Characterization of pancreas-projecting rat dorsal motor nucleus of vagus neurons

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INTRINSIC PANCREATIC GANGLIA form an interconnected nerve plexus lying within the interlobular connective tissue and supply the predominant innervation to the pancreatic ducts, islets, and acini. Previous studies (13, 19) have demonstrated that pancreatic neurons have spontaneous synaptic activity, suggesting they may regulate pancreatic functions intrinsically. Pancreatic neurons, however, also receive an extensive extrinsic innervation. Microinjection of the retrograde transneuronal pseudorabies virus into the pancreas of sympathectomized rats, for example, labeled subsets of neurons in the dorsal motor nucleus of the vagus (DMV) (7, 11, 17, 18, 30). Two morphologically diverse types of cells were stained; one type comprised cells within the medial DMV, the other group contained small round cells with short fibers scattered throughout the DMV (30). Some of these pancreas-projecting DMV neurons appear to have dendrites that extend into the contralateral DMV as well as toward the central canal or area postrema (25). A more systematic morphological analysis of pancreas-projecting DMV cells was not, however, conducted.

Numerous studies have reported diverse morphological features of DMV neurons, mainly in their dendritic extent but also in their somata size or shape (4, 8, 12, 20, 26, 36). We have shown recently that gastric- and intestinal-projecting DMV neurons encompass cells with a vast array of morphological, immunocytochemical, pharmacological, and electrophysiological characteristics (4–6, 9, 15, 27, 33, 37), raising the possibility that pancreas-projecting DMV neurons may also comprise different subpopulations.

Vagal activation exerts direct actions on pancreatic exocrine and endocrine secretion (1, 14, 16, 23, 24). It is well established that the frequency at which the vagus is stimulated determines the type of response in the stomach. For example, Takahashi and Owyang (31), among others, have demonstrated that different frequencies of vagal stimulation release acetylcholine, nitric oxide, or vasoactive intestinal peptide selectively. Similarly, the influence of the vagus on pancreatic exocrine or endocrine function may be dependent on either the frequency of experimental stimulation or the frequency of action potential firing that the DMV neurons are capable of sustaining. Indeed, Berthoud and Powley (2) have shown that, when comparing gastric acid secretion to insulin and glucagon secretion, different parameters of vagal stimulation have divergent effects. Furthermore, electrical stimulation of vagal gastric and hepatic branches increased insulin secretion in an independent and additive manner (1), suggesting that differences exist either in the particular vagal fibers (and by consequence, the originating vagal somata) or in the vagal neuroeffector coupling.

Because all the parasympathetic efferent activity to the pancreas is integrated at the level of the DMV, to gain insights in pancreatic physiology it is essential that this nucleus is investigated in relation to its pancreatic target. Thus, the aims of this study were to characterize the electrophysiological and morphological membrane properties of identified pancreas-projecting neurons in the DMV.

MATERIALS AND METHODS

Retrograde tracers and tissue preparation. The application of retrograde tracers to identified gastrointestinal regions has been described previously (4, 34). Briefly, Sprague-Dawley rats (10–12 days old) of either sex were anesthetized deeply with a 6% solution of halothane with air (400–600 ml/min) in accordance with the National Institutes of Health Guidelines and the approval (protocol #313) of the Pennington Biomedical Research Center-Louisiana State University System Animal Care and Use Committee. During surgery, anesthesia was maintained by placing the head of the rat in a custom-made anesthetic chamber through which the halothane/air mixture was perfused; the depth of anesthesia (abolition of the foot pinch withdrawal reflex) was assessed before and during surgery. Once the

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animals were anesthetized, the abdominal and thoracic areas were cleaned with 70% ethanol, and a laparotomy was performed. Before apposition of retrograde tracers to the pancreas, the spleen was moved toward the upper right flank of the animal; gauze, soaked in sterile saline, was placed on the stomach; and the pancreatic tissue was lifted toward the stomach on top of the gauze. Dil crystals were placed on the body of the pancreas. To restrict the retrograde tracer to the site of application, the tracer was insulated from the abdominal milieu by embedding the Dil crystals in a fast-hardening epoxy compound. After the epoxy compound had dried (3–5 min), the area was examined visually to ensure that the dye had remained affixed to the organ surface and had not made contact with other tissues. The gauze was then removed, the surgical area washed with warm sterile saline solution, the excess solution blotted with cotton tips, the wound closed with 5-0 suture, and the animal was allowed to recover for 10–15 days. For comparison purposes, in some rats, Dil was apposed (using the same technique) along the greater curvature of the stomach or along the antimesenteric border of the duodenum (4).

The method utilized for the tissue slice preparation has already been described (4, 34). Briefly, the rats were placed in a transparent, enclosed anesthetic chamber through which halothane bubbled with air was passed. Once a deep level of anesthesia was attained (abolition of the foot pinch withdrawal reflex), the rat was killed by severing the major blood vessels in the chest. The brain stem and the cerebellum were removed and placed for 2–5 min into a chamber containing oxygenated, ice-cold Krebs solution (see Solutions). After removing the cerebellum, the brain stem was glued to a plastic support and 7 to 9 coronal slices (300-μm thick) containing the DMV were cut. The slices were then incubated and equilibrated for at least 1 h at 32 ± 1°C in oxygenated Krebs solution before electrophysiological recording. Before experimentation, the pancreas was examined visually to ensure that the dye was still apposed and had not diffused in the abdominal milieu. This is particularly important with the use of Dil because this carbocyanine dye diffuses passively into neural tissues rather than being transported actively. If Dil had entered the general circulation or had been spilled in the mesenteric area, fluorescent labeling would have also be present in the area postrema and the specimen discarded. Other positive control studies have also been performed in which Dil crystals were spilled deliberately onto the surface of the stomach, liver, and small intestine. Such spillage provides scattered fluorescence throughout the DMV and the area postrema. Unlike specific labeling, which is extremely bright and intense, labeling derived from spillage is rather faint (signifying that only minute amounts of dye diffused into the neuron). A single slice was then mounted on a custom-made perfusion chamber (volume 500 μl) and kept in place by a nylon web. The slice was maintained at 35 ± 1°C by perfusion with Krebs solution at 2.5 ml/min.

DMV neurons: identification and recordings. Patch-clamp recordings were made only from fluorescently labeled DMV neurons. Carbocyanine dyes (such as Dil) do not cause adverse effects with the brief illuminations used for neuronal identification (4, 10, 21, 22). Typically, after labeling of the pancreas, an average of 1–2 unequivocally labeled neurons were observed in each brain stem slice. Patch-clamp recordings were made from DMV neurons using borosilicate patch pipettes with a tip resistance of 3–7 MΩ once filled with a potassium gluconate intracellular solution (see Solutions). Recordings were corrected manually for liquid junction potential. Only those recordings having a series resistance < 15 MΩ were used. The recording pipette contained Neurobiotin (2.5 mg/ml) to stain the recorded neuron for later morphological analysis.

Neurons were current clamped at ~60 mV and injected with a 5- to 30-ms-long pulse of direct current (DC) sufficient to evoke a single action potential at its offset. For a neuronal recording to be accepted, the membrane had to be stable at the holding potential, the action potential evoked after injection of DC had to have an amplitude of at least 60 mV, and the membrane had to return to baseline at the end of the afterhyperpolarization (AHP).

Measured electrophysiological properties included the membrane input resistance (measured from the current deflection obtained by stepping the membrane from −50 to −60 mV), duration of the action potential at the threshold, the amplitude and duration of the AHP after the firing of a single action potential, and the frequency of action potential firing in response to depolarizing DC pulses of 400 ms duration and intensities ranging from 30 to 270 pA in step increments.

Data and statistical analysis. Data were filtered at 2 kHz, digitized via a Digidata 1320 interface (Axon Instruments, Union City, CA), and stored and analyzed on a personal computer utilizing the pClamp8 software (Axon Instruments). Results are presented as means ± SE. Differences between groups were analyzed with ANOVA followed by Duncan’s multiple range test or χ² test. The level of significance was set at P < 0.05.

Morphological reconstructions. At the conclusion of electrophysiological recording, Neurobiotin was injected into the DMV neuron by passing positive current pulses (0.6 nA, 600 ms on, 1200 ms off) for 10 min through the patch pipette. After injection of Neurobiotin, the pipette was retrieved from the cell, which was allowed to seal for 10–20 min in the perfusion chamber before overnight fixation in Zamboni’s fixative at 4°C (see Solutions). The slice was then cleared in PBS-TX (see Solutions) and kept at 4°C until the injected Neurobiotin was visualized by using a cobalt-nickel enhancement of the Avidin D-horseradish peroxidase (Avidin D-HRP) technique as described previously (4, 20). Briefly, slices were incubated in Avidin D-HRP solution (see Solutions) for 2 h. After 15 min rinse in PBS and subsequent incubation for 15–20 min in Avidin D-HRP and diaminobenzidine solutions (see Solutions), the slice was incubated for 15 additional minutes in the presence of 3% H₂O₂. The slice was then rinsed in PBS, placed on a gelatin-coated coverslip, air dried, cleared in alcohol and xylene, and mounted in Permount.

Three-dimensional reconstructions of individual Neurobiotin-labeled neurons, digitized at a final magnification of ×600, were made by using Neuronucida software (Microbrightfield, Williston, VT). Each reconstruction was verified by using the software for “mathematical completeness.” The optical and physical compression of the slice that may occur is corrected by rescaling the section to 300 μm (the original thickness at time of sectioning).

The morphological features assessed include soma area and diameter, form factor (a measure of circularity for which a value of 1 indicates a perfect circle and 0 indicates a line; form factor = 4πa²/πp², where a is the soma area and p is the perimeter of the soma in the horizontal plane), whether the cell has bipolar or multipolar dendrites, number of segments (i.e., branching of dendrites), branch order, and extension in the x- and y-axes termination of the dendrites (i.e., with at least 1 dendrite ending in apposition to the central canal/fourth ventricle or not). Data analyses were performed as described previously (4, 20).

Solutions composition. Krebs solution was (in mM) 120 NaCl, 26 NaHCO₃, 3.75 KCl, 1 MgCl₂, 2 CaCl₂ and 11 dextrose, maintained at pH 7.4 with O₂-CO₂ (95%-5%). Intracellular solution was (in mM) 128 K gluconate, 10 KCl, 0.3 CaCl₂, 1 MgCl₂, 10 HEPES, 1 EGTA, 2 ATP, 0.25 GTP, adjusted to pH 7.35 with KOH. Zamboni’s fixative was 1.6% (wt/vol) paraformaldehyde, 19 mM KH₂PO₄, and 100 mM Na₂HPO₄·7H₂O in 240 ml saturated picric acid-1600 ml H₂O; adjusted to pH 7.4 with HCl; PBS-TX (in mM) was 115 NaCl, 75 Na₂HPO₄·7H₂O, 7.5 KH₂PO₄ and 0.15% Triton X-100. Avidin D-HRP solution was 0.05% diaminobenzidine in PBS containing 0.5% gelatin supplemented with 0.025% CoCl₂ and 0.02% NiNH₄SO₄.

Chemicals. Chemicals purchased were Neurobiotin and Avidin D-HRP from Vector Labs (Burlingame, CA); Permout from Fisher Scientific (Pittsburgh, PA); Dil from Molecular Probes (Eugene, OR); and all other chemicals from Sigma (St. Louis, MO).
RESULTS

Recordings were limited to cells showing the brightest and most intense DiI fluorescent stain, i.e., the cells whose peripheral terminal fields were the most likely to be on the site of dye apposition. A total of 14 parameters were assessed in 278 neurons recorded in whole cell configuration. 178 neurons from 53 rats were labeled from the body of the pancreas, 50 neurons from 11 rats were labeled from the stomach, and 50 neurons from 8 rats were labeled from the duodenum.

Basic electrophysiological properties. The electrophysiological and morphological properties of pancreatic-projecting neurons, compared with gastric- and duodenal-projecting neurons, are summarized in Table 1. Overall, the electrophysiological properties of pancreatic-projecting neurons were more similar to gastric- than to duodenal-projecting neurons (see, for example, the action potential AHP amplitude and action potential firing at low intensity of current; Figs. 1 and 2).

Interestingly, in 14 of the 178 pancreatic-projecting neurons recorded, we observed the presence of a slowly developing apamin-insensitive AHP (Fig. 1B). This slow AHP (15.5 ± 3.20 and 166.6 ± 39.79 ms rise and decay time, respectively) was not observed in any of the gastrointestinal-projecting DMV neurons studied previously, and represents a biophysical characteristic of a yet unidentified subgroup of pancreatic-projecting DMV neurons. Unfortunately, the rare occurrence of this slow AHP prevented us from conducting any further analysis. Apart from the presence of the slowly developing AHP, the electrophysiological data obtained from this neuronal subgroup were qualitatively and quantitatively similar to those of the majority of pancreatic-projecting neurons and have thus been pooled in our analysis.

Because the absolute amplitude of the AHP varied dramatically among pancreatic-, gastric-, and intestinal-projecting neurons, we investigated the firing rate of DMV neurons after 400-ms-long injections of DC at different intensities. An examination of the relationship between the frequency response and the amount of current injected indicated that pancreatic neurons have a frequency response slower than gastric neurons at higher DC intensities (>150 pA) but faster than intestinal-projecting neurons at all the intensities tested (30–270 pA; P < 0.05; Fig. 2).

Basic morphological properties. Morphological analysis of Neurobiotin-filled neurons was based on reconstructions obtained from 74 pancreas-, 20 gastric-, and 13 intestinal-projecting neurons that maintained complete somato-dendritic characteristics. Data are summarized in Table 1.

Table 1. Electrophysiological and morphological properties of dorsal motor nucleus of the vagus neurons

<table>
<thead>
<tr>
<th>Pancreas</th>
<th>Gastric</th>
<th>Intestinal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Electrophysiological properties</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>172–178</td>
<td>37–50</td>
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<tr>
<td>Rinp, MΩ</td>
<td>434±14.1</td>
<td>387±27.0</td>
</tr>
<tr>
<td>AP duration, ms</td>
<td>3.02±0.07</td>
<td>2.66±0.09*</td>
</tr>
<tr>
<td>AHP amplitude, mV</td>
<td>17.7±0.4</td>
<td>18.0±0.9</td>
</tr>
<tr>
<td>AHP τ, ms</td>
<td>108.5±7.3</td>
<td>67.8±4.6*</td>
</tr>
<tr>
<td>AP at 30pA, s−1</td>
<td>7.8±0.3</td>
<td>8.9±0.9</td>
</tr>
<tr>
<td>AP at 270pA, s−1</td>
<td>29.7±0.9</td>
<td>35.6±2.30*</td>
</tr>
<tr>
<td><strong>Morphological properties</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>69</td>
<td>20</td>
</tr>
<tr>
<td>x-Axis</td>
<td>334.0±13.2</td>
<td>349.9±32.6</td>
</tr>
<tr>
<td>y-Axis</td>
<td>217.3±10.7</td>
<td>195.4±16.0</td>
</tr>
<tr>
<td>Soma area</td>
<td>273.7±11.7</td>
<td>315.7±18.5*</td>
</tr>
<tr>
<td>Soma diameter</td>
<td>24.7±0.7</td>
<td>19.9±0.6*</td>
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<tr>
<td>Form factor</td>
<td>0.74±0.01</td>
<td>0.83±0.02*</td>
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<tr>
<td>Segments</td>
<td>9.68±0.41</td>
<td>19.2±2.0*</td>
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<tr>
<td>Segment length</td>
<td>118.2±4.1</td>
<td>116.7±8.4</td>
</tr>
<tr>
<td>Branch order</td>
<td>3.77±0.14</td>
<td>4.06±0.47</td>
</tr>
</tbody>
</table>

Rinp, input resistance; AP, action potential; AHP, afterhyperpolarization; τ, time.

* P < 0.05 vs. pancreas-projecting neurons; ** P < 0.05 vs. gastric-projecting neurons.
Differences in morphology among pancreas- and gastric-projecting neurons were observed for four cellular features viz, soma diameter, form factor, and area as well as number of segments. Conversely, significant differences in morphology between pancreas- and intestinal-projecting neurons were restricted to the number of segments.

Interestingly, pancreas-projecting neurons could be divided into two subgroups on the basis of their dendritic characteristics. In fact, the majority of neurons could be defined as having a multipolar morphology, i.e., more than two dendrites exiting the cell soma (n = 51; Fig. 3), whereas a minority of neurons could be defined as having a bipolar morphology, i.e., only two dendrites exiting the cell somata (n = 22; Note that one remaining neuron was unassigned). These two neuronal groups differed further in their soma form factor in which multipolar cells had a more circular soma (0.76 ± 0.01) compared with bipolar cells (0.69 ± 0.02; P < 0.05) and in their branch order in which multipolar cells had fewer branches than bipolar cells (3.6 ± 0.17 compared with 4.1 ± 0.24, respectively, P < 0.05). The rostrocaudal or the distribution in the right or left DMV of these neuronal subtypes, however, did not show a preferential localization of either cell type (Fig. 3C). Some of the basic electrophysiological characteristics of bi- and multipolar neurons were also different. In fact, multipolar cells had a shorter-lasting AHP (93.4 ± 9.9 ms vs. 154.4 ± 42.8 ms time constant of AHP decay; P < 0.05) and fired more action potentials (8.8 ± 0.71 and 31.0 ± 2.0 action potentials/s after injection of 30 and 270 pA of DC, respectively) than bipolar cells (7.3 ± 0.94 and 26.8 ± 2.4 action potentials/s after injection of 30 and 270 pA of DC, respectively; P < 0.05). Data are summarized in Table 2.

Finally, pancreas-projecting neurons appeared to show differences in projections of dendrites. In fact, a subgroup comprising 17 neurons differed from the remaining neurons in having at least one dendrite that projected to either the ependymal layer of the central canal or the fourth ventricle. No differences were observed among these subtypes of neurons in Fig. 3. Morphology and localization of identified pancreas-projecting DMV neurons. A: brightfield micrograph at low magnification of a coronal brain stem slice at approximately 1.2 mm from obex. Ax: computer-aided reconstruction of the neuron depicted in A. Note that the cell has a multipolar dendritic arbor. The axonal terminal has been indicated by an arrow. B: brightfield micrograph at low magnification of a brain stem slice at 1 mm from obex. Note that some of the dendrites make contact with the ependymal layer of the 4th ventricle. Bb: computer-aided reconstruction of the neuron depicted in B. Note that the cell has a bipolar dendritic arbor. The axonal terminal has been indicated by an arrow. C: location of DMV neurons in the horizontal plane. Note that neurons with bipolar (○) or multipolar (■) shape are distributed evenly throughout the rostrocaudal and mediolateral extent of both the right and left portions of the DMV. XII, hypoglossus; R, rostral; IV, fourth ventricle; C, caudal; AP, area postrema.
DMV have distinguishable morphological characteristics such as dendrites projecting toward the central canal or the area postrema (25) and that two different types of cells are stained in DMV after tracer injections in the pancreas (30). Here we report that ~30% of pancreas-projecting neurons in the DMV have one or more dendrites making contact with the ependymal layer of the central canal or, more rostrally, the fourth ventricle. Within these anatomical subgroups, however, the electrical properties of DMV neurons were similar. Conversely, we observed significant differences in electrical as well as in morphological properties between pancreas-projecting neurons that had bipolar vs. multipolar morphology. Although we have shown recently that the electrophysiological properties of DMV neurons are intrinsic and not determined by morphological characteristics (20), it is possible that the different shape and complement of electrophysiological properties of these neuronal subpopulations underlies selective modulation of different functions. When considering electrophysiological diversities within the pancreas-projecting DMV neurons, we observed the striking presence of a small but well-defined subgroup of cells (14 of 178) that showed the presence of a slowly developing AHP. This AHP seems to be characteristic of pancreas-projecting neurons; its presence in rat DMV cells has not been reported previously. Although the low frequency of occurrence of cells with the slowly developing AHP prevented us from performing any systematic analysis, the kinetic characteristics of the AHP resembled those of the apamin-insensitive current described in guinea pig DMV neurons (28, 29). It is possible that the presence of the slow AHP represents a means by which pancreatic secretion can be modulated via yet unidentified hormones and/or neurotransmitters that target this current selectively.

Interestingly, the electrophysiological properties of pancreas-projecting neurons are, with a few exceptions, almost indistinguishable from those of gastric-projecting neurons, but they differ from intestinal-projecting neurons. In contrast, the somatic properties of the ensemble of pancreas-projecting DMV neurons are similar to those of intestinal-projecting neurons, whereas they differ from those of gastric-projecting cells. Despite the overall similarities in the mediolateral and dorsoventral extension of the dendritic arborization of pancreas-, gastric-, and intestinal-projecting neurons, pancreatic cells have a significantly lower number of dendritic segments compared with the other target areas.

In summary, our data show that, whereas on average pancreas-projecting neurons differ from gastric- or intestinal-projecting neurons, no single property per se can be used as a distinguishing characteristic. Rather, a systematic analysis of the electrical and morphological properties is necessary.

In conclusion, we have shown that the rat DMV comprises subpopulations of pancreas-projecting neurons. It is tempting to hypothesize that these groups of pancreas-projecting DMV neurons represent classes of parasympathetic preganglionic neurons that regulate either exocrine or endocrine pancreatic functions selectively.

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