CYCLOPHOSPHAMIDE-INDUCED BLADDER INFLAMMATION SENSITIZES AND ENHANCES P2X RECEPTOR FUNCTION IN RAT BLADDER SENSORY NEURONS

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Abstract

We studied sensitization of retrogradely labeled bladder sensory neurons and plasticity of P2X receptor function in a model of cystitis using patch-clamp techniques. Saline (control) or cyclophosphamide (CYP) was given intraperitoneally to rats on days 0, 2 and 4. On day 5, lumbosacral (LS, L6-S2) or thoracolumbar (TL, T12-L2) dorsal root ganglia were removed and dissociated. Bladders from CYP-treated rats showed partial loss of the urothelium and greater myeloperoxidase activity compared with controls. Bladder neurons from CYP-treated rats were increased in size (based on whole cell capacitance) compared with controls and exhibited lower activation threshold, increased action potential width and greater number of action potentials in response to current injection or application of purinergic agonists. Most control LS bladder neurons (>85%) responded to ATP or α,β-metATP with a slowly desensitizing current; these agonists affected only half of TL neurons, producing predominantly fast/mixed desensitizing currents. CYP treatment increased the fraction of TL bladder neurons sensitive to purinergic agonists (>80%) and significantly increased current density in both LS and TL bladder neurons compared with control. Importantly, LS and TL neurons from CYP-treated rats showed a selective increase in the functional expression of heteromeric P2X$_{2/3}$ and homomeric P2X$_3$ receptors, respectively. While desensitizing kinetics were slower in LS neurons from CYP-treated compared with control rats, recovery kinetics were similar. The present results demonstrate that bladder inflammation sensitizes and increases P2X receptor expression and/or function for both pelvic and lumbar splanchnic
pathways, which contribute, in part, to the hypersensitivity associated with cystitis.

**Key words**: cystitis, visceral hypersensitivity, cyclophosphamide, purinergic agonists, whole cell patch clamp
Introduction

About 16% of adult men and women report problems with micturition (Tubaro 2004), including increased urinary frequency, urgency and urinary incontinence. While multiple disorders may contribute to these symptoms, interstitial cystitis (IC)/painful bladder syndrome is especially troublesome because affected individuals report significant discomfort and pain in addition to urinary symptoms (Burkman 2004; Nickel 2004). Pain is the most troubling symptom to IC patients (Parsons 2002) and 94% of registrants in an IC database report pain referred to the pelvic area (Kirkemo et al. 1997). No infectious agent causing IC has been identified, although bladders of many IC patients show signs of inflammation, including edema, vasodilatation and infiltration of mast cells (Burkman 2004; Nickel 2004).

Changes in sensory input from the bladder (i.e., bladder hypersensitivity) play an important role in the development of IC (Ness et al. 2005). IC is associated with an increased release of ATP from bladder urothelial cells (Sun et al. 2001) and ATP is also released from bladder urothelium in response to stretch or bladder distension (Ferguson et al. 1997; Vlaskovska et al. 2001). Importantly, interactions between the urothelium and bladder nerve terminals are thought to regulate micturition and nociception (Andersson 2002; Kirkemo et al. 1997; Meen et al. 2001; Ness et al. 2005; Tubaro 2004; Vlaskovska et al. 2001), and ATP and P2X receptors have been implicated in bladder nociception (Rapp et al. 2005; Ford et al. 2006). For example, both P2X2 and P2X3 receptor knockout mice exhibit bladder hyporeflexia (Cockayne et al. 2000, 2005) and recordings from
bladder primary afferent fibers show attenuated responses to mechanical bladder stimulation (Vlaskovska et al. 2001). P2X2 and P2X3 receptor expression is increased in the urothelium of IC patients (Tempest et al. 2004).

The bladder is innervated by pelvic and lumbar splanchnic nerves with cell bodies, respectively, in lumbosacral (LS, L6-S2) and thoracolumbar (TL, T13-L2) dorsal root ganglia (DRG). Most studies have focused on the role of the pelvic nerve, transection of which abolishes bladder contractions in response to repetitive filling, suggesting that the pelvic nerve is important for the sensation of bladder distension and micturition (Kontani and Hayashi 1997; Meen et al. 2001). However, the lumbar splanchnic nerve is also activated by mechanical and chemical stimulation of the bladder, and may play an important role in regulation of micturition and painful sensations after bladder irritation/damage (Mitsui et al. 2001; Moss et al. 1997). In addition, recent evidence suggests that different nerves innervating a visceral organ mediate different functions (Brierley et al. 2004; Dang et al. 2005a; Lamb et al. 2003). Consistent with this notion, bladder lumbar splanchnic afferents have been reported to respond more vigorously to chemical stimuli than do pelvic nerve counterparts (Moss et al. 1997).

In the present report, we used a well-established model of urinary bladder inflammation (Lanteri-Minet et al. 1995; Bon et al. 1997, 2003 ) to examine the consequences of inflammation on characteristics of TL and LS bladder neurons in the rat, focusing on their sensitivity to purinergic receptor agonists. We hypothesized that changes in the excitability and sensitivity of bladder sensory neurons to endogenous purinergic receptor agonists contribute to bladder
dysfunction, discomfort and pain that characterize bladder disorders such as cystitis. Portions of these data have appeared in a preliminary form (Dang et al. 2005c).
Materials and Methods

Male Sprague-Dawley rats (200-300 g; Harlan, Indianapolis, IN) were used throughout. Rats were housed under a 12-hour light and dark cycle with free access to food and water. Animal handling adhered to the Guide for the Care and Use of Laboratory Animals (National Research Council); the experimental protocol was approved by the Animal Care and Use Committee, The University of Iowa.

Bladder inflammation, histology and myeloperoxidase (MPO) activity

Systemic administration of cyclophosphamide (CYP), which is metabolized to the bladder irritant acrolein (Cox 1979), causes hemorrhagic cystitis in humans and produces a cystitis-like condition in rodents (Lanteri-Minet et al. 1995; Bon et al. 1997, 2003). Saline (control) or CYP (100 mg/kg) was administered systemically (i.p.) on days 0, 2 and 4. On day 5, four rats from each group were euthanized (see below) and the bladders removed, fixed in paraformaldehyde (4%), paraffin-mounted and cut at a thickness of 10 µm. Sections were stained with hematoxylin and eosin (both at 5%), mounted and examined microscopically at 100 and 400 X magnification by a pathologist. To further quantify bladder inflammation, we measured MPO activity in bladder tissue from an additional six rats/group. Rats were anesthetized, the bladders removed rapidly, minced, homogenized in ice-cold 50 mM phosphate buffer (pH 6) containing 0.5% hexadecyltrimethyl-ammonium bromide, centrifuged at 1000 rpm for 5 min and the supernatant retained. In the presence of hydrogen peroxide (30%) and o-dianisidine dihydrochloride (0.5%), the absorbance of the
supernatant was determined with a U2001 photometric reader (Hitachi, Naperville, IL).

*Bladder neuron labeling and bladder inflammation*

Under pentobarbital anesthesia (50 mg/kg i.p.), the bladder was surgically exposed (lower abdominal incision ~1 cm in length) and 1.1’-dioctadecyl-3,3,3,’3-tetramethylindocarbocyanine methanesulfonate (DiI\textsuperscript{(18)}; 100 mg in 2 ml DMSO; Molecular Probes, Eugene, OR) was injected into 6-8 sites within the wall of the bladder base around the trigone using a 30 gauge needle (6 µl per site). Any visible leakage of DiI from the injection site was removed with a cotton swab. Surgery was short in duration (typically < 60 min) and the depth of surgery was assessed by toe pinch extensor withdrawal. The incision was closed with 4.0 silk suture and rats were allowed to recover. Post-operative analgesia was provided by buprenorphine (2 mg/kg i.p.). In four rats, Fast Blue (FB; 5% in saline; EMS-Chemie, Gross Umstadt, Germany) was injected as described above for DiI. Two-three weeks later, saline (control) or CYP (100 mg/kg) was administered systemically (i.p.) on days 0, 2 and 4. On day 5, rats were deeply anesthetized (pentobarbital 150 mg/kg i.p.) and lumbosacral (LS, L6-S2) or thoraco-lumbar (TL, T13-L2) DRG were harvested for acute dissociation and whole cell recordings. After removing DRG, we opened the abdomen, examined the pelvic area for leakage of dye, noted the macroscopic appearance of the bladder, removed the bladder and measured its wet weight after emptying the contents. Euthanasia was accomplished by exsanguination after removal of the bladder.
To confirm the specificity of retrograde labeling for bladder neurons, we injected DiI into the bladder wall of four rats as described above. At the same time, we injected fast blue (FB) into the parietal peritoneum adjacent to the bladder. Two-three weeks later, TL DRGs (T13-L2) were removed, fixed in 4% paraformaldehyde, cryoprotected, cut at a thickness of 10 µm and examined by fluorescence microscopy (see below). We studied only TL DRG because the cell bodies of neurons that innervate the parietal peritoneum (and bladder) are located in thoracolumbar DRGs (Applebaum et al. 1980; Tanaka et al. 2002).

**Cell dissociation and plating**

The general protocols for harvesting DRG and acute cell dissociation have been previously described (Dang et al. 2004, 2005a,b). Briefly, after removal, ganglia were minced and incubated at 37°C, 5% CO2 for 60 min in serum-free, supplemented Neuro-A medium (B27 supplements - 5%; L-glutamine - 0.5 mM; penicillin/streptomycin mixture - 1%; all from Gibco, Invitrogen Corp., Grand Island, NY) containing collagenase (type 4; 2 mg/ml), and trypsin (1 mg/ml; from Worthington Biochemical Corp., Lakewood, NJ). Tissue fragments were gently triturated to encourage cell dissociation. Cells were collected by 5 min centrifugation at 150 X g and washed 3 times with supplemented Neuro-A medium (without enzymes) and resuspended in supplemented, enzyme free Neuro-A medium. The cells were plated on poly-D-lysine-coated coverslips (Becton Dickinson Labware, Bedford, MA) and incubated at 37°C, 5% CO2 for 2-3 hr before electrophysiological studies. Acutely dissociated neurons were round and devoid of any processes, thus reducing potential space-clamp errors. Only
bladder sensory neurons (i.e., Dil- or FB-containing DRG neurons) were studied. All recordings were performed within 10 hr after plating.

Solutions and electrophysiological recordings

Coverslips with cells were transferred to a recording chamber (1ml) superfused continuously (2 ml/min) with external solution containing (in mM): NaCl 140, KCl 5, MgCl₂ 2, CaCl₂ 2, HEPES 10, glucose 10. The pH was adjusted to 7.4 with NaOH (310 mOsm). Under low magnification (50 X), neurons that innervate the bladder were identified by Dil content using a rhodamine filter and clearly showed a bright orange/red color under UV light (excitation wavelength: 530 - 560 nm and barrier filter: 573 - 648 nm). In general, less than 5 s were required to identify a bladder neuron. FB labeled bladder neurons were identified using a UV-2A filter (Nikon, Japan; excitation wavelength: 330-380 nm and barrier filtered: 420 nm) as described above. Fire-polished micropipettes with tip resistances of 1.5-2 MΩ were used for current and voltage clamp recordings. The uncompensated series resistance was generally about 7 MΩ or less. The pipette was filled with an internal solution consisting of (in mM): KCl 130, CaCl₂ 1, MgCl₂ 1, EGTA 10, HEPES 10, Na₂ATP 4, Tris-GTP 0.5, GDP 0.5. The pH was adjusted to 7.2 using KOH (310 mOsm). After establishing the whole cell configuration, the voltage was clamped at -70 mV using an Axopatch 200B amplifier (Axon Instruments, Union City, CA), digitized at 1 kHz (Digidata 1350, Axon Instruments) and controlled by Clampex software (pclamp 9, Axon Instruments). Cell capacitance was obtained by reading the value from the
Axopatch 200B amplifier. Recordings began 2-3 min after establishing whole-cell configuration to ensure stable recording conditions.

In current-clamp mode, resting membrane potential was determined by obtaining the value from the amplifier (Axopatch 200B). Only cells that had a resting membrane potential more negative than -40 mV and generated action potentials with a distinct overshoot above 0 mV in response to depolarizing current injections were studied. AP duration was determined at 50% of the peak amplitude from baseline. We investigated spontaneous activity by recording the baseline activity for 1 min prior to electrical or chemical stimulation. To identify action potential threshold, a series of 10 ms current pulses in 20 pA increments (1 s apart) was injected. The minimum current (pA) required to evoke an AP was determined (rheobase) and the activation threshold (mV) was taken as the greatest membrane potential in the absence of an AP (Gold and Traub, 2004).

To examine firing patterns in gastric sensory neurons, suprathreshold current (2 × rheobase) was injected for 500 ms and the number of APs counted.

Drugs were applied using a fast-step SF-77B superfusion system (Warner Instruments, Hamden, CT) with a 3-barrel pipette placed in close proximity (100 µm) to the cell, allowing complete solution exchange within 25 ms (Dang et al., 2005a). Agonists were applied for 4 s, whereas antagonists were superfused for 30 s prior to the application of agonists. A washout period of 4 min was allowed between agonist applications. Unless mentioned otherwise, drugs and chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO) and prepared fresh from stock solutions on the day of the experiment. All experiments were performed at
room temperature (21-23°C). We used current increases above 20 pA or voltage changes above 4 mV as thresholds to identify responses to purinergic agonists, as these levels exceeded baseline variability by a factor of two. To determine the kinetics of response onset, we measured the time from 10 to 90% of peak amplitude.

**Immunohistochemistry**

To determine the percentage of bladder neurons expressing the P2X$_3$ subunit, we performed immunohistochemistry for P2X$_3$ protein. Fluorogold (FG; 4 %; Biotium Inc., Hayward, CA) was injected into the bladder base in eight rats as described for Dil above. After two-three weeks, saline (n=4) or CYP (n=4) was given as described above. Rats were sacrificed on day 5 and DRG were rapidly removed, fixed in 4% paraformaldehyde for 4 hr and cryoprotected in 30% sucrose for 3 days. Frozen DRGs were sectioned at 10 µm using a Leica 3M 3050 cryostat; one section was selected every 50 µm to minimize double counting. DRG sections were blocked with 3% goat serum in 0.01M PBS (Sigma-Aldrich Co.) for 2 hr prior to incubation in primary antibody (rabbit anti-rat, 1:1000; Alamone Labs, Jerusalem, Israel) for 24 hr, 4°C, after which the primary antibody was aspirated and sections washed 3 X 15 min with 0.01 M PBS. Secondary antibodies (anti-rabbit) conjugated to either Alexa Fluor 488 or 568 (Molecular Probes) were applied for 4 hr and washed 3 X 15 min with 0.01 M PBS. Sections were mounted with fluorescence mount (Sigma-Aldrich Co.) and viewed with a Nikon microscope equipped with separate fluorescence filters. Images were captured with a model 2.3.1 SPOT digital camera (Diagnostic
Instruments, Sterling Heights, MI). All cells positive for FG (i.e., bladder cells) were counted and the number co-stained with P2X<sub>3</sub> antibody determined. For control experiments, primary antibody was omitted or blocked with a corresponding antigenic peptide for 24 hr. No labeling was observed (data not shown).

**Data analyses**

Data are presented as mean ± SEM. Analyses were performed using the software package Graphpad Prism 4 (Graphpad Software, San Diego, CA). Sigmoidal concentration-response curves were generated using the following equation: \( Y = \frac{A}{1 + \exp\left[- \frac{\log(EC50-X)}{B}\right]} \) where \( X \) is the logarithm of concentration, \( Y \) is the response and starts at 0 and goes to \( A \) with a sigmoidal shape. \( B \) is the Hill slope. Desensitization kinetics were fitted with a standard exponential equation: \( Y = K_0 + K_1 \cdot \exp\left(-\frac{t}{\tau}\right) \) where \( Y \) is the current amplitude at time \( t \), \( K_0 \) is the amplitude of the sustained component and \( \tau \) is the time constant. \( K_0 \) and \( K_1 \) represent the contribution to current amplitude from the fast and slow components of the current, respectively. For dichotomous variables, a Chi-square test was employed. Where appropriate, results were evaluated using a one way ANOVA or Wetch’s t-test after logarithmic transformation. Results were considered to be statistically significant when \( P < 0.05 \).
Results

CYP produces bladder damage and inflammation

Grossly, CYP treatment caused darkening of the bladder in every rat, which was not seen after saline treatment. In addition, the weight of bladders from CYP-treated rats was significantly greater than control rats (control: 101±3 g, n = 36; CYP: 175±5 g, n = 30; P < 0.001). Histologically, the bladder wall appeared thickened in CYP-treated rats compared with controls and was associated with partial loss of the urothelium. The remaining urothelial cells in CYP-treated rats were abnormally large compared with controls. Consistent with both gross and microscopic evaluations of the bladder, CYP treatment significantly increased myeloperoxidase activity relative to controls (control: 0.11±0.01, n = 3; CYP: 0.83±0.2 unit/g, n = 3; p < 0.001).

Specificity of labeling with Dil

Dil is lipophilic and leakage or diffusion from the injection site in the bladder wall could result in labeling of sensory neurons innervating adjacent tissues, which would then be misidentified as bladder sensory neurons. To address this concern, we injected FB into the bladder wall of three rats and counted FB-labeled cells on randomly chosen coverslips containing TL or LS DRG cells. Consistent with previous reports using Dil or another tracer (Dang et al., 2005a; Wang et al., 1998), significantly more bladder sensory neurons were found in LS than TL DRG (LS: 120/1812 = 6.8±0.6%; TL: 66/3268 = 2.5±0.4%; P < 0.01); the percentages of FB-labeled bladder neurons were not different than percentages of Dil-labeled bladder neurons (5.4±0.5% and 2.0±0.3%; Dang et
al., 2005a). It is also possible that Dil may spread within the DRG, again resulting
in non-specific labeling. We addressed this concern by injecting Dil into the
bladder wall and FB into the adjacent parietal peritoneum and looked for double-
labeled DRG cells. Because cell bodies of neurons innervating the parietal
peritoneum and the bladder are found in TL DRG (Applebaum et al. 1980;
Tanaka et al. 2002), we only examined TL DRG. Of 89 Dil and 179 FB neurons
examined, only 4 cells contained both dyes, demonstrating that Dil can be used
to reliably and selectively label sensory neurons for whole cell study.

**Bladder inflammation increases cell size and induces hyperexcitability in bladder sensory neurons**

We examined the distribution of cell size and excitability of bladder sensory neurons after bladder inflammation. Using whole-cell capacitance as an index of cell size, neurons were considered small, medium or large if whole-cell capacitance was $\leq 20$, $20.1-60$ or $> 60$ pF, respectively. As illustrated in Fig. 1, virtually all LS bladder neurons were classified as small (34/152, 22%) or medium (117/152, 77%) in size (one cell [1%] was large), consistent with previous reports (Dang et al. 2005a; Yoshimura and de Groat 1999; Yoshimura et al. 2003). In contrast, all TL neurons were medium (70/88, 80%) or large (18/88, 20%) in size.

Because incorporation of lipophilic Dil into the plasma membrane may potentially alter whole cell capacitance, and consequently mislead interpretation of cell size, we injected hydrophilic FB into the bladder wall in three rats and measured capacitance of FB-labeled LS bladder neurons to facilitate comparison
with previous studies (Yoshimura and de Groat 1999; Yoshimura et al. 2003). The mean whole cell capacitance of FB-labeled LS bladder neurons (26.9±1 pF, n = 90) did not differ from that of Dil-labeled LS bladder neurons (27.7±0.6 pF, n = 152; P > 0.05). Similarly, FB-labeled LS neurons were characterized as small (26/90, 28.9%) or medium (64/90, 71.1%) in size (Fig. 1), consistent with results using Dil in the present and an earlier study (Dang et al., 2005a) and previous reports using FB (Yoshimura and de Groat 1999; Yoshimura et al. 2003).

Bladder inflammation significantly shifted the size distribution of LS neurons (small: 8/132, 6%; χ² = 14.9; P < 0.001; medium: 123/132, 93%; χ² = 14.2; P < 0.001; large 1/132; Fig. 1). Consistent with these findings, the mean cell capacitance of LS neurons significantly increased after bladder inflammation compared with control (control, 27.7±0.6 pF vs. CYP, 33.4±1 pF; P < 0.001). While the proportions of medium (88/115, 77%) and large (27/115, 23%) TL bladder neurons were unchanged by CYP treatment, inflammation similarly increased overall mean TL cell size compared with control (control, 45±1.2 pF vs. CYP, 56±2 pF; P < 0.001).

Bladder inflammation alters active and passive membrane properties of bladder sensory neurons.

LS and TL neurons differed in some of their active and passive membrane properties. Membrane potential was more negative in LS than TL neurons, requiring greater current injection for action potential generation (Table 1). Under control conditions, no bladder neurons exhibited spontaneous activity (LS: 0/23; TL: 0/23). Bladder inflammation did not alter resting membrane potential, but
significant numbers of LS (11/31, 35%; $\chi^2 = 10.3; P < 0.01$) and TL (13/29, 45%; $\chi^2 = 13.8; P < 0.001$) neurons from CYP-treated rats were spontaneously active (Fig. 2A & F; Table 1). Spontaneously active LS and TL neurons from CYP-treated rats fired 43.8±14 and 53.6±13 APs in 60 s, respectively, but had resting membrane potentials that did not differ from neurons without spontaneous activity (LS: -50.1±1.8 mV; TL: -45.3±0.7 mV; P > 0.05) or corresponding control groups. However, all spontaneously active neurons exhibited apparent membrane potential oscillations (oscillation amplitude: LS: 5.9±0.4 mV; TL: 4.8±0.3 mV). Bladder inflammation significantly lowered the rheobase of both LS and TL neurons, shifted the AP threshold to more negative potentials, and widened the AP duration (Fig. 2 B & G; Table 1). In addition, suprathreshold current injection (rheobase X 2) in cells from CYP-treated rats evoked significantly more APs compared with control (Table 1; Fig. 2C & H).

**Bladder inflammation enhances responses to purinergic agonists**

In neurons from control rats, ATP (100µM) depolarized all LS neurons tested (23/23), triggering APs in all but 3, whereas ATP depolarized only 13/23 TL neurons, with 6 firing APs (Fig. 2D & I). ATP produced greater magnitude depolarization in LS bladder neurons compared with TL counterparts (LS: 24.5±1.5 mV; TL: 6.2±1.2 mV), which was associated with a greater number of APs (LS: 8.9±0.7; TL: 3.7±1.4; P < 0.05) (Table 1). We also applied the selective homomeric P2X1, P2X3 and heteromeric P2X2/3 receptor agonist α,β-metATP (North, 2002) to the same bladder neurons that were previously challenged with ATP. α,β-metATP (100 µM) depolarized 23/23 and 12/13 ATP-sensitive LS and
TL neurons, respectively (Fig. 2E & J) and triggered APs in all bladder neurons that generated APs in response to ATP (20 LS and 6 TL neurons) (Table 1).

In bladder neurons from CYP-treated rats, ATP depolarized a similar fraction of LS neurons (30/31, 97% vs. 23/23, 100% in control). In TL neurons, however, bladder inflammation significantly increased the proportion of neurons that responded to ATP (26/29, 90% vs. 13/23, 57% in control; \( \chi^2 = 7.5, P < 0.01 \)). As shown in Fig. 2 D & I, ATP caused significantly greater depolarizations, resulting in AP generation in all neurons obtained from CYP-treated animals and a higher firing rate compared with controls (Table 1). Similarly, \( \alpha,\beta\)-metATP triggered greater depolarizations associated with greater numbers of APs in bladder neurons from CYP-treated rats relative to corresponding control groups (Table 1 and Fig. 2E & J).

**Bladder inflammation increases purinergic currents and slows desensitization**

Consistent with findings described above, significantly more LS than TL neurons responded to the purinergic agonists and with different desensitization kinetics (Fig. 3 and Tables 2 & 3). A slowly desensitizing current predominated in LS neurons in response to ATP (46/53, 87%) (Fig. 3B). Only 3 (6%) and 4 (7%) LS neurons expressed fast (Fig. 3A) and mixed desensitizing currents, respectively (Fig. 3C, C’). \( \alpha,\beta\)-metATP triggered similar, but significantly lower peak currents in almost all of the same cells (Tables 2 & 3).

In contrast, ATP produced predominantly mixed currents in 17/33 (52%) TL neurons (Fig. 3F). Ten of 33 (30%; Fig. 3E) and 6/33 (18%; Fig. 3D) TL neurons expressed slow and rapid desensitizing currents in response to ATP,
respectively. In 6 TL neurons, ATP but not the P2X receptor subtype-selective agonist α,β-metATP triggered inward currents that slowly activated and desensitized. Similar to the effects of ATP, α,β-metATP triggered slow, rapid and mixed desensitizing currents in 4 (15%), 6 (22%) and 17 (63%) of 27 TL neurons, but with significantly lower peak current than did ATP (Table 2). Current densities for kinetically distinct currents and corresponding current kinetics in response to purinergic agonists are summarized in Tables 2 and 3, respectively.

Although CYP treatment did not alter the proportions of LS bladder neurons that responded to purinergic agonists (Table 2), LS neurons from CYP-treated rats expressed significantly greater peak current density compared with controls (Table 2; compare Fig. 3B with B*). As in controls, both ATP and α,β-metATP triggered only slowly desensitizing currents in all LS responders (Fig. 3B*). However, neurons obtained from animals with cystitis showed an accelerated time to peak and slower desensitization kinetics after purinergic agonist stimulation compared with controls (Table 3).

Consistent with the current-clamp data, bladder inflammation significantly increased the fraction of TL neurons that responded to purinergic agonists and increased the peak current density compared with controls (Table 2 & 3). ATP triggered slow, fast and mixed desensitizing currents in 7 (15%, Fig. 3E*), 6 (13%; Fig. 3D*) and 34 (72%; Fig. 3F*) of 47 TL neurons, respectively. In 3 TL neurons, ATP but not α,β-metATP triggered current that slowly activated and desensitized. Interestingly, while α,β-metATP produced a slow desensitizing current in a similar proportion of TL neurons (4/44, 9%) after bladder
inflammation, the fractions of rapidly (19/44, 43%; χ² = 5.4; P < 0.05) and mixed
(21/44, 48%; χ² = 7.2; P < 0.01) desensitizing currents in response to α,β-
metATP increased and decreased significantly, respectively, relative to controls.
In contrast to LS neurons, inflammation did not alter the kinetics of P2X receptor-
mediated currents in TL neurons (Table 3).

Bladder inflammation does not alter the concentration-response function of
purinergic agonists

To investigate whether the increase in current density after bladder
inflammation is due to a change in the potency (EC₅₀) or an increase in the
maximum response to purinergic agonists, we examined the concentration-
dependent effects of ATP and α,β-metATP for the principal currents produced in
LS (slow) and TL (fast/mixed) neurons. To control for differences in current
densities, we expressed current as a fraction of relative current at 300 µM (I/Iₘₐₓ).
As shown in Fig. 4A and B, ATP- and α,β-metATP-activated currents were
concentration-dependent for both the slow and rapid (fast) desensitizing currents
with a lower EC₅₀ for ATP (slow desensitizing current: 16.8 ± 3 µM; n = 15; fast
desensitizing current: 17.6 ± 5 µM; n = 8) compared with α,β-metATP (slow
desensitizing current: 43.5 ± 3 µM; n = 20; fast desensitizing current: 46.3 ± 6
µM; n = 7; P < 0.001 for both currents). The EC₅₀s for slow (ATP and α,β-
metATP, respectively: 17.1 ± 4 µM, n = 13; 39.2 ± 4 µM, n = 10) and fast (ATP
and α,β-metATP, respectively: 14.5 ± 3 µM, n = 9; 38.9 ± 5 µM, n = 11)
desensitizing currents did not differ in bladder neurons from CYP-treated rats
compared with control.
To examine whether purinergic agonists increase maximum currents, we plotted current densities against ATP or α,β-metATP concentrations in the same cells as described above. Bladder inflammation significantly increased the maximum current density triggered by application of ATP or α,β-metATP for both kinetically distinct currents compared with control (Fig. 4C & D).

*Bladder inflammation increases peak current produced by α,β-metATP relative to ATP*

To examine whether ATP activates other P2 receptors for the principal currents in LS (slow) and TL (fast/mixed) bladder neurons, we sequentially applied maximum concentrations of ATP (300 µM) or α,β-metATP (300 µM) to some of the same neurons. In bladder neurons from control rats, ATP produced a 58 ± 3.8% (n = 11; P < 0.001; Fig. 4E) greater peak current than did α,β-metATP for the slow desensitizing current. In contrast, ATP and α,β-metATP evoked similar peak currents for the fast desensitizing current (5.5 ± 1.7 %; n = 5; P > 0.05; Fig. 4F & G), demonstrating that activation of other P2 receptors is, in part, responsible for a greater slowly desensitizing current in response to ATP compared with α,β-metATP at lower concentration (30 µM). In neurons from CYP-treated rats, the maximum current produced by ATP relative to α,β-metATP was similar for the rapid desensitizing current (3.2 ± 0.4 %; n = 4; Fig. 4F and G; right traces). However, ATP only produced a 24.1 ± 2 % greater peak current (n = 9; comparison with ATP effects on control LS neurons, P < 0.001; Fig. 4E right traces) than did α,β-metATP, suggesting a shift to the expression of heteromeric P2X$_{2/3}$ channels, which are activated by α,β-metATP.
Bladder inflammation does not alter recovery kinetics of slow or fast desensitizing currents

We next examined whether bladder inflammation alters recovery kinetics for the principal currents found in LS (slow) and TL (fast/mixed) bladder neurons. Based on kinetic studies in our previous report (Dang et al, 2005), application intervals of 3 and 1 min were chosen for fast and slowly desensitizing currents, respectively, to allow a complete recovery of P2X mediated currents. Using a two stimulus protocol, we followed the initial agonist application (4 s) with a second application at increasing inter-stimulus intervals. The fraction of current in response to the second application of α,β-metATP was normalized to the first application and plotted against time. In bladder neurons from control rats, the slow desensitizing current showed little decay and recovered rapidly, whereas the fast desensitizing component required 3 min for complete recovery. Based on single exponential fitting, the slow desensitizing current recovered significantly faster than did the rapid desensitizing current (time constants of 11.6 ± 0.7 s, n = 12, and 44.1 ± 3 s, n = 6, respectively; P < 0.001). Because fast and slow desensitizing currents were rarely seen in LS and TL bladder neurons, respectively, we did not systematically examine their recovery kinetics. In bladder neurons from CYP-treated rats, recovery kinetics for slow and fast desensitizing currents appeared similar compared with control, which was confirmed by single exponential fitting (time constants for slow and fast currents, respectively: 13.5 ± 1.6, n = 6; 45.8 ± 3 s, n = 8; P > 0.05 compared with controls).

Antagonism of α,β-metATP-activated currents by selective antagonists
To establish that α,β-metATP triggers currents by activating P2X receptors, we applied trinitrophenyl-ATP (TNP-ATP), a selective homomeric P2X₁, P2X₃ and heteromeric P2X₂/₃ receptor antagonist (Virginio et al., 1998) to neurons from CYP-treated rats. In the presence of TNP-ATP (100 nM), the α,β-metATP-mediated slow desensitizing current was reversibly inhibited by 75.3 ± 4% (n = 6) relative to control (Fig. 5A; left traces). However, A317491 (10 µM), a potent and selective homomeric P2X₃ and heteromeric P2X₂/₃ receptor antagonist (Jarvis et al. 2002), only attenuated the current by 32.7 ± 2% in the same cells (Fig. 5A; right traces). Both TNP-ATP and A317491 completely and reversibly inhibited the fast desensitizing current in all six neurons tested (examples in Fig. 5B). These results suggest that activation of heteromeric P2X₂/₃ and homomeric P2X₃ receptors trigger the slow and rapid desensitizing currents, respectively.

Bladder inflammation significantly upregulates the fraction of P2X₃-immunopositive TL neurons

Because significantly more TL neurons responded to purinergic agonists with fast desensitizing kinetics after bladder inflammation, we stained cells for P2X₃ immunoreactivity (Fig. 6). In control neurons, 89/107 (83%) LS and 39/97 (40%) TL cells showed P2X₃ immunoreactivity. Bladder inflammation significantly increased the fraction of TL neurons (71/104, 68%; χ² = 16; P < 0.001), but not LS counterparts (87/98, 89 %), that exhibited P2X₃-immunoreactivity.
Discussion

The present report confirms and significantly extends our previously reported findings (Dang et al. 2005a) on differences between the pelvic (cell bodies in LS DRG) and lumbar splanchnic (cell bodies in TL DRG) innervation of the rat urinary bladder. The present results establish that neuronal excitability in both sensory pathways is significantly increased by bladder inflammation as revealed by changes in action potential threshold and spike frequency during current injection. Additional evidence is provided by the observation that about one-third of bladder neurons obtained from animals with inflamed bladders were spontaneously active, whereas control bladder sensory neurons are uniformly silent. Bladder inflammation also increased significantly the peak inward current in response to application of purinergic receptor agonists and increased the proportion of TL neurons that responded to them, which was verified immunohistochemically, and altered the kinetics of currents triggered by purinergic agonists in LS neurons. Importantly, use of selective purinergic receptor ligands revealed a significant shift in the expression of P2X receptors toward heteromeric P2X_{2/3} and homomeric P2X_{3} for LS and TL neurons, respectively, after bladder inflammation.

Consistent with prior observations in bladder, gastric and colon sensory neurons (Bielefeldt et al. 2002; Dang et al. 2004, 2005a; Gold and Traub 2004; Sugiura et al. 2004, 2007), most bladder sensory neurons are medium sized as judged by their capacitance. As previously described for gastric (Dang et al. 2004) and LS bladder neurons (Yoshimura and de Groat 1999), we noted an
increase in the size of LS and TL bladder neurons harvested from animals with inflamed bladders. We did not address the mechanism for the change in cell size, but others have reported similar findings in response to inflammation of the hind paw, which could be blocked by neutralization of NGF (Nicholas et al. 1999). Prior studies have demonstrated a significant increase in NGF during CYP-induced cystitis in rats, suggesting that an increase in NGF in the target area (bladder) may contribute to the observed increase in cell size (Vizzard 2000).

Bladder inflammation significantly increased the excitability of both TL and LS bladder neurons, consistent with a prior study that examined the properties of LS neurons in the same model (Yoshimura and de Groat 1999). Although not the focus of the present study, we and others have previously demonstrated that increased excitability during visceral inflammation is due to an increase in TTX-resistant sodium currents and a decrease in A-type potassium current (Bielefeldt et al. 2002; Dang et al. 2004; Stewart et al. 2003; Yoshimura and de Groat 1999). We also noted the appearance of spontaneous activity in both afferent pathways (i.e., LS and TL DRG neurons), similar to findings for the stomach after gastric inflammation (Dang et al. 2005b). This was associated with apparent membrane potential oscillations in cells from CYP-treated animals. Changes in the properties of voltage-sensitive sodium currents and/or potassium channels are thought to contribute to membrane potential oscillations and may play an important role in the pathogenesis of neuropathic pain (Amir et al. 1999, 2002; Kapoor et al. 1997). In support, we noted a significantly longer duration of the action potential in both LS and TL neurons from CYP-treated rats, suggesting
changes in expression/properties of TTX-resistant and/or A-type potassium channels.

In contrast to control conditions, a greater proportion of TL bladder neurons responded to purinergic agonists after bladder inflammation. Considering the important role of ATP in the regulation of micturition, the present results may provide an explanation for the increased contribution of splanchnic bladder afferents in conveying sensory information during cystitis (Mitsui et al. 2001). In both TL and LS neurons, we observed an increase in peak current density in response to purinergic stimulation, consistent with a reported increase in the peripheral purinergic component in a model of colitis (Wynn et al. 2004). We could not quantify P2X proteins because only ~5-6% of LS DRG neurons are bladder neurons, but it is likely that the increase in peak current density is due to an increase in the expression of purinergic receptors. First, experiments were performed 3 – 10 h after removal and dissociation of DRG neurons. Therefore, G-protein-mediated effects (e.g., changes in phosphorylation status of the channels) cannot explain differences in currents between cells obtained from controls and animals with cystitis, as these effects would have reversed within this time frame. Second, we noted an increase in the proportion of TL neurons responding to purinergic agonists. Together with immunohistochemical evidence showing a significant increase in the proportion of TL neurons immuno-positive for P2X₃, this suggests de novo synthesis of P2X receptors in neurons that do not normally express P2X receptors. Finally, the increase in peak current density was associated with an increase in the maximum current rather than a change in
the EC\textsubscript{50}. Consistent with this interpretation, others have demonstrated an increase in P2X\textsubscript{3} receptor protein after inflammation of the hind limb footpad (Xu and Huang 2002), presumably due to upregulation of P2X receptor transcripts by NGF (Ramer et al. 2001), which is also increased after bladder inflammation (Lamb et al. 2004; Vizzard, 2000). We did not directly examine input resistance of bladder neurons following CYP treatment. However, Yoshimura and De Groat (1999) previously demonstrated an increase in input resistance associated with a reduction in A-type potassium current in bladder neurons following CYP treatment. They concluded that a reduction in A-type potassium current contributes to bladder neuron hyperexcitability following CYP treatment. The present report provides evidence that CYP treatment increases P2X currents, which contribute, in part, to the hyperexcitability of bladder neurons.

Current kinetics and pharmacologic data suggest that the slow desensitizing current (seen predominantly in LS neurons) is primarily due to activation of heteromeric P2X\textsubscript{2/3} receptors, while the fast desensitizing current (predominant in TL neurons) is mediated by homomeric P2X\textsubscript{3} receptors (for a more detailed discussion, see Dang et al. 2005a and Zhong et al. 2003). Both purinergic agonists triggered only a slow desensitizing current in LS neurons from CYP-treated rats, whereas ATP and α,β-metATP produced primarily mixed and fast desensitizing currents in TL counterparts, respectively, suggesting a differential regulation of P2X receptor expression after bladder inflammation. Because the maximum slow desensitizing current activated by ATP relative to α,β-metATP was reduced in bladder LS neurons after bladder inflammation, the
present data suggest an increased expression of heteromeric P2X_{2/3} receptors and further demonstrate the dominance of heteromeric P2X_{2/3} receptors in LS neurons after bladder inflammation. In contrast, after inflammation, significantly more TL neurons expressed only a fast desensitizing current in response to α,β-metATP (compared with ATP), and the current was inhibited by both TNP-ATP and A317491. These results suggest that bladder inflammation upregulates the expression of homomeric P2X_{3} channels in TL neurons, consistent with an increase in the number of P2X_{3}-immunoreactive TL cells and a report by Wynn et al. (2004) that DRG neurons which normally express CGRP, but not P2X_{3} receptors, also expressed P2X_{3} receptors after colon inflammation. Furthermore, because α,β-metATP activated significantly fewer TL neurons that express mixed desensitizing currents after inflammation compared with control, the present findings suggest that heteromeric P2X_{2/3} receptors are downregulated in some TL neurons after bladder inflammation. Because heteromeric P2X_{2/3} receptors desensitize significantly slower and recover faster than do homomeric receptors, we suggest that purinergic signals are predominantly transmitted through the pelvic rather than the splanchnic pathway in the presence of ATP, especially in cystitis in which ATP release is increased substantially (Sun et al. 2001).

As already discussed in the context of changes in peak current density, G-protein-mediated changes in phosphorylation status are unlikely to account for the slower desensitization kinetics observed in LS neurons from CYP-treated rats. Rather, increases in the expression of other P2 receptors may slow desensitization kinetics for the slow desensitizing current. In support of this,
increasing the proportion of $P2X_2$ relative to $P2X_3$ receptor expression progressively slows the desensitizing kinetics of the slow desensitizing current in a heterologous expression system (Liu et al. 2001).

In summary, bladder inflammation produces hyperexcitability and sensitizes bladder neurons in both the pelvic and splanchnic pathways. This hypersensitivity is associated with an increased expression and/or properties of homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors in TL and LS bladder neurons, respectively. The altered $P2X$ receptor expression accounts for greater current density in response to purinergic agonists, and likely contributes to the slowed desensitization kinetics of the slow desensitizing current after bladder inflammation. Considering the reported mechanosensory role for homomeric $P2X_3$ and $P2X_2$ and heteromeric $P2X_{2/3}$ receptors (Cockayne et al. 2000, 2005; Rong et al. 2002; Vlaskovska et al. 2001), and the increased urothelial release of ATP during inflammation (Sun et al. 2001), the present findings suggest that the altered expression of $P2X$ receptors contributes to the enhanced responses of bladder neurons during cystitis.
References


Acknowledgements

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Figure legends

Fig. 1: Distribution of whole-cell capacitance in lumbosacral (LS) and thoracolumbar (TL) bladder sensory neurons from control and CYP-treated rats. No cells had a capacitance less than 20 pF in TL DRGs, whereas significantly fewer LS than TL neurons had a capacitance greater than 60 pF. Bladder inflammation significantly increased the mean capacitance of both LS and TL neurons. Control LS bladder neurons labeled with fast blue (FB) showed a similar distribution of capacitance compared with Dil labeled cells.

Fig. 2: Passive and active properties of bladder neurons from control and CYP-treated rats examined in current clamp mode. The examples illustrate that neither LS (A; left trace) nor TL (F; left trace) neurons were spontaneously active in the absence of bladder inflammation, but about one-third of bladder neurons from CYP-treated rats exhibited spontaneous activity (A and F, right traces). Bladder inflammation reduced the rheobase current for action potential (AP) generation in both LS and TL bladder neurons (B and G, respectively) and increased the width of the AP. C and H show in the same cells that current injection (500 ms) produced significantly more APs after bladder inflammation. Similarly, ATP (D and I) and α,β-metATP (E and J) depolarized LS and TL bladder neurons, and produced greater depolarizations and more APs in cells from CYP-treated rats.

Fig. 3: Purinergic agonist-activated inward currents in bladder sensory neurons were characterized based on desensitization kinetics as fast (A and D), slow (B and E) and mixed (C and F). To illustrate best exponential fits, we expanded the magnitude and time scale of records (e.g., C, left trace) and fitted with either a
single or double exponential \((C^\prime)\). In neurons with mixed current kinetics, a double exponential always produced the best fit. In both LS and TL bladder neurons, the P2X receptor subtype-selective agonist \(\alpha,\beta\text{-metATP}\) produced currents with similar kinetics to those triggered by ATP, but with significantly less current than did ATP in the same cells (right corresponding traces of each pair).

In LS bladder neurons, the slow desensitizing current predominated (87% of 52 cells), whereas mixed currents were more common in TL neurons (52% of 27 cells). In cells from CYP-treated rats, both ATP and \(\alpha,\beta\text{-metATP}\) produced greater magnitude, but only slow desensitizing currents in LS neurons \((B^\prime)\). Bladder inflammation significantly increased the number of TL bladder neurons that responded to these purinergic agonists (see Table 2) and fast \((D^\prime)\), slow \((E^\prime)\) and mixed \((F^\prime)\) desensitizing currents were all greater in magnitude relative to control.

**Fig. 4:** Dose-response relationship for control slow \((A)\) and fast \((B)\) desensitizing currents produced by ATP \((\bullet)\) and \(\alpha,\beta\text{-metATP}\) \((\circ)\), plotted as current density \((\text{pA/pF})\). ATP is significantly more potent than \(\alpha,\beta\text{-metATP}\) for both kinetically distinct currents. Bladder inflammation did not alter the EC\textsubscript{50}s for the slow \((\square)\) or fast \((\circ)\) desensitizing current (see text). When current densities were plotted against agonist concentrations using the same control cells, ATP \((\bullet)\) produced a significantly greater current density than \(\alpha,\beta\text{-metATP}\) \((\circ)\) for the slow \((A)\) but not fast \((B)\) desensitizing current. After bladder inflammation, ATP \((\square)\) and \(\alpha,\beta\text{-metATP}\) \((\circ)\) produced significantly greater current densities for both the slow \((A)\) and fast \((C)\) desensitizing currents. To directly compare the fraction of slow and
fast desensitizing current produced by α,β-metATP relative to ATP, we applied maximal concentrations of ATP (300 µM) or α,β-metATP (300 µM) to the same cells. ATP and α,β-metATP produced similar current amplitudes for the fast desensitizing current (examples in D and E, expanded time scale), but ATP produced 58±3.8 % (n = 11) more current than did α,β-metATP for the slow desensitizing current (F; left traces). In cells from CYP-treated rats, the magnitude of the slow desensitizing current produced by ATP and α,β-metATP was increased and ATP produced significantly less current difference (24.1±2 %; n = 9; C, right traces).

Fig. 5: Antagonism of α,β-metATP-produced currents in neurons from CYP-treated rats. The examples illustrate about 75% antagonism of the slow current by TNP-ATP (100 nM) (A, left traces), but partial antagonism by A317491 (10 µM) (A, right traces) in the same cell. Both antagonists abolished the fast desensitizing current in the same cell (B) at a concentration of 100 nM, which for A317491 is a significantly lower concentration than the incomplete antagonism of the slow desensitizing current (A, right). α,β-metATP-produced currents recovered completely after washout of antagonists.

Fig. 6: Immunohistochemistry of bladder neurons from CYP-treated and control rats. Fluorogold (FG; left column) was used to retrogradely label LS and TL bladder neurons (arrows), which were counterstained with P2X3 antibody (middle column). Merging the left and middle columns confirmed that bladder sensory neurons were immunoreactive for P2X3 (right column; arrows indicate colocalization).
Table 1: Passive and active electrical properties of LS and TL bladder neurons from CYP-treated and control rats.

<table>
<thead>
<tr>
<th></th>
<th>LS</th>
<th>TL</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP (mV)</td>
<td>control</td>
<td>CYP</td>
</tr>
<tr>
<td></td>
<td>-55.02 ± 2.1</td>
<td>-47.78 ± 1.0*</td>
</tr>
<tr>
<td></td>
<td>-55.34 ± 1.6</td>
<td>-49.61 ± 1.3</td>
</tr>
<tr>
<td>spontaneous activity</td>
<td>control</td>
<td>CYP</td>
</tr>
<tr>
<td></td>
<td>0/23 (0 %)</td>
<td>0/23 (0 %)</td>
</tr>
<tr>
<td></td>
<td>11/31 (35 %)†</td>
<td>13/29 (45 %)†</td>
</tr>
<tr>
<td>rheobase (pA)</td>
<td>control</td>
<td>CYP</td>
</tr>
<tr>
<td></td>
<td>146.1 ± 14.5</td>
<td>95.7 ± 7.5*</td>
</tr>
<tr>
<td></td>
<td>66.2 ± 6.2†</td>
<td>50.6 ± 5.5†</td>
</tr>
<tr>
<td>rheobase (pA/pF)</td>
<td>control</td>
<td>CYP</td>
</tr>
<tr>
<td></td>
<td>5.67 ± 0.7</td>
<td>2.54 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td>2.28 ± 0.3†</td>
<td>1.14 ± 0.2†</td>
</tr>
<tr>
<td>AP threshold (mV)</td>
<td>control</td>
<td>CYP</td>
</tr>
<tr>
<td></td>
<td>-24.5 ± 1.4</td>
<td>-23.3 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>-30.1 ± 0.8†</td>
<td>-28.2 ± 1†</td>
</tr>
<tr>
<td>AP amplitude (mV)</td>
<td>control</td>
<td>CYP</td>
</tr>
<tr>
<td></td>
<td>87.9 ± 4.1</td>
<td>87.4 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>93.4 ± 2.2</td>
<td>89.9 ± 3</td>
</tr>
<tr>
<td>AP duration (ms)</td>
<td>control</td>
<td>CYP</td>
</tr>
<tr>
<td></td>
<td>3.1 ± 0.2</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>4.2 ± 0.3†</td>
<td>5.0 ± 0.6†</td>
</tr>
<tr>
<td>rheobase X 2 (APs)</td>
<td>control</td>
<td>CYP</td>
</tr>
<tr>
<td></td>
<td>5 ± 0.9</td>
<td>6.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>10.7 ± 2.1†</td>
<td>9.2 ± 0.7†</td>
</tr>
<tr>
<td>ATP (100 µM)</td>
<td>depolarization (mV)</td>
<td>control</td>
</tr>
<tr>
<td></td>
<td>24.5 ± 1.5</td>
<td>6.2 ± 1.2*</td>
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<tr>
<td></td>
<td>31.9 ± 1.5†</td>
<td>14.1 ± 1.2†</td>
</tr>
<tr>
<td>number of APs</td>
<td>control</td>
<td>CYP</td>
</tr>
<tr>
<td></td>
<td>8.9 ± 0.7</td>
<td>3.7 ± 1.4*</td>
</tr>
<tr>
<td></td>
<td>15.2 ± 2.1†</td>
<td>13.5 ± 3.6†</td>
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<tr>
<td>αβ-metATP (100 µM)</td>
<td>depolarization (mV)</td>
<td>control</td>
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<td></td>
<td>15.3 ± 1.4</td>
<td>5.4 ± 1.1*</td>
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<tr>
<td></td>
<td>25.6 ± 1.3†</td>
<td>13.2 ± 1.2†</td>
</tr>
<tr>
<td>number of APs</td>
<td>control</td>
<td>CYP</td>
</tr>
<tr>
<td></td>
<td>5.2 ± 0.7</td>
<td>2.4 ± 1.3*</td>
</tr>
<tr>
<td></td>
<td>12.9 ± 0.9†</td>
<td>10.3 ± 1.4†</td>
</tr>
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*, P < 0.05 vs. LS counterparts; †, P < 0.05 vs. control (saline) treatment.
Table 2: Purinergic agonist produced current densities for LS and TL bladder sensory neurons from CYP-treated and control rats.

<table>
<thead>
<tr>
<th></th>
<th>LS neurons</th>
<th>TL neurons</th>
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<tr>
<td></td>
<td>% responding</td>
<td>current density (pA/pF)</td>
</tr>
<tr>
<td>ATP</td>
<td>control CYP</td>
<td>96 % (53/55)</td>
</tr>
<tr>
<td></td>
<td>CYP</td>
<td>97 % (59/61)</td>
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<tr>
<td>fast current</td>
<td>control CYP</td>
<td>5.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>CYP</td>
<td>n/a</td>
</tr>
<tr>
<td>slow current</td>
<td>control CYP</td>
<td>67.8 ± 11</td>
</tr>
<tr>
<td></td>
<td>CYP</td>
<td>116.2 ± 12†</td>
</tr>
<tr>
<td>mixed current</td>
<td>control CYP</td>
<td>6.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>CYP</td>
<td>n/a</td>
</tr>
<tr>
<td>% responding</td>
<td>control CYP</td>
<td>95 % (52/55)</td>
</tr>
<tr>
<td></td>
<td>CYP</td>
<td>92% (56/61)</td>
</tr>
<tr>
<td>slow current</td>
<td>control CYP</td>
<td>28.4 ± 4</td>
</tr>
<tr>
<td></td>
<td>CYP</td>
<td>51.5 ± 5†</td>
</tr>
<tr>
<td>mixed current</td>
<td>control CYP</td>
<td>2.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>CYP</td>
<td>n/a</td>
</tr>
</tbody>
</table>

*, P < 0.05 between LS and TL neuron groups; †, P < 0.05 between control and CYP treatment. na, not applicable.
Table 3: Current kinetics for LS and TL bladder sensory neurons from CYP-treated and control rats.

<table>
<thead>
<tr>
<th>current</th>
<th>treatment</th>
<th>ATP (30µM)</th>
<th>αβ-metATP (30µM)</th>
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<tr>
<td></td>
<td></td>
<td>10-90% (ms)</td>
<td>tau 1 (ms)</td>
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<tr>
<td>LS</td>
<td>fast</td>
<td>control</td>
<td>22.9 ± 4</td>
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<td></td>
<td></td>
<td>CYP</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>slow</td>
<td>control</td>
<td>226.7 ± 38</td>
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<tr>
<td></td>
<td></td>
<td>CYP</td>
<td>125.2 ± 21†</td>
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<tr>
<td></td>
<td>mixed</td>
<td>control</td>
<td>26.7 ± 3</td>
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<tr>
<td></td>
<td></td>
<td>CYP</td>
<td>n/a</td>
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<tr>
<td>TL</td>
<td>fast</td>
<td>control</td>
<td>22.7 ± 2</td>
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<td></td>
<td></td>
<td>CYP</td>
<td>19.6 ± 3</td>
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<td></td>
<td>slow</td>
<td>control</td>
<td>258 ± 48</td>
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<td></td>
<td></td>
<td>CYP</td>
<td>212 ± 19</td>
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<tr>
<td></td>
<td>mixed</td>
<td>control</td>
<td>27.6 ± 3</td>
</tr>
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<td></td>
<td></td>
<td>CYP</td>
<td>24.3 ± 2</td>
</tr>
</tbody>
</table>

10-90% rise time; †, P < 0.05 vs. the corresponding control group