Two populations of sympathetic neurons project selectively to mesenteric artery or vein

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two populations of sympathetic neurons project selectively to mesenteric artery or vein. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1263–H1272, 1999.—The objective of this study was to determine whether sympathetic neurons of the inferior mesenteric ganglion (IMG) projecting to mesenteric arteries could be distinguished by their localization, neurochemical phenotype, and electrophysiological properties from neurons projecting to mesenteric veins. In an in vitro intact vasculature-IMG preparation, neurons were labeled following intraluminal injection of Fluoro-Gold or rhodamine beads into the inferior mesenteric artery (IMA) or vein (IMV). The somata of neurons projecting to IMA were localized in the central part of the IMG, whereas those projecting to IMV were localized more peripherally. None of the labeled neurons was doubly labeled. Neuropeptide Y immunoreactivity was found in 18.9% of neurons innervating the IMA, but not in neurons innervating the IMV. Identified neurons were dissociated and characterized using whole cell patch-clamp recording. After direct soma depolarization, all of the labeled arterial and venous neurons were classified as tonic firing, compared with only 40% of unlabeled neurons; the remaining 60% of unlabeled neurons were phasic firing. The results indicate that IMG neurons projecting to mesenteric arteries are distinct from neurons projecting to mesenteric veins.

vasoconstrictor neuron; neuropeptide Y; electrophysiology; retrograde tracing

THE SYMPATHETIC INNERVATION of the vascular system is divided into different functional groups based on the particular vascular bed and its reflex responses. Physiologically, these different groups do not function as one; rather, the activity of the neurons in each pathway is governed separately. Blood flow to the skin, for example, is regulated separately from blood flow to the skeletal muscles or to the abdominal organs (20).

Previous functional studies indicate there is a basis for vessel-specific pathways in the prevertebral sympathetic innervation of the mesenteric circulation (17, 18). Mesenteric arteries and veins show different sensitivities to sympathetic neural activity regardless of whether the nerve stimulation is direct (22) or indirect via baroreceptor or chemoreceptor reflex activation (11, 16). For example, venous capacitance vessels show a greater degree of responsiveness at lower frequencies of stimulation than arterial resistance vessels, which require higher frequencies of stimulation to elicit the same degree of constriction (18). The neurotransmitters used to mediate neuromuscular transmission also differ between mesenteric arteries and veins; in veins, norepinephrine mediates all components of sympathetic vasoconstriction, whereas in arteries, ATP and neuropeptide Y (NPY) play an important role in neurally mediated vasoconstriction (39, 42).

Discrete neuronal phenotypes can be distinguished in prevertebral ganglia on the basis of electrophysiological properties (8), neurochemistry (1, 30, 36), or morphology (3). Functional studies also suggest that the postganglionic outflow from prevertebral ganglia is regionalized with respect to the gastrointestinal tract (28, 29, 32). A defined subpopulation of neurons innervates mesenteric blood vessels (30), but it is not known whether the phenotype of neurons innervating mesenteric artery and vein differ, or whether there is a distribution of neuronal phenotypes in the innervation of the two vessel types.

Previously, vasoconstrictor neurons have been speculated to contain immunoreactivity to NPY (NPY-1R), since the proportion of inferior mesenteric ganglion (IMG) neurons that contain NPY-1R correlates closely with the proportion of neurons proposed to subserve a vasoconstrictor function (25, 31). Further speculation has been made as to the electrophysiological properties of neurons projecting to the mesenteric vasculature; vasoconstrictor neurons have been proposed to display phasic firing patterns, since the anatomic occurrence of this neuronal type and the proportion of phasic neurons in the IMG correlate with the calculated population of vasoconstrictor neurons (4, 31).

MATERIALS AND METHODS

Female guinea pigs (150–250 g) were killed by cervical dislocation and exsanguination. An abdominal laparotomy was performed under aseptic conditions. The abdominal aorta, the IMG, and the inferior mesenteric artery (IMA) and vein (IMV) were gently isolated and excised together with a 3- to 5-cm-long segment of the descending colon, taking care not to stretch the vascular sympathetic innervation. After isolation, the IMG-vascular preparation was placed in a culture dish bathed with oxygenated feeding medium (see Composition of solutions) and pinned under light tension. The colon was then dissected away from the mesenteric vasculature to leave an IMA/IMV preparation with intact IMG vascular innervation.

Retrograde tracing. To provide access to the lumen of the IMA, a small incision was made in the aorta proximal to the branch point of the IMA to allow insertion of a syringe needle (0.164 mm OD, 34 gauge) for intraluminal perfusion. The artery was first rinsed with feeding medium to eliminate...
blood dots, followed by slow perfusion with 5 ml of air then 5 ml of distilled water. This procedure has previously been shown to remove the layer of endothelial cells covering the vessel lumen (34). After the water was flushed out of the artery with feeding medium, the vessel was perfused for 1 min with a solution containing 25% (wt/vol) phenol to induce limited damage of the fine perivascular nerve terminals and promote maximal uptake of retrograde tracers (9). The phenol itself caused the length of the vasculature to shorten by ~6%. The phenol was then rinsed away thoroughly with feeding medium before rhodamine latex microspheres ("beads") 0.02–0.2 μm in diameter or Fluoro-Gold (4%) suspended in neuronal culture feeding medium were injected in the arterial lumen. Retrograde tracers filled approximately one-half of the total length of the vasculature. For example, the total length of arterial vessels in the excised preparation was ~70–80 mm. Fluoro-Gold was seen to fill 35–40 mm of arterial vessels, that is, the primary and secondary branches of the IMA. The tracer was rarely observed to enter into third or higher order arterial or venous branches. After the injection, the needle was carefully removed from the artery, which was then sealed by pinching with forceps. The vein was also subjected to this luminal preparation protocol in the same preparation and injected with Fluoro-Gold (if the artery was perfused with rhodamine beads) or rhodamine beads (if the artery was perfused with Fluoro-Gold). To avoid leakage of retrograde tracers, the open portions of the vein were sealed with a drop of cyanoacrylate glue after the injection.

Controls for the specificity of the retrograde tracing experiments were carried out by applying Fluoro-Gold or rhodamine beads to the external vasculature and associated tissues, thus allowing the retrograde tracers to be taken up by nonvascular as well as vascular nerve endings.

The tissue was then placed in organ culture for 3 days at 37°C (see Composition of solutions), and the organ culture solution was replaced every 24 h. After organ culture, the IMG preparations were then either fixed for immunohistochemical analysis or dissociated for electrophysiological recording.

Immunohistochemical procedures. After organ culture, the IMG together with a small piece of mesenteric artery was dissected free from the rest of the mesenteric vasculature, rinsed in Hank's solution, and fixed in Zamboni's solution (see Composition of solutions) for 24 h at 4°C. The IMG was oriented with a piece of IMA as the landmark along the rostrocaudal axis on a thin (2 mm) slice of fixed liver, which provided mechanical support. The IMG then was embedded in OCT compound (Sakura Finetek) and immersed in a beaker dish containing 2-methylbutanone refrigerated with liquid nitrogen. After fixation, the ganglion was rinsed three times with a solution containing 0.0015% Triton X-100 (PBS-TX; see Composition of solutions) before being stored for 24 h in PBS-TX to ensure complete removal of fixative. The specimen was then rinsed with PBS-TX before being frozen by immersion in a solution of 2-methylbutanone refrigerated with liquid nitrogen. Sections of the ganglion (16–20 μm thick) were cut using a cryostat and mounted on a subbed coverslip.

The coverslip with the IMG was incubated with the primary anti-NPY antibody (1:100, rabbit anti-NPY, porcine) in a humid immunohistochemistry chamber at 37°C for 30 min (43). After incubation, the primary antibody was drained off, and the coverslip was rinsed in PBS-TX-BSA every 15 min for 45 min. The coverslip was back dried before application of the secondary antibody (FITC-labeled goat anti-rabbit IgG, 1:100) and incubated for 30 min at 37°C. The secondary antibody was then drained, and the coverslip was washed as before. After the coverslip was dried, one drop of Fluormount-G mounting medium was applied to the tissue section, which was then mounted on a clean slide. All antibodies were diluted in PBS-TX solution containing 1% (wt/vol) BSA (PBS-TX-BSA).

Immunohistochemical analysis. Slices containing the IMG and the initial segment of the IMA were viewed on an Olympus BH-2 microscope (magnification ×200–400) equipped with ultraviolet (UV) and tetramethylrhodamine isothiocyanate (TRITC) filters for identification of retrogradely labeled neurons and with FITC filters for identification of NPY-IR neurons. The number of neurons in each section was counted. This included retrograde tracer-labeled neurons, NPY-IR neurons, and unlabelled and/or unstained neurons. In some sections, there were no retrogradely filled neurons or no NPY-IR artery or vein neurons. The data for the number of neurons filled from artery or vein were expressed as a percentage of the total number of neurons in each ganglion. The mean percentage of particular types of neurons (i.e., artery neuron, vein neuron, NPY+/−) was the mean of the percentages from each preparation.

Labeled neurons identified with two nuclei in each ganglion lobe were located and then drawn in an "average" or stylized drawing of the ganglia. The ganglion lobe viewed under fluorescent microscope (×400) was divided into four quadrants by superimposing cross-hairs to allow mediolateral and rostrocaudal localization of identified neurons. A circle was drawn that intersected the midway point of each quadrant of the cross-hair; each of the quadrants was then divided into central and peripheral areas. The inner area was taken as a reference for medial region, and the outer area was taken as peripheral region of the IMG. Tissue sections were photographed using an Olympus OM-26 camera on FujiChrome 100 or 400, rated at 100 or 400 ASA, respectively. The photographic images were digitized by scanning the photographs with a Sprint scanner for measuring the cross-sectional area of soma. With the use of Optimas Image Analysis Software (Optimas, Bothell, WA), the cross-sectional area was circumscribed by manually drawing a line on the cell membrane with a cursor. The cross-sectional areas were determined for labeled arterial neurons, venous neurons, and unlabeled neurons obtained from the same ganglion.

Neuronal dissociation and culture methodology. After organ culture, the IMG were dissociated and cultured as described previously (7). Briefly, the IMG was excised, enzymatically dissociated (9 mg/ml papain, 1 mg/ml collagenase, and 4 mg/ml dispase), and plated as a monolayer onto poly-D-lysine-coated glass-bottomed 10-mm well in 35-mm culture dishes (MatTek, Ashland, MA). Cells were maintained in feeding medium (see Composition of solutions) at 37°C in a 5% CO₂ humidified incubator. The feeding medium was replaced every 3 days.

Electrophysiological recordings. Culture dishes containing IMG neurons were transferred to the stage of an Olympus IMT-2 inverted microscope equipped with UV and TRITC fluorescent filters for Fluoro-Gold and rhodamine, respectively, and perfused with Krebs solution (see below); the temperature was held constant at 37°C. The cells were briefly viewed with fluorescent illumination to identify the presence of retrograde tracers; whole cell patch recordings were made both from cells labeled with retrograde tracers and unlabeled neurons, using borosilicate patch pipettes of resistance 3–8 MΩ with an intracellular solution utilizing potassium chloride as the current carrier (see Composition of solutions). Electrophysiological recordings were made using an Axopatch 1D (Axon Instruments, Foster City, CA), and data were filtered at 2 Hz, digitized via a TL-1 DMA interface (Axon...
ANOVA followed by Duncan's multiple-range test, with P indicates dine (DMPH4), and 10 µM each of cytosine arabinoside, glutathione, 0.05 mg/ml 6,7-dimethyl-5,6,7,8-tetrahydropterin-streptomycin, 10 mg/ml ascorbic acid, 0.25 mg/ml fetal calf serum, 2 mM glutamine, 0.3% glucose, 1,000 U/ml penicillin-streptomycin, and 10 µM each of cytosine arabinoside, fluorodeoxyuridine, and uridine. Feeding medium was minimal essential medium supplemented with 2.5 ml guinea pig serum, 2 mM glutamine, 0.3% glucose, 1,000 U/ml penicillin-streptomycin, 10 mg/ml ascorbic acid, 0.25 mg/ml glutathione, 0.05 mg/ml DMpH4, 50 ng/ml nerve growth factor, and 10 µM each of cytosine arabinoside, fluorodeoxyuridine, and uridine. Zamboni’s solution contained 32 g paraformaldehyde, 240 ml saturated picric acid, 5.25 g KH2PO4, 53.6 g Na2HPO4 ·7H2O, and 1,600 ml H2O, pH 7.2. PBS-TX buffer contained 13.5 g NaCl, 40.2 g NaHCO3, 174.5 g HEPES, 1,500 ml H2O, 2.25 ml Triton X-100, and 0.04 g KH2PO4. Krebs solution contained (in mM) 120 NaCl, 26 NaHCO3, 3.75 KCl, 1 MgCl2, 2 CaCl2, and 100 ml Hanks’ balanced salt solution. It contained (in mM) 140.5 KCl, 2 MgCl2, 0.5 EGTA, 5 HEPES, 4 ATP, and 0.25 GTP, pH adjusted to 7.35 with KOH. Hanks’ solution contained 100 ml Hanks’ balanced salt solution (10x), Gibco and 119 mg/100 ml HEPES, pH adjusted to 7.4.

Chemicals. Rhodamine latex microspheres were purchased from Lumafluor (Naples, FL), and Fluoro-Gold was purchased from Fluorochem (England, CO). Rabbit anti-NPY antibody was purchased from Peninsula Labs (Belmont, CA), the FITC-labeled goat anti-rabbit IgG secondary antibody was purchased from ICN Biomedical (Aurora, OH), and the Fluoromount-G mounting medium was purchased from Southern Biotechnology Associates (Birmingham, AL). Papain and collagenase were purchased from Worthington Biochemical (Freehold, NJ). Dispase was purchased from Boehringer Mannheim (Mannheim, Germany). Minimal essential medium, Hanks’ balanced salt solution, penicillin-streptomycin, and l-glutamine were from Gibco (Grand Island, NY). DMpH4 was from Calbiochem (San Diego, CA). Guinea pig serum was from Chemicon (Temecula, CA). All other drugs, chemicals, and reagents were purchased from Sigma Chemical (St. Louis, MO).

RESULTS

Mesenteric vessels are innervated by two distinct populations of IMG neurons. Retrogradely labeled neurons were identified and counted only in those slice preparations where the nuclei were visible. Intraluminal perfusion with the retrograde tracer (either Fluoro-Gold or rhodamine beads in artery or vein) proved effective in selectively labeling a small number of IMG neurons projecting to either arteries or veins. Double labeling was never observed, that is, retrogradely labeled neurons were never found to contain both Fluoro-Gold and rhodamine beads.

The specificity of the technique was confirmed by experiments in which Fluoro-Gold or rhodamine beads were applied to the external vasculature and associated tissues, allowing the retrograde tracers to be taken up by nonvascular as well as vascular nerve endings. In such experiments, a large number of neurons, as well as nonneuronal cells, in all regions of the ganglia was labeled.

The average numbers of labeled arterial neurons (10.1 ± 2.1, n = 19 ganglia) per ganglion were similar to the average number of venous neurons (7.7 ± 2.5, n = 13 ganglia, P > 0.05) after injection of either rhodamine beads or Fluoro-Gold into IMA or IMV. Both rhodamine beads and Fluoro-Gold proved equally effective in labeling neurons projecting from the IMG to the IMA. A total of 42 neurons was later found to contain the retrogradely transported beads in 4 preparations in which rhodamine beads were injected into the IMA. In 15 preparations in which Fluoro-Gold was injected into the IMA, subsequent examination identified a total of 150 Fluoro-Gold-containing neurons. Rhodamine beads proved more effective than Fluoro-Gold in labeling neurons projecting to the IMV, however. A total of 68 neurons from 5 preparations was labeled when rhodamine beads were injected into the IMV, whereas only 32 neurons from 8 preparations were labeled when Fluoro-Gold was injected into the IMV.

Neurons projecting to arteries or veins are located in distinct areas of the IMG. To analyze the location of retrogradely labeled neurons within the IMG, each ganglion was divided into four quadrants, and the location of both Fluoro-Gold- and rhodamine-labeled neurons within each quadrant was marked on a scale map of the IMG. These analyses were carried out independently by two observers. Neurons projecting to the IMA were found to be located predominantly in the central areas of both lobes of the IMG, whereas neurons projecting to the IMV were located more peripherally in both the caudal and rostral lobes of the IMG (Fig. 1).

Neurons projecting to mesenteric veins are larger than those projecting to arteries. The average cross-sectional area of an arterial neuron was 1,198 ± 183 (SE) µm² (n = 7, range 603–1,546 µm), which was significantly smaller than venous neurons whose cross-sectional area was 2,124 ± 162 (SE) µm² (n = 7, range 1,155–3,210 µm; P < 0.05). Both arterial and venous neurons were smaller than unlabeled neurons, which had a cross-sectional area of 3,083 ± 210 (SE) µm² (n = 10, range 2,107–4,200 µm; P < 0.05).

NPY-IR is differentially distributed in IMG neurons projecting to mesenteric arteries and veins. In acute ganglia, that is, IMG not organ cultured, 188 of 880 neurons from 6 ganglia (i.e., 21.4%) contained NPY-IR. A similar proportion of unlabeled neurons (neurons not labeled by injection of retrograde tracers into either the IMA or IMV) from 22 ganglia (942 of 4,942, 18.9%; P = NS) from organ cultured preparations were NPY-IR.
Fig. 1. A: two retrogradely labeled arterial neurons (arrows) after injection of rhodamine beads into inferior mesenteric artery (IMA). TRITC, tetramethylrhodamine isothiocyanate. B: scheme of inferior mesenteric ganglion (IMG) depicting location of labeled arterial vascular neurons shown in A. Dotted box represents borders of photomicrograph. Arterial neurons are shown as solid circles, whereas unlabeled neurons are shown as open circles. Note arterial neurons are located toward center of caudal lobe of IMG. C: retrogradely labeled venous neuron (arrow) after injection of Fluoro-Gold into inferior mesenteric vein (IMV). UV, ultraviolet. D: scheme of IMG depicting location of labeled venous vascular neuron shown in C. Dotted box represents borders of photomicrograph. Venous neuron is shown as solid square, whereas unlabeled neurons are shown as open circles. Note venous neuron is located toward periphery of rostral lobe of IMG. E: scheme of IMG depicting location of retrogradely labeled arterial and venous neurons from 4 guinea pigs after injection of rhodamine beads into IMA and Fluoro-Gold into IMV. Arterial neurons are shown as circles, and venous neurons are shown as squares; corresponding shading for both arterial and venous neurons is used to indicate guinea pig from which results were taken. Note clustering of arterial neurons in center of both lobes of IMG, whereas venous neurons are located more peripherally.
Similarly, 10 of 31 neurons (18.9%; \( n = 9 \) ganglia; \( P = NS \)) projecting to the IMA were found to contain NPY-IR (Fig. 2). Conversely, of those neurons identified as projecting to the IMV (71 neurons from 7 ganglia), none was found to contain NPY-IR (\( P < 0.05 \)).

These data demonstrate that mesenteric arteries are innervated by a distinct population of IMG neurons that differs from the population that projects to mesenteric veins in terms of their location within the ganglion, their size, and NPY-IR.

Electrophysiological properties of vascular neurons. Whole cell electrophysiological recordings were made from 14 retrogradely labeled and 48 unlabeled neurons from the same ganglia. In addition, we compared recordings made in 70 acutely dissociated IMG neurons.

Characterization of firing pattern. Action potentials were initiated by delivering depolarizing current pulses of graded intensity (1-s duration) to neurons clamped at \(-60 \text{ mV}\). The current needed to reach the threshold for action potential firing was \(90.4 \pm 15.9, 47.3 \pm 10.9,\) and \(29.4 \pm 5.2 \text{ pA}\) in unlabeled tonic, arterial, and venous neurons, respectively. These were not significantly different from one another. Neurons were then classified as phasic or tonic on the basis of their firing characteristics in response to depolarizing current at three times threshold (4). In acutely dissociated preparations, 70% (49/70) of neurons discharged action potentials at the beginning of the depolarizing current pulse only and were thus classified as phasic firing (Fig. 3). The remaining acutely dissociated neurons (21 of 70 neurons, i.e., 30%) fired rhythmically and continuously throughout the current pulse and were thus classified as tonic firing (Fig. 3). These two firing patterns have already been described in intact IMG as well as in other autonomic neurons (7). The same ratio of tonic and phasic neurons was observed when whole cell recordings were made from unlabeled organ cultured neurons, that is, 33 of 48 unlabeled neurons (69%) were phasic firing, and the remaining 15 neurons (31%) were tonic firing. In contrast, all of arterial and venous neurons, i.e., 14 of 14 neurons, responded with a tonic firing pattern (\( P < 0.05 \)).

Basic properties. Because all the labeled neurons were tonic firing, we analyzed the basic properties of tonic firing acutely dissociated neurons and compared them with unlabeled and arterial and venous neurons. The input resistance of venous neurons was greater than acutely dissociated, unlabeled, and arterial neurons (\( P < 0.05 \)). The depolarization required to reach threshold for action potential firing in vascular neurons was less than the depolarization in both acute and unlabeled neurons (\( P < 0.05 \)). Significant differences

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Fig. 2. A: retrogradely labeled arterial neuron (solid arrow) after injection of Fluoro-Gold into IMA. B: corresponding field of view illustrating immunoreactivity to neuropeptide Y (NPY) (dotted arrow). Note arterial neuron in A is also NPY immunoreactive. C: retrogradely labeled venous neuron (solid arrow) after injection of Fluoro-Gold into IMV. D: corresponding field of view illustrating immunoreactivity to NPY (dotted arrow). Note that 2 NPY-immunoreactive neurons do not contain Fluoro-Gold, whereas retrogradely labeled venous neurons are not NPY immunoreactive.
were not found when comparing the resting membrane potential or the action potential amplitude among all four neuronal groups (P = NS, see Table 1).

Voltage-dependent ionic currents. The presence or absence of several voltage-dependent ionic currents [M current (I_M) and A current (I_A) and inward rectification; Fig. 3] were examined in acutely dissociated, arterial, venous, and unlabeled neurons.

I_M was analyzed in voltage clamp with the following protocol: neurons were voltage-clamped at −30 mV and then step hyperpolarized by increments of 10 mV for a duration of 2.3 s every 2–18 s until a final voltage of −100 mV was attained. The identity of I_M was confirmed with the use of the nonselective ion channel blocker barium (2 mM). I_M was found to be present in 10 of the 24 acute tonic neurons tested (i.e., 41%), but only in 4 of 14 (i.e., 29%) of the unlabeled tonic neurons (P > 0.05 compared with acute neurons) and only in 3 of 14 (i.e., 21%) of the neurons projecting to either artery or vein (P > 0.05 compared with acute neurons).
RESULTS

The protocols for $I_A$ were conducted in the presence of tetrodotoxin (0.1 μM) to block the transient sodium current and cobalt (2 mM) to block calcium currents. $I_A$ inactivation was analyzed in voltage clamp with the following protocol: neurons were voltage-clamped at −50 mV and then subjected to −5 s conditioning pulses at different hyperpolarized potentials starting from −60 mV and progressing in 10-mV steps up to −110 mV. This was followed by clamping the neurons at −40 mV, thus preventing the activation of the potassium delayed rectifier. The identity of $I_A$ was confirmed with the use of the nonspecific antagonist 4-aminopyridine (1 mM). $I_A$ was found to be present in 8 of the 20 acute tonic neurons tested (i.e., 40%) and in 4 of 15 (i.e., 27%) of the unlabeled tonic neurons ($P < 0.05$ compared with acute tonic neurons). The percentage was increased in vascular neurons, with $I_A$ being present in 7 of the 12 neurons analyzed. No differences in the proportion of neurons expressing $I_A$ were observed between arterial and venous projecting neurons ($P > 0.05$ compared with acute neurons).

Inward rectification. The inward rectification current was analyzed in voltage clamp with the following ramp protocol: neurons were clamped at −60 mV and then step hyperpolarized to −100 mV for 3 s before being depolarized to 0 mV over 14.4 s. The rate of the ramp was 7 mV/s. The inward rectification was defined as the cesium-sensitive inward deflection that was observed at potentials negative to $-71 \pm 2.2$ mV ($n = 6$). Although no statistically significant differences were observed in the occurrence of inward rectification among the acute, unlabeled, and venous neurons, the current was present in 8 of 14, 6 of 13, and 3 of 7 neurons, respectively, a statistically significantly larger occurrence was observed in arterial neurons, with the current being present in 4 of the 5 arterial neurons analyzed ($P < 0.05$; data not shown, but see the DISCUSSION).

These data demonstrate that IMG neurons projecting to the mesenteric vasculature are comprised exclusively of tonic firing neurons. In addition, neurons projecting specifically to mesenteric arteries are more likely to display inward rectification.

DISCUSSION

This study demonstrates that neurons of the IMG projecting to mesenteric arteries can be distinguished from neurons projecting to mesenteric veins by their localization within the ganglion, their immunoreactivity to NPY, and their electrophysiological properties. Not only are arterial neurons different from venous neurons, but vascular neurons differ from nonvascular with respect to these properties. These findings further substantiate the idea that prevertebral sympathetic ganglion neurons are influenced by their selected target organ.

Labeling of vascular neurons. An average of nine neurons per ganglion was labeled following injection of retrograde tracer into the IMA or IMV. Such a small number of labeled neurons is a likely indication that the labeling technique was selective. Had the fibers of passage or nerve terminals been damaged during the surgical procedures, the numbers of labeled neurons would have been much greater. Indeed, in control experiments when retrograde tracers were placed on the surface of the mesentery and on the exterior of the blood vessels where they could contact both the nonvascular nerves and fibers of passage, a significantly larger population of IMG neurons as well as nonneuronal cells throughout both lobes of the IMG were labeled.

Viscerotopic localization of vascular neurons. The organization of sympathetic ganglia with respect to innervated organs has important implications for the degree to which sympathetic outflow is specific for a particular organ or generalized for groups of organs. Although some of the discrimination of global vs. specific sympathetic activation is organized in the

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Table 1. Electrophysiological properties of identified neurons

<table>
<thead>
<tr>
<th></th>
<th>Acute Neurons</th>
<th>Unlabeled Neurons</th>
<th>Arterial Neurons</th>
<th>Venous Neurons</th>
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<td>n</td>
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<td>15</td>
<td>6</td>
<td>8</td>
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<td>$E_m$, mV</td>
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<td>−47 ± 2.2</td>
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<td>$R_{in}$, MΩ</td>
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<td>416 ± 128</td>
<td>339 ± 103</td>
<td>623 ± 109</td>
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<tr>
<td>Action potential threshold, $\Delta$ mV</td>
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<td>15 ± 0.6</td>
<td>10 ± 1.0*</td>
<td>12 ± 1.0*</td>
</tr>
<tr>
<td>Action potential amplitude, mV</td>
<td>115 ± 3.0</td>
<td>103 ± 3.4</td>
<td>97 ± 6.5</td>
<td>105 ± 11.9</td>
</tr>
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Values are means ± SE; $n$, no. of neurons. $E_m$, membrane potential; $R_{in}$, input resistance. *$P < 0.05$ compared with acute neurons.

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Fig. 3. Action potential firing patterns of IMG neurons current clamped at −60 mV after injection of depolarizing current pulses (3 × rheobasic strength). A: phasic firing behavior from an unlabeled neuron. Neuron fires an action potential only at onset of depolarizing current pulse. B: tonic firing behavior from an arterial neuron. Neuron fires action potentials throughout duration of depolarizing current. Amplitude of final action potential is shortened due to sampling rate of electrophysiology software. C: inward rectification in an arterial tonic firing neuron. Inward rectification was identified from a ramp protocol. Neurons were voltage clamped at −60 mV before being hyperpolarized to −100 mV for 3.6 s. Neurons were then steadily depolarized to 0 mV over 14.4 s before being step hyperpolarized back to holding potential of −60 mV (see voltage trace below). Inward rectification was observed over voltage range −100 to −60 mV. Currents at onset and offset of ramp protocol have been removed. D: M current protocol in same arterial neuron as C. Neurons were voltage clamped at −30 mV step hyperpolarized by increments of 10 mV for a duration of 2.3 s every 2−18 s until a final voltage of −110 mV was reached (see voltage trace below). Note inward and outward current relaxations characteristic of M current in addition to inward rectification, seen as an increasingly larger current deflections to voltage steps negative to −70 mV. E: lack of inward rectification in an unlabeled tonic firing neuron. F: M current protocol in same unlabeled neuron as E. M current was present in this neuron. Because an M current was present in both arterial and unlabeled neuron, inward rectification observed in arterial neuron (C) but not unlabeled neuron (E) cannot be attributed to M current.
central nervous system (21), this would be expected to be modified by the anatomic characteristics of pathways in the ganglia (19). In both the IMG, as shown in this study, and in paravertebral ganglia (9), arteries and veins are innervated by separate sympathetic neurons. Whereas artery and vein neurons are segregated in different regions of the IMG, they are randomly mixed within individual paravertebral ganglia, but segregated among ganglia at different spinal levels (9). This would extend previous observations that sympathetic ganglion neurons are often localized anatomically in relation to their efferent output (2) as well as their synaptic input (23, 28, 33), thus providing for separate or different activation of different functional subgroups within the ganglion. This same principle has been found to hold true in pelvic ganglia (24, 38). With respect to vascular neurons, this organization could account for the different physiological regulation of arteries and veins (17, 18).

Size of vascular neurons. A difference in the size of arterial and venous neurons provides additional support for the idea that these are separate subpopulations of neurons. Differences in size of sympathetic neurons have been correlated with other differentiating properties such as neurochemical makeup (24), electrophysiological properties (3), and innervation target (12). In the superior cervical ganglion, presumed vasomotor neurons (contain NPY) are smaller than pilomotor neurons (do not contain NPY) (12). However, the grouping of several of these properties in a characteristic neuron type has been difficult to establish (25). For example, in the IMG, we found that arterial neurons were smaller than either venous or unlabeled neurons, although they were both tonic firing. In contrast, in the celiac ganglia of the guinea pig, tonic neurons are larger than phasic neurons (3) without reference to their target.

Immunohistochemistry of vascular neurons. The presence of NPY is often taken as a marker of vasomotor neurons in sympathetic ganglia because of the presence of NPY in nerve fibers surrounding blood vessels (15) and its preponderance in sympathetic ganglion cells (30, 31). We found that NPY is not preferentially localized in arterial neurons when compared with its incidence in all ganglionic neurons, generally ~20% of neurons in IMG (31, 36), and that it is not found in venous neurons. In the one other study where artery and vein neurons were identified by placing retrogradely transported tracers on blood vessels, 94% of arterial neurons but only 17% of venous neurons in lumbar paravertebral ganglia contained NPY (9). Such an absence of NPY-IR in venous neurons raises the question of whether NPY-IR can be demonstrated around mesenteric veins. The distribution of NPY around veins has not received the detailed attention that it has around arteries. However, sparse NPY-IR has been demonstrated to surround large veins of several organs (10, 37), including the gastrointestinal tract. It is likely that the extremely low density of NPY-positive innervation is a reflection of the lower incidence of NPY-positive venous neurons in the ganglia.

The question arises, therefore, as to whether the proportion of vascular neurons immunoreactive for NPY has been underestimated in our study. Such miscalculations are unlikely to arise from technical limitations of the anti-NPY antibody, since the overall proportion of NPY-IR in acutely fixed IMG was consistent from ganglion to ganglion (21%) and corresponded well with previously published reports (31, 36). Similarly, the proportion of NPY-IR neurons is not different in ganglia after organ culture (13, 24). The presence of NPY-IR cannot, therefore, be taken as a characteristic feature of vasoconstrictor neurons (12–14, 35).

Electrophysiological properties of vascular neurons. Neurons in sympathetic ganglia can be classified electrophysiologically as tonic or phasic firing based on their response to direct somal depolarization (5, 7, 26, 41). In the present study, all vascular neurons, whether arterial or venous, were tonic. Our data provide the first direct evidence linking a particular IMG neuronal category, i.e., tonic firing neurons, with a specific function, i.e., innervation of mesenteric vasculature. These data are in contrast to previous studies which speculated that phasic neurons are involved in vasoconstriction because of the preponderance of phasic neurons in paravertebral ganglia, a majority of which subserve primarily vasomotor functions (31). It is unlikely that the electrophysiological homogeneity of the retrogradely labeled vascular neurons in the present study was a product of the organ culture procedure, however, because 1) phasic neurons were among the unlabeled population, and 2) the proportion of phasic vs. tonic neurons was the same in neurons dissociated from ganglia immediately after removal from the animal (70 vs. 30%, respectively) as it was in neurons dissociated from ganglia that were organ cultured (69 vs. 31%, respectively). Finally, the basic passive properties of the labeled neurons did not differ from those of unlabeled or acutely dissociated neurons.

The voltage-sensitive currents examined that could account for the tonic firing characteristics of vascular neurons were compared among the four groups of identified neurons, i.e., arterial, venous, unlabeled, and acutely dissociated neurons. Although I M is considered to impose phasic firing behavior on sympathetic neurons (4, 40), the presence of a small I M has also been demonstrated in tonic firing neurons (4), so its presence in tonic firing neurons of the present study should not be considered surprising. Similarly, the high percentage of tonic neurons expressing I A is not unexpected, given the association of the I A with tonic firing neurons (6).

Interestingly, venous neurons differed electrophysiologically from arterial neurons in that arterial neurons were more likely to show inward rectification. By closing upon depolarization, inwardly rectifying potassium channels prolong membrane depolarizations (4). Tonic firing celiac ganglia neurons receive small, sub-threshold fast as well as slow excitatory synaptic inputs (32). The presence of inward rectification in
tonic arterial neurons may be of significance, since the membrane depolarization to excitatory synaptic inputs would be larger, hence less synaptic excitation would be required to reach action potential threshold. Indeed, the depolarization required to reach threshold for action potential firing is significantly less in arterial neurons when compared with the other neuronal groups. In addition, mesenteric arteries require higher frequencies of nerve stimulation not only to induce the same degree of norepinephrine-mediated vasoconstriction as veins, but also to induce the release of nonnoradrenergic mediators such as NPY, which play important additional roles in sympathetic vasoconstriction (42). Inward rectification would allow arterial neurons to fire action potentials at these required higher frequencies.

In conclusion, our results support the concept of differential sympathetic control of arteries and veins (16, 18, 22, 27). Arterial and venous neurons are separate subpopulations within the IMG. Their different location within the IMG provides a structural basis for their separate activation by synaptic inputs. In addition, their different neurochemical and electrophysiological properties could be the basis for distinctly different responses to synaptic activation. A more complete understanding of the impact of the structure and function of arterial and venous neurons on the neurogenic regulation of artery and vein awaits further study.

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