Functional vagal input to chemically identified neurons in pancreatic ganglia as revealed by Fos expression

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Wang, J Iuliuin, Huiyuan Zheng, and Hans-Rudolf Berthoud. Functional vagal input to chemically identified neurons in pancreatic ganglia as revealed by Fos expression. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E958–E964, 1999.—The importance of neural elements in the control of both endocrine and exocrine pancreatic secretory functions and their coordination with gastrointestinal, hepatic, and general homeostatic functions is increasingly recognized. To better characterize the vagal efferent input to the pancreas, the capacity of electrical vagal stimulation to induce expression of c-Fos in neurochemically identified neurons of intrapancreatic ganglia was investigated. At optimal stimulation parameters, unilateral stimulation of either the left or right cervical vagus induced Fos expression in ~30% of neurons in the head and 10–20% of neurons in the body and tail of the pancreas. There was no Fos expression if no stimulation or stimulation with a distally cut vagus was applied. Large proportions of neurons contained nitric oxide synthase as assessed with NADPH diaphorase histochemistry (88%) and choline acetyltransferase. The proportion of nitricergic and nonnitricergic neurons receiving vagal input was not different. It is concluded that a significant proportion of pancreatic neurons receives excitatory synaptic input from vagal preganglionic axons and that many of these vagal preganglionic neurons can produce nitric oxide and acetycholine.

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AS EARLY AS 1869, Langerhans observed that the pancreatic islets were innervated. Since then, neural elements in the various compartments of the mammalian pancreas have been extensively studied at the anatomic, neurochemical, and functional levels. Extrinsic motor innervation of the pancreas is provided by both the sympathetic nervous system, with noradrenergic postganglionic neurons containing also neuropeptide Y or galanin (8, 29) located in the celiac and superior mesenteric ganglia (11), and the parasympathetic system, with vagal preganglionic neurons located in the dorsal vagal nucleus in the medulla (11, 22). In addition, substance P and calcitonin gene-related peptide containing dorsal root afferents innervate blood vessels, islets, and acinar tissue (8, 29), whereas vagal afferents originating in the nodose ganglia innervate primarily pancreatic islets but not ganglia (19). Analogous to the myenteric and submucosal enteric nerve plexuses, there is an intrinsic pancreatic neural plexus, consisting of interlobular ganglia distributed throughout the organ and connected by intrapancreatic nerve bundles (6). The neurons in these ganglia could be considered parasympathetic (vagal) postganglionic neurons; however, they are likely to receive other inputs and to serve an overall pancreatic integrative function (25).

Vagal efferent innervation is thought to play an important role in both exocrine and endocrine pancreatic functions during the ingestion, digestion, and absorption of food. During the appetitive and cephalic consummatory phases of food ingestion, vagal activation has been demonstrated to produce the early phase of insulin secretion (16, 28). There is also recent evidence that pancreatic exocrine secretion activated by intestinal food via the release of secretin and cholecystokinin depends on intact vagal innervation to the pancreas (13). Moreover, the dorsal motor nucleus of the vagus may constitute an important link in negative feedback control of exocrine pancreas secretion by pancreatic polypeptide and other peptides (20). Complete characterization of the vagal efferent outflow to the pancreas is, therefore, important for understanding how such negative feedback signals operate.

Stimulation of vagal preganglionic fibers can directly affect pancreatic endocrine and exocrine secretion, as demonstrated in isolated perfused pancreas preparations with an intact vagal supply (3, 10) and other in vivo studies (4). Anatomically, the presence of vagal preganglionic axon terminals in interlobular pancreatic ganglia of the rat has been demonstrated after anterograde labeling of vagal motor neurons in the dorsal motor nucleus with the carbocyanine dye Dil (6). However, it is difficult to quantitatively determine vagal input to pancreatic ganglia, because labeling is rarely complete and because it is impossible to establish the presence of synaptic contacts on individually identified neurons with light microscopy.

The aims of the present investigation were to determine the quantitative aspect of functional vagal preganglionic input to intrapancreatic neurons and to identify some of the neurochemical phenotypes of such vagally innervated neurons. Electrical stimulation of the cervical vagi was used to stimulate all vagal preganglionic neurons, and the expression of the immediate-early gene product c-Fos was used as an indicator of excitatory synaptic input to postganglionic neurons. Fos immunohistochemistry was then combined with NADPH diaphorase histochemistry or choline-acetyltransferase immunohistochemistry to identify some of the neurochemical phenotypes of vagally activated neurons. This method was feasible because there was no spontaneous expression of Fos and because parallel studies in the gastrointestinal tract have shown that
most, if not all, enteric neurons can express Fos (32). The results of the present study show that a significant proportion of neurons, higher than indicated by the anatomic tracing method, receives excitatory vagal input, and that many of these postganglionic neurons are nitricergic and cholinergic.

**MATERIALS AND METHODS**

Adult male Sprague-Dawley rats weighing 200–300 g were housed in acrylic cages under standard laboratory conditions (12:12-h light-dark cycle, lights on at 0700, 22 ± 3°C), and normal laboratory chow and tap water were available ad libitum. Five days before tests, all animals received an injection of Fluorogold (1.5–2 mg in 1.2 ml sterile saline ip) to label all neurons in autonomic ganglia (21).

Rats were anesthetized with pentobarbital sodium (60–65 mg/kg ip) or a subcutaneous mixture of ketamine (100 mg/kg), xylazine (5 mg/kg), and acepromazine (2 mg/kg); atropine (1 mg/kg ip) was administered to block muscarinic cholinergic transmission; and the animal was put on a homeothermic heating blanket. When the animals were fully unresponsive to an ear pinch, a tracheal catheter (PE-90) was inserted to ease respiration. Then either the left (n = 27) or right (n = 5) cervical vagus was exposed by blunt dissection, and a silk suture was tied around the nerve just beneath the nodose ganglion. The nerve was then cut above the suture and laid on a bipolar platinum hook electrode held in place by a micromanipulator. The slightly elevated nerve-electrode assembly was then immersed in prewarmed paraffin oil. After a resting period of 10 min, electrical stimulation was started with rectangular pulses of 1- to 2-mA intensity and 0.5-ms pulse duration at 8 Hz (n = 3), 16 Hz (n = 6), 24 Hz (n = 10), or 48 Hz (n = 6), and pulse trains of 10 s on and 10 s off for 30 min. Control stimulation (n = 7) consisted of the same procedure, but before stimulation (16 or 24 Hz), the cervical vagal trunk was severed ~10 mm below the electrode. At the end of the stimulation, the wound was closed with a wound clip, and the rats were left untouched on the heating pad.

One hour after termination of stimulation, rats were euthanized with an overdose of pentobarbital sodium and transcardially perfused with 0.2 liter of heparinized (20 U/ml) cold phosphate buffered saline (pH 7.4) solution followed by 0.5 liter of 4% paraformaldehyde solution. The pancreas and other visceral organs were extracted, and the pancreas was divided into three parts about equal in size: the head, bordering the duodenum, pylorus, and antral stomach; the body, attached to the greater curvature of the gastric corpus; and the tail, attached to the spleen. After 4–15 h of postfixation in the same fixative, the samples were immersed in 25% sucrose-PBS overnight, mounted on a cryostat chuck, and serially sectioned at 25 µm in a cryostat. Every third section was mounted onto poly-L-lysine-coated slides and stored at −20°C until processing for immunocytochemistry.

Tissue sections were washed in PBS, and for NADPH diaphorase (NADPH-d) histochemistry they were incubated in 0.1 M phosphate buffer (pH 7.4) containing 0.3% Triton X-100, 0.5 mg/ml β-NADPH, and 0.1 mg/ml nitroblue tetrazolium (the latter two from Sigma Chemical) at 37°C for 1 h. After three rinses in PBS, the slides were then processed for c-Fos immunohistochemistry. Initially, the sections were pre-treated with a solution of 1.5% hydrogen peroxide, 20% methanol, and 0.2% Triton X-100 in PBS for 20 min to decrease endogenous peroxidase and then were rinsed with PBS. Sections were then immersed in a blocking solution (5% normal goat serum, 1% bovine serum albumin, and 0.5% Triton X-100 in PBS) for 2 h to inhibit nonspecific binding. The slides were incubated in rabbit c-fos primary antibody (AB-5, Oncogene Sciences, Cambridge, MA), which was diluted 1:12,500 for 40–60 h at 7°C in a humid chamber. Thereafter, sections were washed in PBS-GEL (0.1% gelatin in PBS) and incubated for 2 h at room temperature in biotinylated secondary antibody (goat anti-rabbit IgG, Jackson Immunoresearch Labs, West Grove, PA) at 1:500 dilution. After three rinses, the sections were incubated for 90 min in an avidin-biotin complex (Vectastain ABC Elite kit, Vector Labs) at one-half of the recommended dilution and visualized using a Ni-enhanced DAB substrate kit (Pierce Chemical, Rockford, IL), after which the slides were again washed three times in PBS.

Sections to be double-labeled for choline acetyltransferase were not stained for NOS but were subjected to the c-Fos protocol described in the previous paragraph. After the final PBS rinse following DAB, the slides were incubated in a rabbit polyclonal antibody [anti-choline acetyl transferase (ChAT) Peptide 3, gift of Dr. Michael Schemann, Hannover, Germany], which was diluted to 1:500 overnight at 7°C in the humid chamber. The next day, the sections were washed in PBS-GEL three times before incubation in Texas Red-conjugated secondary antibody (goat anti-rabbit) from Jackson Labs at a 1:300 dilution in PBS with 0.5% Triton X-100 added. After 2 h at room temperature, the slides were again washed, cleared with 70% glycerol, and then covered with 100% glycerol to which 5% n-propyl gallate had been added as an anti-fade reagent. Specificity tests for immunohistochemistry processing were accomplished by omission of the c-Fos or ChAT primary antibody.

Because the Fluorogold neuronal counterstain was substantially quenched by the immunohistochemical processing, all sections were first inspected under the epifluorescence microscope by use of an ultraviolet excitation filter block before processing. Fluorogold-labeled neurons were counted, and sketches of the location of single neurons and ganglia were made for easier retrieval when reinspected after processing. This two-step method had the advantage that slides with sections that did not contain significant numbers of neurons were excluded from immunohistochemical processing.

After NADPH-d and immunohistochemical Fos processing, ≥100 neurons for each animal and pancreas tier were identified under the brightfield microscope, and their Fos and NOS status was determined. Staining intensity of Fos-positive neuronal nuclei ranged from light brown to almost black and was clearly distinguishable from the absence of any stain in Fos-negative neurons. NADPH-d-positive (that is, NOS) neurons were characterized by a light blue-to-dark blue stain in Fos-negative neurons. NADPH-d-positive (that is, NOS) neurons were characterized by a light blue-to-dark blue stain confined to the cytoplasm, so that double-labeled neurons could easily be distinguished on the basis of both color and location. Neurons were only counted when staining intensity was above background and when at least part of the nucleus, stained or unstained, was contained in the section.

Total counts were expressed as proportion of Fos-positive neurons for each animal and pancreas tier, and these percentage scores were statistically evaluated using a repeated-measures model with test-of-effect slices and Bonferroni-adjusted post hoc comparisons.

**RESULTS**

Single neurons and ganglia consisting of up to 30 neurons were found throughout the three parts of the pancreas. In most cases the actual size of the ganglia could not be determined in our sectioned material. An average number of 3–4 neurons were found in the random 25-µm sections. Ganglia were often found

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associated with blood vessels or within larger nerve bundles but were also occasionally found near islets, along the pancreatic and bile ducts, or at more peripheral locations of pancreatic lobules (Fig. 1).

Estimation of extent of vagal input. In nonstimulated animals and after control stimulation (distally cut vagal trunk), there was no Fos-like immunoreactivity in any neurons or other cells. A very light, brown DAB reaction product was seen in larger nerve bundles and some portions of islets, and because this was not the case when the primary antibody was omitted, it likely represented nonspecific binding. In contrast, unilateral electrical vagal stimulation produced staining in neuronal nuclei ranging from light brown to black, particularly with 16 and 24 Hz (Fig. 1, A–C). No other cell types showed Fos induction. A complete frequency-response relationship for the three pancreatic subdivisions is depicted in Fig. 2. With left cervical vagal stimulation, the proportion of neurons induced to express nuclear Fos was highest with 16- and 24-Hz stimulation and very low with 8- and 48-Hz stimulation [main effect of frequency: F(4, 25) = 12.0, P < 0.0001], and expression was highest in the head of the pancreas [main effect of location: F(2, 25) = 31.7, P < 0.0001]. There was a marginally significant interaction [F(6, 25) = 2.56, P < 0.05], meaning that there were differences in the frequency-response relationship across the three parts of the pancreas. In the head of the pancreas roughly 30%, and in the rest of the pancreas ∼10–20%, of all neurons were Fos positive when optimal stimulation parameters were used to stimulate the left cervical vagus. Increasing the intensity of the stimulation current from the nominal 1 mA to 2 mA did not significantly increase the proportion of Fos-positive neurons in any part [Fig. 3; main effect of current intensity: F(1, 25) = 0.42, not significant (NS)].

Stimulation of the right cervical vagus with 24-Hz frequency and 1-mA intensity induced Fos in a similar proportion of neurons in the head and tail compared with left stimulation (Fig. 4). In the middle part of the
pancreas (body), right cervical vagal stimulation activated a significantly ($P < 0.01$) higher proportion of neurons.

Vagal input to subpopulations of neurochemically identified pancreatic neurons. Nitrergic neurons were identified by NADPH-d histochemistry (Fig. 1, D and E), and cholinergic neurons were identified with an antibody directed against choline acetyltransferase (Fig. 1, F and G). Roughly 90% of neurons were NADPH-d positive, and this proportion was similar in the three parts of the pancreas (Table 1). Vagal stimulation induced Fos expression in a similar proportion of nitrergic and nonnitrergic neurons in each of the three parts (Table 1), but as found in the overall analysis of Fig. 2, this proportion was highest in the head and lowest in the tail. An example of a Fos-positive nitrergic neuron is depicted in Fig. 1, E and E'.

A large proportion of neurons were ChAT positive, with very weak to strong staining. No attempt was made to quantitate the exact number because of a difficulty in distinguishing the very weakly stained from ChAT-negative neurons (Fig. 1, F and G). An example of vagal stimulation-induced Fos expression in ChAT-positive neurons in the head of the pancreas is shown in Fig. 1, G and G'.

**DISCUSSION**

Before we consider the functional implications of the present findings, we will discuss some methodological issues. As in many recent studies, we used the expression of Fos as an indicator of synaptic neuronal activation, and we need to be aware of certain limitations of this method. First, the method seems only valid for excitatory but not inhibitory synaptic input. This is because a rise in intracellular calcium is one step in the cascade leading to Fos expression (12), and inhibitory postsynaptic potentials do not increase intracellular calcium. However, this shortcoming of the Fos method may not pose a problem for the vagal efferent system, because vagal preganglionic lesions seem to be strictly excitatory. In a guinea pig in vitro gastric wall preparation with vagal nerve attached, no inhibitory postsynaptic potentials were ever recorded in myenteric plexus neurons upon vagal stimulation (24). This is not to say that there are no inhibitory effects of vagal efferent

**Table 1. Proportion of NADPH diaphorase-positive (nitrergic) neurons and vagally activated (Fos-positive) neurons in different parts of the pancreas**

<table>
<thead>
<tr>
<th>Part of Pancreas</th>
<th>Total No. of Neurons Counted</th>
<th>NADPH-d Neurons</th>
<th>Non-NADPH-d Neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Numbers*</td>
<td>Vagally activated %</td>
<td>Numbers*</td>
</tr>
<tr>
<td>Head (n = 5)</td>
<td>609</td>
<td>86 ± 3</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>Body (n = 4)</td>
<td>508</td>
<td>89 ± 3</td>
<td>16 ± 10</td>
</tr>
<tr>
<td>Tail (n = 5)</td>
<td>657</td>
<td>85 ± 4</td>
<td>10 ± 6</td>
</tr>
</tbody>
</table>

Results from left cervical vagal stimulation at 24 Hz frequency and 1- or 2-mA intensity are shown as total numbers or means ± SE for nos. of parts examined. NADPH-d, NADPH diaphorase. *Sum of neurons for all animals in respective group. †Double-labeled neurons.
outflow, but that they are accomplished by either centrally initiated decreases in tonic preganglionic activity or activation of inhibitory postganglionic neurons.

The second problem could be an inability of certain neurons to express Fos under any stimulation condition. Although we cannot rule this out for pancreas neurons, we have shown earlier that virtually all gastric enteric neurons, which share a common origin with pancreatic neurons, are capable of expressing Fos upon vagal stimulation (32). In addition, only one class of enteric neurons, the calbindin containing sensory neurons in the guinea pig small intestine, does not express Fos (23), but no such neurons were found in the guinea pig pancreas (15). At present there is no stimulus available that activates all neurons in the pancreas and could serve as a positive control for the ability to express Fos.

Another concern is the possibility of activation via antidromic stimulation of vagal afferent fibers and the potential release of excitatory transmitters within pancreatic ganglia. This is very unlikely, because few vagal afferent terminals have been found in pancreatic ganglia (19), and in the stomach, where intraganglionic terminals are plentiful, intentional antidromic stimulation of vagal afferents did not induce Fos in a significant number of myenteric plexus neurons (31).

Finally, it is also unlikely that a significant number of activated neurons were not directly innervated by vagal preganglionic fibers but were excited via postganglionic neurons acting as interneurons. There may be few interneurons in pancreatic ganglia, and by using atropine pretreatment, at least cholinergic transmission was blocked. Taken together, these arguments suggest that the Fos method is a useful tool to determine the quantitative aspect of vagal preganglionic input to pancreas neurons.

Density of vagal input. With use of optimal electrical stimulation of either side of the vagal system, ~30% of neurons in pancreatic ganglia were functionally activated as indicated by the expression of nuclear Fos. Under the assumption that each vagus innervates a different population of pancreas neurons, this would suggest that slightly more than one-half of all neurons receive excitatory vagal preganglionic input. It is, however, possible that the same neuron receives overlapping input from both sides. This may be indicated by the fact that there was very little regional difference in the proportion of activated neurons between left and right vagal stimulation. Only in the middle part of the pancreas was Fos activation different, which suggests that the terminal fields of the two sides are not completely overlapping. However, because it is likely that the ventral and dorsal vagal trunks innervate the pancreas along the ventral and dorsal gastroduodenal arteries, our division into head, body, and tail would not be able to distinguish between dorsal and ventral. Only bilateral vagal stimulation will ultimately be able to resolve this issue.

Given the multiple and powerful physiological effects in both the endocrine and exocrine pancreas, such a considerable density of innervation is not surprising. In the neighboring gastric myenteric plexus, nearly every neuron, and in the duodenum ~30% of the neurons, receives vagal input as demonstrated with the Fos method (32). In the only other attempt to quantify vagal innervation of the pancreas with morphological methods, it was found that anterogradely labeled vagal preganglionic terminals innervated ~20% of the pancreatic ganglia near the duodenum and <10% in the rest of the pancreas, and it was not possible to determine exactly the proportion of innervated neurons in each ganglion (6). Thus, although the density of innervation gradient between head and tail was similar to the one found in the present study, the overall estimate of innervation was considerably less. This difference is likely due to incomplete labeling in the vagal motor nucleus and/or absence of anterograde transport in very fine axons.

Clearly, a significant portion (40–70%, depending on the degree of overlap) of neurons does not seem to receive excitatory vagal input, and the question as to their function arises. They may receive input from other sources, such as sympathetic fibers or the enteric nervous system, or they may be interneurons that did not get sufficiently activated by vagal activation. Theoretically they could be sensory neurons, although as we have discussed, at least in the guinea pig, one of the neurochemical markers for sensory neurons, calbindin, is not found in pancreatic ganglia of that species.

Characteristics of preganglionic neurons. Vagal preganglionic neurons have been distinguished by their caliber and conduction velocity as well as by their neurochemical phenotype. In particular, larger-diameter relatively fast conducting fibers have been found to have a lower threshold for electrical stimulation, and thinner, more slowly conducting fibers have a higher threshold (1, 17). At supramaximal stimulus intensities, as utilized in the present study, the thinner high-threshold fibers cannot follow higher frequencies of stimulation as well as the thicker low-threshold fibers do. In functional studies measuring several physiological responses to vagal stimulation, it was found that whereas frequencies of ~1–2 Hz were optimal to stimulate gastric acid secretion, 8- to 16-Hz frequencies were necessary to obtain maximal insulin secretion, and still higher frequencies were necessary to obtain maximal cardioinhibition (5). The 16–24 Hz necessary for maximal expression of Fos in the present study is slightly higher than the 8–16 Hz for maximal insulin secretion. This may suggest that fibers leading to other pancreatic responses, such as exocrine secretion, may be of a larger caliber than the ones leading to insulin secretion. The almost complete ineffectiveness of 48 Hz to induce Fos expression was surprising, particularly because this frequency was optimal in the gastric myenteric plexus (32). It was likely due to a blocking effect known to occur in thin fibers, essentially preventing the axons from firing action potentials after a brief initial period that is not long enough to induce Fos expression.

Characteristics of postganglionic neurons. In confirmation of earlier studies in rats (25, 26, 30), we found...
that a large proportion of neurons in the pancreas contains NADPH-d and can thus be considered nitricergic. Because a large proportion also contains choline acetyltransferase, many of the neurons must be both cholinergic and nitricergic, as has been shown in the guinea pig (14, 15). Our results further show that many of the nitricergic neurons are functionally innervated by vagal preganglonics but that there is no preferential innervation, in that nonnitricergic neurons are innervated to a similar extent. Although the physiological effects, such as muscarinic stimulation of insulin, glucagon, pancreatic polypeptide, and exocrine secretion by cholinergic neurons, are well known (18), the effects of nitric oxide released from nitricergic neurons in the pancreas are less clear. NOS blockers have been shown to decrease insulin secretion in the conscious calf (9), the dog (7), the perfused human pancreas (2), and perfused rat islets (27). In particular, the insulin-releasing effects of both electrical vagal stimulation (10) and sham feeding (7), which is known to activate the vagal system, can be strongly inhibited by NOS blockers, suggesting that endogenous NOS is an important mediator of neurally stimulated insulin secretion. However, because both constitutive and inducible forms of NOS are localized in vascular endothelial cells as well as certain islet cells, and because the effect can be obtained from perfused islets (27), it is not clear what the role of the neuronal NOS is. An electrophysiological study in isolated cat pancreatic ganglia suggests that, in addition to effects in islets or acinar tissue, neuronally released NOS may have complex modulatory effects on other neurons via the numerous varicose axon terminals present in pancreatic ganglia (25).

In conclusion, we have demonstrated that 30–60% (depending on assumptions about overlap between left and right vagal innervation) of the neurons in intrapancreatic ganglia receive excitatory functional vagal input. The majority of the vagally activated neurons contain both ChAT and NOS and can potentially produce nitric oxide and acetylcholine. The relative roles and interactions of these two neurotransmitters in the many vagally induced effects on the endocrine and exocrine pancreas remain to be identified.

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