Differential contribution of Kv4-containing channels to A-type, voltage-gated potassium currents in somatic and visceral dorsal root ganglion neurons

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Yunoki T, Takimoto K, Kita K, Funahashi Y, Takahashi R, Matsuyoshi H, Naito S, Yoshimura N. Differential contribution of Kv4-containing channels to A-type, voltage-gated potassium currents in somatic and visceral dorsal root ganglion neurons. J Neurophysiol 112: 2492–2504, 2014. First published August 20, 2014; doi:10.1152/jn.00054.2014.—Little is known about electrophysiological differences of A-type transient K\(^+\) (K\(_A\)) currents in nociceptive afferent neurons that innervate somatic and visceral tissues. Staining with isoelectric B4 (IB4)-FITC classifies L6-S1 dorsal root ganglion (DRG) neurons into three populations with distinct staining intensities: negative to weak, moderate, and intense fluorescence signals. All IB4 intensely stained cells are negative for a fluorescent dye, Fast Blue (FB), injected into the bladder wall, whereas a fraction of somatic neurons labeled by FB, injected to the external urethral dermis, is intensely stained with IB4. In whole-cell, patch-clamp recordings, phrixotoxin 2 (PaTx2), a voltage-gated K\(^+\) (Kv)4 channel blocker, exhibits voltage-independent inhibition of the K\(_A\) current in IB4 intensely stained cells but not the one in bladder- innervating cells. The toxin also shows voltage-independent inhibition of heterologously expressed Kv4.1 current, whereas its inhibition of Kv4.2 and Kv4.3 currents is voltage dependent. The swapping of four amino acids at the carboxyl portion of the S3 region between Kv4.1 and Kv4.2 transfers this characteristic. RT-PCRs detected Kv4.1 and the long isoform of Kv4.3 mRNAs without significant Kv4.2 mRNA in L6-S1 DRGs. Kv4.1 and Kv4.3 mRNA levels were higher in laser-captured, IB4-stained neurons than in bladder-afferent neurons. These results indicate that PaTx2 acts differently on channels in the Kv4 family and that Kv4.3 subunits functionally participate in the formation of K\(_A\) channels, yet molecular correlates of K\(_A\) currents in distinct target tissues, cell morphologies, and other properties still remain unclear.
We therefore set out to identify cellular and electrophysiological characteristics of \( \kappa_\alpha \) channels in DRG neurons innervating somatic and visceral tissues. Here, we show that IB4 intensely positive neurons innervate the somatic tissue but not the bladder. Moreover, phrixotoxin 2 (PaTx2), a Kv4 channel blocker, exhibits distinct voltage-dependent inhibitions of heterologously expressed Kv4.x currents. With the use of this toxin, we identify further the functional contribution of Kv4.1/erologously expressed Kv4.x currents. With the use of this blocker, exhibits distinct voltage-dependent inhibitions of h...

**Glossary**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>( \tau_\text{act} )</td>
<td>Time constants of current activation</td>
</tr>
<tr>
<td>( \tau_\text{decay} )</td>
<td>Time constants of decay</td>
</tr>
<tr>
<td>( D )</td>
<td>Concentration of toxins (M)</td>
</tr>
<tr>
<td>( G/G \text{ max (control)} )</td>
<td>Peak ( K^+ ) conductance relative to the maximum conductance in control</td>
</tr>
<tr>
<td>( h )</td>
<td>Hill coefficient</td>
</tr>
<tr>
<td>( I/I \text{ max} )</td>
<td>Normalized peak ( K^+ ) current amplitude for inactivation curve analysis</td>
</tr>
<tr>
<td>( I_c )</td>
<td>Mean amplitude of depolarization-induced current in control (in the absence of toxins)</td>
</tr>
<tr>
<td>( k )</td>
<td>Slope factor</td>
</tr>
<tr>
<td>( K_i )</td>
<td>Apparent dissociation constant</td>
</tr>
<tr>
<td>( L )</td>
<td>Fractional current amplitude remaining when the affinity sites are fully occupied</td>
</tr>
<tr>
<td>( V_h )</td>
<td>Voltage at half-maximal conductance</td>
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</table>

**MATERIALS AND METHODS**

**Animal Preparation and DRG Cell Dispersion**

Adult female Sprague-Dawley rats (200–250 g; Hilltop, Scottsdale, PA) were used. All animal experiments were carried out in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and with the guidelines by the U.S. National Institutes of Health regarding the care and use of animals for experimental procedures. Fast Blue (FB; 2% w/v; EMS-Chemie, Zürich, Switzerland) was injected into the bladder wall to identify afferent neurons innervating the bladder as visceral neurons by retrograde axonal transport of the fluorescent dye, as described previously (Hayashi et al. 2009; Yoshimura and de Groat 1999). Briefly, the bladder was exposed by a midline lower-abdominal incision, and the dye was injected with a 30-gauge needle at four to six sites (20 \( \mu l \) total vol) on the surface of the bladder under isoflurane anesthesia (1–2%). At each injected site, the needle was kept in place for 20–30 s, and any leakage of dye was removed by application of cotton swab. The injection site was then rinsed with saline, and the incision was closed. In a separate group of animals, we injected FB into the dermis surrounding the external urethral orifice at four sites (20 \( \mu l \) total vol) on the bladder wall to identify afferent neurons innervating the bladder as somatic neurons of the S3 region (S3b) to test its roles in voltage dependence of the toxin effects (see Fig. 6). Replacement of the four-amino acid sequences between Kv4.1 and Kv4.2 was done using a two-step, overlapped PCR with primers containing a part of the other channel subunit sequence. Obtained constructs were verified by DNA sequencing.

**Chinese hamster ovary (CHO)-K1 cells on an 8-mm coverslip** were transfected with expression constructs for Myc-tagged mouse Kv4.1, rat Kv4.2, and rat Kv4.3 at the cDNA ratio 1:4–1:8 using Lipofectamine PLUS (Invitrogen, Life Technologies, Grand Island, NY). We also transfected rat Kv1.4 and rat Kv2.1 on CHO-K1 cells. A small amount of Emerald-C1 (10 ng/dish) was included in transfection for visualization of transfected cells (Takimoto et al. 2002). One or 2 days after transfection, at ~10% confluence, the membrane currents of the cells were recorded using patch-clamp methods.

**Whole-Cell, Patch-Clamp Recording Procedure**

The setup of the patch-clamp experimental system used was essentially the same as described previously (Hayashi et al. 2009; Yoshimura and de Groat 1999). Briefly, a whole-cell, voltage-clamp recording was performed on dispersed, native DRG neurons or cultured CHO-K1 cells with an Axopatch-700B patch-clamp amplifier, and data were acquired and analyzed by pCLAMP 8 software at a sampling rate of 1,000/s (Axon Instruments, Union City, CA). The filter was set to ~3 dB at 2,000 Hz, and the P/N protocol was used to subtract leak currents. Whole-cell input capacitance was neutralized directly from the amplifier. Current traces were low-pass filtered by the digital filter of the data acquisition program (pCLAMP 8). Patch electrodes were fabricated from borosilicate capillary tubing and had resistances of 3–7 MΩ when filled with the internal solution. During recordings, isolated DRG neurons and CHO-K1 cells were superfused with bath solution at a flow rate of 2.0 ml/min in a chamber with a 0.30-ml vol at room temperature (20–22°C). Patch pipettes were filled with...
with a solution containing the following (in mM): 140 KCl, 1.0 CaCl₂, 2.0 MgCl₂, 11 EGTA, 2.0 ATP, 0.40 GTP, and 10 HEPES, titrated to pH 7.4 with Tris base.

For patch-clamp experiments in native DRG neurons, we selected two populations of single cells: 1) FB-positive bladder afferent neurons smaller than 30 μm in diameter and 2) intensely stained cells with IB4-FITC. Note that there was no overlap in these two populations (see Table 3). We obtained outward K<sub>P</sub> currents by de polarizing step pulses from a holding potential of −40 mV for 500 ms, and the outward K<sub>A</sub> current was obtained by subtracting K<sub>P</sub> from the outward currents activated from the holding potential of −120 mV (I<sub>−120</sub>), as established previously (Hayashi et al. 2009; Yoshimura and de Groat 1999).

The extracellular solution, which contained the following (in mM): 150 choline-Cl, 5.0 KCl, 0.03 CaCl₂, 3.0 MgCl₂, 10 D-glucose, and 10 HEPES/Tris-base (pH 7.4), was used to suppress inward Na<sup>+</sup> and Ca<sup>2+</sup> currents. At the end of experiments, all neurons investigated their capsaicin (1 μM) sensitivity by switching the bath solution to the following solution (in mM): 150 NaCl, 5.0 KCl, 2.5 CaCl₂, 1.0 MgCl₂, 10 D-glucose, and 10 HEPES/Tris-base (pH 7.4). Only the capsaicin-sensitive cells were enrolled into analysis.

In Kv channel-expressed CHO cells, Kv currents were evoked by de polarizing voltage pulses from −100 mV. The extracellular solution included the following (in mM): 150 NaCl, 5.0 KCl, 2.5 CaCl₂, 1.0 MgCl₂, 10 D-glucose, and 10 HEPES/Tris-base (pH 7.4).

**Drugs**

All drugs were dissolved directly into the extracellular solution. PaTx2 was applied to the cell by injection into the bath solution. Recombinant PaTx2 was purchased from Alomone Labs (Jerusalem, Israel). The rest of the chemicals were purchased from Sigma-Aldrich.

**Equation for Curve Fitting of the Steady-State Activation and Inactivation of K<sub>₄</sub> and Kv4 Currents**

The peak outward current amplitude, evoked by depolarization from the holding potential of −100 mV to various potentials, from −60 to +50 mV every 10 mV, was recorded to estimate the peak K<sup>+</sup> conductance. The duration from a pulse to the next pulse was 5 s. The steady-state activation curve was plotted as the G/G max (control) vs. the test potential (V) concerning the theoretical equilibrium potential of K<sup>+</sup> in 20°C. The data were fitted by the modified Boltzmann equation

\[
G/G\text{ max (control)} = 1/(1 + \exp [(Vh - V)/k])
\]

Steady-state inactivation was determined by depolarizing voltage steps to +20 mV, following 1 s conditioning prepulses from −120 to +20 mV. The inactivation curve was plotted as the I/I max vs. the potential of the conditioning prepulse (V). The data were fitted by the modified Boltzmann equation

\[
I/I\text{ max} = 1/(1 + \exp [(V - Vh)/k])
\]

**Table 1. PCR primers for DRG RNAs**

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank #</th>
<th>Sequence</th>
<th>Position</th>
<th>Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kv4.1</td>
<td>XM_217601</td>
<td>ACTAGCCGGCTGCTGCGGAGGA AGAGGGGAGAAGAGGTCCTGGA</td>
<td>1,683–1,702</td>
<td>271</td>
</tr>
<tr>
<td>Kv4.2</td>
<td>NM_031730</td>
<td>TGGGGCACTGCCCTGCAATTTGAGAGAAGAGGTCCTGGA</td>
<td>1,953–1,934</td>
<td>579</td>
</tr>
<tr>
<td>Kv4.3</td>
<td>NM_031739</td>
<td>GAGGACAGCGCCGCTGCAATTTGAGAGAAGAGGTCCTGGA</td>
<td>1,406–1,385</td>
<td>217 (S)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_017008</td>
<td>TATGATGACATCAAGAAGGTGCAAGGCGGTGGTGCTGTA</td>
<td>828–847</td>
<td>579</td>
</tr>
</tbody>
</table>

DRG, dorsal root ganglion; Kv, voltage-gated K<sup>+</sup>; S, short splicing isoform of Kv4.3; L, long splicing isoform of Kv4.3.
test (see Figs. 3 and 7, and see Table 6). Changes were considered significant at \* \( P < 0.05 \), and \** \( P < 0.01 \). Data are expressed as mean with the SE, except those (see Table 5) that were expressed as mean with SD.

RESULTS

Characterization of Bladder and Cutaneous DRG Neurons

We first examined IB4-FITC binding to acutely dissociated, live L6-S1 DRG neurons from animals that had been injected with FB at the bladder walls. Dissociated, single DRG neurons (530 cells) were observed as round- to oval-shaped cells, in which 11\% (59 cells) were FB-positive bladder afferent neurons (Fig. 1A). Staining with IB4-FITC provided various fluorescence intensities. We classified cells based on the IB4-staining level into three categories: cells showed no or negligible fluorescent staining (IB4:0), whereas others exhibited moderate to intense signals (moderate, IB4:1; intense, IB4:2; Fig. 1 and Table 3). The analysis of 530 cells from three rats in this fashion indicated that \( \frac{1}{3} \) of cells were moderately stained with IB4, whereas \( \frac{2}{10} \% \) of cells were intensely stained (Table 3). IB4-stained cells were distributed in various sizes of DRG neurons (Fig. 1B). We also verified visual evaluation of IB4-FITC signal intensity by measuring the fluorescence intensity of 92 DRG neurons, randomly photographed during experiments. The fluorescence intensity of grades 0, 1, and 2 cells, judged by visual evaluation, was <20\% (32 of 92 cells; 34.8\%), between 20 and 70\% (41 of 92 cells; 44.6\%), and >70\% (19 of 92 cells; 20.6\%) of the highest value among DRG neurons, respectively, demonstrating that our visual evaluation method is appropriate to classify DRG neurons based on IB4-staining intensity.

FB-positive bladder afferent neurons showed only negative to weak (IB4:0, 35/59 cells = 59\%) or moderate (IB4:1, 24/59 cells = 41\%) IB4-FITC staining and were never intensely stained with IB4-FITC (Fig. 1A).

L6-S1 DRG also contains somatic afferent neurons innervating the external skin area surrounding the urethra through the pudendal nerve. Therefore, we analyzed a total of 260 cells from three rats that had been injected with FB at the external urethral dermis (Fig. 2 and Table 4). Among those, 34 cells (13\%) were FB-positive cutaneous afferent neurons. As expected, three IB4-staining categories represented similar proportions to the above experiments. Likewise, no apparent

| Table 2. Real-time PCR primers and probes for laser-captured cells |
|-----------------|-----------------|-----------------|
| Gene           | GenBank #       | Sequence        | Position        |
| Kv4.1          | NM_001105748    | 5’ Primer       | GCACCAGGAAGCAAACTTC  |
|                |                 | 3’ Primer       | GTACAGGAAGGAATGACC  |
|                |                 | Probe           | TGATGCTTACCACATCGCA  |
| Kv4.3          | NM_031739       | 5’ Primer       | AGAGACAGCTATCGCATCG  |
|                |                 | 3’ Primer       | AGAGAGGCTTCTGCTGTA  |
|                |                 | Probe           | TGAGCAAGACTGATGAGACTCA  |
| GAPDH          | NM_017008       | 5’ Primer       | GATAGGCCAACATCGCTTC  |
|                |                 | 3’ Primer       | GCCGTCAGCCTCGTCTCA  |

Fig. 1. Morphological characterization of dissociated L6-S1 dorsal root ganglion (DRG) neurons from rats injected with Fast Blue (FB) into the bladder wall. A: single DRG neurons in the same field, observed by light-field and fluorescent microscope, are shown in the top and bottom, respectively. Top: an arrow indicates a FB-positive bladder afferent neuron identified under the fluorescent light. Bottom: single DRG neurons with various green-staining intensities of isolecitin B4 (IB4)-FITC. The numbers (0–2) beside the cells indicate the intensity of staining: 0, no or weak (negative) staining; 1, moderate staining; 2, intense staining. B: cell-size distributions of FB-positive and -negative DRG cells with different IB4 staining intensities (grades 0–2). Note that there is no grade 2-stained cells in FB-positive bladder afferent neurons.

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correlation between IB4-staining intensity and cell size was observed. However, FB-positive cutaneous afferent neurons included many IB4-stained cells, and >¼ of FB-positive cells were intensely stained with IB4-FITC (IB4:2, 9/34 cells). Thus intense IB4-positive cells constitute a subpopulation of somatic afferent neurons innervating the urethral external skin, whereas visceral afferent neurons innervating the bladder consist of IB4-negative to moderately stained cells. Hence, afferent neurons with distinct IB4-staining intensities may preferentially innervate either cutaneous or visceral tissues.

Distinct Effects of PaTx2 on $K_A$ Currents in Bladder Afferent and IB4-Positive Neurons

$K_A$ currents play important roles in controlling activity of excitable cells, including sensory neurons. We wished to identify functional differences in $K_A$ currents between cutaneous and visceral afferent neurons. We chose two populations of L6-S1 DRG neurons, <30 μm in diameter, from rats injected with FB in the bladder: 1) IB4 intensely stained cells and 2) FB-positive cells. The former represents a subpopulation of somatic afferent neurons, whereas the latter corresponds to a fraction of bladder afferent neurons. There was no overlap in these two neuron types. All of the tested cells in the two groups showed the response to capsaicin (1 μM) to induce inward current, indicating that they were capsaicin-sensitive C-fiber afferent cells. The peak current density of the capsaicin-induced inward current was 80 ± 4.7 pA/pF ($n = 24$) in IB4 intensely stained neurons and 89 ± 12 pA/pF in bladder afferent neurons ($n = 11$).

$K_v$ currents were recorded using two holding potentials to obtain $K_{DR}$ and $K_A$ current components. $K_{DR}$ currents were evoked from a holding potential of −40 mV, whereas $K_A$ currents were obtained by subtracting $K_{DR}$ currents from $I_{−120}$ as we reported previously (Hayashi et al. 2009; Yoshimura and de Groat 1999). Peak amplitudes of $K_A$ and $K_{DR}$ were larger in IB4 intensely stained cells than those in bladder afferent cells ($P < 0.01$, Mann-Whitney test; Table 5). The $V_h$ in steady-state activation and inactivation was lower in IB4 intensely stained cells than those in bladder afferent neurons ($P < 0.01$ for steady-state activation, and $P < 0.01$ for steady-state inactivation, Mann-Whitney test; Table 5). These differences indicate that the bladder afferent cells possess a typical slowly inactivating $K_A$ current, as we have reported previously (Yoshimura and de Groat 1999; Yoshimura et al. 1996), whereas IB4 intensely positive cells display $K_A$ currents with faster kinetics.

Recent studies indicate that $K_v4.x$ subunits are significantly expressed in DRG neurons (Matsuyoshi et al. 2012; Phuket and Covarrubias 2009). To identify the functional contribution of $K_v4$ channels to $K_A$ currents in DRG neurons, we tested the effect of a $K_v4$ blocker, PaTx2, on the currents. Application of PaTx2 at 1 μM significantly inhibited the $K_A$ current in IB4 intensely positive cells ($P < 0.01$ for PaTx2, 500 nM; $P < 0.01$ for PaTx2, 1,000 nM; Wilcoxon signed-rank test) without significant influence on the $K_{DR}$ current (96 ± 0.85% of the predrug value for PaTx2, 1,000 nM; $n = 13$; Fig. 3, A, B, and D). In contrast, PaTx2 produced no effect on the $K_A$ currents in FB-positive bladder afferent neurons (Fig. 3, C and D). These results indicate that $K_v4$ family channels contribute to the $K_A$ current in IB4 intensely stained afferent neurons that represent a subpopulation of somatic afferent cells. In contrast, these

**Table 3. IB4 staining intensity of bladder afferent neurons**

<table>
<thead>
<tr>
<th>IB4:0</th>
<th>IB4:1</th>
<th>IB4:2</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB-negative neurons</td>
<td>168 (32%)</td>
<td>198 (37%)</td>
</tr>
<tr>
<td>Bladder afferent neurons</td>
<td>35 (6.6%)</td>
<td>24 (4.5%)</td>
</tr>
<tr>
<td>Total neurons</td>
<td>203 (38%)</td>
<td>222 (42%)</td>
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</table>

**Table 4. IB4 staining intensity of skin afferent neurons**

<table>
<thead>
<tr>
<th>IB4:0</th>
<th>IB4:1</th>
<th>IB4:2</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB-negative neurons</td>
<td>90 (35%)</td>
<td>96 (37%)</td>
</tr>
<tr>
<td>Skin afferent neurons</td>
<td>11 (4.2%)</td>
<td>14 (5.4%)</td>
</tr>
<tr>
<td>Total neurons</td>
<td>101 (39%)</td>
<td>110 (42%)</td>
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Total 530 cells. Fast Blue (FB) was injected into the bladder wall at 4–6 sites (20 μl total vol) in each animal. IB4, isolecitin B4.

**Fig. 2. Morphological characterization of dissociated L6-S1 DRG neurons from rats injected with FB into the skin around the urethral orifice. A: single DRG neurons in the same field observed by light-field and fluorescent microscope are shown in the top and bottom, respectively. Top: arrows indicate FB-positive cutaneous afferent neurons identified under the fluorescent light. Bottom: single DRG neurons with various green-staining intensities of IB4-FITC. The numbers (0–2) beside the cells indicate the intensity of staining, as described in Fig. 1. B: cell-size distributions of FB-positive and -negative DRG cells with different IB4-staining intensities (grades 0–2). Note that grade 2-stained cells are observed in FB-positive cutaneous afferent neurons.**
Table 5. Electrophysiological properties of IB4 intensely positive and bladder afferent neurons

<table>
<thead>
<tr>
<th></th>
<th>IB4 Intensely Positive Cells</th>
<th>Bladder Afferent Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td>Diameter, µm</td>
<td>27 ± 2.1</td>
<td>24 ± 2.2*</td>
</tr>
<tr>
<td>Input capacitance, pF</td>
<td>35 ± 3.7</td>
<td>28 ± 3.3*</td>
</tr>
<tr>
<td>I_{A} peak at 0 mV, nA</td>
<td>2.5 ± 0.63</td>
<td>0.73 ± 0.14*</td>
</tr>
<tr>
<td>I_{DR} peak at 0 mV, nA</td>
<td>1.3 ± 0.39</td>
<td>0.75 ± 0.22*</td>
</tr>
<tr>
<td>Steady-state activation V_h, mV</td>
<td>−58 ± 4.4</td>
<td>−31 ± 0.24*</td>
</tr>
<tr>
<td>Steady-state inactivation V_i, mV</td>
<td>−89 ± 4.3</td>
<td>−75 ± 4.3*</td>
</tr>
<tr>
<td>τ act at 0 mV, ms</td>
<td>1.9 ± 0.20</td>
<td>3.5 ± 0.72*</td>
</tr>
<tr>
<td>τ decay at 0 mV, ms</td>
<td>160 ± 19</td>
<td>210 ± 17*</td>
</tr>
</tbody>
</table>

Values are means ± SD. I_{A}, A-type transient K⁺ current; I_{DR}, delayed rectifier-type K⁺ current. *P < 0.01 vs. IB4 intensely positive cells, Mann-Whitney test.

depolarizing pulses (Fig. 4A). No apparent voltage shift was seen in the normalized steady-state activation curves before and after application of PaTx2 (Fig. 4B). The toxin produced no significant changes in time constants for activation and inactivation, although it tended to slow both processes at all voltages (Fig. 4, C and D). Thus PaTx2 voltage independently blocks the K_A current in IB4 intensely stained cells without apparent effects on activation or inactivation.

Distinct Effects of PaTx2 on Heterologously Expressed Kv4 Channels

The lack of voltage dependence of the toxin action on the native K_A current stimulated us to test whether the toxin might similarly inhibit heterologously expressed Kv4 currents in a voltage-independent fashion. We expressed N-terminally Myc-tagged rat Kv4.1, Kv4.2, or Kv4.3 channels in CHO cells. Transient outward K⁺ currents were evoked by depolarizing voltage pulses in channel cDNA-transfected cells (Fig. 5, A–C). The inactivation time courses were slightly slower than those obtained with the corresponding channels without a tag (time constants of inactivation at 30 mV were 56.3 ± 6.8 ms for wild-type Kv4.3 and 104.7 ± 22.3 ms for Myc-tagged Kv4.3, unpublished observation). The toxin at 500 nM significantly reduced the peak amplitude of all Kv4.x currents (P < 0.01 for all Kv4.x currents evoked with depolarization to 0 mV.

Fig. 3. Effects of phrixotoxin 2 (PaTx2) on A-type transient K⁺ (K_A) currents in IB4 intensely stained and bladder afferent neurons. K_A current (I_A) trace was obtained by subtracting the delayed rectifier-type K⁺ (K_DR) current (I_{DR}) from the outward currents activated from the holding potential of −120 mV (I_{120}). I_{DR} and I_A were evoked by a depolarizing voltage step to 0 mV from the holding potential of −40 mV and −120 mV, respectively. A and C: representative I_{120}, I_{DR}, and I_A before (Control) and after application of 1 µM PaTx2 in an IB4 intensely positive neuron (A) and a bladder afferent neuron (C). B: the time course of PaTx2 effects on peak I_A amplitudes in an IB4 intensely positive neuron. D: the concentration-response relationship of PaTx2. Relative peak I_A amplitudes were determined using the level before application of PaTx2 (control) as 1 from the same cell. In IB4 intensely positive cells, PaTx2 decreased the K_A current in a concentration-dependent manner: 300 nM, 0.93 ± 0.024; 500 nM, 0.87 ± 0.019; 1,000 nM, 0.74 ± 0.040 (n = 4–18). Peak I_A amplitudes at 500 and 1,000 nM PaTx2 were significantly different from the control level in IB4 intensely positive cells (**P < 0.01 for PaTx2, 500 nM; ***P < 0.01 for PaTx2, 1,000 nM, Wilcoxon signed-rank test). In contrast, PaTx2 exhibited no significant changes in peak I_A amplitudes in bladder afferent neurons: 500 nM, 0.98 ± 0.0050; 1,000 nM, 0.94 ± 0.023 (n = 6–11). The data are expressed as means ± SE.
and +30 mV, Wilcoxon signed-rank test; Fig. 5, A–C). The estimated $K_i$ values were 230 nM, 120 nM, and 110 nM for Kv4.1, Kv4.2, and Kv4.3 currents evoked with depolarization to +30 mV, respectively (Table 6). No detectable effects were seen with Kv1.4 or Kv2.1 currents at 500 nM (data not shown). Hence, PaTx2 is specific for Kv4 family channels in this concentration range.

Importantly, we found that PaTx2 produced distinct inhibitory actions on Kv4 currents. The toxin similarly reduced Kv4.1 current elicited by pulse voltages at 0 and +30 mV, whereas it caused more pronounced inhibition of Kv4.2 and Kv4.3 currents at the lower pulse voltage (Fig. 5D). The toxin did not apparently change the voltage dependence of steady-state activation (Fig. 5A) or inactivation (Fig. 5E) for the Kv4.1 current. In addition, no significant changes in the time constants for activation or inactivation were observed at any pulse voltages for the current (Fig. 5A). In contrast, the toxin caused clear changes in these parameters for Kv4.2 and Kv4.3 currents. The steady-state activation (Fig. 5, B and C) and inactivation (Figs. 5E) curves were shifted to more positive voltages with significant changes in the time constants for activation and inactivation (Fig. 5, B and C). These results indicate that the toxin voltage independence and dependently inhibited Kv4.1 and Kv4.2/Kv4.3 currents, respectively. It should be noted, however, that Kv4.3 current exhibited a complex behavior upon the toxin treatment and might involve, in part, voltage-independent inhibition by the toxin. For instance, the current showed a less obvious, positive shift in the steady-state inactivation (Fig. 5E).

The observed different inhibitory modes of Kv4 channels might arise from sequence differences in their interaction site with PaTx2. Specifically, the latter part of the third transmembrane S3 is a major interaction site with gating modifier toxins. Sequence alignment of Kv4 polypeptides revealed divergence of four amino acids in this portion (Fig. 6A). Therefore, we set out to test if swapping this portion between Kv4 proteins might transfer voltage dependence of the toxin action. Since Kv4.1 and Kv4.2 channels show apparent differences in the toxin inhibition, we generated the two-channel proteins with the four-amino acid sequence of the other at the corresponding site (Kv4.1-VMTD and Kv4.2-FVPK). Kv4.1-VMTD exhibited most of the characteristics of the wild-type Kv4.2 channel in the toxin inhibition. The toxin produced larger inhibition at lower pulse voltage (Fig. 6D) and shifted steady-state activation and inactivation curves to the right (Fig. 6, B and E). Time constants for inactivation were also reduced at lower voltages.

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Fig. 4. Effects of PaTx2 on steady-state kinetic parameters of $I_{h}$ in IB4 intensely stained neurons. $I_{h}$ current trace was obtained by subtracting KDR from $I_{h}$. Membrane depolarization was evoked by test pulses from −80 to +10 mV with a 10-mV interval from the holding potential of −40 mV ($F_{h}$) and −120 mV ($F_{h,120}$). A: representative $I_{h}$ current traces show inhibition by PaTx2 and its recovery. $I_{h}$ in IB4 intensely positive DRG cells was suppressed by application of 1 µM PaTx2 and was almost totally recovered after washing PaTx2 by perfusion. B: relative conductance levels ($G/G_{\text{max}}$ control) are plotted against the depolarizing test potential ($V$). Open and filled circles indicate the values before and after application of PaTx2, respectively. Solid lines were obtained with the normalized PaTx2 data using the maximum conductance value. C: $\tau$ act are plotted against $V$. D: $\tau$ decay are plotted against $V$. Note that time-constant ($\tau$) values for activation and inactivation were obtained using a single exponential equation. The data are expressed as means ± SE ($n = 5$).

Fig. 5. Effects of PaTx2 on expressed voltage-gated K$^+$ (Kv4) currents. Kv4.1, Kv4.2, and Kv4.3 currents were recorded in Chinese hamster ovary-K1 cells transfected with Myc-tagged rat Kv4.x expression vectors in a whole-cell configuration. K$^+$ currents were elicited by 300 ms test pulses from a holding potential of −100 mV, before and after application of 200 nM PaTx2, except that the data with 500 nM toxin are shown in Ai, Bi, Ci, and E. Open and closed circles represent before and after application of 200 nM PaTx2 ($n = 5–8$). A–C: representative Kv4 current traces; ii: steady-state activation curves; iii: $\tau$ act; and iv: $\tau$ decay. Broken lines (in ii) were generated with the PaTx2 data normalized to the maximum conduction value. $\tau$ Values for activation and inactivation were obtained using a single exponential equation. D: PaTx2 dose-dependent inhibition of Kv4 currents at 0 mV and 30 mV. E: steady-state inactivation.
Similarly, Kv4.2-FVPK showed voltage-independent inhibition by the toxin. The toxin similarly decreased Kv4.2-FVPK current at different pulse voltages (Fig. 6D) without an apparent shift in the steady-state inactivation curve and time constants (Fig. 6, E and B) These results indicate that the four-amino acid portion at the end of S3 determines voltage dependence of the inhibition by PaTx2.

Expression of Kv4 mRNAs in DRG Neurons

We performed RT-PCRs to detect the expression of Kv4 mRNAs. Abundant expression of Kv4.1 and the long isoform of Kv4.3 mRNAs were seen in L6-S1 DRGs (Fig. 7A). In contrast, a very low level of the Kv4.2 message was detected. These findings indicate that DRG neurons are largely devoid of Kv4.2.

We then compared the expression level of Kv4.1 and Kv4.3 mRNAs between bladder afferent neurons and IB4-stained DRG neurons using LCM methods (Fig. 7B). Although the difference in IB4-staining intensity (e.g., intense vs. moderate staining) is less obvious in DRG sections (Fig. 7B) compared with the cell culture system (Figs. 1 and 2), we laser captured DRG cells, which were not labeled by FB injected into the bladder wall, with relatively strong IB4 staining. Real-time PCR analysis detected higher expression of Kv4.1 and Kv4.3 mRNAs in laser-captured, IB4-stained neurons than in FB-labeled bladder afferent neurons (n = 6 rats; P < 0.05 for both Kv4.1 and Kv4.3, Wilcoxon signed-rank test; Fig. 7, D and E).

Taken together, the findings suggest that selective expression of the pore-forming Kv4.1 and possibly Kv4.3 in IB4 intensely stained afferent neurons contributes to the observed voltage-independent inhibition of the K_A current.

DISCUSSION

IB4 Staining of Bladder and Cutaneous Afferent Neurons in Rats

Several groups have reported various percentages of IB4-positive cells in adult rat bladder afferents (L6-S1): 14% (adult female) (Yoshimura et al. 2003), 29% (adult male) (Bennett et al. 1996), 48% (adult male) (Hwang et al. 2005), and 61% (adult male) (Dang et al. 2005). Dang et al. (2005) reported the highest rate of IB4 staining in bladder afferents, possibly due to the high-intensity staining by IB4-Alexa Fluor 488 used in their study, whereas IB4-FITC was used in other studies, including our current study. The former two groups also reported that the percentage of IB4-positive cells is higher in somatic afferent neurons than in bladder afferents: 27% (distal urethra, L6-S1 DRG) (Yoshimura et al. 2003) and 43% (medial ankle, L3 DRG) (Bennett et al. 1996). Other studies have also found high percentages of IB4-positive cells in cutaneous somatic afferent neurons from lumbar DRG of adult rats: 48% (knee) (Ivanavicius et al. 2004), 44% (vibrissal pad area) (Ambalavanar et al. 2003) using IB4-FITC, and 70% (footpads) (Lu et al. 2001) using IB4-Cy3.

In our current experiments with single, live DRG neurons, membrane staining of IB4-FITC was compared very clearly with an image of fixed and sliced sections. This condition with live cells allowed us to classify the level of IB4 staining. Instead of defining individual neurons as positive or negative, we therefore divided cells into three categories: grades 0 (no or weak staining), 1 (moderate staining), and 2 (intense staining). According to this classification, we found that bladder afferent neurons lack the IB4 intensely stained (grade 2) cells. On the contrary, skin afferent neurons contained significant IB4 intensely stained (grade 2) cells (24%). These results strongly suggest that IB4 intensely stained (grade 2) cells are not visceral afferents but represent a subpopulation of somatic afferent neurons. On the other hand, IB4 moderately stained (grade 1) cells were included in both bladder and skin afferent populations. Taken together, various percentages of IB4-positive cells in visceral and somatic DRG neurons in the previous studies might be attributable to different criteria used to identify IB4-positive cells. In particular, the previous studies might include or exclude in the positive category IB4 moderately stained cells, which were categorized as grade 1 in the current study.
A

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<tr>
<th>LFAAPSRRC</th>
<th>FLRSLID</th>
<th>VMSLIDVAIL</th>
<th>PYYIGL</th>
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B

Kv4.1-VMTD

i

![Control](image1)  
![PaTx2 500 nM](image2)

ii

G/Gmax (control)

![Graph](image3)

iii

τ_{act} (ms)

![Graph](image4)

iv

τ_{decay} (ms)

![Graph](image5)

C

Kv4.2-FVPK

i

![Control](image6)  
![PaTx2 500 nM](image7)

ii

G/Gmax (control)

![Graph](image8)

iii

τ_{act} (ms)

![Graph](image9)

iv

τ_{decay} (ms)

![Graph](image10)

D

Kv4.1-VMTD

Relative amplitude

![Graph](image11)

Concentration of PaTx2 (Log M)

Kv4.2-FVPK

Relative amplitude

![Graph](image12)

Concentration of PaTx2 (Log M)

E

Kv4.1-VMTD

Vmax

![Graph](image13)

Holding potential (mV)

Kv4.2-FVPK

Vmax

![Graph](image14)

Holding potential (mV)
Electrical and Pharmacological Properties of $K_A$ Currents in Bladder Afferent and IB4 Intensely Positive Neurons

The IB4 staining results indicate that DRG cells, intensely stained with IB4-FITC, are a subpopulation of somatic afferent neurons but not visceral afferent neurons innervating the bladder. Thus the combination of FB injection into the bladder wall with IB4 staining of live, dissociated L6-S1 DRG cells allowed us to examine the differences in potassium currents in the same preparation. In this preparation, FB-positive cells were bladder-innervating visceral afferent neurons, whereas IB4 intensely stained neurons should represent a fraction of IB4-positive somatic afferent neurons. We found the following using these techniques: That 1) IB4 intensely stained DRG neurons exhibit a larger amplitude of $K_A$ currents than FB-labeled bladder afferent neurons, 2) activation and inactivation of $K_A$ currents in IB4 intensely stained DRG neurons are faster with more hyperpolarized, half-maximum potentials than those in bladder afferent neurons, and 3) the Kv4 channel blocker PaTx2 suppresses $K_A$ currents in IB4 intensely stained DRG neurons but has no effect on the current in bladder afferent neurons. Thus bladder afferent and IB4-positive neurons contain the $K_A$ current with distinct kinetic and pharmacological properties. Bladder afferent neurons appear to contain an almost exclusive, slow-inactivating current, whereas IB4-positive neurons contain fast-inactivating currents. The results with PaTx2 further suggest that Kv4 channels are responsible for the fast-inactivating current in IB4-positive afferents that corresponds to a subpopulation of somatic afferents.

Previous studies have shown that $K_A$ currents in sensory neurons, including DRG cells, consist of slow- and fast-inactivating components (Akins and McCleskey 1993; Everill and Kocsis 1999; Everill et al. 1998; Gold et al. 1996; McFarlane and Cooper 1991; Yoshimura et al. 1996). Slow-inactivating $K_A$ currents are seen in capsaicin-sensitive C-fiber afferent neurons and are sensitive to dendrotoxin, a blocker of Kv1.1- and Kv1.2-containing channels. Furthermore, Kv1.4 subunits are significant in small-sized DRG neurons, including bladder afferent neurons (Hayashi et al. 2009; Rasband et al. 2001; Takahashi et al. 2013; Yang et al. 2004). Therefore, Kv1 family channels, including the inactivating, ball-containing Kv1.4 subunit, are thought to carry the slow-inactivating $K_A$ current in C-fiber DRG neurons. The slow kinetics and insensitivity to PaTx2 of the $K_A$ current in bladder afferents are consistent with this idea. In contrast, the fast-inactivating $K_A$ current is generally seen in capsaicin-insensitive, myelinated A-fiber bladder afferent neurons (Yoshimura et al. 1996).
show here that IB4 intensely stained cells that respond to capsaicin exhibit prominent \(K_A\) currents with faster kinetics and PaTx2 sensitivity. Thus an IB4 intensely positive subpopulation of somatic C-fiber DRG neurons appears to possess fast-inactivating \(K_A\) currents carried by Kv4 family channels.

**Voltage-Dependent and -Independent Inhibition of Kv4 Channels by PaTx2**

PaTx2 is a member of gating-modifying toxins that include hanatoxins and heteropoda toxins (Diochot et al. 1999; Escoubas et al. 2002; Sanguinetti et al. 1997; Swartz and MacKinnon 1995). These toxins act on a nonpore region to exhibit voltage-dependent inhibition of target channel currents (Corzo and Escoubas 2003; Norton and Pallaghy 1998; Swartz 2007; Zarayskiy et al. 2005). With the use of heterologously expressed Kv4.x currents, we found that PaTx2 shows distinct voltage dependencies for their inhibition. The toxin altered the steady-state activation and inactivation curves of the Kv4.2 current to a positive potential. The toxin also increased time-constant values in the raising and decaying phases of Kv4.2 current. These results indicate that the inhibition of Kv4.2 current by the toxin is mainly voltage dependent. On the other hand, the toxin caused little shift in the normalized, steady-state activation and inactivation curves of the Kv4.1 current without apparent changes in the time-constant values. Therefore, the toxin inhibits the Kv4.1 current in a voltage-independent fashion. The inhibition of the Kv4.3 current by the toxin seemed to include both inhibitory mechanisms. A similar voltage-dependent and -independent blockade of Kv4.6 currents has been reported recently with heteropoda toxin 2 (Desimone et al. 2011). Similar to this report, we found that four amino acids at the end of S3 primarily determine voltage dependence of the toxin action. Thus gating-modifying toxins, including PaTx2, detect subtle differences in pore-forming subunit sequences at the specific region to produce distinct inhibitory mechanisms. Hence, these toxins appear to be useful tools to identify molecular correlates of the native currents.

We took advantage of the two inhibitory fashions by PaTx2 to determine molecular correlates of the \(K_A\) current in IB4 intensely positive afferent DRG neurons that correspond to a subpopulation of somatic afferent cells. PaTx2 did not shift the normalized steady-state activation curve with minor changes in the time constants. Thus the toxin inhibition of the native \(K_A\) current is mostly voltage independent. These findings support the idea that the \(K_A\) channel in IB4 intensely stained DRG cells contains Kv4.1 and possibly Kv4.3 subunits. Our RT-PCR data are consistent with a less-obvious contribution of Kv4.2 in DRG neurons and revealed higher Kv4.1 and Kv4.3 mRNA levels in laser-captured, IB4-positive neurons than in bladder afferent neurons. There are certainly limitations in this interpretation. For instance, different Kv4.x subunits can form heteromeric channel complexes. The inhibitory fashion of heteromeric Kv4.x channel currents by the toxin might not linearly reflect the subunit composition. However, PaTx2 is considered to bind to each subunit in the tetrameric channel. Therefore, the obtained voltage-independent inhibition of the \(K_A\) current likely represents the overall subunit ratio in the native, IB4 intensely positive afferent cells.

Previous immunochemical studies indicate the presence of Kv4.3 subunits in DRG cells. For example, Kv4.3-immunoreactive proteins were found selectively in the somata of a subset of nonpeptidergic (i.e., CGRP-negative), nociceptive DRG neurons (Chien et al. 2007). Although less is known about the protein expression of the Kv4.1 subunit, due to the lack of a high-quality commercial antibody against this protein, the expression of Kv4.1 mRNA has been reported in small-sized DRG neurons using a single-cell RT-PCR method (Phuket and Covarrubias 2009). We also recently reported that Kv4.1 mRNA is expressed, not only in small-sized cells but also in all sizes of rat DRG neurons using in situ hybridization techniques (Matsuyoshi et al. 2012). Our LCM study demonstrated further that Kv4.1 and Kv4.3 mRNA expression is higher in IB4-positive afferent neurons than that in bladder afferent neurons. However, the obtained Kv4 subunit mRNA levels do not necessarily indicate the functional contribution of Kv4.1 or Kv4.3 subunits to the \(K_A\) current. In this regard, our electrophysiological data demonstrate the presence of the PaTx2-sensitive \(K_A\) current in IB4 intensely stained neurons constituting a subpopulation of somatic afferent cells. The lack of voltage-dependent inhibition of this \(K_A\) current supports a large contribution of Kv4.1 subunits to this current. It is important to note that IB4 intensely positive cells constitute a smaller fraction of somatic afferent neurons than those moderately stained. Thus further studies with IB4-negative and -moderately stained cells are needed to obtain a more complete view on molecular correlates of \(K_A\) currents between somatic and visceral afferent neurons.

In conclusion, the reduction of \(K^+\) channel activity, including slow- and fast-inactivating \(K_A\) currents, is one of the important mechanisms for hyperexcitability and chronic pain. The present study identified that Kv4.1 and possibly Kv4.3 subunits functionally contribute to \(K_A\) channels in IB4 intensely stained neurons that correspond to a subpopulation of somatic afferent cells. Molecular correlates of \(K_A\) channels in different afferent pathways could help to develop suitable molecular targets for the treatment of pain conditions of somatic and visceral organs.

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**DISCLOSURES**

There is no conflict of interest and no commercial affiliation in the present study.

**AUTHOR CONTRIBUTIONS**

Author contributions: K.T., H.M., S.N., and N.Y. conception and design of study. T.Y., K.K., Y.F., and R.T. performed experiments; T.Y. and Y.F. analyzed data; T.Y., K.T., and N.Y. interpreted results of experiments; T.Y. prepared figures; T.Y. and N.Y. drafted manuscript; K.T. and N.Y. edited and revised manuscript; K.T. and N.Y. approved final version of manuscript.

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Phuket J, Covarrubias L. The expression of Kv4.1 subunits in small-sized cells but also in all sizes of rat DRG neurons using in situ hybridization techniques (Matsuyoshi et al. 2012). Our LCM study demonstrated further that Kv4.1 and Kv4.3 mRNA expression is higher in IB4-positive afferent neurons than that in bladder afferent neurons. However, the obtained Kv4 subunit mRNA levels do not necessarily indicate the functional contribution of Kv4.1 or Kv4.3 subunits to the \(K_A\) current. In this regard, our electrophysiological data demonstrate the presence of the PaTx2-sensitive \(K_A\) current in IB4 intensely stained neurons constituting a subpopulation of somatic afferent cells. The lack of voltage-dependent inhibition of this \(K_A\) current supports a large contribution of Kv4.1 subunits to this current. It is important to note that IB4 intensely positive cells constitute a smaller fraction of somatic afferent neurons than those moderately stained. Thus further studies with IB4-negative and -moderately stained cells are needed to obtain a more complete view on molecular correlates of \(K_A\) currents between somatic and visceral afferent neurons.

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Kv4-containing channels in somatic C-fiber afferent neurons


