Neural regulation of glucagon-like peptide-1 secretion in pigs

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Hansen, Lene, Sarah Lampert, Hitoshi Mineo, and Jens J. Holst. Neural regulation of glucagon-like peptide-1 secretion in pigs. Am J Physiol Endocrinol Metab 287: E939–E947, 2004; doi:10.1152/ajpendo.00197.2004.—Glucagon-like peptide (GLP)-1 is secreted rapidly from the intestine postprandially. We therefore investigated its possible neural regulation. With the use of isolated perfused porcine ileum, GLP-1 secretion was measured in response to electrical stimulation of the mixed, perivascular nerve supply and infusions of neuroactive agents alone and in combination with different blocking agents. Electrical nerve stimulation inhibited GLP-1 secretion, an effect abolished by phentolamine. Norepinephrine inhibited secretion, and phentolamine abolished this effect. GLP-1 secretion was stimulated by isoproterenol (abolished by propranolol). Acetylcholine stimulated GLP-1 secretion, and atropine blocked this effect. Dimethylphenylpiperazine stimulated GLP-1 secretion. In chloralose-anesthetized pigs, however, electrical stimulation of the vagal trunks at the level of the diaphragm had no effect on GLP-1 or GLP-2 and weak effects on plasma glucose and GLP-1 to an oral glucose load and reduces the GLP-1 response (5). Studies using the human NCI-H716 cell line (1, 37) have shown that cholinergic agonists stimulated GLP-1 release and suggested that M1 and M2 muscarinic receptors are involved. Studies using the human NCI-H716 cell line (1, 37) have shown that cholinergic agonists stimulated GLP-1 release and suggested that M1 and M2 muscarinic receptors are involved. Studies using the human NCI-H716 cell line (1, 37) have shown that cholinergic agonists stimulated GLP-1 release and suggested that M1 and M2 muscarinic receptors are involved. Studies using the human NCI-H716 cell line (1, 37) have shown that cholinergic agonists stimulated GLP-1 release and suggested that M1 and M2 muscarinic receptors are involved.

GLUCAGON-LIKE PEPTIDE (GLP)-1 is a peptide primarily produced in the lower part of the gut (reviewed in Ref. 22). It is known as an incretin hormone (stimulating insulin secretion) (27) and thought to be part of the “ileal brake” mechanism (inhibition of upper gut motility and secretion elicited by the presence of unabsorbed nutrients in the ileum) (28, 32, 44). Thus infusions of GLP-1 have been shown to reduce appetite and energy intake in humans (14). Relatively little is known about the mechanisms that regulate GLP-1 secretion in pigs and humans. The presence of unabsorbed nutrients in the lumen seems to be an important stimulus for GLP-1 secretion in rats (38), pigs (23), and humans (28). However, the response to a meal is usually rapid, with increases in the plasma concentration occurring within a few minutes after the start of meal ingestion (6, 10, 24, 34, 38), before the bulk of the meal is thought to have reached the lower gut. This suggests that a neural and/or an endocrine pathway from the upper part of the gastrointestinal tract to the lower gut may exist.

In rats, glucose-dependent insulinotropic peptide (GIP) has been shown to stimulate GLP-1 secretion (9, 20, 35, 38), although recent studies have indicated that a neural pathway involving the vagus nerve (2, 39) might predominate compared with a direct endocrine pathway. Infusions of muscarinic cholinergic agonists into isolated perfused rat ileum and colon resulted in stimulation of GLP-1 secretion (9, 19, 35), and studies in anesthetized rats and in fetal rat intestinal cells suggested that both M1 and M2 muscarinic receptors could be involved in control of GLP-1 release (2). Catecholamines could also be part of a neural stimulatory pathway in rats, as infusion of a β-adrenergic agonist stimulated GLP-1 secretion in isolated perfused rat ileum and colon (9, 35).

In humans and pigs, none of the known duodenal peptides (including GIP), in normal physiological postprandial concentrations, are capable of stimulating GLP-1 secretion (13, 17, 31). Infusion of atropine in humans delays the increase of both plasma glucose and GLP-1 to an oral glucose load and reduces the GLP-1 response (5). Studies using the human NCI-H716 cell line (1, 37) have shown that cholinergic agonists stimulated GLP-1 release and suggested that M1 and M2 muscarinic receptors are involved. In isolated perfused porcine ileum, we have previously found that GLP-1 secretion could be increased by infusion of acetylcholine (ACh) during coinfusion of phenolamine (an α-adrenergic blocker) (16). All this suggests that ACh could be a transmitter in a neural stimulatory pathway for GLP-1 secretion. However, a more recent study in conscious pigs (6) indicated that the vagus nerve is not involved in control of GLP-1 release.

We therefore investigated the neural regulation of GLP-1 secretion in pigs, using isolated perfused preparations of porcine ileum as an experimental model. We studied the effects of different neurotransmitters as well as electrical stimulation of periarterial intestinal nerves, alone and in combination with pharmacological blockade of different neural receptors. Finally, we studied the effects of electrical stimulation of the vagus nerves on the secretion of GLP-1 in anesthetized pigs.

MATERIALS AND METHODS

This study conformed to the Danish legislation governing animal experimentation (1987) and was carried out after permission was granted by the Animal Experiments Inspectorate of the Danish Ministry of Justice.

Perfusion Experiments

Danish LYY strain pigs (15–20 kg) were fasted overnight but allowed free access to drinking water. After premedication with ketamine (10 mg/kg Ketalar; Parke-Davis, Morris Plains, NJ) and induction with Penithal (250 mg tiomebumal-sodium; SAD, Copenhagen, Denmark), the animals were anesthetized with α-chloralose (100 mg/kg; Merck, Darmstadt, Germany) and N2O-O2. This anesthesia is thought to have a minimal depressant effect on peripheral autonomic nervous activity. An 80-cm segment of the central ileum, including its arterial and venous supply, was isolated. During the isolation process, great care was taken to preserve all visible nerve fibers to the segment (these usually form a dense network around the...
supplying artery). The segment was perfused in a single pass system as previously described (4) with the use of a gassed (5% CO₂ in O₂) Krebs-Ringer-bicarbonate perfusion medium. The medium contained, in addition, 0.1% human serum albumin (Behringwerke, Marburg, Germany), 5% dextan T-70 (Pharmacia Biotech, Uppsala, Sweden), 7 mmol/l glucose, a mixture of amino acids (5 mmol/l Vamin; Pharmacia Biotech), and 15–20% freshly washed bovine erythrocytes (obtained 2–3 days earlier at a local abattoir). A cycloxygenase inhibitor (1 mg/ml indomethacin, Conforidit; Dumex, Copenhagen, Denmark) was added to the medium to prevent generation of prostaglandins in the perfusion system. The gut lumen was perfused with preheated perfusate (without erythrocytes) at 3 ml/min. A bipolar platinum electrode, shaped like a hook, was carefully positioned around the supplying artery and the network of nerve fibers surrounding it, without damage to the fibers, and was kept in place by loose ligature. Perfusion pressure was recorded constantly via a sidearm to the arterial catheter by a Statham transducer connected to an amplifier and a recorder. Motor activity of the gut was estimated visually, but contractions of the gut were also reliably reflected as short-lasting increments in perfusion pressure (spikes), validated previously in experiments involving intraluminal manometry (42). The frequency of such increments (which did not increase the basal perfusion pressure) was recorded. The venous effluent was collected for 1-min periods and centrifuged at 4°C within a few minutes. The supernatants were frozen immediately for subsequent radioimmunoassay.

**Experimental protocol.** Preparations of isolated perfused ileum were prepared from a total of 50 pigs. In all experiments, a 30-min equilibration period was allowed at the start. The experimental protocol consisted of a primary stimulus applied alone and in combination with different pharmacological blocking agents, and, in addition, a control experiment was carried out.

**Primary stimulus.** Electrical nerve stimulation of periarterial nerves was carried out using frequencies of 4, 8, and 16 Hz (all 10 mA, 4-ms impulse duration). A frequency of 8 Hz resulted in an ~50% increase in perfusion pressure, confirming activation of the sympathetic innervation. A higher frequency (16 Hz) did not result in a further increase in perfusion pressure, whereas a lower frequency (4 Hz) increased perfusion pressure by only ~25%. Therefore, 8 Hz were chosen for most experiments. ACh (final concentration 10⁻⁶ M; Sigma, St. Louis, MO) was infused for activation of cholinergic receptors, dimethylphenylpiperazine (DMPP; final concentration 10⁻³ M; Sigma) for activation of nicotinic cholinergic receptors, isoprotrenol (Iso; final concentration 10⁻⁵ M; DAK Laboratories, Copenhagen, Denmark) for activation of β-adrenergic receptors, and, finally, nor-epinephrine (NE; final concentration 10⁻⁶ M; DAK Laboratories) for activation of α-adrenergic receptors.

**Blocking agents.** Phentolamine (final concentration 10⁻⁵ M Regitin; Novartis Healthcare, Copenhagen, Denmark) was employed for α-adrenergic blockade, propranolol (final concentration 10⁻⁵ M; Nycomed, Roskilde, Denmark) for β-adrenergic blockade, and atropine (final concentration 10⁻⁶ M; SAD) for muscarinic cholinergic blockade. Hexamethonium (final concentration 3 × 10⁻⁵ M; Sigma) was used in combination with phentolamine and atropine to provide additional nicotinic cholinergic blockade.

**Protocol design.** Phentolamine was applied as first blocking agent in 24 experiments in combination with 8 Hz, ACh, and NE as primary stimulus. Atropine was applied as first blocking agent in 12 experiments with 8 Hz, ACh, and DMPP as primary stimulus. A combination of phentolamine and atropine was also applied, after administration of either atropine or phentolamine, with 8 Hz and ACh as primary stimulus. In three experiments, phentolamine and atropine were applied together as combined first blocking agents. Hexamethonium was applied as third blocking agent in addition to both phentolamine and atropine. Propranolol was applied as first blocking agent in 11 experiments with 8 Hz, ACh, and Iso as primary stimulus. All primary stimuli were applied for 5-min periods with at least 10 min separating the different primary stimuli and in random order. The blocking agents were applied at least 10 min before the primary stimulus (for atropine, at least 20 min before). The blockade was continued to the end of the experiment when first started.

**Control experiment.** In 14 of the perfusion experiments, a control stimulation was carried out to evaluate the relative magnitude of the changes elicited by nerve stimulation. As control stimulus we used neomedin C (NC; the COOH-terminal decapptide of gastrin-releasing polypeptide or "mammalian bombesin"); Peninsula Europe, Merseyside, St. Helens, UK) infused at 10⁻⁸ M (final concentration). NC has earlier been reported to be a strong stimulus for GLP-1 secretion (7, 9, 16, 20, 33, 35).

**Analysis of the perfusion effluent.** All effluent fractions were analyzed for GLP-1 by use of a previously described radioimmunoassay (34). The assay employs the antibody 89390, which has an absolute requirement for the amidated COOH terminus of the GLP-1 molecule and therefore recognizes both GLP-1 (7–36) amide and the inactive metabolite GLP-1 (9–36) amide, both of which are released in response to activation of the L cells (15).

**Calculations and statistical evaluation.** Because of the constant perfusion flow, the hormone effluent concentrations parallel secretion rates. Changes in GLP-1 secretion and perfusion pressure are presented as percentage of basal outputs (mean of 5-min periods imme-
GLP-1 secretion, perfusion pressure, and motor activity

Table 1. GLP-1 secretion, perfusion pressure, and motor activity

<table>
<thead>
<tr>
<th></th>
<th>GLP-1 Secretion</th>
<th>Perfusion Pressure</th>
<th>Motor Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean ± SE (%) of basal</td>
<td>P value</td>
</tr>
<tr>
<td>4 Hz</td>
<td>10</td>
<td>122 ± 10.8 ± 8.9</td>
<td>0.061</td>
</tr>
<tr>
<td>8 Hz</td>
<td>37</td>
<td>84 ± 2.3 ± 6.9 ± 6.9</td>
<td>0.000</td>
</tr>
<tr>
<td>16 Hz</td>
<td>19</td>
<td>79 ± 9.3</td>
<td>0.016</td>
</tr>
<tr>
<td>8 Hz + phentolamine</td>
<td>19</td>
<td>106 ± 5.8 ± 5.8 ± 5.8</td>
<td>0.249</td>
</tr>
<tr>
<td>8 Hz + propranolol</td>
<td>5</td>
<td>79 ± 6.9 ± 6.9 ± 6.9</td>
<td>0.020</td>
</tr>
<tr>
<td>8 Hz + atropine</td>
<td>12</td>
<td>97 ± 3.6</td>
<td>0.457</td>
</tr>
<tr>
<td>8 Hz + phentolamine + atropine</td>
<td>21</td>
<td>107 ± 4.2 ± 4.2 ± 4.2</td>
<td>0.066</td>
</tr>
<tr>
<td>8 Hz + phentolamine + atropine + hexamethonium</td>
<td>13</td>
<td>108 ± 5.2 ± 5.2 ± 5.2</td>
<td>0.091</td>
</tr>
<tr>
<td>ACh</td>
<td>16</td>
<td>153 ± 15.4 ± 15.4 ± 15.4</td>
<td>0.003</td>
</tr>
<tr>
<td>ACh + phentolamine</td>
<td>10</td>
<td>145 ± 8.3 ± 8.3 ± 8.3</td>
<td>0.001</td>
</tr>
<tr>
<td>ACh + propranolol</td>
<td>5</td>
<td>151 ± 17.0 ± 17.0 ± 17.0</td>
<td>0.036</td>
</tr>
<tr>
<td>ACh + atropine</td>
<td>9</td>
<td>89 ± 6.2 ± 6.2 ± 6.2</td>
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<tr>
<td>ACh + phentolamine + atropine</td>
<td>8</td>
<td>101 ± 5.4 ± 5.4 ± 5.4</td>
<td>0.925</td>
</tr>
<tr>
<td>ACh + propranolol + hexamethonium</td>
<td>7</td>
<td>99 ± 5.4 ± 5.4 ± 5.4</td>
<td>0.913</td>
</tr>
<tr>
<td>DMPP</td>
<td>15</td>
<td>115 ± 2.6</td>
<td>0.027</td>
</tr>
<tr>
<td>DMPP + atropine</td>
<td>7</td>
<td>99 ± 2.4</td>
<td>0.788</td>
</tr>
<tr>
<td>Iso</td>
<td>6</td>
<td>130 ± 3.3</td>
<td>0.000</td>
</tr>
<tr>
<td>Iso + propranolol</td>
<td>6</td>
<td>106 ± 8.3</td>
<td>0.463</td>
</tr>
<tr>
<td>NE</td>
<td>15</td>
<td>83 ± 4.8</td>
<td>0.001</td>
</tr>
<tr>
<td>NE + phentolamine</td>
<td>6</td>
<td>117 ± 9.9</td>
<td>0.125</td>
</tr>
</tbody>
</table>

4, 8, and 16 Hz, 4-, 8-, and 16 Hz nerve stimulation; DMPP, diisopropylfluorophosphate; Iso, isoproterenol; NE, norepinephrine; GLP, glucagon-like peptide. aP < 0.05 compared with response to 8 Hz or ACh or DMPP or Iso or NE. bP < 0.05 compared with response to 8 Hz + phentolamine or ACh + phentolamine or NE + phentolamine. cP < 0.05 compared with response to 8 Hz + propranolol or ACh + propranolol or ISO + propranolol. dP < 0.05 compared with response to 8 Hz + atropine or ACh + atropine or DMPP + atropine. eP < 0.05 compared with response to 8 Hz + phentolamine + atropine + hexamethonium or ACh + phentolamine + atropine + hexamethonium. fANOVA, P < 0.001, post hoc: effect of 4 Hz is significantly different from 8 Hz and 16 Hz (both P < 0.001). gANOVA, P < 0.05, post hoc: effect of 4 Hz is significantly different from 16 Hz (P < 0.01) and effect of 4 Hz compared to 8 Hz (P = 0.059). hANOVA, P < 0.001, post hoc: effect of 16 Hz is significantly different from 4 and 8 Hz (both P < 0.001). iLow amplitude.

Nervous control of GLP-1 secretion

Data were collected at −15, −10, −5, 0, 1, 2, 3, 4, 5, 7, 10, and 15 min. Nerve stimulation was carried out from 1 to 5 min, and the protocol was repeated after administration of atropine (5 mg atropine; SAD). In one experiment, nerve stimulation was only carried out after atropine.

Plasma analysis. Plasma samples were analyzed for GLP-1 as above and, in addition, for GIP, GLP-2, vasodepressin intestinal polypeptide (VIP), and somatostatin with the use of previously described specific radioimmunoassays (3, 12, 18, 26, 34). The GIP assay was carried out using antiserum R65, which recognizes all molecules containing the central sequence of GIP regardless of COOH- or NH2-terminal truncations or extensions. The GLP-2 assay was carried out using antiserum 92160, which recognizes the NH2-terminus of GLP-2 and, therefore, only fully processed, biologically active GLP-2 molecules. Somatostatin was assayed using antiserum 1758, which recognizes both somatostatin-14 and -28. The detection limits of all assays were <5 pmol/l.

Calculations and statistical evaluation. All results are expressed as means ± SE. Changes in concentrations due to nerve stimulation are presented as the mean responses (time points 1–5 min) in percentage over basal. Basal values are defined as the means of results from time points −15, −10, −5, and 0 min. Statistical analysis was performed using nonparametric statistics. The effects of splanchnicotomy were analyzed by comparing basal and responses to nerve stimulation in percentage by using the Mann-Whitney U-test. The significance of an observed effect due to nerve stimulation during the experiment was tested using Friedman ANOVA, followed by testing each time point against basal using Wilcoxon matched-pair test. The significance of the response to nerve stimulation in percentage was tested against 100%, and the difference between portal and arterial blood was tested.
both by use of Wilcoxon matched-pair test. The significance of the effects of atropine was tested using the Mann-Whitney U-test.

**Statistics**

All calculations were performed using the software STATISTICA (STATISTICA for Windows; StatSoft, Tulsa, OK). Data are presented as means ± SE. *P* values < 0.050 were considered significant.

**RESULTS**

**Perfusion Experiments**

In the basal, nonstimulated state at the start of the experiments (*n* = 50), the effluent concentration of GLP-1 averaged 25.4 ± 2.8 pmol/l (corresponding to a secretion rate of 609.8 ± 67.6 fmol/min), the perfusion pressure was 43.3 ± 1.5 mmHg, and the motor activity was 1.78 ± 0.20 spikes/min.

*Control experiment.* Infusion of NC at $10^{-8}$ M (*n* = 14, Fig. 1) strongly stimulated GLP-1 secretion to a mean of 392 ± 51% (*P* < 0.001) compared with basal. NC also increased perfusion pressure to 106 ± 2% (*P* = 0.009) of basal pressure and increased motor activity to 6.01 ± 0.47 spikes/min (*P* < 0.001 compared with basal activity).

*Effects of pharmacological blockers in the basal state.* The intrinsic effects of the different pharmacological blocking agents were investigated in the experiments where they were given as first blocking agent. GLP-1 secretion was not significantly affected by addition of phentolamine, propranolol, atropine, and the combination of atropine and phentolamine compared with preinfusion levels. Perfusion pressure was increased slightly by phentolamine (2.68 ± 0.74 mmHg, *P* < 0.010), propranolol (3.62 ± 1.41 mmHg, *P* < 0.050), and atropine (1.27 ± 0.47 mmHg, *P* < 0.050), whereas the combination of atropine and phentolamine had no effect (2.4 ± 1.44 mmHg, *P* = 0.238), compared with preinfused levels. Motor activity was strongly inhibited by atropine (by 1.51 ± 0.45 spikes/min, *P* < 0.010) compared with preinfusion levels. Addition of hexamethonium had no additional effect. Neither phentolamine nor propranolol had any effect on motor activity.

*Nerve stimulation.* The effects of different frequencies are shown in Table 1. Compared with basal prestimulatory levels, electrical nerve stimulation at 4 Hz had no significant effect, whereas both 8- and 16-Hz nerve stimulation inhibited GLP-1
secretion. Comparison of the effects on GLP-1 secretion of the different frequencies by ANOVA (Table 1) showed that the effect of 4 Hz was significantly different from both 8 and 16 Hz, whereas there was no significant difference between 8 and 16 Hz. Perfusion pressure was significantly increased by all frequencies compared with basal levels, and comparison of the different effects by ANOVA showed that the effect of 4 Hz was significantly lower than the effect of 16 Hz (Table 1). Motor activity was not significantly affected by the different frequencies compared with prestimulatory levels. However, comparing the effects by ANOVA (Table 1) showed that motor activity was increased during 16-Hz compared with both 4- and 8-Hz stimulation.

The effects of the different pharmacological blocking agents in combination with 8-Hz nerve stimulation are shown in Table 1 and Fig. 2. Addition of phentolamine during 8-Hz nerve stimulation (Table 1 and Fig. 2) stimulated motor activity (P < 0.001), abolished the inhibition of GLP-1 secretion (P < 0.010), and strongly reduced the effect on perfusion pressure (P < 0.010) compared with the effect of 8 Hz alone. Further addition of atropine to the combination of phentolamine and 8-Hz nerve stimulation did not change the effect on GLP-1 secretion or perfusion pressure but decreased motor activity compared with 8 Hz plus phentolamine (P < 0.001), although motor activity was still increased compared with prestimulatory levels (P < 0.001). Addition of hexamethonium together with phentolamine and atropine during 8-Hz nerve stimulation had no further effect on GLP-1 secretion, motor activity, or perfusion pressure. Addition of only atropine during 8-Hz nerve stimulation (Table 1) abolished the inhibitory effect of 8-Hz nerve stimulation on GLP-1 secretion (P < 0.050 compared with the effect of 8 Hz alone). Addition of only propranolol slightly decreased perfusion pressure compared with the effect of 8-Hz nerve stimulation alone (P < 0.050) but did not change the effect on GLP-1 secretion and motor activity.

ACh. Infusion of ACh stimulated GLP-1 secretion and perfusion pressure (Fig. 3 and Table 1) and increased motor activity (Table 1). Coinfusion of atropine (Fig. 3 and Table 1) abolished the effect on GLP-1 secretion (P < 0.050) and tended to reduce the motor activity (P = 0.069). Neither addition of phentolamine nor of propranolol nor of hexamethonium influenced the effect of ACh infusion alone or the effect of ACh in combination with atropine.

DMPP. Infusion of DMPP increased GLP-1 secretion, perfusion pressure, and motor activity (Table 1). Coinfusion of atropine tended to reduce the effect on GLP-1 secretion (P = 0.066). Atropine abolished the effect on motor activity (P < 0.001) but did not change the effect on perfusion pressure.

Iso. Infusion of Iso (Table 1) stimulated GLP-1 secretion and decreased perfusion pressure but had no effect on motor activity. Addition of propranolol abolished the effect of Iso on GLP-1 secretion (P < 0.050) and perfusion pressure (P < 0.001) compared with infusion of Iso alone.

NE. Infusion of NE (Table 1) inhibited GLP-1 secretion while increasing perfusion pressure. Coinfusion of phentolamine abolished the effect on GLP-1 secretion and perfusion pressure (both P < 0.001). NE alone or in combination with phentolamine had no effect on motor activity.

In Vivo Experiments

Splanchnicotomy had a minimal effect on the hormone results, if any. In the following, the results from experiments with cut and intact splanchnic nerves were therefore combined.
A summary of the results of electrical stimulation of the vagus nerves is shown in Table 2 and Figs. 4–6. Portal plasma concentrations of the hormones were generally higher ($P = 0.01–0.05$) than the arterial plasma concentrations. Atropine decreased basal VIP concentration in both portal venous and arterial plasma and also increased heart frequency (for all, $P = 0.028$). For values see Table 2. However, addition of atropine had no significant effects on the other measured parameters.

As shown in Fig. 4 and in Table 2, VIP release increased markedly in response to electrical stimulation of the vagus nerve. There was no significant effect of atropine, but the portal response was larger than the arterial response (artery vs. portal vein: $P = 0.028$ without atropine, and $P = 0.018$ with atropine).

The GIP response is shown in Fig. 5 and Table 2. There was a late, small increase in the experiments without atropine that reached significance at 7–15 min in the artery (to $152 \pm 18\%$, $P = 0.028$) and in the portal vein (to $139 \pm 14\%$, $P = 0.046$) and an even smaller arterial response after atropine (to $114 \pm 5\%$, $P = 0.046$), whereas the portal vein response was insignificant ($106 \pm 4\%$, $P = 0.091$), compared with prestimulatory basal release.

The somatostatin response to vagus stimulation is shown in Fig. 6 and Table 2. There was a small, significant increase in the arterial plasma concentration in the experiments without atropine at times of 5–15 min (increase $139 \pm 9\%$, $P = 0.028$ compared with basal, prestimulatory values by Friedman analysis) but no changes after atropine and in portal venous plasma.

GLP-1 and GLP-2 responses are shown in Table 2, but neither was affected by nerve stimulation.

Blood pressure decreased during nerve stimulation (Table 2) both without and with atropine. Heart frequency (Table 2) was unaffected by vagal stimulation.

**DISCUSSION**

The present study was carried out to evaluate the importance of the extrinsic neural regulation of the intestinal secretion of GLP-1 in pigs. As mentioned above, several experimental findings have suggested that neural regulation could be of importance, and a variety of neuroactive agents have been...
shown to influence GLP-1 secretion in previous studies (7, 9, 19, 35). We chose for our studies an experimental model, the isolated perfused porcine ileum, that, judging from a series of previous studies, should be particularly suitable for this purpose. First of all, pigs appear to have a meal response of GLP-1 secretion (24) similar to that of humans. Because of this and numerous other similarities between porcine and human physiology (21, 30), we considered pigs to constitute relevant animal models. Second, the perfused ileum has been shown to release large amounts of GLP-1 in response to introduction of glucose into the lumen (15, 33). This corresponds well to observations in humans, where intestinal carbohydrate administration is known to constitute a strong stimulus for GLP-1 secretion (10, 28). Third, this preparation has been instrumental in elucidating the release and effects of a variety of transmitters of the enteric nervous system, including tachykinins, galanin, VIP, and calcitonin gene-related peptide (36, 40, 41). These studies also indicated that several components of the intestinal innervation might be activated on electrical stimulation of the mixed periarterial nerves supplying the prepara-
tion. Vascular and motor reactions of the preparation before and after application of pharmacological blocking agents indicated that electrical nerve stimulation strongly activates sympathetic nerve fibers, the effects of which can be removed by α-adrenergic blockade. Under the latter condition, excitatory cholinergic and noncholinergic mechanisms become unmasked and are then amenable to further pharmacological analysis. For instance, the release of the neuropeptide VIP, the most abundant neuropeptide of the gut, is inhibited by nerve stimulation, but the response is reversed to a marked increase after α-adrenergic blockade (29). Further muscarinic as well as nicotinic antagonism will abolish this secondary response, revealing its cholinergic nature. We were therefore confident that by applying the same experimental approach in these studies of GLP-1 secretion in pigs, it would be possible to reveal the nature and extent of its neural regulation.

To evaluate the significance of the observed responses, we included intra-arterial infusions of the neuropeptide NC. NC is a generally excitatory neuropeptide that is widely distributed in the enteric nervous system and has been demonstrated to cause a strong release of GLP-1 in vitro (7, 9, 20, 32, 35). Indeed, in the present series of experiments, NC caused a highly significant fourfold augmentation of GLP-1 secretion. In previous experiments, it was found that neutralization of the inhibitory effects of paracrine somatostatin would cause a similar increase in GLP-1 secretion (16). Stronger stimuli for GLP-1 secretion in pigs are not known, and therefore, as a working hypothesis, we consider the response to NC as being close to a maximal response.

However, recognizing the complexity of the response to mixed intestinal nerve stimulation, we also included a series of in vivo experiments, allowing selective and strong stimulation of the vagal nerve trunks at a site where the stimulation does not directly influence cardiac function (at the level of the gastroesophageal junction). This was done without and with sectioning of the splanchnic nerves (reducing sympathetic effects elicited by nerve stimulation) and without and with atropine to expose noncholinergic mechanisms.

The major result of electrical stimulation of the mixed intestinal nerves was an α-adrenergic inhibition of secretion (because it could be abolished by phentolamine), interpreted to reflect activation by nerve stimulation of postganglionic sympathetic nerve fibers and release of NE and activation of α-adrenergic inhibitory receptors. In agreement with this interpretation, NE alone also inhibited GLP-1 secretion. We could confirm a stimulatory effect of β-adrenergic stimulation (8, 9, 35), but removal of the α-adrenergic inhibition did not result in a stimulated secretion during nerve stimulation, indicating that β-adrenergic activation via NE release from sympathetic nerve endings does not play a role in GLP-1 secretion. The target cells for NE effects on the gut are often thought to be neurons of the enteric plexuses, but an additional inhibitor of GLP-1 secretion that could transmit the effects of NE has not been identified. Could it be somatostatin? Somatostatin inhibits GLP-1 secretion but is released by nerve stimulation after α-adrenergic blockade (but not before) (16) and is therefore an unlikely transmitter. Rather, inhibition of intrinsic cholinergic neurons could explain the effect of NE and nerve stimulation (see below).

Thus ACh at $10^{-6}$ M stimulated GLP-1 secretion by 153%, an effect that was unaffected by phentolamine addition but
abolished by atropine. This concentration of ACh is considered maximal for activation of cholinergic effects and causes strong motor activity and a release of large amounts of transmitter such as VIP (29). Higher concentrations (10^{-5} M) elicit tonic contractions of the gut and rising perfusion pressures and are considered unphysiological. Compared with NC, ACh is less potent and less efficacious but still could explain the fast GLP-1 response to a meal. However, nerve stimulation after α-adrenergic blockade did not result in a stimulation of GLP-1 secretion. We interpret this to indicate that extrinsic cholinergic excitatory mechanisms for GLP-1 secretion are of minor significance. This notion is strongly supported by the results of the selective stimulation of the vagus nerves in vivo. In these experiments, the nerve stimulation, as expected (11), elicited a marked atropine-resistant release of VIP, attesting to the successful activation of the vagus nerves. But again, there was no effect on GLP-1 secretion. The measurements of GLP-2 were included because in pigs, part of GLP-1 is processed to GLP-1-(7–37) rather than GLP-1-(7–36) amide (16) and might therefore escape detection. However, GLP-2 secretion was also unaffected. In view of the proposition that GLP-1 might be released secondary to a duodenal release of GIP (2), we also measured GIP. Indeed, there was a small but rather late release of GIP that was accompanied by neither a release of GLP-1 nor a release of GLP-2. In addition, because a release of somatostatin could conceal a possible GLP-1 response (16), we also measured somatostatin. However, only very small changes in somatostatin concentrations were observed during the experiments without atropine, none were observed with atropine, and neither experiment was accompanied by an effect on GLP-1 secretion.

By sampling from the portal vein, we made sure that even very small effects of nerve stimulation would be detected. In addition, we used a frequency that in a previous experiment elicited near-maximal effects (11). It is of interest that VIP at 10^{-5} M actually stimulates GLP-1 secretion from our perfused ileum preparation (Holst JJ, unpublished studies), but, despite the large release of VIP during nerve stimulation, there was no GLP-1 secretion. VIPergic neurons, essential for hyperemic responses and for descending relaxation in peristalsis, therefore, do not appear to participate in an extrinsic neural pathway for GLP-1 secretion. The same seems to apply to NC (and gastrin-releasing peptide), which is also released during vagus stimulation in pigs (25).

Although the extrinsic parasympathetic innervation thus seems to be of little importance for GLP-1 secretion in pigs, its secretion may still be regulated by neurons of the intrinsic enteric nervous system. The stimulatory effects of ACh and the presence of muscarinic receptors on the L cells (1, 2) would be in agreement with this. A tonic stimulatory effect of GLP-1 secretion by cholinergic intrinsic neurons could also explain the loss of inhibitory effects of nerve stimulation after atropine, given that the inhibitory effects of sympathetic nerve stimulation are mainly exerted via inhibition of excitatory neurons of the enteric ganglia, as discussed above. Also, tachykinins stimulate the secretion of GLP-1 (43) and are released from the perfused porcine ileum during nerve stimulation plus cholecystokinin plus atropine (40). However, as shown in the present experiments, under these conditions there was no increase of GLP-1 secretion. The overall result of this study, therefore, is that in pigs, the extrinsic intestinal innervation is predominantly inhibitory with respect to GLP-1 secretion. Conversely, this implies that lessening of the sympathetic intestinal tone could provide a stimulus for GLP-1 secretion. In addition, GLP-1 secretion has been shown to be tightly controlled by paracrine, somatostatinergic mechanisms. Regulation of this paracrine activity, therefore, is an additional means for regulation of GLP-1 secretion. It is also clear that transmitters of the intrinsic enteric nervous system, notably ACh and possibly NC, increase secretion and may therefore play a role in GLP-1 secretion elicited by local reflexes. The conditions under which such a release might occur remain to be identified.

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