretion is under the control of specific muscarinic receptor subtypes expressed by the rat L cell.

Infusion of atropine, a nonspecific muscarinic receptor antagonist, 20 min before intraduodenal administration of corn oil completely blocked nutrient-induced GLP-1 secretion. This finding clearly demonstrates the involvement of cholinergic muscarinic receptors in the control of postprandial GLP-1 secretion in the rat. Furthermore, as atropine does not delay intestinal transit in the anesthetized rat (21), this effect was probably not due to any alterations in nutrient fluxes through the gut. In addition, a previous study has shown that atropine inhibits the stimulatory effect of a cholinergic agonist on GLP-1 secretion in the isolated vascularly perfused rat ileum (27). These findings are also similar to the results of a study in humans showing that atropine reduces the integrated GLP-1 response to an oral glucose load (23). In contrast, D'Alessio *et al.* (28) reported that nicotinic blockade, but not muscarinic blockade, prevents nutrient-induced GLP-1 secretion in rhesus macaques. We have also shown that hexamethonium (a cholinergic nicotinic receptor antagonist) strongly inhibits GLP-1 secretion induced by intraduodenal fat or a mixed meal in rats (1). Thus, in rat, monkey, and human, GLP-1 secretion is regulated by cholinergic receptors. However, the involvement of muscarinic receptors in the control of this secretion appears to be species specific.

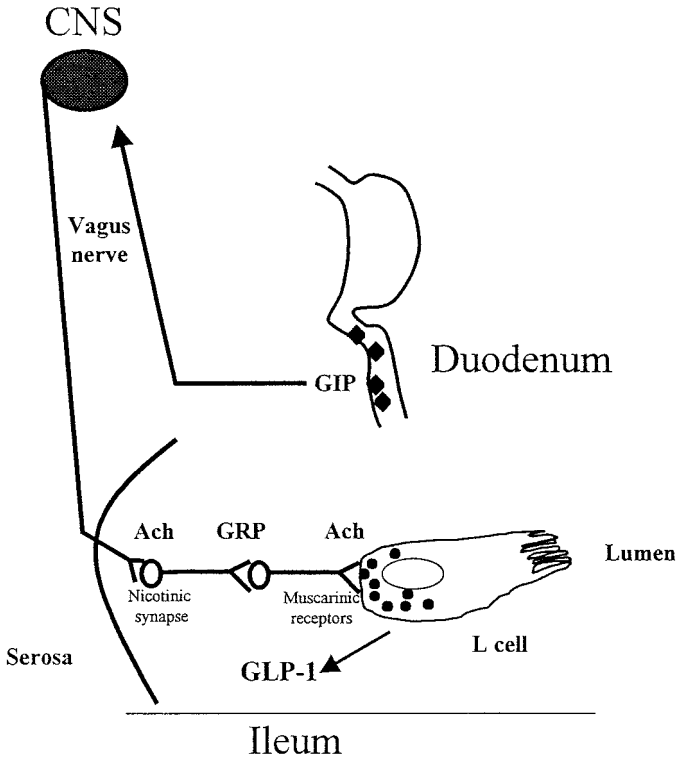

FIG. 6. Model of the indirect mechanism underlying GLP-1 secretion from rat L cells after food ingestion. Entry of chyme into the duodenum induces the release of GIP from duodenal endocrine K cells. GIP stimulates the afferent branch of the vagus nerve to the vagus center, which then sends a stimulatory signal via the efferent, celiac branch of the vagus nerve to nicotinic receptors on GRPergic neurons in the enteric nervous system. These neurons signal the L cells, in turn, through activation of cholinergic neurons, leading to activation of muscarinic receptors on the L cell.

Five different isoforms of muscarinic receptors have been described (M1–M5), although M4 and M5 have been reported to localize mainly in the brain (29). We therefore also performed experiments to establish which of the muscarinic receptor subtypes (M1, M2, or M3) is involved in regulating GLP-1-(7–36 amide) secretion *in vivo* in rats. As shown in Fig. 1, pirenzepine (an M1 antagonist) completely inhibited corn oil-induced GLP-1 secretion. By contrast, neither gallamine (an M2 antagonist) nor 4-DAMP (an M3 antagonist) had a significant effect on GLP-1 secretion. Furthermore, double immunostaining of the rat ileum for GLP-1 and M1, M2, or M3 muscarinic receptors showed that L cells express all three muscarinic receptor subtypes, suggesting that acetylcholine acts directly on muscarinic receptors localized at the level of the L cell. Finally, a direct role for muscarinic receptors in regulating GLP-1 secretion in the rat was established using FRIC cultures. This study confirmed the results of the *in vivo* experiments, in that the M1 receptor was found to be the major regulator of GLP-1 secretion from the L cell *in vitro*. Thus, although both the rat ileal L cell and the fetal rat intestinal L cell express multiple subtypes of muscarinic receptors, only the M1 receptor appears to play a role in GLP-1 secretion in adult rats. These findings are consistent with the mechanism of action of the M1 muscarinic receptor to activate PLC/calcium (30), a pathway that is known to stimulate GLP-1 secretion from the L cell (12, 15).

Interestingly, in FRIC cultures both M1 and M2 receptors seem to be important in regulating GLP-1 secretion, suggesting that there are some differences in the control of the L cell between fetal and adult rats. Furthermore, the M3 receptor antagonist, 4-DAMP, significantly stimulated GLP-1 secretion from FRIC cultures, whereas there was no effect of 4-DAMP on nutrient-induced GLP-1 secretion in the adult rat. As we were most interested in the factors regulating postprandial secretion of GLP-1, we did not determine the effect of any muscarinic antagonist on basal GLP-1 secretion. However, the findings with 4-DAMP suggest that either this antagonist exerts partial agonist activity on the M3 receptor in the fetal L cell or, alternatively, this receptor is inhibitory in the fetal, but not the adult, L cell.

The neuropeptide, GRP, has been shown to potently stimulate GLP-1 secretion *in vitro* as well as *in vivo* in humans and several animal species (14, 16, 17, 24, 27, 31). Consistent with a role for GRP in the regulation of postprandial GLP-1 secretion, the administration of a GRP antagonist was found to completely inhibit intraduodenal fat-induced GLP-1 secretion *in vivo* in rats (24). Furthermore, Persson *et al.* (31) have shown that mice lacking the GRP receptor have reduced GLP-1 and insulin responses as well as oral glucose intolerance, showing that intact GRP receptors are required for normal GLP-1 release. No study to date has reported on the interaction between the muscarinic and GRP systems in regulating GLP-1 secretion, but as both muscarinic and GRP antagonists completely inhibit fat-induced GLP-1 secretion *in vivo* in rats, these findings suggest that the two pathways are connected. When taken together with the results of studies of the role of the endocrine hormone GIP (18, 19) as well as of nicotinic receptors (1) in the regulation of GLP-1 secretion in the rat, the results of the present study lead us to propose the following pathway, as summarized in Fig. 6. Arrival of chyme in the duodenum stimulates GIP secretion by local endocrine K cells. This, in turn, stimulates the afferent vagus nerve to the central nervous system, thereby activating the efferent vagus (celiac) nerve to the ileum. Acting via a nicotinic synapse, this stimulates the release of GRP by intrinsic neurons, which, in turn, release acetylcholine from cholinergic neurons in the enteric nervous system to activate muscarinic receptors on the L cell. This complex interplay between nutrients in the duodenum and the ileal L cell, as mediated by neuro/endocrine modulators in the central and enteric nervous systems, thereby results in a highly integrated GLP-1 response to ingested nutrients.

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References

1. **Anini Y, Fu-Cheng X, Cuber JC, Kervran A, Chariot J, Roze C** 1999 Comparison of the postprandial release of peptide YY and proglucagon-derived peptides in the rat. Pflugers Arch 438:299–306
2. **Elliott RM, Morgan LM, Tredger JA, Deacon S, Wright J, Marks V** 1993 Glucagon-like peptide-1 (7–36)amide and glucose-dependent insulinotropic polypeptide secretion in response to nutrient ingestion in man: acute postprandial and 24-h secretion patterns. J Endocrinol 138:159–166
3. **Drucker DJ, Philippe J, Mojsov S, Chick WL, Habener JF** 1987 Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. Proc Natl Acad Sci USA 84:3434–3438
4. **Mojsov S, Weir GC, Habener JF** 1987 Insulinotropin: glucagon-like peptide I (7–37) co-encoded in the glucagon gene is a potent stimulator of insulin release in the perfused rat pancreas. J Clin Invest 79:616–619
5. **Kreymann B, Williams G, Ghatei MA, Bloom SR** 1987 Glucagon-like peptide-1 7–36: a physiological incretin in man. Lancet 5:1300–1304
6. **Komatsu R, Matsuyama T, Namba M, Watanabe N, Itoh H, Kono N, Tarui S** 1989 Glucagonostatic and insulinotropic action of glucagonlike peptide I-(7–36)-amide. Diabetes 38:902–905
7. **Schirra J, Katschinski M, Weidmann C, Schafer T, Wank U, Arnold R, Goke B** 1996 Gastric emptying and release of incretin hormones after glucose ingestion in humans. J Clin Invest 97:92–103
8. **Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD, Wilding JP, Smith DM, Ghatei MA, Herbert J, Bloom SR** 1996 A role for glucagon-like peptide-1 in the central regulation of feeding. Nature 379:69–72
9. **Stoffers DA, Keiffer TJ, Hussain MA, Drucker DJ, Bonner-Weir S, Habener JF, Egan JM** 2000 Insulinotropic glucagons-like peptide 1 agonists stimulate expression of homeodomain protein IDX-1 and increase islet size in mouse pancreas. Diabetes 49:741–748
10. **Nauck MA, Kleine N, Orskov C, Holst JJ, Willms B, Creutzfeldt W** 1993 Normalization of fasting hyperglycemia by exogenous glucagons-like peptide1(7–36)amide in type 2 (non insulino-dependent) diabetic patients. Diabetologia 36:741–744
11. **Drucker DJ** 2001 Development of glucagon-like peptide-1-based pharmaceuticals as therapeutic agents for the treatment of diabetes. Curr Pharm Des 11:1399–1412
12. **Brubaker PL** 1988 Control of glucagon-like immunoreactive peptide secretion from fetal rat intestinal cultures. Endocrinology 123:220–226
13. **Rocca AS, Brubaker PL** 1995 Stereospecific effects of fatty acids on proglucagon-derived peptide secretion in fetal rat intestinal cultures. Endocrinology 136:5593–5599
14. **Brubaker PL** 1991 Regulation of intestinal proglucagon-derived peptide secretion by intestinal regulatory peptides. Endocrinology 128:3175–3182
15. **Brubaker PL, Schloos J, Drucker DJ** 1998 Regulation of glucagon-like peptide-1 synthesis and secretion in the GLUTag enteroendocrine cell line. Endocrinology 139:4108–4114
16. **Dumoulin V, Dakka T, Plaisancie P, Chayvialle JA, Cuber JC** 1995 Regulation of glucagon-like peptide-1-(7–36) amide, peptide YY, and neurotensin secretion by neurotransmitters and gut hormones in the isolated vascularly perfused rat ileum. Endocrinology 136:5182–5188
17. **Plaisancie P, Bernard C, Chayvialle JA, Cuber JC** 1994 Regulation of glucagon-like peptide-1-(7–36) amide secretion by intestinal neurotransmitters and hormones in the isolated vascularly perfused rat colon. Endocrinology 135: 2398–2403
18. **Rocca AS, Brubaker PL** 1999 Role of the vagus nerve in mediating proximal nutrient-induced glucagon-like peptide-1 secretion. Endocrinology 140:1687–1694
19. **Roberge JN, Brubaker PL** 1993 Regulation of intestinal proglucagon-derived peptide secretion by glucose-dependent insulinotropic peptide in a novel enteroendocrine loop. Endocrinology 133:233–240
20. **Roberge JN, Brubaker PL** 1991 Secretion of proglucagon-derived peptides in response to intestinal luminal nutrients. Endocrinology 128:3169–174
21. **Fu-Cheng X, Anini Y, Chariot J, Castex N, Galmiche JP, Roze C** 1997 Mechanisms of peptide YY release induced by an intraduodenal meal in rats: neural regulation by proximal gut. Pflugers Arch 433:571–579
22. **Abello J, Ye F, Bosshard A, Bernard C, Cuber JC, Chayvialle JA** 1994 Stimulation of glucagon-like peptide-1 secretion by muscarinic agonist in a murine intestinal endocrine cell line. Endocrinology 134:2011–2017
23. **Balks HJ, Holst JJ, von zur Muhlen A, Brabant G** 1997 Rapid oscillations in plasma glucagon-like peptide-1 (GLP-1) in humans: cholinergic control of GLP-1 secretion via muscarinic receptors. J Clin Endocrinol Metab 82:786–790
24. **Roberge JN, Gronau KA, Brubaker PL** 1996 Gastrin-releasing peptide is a novel mediator of proximal nutrient-induced proglucagon-derived peptide secretion from the distal gut. Endocrinology 137:2383–2388
25. **Steinle JJ, Smith PG** 2000 Presynaptic muscarinic facilitation of parasympathetic neurotransmission after sympathectomy in the rat choriod. J Pharmacol Exp Ther 294:627–632
26. **Huang THK, Brubaker PL** 1995 Synthesis and secretion of glucagon-like peptide-1 by fetal rat intestinal cells in culture. Endocrine 3:499–503
27. **Herrmann-Rinke C, Voge A, Hess M, Goke B** 1995 Regulation of glucagon-like peptide-1 secretion from rat ileum by neurotransmitters and peptides. J Endocrinol 147:25–31
28. **D'Alessio DA, Kieffer TJ, Taborsky Jr GJ, Havel PJ** 2001 Activation of the parasympathetic nervous system is necessary for normal meal-induced insulin secretion in rhesus macaques. J Clin Endocrinol Metab 86:1253–1259
29. **Levey AI** 1993 Immunological localization of m1–m5 muscarinic acetylcholine receptors in peripheral tissues and brain. Life Sci 52:441–448
30. **Eglen RM, Choppin A, Watson N** 2001 Therapeutic opportunities from muscarinic receptor research. Trends Pharmacol Sci 22:409–414
31. **Persson K, Gingerich RL, Nayak S, Wada K, Wada E, Ahren B** 2000 Reduced GLP-1 and insulin responses and glucose intolerance after gastric glucose in GRP receptor-deleted mice. Am J Physiol 279:E956–E962