Bladder hyperactivity and increased excitability of bladder afferent neurons associated with reduced expression of Kv1.4 α-subunit in rats with cystitis

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Hayashi Y, Takimoto K, Chancellor MB, Erickson KA, Erickson VL, Kirimoto T, Nakano K, de Groat WC, Yoshimura N. Bladder hyperactivity and increased excitability of bladder afferent neurons associated with reduced expression of Kv1.4 α-subunit in rats with cystitis. Am J Physiol Regul Integr Comp Physiol 296: R1661–R1670, 2009. First published March 11, 2009; doi:10.1152/ajpregu.91054.2008.—Hyperexcitability of C-fiber bladder afferent pathways has been proposed to contribute to urinary frequency and bladder pain in chronic bladder inflammation including interstitial cystitis. However, the detailed mechanisms underlying hyperexcitability after bladder inflammation are not fully understood. Thus, we investigated changes in the properties of bladder afferent neurons in rats with bladder inflammation induced by intravesical application of hydrochloric acid. Eight days after the treatment, bladder function and bladder sensation were analyzed using cystometry and an electrodiagnostic device of sensory function (Neurometer), respectively. Whole cell patch-clamp recordings and immunohistochemical staining were also performed in dissociated bladder afferent neurons identified by a retrograde tracing dye, Fast Blue, injected into the bladder wall. Cystitis rats showed urinary frequency that was inhibited by pretreatment with capsaicin and bladder hyperalgesia mediated by C-fibers. Capsaicin-sensitive bladder afferent neurons from sham rats exhibited high thresholds for spike activation and a phasic firing pattern, whereas those from cystitis rats showed lower thresholds for spike activation and a tonic firing pattern. Transient A-type K+ current density was not altered after cystitis. The expression of voltage-gated K+ currents in capsaicin-sensitive bladder afferent neurons was significantly smaller in cystitis rats than in sham rats, although sustained delayed-rectifier K+ currents were reduced in bladder afferent neurons from cystitis rats. These data suggest that bladder inflammation increases bladder afferent neuron excitability by decreasing expression of Kv1.4 α-subunits. Similar changes in capsaicin-sensitive C-fiber afferent terminals may contribute to bladder hyperexcitability and hyperalgesia due to acid-induced bladder inflammation.

Voltage-gated K+ (Kv) currents are major determinants of neuronal excitability. Kv currents in sensory neurons are divided into two major categories; i.e., sustained delayed rectifier-type K+ (KDR) and transient A-type K+ (KA) currents (19, 21, 30, 57). KA currents in sensory neurons including dorsal root ganglion (DRG) cells can be further subdivided into at least two different subtypes based on their inactivation kinetics (i.e., fast- and slow-decaying KA currents) (3, 17, 37). A reduction in KDR and/or KA currents is reportedly involved in hypersensitivity of afferent pathways under various pathological conditions (1, 18). We have also reported that bladder inflammation for 2 wk increased excitability of capsaicin-sensitive DRG neurons innervating the urinary bladder due to a reduction in slow-decaying KA currents without affecting KDR currents (59).

Kv channels are formed by subfamily-specific tetramerization of channel subunits composed of ion-conducting α-subunits and auxiliary β-subunits (32). For instance, individual expression of Kv1.4 and other Kv1-family α-subunits generates homotetramers with rapidly-inactivating and sustained current kinetics, respectively (52). Coexpression of Kv1.4 and other Kv1-family α-subunits results in formation of heteromeric complexes with intermediate current kinetics (42). Transcripts for various α-subunits in Kv1 subfamily have been identified in DRG (24, 25, 56). Previous studies have indicated that Kv1.1 and Kv1.2 α-subunits are expressed in both small- and medium/large-sized DRG neurons; whereas Kv1.4 α-subunits are preferentially expressed in small-sized DRG neurons, the majority of which also express TRPV1, indicating that Kv1.4 constitutes a major fraction of α-subunits in small-sized, nociceptive DRG neurons (43). Furthermore, slow-decaying KA currents, but not fast-decaying Ka currents, in DRG neurons are partially reduced by α-dendrotoxin, a blocker for Kv1.1 and Kv1.2 channels (17, 18, 21, 40, 56). These findings suggest that slow-decaying KA currents in C-fiber sensory neurons are generated by heteromeric channels formed with Kv1.4 and Kv1.1/Kv1.2 α-subunits.

Previous studies have shown reductions in Kv α-subunits in rats with neuropathic pain induced by spinal nerve ligation (43) and sciatic nerve injury (25, 56). However, a molecular mechanism responsible for a reduction of KA currents and hyperexcitability of bladder afferent pathways after bladder inflammation remains to be elucidated. Hence, the present study was performed to clarify the mechanisms underlying bladder dysfunction and hyperexcitability of bladder afferent neurons in cystitis rats, especially focusing on Kv channel function.

IT HAS BEEN DEMONSTRATED THAT afferent pathways innervating the urinary bladder consist of myelinated Aδ-fibers and unmyelinated C-fibers and that hyperexcitability of C-fibers in bladder afferent pathways contributes to bladder overactivity and/or bladder pain under pathological conditions such as painful bladder syndrome/interstitial cystitis (PBS/IC) (60, 61).

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MATERIALS AND METHODS

All animal experiments were in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Pittsburgh and Taisho Pharmaceutical, Japan, and with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Female Sprague-Dawley rats (170–220 g) (Hilltop, Pittsburgh, PA; Charles River Laboratories, Japan) were used for this study and were housed under a 12:12-h light-dark cycle with free access to water and food.

Production of cystitis model. Bladder inflammation was induced according to a previously used method (26). Briefly, a polyethylene catheter (PE-50) was inserted transurethrally into the bladder under pentobarbital (50 mg/kg ip) anesthesia. Following withdrawal of urine from the bladder, 0.4 N hydrochloric acid (HCl) was infused into the bladder in a volume of 0.2 ml and kept at least for 90 s. Sham rats were treated with saline instead of HCl. An antibiotic (Cefazolin; 7 mg/kg) and an analgesic (buprenorphine, 0.2 mg/kg) were injected intramuscularly after the intravesical treatment to suppress posttreatment infection and pain, respectively. Experiments were performed 8 days after intravesical treatment.

For whole cell patch-clamp recordings and immunohistochemical studies, Fast Blue (1% wt/vol) (EMS Chemie, Zürich, Switzerland) was injected into the bladder wall 4–5 days before HCl instillation into the bladder to identify afferent neurons innervating the bladder by retrograde axonal transport of the fluorescent dye as previously described (59). 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 (total volume, 20 µl) on the surface of the bladder under isoflurane anesthesia. At each injection 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.

Histological analysis. Under pentobarbital (80 mg/kg ip) anesthesia, the bladder was removed and fixed with 4% paraformaldehyde in 0.1 M PBS. Transverse sections were then prepared from the middle part of the bladders, embedded in paraffin wax, cut into 4-µm sections, and then stained with hematoxylin–eosin, Alcian blue-safranine (for mast cells), or Luna (for eosinophils) staining. The numbers of mast cells and eosinophils were counted in the entire field of a randomly selected section of the bladder with ×100 magnification in a blinded fashion.

Continuous cystometry. A PE-50 catheter was implanted through the dome of the bladder under urethane anesthesia (1.0 g/kg sc). The catheter was connected to a pressure transducer for recording intravesical pressure and a syringe pump for infusing saline (3.0 ml/h) into the bladder. Micrutiition volume was continuously measured using an electrical scale positioned under the animal to collect fluid released from the urethral orifice. Intravesical pressure and micrutiition volume were recorded using a PowerLab System (AD Instruments, Bella Vista, Australia). In some animals, capsaicin was subcutaneously administered in three divided doses (50 and 25 mg/kg on the first day and 50 mg/kg on the second day) over a 2-day period to desensitize the majority of C-fiber afferents that are sensitive to capsaicin 5 days before cystometry (47). C-fiber desensitization was confirmed by negative responses to an eye wipe test before the experiments (53).

Measurement of bladder sensation. Two silver wire electrodes attached to a specially designed balloon catheter was inserted into the empty bladder through the urethral orifice under isoflurane anesthesia. The tip of one silver wire electrode was positioned on the surface of the balloon to have contact with the intraluminal surface of the bladder when the balloon catheter was inflated by filling with saline (0.2 ml). The other electrode tip was positioned on the catheter distal to the balloon to have contact with the bladder neck. Rats were then placed in a Bollman-type cage and recovered from anesthesia. Electrical stimuli of two different sine-wave pulses (250 or 5 Hz) with increasing intensities (range: 0–300 µA) were applied through the electrodes using a Neurometer CPT/C (Neurotron, Baltimore, MD) (4), and the animal responses were recorded (2, 27, 39). When vocalization and/or sudden body movements were observed as current intensities were increased, the stimulation was immediately stopped and the intensity was recorded as current perception thresholds (CPT) of bladder sensation at each frequency of stimulation (27, 39).

Whole cell patch-clamp recordings. Freshly dissociated neurons from L6-S1 DRG were prepared from isoflurane-anesthetized rats as described previously (59). Briefly, L6-S1 DRG were removed, minced, and incubated for 25 min at 35°C in 5 ml DMEM (Sigma, St. Louis, MO) containing 0.3 mg/ml trypsin (Type III, Sigma), 1 mg/ml collagenase (Type I, Sigma), and 0.1 mg/ml deoxyribonuclease (Type IV, Sigma). Trypsin inhibitor (Type II-S, Sigma) was then added to neutralize the enzyme activity. Individual DRG cell bodies were isolated by trituration and plated on poly-l-lysine-coated 35-mm culture dishes. Whole cell patch-clamp recordings were performed at room temperature (20–22°C) on each Fast Blue-positive neuron within 10 h after dissociation. The internal solution contained (in mM): 140 KCl, 1 CaCl2, 2 MgCl2, 11 EGTA, 10 HEPES, 2 Mg-ATP, and 0.4 mM GTP adjusted to pH 7.4 with KOH. Patch electrodes had resistances of 3–5 MΩ when filled with the internal solution. Neurons were superfused at a flow rate of 2.0 ml/min with an external solution containing (in mM): 150 NaCl, 5 KCl, 2.5 CaCl2, 1 MgCl2, 10 HEPES, and 10 d-glucose, adjusted to pH 7.4 with NaOH. For the isolation of K+ currents following the evaluation of action potential characteristics, the external solution was changed to one containing (in mM): 150 choline-Cl, 5 KOH, 0.03 CaCl2, 10 HEPES, 3 MgCl2, and 10 d-glucose, adjusted to pH 7.4 with Tris-base. All recordings were made with an Axopatch 700B patch-clamp amplifier and controlled by Clampex software (Axon Instruments, Sunnyvale, CA). Data were then analyzed by pCLAMP software (Axon Instruments). Cell membrane capacitances were obtained by reading the value for whole cell input capacitance neutralization directly from the amplifier. In current-clamp recordings, data are presented from neurons that exhibited resting membrane potentials greater than −40 mV and action potentials that overshoot 0 mV. In voltage-clamp recordings, the filter was set to −3 dB at 2,000 Hz. Leak currents were subtracted by P/4 pulse protocol, and the series resistance was compensated by 50–60%. The voltage error did not exceed 5 mV after compensation of the series resistance, and a charging time constant of the voltage clamp was <300 µs, which was faster than gating properties of outward K+ currents in this study. In a protocol examining firing characteristics, action potentials were elicited by depolarizing current pulses (duration 800 ms) at intensities that were kept at just suprathreshold for inducing a single action potential during a 50-ms depolarizing stimulus pulse. Capsaicin-sensitive neurons were identified by capsaicin (500 nM)-induced inward currents in voltage-clamp recordings at the end of patch-clamp recordings in each cell. Capsaicin was dissolved in the normal external solution containing 10% ethanol and 10% Tween 80 at a concentration of 5 mM and then diluted in the external solution before experiments. No effects were detected by application of ethanol and Tween 80 in concentrations as high as 0.2%.

Immunohistochemistry. Eight days after HCl or vehicle treatment, rats were deeply anesthetized with pentobarbital (80 mg/kg ip) and perfused through the left ventricle with 300 ml cold oxygenated PBS, followed by a fixative solution consisting of 4% paraformaldehyde in 0.1 M PBS. L6 DRGs were then removed and postfixed for 8 h in the same fixative solution. The tissues were placed in PBS containing increasing concentrations of sucrose (10, 20, and 30%) at 4°C for cryoprotection, frozen in mounting medium, and sectioned at 30-µm thickness. After mounting on gelatin-coated slides, the sections were washed and incubated with antibodies for Kv1.2 or Kv1.4 α-subunits (Alomone Lab, Israel) for 24 h at 4°C, followed by visualization with anti-rabbit IgG antibody conjugated to Cy3 for 2 h at room temperature. Images were obtained with a fluorescent microscope and the IPLab Spectrum.
In randomly selected DRG sections (6 sections per rat, n = 3 rats), labeling intensity measurements were made on all cell profiles that exhibited a nucleus using Scion Image software (Scion, Frederick, MD). Kv α-subunit staining intensity of each neuron was estimated by subtracting nonspecific background staining. For the measurement of labeling intensity, the nuclear region was excluded. Mean labeling intensity of Kv α-subunits was calculated in dye-labeled bladder afferent neurons as well as unlabeled afferent neurons, and the ratio of mean labeling intensity of bladder afferent neurons vs. unlabeled afferent neurons was obtained in each DRG section. The staining density ratio (dye-labeled vs. unlabeled cells) in each section was then averaged in randomly selected DRG sections in each animal, and thereafter the mean ratio in each animal was averaged again in either normal or HCl-induced cystitis group of animals. These analytical methods for Kv α-subunit staining were used to avoid comparing labeling intensity of dye-labeled afferent neurons between different DRG sections, which can be affected by different staining conditions and nonlinear fluorescent signal decay among sections.

Statistical analysis. Data are presented as means ± SE. Statistical comparisons between two groups were performed by Wilcoxon test or two-tailed unpaired Student’s t-test. Two-way ANOVA followed by two-tailed unpaired Student’s t-test was used for statistical comparisons in K⁺ currents obtained during whole cell patch-clamp experiments. For all comparisons, a value of P < 0.05 was considered to be significant differences.

RESULTS

Intravesical application of HCl induces bladder inflammation with urinary frequency and bladder hyperalgesia. Fig. 1A shows photomicrographs of hematoxylin-eosin-stained bladder tissues from sham and cystitis rats 8 days after intravesical application with HCl. Edema and infiltration of inflammatory cells were observed predominantly in the suburothelial layer of the bladder from HCl-treated rats compared with sham-treated rats. Infiltrated mast cells and eosinophils, which were respectively identified by Alcian blue-safranin and Luna staining, were counted in the entire field of a randomly selected transverse section of the bladder, which contained urothelial, suburothelial, and muscle layers, from sham and cystitis rats. The number of eosinophils reached a peak at 4 days after intravesical HCl application and was still significantly increased at 8 days (Fig. 1B). The number of mast cells in the bladder was also significantly increased in cystitis rats compared with sham rats at 8 days after intravesical HCl application (Fig. 1B). These results indicate that intravesical application of HCl induces sustained bladder inflammation over 1 wk, characterized by infiltration of inflammatory cells mainly in the suburothelial layer of the bladder.

Fig. 1. Bladder inflammation induced by intravesical application of HCl (0.4 N, 0.2 ml) in the rat. A: photomicrographs of hematoxylin-eosin-stained bladder sections from sham and cystitis rats (8 days after HCl injection). Magnification (upper: ×20, lower: ×100). Scale bars (upper: 200 µm, lower: 40 µm). B: time course (1–8 days after the treatment) of the changes in the number of mast cells and eosinophils, which were identified by Alcian blue-safranin and Luna staining, respectively, per entire transverse section of the bladder wall in sham and cystitis rats. Data are means ± SE in sham (n = 4–7) and cystitis groups (n = 4–9). *P < 0.05, **P < 0.01 compared with the sham group at each time point.
Cystometry was performed by continuous saline infusion (3.0 ml/h) into the bladder under urethane anesthesia to evaluate bladder function in sham and cystitis rats (8 days after HCl treatment). HCl-induced cystitis rats showed urinary frequency, which was characterized by shortened intercontraction intervals (cystitis rats: 353 ± 41 s vs. sham rats: 668 ± 95 s, P < 0.05) and decreased micturition volume (cystitis rats: 0.38 ± 0.04 ml vs. sham rats: 0.62 ± 0.10 ml, P < 0.05) (Fig. 2). To examine the contribution of capsaicin-sensitive C-fiber afferent pathways to urinary frequency in HCl-induced cystitis rats, C-fiber desensitization was induced by pretreatment with capsaicin (125 mg/kg sc) 5 days before the experiments. In capsaicin-pretreated groups, no significant changes in intercontraction intervals (883 ± 133 vs. 687 ± 114 s) and micturition volume (0.72 ± 0.11 vs. 0.61 ± 0.08 ml) were observed between HCl-untreated and HCl-treated rats (Fig. 2B). These results indicate that activation of capsaicin-sensitive C-fiber afferent pathways contributes to urinary frequency in HCl-induced cystitis rats. Other cystometric parameters, such as maximal voiding pressure and threshold pressure, were not different among any groups. The contributions of two types of bladder sensory pathways (i.e., A δ- and C-fiber afferents) were evaluated by measuring CPTs in μA using a Neurometer electrodiagnostic device (Neurotron) (Fig. 3). This device utilizes neuroselective electrical stimuli with different frequencies (5, 250, and 2,000 Hz) to perform quantitative assessments of subpopulations (C-, A δ- and Aβ-fibers) of sensory nerve fibers (4). When stimuli at 250 Hz, which reportedly activate Aβ- and Aδ-fiber afferent fibers (29), were applied to the inner surface of the rat bladder using an electrode-mounted balloon catheter, the CPT values, which were the minimal current intensities to induce vocalization and/or sudden body movements of animals, tended to be decreased in cystitis rats (n = 8) compared with sham rats (n = 8); however, the differences were not statistically significant. When the stimulation was applied at 5 Hz to activate C-fiber afferents in addition to A-fiber axons, which were selectively activated by 250 Hz stimulation. White and black bars represent the sham (n = 8) and cystitis rat group (n = 8), respectively. Data are means ± SE. **P < 0.01 compared with the sham rats group.

Increased excitability of afferent neurons innervating the bladder in cystitis. Because all of the above results indicate that rats with HCl-induced cystitis exhibited bladder hyperactivity/
We have previously reported that slow-decaying, transient outward $K^+$ currents (slow-decaying $K_A$ currents) are expressed in small-sized, capsaicin-sensitive bladder afferent neurons and that a reduction in slow $K_A$ currents by an application of 4-aminopyridine increased excitability of these neurons as evidenced by lower thresholds for spike activation and tonic firing (57, 59). Thus, in this study, we compared $K^+$ current density in capsaicin-sensitive bladder afferent neurons from sham and cystitis rats (Fig. 5A). $K^+$ currents were measured following replacement of external solution to the one that suppressed $Na^+$ and $Ca^{2+}$ currents after the evaluation of action potential characteristics in the same cell. Because in our previous studies, slow-decaying $K_A$ currents were activated by depolarizing voltage steps from hyperpolarized membrane potentials and were almost completely inactivated when the membrane potential was maintained at a depolarized level more than $-40$ mV in C-fiber bladder afferent neurons (57, 59), an estimate of the density of slow $K_A$ currents and sustained $K_{DR}$ currents was obtained by the difference in the currents activated by a depolarizing voltage pulse from two differential holding potentials ($-120$ and $-40$ mV). As shown in Fig. 5B, peak current densities of slow-decaying $K_A$ and sustained $K_{DR}$ currents were increased during membrane depolarization in capsaicin-sensitive bladder afferent neurons from both sham and cystitis rats. However, the peak current densities of slow $K_A$ were lower in neurons from cystitis rats than in those from sham rats. Significant differences in current density were detected at depolarizing pulses greater than $+10$ mV. However, the peak current density of sustained $K_{DR}$ currents in capsaicin-sensitive bladder afferent neurons was not different between sham and cystitis rats. These results indicate that a reduction in slow $K_A$ currents contributes to hyperexcitability of bladder afferent neurons in rats with HCl-induced cystitis.

**Reduction in Kv1.4 $\alpha$-subunit expression in DRG neurons innervating the bladder of cystitis rats.** In small-sized DRG neurons, several Kv1-family subunits including Kv1.4 are abundantly expressed, and immunoreactivity of Kv1.4 and

### Table 1. Electrophysiological properties of capsaicin-sensitive bladder afferent neurons from sham and cystitis rats

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Cystitis</th>
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<tr>
<td>No. of cells</td>
<td>19</td>
<td>28</td>
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<tr>
<td>Diameter, $\mu$m</td>
<td>$23.5\pm1.5$</td>
<td>$28.0\pm1.1^*$</td>
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<tr>
<td>Input capacitance, $pF$</td>
<td>$29.8\pm2.1$</td>
<td>$40.5\pm2.5\dagger$</td>
</tr>
<tr>
<td>Membrane potentials, mV</td>
<td></td>
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<tr>
<td>Resting</td>
<td>$-49.6\pm1.8$</td>
<td>$-48.0\pm1.0$</td>
</tr>
<tr>
<td>Spike threshold</td>
<td>$-18.8\pm1.3$</td>
<td>$-28.6\pm1.1\dagger$</td>
</tr>
<tr>
<td>Peak</td>
<td>$42.9\pm2.9$</td>
<td>$45.4\pm2.4$</td>
</tr>
<tr>
<td>Spike duration, ms</td>
<td>$4.3\pm0.3$</td>
<td>$4.3\pm0.4$</td>
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<tr>
<td>No. of action potentials, 800-ms depolarization</td>
<td>$1.3\pm0.3$</td>
<td>$9.7\pm1.2\dagger$</td>
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Data are means ± SE. $^*P < 0.05$, $\dagger P < 0.01$ vs. sham rats.
TRPV1 (capsaicin receptor) is highly colocalized (43, 56). Gating properties and toxin sensitivity of slow-decaying $K_A$ currents suggest that this current is carried by Kv1.4-containing channel complexes (42, 43, 52, 56). To examine whether a reduction in slow $K_A$ currents in bladder afferent neurons after cystitis is associated with decreased expression of these channels, the levels of Kv1.2 and 1.4 $\alpha$-subunits were evaluated using immunohistochemical methods in sham and cystitis rats (8 days after HCl treatment). We randomly selected L6 DRG sections (3 rats from each group, 6 sections/animal) and compared staining densities of Kv1.2 or 1.4 $\alpha$-subunits in Fast Blue-labeled DRG neurons innervating the bladder and unla- beled DRG neurons in the same section. The ratio of staining density of Kv1.4 $\alpha$-subunit in bladder afferent neurons vs. unlabelled DRG neurons was significantly decreased in cystitis rats ($n = 181$ cells) when compared with sham rats ($n = 153$ cells) that exhibited similar levels of Kv1.4 $\alpha$-subunit staining in Fast Blue-labeled and unlabeled neurons (Fig. 6). In contrast, in both groups of animals, similar levels of staining density of Kv1.2 $\alpha$-subunits were observed in Fast Blue-labeled and unlabeled neurons ($n = 144$ and $n = 135$ cells for sham and cystitis groups, respectively) (Fig. 6). These results indicate that decreased expression of Kv1.4 $\alpha$-subunit, but not Kv1.2 $\alpha$-subunit, is associated with the reduction of slow-decaying $K_A$ currents in bladder afferent neurons from cystitis rats.

**DISCUSSION**

The results of this study indicate that 1) HCl-induced cystitis resulting in bladder tissue inflammation lasting over 1 wk elicits urinary frequency due to an activation of capsaicin- sensitive C-fiber afferents pathways and is associated with decreased thresholds for C-fiber-mediated bladder sensation; 2) capsaicin-sensitive bladder afferent neurons obtained from L6-S1 DRG of HCl-induced cystitis rats shows hyperexcitability as evidenced by lower thresholds for spike activation, tonic firing pattern, and a reduction in slow-decaying $K_A$ current density; and 3) the expression of Kv1.4 $\alpha$-subunits is reduced in bladder afferent neurons from HCl-induced cystitis rats. To our knowledge, this is the first report showing the association of functional and immunohistochemical changes in voltage-gated $K^+$ channels that are responsible for hyperexcitability of afferent neurons innervating inflamed visceral organs such as the urinary bladder.

Afferent pathways innervating the urinary bladder consist of myelinated Aδ-fibers and unmyelinated C-fibers (15, 35, 54). In normal rats, conscious voiding is dependent on Aδ-fibers and C-fibers afferents pathways are mechanosensitive, whereas C- fibers afferents are responsible for bladder nociceptive responses (9, 10, 34, 48, 49). In the present study, urinary frequency observed in HCl-induced cystitis rats was suppressed by pretreatment with a high dose of capsaicin, a C-fiber neurotoxin that induces desensitization of capsaicin-sensitive C-fiber afferents, indicating that bladder inflammation over 1 wk can activate C-fiber afferent pathways to induce urinary frequency. This assumption is also supported by the present results using Neurometer CPT/C. HCl-induced cystitis rats had a significantly lowered threshold current intensity for electrical stimuli at 5 Hz applied onto the bladder luminal surface. This frequency is known to activate both C-fibers and A-fibers in somatic pathways (29). On the other hand, current thresholds for the behavioral responses induced by 250 Hz stimulation, which are known to activate Aβ- and Aδ-fiber afferents without significant excitation of C-fibers (29), were not significantly different in sham and cystitis rats. A recent study by Abouassaly et al. (2) also showed that the sensory perception thresholds at 250- and 5-Hz stimulation are useful to examine...
Afferent hyperexcitability in cystitis rats. The present study also indicates that capsaicin-sensitive bladder afferent neurons become hyperexcitable when the bladder is irritated by intravesical HCl instillation. Our previous study showed that the majority (>80%) of bladder afferent neurons from rats that were not stained with antibody against neurofilament-rich A- and C-fibers respond to capsaicin, whereas only a 200-kDa subunit of neurofilament, which is a marker of nociceptive neurons from rats that were not stained with antibody against vesicular acetylcholine transporter, which is a marker of cholinergic neurons, respond to capsaicin (58). Thus, it is assumed that capsaicin-sensitive bladder afferent neurons mainly represent C-fiber afferent cells. The hyperexcitability of capsaicin-sensitive bladder afferent neurons characterized by lower thresholds for spike activation and tonic pattern of firing in HCl-induced cystitis rats is further evidence for enhanced C-fiber bladder afferent activity although hyperexcitability of Aδ-fiber bladder afferents cannot be completely excluded because of a tendency of a decline in Neurometer CPT values at 250-Hz stimulation, which can activate A-fiber afferent pathways without C-fiber activation, in cystitis rats.

Voltage-gated K+ currents in sensory neurons are divided into two major categories; i.e., sustained KvDR currents and transient KvA currents (19, 21, 56, 57, 59). Transient KvA currents in sensory neurons including DRG cells can be further subdivided into at least two different subtypes based on their inactivation kinetics (i.e., fast- and slow-decaying KvA currents) (3, 17–19, 37, 57). It has also been reported that the slow-decaying KvA current, which was also termed the Kp current by Everill et al. (17), is preferentially expressed in small-sized DRG neurons that exhibit tetrodotoxin-resistant action potentials with inflections and responded to capsaicin (19, 57, 59). We have previously reported that application of 4-aminopyridine, a blocker for the KvA channel, increased excitability of bladder afferent neurons as evidenced by changes in the cell firing pattern from phasic to tonic during long-duration membrane depolarization (59). Thus, slow-decaying KvA currents are likely to be involved in reducing excitability in small-sized, nociceptive C-fiber DRG neurons including bladder afferent neurons, although alterations in other mechanisms including sodium (Na+) channels (13) or TRP receptors (38) reportedly contribute to inflammation-induced afferent hyperexcitability.
There is also the possibility that changes in Kv1.4 and other ion-gated channels might interact with each other to induce bladder afferent hyperexcitability.

In an earlier study, we discovered that bladder inflammation for 2 wk, which was induced by repeated systemic injections of cyclophosphamide, resulted in increased excitability of capsaicin-sensitive bladder afferent neurons due to a significant reduction in slow-decaying K_{A} current density (59). The present study confirmed these changes in another rat model of cystitis, and further demonstrated that hyperexcitability of bladder afferent neurons was associated with bladder tissue inflammation, urinary frequency, and bladder hyperalgesia. Since we first reported the above findings of slow-decaying K_{A} currents in bladder inflammation (59), similar results showing the close relationship between hyperexcitability of afferent pathways and a reduction in K_{A} channel function following tissue inflammation have been reported in visceral afferent neurons innervating gastrointestinal organs, such as gastric afferent neurons from rats with gastric ulcers (14), intestinal afferent neurons from guinea pigs with ileitis (50), and pancreatic afferent neurons from rats with pancreatitis (55). Thus, the reduction in slow-decaying K_{A} currents seems to be one of the key events resulting in hyperexcitability of C-fiber afferent neurons innervating visceral organs.

In this study, we also found that a reduction in slow-decaying K_{A} channel activity was associated with decreased expression of Kv1.4 α-subunit in bladder afferent neurons from cystitis rats. Voltage-gated K^{+} channels are composed of homo- or heterotetramers of α-subunits that form K^{+} ion conducting pores (31, 33, 45). Over a dozen genes encoding the pore-forming α-subunits of voltage-gated K^{+} channels have been isolated from mammalian tissues, and have been divided into several subfamilies (20). Previous reports have indicated that Kv1 α-subunits including Kv1.1, Kv1.2, and Kv1.4 could be major components of voltage-gated K^{+} channels in DRG neurons. Kv1.1 and Kv1.2 α-subunits are expressed in both small- and medium/large-sized DRG neurons, whereas the expression of Kv1.4 α-subunits is predominantly seen in small-sized DRG neurons (43). In addition, Kv1.4 α-subunits and TRPV1 capsaicin receptors are highly colocalized in small-sized DRG neurons (5, 43). It is known that a homotetramer of Kv1.4 α-subunits exhibits rapid activation and prominent inactivation processes but is insensitive to α-dendrotoxin (DTX), a specific inhibitor for Kv1.1 and Kv1.2 (23). However, previous studies have demonstrated that heteromeric channels containing Kv1.4 and DTX-sensitive Kv1.1/ Kv1.2 α-subunits are also toxic sensitive (12, 44) and that these heteromeric complexes exhibit inactivation much slower than the Kv1.4 homomeric channels, reminiscent of slow-decaying K_{A} in DRG neurons (42). We have previously reported that DTX partially suppressed slow-decaying K_{A} currents in capsaicin-sensitive, small-sized DRG neurons (56). Thus, it seems reasonable to assume that assembly with Kv1.4 and other DTX-sensitive Kv α-subunits, such as Kv1.1 and/or Kv1.2, contributes to formation of slow-decaying K_{A} channels in capsaicin-sensitive C-fiber afferent neurons, including those innervating the urinary bladder. Although we did not examine whether reduced Kv1.4 expression in bladder afferent neurons occurs in C- or Aδ-fiber cells following bladder inflammation, high capsaicin sensitivity of C-fiber bladder afferent neurons (58) and the significant overlap of Kv1.4 and TRPV1 expression in small-sized DRG neurons (5, 43) suggest that changes in Kv1.4 expression is more likely to occur in capsaicin-sensitive C-fiber bladder afferent neurons than in Aδ-fiber bladder neurons, although there is a possibility that Kv1.4 expression in the small number of capsaicin-sensitive Aδ-fiber bladder afferent neurons is also decreased after bladder inflammation. Taken together, it seems reasonable to assume that reduced expression of Kv1.4 α-subunits in bladder afferent neurons following bladder inflammation is a molecular mechanism responsible for the reduction in slow-decaying K_{A} currents and the resulting hyperexcitability of capsaicin-sensitive C-fiber bladder afferent neurons and C-fiber-mediated bladder hyperactivity/hyperalgesia in rats with HCl-induced cystitis.

PBS/IC is a debilitating chronic disease characterized by suprapubic pain related to bladder filling, coupled with urinary frequency, without proven urinary infection or other obvious pathology (8, 16, 41, 60). While the etiology is unknown, theories explaining the pathophysiology of PBS/IC include an altered urothelial barrier, neurogenic inflammation including mast cell infiltration and afferent sensitization (6, 7, 36, 61). A recent study using cats with naturally occurring feline-type IC has demonstrated that capsaicin-sensitive DRG neurons exhibited an increase in cell size and had increased firing rates to depolarizing current pulses due to a reduction in low-threshold K^{+} currents elicited by membrane depolarization between −50 to −30 mV (46), as similarly found in our present and previous studies using cystitis rats. Therefore, the reduction in K_{A} currents due to decreased Kv1.4 α-subunit expression resulting in afferent hyperexcitability might be involved in the pathogenesis of visceral hypersensitive disorders, such as PBS/IC, although further studies are needed to clarify this point. In our preliminary results (data not shown), a reduction of Kv1.4 expression in L6-S1 DRG using intrathecal application of small-interfering RNA induces frequent voiding in normal rats (22). Overall, voltage-gated K^{+} channels composed of Kv1.4 α-subunits might represent an interesting target for the development of new antinociceptive drugs that can activate or facilitate slow-decaying K_{A} channels and thereby reduce the symptoms of PBS/IC.

REFERENCES


AFFERENT HYPEREXCITABILITY IN CYSTITIS RATS


