Effects of pancreatic polypeptide on pancreas-projecting rat dorsal motor nucleus of the vagus neurons

Kirsteen N. Browning, F. Holly Coleman, and R. Alberto Travagli

Department of Neuroscience, Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, Louisiana

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Browning, Kirsteen N., F. Holly Coleman, and R. Alberto Travagli. Effects of pancreatic polypeptide on pancreas-projecting rat dorsal motor nucleus of the vagus neurons. Am J Physiol Gastrointest Liver Physiol 289: G209–G219, 2005. First published April 7, 2005; doi:10.1152/ajpgi.00560.2004.—We investigated the pre- and postsynaptic effects of pancreatic polypeptide (PP) on identified pancreas-projecting neurons of the rat dorsal motor nucleus of the vagus in thin brain stem slices. Perfusion with PP induced a TTX- and apamin-sensitive, concentration-dependent outward (22% of neurons) or inward current (21% of neurons) that was accompanied by a decrease in input resistance; PP was also found to affect the amplitude of the action potential afterhyperpolarization. The remaining 57% of neurons were unaffected. PP induced a concentration-dependent inhibition in amplitude of excitatory (n = 22 of 30 neurons) and inhibitory (n = 13 of 17 neurons) postsynaptic currents evoked by electrical stimulation of the adjacent nucleus of the solitary tract, with an estimated EC50 of 30 nM for both. The inhibition was accompanied by an alteration in the paired pulse ratio, suggesting a presynaptic site of action. PP also decreased the frequency, but not amplitude, of spontaneous excitatory (n = 6 of 11 neurons) and inhibitory currents (n = 7 of 9 neurons). In five neurons, chemical stimulation of the area postrema (AP) induced a TTX-sensitive inward (n = 3) or biphasic (outward and inward) current (n = 2). Superfusion with PP reversibly reduced the amplitude of these chemically stimulated currents. Regardless of the PP-induced effect, the vast majority of responsive neurons had a multipolar somata morphology with dendrites projecting to areas other than the fourth ventricle or the central canal. These results suggest that pancreas-projecting rat dorsal motor nucleus of the vagus neurons are heterogeneous with respect to their response to PP, which may underlie functional differences in the vagal modulation of pancreatic functions.

brain stem; parasympathetic; gastrointestinal

CHOLINERGIC NEURONS OF THE dorsal motor nucleus of the vagus (DMV) control parasympathetic function to the gastrointestinal tract by impinging on postganglionic excitatory cholinerergic or inhibitory nonadrenergic, noncholinergic (NANC) neurons (2, 18, 51). The DMV also provides the parasympathetic innervation to the pancreas; microinjection of the retrograde transneuronal Bartha virus into the pancreas of sympathectomized rats results in labeling of two morphologically different types of neurons in the DMV; one group of neurons has a multipolar soma morphology, whereas the other group of cells has bipolar soma (14, 21, 28, 29, 46). The pancreas-projecting DMV neurons can further be distinguished on the basis of their dendritic arborization with one group projecting toward, whereas the other group projects to regions other than, the central canal or fourth ventricle (8, 42, 46).

Vagal activation affects directly both the exocrine as well as the endocrine pancreas (6, 24, 27, 38, 39, 41). Indeed, it has been shown that different frequencies of stimulation affect either endocrine or exocrine secretion (3, 5), raising the possibility that selective types of stimulation or different vagal preganglionic cells can affect these functions differently. After ingestion of a meal, pancreatic polypeptide (PP) is released from the pancreas via a vagally-dependent, atropine-sensitive mechanism (reviewed in Ref. 40); circulating PP then potently inhibits exocrine secretion (reviewed in Refs. 23, 55). This inhibition of pancreatic exocrine secretion is not, however, due to a direct effect of PP on the pancreatic acini. In fact, PP does not affect secretion in an in vitro acinar preparation, and there are no PP receptors on the acinar or ductal cells (30, 37, 41, 45, 55). Rather, circulating PP acts centrally to enhance gastric secretion and motility via actions at the level of efferent vagal fibers. Physiologically identified gastric-projecting neurons are excited by PP (32–34), and recent studies in rats have shown that PP (Y4) receptors are located in the dorsal vagal complex [DMV, i.e., DMV and nucleus of the solitary tract (NTS)] (17, 56), where they are distinguishable from the neuropeptide Y (NPY) and peptide YY (PYY) binding sites (Y1 and Y2 receptors) (25, 54). As with the effects of PP on gastric function, the reduction of exocrine pancreatic secretion induced by systemic administration of PP occurs via actions in the DVC and is dependent on an intact vagal innervation (17, 43, 55). The response of identified pancreas-projecting DMV neurons to PP, however, has not been assessed.

The study of the effects of PP on synaptic activity within the DVC is also of physiological relevance, because the in vivo inhibitory effects of PP are enhanced by treatments, such as 2-deoxy-glucose or CCK, known to increase pancreatic baseline secretion (39). Recently, it has been hypothesized that synaptic connections within the DVC and, in particular, between the area postrema and the DMV play a relevant role in the physiological actions of PP (17). The effects of PP on the synaptic responses of identified pancreas-projecting DMV neurons, however, have not been assessed.

The aims of this study were 1) to investigate the in vitro effects of PP on the membrane of identified rat pancreas-projecting DMV neurons 2) to determine the effects of PP on excitatory and inhibitory synaptic transmission within the DVC to identified pancreatic-projecting DMV neurons, and 3) to determine whether cells responding to PP have distinguishing membrane characteristics.

METHODS

Retrograde tracers and tissue preparation. As described previously for other visceral regions including the pancreas (8, 11, the retrograde tracer DiI was applied to the pancreas of Sprague-Dawley rats. Briefly, rats (12–14 days old) of either sex were anesthetized deeply (3% Isoflurane with air, 600 ml/min) in accordance with the National Institutes of Health guidelines and the Pennington Biomedical Research Center-Louisiana State University System Animal Care and Use Committee. A deep level of anesthesia (abolition of the foot pinch withdrawal reflex) was maintained throughout the surgical procedure. The abdominal and thoracic areas were cleaned with 70% ethanol before performing a laparotomy. The spleen was reflected toward the upper right flank of the rat before gauze, soaked in sterile saline, was placed on the stomach. The pancreas was then placed on top of the gauze, and DiI crystals were apposed to the body of the pancreas. To restrict it to the site of application, the neuronal tracer was embedded in place using a fast-hardening epoxy resin that was allowed to dry for 3–5 min before the pancreas was replaced; then the gauze was removed, and the entire surgical area was washed with warmed sterile saline. The excess solution was blotted with cotton tips, the wound closed with 5–0 suture, and the animal was allowed to recover for 10–15 days.

The method to prepare the tissue slices has already been described (11, 49). Briefly, rats were anesthetized deeply with isoflurane (5%) before being killed by severing the major blood vessels in the chest. The brain stem was removed and placed into oxygenated, ice-cold Krebs solution (see below). After being glued to a plastic support, five to six coronal slices (300 μm thick) containing the DMV were cut using a vibratome. The slices were incubated and equilibrated for at least 1 h in oxygenated Krebs solution (32 ± 1°C) prior to electrophysiological recording. In each instance, the pancreas was examined visually to ensure that the dye had not moved from its site of application and had not diffused into the abdominal milieu. 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 visualized with a Nikon E600FN equipped with TRITC filters. Provided the period of illumination used for neuronal identification is brief, carbocyanine dyes (such as DI) do not cause adverse effects (11, 20, 35). After labeling of the pancreas, typically 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 when filled with a potassium gluconate intracellular solution (see below). Recordings were corrected manually for liquid junction potential, and only those recordings having a series resistance of <15 MΩ were used. Neurobiotin (2.5 mg/ml) was included in the recording pipette to stain the neuron for later morphological analysis. For a neuronal recording to be accepted, the membrane had to be stable at the holding potential, the action potential evoked following injection of direct current (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.

Neurons were voltage clamped at −50 mV before superfusion with PP (0.3–1,000 nM) for a period of time sufficient for the response to reach plateau. DMV neurons were classified as PP responders if perfusion with 100 nM PP induced a current of at least 20 pA in the absence of 0.002% AvidinD-HRP in PBS, 1% Triton X-100, and 0.5% gelatin supplemented with 0.025% CoCl2 and 0.02% Ni(H2O)6Cl2. PP (100 nM) was then applied by superfusion; once the response reached plateau, the membrane potential was restored to control values by injection of DC before the protocols were repeated. Only one cell per slice was tested.

Evoked and spontaneous synaptic currents. Tungsten bipolar stimulating electrodes (WPI, Sarasota, FL) placed in the adjacent NTS (subnucleus centralis, medialis, or commissuralis) were used to evoke excitatory or inhibitory postsynaptic currents (EPSCs or IPSCs, respectively) in the recorded DMV neuron. When evoking EPSCs, neurons were current clamped at −60 mV (i.e., close to ECl), when evoking IPSCs, neurons were current clamped at −50 mV, and the perfusing solution contained 1 mM kynurenic acid (to block glutamatergic currents, 50).

Spontaneous IPSCs were recorded in neurons voltage clamped at −50 mV using a perfusing solution containing 1 mM kynurenic acid and intracellular solution containing KCl; conversely, spontaneous excitatory events were recorded at −60 mV holding potential with a potassium gluconate intracellular solution.

Data and statistical analysis. Data were filtered at 2 kHz, digitized via a Digidata 1320 interface (Axon Instruments, Union City, CA), stored, and analyzed on a PC using the pClamp8 software (Axon Instruments). Results are represented as means ± SE. Each neuron served as its own control, i.e., the neuron was assessed before and after drug application and analyzed using a paired t-test with significance set at P < 0.05.

Morphological reconstructions. At the conclusion of electrophysiological recording, Neurobiotin was injected into the DMV neuron as described previously (11, 31), and the brain stem slice was fixed overnight in Zamboni’s fixative at 4°C. The fixative was cleared from the slice with multiple washes of PBS-TX (see below), and the injected Neurobiotin was visualized using a cobalt-nickel enhancement of the Avidin d-Horseradish Peroxidase (Avidin d-HRP) technique as described previously before mounting in Permount (11, 31).

As described previously (11, 31), Neureolucida software (Microbrightfield, Williston, VT) was used to make three-dimensional reconstructions of the individual Neurobiotin-labeled neurons, digitized at a final magnification of ×600. Each reconstruction was verified using the software for “mathematical completeness” with optical and physical compression of the slice corrected by rescaling the section to the original thickness at the time of sectioning (300 μm).

Included in the morphological features assessed were 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 = 4πa × 1/p2, where a = soma area and p = 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/4th ventricle or not). Data analysis was performed as described previously (11, 31).

Solutions composition. Krebs solution consisted of (in mM) 120 NaCl, 26 NaHCO3, 3.75 KCl, 1 MgCl2, 2 CaCl2, and 11 dextrose, maintained at pH 7.4 with 95% O2-CO2 (95–5%). Potassium gluconate intracellular solution consisted of (in mM) 128 K gluconate, 10 KCl, 0.3 CaCl2, 1 MgCl2, 10 HEPES, 1 EGTA, 2 ATP, and 0.25 GTP, adjusted to pH 7.35 with KOH. Potassium chloride intracellular solution consisted of (in mM) 10 KCl, 1 MgCl2, 10 HEPES, 10 EGTA, 2 ATP-Na, and 0.25 GTP-Na, adjusted to pH 7.35 with HCl. Zamboni’s fixative consisted of 1.6% (wt/vol) paraformaldehyde, 19 mM KH2PO4, and 100 mM Na2HPO4·7H2O in 240 ml saturated picric acid·1,600 ml H2O, adjusted to pH 7.4 with HCl. PBS-TX consisted of (in mM) 115 NaCl, 75 Na2HPO4·7H2O, 7.5 KH2PO4, and 0.15% Triton X-100. Avidin d-HRP solution consisted of 0.002% Avidin d-HRP in PBS, 1% Triton X-100, and 0.05% DAB in PBS containing 0.5% gelatin supplemented with 0.025% CoCl2 and 0.02% Ni(H2O)6Cl2.
Chemicals. Neurobiotin and Avidin d-HRP were purchased from Vector Labs (Burlingame, CA); Permount was purchased from Fisher Scientific (Pittsburgh, PA); DiI was purchased from Molecular Probes (Eugene, OR); rat-PP was purchased from Bachem (King of Prussia, PA); all other chemicals were purchased from Sigma (St. Louis, MO).

RESULTS

To limit spurious results, only those cells showing the brightest and most intense DiI fluorescence were used for recording. The postsynaptic response to PP was tested on 142 pancreas-projecting DMV cells; in 73 neurons, we obtained the full spectrum of electrophysiological parameters, and 50 neurons were filled sufficiently with Neurobiotin to provide the full spectrum of morphological parameters. The presynaptic effects of PP were tested on 74 pancreas-projecting rat DMV neurons; morphological reconstructions were obtained in 15 of these neurons.

Postsynaptic effects of PP. In 22% of neurons tested (32 of 142), PP induced an outward current, whereas a further 20% of neurons (29 of 142) responded with an inward current. Biphasic responses (i.e., inward followed by outward current or vice versa) were never observed. The remaining 57% of neurons (81 of 142) showed no postsynaptic response to PP. The inward and outward currents induced by PP were concentration dependent (10–300 nM) and had similar estimated EC50 concentrations of 30 nM (Fig. 1, A and B). The maximal amplitude of the PP-induced outward current, however, was larger than that of the PP-induced inward current (41.6 ± 9.34 vs. 24.0 ± 2.57 pA, respectively; P < 0.05).

The PP-induced outward and inward currents were unaffected by superfusion with the synaptic blocker, TTX (1 μM; Fig. 1C). For example, in control conditions, perfusion with 100 nM PP induced a 36.0 ± 6.02-pA outward current that reversed following washout. After 10 min perfusion with Krebs solution containing TTX, PP induced a 37.0 ± 4.9-pA outward current (P > 0.05 vs. PP alone; n = 3). Similarly, in four neurons in which 100 nM PP induced an inward current, the amplitude of the current was unaffected by perfusion with TTX (28.2 ± 1.1 pA in control conditions vs. 25.7 ± 2.5 pA in the presence of TTX; P > 0.05).

In neurons in which perfusion with 100 nM PP induced an outward current, the input resistance (measured between −50 and −60 mV) was 516 ± 96.5 MΩ in control and 379 ± 74.7 MΩ following perfusion with PP (73.9 ± 5.1% of control; P < 0.05; n = 4). The reversal potential of the outward current induced by 100 nM PP was assessed in four neurons voltage clamped at −50 mV and subjected to 200 ms-long steps, in −10-mV increments, every 10 s up to −120 mV, in the presence and absence of PP. The reversal potential of the PP-induced outward current was between −90 and −100 mV, i.e., close to the potassium equilibrium potential (EK; Fig. 2). In neurons in which perfusion with 100 nM PP induced an inward current, the input resistance was 393 ± 49.8 MΩ in control and 262 ± 46.2 MΩ following perfusion with PP (66.7 ± 7.2% of control; P < 0.05; n = 5) with an estimated reversal potential close to 0 mV.

The basic electrophysiological and morphological properties of pancreas-projecting neurons responsive to PP are summarized in Table 1. Briefly, neurons that were inhibited by PP were found to have a broader action potential than nonresponsive neurons but a smaller action potential afterhyperpolarization. Neurons responsive to PP (inward or an outward current) differed only in the amplitude of their action potential afterhyperpolarization (see Table 1).

The effects of PP on action potential characteristics were assessed in 11 responsive neurons, six of which were depolarized and the remaining five were hyperpolarized. In all neurons, measurements of the action potential characteristics in the presence of PP were conducted after returning the membrane potential to baseline values via DC injection. In neurons in which PP induced a membrane depolarization, the action potential duration was unaffected (2.22 ± 0.21 and 2.37 ± 0.18 ms in control and PP, respectively; P > 0.05), but both the afterhyperpolarization (AHP) amplitude and duration were...
duration were unaffected (2.38 ± 0.05 vs. neurons hyperpolarized by PP, the action potential and AHP was evoked following DC injection (Fig. 3). Conversely, in neurons hyperpolarized by PP, the action potential and AHP duration were unaffected (2.38 ± 0.24 and 90.0 ± 11.0 ms in control vs. 2.18 ± 0.20 and 102.6 ± 18.0 ms in PP, respectively; *P > 0.05), but the AHP amplitude was increased from 18.0 ± 1.8 to 21.0 ± 1.6 mV in control and PP, respectively (P < 0.05), whereas fewer action potentials were evoked following DC injection (Fig. 3).

Because the effects of PP on pancreas-projecting neurons seems to involve an action on the AHP, which, in DMV neurons is composed, at least in part, of an apamin-sensitive calcium-dependent potassium current (44, 50), we tested the response to PP in the presence and absence of apamin. In six cells that responded to PP (4 with an outward and 2 with an inward current), 10 min of perfusion with 100 nM apamin

Table 1. Electrophysiological and morphological properties of pancreatic neurones tested with PP (postsynaptic)

<table>
<thead>
<tr>
<th></th>
<th>Nonresponsive to PP (n = 73)</th>
<th>Responsive to PP (n = 28)</th>
<th>Responsive to PP (total) (n = 55)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R_{\text{nap}} \Omega</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>437±20.5</td>
<td>459±44.6</td>
<td>475±28.6</td>
</tr>
<tr>
<td>AP duration, ms</td>
<td>3.03±0.14</td>
<td>3.25±0.22</td>
<td>3.55±0.3</td>
</tr>
<tr>
<td>AHP amplitude, mV</td>
<td>18.5±0.7</td>
<td>18.4±1.0</td>
<td>17.0±0.7</td>
</tr>
<tr>
<td>AHP τ, ms</td>
<td>119.6±12.7</td>
<td>99.1±15.6</td>
<td>89.6±9.3</td>
</tr>
<tr>
<td>AP at 30 pA</td>
<td>7.3±0.5</td>
<td>6.6±0.7</td>
<td>7.8±0.7</td>
</tr>
<tr>
<td>AP at 270 pA</td>
<td>28.1±1.6</td>
<td>28.0±2.1</td>
<td>29.4±1.7</td>
</tr>
</tbody>
</table>

Morphological Properties

<table>
<thead>
<tr>
<th></th>
<th>Nonresponsive to PP (n = 25)</th>
<th>Responsive to PP (n = 13)</th>
<th>Responsive to PP (total) (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>x-Axis</td>
<td>338.4±27.8</td>
<td>301.4±26.6</td>
<td>290.0±18.2</td>
</tr>
<tr>
<td>y-Axis</td>
<td>222.6±18.5</td>
<td>157.1±12.5*</td>
<td>168.0±11.7*</td>
</tr>
<tr>
<td>Soma area</td>
<td>268.3±19.5</td>
<td>253.9±21.6</td>
<td>256.6±16.9</td>
</tr>
<tr>
<td>Soma diameter</td>
<td>24.7±1.3</td>
<td>23.3±2.4</td>
<td>23.9±1.3</td>
</tr>
<tr>
<td>Form factor</td>
<td>0.78±0.02</td>
<td>0.72±0.02*</td>
<td>0.72±0.02*</td>
</tr>
<tr>
<td>No. segments</td>
<td>9.76±0.98</td>
<td>9.7±0.93</td>
<td>9.24±0.58</td>
</tr>
<tr>
<td>Segment length</td>
<td>121.6±9.3</td>
<td>110.0±8.6</td>
<td>106.3±5.1</td>
</tr>
<tr>
<td>Branch order</td>
<td>3.90±0.24</td>
<td>3.58±0.3</td>
<td>3.56±0.25</td>
</tr>
</tbody>
</table>

Values are means ± SE. AHP, afterhyperpolarization; AP, action potential; τ, time constant; R_{\text{nap}}, input resistance. *P < 0.05 vs. nonresponsive neurones; †P < 0.05 vs. neurons depolarized by pancreatic polypeptide (PP).

Fig. 2. Current-voltage relationship for the PP-induced current. Left: representative traces showing the control response of a pancreas-projecting DMV neuron voltage clamped at −50 mV and stepped to −120 mV in 10-mV increments for 200 ms every 5 s. Right: same protocol as left but in the presence of 100 nM PP. Note that PP induced an outward current. Current-voltage relationship for the traces depicted above. Note that the reversal potential for the PP-induced current is −100 mV, i.e., close to E_{K}.
decreased the amplitude of the PP-induced current to 19.1 ± 10.81% of control (P < 0.05; Fig. 3, C and D).

Presynaptic effects of PP. EPSCs were evoked by electrical stimulation of the subnuclei centralis, medialis, and/or commissurals of the NTS and recorded in 30 identified pancreas-projecting DMV neurons. Perfusion with PP (3–1,000 nM) induced a concentration-dependent inhibition in evoked EPSC amplitude in 22 of the 30 neurons tested (i.e., 73%; Fig. 4A) with an estimated IC50 of 30 nM (Fig. 4C). At 300 nM, the maximal inhibition of EPSC amplitude was 36.3 ± 5.6% (n = 4). The effect of PP did not appear to desensitize during the perfusion time, and tachyphylaxis was not observed when a second perfusion with PP was conducted following 10–15 min washout.

IPSCs were evoked by electrical stimulation of the NTS in 17 DMV neurons. Perfusion with PP (3–1,000 nM) induced a concentration-dependent inhibition in evoked IPSC amplitude in 13 of the 17 neurons tested (i.e., 76%; Fig. 4B) with an estimated IC50 of 30 nM (Fig. 4C). At 300 nM, the maximal inhibition of IPSC amplitude was 22.7 ± 4.2% (n = 3). The effect of PP did not appear to desensitize during the perfusion time, and tachyphylaxis was not observed when a second perfusion with PP was conducted 10–15 min later.

The ratio of the amplitude of two postsynaptic currents evoked a few milliseconds apart is used to determine whether a drug is acting at a pre- or postsynaptic site, with a change in the ratio being taken as indicative of a presynaptic site of action (9, 16, 52). When two EPSCs were evoked 50–200 ms apart, in cells where PP had an effect on the evoked currents, PP decreased the amplitude of the first current (C1) more relative to that of the second current (C2) such that the paired pulse ratio (C2/C1) increased. For example, in the presence of 100 nM PP, the paired pulse ratio of evoked EPSCs increased from 0.76 ± 0.05 to 0.98 ± 0.08 (n = 15, P < 0.05; data not shown). Similarly, in the presence of 100 nM PP, the paired pulse ratio of evoked IPSCs increased from 0.72 ± 0.12 to 0.77 ± 0.14 (n = 4, P < 0.05; data not shown).

Spontaneous glutamatergic events were studied in 11 DMV neurons. In 6 of the 11 neurons, perfusion with 100 nM PP reduced the frequency of spontaneous EPSCs from 6.95 ± 1.43 to 4.46 ± 1.34 events/s (i.e., 58.1 ± 7.85% of control; P < 0.05), leaving the amplitude of the events unaltered (38.6 ±
5.46 pA in control and 39.1 ± 5.32 pA in PP; *P > 0.05; Fig. 5, A and C).

Spontaneous GABAergic events were studied in nine DMV neurons. In seven of the nine neurons, perfusion with 100 nM PP reduced the frequency of spontaneous IPSCs from 3.29 ± 1.17 to 1.53 ± 0.59 events/s (i.e., 50.9 ± 8.8% of control; *P < 0.05), leaving the amplitude of the events unaltered (84.4 ± 12.9 pA in control and 74.1 ± 10.12 pA in PP; *P > 0.05; Fig. 5C).

The effects of PP (100 nM) on the response induced by chemical stimulation of the area postrema were studied in five pancreas-projecting DMV neurons. A pipette containing KCl was placed over the area postrema, and a picospritzer (Parker Instruments, Fairfield, NJ) was used to apply pulses of KCl...
The basic membrane characteristics (input resistance, action potential duration at threshold, afterhyperpolarization amplitude and kinetics of decay, and action potential firing frequency) were compared between pancreas-projecting neurons with PP-responsive (n = 43) and nonresponsive (n = 20) synaptic inputs (Table 2). No differences were observed in the basic membrane properties of these two groups of neurons.

Table 2. Electrophysiological and morphological properties of pancreatic neurons tested with PP (presynaptic)

<table>
<thead>
<tr>
<th>Electrophysiological Properties</th>
<th>Neurons Presynaptically Inhibited by PP (n = 43)</th>
<th>Neurons Presynaptically Unresponsive to PP (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input resistance, MΩ</td>
<td>408 ± 29</td>
<td>418 ± 25</td>
</tr>
<tr>
<td>Action potential duration, ms</td>
<td>3.1 ± 0.16</td>
<td>3.2 ± 0.16</td>
</tr>
<tr>
<td>AHP amplitude, mV</td>
<td>19.4 ± 1.4</td>
<td>17.6 ± 0.9</td>
</tr>
<tr>
<td>AHP duration, ms</td>
<td>72.9 ± 10.8</td>
<td>87.2 ± 9.7</td>
</tr>
<tr>
<td>Action potentials at 30 pA</td>
<td>6.7 ± 0.7</td>
<td>8.5 ± 0.7</td>
</tr>
<tr>
<td>Action potentials at 270 pA</td>
<td>31.2 ± 3.2</td>
<td>32.2 ± 2.0</td>
</tr>
</tbody>
</table>

Values are means ± SE. P < 0.05 vs. neurons presynaptically inhibited by PP.

Morphological reconstructions. Of the 142 neurones in which we tested the postsynaptic effects of PP, we were able to obtain complete morphological reconstructions of 50 cells (25 nonresponsive and 25 responsive to PP). We reported previously (8) that pancreas-projecting neurons had either a bipolar or a multipolar somata morphology. In the present study, differences were not found in terms of morphological differences between responsive and nonresponsive cells (in both groups, 17 of 25 neurones were multipolar; χ²-test P > 0.05; Fig. 7A). Surprisingly, when considering the orientation of dendritic projections, neurones unresponsive to PP were most likely to project to the ependymal layer of the central canal or fourth ventricle than neurones responding to PP (independently of whether they responded with an inward or an outward current). In fact, 11 of 25 nonresponsive neurones projected to the ependymal layer compared with 4 of 24 responding neurones (χ²-test P < 0.05; note: 1 neuron had its dendrites severed and was thus excluded from the count). Differences were not found, however, in the rostrocaudal distribution of PP responsive vs. nonresponsive neurones (Fig. 7B).

Of the 74 pancreas-projecting neurones in which the responsiveness of synaptic inputs to PP was assessed, a complete morphological analysis was obtained in 15 cells. Of those, PP inhibited synaptic input to 11 neurones with the remaining four neurones showing no effect. Of the 11 neurones in which PP inhibited synaptic transmission, the majority (10 of 11) had a multipolar rather than a bipolar morphological shape with dendrites that projected away from rather than toward the central canal or fourth ventricle (7 of 10 neurones). Differences in morphological properties were not observed in the distribution along the rostrocaudal axis of neurones with PP-responsive vs. nonresponsive synaptic inputs (n = 32, 10 neurones with...
inputs nonresponsive to PP, 22 neurons with inputs responsive to PP; Fig. 7C).

**DISCUSSION**

In this study, we have shown that, in an in vitro preparation of juvenile rats, 1) perfusion of identified pancreas-projecting neurons with PP induces either an inward (21%) or an outward (22%) current in distinct subsets of neurons, leaving the remaining 57% unaffected; 2) perfusion with PP decreases synaptic transmission between the NTS and the DMV; and 3) the effects of PP are more likely to occur in pancreas-projecting DMV neurons with multipolar somata morphology and dendrites projecting in directions other than the ependymal layer of the fourth ventricle.

On the basis of our data, we suggest that portions of the vagal-mediated effects of PP on pancreatic functions are prompted by both the direct modulation of subsets of DMV preganglionic neurons as well as by inhibition of discrete brain stem circuits. The pharmacological diversity of the responses of DMV neurons may indicate functional differences in the vagal control of pancreatic functions. Our conclusions are based on the following evidence.

Perfusion with PP affects a subpopulation (~40%) of identified pancreas-projecting DMV cells in which PP induced concentration-dependent excitation or inhibition of the neuronal membrane. The inhibitory effects of PP seem to be mediated by an increase in a potassium conductance, because the PP-induced outward current was associated with a decrease in input resistance, had a reversal potential close to equilibrium potential ($E_K$), and the amplitude of the fast, apamin-sensitive, calcium-dependent AHP that develops at the termination of the action potential was increased significantly. Indeed, pretreatment with apamin reduced significantly the amplitude of the outward PP-induced current.

The PP-induced inward current was similarly sensitive to apamin, suggesting the involvement of the closure (or at least the lack of opening) of a calcium-dependent potassium conductance, most likely the fast, apamin-sensitive AHP that has already been described in DMV neurons (11, 44). The PP-induced inward current, however, was also associated with a decrease in input resistance, which, coupled with an extracellular potential positive to 0 mV, would also imply the involvement of either a nonselective cationic conductance or the opening of channels (e.g., calcium or sodium) that have a positive reversal potential. Unfortunately, the low occurrence of PP-induced inward currents prevented us from conducting a more thorough study of the underlying ionic mechanisms.

In a subpopulation of pancreas-projecting DMV neurons, PP inhibits, in a concentration-dependent manner, EPSCs and IPSCs evoked by electrical stimulation of the adjacent NTS. When pairs of EPSCs or IPSCs were evoked, PP induced an alteration in the ratio of the current amplitudes and, additionally, PP reduced the frequency but not amplitude of spontaneous EPSCs or IPSCs. Both effects are strongly suggestive of a presynaptic site of action, i.e., on synaptic terminals apposing DMV neurons.

Recently, we have suggested that pancreas-projecting preganglionic parasympathetic neurons within the DMV can be distinguished from neurons innervating other areas of the gastrointestinal tract by a combination of their electrophysiological and morphological properties (8). Our present results show that the electrophysiological properties of the subgroup of PP-responsive pancreas-projecting neurons are not different from neurons unresponsive to PP (with the exception of neurons hyperpolarized by PP, which showed a broader action potential and a smaller AHP). Furthermore, their distribution throughout the rostrocaudal extent of the brain stem does not differ from neurons unresponsive to PP. We observed, however, that the majority of DMV neurons responsive to PP had a multipolar somata shape with dendritic projections oriented away from the central canal or fourth ventricle.

Although this observation is rather puzzling, because PP effects are supposedly determined by circulating PP rather than peptide release by local nerve terminals, one should consider that large portions of the DVC are either outside (i.e., the area postrema) or have a leaky blood-brain barrier (i.e., DMV and...
NTS) (15), which can be crossed by PP (1). It is thus possible that both DMV neurons and the synaptic circuits modulated by PP comprise one or more components that are accessible to circulating PP.

Our results, showing that perfusion with PP reduces significantly the synaptic currents evoked by stimulation of the area postrema, support recent in vivo data by Deng and colleagues (17). In their work, Deng et al. (17) reported that ablation of the area postrema and, likely, of some of the synaptic connections between NTS and DMV significantly reduced the effects of PP on basal pancreas exocrine secretion. Interestingly, the same work also reported that PP increased its inhibitory effects on 2-deoxyglucose (2-DG) stimulated pancreas secretion in rats that underwent area postrema ablation. This observation would suggest that synapses other than the area postrema-DMV circuit are also involved in the modulatory effect of PP on pancreatic exocrine functions. Indeed, in the present work, we showed that PP also modulates both excitatory as well as inhibitory transmission between the NTS and the DMV.

We have shown previously that the actions of several neurotransmitters or neuromodulators including other members of the pancreatic polypeptide family, i.e., NPY and PYY, do not inhibit GABAergic synaptic transmission to gastric-projecting DMV neurons unless the activity of the cAMP-protein kinase A pathway is stimulated either by direct activation of adenylate cyclase (e.g., forskolin) or by neurotransmitters positively coupled to adenylate cyclase (e.g., CCK, thyrotropin-releasing hormone) (13, 48, 51). It is interesting to note, then, that PP can inhibit GABAergic synaptic transmission to pancreas-projecting DMV neurons in naïve brain stem slices, without the prior pharmacological enhancement of cAMP levels. PP acts exclusively at Y4 receptors that, as with the other receptors of the PP family, are negatively coupled to adenylate cyclase through Gi/o (36). It remains to be seen whether these inhibitory actions of PP on GABAergic synapses reflect intrinsic differences in the coupling of the Y4 receptor.

Physiological significance. It has been well established that the frequency of vagal stimulation determines the type of response in the stomach; different frequencies of stimulation, for example, release acetylcholine, nitric oxide, or vasoactive intestinal peptide selectively (47). Similarly, it is also well established that the vagus nerve controls the pancreas in a heterogeneous manner (3, 5, 38, 39, 41, 53) and, similar to gastric functions, the influence of the vagus nerve on pancreatic exo- or endocrine function may depend on the frequency of experimental stimulation or on the frequency of action potential firing the DMV neuron can sustain. Indeed, Berthoud et al. (5) 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 the vagal gastric and hepatic branches increased insulin secretion in an independent and additive manner (3), suggesting that differences may exist either in the particular vagal fibers (and, by consequence, in the original vagal soma) or in the vagal neuroeffector coupling. In this study, we have provided evidence for the presence of at least three pharmacological subgroups of pancreas-projecting neurons, i.e., those that respond to PP with either an excitation, with an inhibition, and those that are insensitive to PP. It is tempting to speculate that these differing groups of pancreas-projecting DMV neurons represent classes of parasympathetic preganglionic neurons that regulate either exocrine (i.e., the neurons responsive to PP) or endocrine (the neurons unresponsive to PP) pancreatic functions selectively.
PP modulates vagal pancreatic functions both via a direct effect on the membrane of DMV neurons as well as indirectly via modulation of the synaptic currents impinging on them. On the basis of these results, we hypothesize that distinct DMV cells groups form clusters of neurons that selectively control pancreatic functions.

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GRANTS

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