**KCa1.1 channel contributes to cell excitability in unmyelinated but not myelinated rat vagal afferents**

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Li B-Y, Glazebrook P, Kunze DL, Schild JH. KCa1.1 channel contributes to cell excitability in unmyelinated but not myelinated rat vagal afferents. Am J Physiol Cell Physiol 300: C1393–C1403, 2011. First published February 16, 2011; doi:10.1152/ajpcell.00278.2010.—High conductance calcium-activated potassium (BKCa) channels can modulate cell excitability and neurotransmitter release at synaptic and afferent terminals. BKCa channels are present in primary afferents of most, if not, all internal organs and are an intriguing target for pharmacological manipulation of visceral sensation. Our laboratory has a long-standing interest in the neurophysiological differences between myelinated and unmyelinated visceral afferent function. Here, we seek to determine whether there is a differential distribution of BKCa channels in myelinated and unmyelinated vagal afferents. Immunocytochemistry studies with double staining for the BK-type KCa1.1 channel protein and isletcin B4 (IB4), a reliable marker of unmyelinated peripheral afferents, reveal a pattern of IB4 labeling that strongly correlates with the expression of the KCa1.1 channel protein. Measures of cell size and immunostaining intensity for KCa1.1 and IB4 cluster into two statistically distinct (P < 0.05) populations of cells. Smaller diameter neurons most often presented with strong IB4 labeling and are presumed to be unmyelinated (n = 1,390) vagal afferents. Larger diameter neurons most often lacked or exhibited a very weak IB4 labeling and are presumed to be myelinated (n = 58) vagal afferents. Complementary electrophysiological studies reveal that the BKCa channel blockers charybdotoxin (ChTX) and iberiotoxin (IbTX) bring about a comparable elevation in excitability and action potential widening in unmyelinated neurons but had no effect on the excitability of myelinated vagal afferents. This study is the first to demonstrate using combined immunohistochemical and electrophysiological techniques that KCa1.1 channels are uniquely expressed in unmyelinated C-type vagal afferents and do not contribute to the dynamic discharge characteristics of myelinated A-type vagal afferents. This unique functional distribution of BK-type KCa channels may provide an opportunity for afferent selective pharmacological intervention across a wide range of visceral pathophysiologies, particularly those with a reflexogenic etiology and pain.

sensory neurons; visceral function; charybdotoxin; iberiotoxin; A- and C-type vagal afferents

**IMMUNOHISTOCHEMICAL AND CELLULAR** electrophysiological studies have demonstrated that large conductance (BK-type) Ca2+-activated K+ (KCa) channels are present in primary vagal afferent neurons (8, 14, 22, 30). Beyond participating in membrane repolarization and contributing to the trajectory of the after hyperpolarization, the integrative physiological function associated with this subtype of KCa channel remains poorly understood. Recently, it has been shown that prostaglandin-

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Here, using immunohistochemical techniques, we present evidence for a preferential expression of the KCa1.1 BK-type channel subtype in unmyelinated vagal afferent neurons of male rat. The functional implications for such an expression pattern are further investigated using an intact ganglion preparation for patch-clamp electrophysiological study of action potential dynamics from vagal neurons of known afferent conduction velocity (CV). Application of the BK-type channel antagonists ChTX and iberiotoxin similarly broadens the somatic action potential and increase the excitability of unmyelinated vagal afferents, while having no measurable electrophysiological effects on myelinated afferents. We further document that this differential expression extends from neonatal (P5–9) through maturation to adult rat (>250 g) albeit with subtle functional differences suggestive of ontogeny in the voltage- and/or Ca\(^{2+}\)-dependent properties of the BK-type KCa channel. In addition to demonstrating a fiber-specific distribution of BK-type channels, these experimental observations provide further clarity concerning the differential ionic mechanism for integration of sensory information arising from unmyelinated and myelinated vagal afferents in rat.

**MATERIALS AND METHODS**

Sprague-Dawley (Harlan, Indianapolis) rat pups (5- to 9-day-old) of either gender or adult males (>250 g) were used in all electrophysiological studies. Young adult male (P49) Sprague-Dawley rats were used for immunocytochemistry. All protocols were approved by the Institutional Animal Care and Use Committees of the Purdue School of Science; Indiana University Purdue University, Indianapolis; or Case Western Reserve University.

**Immunocytochemistry.** Left side vagal ganglia were isolated, quick frozen, and cryosectioned. Horizontal serial sections of 8-μm thickness were collected onto glass slides and fixed with cold 4% paraformaldehyde for 30 min. Sections were blocked for nonspecific staining in PBS containing 10% normal donkey serum, 0.3% Triton-X 100 (Pierce), and 1% BSA (Jackson ImmunoResearch Laboratories) followed by overnight incubation in a primary antibody cocktail consisting of anti-BKCa (1:50; mouse; NeuroMab) or anti-BKCa (1:200; rabbit; Alomone) in PBS containing 0.3% Triton-X 100 and 1% BSA. Secondary antibodies (1:400) from appropriate hosts (Jackson Laboratories) labeled with rhodamine red-X were diluted in PBS contain- ing 0.3% Triton-X 100 and 10% donkey serum for 90 min at room temperature. Before imaging the slides were incubated with Lectin from Bandeiraea simplicifolia (Griffonia simplicifolia) conjugated to FITC (IB4; Sigma) 2:100 for 10 min. The slides were washed briefly in PBS and coverslipped using Vectashield mounting medium with DAPI. Imaging was carried out using a Nikon E600 microscope with a SPOT RT digital camera and its associated software (Diagnostic Instruments).

The resultant 8-bit images were imported into Metamorph (Version 6.1, Molecular Devices). The flattened background command was utilized to mitigate false variations in brightness. Only those neurons with a DAPI-labeled nucleus were selected and manually outlined for area measurements. This database of normalized measures was interrogated using an automated cluster analysis algorithm (MATLAB, The MathWorks) to determine if the joint distribution of these measures could be segregated into two statistically distinct subpopulations. The difference between the identified subgroups was further tested by analysis of variance and Tukey’s tests (α = 0.05).

**Preparation of intact ganglia for patch recording of vagal neurons.** Slices of vagal ganglia with intact axons were prepared in a manner previously described (27). Briefly, anesthetized animals were guillotined at the midauxillary region, preserving a length of vagus nerve suitable for measure of fiber CV. The entire vagal ganglion with attached nerve trunk was excised under stereomicroscopy (>40), and the tissue immediately was placed in chilled (4°C) recording solution (see below). The interior of the ganglion was exposed by taking a small slice from the surface of the capsule, and the ganglion and attached nerve were placed in a solution of Earle’s balance salt solution (Sigma, St. Louis, MO) containing type II collagenase (1.0 mg/ml) for 40–45 min at 37°C followed by a solution containing Trypsin-3X (5 mg/ml) for an additional 20–22 min at 37°C. The ganglion and attached nerve were moved to the recording chamber and perfused with room temperature recording solution for at least 1 h before the study.

**Preparation of isolated vagal ganglion neurons for patch recording.** All procedures for dissection and enzymatic isolation of vagal ganglion neurons (VGN) were identical to those previously reported (40). Briefly, for each day of experiments the entire vagal ganglia from at least two rat pups were excised bilaterally and placed in a chilled (4–8°C) vagal complete media (VCM) consisting of DMEM F-12 (Invitrogen), 5% FBS (HyClone), 0.01% penicillin-streptomycin (Sigma), and 0.1% MITO + serum extender (Becton Dickinson). The whole ganglia were digested using Trypsin-3X (5 mg/ml; Worthington) for 30 min at 37°C. The enzymatic solution was replaced with VCM, and the ganglia were titrated with an aspiration pipette. The cell bodies were plated on poly-n-lysine (Sigma) coated coverslips for 3–6 h at 37°C in a high humidity, 97% room air, and 3% CO\(_2\) environment before patch recording.

**Recording solutions.** For all current-clamp recordings of action potential discharge, the extracellular solution consisted of the following (in mM): 137 NaCl, 5.4 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 10 glucose, and 10 HEPES with pH adjusted to 7.35 using 1 N NaOH. For all voltage-clamp recordings of K\(^+\) currents, the extracellular solution consisted of the following (in mM): 137 NaCl, 5.4 KCl, 1 MgCl\(_2\), 10 glucose, 10 HEPES, and nominally Ca\(^{2+}\)-free with pH adjusted to 7.3 using 1 N HCl. The pipette solution for both recording protocols contained the following (in mM): 140 potassium aspartate, 3 MgCl\(_2\), 4 BAFTA-K, 10 HEPES, and 0.25 CaCl\(_2\) for a final buffered intracellular calcium concentration of 10 mM with pH adjusted to 7.3 using 1.3 ml of 1 N KOH per 100-ml stock for a final intactcell potassium ion concentration of ~153 mM. Just before recording, 2 mM Mg-ATP was added to the pipette solution from a stock solution. Osmolarities of all extracellular and pipette solutions were adjusted using n-manitol (Sigma) to 310 and 290, respectively. A stock of 100 nM ChTX (Alomone) or 100 nM of iberiotoxin (IBTX; Alomone) was prepared fresh for each day of experiments and applied using a perfusion pipette or bath perfused depending on whether the patched VGN was from an isolated or intact ganglion preparation, respectively. All recordings were carried out at room temperatures (20–23°C).

**Electrophysiological techniques.** The whole cell patch technique was used to carry out the voltage and current-clamp recording protocols using an Axoclamp 700A. Borosilicate glass pipettes (Sutter) were pulled and polished down to a resistance of 1–2 MΩ. Following correction for all offsets, a giga-ohm seal was formed and the pipette capacitance was compensated. Upon going whole cell the total cell capacitance (30–50 pF) and electrode access resistance (3–5 MΩ) were also compensated (70–80%). For current-clamp recordings using an intact ganglion, a Pt-IR bipolar stimulation electrode was positioned a measured distance from the recording electrode. A 500-μs monophasic current pulse was used to depolarize the afferent
fiber arising from the VGN under study. The time between the stimulus artifact and arrival of the propagating action potential at the patch electrode was used in calculating fiber CV. A VGN was classified as a myelinated (A-type) or unmyelinated (C-type) afferent if fiber CV was >10 m/s or <2 m/s, respectively (27). Single somatic action potentials were also elicited using a 500-μs current pulse delivered through the patch electrode while step depolarizing currents were used to evoke repetitive discharge. For isolated VGN, we have previously demonstrated a methodology whereby select measures of action potential wave shape can be reliably used to classify an isolated neuron as either a myelinated A-type or unmyelinated C-type afferent (28).

All voltage-clamp recordings were preceded by one of these current-clamp protocols for afferent classification before exchange of the bathing solution for one suitable for recording whole cell K+ currents. The voltage-clamp protocols consisted of 300-ms steps delivered at 3-s intervals from a holding potential of −80 mV to +40 mV in increments of 10 mV. Recordings were low pass filtered to 10 KHz and digitized at 50 KHz. The experimental protocols, data collection, and preliminary analysis were carried out using pCLAMP 9 and the Digitida 1322A (Axon Instruments). Corrections for liquid junction potentials were taken into consideration before final analysis of the data. Aged means were tabulated as means ± SD. A two-sided paired Student’s t-test was used to assess statistical significance (P < 0.05) of the impact of the channel antagonists.

RESULTS

The immunohistochemical and electrophysiological studies were carried out independently in the Kunze and Schild laboratories, respectively.

Immunohistochemical staining of KCa1.1 in vagal neurons. The immunohistochemical studies made use of 26, 8-μm thick horizontal sections. Fluorescence illumination of the BK-type KCa (KCNMA1) antibody shows diffuse labeling of all neurons throughout the ganglia, albeit with varying levels of intensity from section to section (Fig. 1, A and A’, arrows). No obvious differences in labeling intensity are noted between the mouse (NeuroMab) and rabbit (Alomone) antibodies, and therefore the two results are combined for purposes of analysis. Those VGN with the largest cross-sectional diameters exhibit the lightest staining for not only the KCa1.1 channel but also IB4, a reliable marker for sensory neurons with unmyelinated fibers (Fig. 1, B and B’, arrows). Superposition of these images clearly demonstrates a lack of dual labeling in these larger, presumably myelinated VGN (arrows). In stark contrast, nearly all of the smaller diameter VGN are prominently labeled for both the BK-type KCa1.1 antibody (red) and IB4 lectin (green). Superposition of these images shows dual labeling in the smaller, presumably unmyelinated VGN (Fig. 1, C and C’, yellow). These images provide strong, albeit qualitative, evidence that KCa1.1 is more prominently expressed in unmyelinated than myelinated VGN. However, the subjective nature of such visual inspection and the inherent variability in the fluorescence emissions between sections (Fig. 1, left and right, presents two extreme examples) necessitate a more rigorous quantification of KCa1.1 distribution relative to afferent fiber type.

Measures of circumscribed cell area and average pixel intensity associated with the immunostaining for the KCa1.1 channel protein (Fig. 1, A and A’, red) and IB4 (Fig. 1, B and B’, green) are carried out on each neuron (n = 1,448) with clear nuclear staining (DAPI). Measures from each tissue slice are normalized for each tissue slice and sorted into a hierarchical cluster tree. The clustering algorithm reveals two sub-populations of neurons with joint distributions of cell area (or equivalent diameter) and intensity of KCa1.1 and IB4 immunostaining that are statistically distinct (Fig. 2, spheres demarcate 2 SD from the population mean, i.e., P < 0.05). The larger subgroup (n = 1,390) has an average cell area of 615.4 ± 216 μm² (27.5 ± 5 μm diameter) and normalized IB4 and KCa1.1 immunostaining intensities of 0.53 ± 0.2 and 0.51 ± 0.2, respectively. The smaller subgroup has an average cell area of 1,315.9 ± 211 μm² (40.8 ± 3 μm diameter) and normalized IB4 and KCa1.1 immunostaining intensities of 0.18 ± 0.1 and 0.25 ± 0.2, respectively. A two-tailed Student’s t-test shows that the measurement means for these two subgroups are significantly different (P < 0.01). Additional analysis using an ANOVA and Tukey’s test confirms that all but two combinations of measurement means are significantly different at the 0.05 level. The two combinations that fail the Tukey’s test are the normalized IB4 and KCa1.1 immunostaining intensities for each individual subgroup, which can reasonably be expected should these two markers similarly vary in intensity according to neuronal cell type.

Functional impact of BK-type KCa channel antagonists on vagal afferents of known fiber type. Following the formation of a successful patch the intrinsic resting membrane potential (RMP) is measured for each VGN. This intrinsic RMP is then adjusted to −60 mV using a small magnitude (5–10 pA) depolarizing background current. A uniform RMP increases the likelihood that from cell to cell all subtypes of voltage-dependent ion channels present in the VGN are operating from a consistent gating probability (39). A common objective for both the current-clamp and voltage-clamp recordings is to determine if the BK-type KCa1.1 channel antagonists have similar effects on the action potential discharge characteristics and whole cell K+ current of myelinated and unmyelinated VGN.

Differential effects of ChTX on action potential discharge of A- and C-type VGN from adult male rat. Electrical stimulation of the vagus nerve elicits somatic action potentials from VGN in the attached ganglion (Fig. 3). Vagal afferents with fiber CV excess of 10 m/s and narrow (<1.0 ms) somatic action potentials with upstroke and downstroke velocities in excess of 200 mV/ms and −150 mV/ms, respectively, are classified as myelinated A-type VGN. Those with fiber CV <2 m/s and broad (>2.0 ms) somatic action potentials with upstroke and downstroke velocities generally less than 55 and −30 mV/ms, respectively are classified as unmyelinated C-type VGN. Bath application of 100 nM ChTX has no measurable effect on the shape of nerve evoked A-type action potentials (Fig. 3A) or the CV of myelinated vagal afferent fibers, i.e., 13.8 ± 3.3 m/s before and 13.8 ± 3.7 m/s after ChTX (n = 5; P > 0.5). Bath application of 100 nM ChTX increases the average duration of nerve evoked C-type action potentials from 2.5 ± 0.5 to 3.0 ± 0.7 ms (Fig. 3B; n = 9; P < 0.05). Interestingly, loss of the BK-type KCa1.1 current has no significant effect on the CV of unmyelinated vagal afferent fibers even though the intact vagus nerve is continuously exposed to ChTX, i.e., 0.65 ± 0.8 m/s before and 0.64 ± 0.8 m/s after application of ChTX (n = 9; P > 0.5).

Somatic excitability of VGN is investigated using a high intensity, short duration (500 μs) current pulse and a low intensity, long duration (500–1,000 ms) current step delivered through the patch electrode. Generally pulse magnitudes less than 1 nA elicit a somatic action potentials from A-type VGN
with voltage trajectories quite similar to those evoked using nerve stimulation (Fig. 4A). Low magnitude step currents of 150 pA consistently elicit sustained, high-frequency repetitive discharge from all myelinated A-type vagal afferent neurons (44.5 ± 7 Hz; Fig. 4C). Application of 100 nM ChTX has no measurable effect on the shape of the A-type action potential nor is repetitive discharge significantly altered (46.0 ± 7 Hz; \( P > 0.5; n = 5 \)). In contrast, C-type VGN require pulse...
magnitudes of 2–3 nA to elicit somatic action potentials but membrane voltage trajectories remain quite similar to those evoked by nerve stimulation (Fig. 4B). High magnitude step currents of 600 pA consistently elicits sustained, low frequency repetitive discharge from all unmyelinated C-type VGN (1.24 ± 0.5 Hz, Fig; 4D). As observed with nerve stimulation, application of 100 nM ChTX brings about a significant broadening of the evoked somatic action potential from 2.60 ± 0.6 to 3.09 ± 0.7 ms (P < 0.05; n = 9). In the presence of the K<sub>Ca</sub> channel antagonist, the same step current magnitude evokes a significantly higher rate of repetitive discharge in C-type VGN (10.8 ± 4 Hz; P < 0.01; n = 9). A summary of the impact ChTX has on the trajectory of membrane voltage and excitability of myelinated A-type and unmyelinated C-type vagal afferents is provided in Table 1.

Differential effects of IbTX on action potential discharge of A- and C-type VGN from adult male rat. Iberiotoxin is a potent and highly selective blocker of the BK<sub>Ca</sub> channel molecularly identified as KCa1.1. While ChTX antagonizes this channel protein, it has also been shown to block an intermediate KCa conductance (KCa3.1) as well as a few subtypes of...
### Table 1. Impact of 100 nM ChTX and 100 nM IbTX on AP waveform characteristics of VGN identified as myelinated A-type and unmyelinated C-type neurons

<table>
<thead>
<tr>
<th>Rat VGN</th>
<th>Isolated, 5- to 9-day-old pup</th>
<th>Intact Ganglia, adult &gt;250 g</th>
<th>Intact Ganglia, adult &gt;250 g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A type (n = 5)</td>
<td>C type (n = 6)</td>
<td>A type (n = 5)</td>
</tr>
<tr>
<td></td>
<td>CNTRL</td>
<td>ChTX</td>
<td>CNTRL</td>
</tr>
<tr>
<td>RMP</td>
<td>−61.8 ± 3</td>
<td>−59.3 ± 3</td>
<td>−65.5 ± 5</td>
</tr>
<tr>
<td>APFT</td>
<td>−42.1 ± 3</td>
<td>−41.6 ± 2</td>
<td>−27.2 ± 3</td>
</tr>
<tr>
<td>APFF</td>
<td>40.6 ± 8</td>
<td>41.2 ± 7</td>
<td>1.25 ± 0.4</td>
</tr>
<tr>
<td>APD₀</td>
<td>0.8 ± 0.1</td>
<td>0.90 ± 0.1</td>
<td>2.54 ± 0.6</td>
</tr>
<tr>
<td>APPEAK</td>
<td>40.5 ± 3</td>
<td>40.9 ± 5</td>
<td>45.3 ± 3</td>
</tr>
<tr>
<td>AHP</td>
<td>18.4 ± 5</td>
<td>17.7 ± 5</td>
<td>75.7 ± 23</td>
</tr>
<tr>
<td>AHPPEAK</td>
<td>−65.8 ± 2</td>
<td>−66.9 ± 1</td>
<td>−73.4 ± 2</td>
</tr>
<tr>
<td>UVAP₀</td>
<td>202.0 ± 33</td>
<td>215.0 ± 43</td>
<td>47.9 ± 12</td>
</tr>
<tr>
<td>DVAPEAK</td>
<td>−81.1 ± 7</td>
<td>−82.5 ± 10</td>
<td>−33.7 ± 8</td>
</tr>
</tbody>
</table>

Data are means ± SD. ChTX, charybdotoxin; IbTX, iberiotoxin; AP, action potential; VGN, vagal ganglion neurons; CNTRL, control; RMP, resting membrane potential (mV); APFT, AP firing threshold (mV); APFF, AP firing frequency (Hz), C-type 600 pA and A-type 300 pA step currents; APD₀, AP duration at 50% deflection (ms); APPEAK, AP peak deflection (mV); AHPPEAK, peak after hyperpolarization (mV); AHP₀, 80% of recovery time to RMP after AHPPEAK (ms); UVAP₀, upstroke velocity at APD₀ (mV/ms); DVAPEAK, downstroke velocity at APD₀ (mV/ms). *P < 0.05, †P < 0.01 vs. CNTRL.

Likewise, IbTX broadens the action potentials of unmyelinated A-Type VGN. As with ChTX, 100 nM IbTX has no effect on APPEAK (ms); UVAP₀, upstroke velocity at APD₀ (mV/ms); DVAPEAK, downstroke velocity at APD₀ (mV/ms). *P < 0.05, †P < 0.01 vs. CNTRL.
Differential effects of ChTX on action potential discharge of A- and C-type VGN from neonatal rat. To determine if the afferent-specific effectiveness of the BK-type K<sub>Ca</sub> channel antagonists was age related comparable electrophysiological studies were carried out in VGN isolated from neonatal rats (5- to 9-days-old). In response to a brief (500 μs) current pulse delivered through the patch electrode, VGN classified as myelinated A-type exhibited low threshold (~42.1 ± 3 mV), brief duration (0.89 ± 0.1 ms) somatic action potentials (Fig. 6A; n = 5). Depolarizing step current injections of 300 pA consistently evoked stable, high frequency action potential discharge (40.6 ± 8 Hz). All measures of action potential discharge characteristics were similar to those from adult A-type neurons albeit somewhat less robust, e.g., measures of upstroke velocity and downstroke velocity of A-type VGN from neonatal rat were somewhat slower than those from adult rat (Table 1). As in the adult preparations, application of 100 nM ChTX did not bring about any significant changes across a comprehensive assessment of action potential wave shape dynamics and repetitive discharge characteristics (Table 1). Consistent with recordings from adult rats, VGN from neonatal rat classified as unmyelinated C-type exhibited much higher threshold for discharge (~27.2 ± 3 mV) and broad duration (2.54 ± 0.6 ms) somatic action potentials with a prominent hump over the time course of repolarization (Fig. 6B; n = 6). Much larger magnitude depolarizing step current injections of 600 pA were required to evoke repetitive action potential discharge, albeit at a much lower average frequency of 1.25 ± 0.4 Hz. Unlike myelinated A-type VGN, all unmyelinated C-type VGN exhibited a number of distinct changes in wave shape and excitability when exposed to 100 nM ChTX (Table 1). Most notable was a nearly 30% increase in the action potential duration from 2.54 ± 0.6 to 3.25 ± 0.5 ms (P < 0.01) along with a concomitant decrease in the downstroke velocity from ~33.7 ± 8 to ~18.5 ± 5 mV/ms (P < 0.01). Loss of the BK-type K<sub>Ca</sub> current also resulted in a significant increase in the action potential upstroke velocity from 47.9 ± 12 to 57.3 ± 17 mV/ms (P < 0.05), which resulted in a significant increase in the peak amplitude of the action potential from 45.3 ± 3 to 47.9 ± 3 mV (P < 0.05). Collectively, these changes led to a substantial increase in excitability of unmyelinated C-type VGN, as the same 600-pA step current magnitude now elicited sustained repetitive discharge frequencies nearly 10 times faster than control (11.2 ± 4.7 vs. 1.25 ± 0.4 Hz; P < 0.01), an effect that closely paralleled those ChTX-mediated changes in excitability observed using an adult preparation. However, RMP, upstroke velocity (UV<sub>APD50</sub>), and action potential peak voltage (AP<sub>PEAK</sub>) were all significantly altered in the presence of ChTX in C-type (but not A-type) neonatal VGN (Table 1). These effects were not observed in unmyelinated C-type VGN from adult rats. A comparison of the pooled measures of action potential discharge (Table 1) suggests such subtle functional differences may be the consequence of as yet unrecognized neurobiological factors in the developmental expression of the voltage- and/or Ca<sup>2+</sup>-dependent properties of the BK-type K<sub>Ca</sub> channel.

Differential effect of ChTX on the whole cell K<sup>+</sup> current of A- and C-type VGN from neonatal rat. Given the difference in the effectiveness of the BK-type K<sub>Ca</sub> channel antagonists between myelinated and unmyelinated vagal afferents, a voltage-clamp study was carried out to determine if myelinated VGN lacked a significant BK-type K<sub>Ca</sub> current. An initial current-clamp recording was carried out using the extracellular action potential recording solution to reliably classify the cell under study as a myelinated or unmyelinated VGN (28). The extracellular solution was then exchanged for one suitable for recording whole cell K<sup>+</sup> currents. From a holding potential of −80 mV, 400-ms voltage-clamp steps were applied in increments of +10 mV to a maximum of +40 mV. This protocol elicited a rapidly activating and sustained outward K<sup>+</sup> current (Fig. 7A). This protocol was repeated in the presence of 100 nM ChTX, which resulted in a marked reduction in the whole cell K<sup>+</sup> current. When these traces were digitally subtracted from the control traces, the voltage- and time-dependent profile of the ChTX-sensitive K<sup>+</sup> current was revealed to have comprised ~25% of the whole cell K<sup>+</sup> current under control conditions (Fig. 7B). The normalized current-voltage (I-V) profiles from a sample (n = 8) of VGN identified unmyelinated afferents showed that ChTX significantly reduced the outward whole cell K<sup>+</sup> current at voltage-clamp steps to 0 mV and above (Fig. 7C). Consistent with the action potential recordings
Kv1.2 channel subtypes (17, 18, 22). Nanomolar concentrations of ChTX block the large conductance BK-type KCa1.1 and the potential for a differential distribution of modulatory β-subunits. The combined results of our immunohistochemical studies (Figs. 1 and 2) and electrophysiological recordings documenting the afferent-specific effectiveness of ChTX and IbTX (Figs. 3–7) are suggestive of a marked functional expression of KCa1.1 in unmyelinated C-type vagal afferents with little to no functional expression of this channel subtype in myelinated A-type VGN. However, KCa1.1 channels are known to associate with four different modulatory β-subunits (β1–4; KCNMB1–4) (51). One of these subunits, β4, can render the calcium-activated potassium channel subunit α1 (KCa1.1) resistant to nanomolar concentrations of ChTX and IbTX (33). It is plausible to consider that the complete insensitivity of A-type VGN to the effects of ChTX and IbTX comes about through an association of KCa1.1 with the β4-subunit. Such a

![Fig. 7. Voltage-clamp evidence for differential expression of a BK-type KCa1.1 channel. A: from a cell classified as an unmyelinated C-type VGN, depolarizing voltage-clamp steps revealed a large, sustained outward K+ current (i,K(total)). B: K+ current eliminated with the application of 100 nM ChTX (subtracted records, see text). For A and B, the activation protocol consisted of 300-ms voltage-clamp steps at 3-s intervals from a Vhold of −80 mV to +40 mV in increments of +10 mV. C: normalized current-voltage (I-V) relationship for the whole cell K+ currents from cells classified as unmyelinated C-type VGN under control (CNTRL, n = 8) conditions and in the presence of 100 nM ChTX. D: normalized I-V relationship for the whole cell K+ current from cells classified as a myelinated A-type VGN under control (CNTRL, n = 8) conditions and in the presence of 100 nM ChTX. For C and D, data are means ± SD of measures made 250 ms into the clamp step (dotted trace, Fig. 7A) with *P < 0.05 and **P < 0.01 vs. CNTRL.](https://www.ajpcell.org/article/S0361-8588(11)00001-7/abstract)
hypothesis implies that the subunit compositions of the BK channels in unmyelinated and myelinated afferents may be different. This interpretation would be consistent with recent studies showing that the heterogeneity in BKCa current function in cutaneous neurons may result from, at least in part, a differential distribution of β-subunits (55). Indole-diterpenes such as paxilline can inhibit the IbTX-insensitive β4-subunit isoform of the KCa1.1 channel (25). Therefore, further investigation using paxilline as a selective antagonist of KCa1.1 may provide a means to clarify and add support to the conclusions presented in this study.

**Literature support for a differential expression of KCa1.1 across vagal sensory afferent terminals.** It has long been recognized that unmyelinated vagal afferents exhibit a far broader sensitivity to physiologically relevant chemicals such as inflammatory mediators than myelinated vagal afferents (23). The physiological implications of this fact remain somewhat elusive. However, recent results from intact in vitro and in situ preparations are beginning to demonstrate that KCa channels and in particular the BK-type KCa1.1 generally are not functionally involved in the process of sensory reception at the peripheral afferent terminal ending of mechanosensitive myelinated vagal afferents although some exceptions can be noted for particular species and organ systems (16, 32, 37, 54). Much less is known concerning the ion channel subtypes providing a functional contribution to the sensory encoding process at the peripheral terminals of unmyelinated vagal afferents due, in large part, to recording difficulties associated with these fragile and small diameter fibers. However, there are intriguing data from airway afferent preparations that BK-type KCa channels may play an important role in prejunctional modulation of neuropeptides released from unmyelinated peripheral terminals, the implication being that BK-type KCa channels may play a critically important role in the response of sensory nerve terminals to neurogenic inflammation in addition to the more widely recognized impact on discharge threshold through modulation of resting membrane potential (10, 11, 44, 45, 52, 53).

Reynolds et al. (37) used an in vitro rat aortic arch preparation for recording the pressure-dependent discharge of single, identified myelinated baroreceptor fibers. Neither intraluminal exposure of 100 nM ChTX for up to 30 min nor direct application of the drug to the adventitial tissue area pervaded by the afferent terminal endings altered the pressure encoding characteristics of myelinated baroreceptors (37). Single fiber recordings of low threshold, myelinated vagal mechanoreceptors and the effects of BK-type KCa antagonists have also been carried out utilizing esophageal and airway in vitro preparations most often from guinea pig. Zagorodnyuk et al. (54) demonstrated that 100 nM of iberiotoxin, a potent and selective antagonist for KCa1.1, failed to alter stretch-evoked firing. Interestingly, 100 nM ChTX did produce a modest increase in spontaneous and stretch-evoked firing of myelinated esophageal afferents but obviously not as a result of KCa1.1 blockade. This is an observation supported by others (32, 54) and perhaps suggests that low threshold mechanoreceptors of guinea pig esophagus and airway, perhaps unlike the rat, functionally expressed KCa1.2 channels at the peripheral termination of myelinated mechanosensory afferents along with other subtypes of dendrotoxin-sensitive ion channels.

Noceptive and mechanosensitive unmyelinated vagal afferents prominently participate in centrally mediated reflexes in addition to releasing neuropeptides at their peripheral terminals, playing an important role in mediating localized neurogenic inflammation and afferent sensitization throughout the viscera (3, 7, 10, 12, 45). Studies investigating the potential contribution that KCa1.1 channels may make to the neural encoding of arterial pressure by unmyelinated rat aortic baroreceptors have yet to be carried out. However, there are numerous autocrine and paracrine chemical factors known to impact the sensitivity of unmyelinated baroreceptors that can also have modulatory effects on BK-type KCa channel gating both through intracellular signaling molecules and pathways (9, 13). Far more direct evidence of a role for BK-type KCa channels in mediating unmyelinated afferent function is available from studies of guinea pig airway afferents. It has long been recognized that ChTX-sensitive KCa channels exhibit the capacity for prejunctional modulation of the release of peptides from capsaicin-sensitive unmyelinated airway afferents (35, 44). More recently, Yoshihara et al. (52, 53) have demonstrated a similar role for ChTX-sensitive KCa1.1 channels in mediating the effects of cannabinoid and neuroactive steroid receptor agonists in unmyelinated guinea pig airway afferents.

**Conclusions.** This study provides compelling evidence that a BK-type KCa current makes a functional contribution to the neurophysiological properties of unmyelinated but not myelinated vagal afferent neurons from healthy rats. Labeling for IB4-containing neurons that are presumably mostly unmyelinated C-type afferents significantly coexpressed with markers for the BK-type KCa1.1 channel protein (KCNMA1). The implication being that those cells strongly expressing the BK-type KCa1.1 channel protein are presumably mostly unmyelinated C-type afferents (Figs. 1 and 2). Current and voltage-clamp recordings documenting the effects of ChTX and IbTX on action potential discharge and the whole cell K+ current were examined using rat vagal afferent neurons of known fiber type (28). In both neonatal (P5–9) and adult male (>250 g) rat preparations, neither 100 nM ChTX nor 100 nM IbTX had any effects on numerous measures of neuronal discharge and whole cell K+ current I-V characteristics in myelinated A-type neurons, while the same antagonist concentration consistently broadened somatic action potentials, increased neuronal excitability, and decreased the whole cell K+ current in unmyelinated C-type vagal neurons (Figs. 3–7 and Table 1).

Advances in the understanding of the molecular structure and chemical sensitivities of BK-type KCa channels have resulted in pharmacological agents that offer considerable potential as therapeutic interventions for pathophysiological conditions of the central nervous system and pulmonary afferents (19, 45). Much less attention has been directed toward understanding the role of BK-type KCa channels in other areas of visceral sensory afferent function. However, recent investigations surrounding the selective modulation of BK-type KCa channels have shown the potential for novel pharmacological treatments for a wide range of visceral organ system diseases (42, 43). Collectively, our results are highly suggestive of a preferential expression of the KCa1.1 channel in unmyelinated but not myelinated vagal afferents of male rat. Given the vastly different neurophysiological properties and reflexogenic capacities of these distinct classes of sensory afferents, such a biophysical dichotomy would greatly enhance the potential for
fibers-specific manipulation of autonomic nervous system function and dysfunction.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


