Phenotypic characterization of gastric sensory neurons in mice

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Recent studies suggest that the capsaicin receptor [transient receptor potential vanilloid (TRPV1)] may play a role in visceral mechanosensation. To address the potential role of TRPV1 in vagal sensory neurons, we developed a new in vitro technique allowing us to determine TRPV1 expression directly in physiologically characterized gastric sensory neurons. Stomach, esophagus, and intact vagus nerve up to the central terminations were carefully dissected and placed in a perfusion chamber. Intracellular recordings were made from the soma of nodose neurons during mechanical stimulation of the stomach. Physiologically characterized neurons were labeled iontophoretically with neurobiotin and processed for immunohistochemical experiments. As shown by action potential responses triggered by stimulation of the upper thoracic vagus with a suction electrode, essentially all abdominal vagal afferents in mice conduct in the C-fiber range. Mechanosensitive gastric afferents encode stimulus intensities over a wide range without apparent saturation when punctate stimuli are used. Nine of 37 mechanosensitive vagal afferents expressed TRPV1 immunoreactivity, with 8 of the TRPV1-positive cells responding to stretch. A small number of mechanosensitive gastric vagal afferents express neurofilament heavy chains and did not respond to stretch. By maintaining the structural and functional integrity of vagal afferents up to the nodose ganglion, physiological and immunohistochemical properties of mechanosensory gastric sensory neurons can be studied in vitro. Using this novel technique, we identified TRPV1 immunoreactivity in only one-fourth of gastric mechanosensitive neurons, arguing against a major role of this ion channel in sensation of mechanical stimuli under physiological conditions.

vagal afferents can be differentiated into low- and high-threshold fibers (25, 34, 35). This is consistent with the generally held notion that information regarding potentially injurious stimuli is relayed by specialized spinal nociceptors (10). However, both vagal and spinal sensory neurons sensitize in response to experimental inflammation associated with visceral hypersensitivity (3, 4, 33). Moreover, psychophysical studies in humans demonstrate that pharmacological modulation of gastric sensory-motor function shifts perception of low- and high-intensity stimuli, suggesting that summation of afferent input (i.e., intensity) rather than activation of specific pathways (i.e., specificity) is important in triggering sensations of discomfort and pain (49).

Several potential candidate molecules have been identified that may be involved in mechanosensation and its modulation. Among those are members of the transient receptor potential (TRP) family of ion channels, a group of nonselective cation channels first identified in Drosophila that may contribute to the transduction of mechanical stimuli (29). Several recent studies suggest that the capsaicin receptor [TRP vanilloid (TRPV1)], a nonselective cation channel activated by heat, protons, and endogenous lipid mediators, may directly or indirectly modulate mechanosensory function of the gastrointestinal tract. Mechanosensitive fibers of TRPV1-knockout mice exhibit blunted responses to graded jejunal distension or colorectal stretch (24, 41). Immunohistochemical experiments have demonstrated a higher density of TRPV1-immunoreactive fibers in biopsies of the rectal mucosa obtained from patients with rectal hypersensitivity or urgency (11). The pungent vanilloid capsaicin, which activates TRPV1, acutely sensitizes humans to gastric distension (28). Molecular and functional data support the presence of several different TRP channels, including TRPV1, in nodose neurons (57). We recently showed (13) that half of gastric nodose neurons studied in vitro respond to capsaicin. Berthoud et al. (2) reported that about one-third of vagal afferents innervating the stomach respond to mucosal application of capsaicin. To more directly determine the role of TRPV1-positive cells in gastric mechanosensation, we developed a novel experimental approach combining the characterization of mechanosensory properties in vitro with immunohistochemical studies of identified gastric nodose neurons.

METHODS

Experiments were performed with male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) weighing 15–20 g. Animal handling adhered to the Guide for the Care and Use of Laboratory Animals (National Research Council); all procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Mice were housed under a 12:12-h light-dark cycle with free access to food and water.

Mice were anesthetized with an intraperitoneal injection of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) and perfused transcardially with chilled (4°C) and oxygenated (95% O2-5% CO2) artificial cerebrospinal fluid (ACSF) in which most sodium cations had been replaced with sucrose (in mM: 253 sucrose, 1.9 KCl, 1.2 KH2PO4, 1.3 MgSO4, 2.4 CaCl2, 26.0 NaHCO3, 10.0 D-glucose) to...
decrease excitotoxicity. The abdominal cavity was exposed through a midline incision, and stomach, liver, spleen, and mediastinal structures were rapidly dissected up to the thoracic inlet. The vertebral column was then cut immediately distal of the thoracic inlet and head, and mediastinal and upper abdominal organs were placed in a dish and submerged in the same ice-cold solution for further dissection. Liver, spleen, lungs, diaphragm, and heart were removed. The skull was opened, and the nodose ganglion was exposed in the jugular foramen and carefully dissected free. The cervical vagus was isolated and freed of connective tissue down to the origin of the recurrent laryngeal nerve. The proximal esophagus was cut, and the distal end was freed from the posterior aspect of the vertebral column, leaving the continuity of the stomach with intact vagus up to central filaments projecting to the brain stem. Finally, the stomach was opened along the greater curvature and cleaned of its contents. The entire preparation was then transferred into a recording chamber, which was constantly perfused at a rate of 50 ml/min with ice-cold oxygenated ACSF without sucrose and with 127 mM NaCl (concentration of other constituents as described above). The stomach (mucosal side up) was secured with minuten pins, and the solution was slowly warmed to 32°C for electrophysiological recording. Most preparations were performed with the right vagus, with dissections of the left vagal pathway in only 15% of the experiments.

Physiological characterization of vagal neurons. A suction electrode was placed on the proximal vagus (~6–8 mm distal of the nodose neuron. Brief current pulses (0.5 ms; 5–10 mA) were administered repeatedly at 0.3 Hz to identify neurons driven by vagal stimulation. The search stimulus triggered visible contractions of the esophagus, confirming the functional integrity of the efferent vagal pathway. Nodose neurons were impaled intracellularly with glass microelectrodes (tip resistance >100 MΩ) filled with 1 M potassium acetate containing 5% neurobiotin (Vector Laboratories, Burlingame, CA). Intracellular penetrations caused a sudden voltage change and showed an action potential with a latency of ~15 ms after the electrical search stimulus if cells were driven by vagal stimulation. Cells with a resting membrane potential of at least −35 mV and an action potential overshoot (peak >0 mV) were selected for further characterization (16). Cells were studied with an Axoclamp 2B amplifier, digitized (CED Micro 1401; Cambridge Electronic Design, Cambridge, UK) with a sampling rate of 44 kHz, and stored on a personal computer with Spike2 software (Cambridge Electronic Design). Resting membrane potential, spontaneous action potential frequency, action potential amplitude and half-width, and amplitude and duration of the afterhyperpolarization were determined for all cells. The conduction velocity was calculated, using the distance between the suction electrode and the nodose ganglion and the latency between search stimulus and onset of the action potential.

After stable recordings were established, the stomach was gently probed with a blunt glass probe (1 mm in diameter) to determine the number and location of receptive fields within the stomach. If cells responded to this gentle pressure, the size of the receptive field(s) and the response properties were further characterized with calibrated von Frey filaments (2.83–4.56 mN). Distinct receptive fields had to be separated by an unresponsive area that measured at least 2 mm. To allow comparison with previously published work, we used weight (0.07–4 g) rather than the force exerted by the filaments when displaying results. Finally, the stomach was stretched by 2 mm in the longitudinal and circular directions with a small forceps. Responses were analyzed by determining the number of action potentials in 100-ms intervals for 1 s after the onset of the stimulus.

After physiological characterization, the approximate location of the neuron within the ganglion was noted and the cell was iontophoretically labeled with neurobiotin (500–ms current pulses at 10–20 nA for 60–100 s). At the end of the experiment, the nodose ganglion was removed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB).

Immunohistochemistry. After a fixation time of ~30 min, the ganglion was transferred into 0.1 M PB with 25% sucrose and stored at 4°C for at least 18 h before embedding in 10% gelatin dissolved in 0.1 M PB, which was hardened with 4% paraformaldehyde until firm. Sections (35 μm) were cut on a sliding microtome, washed in three times in 0.1 M PB, and placed in blocking solution containing 5% normal goat serum and 0.25% Triton X-100. Sections were then placed in a solution with fluorescently tagged avidin to label neurobiotin-filled cells (Molecular Probes, Eugene, OR). Next, floating sections were incubated overnight at room temperature with antibodies against TRPV1 (1:50; catalog no. PC420, Calbiochem, San Diego, CA) and/or the neurofilament heavy chain (1:400; N52, Sigma Biochemicals, St. Louis, MO; monoclonal antibody against COOH terminus of the pig neurofilament 200) and/or Alexa Fluor 647-conjugated isolecitin Griffonia simplicifolia IB4 (IB4, 1:100; Molecular Probes). After being washed three times in 0.1 M PB, sections were incubated for 2 h at room temperature with Alexa Fluor 488-conjugated avidin (1:500; Molecular Probes) to visualize the iontophoretically injected neurobiotin and secondary antibodies, donkey anti-rabbit Cy3 or anti-Cy5 mouse (1:150; Jackson Immunoresearch, West Grove, PA). The sections were then washed three times in 0.1 M PBS and mounted in 0.1% gelatin. Labeling was analyzed with an Olympus confocal microscope (Leica Microsystems, Wetzlar, Germany) and sequential scanning to prevent bleed-through of the different fluorophores. Alexa Fluor 488-conjugated avidin was visualized at an excitation wavelength of 495 nm (emission filter 520 nm) and the Cy3- and Cy5-coupled antibodies with excitation wavelengths of 550 nm (emission filter 570 nm) and 650 nm (emission filter 670 nm), respectively. We measured the size of gastric sensory neurons by determining the perimeter with imaging processing software (Image J 1.29, Wayne Rasband, National Institutes of Health). The very low background staining of the Alexa Fluor 488-conjugated avidin did not permit measurement of unlabeled cells. As most nodose neurons bind the lectin IB4 (22), we used the merged immunohistochemical image of double-labeled sections (IB4 and TRPV1) to determine the cell perimeter for such unlabeled cells. Only cells with a visible nucleus were used for this analysis.

Statistical analysis. All data are given as means ± SE. Results were analyzed with the Mann-Whitney rank-sum test, two-way ANOVA, or Fisher exact test where appropriate. A value of P < 0.05 was considered statistically significant.

RESULTS

A total of 863 nodose neurons responding to the electrical search stimulus were studied. All but 13 cells (98%) were C fibers with conductance velocities of 1 m/s or less (Fig. 1A). Cells with resting membrane potentials of ~45 mV or less had lower peak amplitudes of the action potential and significantly longer afterhyperpolarizations compared with the remaining cells (Table 1). With gentle probing of the stomach as the search stimulus, 79% (9%) of the cells responded to gastric stimulation. The receptive fields were punctate, measuring ~1–2 mm² in diameter. Five cells (6%) responded to probing of more than one site separated by at least 2 mm. Most of the receptive fields were in the forestomach, with the neurons in the right and left ganglion being activated by stimulation of the dorsal or ventral aspect of the gastric wall, respectively (Fig. 2A). Gastric sensory neurons were located throughout the nodose ganglion, with 43% found in the distal third, 41% in the middle, and 16% in the proximal third of the nodose ganglion (Fig. 2B).

Basic electrophysiological properties of gastric vagal neurons. All of the identified gastric sensory neurons conducted in the C-fiber range with a mean of 0.53 ± 0.02 m/s, which did
not differ significantly from nodose neurons not responding to mechanical stimulation of the stomach (Fig. 1A). Stably impaled gastric cells had a resting membrane potential of $-48.3 \pm 1.4\,\text{mV}$, which again was similar to results obtained in the nonresponsive neurons studied (Fig. 1B). Whereas essentially all nonresponsive cells were quiescent at rest, $44\,(56\%)$ of the gastric nodose neurons generated action potentials at rest at a frequency of $1.1 \pm 0.1\,\text{Hz}$. As shown in Fig. 1C, action potentials showed a distinct hump on the falling phase in $35\%$ of the gastric neurons. The action potential half-width ($2.2 \pm 0.1\,\text{ms}$) did not differ between gastric nodose neurons and neurons not responding to gastric distension (Fig. 1D). All but six ($8\%)$ of the gastric neurons had a distinct afterhyperpolarization with an average amplitude of $16 \pm 1\,\text{mV}$ and a duration of $19 \pm 2\,\text{ms}$ (Fig. 3). The histogram in Fig. 3B shows that the afterhyperpolarization did not differ between gastric nodose neurons and neurons not responding to gastric stimulation.

We next examined whether different active and passive membrane properties correlated. As conduction velocity showed relatively homogeneous distribution with all gastric sensory neurons conducting in the C-fiber range, we did not look for correlations between this property and other variables. The resting membrane potential did not correlate with the half-width of the action potential ($r^2 = 0.006, P = 0.57$; Fig. 4A) or the duration of the hyperpolarization ($r^2 = 0.07, P =$

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Table 1. Different active and passive membrane properties of nodose neurons based on resting membrane potential

<table>
<thead>
<tr>
<th>Property</th>
<th>Cells with $V_m \leq -45,\text{mV}$</th>
<th>Cells with $V_m &gt; -45,\text{mV}$</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_m$, mV</td>
<td>$-55.3\pm 0.4$</td>
<td>$-40.5\pm 0.2$</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>CV, m/s</td>
<td>$0.6\pm 0.02$</td>
<td>$0.55\pm 0.01$</td>
<td>$P = 0.1$</td>
</tr>
<tr>
<td>AP amplitude, mV</td>
<td>$73.3\pm 0.8$</td>
<td>$65.0\pm 0.8$</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td>AP duration, ms</td>
<td>$1.99\pm 0.04$</td>
<td>$2.0\pm 0.04$</td>
<td>$P = 0.96$</td>
</tr>
<tr>
<td>AHP amplitude, mV</td>
<td>$13.3\pm 0.5$</td>
<td>$19.1\pm 0.4$</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td>AHP duration, ms</td>
<td>$16.7\pm 0.9$</td>
<td>$21.3\pm 0.7$</td>
<td>$P &lt; 0.01$</td>
</tr>
</tbody>
</table>

Values are means $\pm$ SE. $V_m$, resting membrane potential; CV, conduction velocity; AP, action potential; AHP, afterhyperpolarization.
0.06; Fig. 4C). In contrast, there was a relationship between the resting membrane potential and the amplitude of the action potential \( r^2 = -0.21, P < 0.01 \); Fig. 4B) and afterhyperpolarization \( r^2 = 0.26, P < 0.01 \); Fig. 4D). Although action potential duration and amplitude were not correlated \( r^2 = 0.04, P = 0.15 \), afterhyperpolarization amplitude significantly correlated with duration \( r^2 = 0.26, P < 0.01 \).

Response properties of gastric vagal neurons. As shown in Fig. 5, mechanical stimulation typically triggered a distinct response with a rapid increase in action potential frequency. Within 1 s after stimulus onset, the maximal action potential frequency fell to 44 ± 8% from its peak of 20.6 ± 2.7 Hz (Fig. 6). We used calibrated von Frey filaments to characterize the mechanical properties of gastric vagal neurons. The high flow

Fig. 3. Afterhyperpolarization (AHP) in nodose neurons. A: representative voltage tracings of gastric sensory neurons without (top) and with (bottom) distinct AHP. B: histogram illustrates the distribution of AHP duration in all nodose (filled bars) and gastric sensory neurons (open bars).

Fig. 4. Correlation between resting membrane potential and active membrane properties. A: scatter plots for all nodose neurons and gastric nodose neurons reveal no relationship between resting membrane potential and action potential duration. B: there was a significant inverse relationship between the resting membrane potential and the peak amplitude of the action potential. Both duration (C) and amplitude (D) of the AHP significantly correlated with the resting membrane potential.
rate of the oxygenated ACSF did not allow reproducible application of filaments with a force of \( \frac{8.33}{1021} \) mN (0.07 g). The response rate significantly rose with increasing filament force and did not show saturation at 4,56 mN (4.0 g; Fig. 5). To minimize local damage, stimulus strength was not increased beyond this point. On the basis of previous reports a 3.61-mN (0.4 g) von Frey hair was used to classify neurons as high (if they did not respond) or low (if they did respond) threshold (30, 37). Twenty-one of 33 (64%) completely studied cells responded to filament forces \( \leq 3.61 \text{ mN (0.4 g)} \) with a mean threshold of 0.126 ± 0.01 g compared with 0.77 ± 0.3 g for cells requiring stronger stimulation. The peak response to mechanical stimulation was significantly higher (30 ± 2 vs. 16 ± 4 Hz; \( P < 0.02 \)) in neurons with lower response threshold. However, resting membrane potential (−52 ± 2.7 vs. −51 ± 2.8 mV; \( P = 0.91 \)), action potential frequency at baseline (1.4 ± 0.2 vs. 1.1 ± 0.2 Hz; \( P = 0.3 \)), action potential duration (2.2 ± 0.2 vs. 1.7 ± 0.2 ms; \( P = 0.26 \)), afterhyperpolarization duration (17.8 ± 3.5 vs. 26.8 ± 8.3 ms; \( P = 0.47 \)), and adaptation during a 1-s stimulus (51 ± 10 vs. 37 ± 15% of peak; \( P = 0.37 \)) did not differ between cells with low and high threshold to gastric stimulation, respectively.

All but eight vagal sensory neurons (24%) exhibited at least some adaptation during continuing mechanical stimulation. The small number of neurons without adaptation limits the power of a statistical analysis. Considering this important caveat, nonadapting and adapting cells did not significantly differ in terms of resting membrane potential (−47 ± 3 vs. −55 ± 3 mV; \( P = 0.12 \)), duration of the action potential

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**Fig. 6.** Adaptation in response to different mechanical stimuli. A: representative responses to mechanical stimulation with a von Frey filament (4.17 mN). The example in the top trace continues to fire throughout the entire stimulus, whereas the bottom trace shows rapid adaptation. B: time-dependent change in action potential frequency during ongoing stimulation, revealing significant adaptation at all stimulus intensities tested. C: adaptation was similar for stretch stimulation (●) and punctate stimulation with 4.56-mN von Frey hairs (○). Although the peak response to stretch was significantly lower compared with the punctate stimulus, there was no difference in adaptation.
(2.5 ± 0.4 vs. 1.8 ± 0.2 ms; P = 0.15) or afterhyperpolarization (20.5 ± 5.7 vs. 18.7 ± 4.7 ms; P = 0.24), response threshold (0.35 ± 0.2 vs. 0.23 ± 0.03 g; P = 0.96), or peak response frequency (17 ± 5 vs. 26 ± 4 Hz; P = 0.15). Although the number of action potentials per 100 ms clearly depended on the stimulus strength (F = 18.5, P < 0.01) and decreased over time (F = 42.7, P < 0.01), the stimulus intensity did not significantly affect the rate of decline (F = 0.5, P = 0.99; Fig. 6B).

In 52 cells, qualitative responses to stretch were tested, with 40 (77%) responding with a frequency of 14 ± 2 Hz. Compared with the maximal response during stimulation with calibrated filaments, the action potential frequency was significantly lower (F = 12.8, P < 0.01). As shown in Fig. 6C, stretch responses showed a similar adaptation with time-dependent decrease of spike frequency (F = 8.3, P < 0.01) at a rate that did not differ from that observed during von Frey hair application (F = 0.18, P = 0.98). Cells not responding to stretch did not differ from stretch-responsive cells in resting membrane potential (−48 ± 4 vs. −48 ± 2 mV; P = 0.93), duration of action potential (2.3 ± 0.2 vs. 2.0 ± 0.2 ms; P = 0.26) or afterhyperpolarization (22 ± 4 vs. 17 ± 3 ms; P = 0.33), and peak frequency during von Frey filament stimulation (17 ± 10 vs. 22 ± 3 Hz; P = 0.53) or adaptation (18 ± 12 vs. 49 ± 9%; P = 0.13). However, there was a trend to higher thresholds for von Frey filaments in neurons not responding to stretch (1.06 ± 0.6 vs. 0.22 ± 0.05 g; P = 0.09).

Neurochemical properties of gastric vagal neurons. Of the total sample of 79 neurons with gastric receptive fields, iontophoretically injected neurobiotin allowed unambiguous identification in 38 cases (Fig. 7). A total of 37 cells tested well for immunoreactivity for TRPV1, with 9 (24%) showing immunoreactivity. There was no significant difference between TRPV1-positive and -negative cells in resting membrane potential (−51.0 ± 2.2 vs. −50.1 ± 2.3 mV; P = 0.75; TRPV1 positive vs. TRPV1 negative), duration of action potential

Fig. 7. Neurochemical characterization of gastric sensory neurons. A: a single neuron labeled with neurobiotin. B: immunohistochemical staining for transient receptor potential vanilloid (TRPV)1 labels about half of the cells. C: merged image shows that the gastric neuron studied was TRPV1 negative. Most nodose neurons react with the plant lectin IB4 (D), with the merged image (E) demonstrating IB4 staining in the gastric neuron. F demonstrates a significant overlap between IB4 and TRPV1 staining in nodose neurons.
TRPV1-positive and -negative cells (0.27). Eleven of the TRPV1-negative cells (39%) did not respond to stretch, whereas only one of the TRPV1-positive cells was unresponsive to circular and longitudinal stretch.

As shown in Fig. 8A, cell sizes did not differ between TRPV1-positive and -negative (P = 0.32). We also compared the size of all gastric mechanosensory neurons identified by neurobiotin labeling with that of 106 unlabeled nodose neurons with clearly defined circumference and similarly did not observe differences in cell size.

The plant lectin IB4 labels small, nonpeptidergic spinal neurons and can be found in a subgroup of nodose neurons (20, 47). In preliminary experiments, we noted IB4 staining in ~45% of retrogradely labeled gastric sensory neurons (unpublished data). Six of 14 intracellularly filled neurons bound the plant lectin IB4. IB4-positive neurons had a lower resting membrane potential (~59.5 ± 5.4 mV vs. ~45.6 ± 3.9 mV; P < 0.05; IB4 negative vs. IB4 positive) but did not differ from IB4-negative cells in terms of the duration of action potential (1.7 ± 0.3 vs. 2.2 ± 0.5 ms; P = 0.47) and afterhyperpolarization (7.8 ± 4.6 vs. 15.4 ± 2.6 ms; P = 0.16), resting activity (0.9 ± 0.4 vs. 1.4 ± 0.3 Hz; P = 0.36), or peak response to mechanical stimulation (24 ± 5 vs. 22 ± 6 Hz; P = 0.79). All IB4-negative and all but one of the IB4-positive cells responded to stretch. Even though all gastric neurons had conduction velocities in the C-fiber range, 25% of the retrogradely labeled gastric sensory neurons showed immunoreactivity for neurofilament heavy chains (unpublished data). We thus tested N52 immunoreactivity in triple-labeling experiments in 14 cells, 2 of which were N52 positive. As already described above, both of these conducted in the C-fiber range and did not respond to stretch. The cell bodies were located in the distal and middle sections of the nodose ganglion.

Thirteen cells were tested for TRPV1 immunoreactivity and IB4 labeling. Three TRPV1-positive cells were also positive for IB4, whereas only 3 of the 10 TRPV1-negative cells showed lectin binding. Of 14 neurons stained for TRPV1 and IB4, whereas only 3 of the 10 TRPV1-negative cells showed immunoreactivity for neurofilament heavy chains and do not respond to stretch. However, nearly half of the pulmonary afferents in the rat had conduction velocities exceeding 15 m/s (21). Similarly, one-fifth of guinea pig and nearly two-thirds of opossum esophageal afferents respond to stretch. The cell bodies were located in the distal and middle sections of the nodose ganglion.

DISCUSSION

We developed a new in vitro technique that allows a detailed physiological and neurochemical characterization of gastric vagal afferents. The key findings of our initial investigation using this approach are that 1) essentially all abdominal vagal afferents in mice conduct in the C-fiber range; 2) mechanosensitive gastric afferents encode stimulus intensities over a wide range without apparent saturation when punctate stimuli are used; 3) about one-fourth of the mechanosensitive vagal afferents express TRPV1 immunoreactivity, with ~90% of TRPV1-positive cells responding to stretch; and 4) a small number of mechanosensitive gastric vagal afferents express neurofilament heavy chains and do not respond to stretch.

Conduction velocity of vagal afferents in the mouse. Placement of a suction electrode onto the upper thoracic vagus allowed us to determine the conduction velocity of successfully impaled neurons independent of their receptive field within the stomach. With this paradigm, only 2% of the nodose neurons had conduction velocities in the range typical for Aδ fibers. Although our data are consistent with morphological studies, physiological experiments performed in other species and/or with other approaches have described a more heterogeneous nature of vagal afferents with conduction velocities in the C- and Aδ-fiber range. Ultrastructural examinations of the rat thoracic and abdominal vagus demonstrated that >95% of the fibers are unmyelinated, with the cervical vagus proximal to the recurrent laryngeal nerve containing a significant number of myelinated axons (23, 40). In line with these results, the compound action potential of the cervical vagus shows at least two distinct voltage responses with conduction velocities falling into the range typical for C and Aδ fibers (17, 31). More detailed physiological data have been obtained with single-fiber recordings from the cervical vagus and extracellular recordings in the nodose ganglion. In the mouse, rabbit, and guinea pig, 90–95% of vagal afferents are C fibers (26, 46, 50). However, nearly half of the pulmonary afferents in the rat had conduction velocities exceeding 15 m/s (21). Similarly, one-fifth of guinea pig and nearly two-thirds of opossum esophageal afferents respond to stretch, whereas only one of the TRPV1-positive cells was unresponsive to circular and longitudinal stretch.

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The plant lectin IB4 labels small, nonpeptidergic spinal neurons and can be found in a subgroup of nodose neurons (20, 47). In preliminary experiments, we noted IB4 staining in ~45% of retrogradely labeled gastric sensory neurons (unpublished data). Six of 14 intracellularly filled neurons bound the plant lectin IB4. IB4-positive neurons had a lower resting membrane potential (~59.5 ± 5.4 mV vs. ~45.6 ± 3.9 mV; P < 0.05; IB4 negative vs. IB4 positive) but did not differ from IB4-negative cells in terms of the duration of action potential (1.7 ± 0.3 vs. 2.2 ± 0.5 ms; P = 0.47) and afterhyperpolarization (7.8 ± 4.6 vs. 15.4 ± 2.6 ms; P = 0.16), resting activity (0.9 ± 0.4 vs. 1.4 ± 0.3 Hz; P = 0.36), or peak response to mechanical stimulation (24 ± 5 vs. 22 ± 6 Hz; P = 0.79). All IB4-negative and all but one of the IB4-positive cells responded to stretch. Even though all gastric neurons had conduction velocities in the C-fiber range, 25% of the retrogradely labeled gastric sensory neurons showed immunoreactivity for neurofilament heavy chains (unpublished data). We thus tested N52 immunoreactivity in triple-labeling experiments in 14 cells, 2 of which were N52 positive. As already described above, both of these conducted in the C-fiber range and did not respond to stretch. The cell bodies were located in the distal and middle sections of the nodose ganglion.

Thirteen cells were tested for TRPV1 immunoreactivity and IB4 labeling. Three TRPV1-positive cells were also positive for IB4, whereas only 3 of the 10 TRPV1-negative cells showed lectin binding. Of 14 neurons stained for TRPV1 and IB4, whereas only 3 of the 10 TRPV1-negative cells showed immunoreactivity for neurofilament heavy chains and do not respond to stretch. However, nearly half of the pulmonary afferents in the rat had conduction velocities exceeding 15 m/s (21).
geal afferents had conductance velocities in the Aβ-fiber range (44, 53). For the abdominal vagus, rat gastric afferents studied in vivo all conducted in the C-fiber range (14, 25, 35, 36). However, in the ferret 60% of the vagal afferents innervating the stomach were Aβ fibers (7, 37). Using a different approach with extracellular recordings of mechanosensitive afferents within the nodose ganglion of the cat, Gaige et al. (19) reported that all cells innervating the intestine conducted in the C-fiber range. The reason for these apparent discrepancies is not clear. Experiments were performed in different species from mouse to cat, which may explain some, but not the entire, variability. In view of reported differences between the rostral and caudal portion of the nodose ganglion in other species (53), we typically searched the nodose ganglion in its entirety, thus arguing against a systematic bias due to the experimental approach. Interestingly, cell bodies of the two neurofilament-positive gastric neurons were not located in the rostral third of the ganglion. Technical factors likely contribute to the differences in experimental results. For example, small-diameter fibers may lead to relatively low signal intensity, thus potentially biasing teased fiber recordings toward larger and more rapidly conducting fibers. We do not expect a systematic bias in the opposite direction (i.e., preferential identification of neurons with unmyelinated axons) in our study. In dorsal root ganglion neurons, the soma of unmyelinated neurons is typically small in diameter (27). Whereas a relationship between soma size and conduction velocity may not hold for visceral sensory neurons, the use of sharp electrodes would not lead to a systematic bias in favor of such small cells, as those are actually more difficult to impale.

Mechanosensitive gastric vagal afferents in the mouse. The main goal of our investigation was a comprehensive characterization of gastric vagal sensory neurons. Consistent with prior studies using the teased fiber technique, vagal afferent neurons innervating the stomach either were quiescent or had a low resting activity (7, 25, 35, 38, 55). The basic electrophysiological properties are in line with previously published results obtained with sharp electrodes in isolated neurons or in the intact nodose ganglion (1, 31, 46, 50). Approximately one-third of the cells had a distinct hump on the falling phase of the action potential. Importantly, action potential duration did not correlate significantly with the resting membrane potential, suggesting differences in intrinsic properties, such as differential ion channel expression, among nodose neurons. However, all of the gastric and nearly all of the nodose neurons as a whole conducted in the C-fiber range, thus raising questions about the validity of somatic action potential morphology as a surrogate marker to identify A- and C-type neurons in nodose neurons (43). Unlike previous studies of the guinea pig nodose neurons (50), we did not observe prolonged afterhyperpolarizations in our sample. Although most cells generated relatively short hyperpolarizations, ~10% of the cells did not have a distinct afterhyperpolarization. Considering the potential importance of the afterhyperpolarization in response pattern and frequency, we compared properties of mechanosensitive gastric neurons that exhibited significant adaptation during continuing mechanical stimulation to those that showed little adaptation (50). The duration and amplitude of afterhyperpolarization did not differ between these two groups. Moreover, there was no correlation between the degree of adaptation and duration ($r^2 = 0.01$) or amplitude ($r^2 = 0.03$) of the afterhyperpolarization.

Using blunt probing of the entire stomach, we noted receptive fields throughout the entire stomach, with a preferential localization closer to the lesser curvature, which corresponds with the branching pattern of vagal afferent fibers and density of intermuscular arrays, but less so with the intraganglionic laminar endings (18, 51). Nearly half of the receptive fields were located in cardia and fundus. This differs from results obtained with gastric distension as the search stimulus in vivo, which primarily activates afferents with receptive fields in corpus or antrum (14, 25, 35, 45). The receptive fields were punctate and typically measured <2 mm² in size, which is consistent with results obtained in vitro and in vivo (2, 6, 54–56). In only 5% of the gastric sensory neurons examined did we find more than a single receptive field within the stomach. These results are lower than previously reported data obtained in rats (17%) or guinea pigs, where half of the mechanosensitive afferents had more than one “hot spot” as defined by responses to punctate stimulation with von Frey filaments (2, 55). In the guinea pig, these distinct hot spots were often found in close proximity, located adjacent to different intraganglionic laminar endings of a single branching axon. Considering our operational definition of multiple receptive fields (separation by at least 2 mm) and the obvious differences in animal size, our results clearly represent the lower limit of multiple receptive fields for a single afferent neuron.

Consistent with data obtained with the teased fiber technique, vagal afferents responded to a wide range of stimulus intensities without apparent saturation (25, 35, 37, 39). In the guinea pig esophagus, Yu et al. (53) recently described a subgroup of myelinated vagal fibers that responded to esophageal distension with apparent saturation at low distension pressures. We (25) and others (36, 38, 39) were not able to identify similarly behaving mechanosensory afferents in the mouse or rat stomach. In the absence of direct species comparisons using identical experimental approaches, this discrepancy may be due in part to species differences. We chose a stimulation paradigm that relied on focal application of a defined force, allowing us to localize receptive fields, reproducibly trigger responses, and determine response thresholds. It is certainly difficult to relate in vitro data obtained through stretch or punctate stimulation to distension of a hollow organ. However, consistent with results obtained with the teased fiber technique (38, 39), our findings do not show an apparent saturation with increasing stimulation intensity, thus suggesting that vagal fibers may encode stimuli into the noxious range.

Detailed physiological studies in the proximal and distal gastrointestinal tract separated afferent fibers based on their differential responses to stretch and stimulation with von Frey hairs (8, 24, 30, 37, 39, 53). In the mouse stomach, mucosal and tension receptors have been identified experimentally based on their response patterns to mucosal stroking and circular tension, respectively (38, 39). As stronger punctate stimuli distort the underlying tissue, thereby activating stretch and tension receptors, low filament forces are required to more clearly distinguish between these groups. However, the flow rate required to maintain viability of our preparation did not allow us to reliably apply stimulus intensities below 0.07 g (2.83 mN). Consistent with prior reports, we noted differences...
in threshold and/or response pattern and rate (6, 14, 39). Two-thirds of the cells responded with a train of rapidly adapting action potentials to the lowest filament force. The properties of these cells resemble the “mucosal afferents” that have been described in the ferret and mouse (37–39). Although experiments performed with the teased fiber technique showed a lower resting activity in mucosal afferents, we did not observe differences in the resting activity of these cells compared with neurons responding to higher stimulation intensities and stretch.

None of the mechanosensitive nodose neurons displayed the typical properties of “tension” fibers described in ferrets and mice, with rhythmic baseline activity and slowly adapting responses to stretch (37–39). Considering the distortion of underlying tissue with stronger filament strength, it is not surprising that response patterns did not differ qualitatively between stretch and punctate stimulation with von Frey filaments, with both showing significant adaptation with a comparable time course. Only a small number of neurons displayed little adaptation during the continuing stimulus. However, other parameters, most notably the activation threshold for punctate stimuli, did not differ significantly from the remaining mechanosensitive gastric neurons, thus arguing against interpreting these nonadapting neurons as the correlate of “tension” receptors seen in teased fiber preparations. Whereas we occasionally saw low-amplitude spikes with a peak of <20 mV, it is unlikely that action potential failures within the soma are the main reason for the discrepancies between different experimental approaches.

**Neurochemical properties of vagal gastric sensory neurons.** Considering recent findings suggesting a special role for TRPV1 in mechanosensation of gastrointestinal afferents (24, 41), we focused on TRPV1 immunoreactivity of identified gastric sensory neurons. Staining for TRPV1 was successful in 37 cells, with 9 showing clear immunoreactivity for TRPV1. With capsaicin responses used as a surrogate marker for TRPV1 expression, a comparable fraction of mechanosensitive gastric afferent fibers responded to mucosal application of capsaicin (7, 37). We did not perform similar experiments to examine capsaicin responses in identified neurons, as the associated manipulation in the experimental chamber led to loss of intracellular access. Electrophysiological experiments in isolated neurons suggest that a higher fraction of gastric sensory neurons express the capsaicin receptor, but this may reflect an increased TRPV1 expression resulting from the dissociation and culturing of cells in vitro (13). Moreover, our results and those obtained with the teased fiber technique relied on a search strategy employing a mechanical stimulus, thus providing a low estimate of capsaicin-responsive neurons because we would not detect neurons activated by other stimulus modalities, such as temperature or chemical, unless they also responded to mechanical stimulation. Considering the fact that the majority of gastric mechanosensory neurons do not exhibit TRPV1 immunoreactivity, our data clearly rule out a direct role for TRPV1 in mechanosensation. However, there is a very real possibility that TRPV1 modulates the stretch response as indicated by studies in TRPV1−/− mice that show a blunted response to visceral distension (5, 24, 41). That is, there is mounting evidence that TRPV1 is not necessary for detection of a range of stimuli like heat (52) and mechanical probing, but its presence is required for the full range of responses or for the development of hyperalgesia in pathological states like inflammation (9, 32). The exact mechanisms underlying such an effect of TRPV1 are unclear. Current evidence does not support direct gating of this ion channel by stretch (29). In the absence of a direct effect, activation of TRPV1, for example through endogenous ligands, may alter neuron excitability, thereby indirectly affecting mechanosensation (15, 48).

We also examined the presence of two other neurochemical markers, the lectin IB4, which is often used as a surrogate marker for nonpeptidergic, nociceptive spinal afferent neurons (47), and the presence of the neurofilament heavy chain, generally considered a marker for myelination (27). In mouse spinal afferents, TRPV1 immunoreactivity is rarely seen in IB4-positive neurons (typically <5% of IB4-positive afferents express TRPV1) (58). This is true for both somatic and visceral afferents (12). In addition, very few spinal visceral afferents express IB4, and we have found this to be true for the colon (12), stomach, and pancreas (unpublished data; Fig. 7F). Thus the fact that 6 of 14 gastric afferents expressed IB4 staining and that half of these also expressed TRPV1 suggests that gastric node afferents have chemical phenotypes that are unique compared both to somatic and visceral spinal afferents. Systematic studies with larger sample sizes are needed to confirm whether placode-derived nodose neurons have a neurochemical phenotype distinct from that typically seen in the neurocrest-derived dorsal root ganglion neurons innervating skin or viscera.

Even though all of the gastric neurons conducted in the C-fiber range, 2 of 14 cells stained positive for N52 and did not respond to stretch. Consistent with these findings, ~25% of retrogradally labeled gastric nodose neurons show immunoreactivity for N52 (unpublished data). Ruan et al. (42) recently reported neurofilament immunoreactivity in nearly one-third of mouse nodose neurons. The present results show that, at least for mouse nodose ganglia, N52 staining is not a reliable marker for myelinated fibers.

In conclusion, we developed a novel approach allowing comprehensive characterization of vagal afferent neurons. By combining physiological and neurochemical characterization, we conclude that TRPV1 does not play a direct role in mechanosensation within the stomach. Considering the potential role of TRPV1 in sensitizing responses to mechanical or other stimuli (24, 41), future experiments will address whether such sensitizing effects are limited to neurons expressing TRPV1.

**REFERENCES**


