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Differential purinergic signaling in bladder sensory neurons of naïve and bladder inflamed mice

Xiaowei Chen and G.F. Gebhart

Center for Pain Research, Department of Anesthesiology University of Pittsburgh, Pittsburgh, PA 15213

Abstract

This study explored purinergic signaling in lumbosacral (LS) and thoracolumbar (TL) dorsal root ganglion neurons innervating the urinary bladder. In naïve mice, a greater proportion of LS (93%) than TL (77%) bladder neurons responded to purinergic agonists. Three types of purinergic currents were identified: 'sustained' (homomeric P2X2) currents were detected only in LS neurons, rapidly activating, 'slow' deactivating (heteromeric P2X2/3) currents predominated in both LS and TL neurons, and 'fast' activating/deactivating (homomeric P2X3) currents were detected only in TL neurons. Relative to TL bladder neurons, slow current density was greater in LS neurons, which also had a more negative action potential threshold and generated more action potentials in response to purinergic agonists (suggesting greater excitability of LS neurons). Single cell nested PCR documented P2X2 and P2X3 subunit expression in both TL and LS bladder neurons.

Relative to saline treatment, bladder wall thickness and weight increased after cyclophosphamide (CYP) treatment. Both LS and TL neuron excitability increased (rheobase was decreased and responses to purinergic agonists increased) after CYP treatment. The proportion of sustained currents in LS bladder neurons increased four-fold after CYP bladder inflammation. Although proportions of slow and fast purinergic currents in TL neurons were unchanged by CYP treatment, the fast current density was greater than in saline-treated mice. These results in mouse, as previously described in rat, reveal differential purinergic signaling in TL and LS bladder neurons. The predominant currents and significant changes after inflammation, however, occur in different ganglia/sensory pathways in mouse and rat.

Keywords

bladder; purinergic; P2X; ATP; cyclophosphamide; inflammation; single cell PCR; patch clamp

Introduction

A unique feature of visceral innervation is that each organ is innervated by two nerves [18]. The different nerves have some similar, but also different functions. For example, the

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Correspondence: G.F. Gebhart, PhD Center for Pain Research W1444 BST 200 Lothrop St. Pittsburgh, PA 15213 gebhartgf@upmc.edu
Ph: 412-383-5911 Fax: 412-383-5466.

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mechanosensitivity and location of receptive endings of the pelvic and lumbar splanchnic innervations of the urinary bladder [51] and colon [6] in the mouse have been directly compared and documented as significantly different. Other studies that have examined cell bodies in dorsal root or nodose ganglia of different nerves innervating the same organ have similarly revealed significant differences in gastric [15,36], airway [44,22] and urinary bladder [14] sensory neurons. The importance of these findings relates to resolution of potential mechanisms that may underlie functional visceral disorders (e.g., irritable bowel syndrome, painful bladder syndrome/interstitial cystitis [PBS/IC], etc.), all of which are characterized by discomfort and pain in the absence of gross pathology.

In addition, PBS/IC is further characterized by urge and increased urination frequency and can lead to chronic pelvic pain [7,28,41]. Among potential endogenous mediators of bladder discomfort and pain, adenosine triphosphate (ATP) has been identified as important. ATP is released from bladder urothelium during distension or chemical stimulation [17,3] and release is increased in PBS/IC patients [37,38]. Purinergic ionotropic (P2X) receptors have been implicated in bladder overactivity and disorders of sensation in humans [23,30,40] and animals [16,52]. In addition, P2X2, P2X3 and P2X2/P2X3 double knockout mice exhibit reduced bladder reflexes and decreased afferent nerve activity in response to bladder distension [10,9,48].

Sensory information from the bladder is conveyed to the spinal cord via lumbar splanchnic and pelvic pathways [45,42,43]. The cell bodies of these afferent pathways are located, respectively, in thoracolumbar (TL) and lumbosacral (LS) dorsal root ganglia [1,2]. In the present report, we characterized the purinergic sensitivity of the lumbar splanchnic and pelvic innervations of the mouse urinary bladder by study of their cell bodies in TL and LS dorsal root ganglia in the absence and presence of urinary bladder inflammation (produced by systemic administration of cyclophosphamide; [4]).

Methods

Animals

Male C57BL/6 mice (6-8 weeks; Taconic Labs, Germantown, NY) were used for most experiments; P2X3 knockout mice generated on a 129Ola $\times$ C57BL/6 genetic background were also used [10]. P2X3 knockout mice were obtained from The Jackson Laboratory courtesy of Debra A. Cockayne, Roche Bioscience, Palo Alto, CA. Mice were housed in polypropylene cages with *ad libitum* access to food and water. All protocols were reviewed and approved by the Institutional Animal Care & Use Committee, the University of Pittsburgh.

Bladder neuron retrograde labeling and bladder inflammation

Mice were anesthetized with 2% inhaled isoflurane (Hospira Inc., Lake Forest, IL), the bladder exposed via a lower abdominal incision ~5 mm in length and 10 μ l of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiIC₁₈(3); Molecular Probes, Eugene, OR) in 0.2mg/ml in DMSO was injected into 3-4 sites within the bladder wall and the base around the trigone using a 30 gauge needle. Sterile cotton-tipped applicators were applied to injection sites to absorb any DiI that leaked from injection sites. The incision was closed (6.0 silk, Ethicon Inc., Somerville, NJ) and post-operative analgesia provided after surgery (buprenorphine, 0.05mg/kg, i.p.; Bedford labs, Bedford, OH). Two weeks after retrograde labeling, mice were treated intraperitoneally either with saline or cyclophosphamide (CYP; 100 mg/kg, dissolved in saline; Sigma-Aldrich Inc., St. Louis, MO) daily for 5 days; mice were sacrificed by CO₂ inhalation on day 6 and dorsal root ganglia (DRG) were removed. Systemic administration of CYP, which is metabolized to the bladder irritant acrolein [13], can cause hemorrhagic cystitis in humans as an adverse event and produces a cystitis-like condition in rodents (e.g., [4]).

Cell dissociation and electrophysiological recordings

Lumbosacral (LS; L6-S2) and thoracolumbar (TL; T13-L2) DRG were harvested for electrophysiological whole cell recordings. After dissection of DRG, the pelvic area around the bladder was examined for evidence of DiI leakage; leakage was rarely noted and DRG from mice with evidence of leakage were not used. LS and TL DRG were incubated at 37°C, 5% CO₂ for 45 min in DMEM media containing 1% penicillin/streptomycin (Invitrogen Corp., Grand Island, NY), 1 mg/ml type 4 collagenase and 1 mg/ml neutral protease (Worthington Biochemical Corp., Lakewood, NJ). Tissue was gently triturated and collected after 5 min centrifugation at 120 X g, washed 3 times and re-suspended in DMEM media containing 0.5 mM L-glutamine, 1% penicillin/streptomycin and 10% fetal bovine serum. The cells were plated on poly-D-lysine-coated coverslips (Becton Dickinson Labware, Bedford, MA) and incubated at 37°C, 5% CO₂ for 14-16 hrs. Only DiI positive bladder sensory neurons were studied. All recordings were performed within 24 hours after plating.

Coverslips were transferred to a recording chamber perfused with an external solution (in mM) consisting of NaCl 140, KCl 5, MgCl₂ 1, CaCl₂ 2, glucose 10, and HEPES 10 at pH 7.4, 310 mOsm. Glass micropipette tips were heat-polished to resistances ~ 2-3 MΩ and filled with an internal solution (in mM) consisting of KCl 130, NaCl 4, CaCl₂ 0.2, HEPES 10, EGTA 10, MgATP 2, and NaGTP 0.5 at pH 7.25, 300 mOsm. After establishing the whole-cell configuration, membrane voltage was clamped at -70 mV using an Axopatch 200B amplifier, digitized at 1 kHz (Digidata 1350), and controlled by Clampex software (pClamp 9) (all from Axon Instruments, Union City, CA). Cell capacitance was obtained by reading the value from the Axopatch 200B amplifier. In current clamp experiments, only neurons that had a resting membrane potential more negative than -50 mV and a distinct action potential overshoot >0 mV were studied.

Drugs were applied through a 3-barrel glass pipette placed close (~100 μm) to the cell using a fast-step SF-77B superfusion system (Warner Instruments, Hamden, CT). Agonists were applied for 4 sec at an interval of 2 min; antagonists were superfused for 30 sec before the application of agonists. Drugs were obtained from Sigma-Aldrich, Inc. and prepared fresh from stock solutions on the day of the experiment. All experiments were performed at room temperature (21–23°C).

Single cell RT- PCR

Bladder DRG neurons were retrogradely labeled and mice sacrificed as described above. LS and TL DRG were removed and plated as described above for electrophysiological study. After 14-16 hr incubation, coverslips were placed in the patch clamp recording chamber and perfused with sterilized external solution. DiI positive neurons were collected with glass pipettes (tip diameter ~ 60-80 μm) by gentle suction and expelled into 0.2 ml microcentrifuge tubes containing 2.5 μl lysis buffer consisting of 1X first-strand buffer, 2U RNaseOut, 10 μM dNTP, 0.5% IGEPAL, and 0.05 μg/μl Oligo(dT)12-18 primer. Tubes were incubated at 65°C for 1.5 minutes and held at room temperature for 2 min. Another 2.5 μl of RT-PCR buffer consisting of 50 U SuperScript II reverse transcriptase, 1X first-strand buffer, 2U RNaseOut, and 10 μM dNTP was rapidly added to each tube. Tubes were incubated at 37°C for 20 min, then at 65°C for 10 min to generate first strands of cDNA sequence. Negative controls were tubes without labeled neurons or processed with RT-PCR buffer not containing reverse transcriptase. Only RT-PCR products of the batches that passed tests of negative controls underwent further PCR steps. All single cell RT-PCR reagents were purchased from Invitrogen Corp.

Multiplex PCR and gene specific nested PCR

P2X2 and P2X3 genes were amplified through two rounds of PCR (multiplex and nested PCR) from the cDNA library of individual mouse bladder neurons. In the first round, two external

primers of targeted genes were added together into a 25 μ l volume PCR solution containing 1X PCR buffer, 0.2mM dNTP, 1.6 μ M primers of each gene and 1U Taq Polymerase. In the second round, 1 μ l of first round PCR amplicons served as a template and two internal primers of individual genes were added to the 25 μ l PCR system. Both multiplex and nested PCR used the following PCR conditions: 1 cycle of 10 min at 95 °C; 32 cycles of 94 °C/30 sec, 52°C/30 sec, and 72 °C/30 sec before a final extension step at 72 °C for 10 min, after which 10 μ l of the nested PCR products was electrophoresed on a 2% (w/v) agarose gel at 100V for 25 min. After electrophoresis, the gel was stained with 0.005% ethidium bromide and bands of PCR products were visualized under UV light. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal positive control. Single cell RT-PCR products that failed to pass internal positive tests were eliminated from the study. All primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and are listed in table 1.

Bladder histology and myeloperoxidase (MPO) assay

Urinary bladders were removed from CYP- and saline-treated mice deeply anesthetized with 5% inhaled isoflurane. Each bladder was longitudinally cut into two parts, one part for histological examination and the other for MPO assay. For histological evaluation, bladder tissue was fixed in 4% formaldehyde, embedded in paraffin and cut at 10 μ m thickness. Tissue sections were stained with 5% hematoxylin and eosin and evaluated by a pathologist (R. H. Garman, DVM, DACVP, Consultants in Veterinary Pathology, Inc.). For the MPO assay, bladder tissue was rinsed with saline, patted dry and homogenized in ice-cold 50 mM phosphate buffer containing 0.5% hexadecyltrimethyl-ammonium bromide. After three repeated freeze (dry ice) - thaw (37°C water bath) cycles, samples were centrifuged at room temperature for 1min at 200 X g. Absorbance of the supernatant (10 μ l diluted to 300 μ l with an indicator solution containing 0.05% hydrogen peroxide and 0.5% o-dianisidine dihydrochloride) was measured photometrically (SpectraMax Plus 384, Molecular Devices,). All drugs were purchased from Sigma-Aldrich, Inc.

Data analysis

We used Graphpad Prism 5 (Graphpad Software, San Diego, CA) to perform statistical analyses. Data are presented as mean $\pm$ SEM. Comparisons were made using Student's t-test for parametric measures of LS and TL bladder neurons or saline vs. CYP treatment groups. For dichotomous variables, we used Fisher's exact test. Results were considered statistically significant when $P < 0.05$.

Results

Cell density and size distribution of bladder sensory neurons

To estimate the proportion of bladder sensory neurons contained in LS and TL DRG, we randomly chose 4 LS DRG and 4 TL DRG coverslips from 4 mice. Each coverslip was divided into four quadrants. Numbers of DiI-labeled cells and total cells were counted in a randomly selected viewing field (10X objective) in each quadrant. DiI-labeled cells represented $6.0 \pm 0.4\%$ (77/1276) of L6-S2 DRG cells, a proportion significantly greater than the $2.4 \pm 0.2\%$ (40/1635) of DiI-labeled T13-L2 DRG cells. These proportions do not differ from similar data collected in the rat [14].

Unlike in the rat, however, where TL bladder neuron capacitance was significantly greater than LS bladder neuron capacitance, mean whole cell capacitance (as an index of cell size) did not differ between mouse LS (31.1 ± 1.0 pF) and TL (32.3 ± 1.1 pF) bladder sensory neurons. The distributions of cell size (supplemental Fig 1) also did not differ; most cells ($\sim 70\%$) were medium in size (20-45 pF), as in the rat [14]. In the rat, the proportion of small sized (< 20 pF)

bladder sensory neurons was significantly greater and the number of large sized (>45 pF) cells significantly less in LS than TL DRG ([16]; Fig S1 presents data for the mouse).

Characterization of bladder sensory neuron responses to purinergic receptor agonists

A total of 205 bladder sensory neurons from naïve mice were studied. ATP (30 μ M) was applied to cells as a non-selective P2X receptor agonist; α,β -methylene ATP (α,β -met ATP, 30 μ M) was employed as a P2X3 receptor-selective agonist (homomeric P2X3 and heteromeric P2X2/3). Agonists were applied for 4 sec at 2 min intervals. Overall, 93.0% (106/114) of LS and 76.9% (70/91) of TL bladder neurons responded to ATP (LS vs TL, $P < 0.05$); 83.3% (95/114) of the same LS and 76.9% (70/91) of the same TL bladder neurons also responded to α,β -met ATP (LS vs TL, $P < 0.05$) (Table 2). Among purinoceptive bladder neurons, the overwhelming majority of LS (89.6%, 95/106) and TL (74.3%, 52/70) (Table 2) neurons exhibited slowly desensitizing currents in response to both ATP and α,β -met ATP (examples given in Fig. 1A, C). The remaining LS bladder neurons (10.4%, 11/106) demonstrated a sustained current without an obvious desensitizing phase during agonist application. Sustained currents in LS bladder neurons were evoked only by ATP and not by α,β -met ATP (example in Fig. 1B). Neither ATP nor α,β -met ATP generated sustained currents in TL bladder sensory neurons; instead, both agonists produced rapidly activating, rapidly inactivating fast currents (25.7%, 28/70, example in Fig. 1D).

Table 3 summarizes the properties of purinergic agonist-evoked currents in LS and TL bladder sensory neurons from naïve mice. The activation (time to peak) and desensitization (desensitizing time constant) kinetics of slow currents were not different between LS and TL bladder neurons. However, the current density (current amplitude normalized by whole cell capacitance) of the slow current in LS neurons was significantly greater than in TL neurons in response to ATP ($P < 0.01$) or α,β -met ATP ($P < 0.05$). A sustained current was produced only in LS neurons and a fast current was produced only in TL neurons, suggesting significant differences in P2X receptor subunit composition between LS and TL bladder neurons.

Characterization of P2X receptor subtypes in bladder sensory neurons

Seven ionotropic P2X receptor family subunits (P2X1 to P2X7) have been reported and cloned [29,39]. P2X2 and P2X3 subunits predominate in sensory neurons and form functional homomeric P2X2 receptors, heteromeric P2X2/3 receptors or homomeric P2X3 receptors, each of which has a distinct pharmacological character [19]. In unidentified rat DRG neurons, the predominant response evoked by purinergic agonists is transient and thought to be mediated by activation of homomeric P2X3 receptors. Slow desensitizing (*via* P2X2/3 receptors) and biphasic (*via* a mixture of P2X3 and P2X2/3 receptors) responses are less frequently observed [20].

To examine which P2X receptor subunit(s) mediates purinergic currents in mouse bladder TL and LS neurons, we employed the P2X1, P2X3 and P2X2/3 receptor-selective antagonist TNP-ATP (0.1 μ M) or the non-selective P2X receptor antagonist PPADS (10 μ M). TNP-ATP significantly attenuated the predominant slow current evoked by ATP (by a mean $57.2 \pm 2.8\%$ in TL and $54.6 \pm 2.4\%$ in LS neurons; an example is given in Fig 2A) or α,β -met ATP (by a mean $56.1 \pm 3.8\%$ in TL and $54.5 \pm 4.2\%$ in LS neurons). PPADS similarly attenuated the slow current evoked by ATP (by a mean $77.7 \pm 3.0\%$; an example is given in Fig 2B) or α,β -met ATP (by a mean $82.0 \pm 2.1\%$), revealing that these slow currents are mediated by heteromeric P2X2/3 receptors. Sustained currents evoked by ATP, present only in LS bladder neurons, were not affected by TNP-ATP (Fig 2C) but were significantly attenuated by PPADS (by a mean $82.0 \pm 5.6\%$; an example is given in Fig 2D), suggesting they are mediated by a homomeric P2X2 receptor. Fast currents, present only in TL bladder neurons, are usually associated with

homomeric P2X3 receptors that we confirmed using TNP-ATP (an example is given in Fig 2E).

To further evaluate these currents, we studied LS and TL bladder neurons taken from P2X3 knockout mice. No slow or fast currents were produced in LS or TL neurons by either purinergic agonist; only a sustained current was produced (examples in Fig. 3), consistent with what has been previously reported [9]. Ten of 12 (83.3%) LS bladder neurons responded to ATP with a sustained current (Fig 3A); none responded to α,β -met ATP. TL bladder neurons (n=8) did not respond to either ATP or α,β -met ATP (Fig 3B). The presumptive P2X2 homomeric sustained current produced by ATP in LS bladder neurons from P2X3 knockout mice was attenuated by PPADS, but not TNP-ATP as were bladder neurons from C57BL/6 mice (examples are given in Fig 3C, D). These outcomes confirm that activation of homomeric P2X2 receptors is responsible for the sustained ATP-produced current in LS bladder neurons whereas purinergic currents in TL bladder neurons are mediated through heteromeric P2X2/3 and homomeric P2X3 receptors.

Electrophysiological properties of LS and TL bladder sensory neurons

We also examined the active and passive membrane properties of 32 LS and 30 TL bladder neurons from naïve mice by injecting currents and applying agonists in whole-cell current clamp mode (Table 4). Internal resistance was calculated according to the *I/V* relationship by injecting a series of hyperpolarizing pulses (−300 to 0 pA, 50 ms) in 50pA increments. To determine rheobase, a series of 20 ms current pulses in 20pA increments (1s apart) was injected. The maximum current (pA) that did not evoke an action potential was taken as rheobase. Action potential (AP) threshold was determined from the inflection point where membrane potential started to dramatically rise, AP amplitude was measured from peak RMP to the peak of the AP, AP overshoot was the amplitude from 0 mV to the peak of the AP, AP duration was determined at 50% of the AP amplitude and the AP falling rate was the velocity of change in potential from the AP peak to RMP.

LS and TL bladder neurons from naïve mice did not differ in any membrane property with the exception of AP threshold. LS neurons had a significantly more negative mean AP threshold (-34.1 ± 0.5 mV) than TL neurons (-28.5 ± 0.8 mV, $P < 0.01$; Table 4), suggesting that LS neurons are generally more easily excited. When 30 μ M ATP was applied, a significantly greater proportion of LS neurons fired APs than did their TL counterparts (LS: 21/32, 67.5%; TL: 10/30, 33.3%, $P < 0.05$), suggesting that LS bladder neurons are more sensitive to purinergic agonists at the concentration tested. Spontaneous activity was not observed in either LS or TL bladder neurons before or after agonist application.

Bladder inflammation and tissue damage after CYP treatment

Bladder sensations and fullness are perceived in PBS/IC subjects at lower intravesical volumes, resulting in a leftward shift of the normal psychophysical function (i.e., bladder hypersensitivity; [27]). CYP treatment produces in mice comparable bladder hypersensitivity ([4]), previously documented in the rat to be associated with significant changes in purinergic signaling in bladder sensory neurons [16,21,52]. We thus used CYP in these studies to produce a mild bladder inflammation to evaluate changes in purinergic signaling in mouse bladder sensory neurons

Relative to saline-treated controls, CYP-treated bladders generally had thick walls accompanied by visibly decreased lumen volume. Mean bladder weight after CYP treatment was significantly greater (38.6 ± 1.3 mg) than bladders taken from saline-treated mice (23.7 ± 0.7 mg, $P < 0.005$). Histological examination of bladders from CYP-treated mice revealed mild submucosal edema and unfolding of the urothelium, neither of which was apparent in bladders

from saline-treated mice (supplemental Fig 2). Despite the histological insult produced by CYP, myeloperoxidase activity did not differ between bladders from CYP- and saline-treated mice (data not shown).

Bladder sensory neuron excitability is increased after CYP treatment

As above, active and passive membrane properties of LS and TL bladder neurons were determined in current clamp mode in cells taken from CYP- and saline-treated mice. As presented in Table 4, rheobase was significantly lower in both LS ($P<0.05$) and TL ($P<0.05$) bladder neurons from CYP-treated relative to saline-treated mice (examples given in Fig 4A-D). The magnitude of membrane depolarization produced by α,β -met ATP (but not by ATP) was significantly increased in LS and TL bladder neurons after CYP treatment (examples given in Fig 4E-H). No bladder neurons from CYP-treated mice exhibited spontaneous activity.

P2X receptor mediated currents are changed after CYP treatment

As presented in Table 2, 93.3% of LS bladder neurons from CYP-treated mice responded to ATP, similar to LS neurons from saline-treated mice (96.9%). However, the proportions of LS bladder neurons that exhibited sustained and slow currents to application of ATP were significantly different in CYP- treated relative to saline-treated mice. A four-fold greater proportion ($P<0.01$) of LS neurons from CYP-treated mice (42.9%) exhibited sustained currents than did LS neurons from saline-treated mice (9.7%). Correspondingly, the proportion of LS neurons from CYP-treated mice that gave slow currents (57.1%) was significantly reduced ($P<0.01$) relative to the 90.3% observed in saline-treated mice. α,β -met ATP evokes only slow currents in LS bladder neurons and the proportion of LS neurons from CYP-treated mice (53.3%) that responded to α,β -met ATP was also significantly decreased relative to saline-treated mice (87.5%; $P<0.01$). The activation and desensitization kinetics of the slow currents evoked by ATP/ α,β -met ATP were not different between neurons from saline- and CYP-treated mice. The current densities of both the sustained and slow currents in LS neurons from CYP-treated mice were slightly increased, but not significantly greater than those of saline controls (Table 5). These results suggest that the subunit composition of functional P2X receptors is altered by bladder inflammation, with a greater contribution made by homomeric P2X₂ receptors in pelvic nerve LS neurons after inflammation.

The effect of bladder inflammation on TL bladder neurons was less remarkable than on their LS counterparts. The proportions of TL bladder neurons that responded to either of the purinergic agonists were not significantly different between CYP- and saline-treated mice (Table 2). Although the proportions of responses did not differ, the current density of the fast current evoked by both agonists was greater in neurons from CYP-treated mice while current density of the slow response to agonists was significantly less in neurons from CYP-treated mice (both relative to saline-treated mice; Table 5).

P2X receptor expression in bladder sensory neurons

Because the numbers of bladder sensory neurons contained in TL and LS DRG are relatively few, we employed single cell RT-PCR and single cell nested PCR to examine P2X receptor expression in bladder sensory neurons. When cDNA was harvested after single cell RT-PCR, the mouse GAPDH gene was amplified by regular PCR as an internal control. Only cells having a thick band of GAPDH amplicon were further processed by nested PCR. Negative results of GAPDH amplification were thought to have an unsuccessful reverse transcription reaction and thus abandoned. The remaining bladder neurons were amplified by two rounds of PCR cycles with external primers, then internal primers, respectively. Figure 5A shows an example of positive single cell RT-PCR amplicons of P2X₂ and P2X₃ mRNA. Product length corresponded with the expected size of the targeted region. Each group consisted of 45 cells taken from 3 mice.

The P2X2 subunit transcript was more abundant in LS bladder neurons from saline-treated animals ($91.1\% \pm 2.2\%$) than in TL counterparts ($46.7\% \pm 6.7\%$; $P < 0.01$). After CYP treatment, the frequency of P2X2 transcript expression in TL bladder neurons significantly increased to $73.3\% \pm 6.7\%$ relative to saline-treated mice ($46.7\% \pm 6.7\%$; $P < 0.05$). The P2X3 subunit transcript was abundant in both LS (saline: $91.1\% \pm 5.8\%$; CYP: $83.7\% \pm 10.4\%$) and TL bladder neurons (saline: $93.3\% \pm 3.8\%$; CYP: 100%), and its expression was not affected by CYP treatment (Fig. 5B).

Figure 5C shows the proportions of LS and TL bladder sensory neurons that expressed only P2X2, only P2X3 or both P2X2 and P2X3 transcripts, representing neurons that express homomeric P2X2, homomeric P2X3 and heteromeric P2X2/3 receptors, respectively. Consistent with the purinergic-evoked currents in LS and TL neurons, P2X2 and P2X3 transcript co-expression was predominant in both LS (saline: $82.2\% \pm 2.2\%$; CYP: $76.8\% \pm 9.8\%$) and TL (saline: $51.1\% \pm 8.0\%$; CYP: $73.3\% \pm 6.7\%$) neurons. The proportions of LS bladder neurons expressing only P2X2 transcripts (saline-treated, $6.7\% \pm 3.8\%$; CYP-treated, $14.0\% \pm 7.3\%$) or only P2X3 transcripts (saline-treated, $4.5\% \pm 2.2\%$; CYP-treated, $4.6\% \pm 2.3\%$) were relatively low. No TL bladder neurons expressed only P2X2 transcripts, consistent with ATP-produced sustained currents only in LS bladder neurons. The proportions of TL bladder neurons expressing only P2X3 transcripts (saline: $49.0\% \pm 8.0\%$; CYP: $26.7\% \pm 6.7\%$) was significantly greater than their LS counterparts (both $P < 0.05$), consistent with agonist-produced fast currents only in TL bladder neurons. The proportion of co-expression of P2X2 and P2X3 transcripts in bladder neurons was not affected by CYP treatment.

Discussion

In the present study, we show that mouse bladder sensory neurons in the pelvic (LS) and splanchnic (TL) pathways – like their counterparts in the rat – exhibit (1) different responses to purinergic agonists based on kinetics of activation/inactivation and pharmacologic antagonism of the inward currents produced and (2) changes in those purinergic-evoked currents after bladder inflammation. Significantly, the predominant currents produced differ between the mouse and rat and the significant changes after bladder inflammation occur in different ganglia/sensory pathways in mouse and rat.

Differences between LS and TL bladder sensory neurons

Bladder afferents in pelvic and lumbar splanchnic pathways are both involved in chemo- and mechano-sensation, including nociception [1,24,25,33,32,35,26]. However, these bladder afferent pathways are differentially sensitive to mechanical and chemical stimuli [14,51] as well as in purinergic signaling, which may underlie the different functional aspects associated with these pathways.

Active and passive membrane properties of LS and TL bladder sensory neurons differed in two ways. The AP threshold of LS bladder neurons was significantly lower than in TL counterparts and a greater proportion of LS neurons generated APs in response to ATP than did TL neurons, suggesting that pelvic nerve bladder afferents may be more easily excitable by membrane depolarization or ATP (e.g., when released from urothelial cells) and therefore more sensitive to bladder filling. In support, a single fiber study of mouse bladder afferents found the proportion of stretch-sensitive afferents to be two-fold greater in the pelvic than lumbar splanchnic bladder innervation[51].

The predominant agonist-evoked current in both LS and TL bladder neurons was a P2X2/P2X3-mediated slow current. Unexpectedly, the P2X2-mediated sustained current was evident only in LS neurons whereas the P2X3-mediated fast current was evident only in TL neurons. These outcomes suggest that [1] all purinoceptive mouse LS bladder neurons express the P2X2

subunit, with ~90% also expressing the P2X3 subunit, and [2] all purinoceptive TL bladder neurons express the P2X3 subunit with the significant majority also expressing the P2X2 subunit. That these inward currents represent the composition of P2X subunits is supported by single cell nested PCR (see below).

Effects of CYP/bladder inflammation

In previous studies, blockage of P2X receptors was reported to reduce bladder overactivity *in vivo* [21] as well as hypersensitivity of bladder afferents in response to mechanical and electrical stimuli *in vitro* [52]. Bladder inflammation by CYP in the rat also induces changes in activation of ERK in the urothelium, expression of CGRP and substance P in LS DRG and Fos protein expression in LS spinal cord [12,46,47]. These outcomes as well as previous study of bladder neurons [16] suggest significant inflammation-induced changes in bladder neuron excitability.

In support, we found in the present study that CYP-induced bladder inflammation significantly increased mouse LS and TL bladder neuron excitability as evidenced by a significant decrease in rheobase and changes in responses to purinergic agonists. In LS neurons, more than 40% exhibited P2X2 homomeric sustained currents after CYP treatment relative to ~10% of neurons from saline-treated (or naïve) mice. In TL neurons, in contrast, there were no changes in proportions of purinergic-evoked currents after CYP treatment relative to saline treatment; the function of the homomeric P2X3 receptor (P2X3 fast current density), however, was significantly increased after bladder inflammation.

The significant increase in P2X2 homomeric currents in LS bladder neurons after CYP treatment suggests that a homomeric P2X2 receptor is involved in sensitization of pelvic nerve bladder afferents, consistent with reduced urinary bladder reflexes and decreased pelvic nerve afferent responses to bladder distension in P2X2 knockout mice [9]. With respect to P2X3, the amplitude of the P2X3 homomeric fast current was significantly enhanced and its desensitizing time constant decreased in TL bladder neurons after CYP treatment, suggesting a role in sensitization of the splanchnic pathway. These results support findings of reduced urinary bladder reflexes in P2X3 knockout mice [9,10] and the effect of P2X2/3 and P2X3 antagonists (e. g., TNP-ATP and A-317491) on bladder overactivity and sensitization of bladder afferents in CYP-treated rats [21,52].

P2X2 and P2X3 transcripts are abundantly expressed in mouse bladder sensory neurons

Transcription and expression of P2X receptor subunits are variable between species, organs and ganglia [29,8]. In rodent DRG neurons, P2X3 mRNA and protein is more abundant than other P2X subunits, especially in small-to-medium size neurons that do not express neuropeptides; the P2X2 subunit is detected in both small and large DRG neurons [49,50,5,11]. In the present study, P2X3 receptor expression was >90% in both LS and TL bladder neurons, consistent with electrophysiological studies of bladder afferents [31] and immunohistochemical localization in bladder sensory neurons [16] and nerve terminals in the suburothelial nerve plexus [10,34].

We also found P2X2 receptor expression to be high in LS bladder neurons, but less (by ~50%) in TL neurons. After CYP-treatment, P2X2 gene transcription in TL bladder neurons significantly increased, suggesting enhanced purinergic signaling at the transcriptional level. P2X2 receptor expression in LS bladder neurons, however, did not change after CYP-treatment, which appears to be inconsistent with the four-fold increase in homomeric P2X2 currents noted after CYP treatment. This may reflect the high expression frequency of both P2X2 and P2X3 mRNA in LS bladder neurons (>90%) in naive states. Because of the high proportion of bladder neurons in which P2X mRNA was detected in both saline- and CYP-

treated mice, the increase in P2X3 homomeric current density in TL neurons and the increase in P2X2 currents in LS neurons, post-transcriptional modulation of P2X receptors and/or interaction with other molecules/signaling pathways appear to be important in bladder neurons and sensitization of bladder afferents.

Species differences in purinergic signaling in bladder sensory pathways

Purinergic currents in bladder sensory neurons under both physiological and bladder-inflamed conditions in mice (C57BL/6, Taconic, Germantown, NY) differ from those previously reported in the rat (Sprague-Dawley, Harlan, Indianapolis, IN) [16]. The predominant purinergic current in naïve rat LS bladder neurons was a P2X2/3 heteromeric slow current (87% of rat purinoceptive neurons). No P2X2 homomeric sustained currents were noted, but low percentages (6%) of P2X3 homomeric fast currents and mixed (rapidly activating and mixed desensitizing) currents (7%) were noted, neither of which were found in LS bladder neurons in either naïve, saline- or CYP treated mice.

The predominant (50 – 60%) purinergic-evoked current in rat TL bladder neurons was the mixed current, with about one-third of rat TL bladder neurons exhibiting a P2X2/3 heteromeric slow current (the predominant current [75%] in mouse TL bladder neurons). P2X2 homomeric sustained currents are present in mouse LS bladder neurons, but not in either rat LS or TL bladder neurons, and P2X3 homomeric fast currents are present only in mouse TL bladder neurons, but in both LS and TL rat bladder neurons.

Results after CYP treatment in the mouse and rat are consistent in that purinergic currents are changed, albeit associated with different P2X subunits and different bladder sensory pathways. After bladder inflammation in the rat, the only purinergic current evoked in LS bladder neurons was a P2X2/3 heteromeric slow current (increasing from 87% to 100% of neurons studied); fast and mixed currents noted above were absent. That may suggest enhanced expression/function of the P2X2 subunit in rat LS bladder neurons, but this was not determined [16]. In rat TL bladder neurons, the proportion of neurons exhibiting P2X3 homomeric fast currents doubled from 22% (saline) to 43% after CYP treatment whereas the P2X2/3 heteromeric slow current was reduced by about 50%. In the present study, we noted no significant changes in purinergic-evoked currents in mouse TL bladder neurons with the exception of an increase in P2X3 fast current density, suggesting a greater contribution of homomeric P2X3 receptors in mouse and rat TL bladder neurons after bladder inflammation.

In summary, the present study supports an important role for both P2X2 and P2X3 receptor subunits in bladder sensory transmission. The significance of differences in purinergic signaling in bladder sensory pathways between rat and mouse is not clear, but may suggest greater roles of the splanchnic/TL and pelvic/LS pathways, respectively, in bladder hypersensitivity/reflexia after bladder inflammation. Given the interest in developing P2X receptor antagonists for conditions of bladder inflammation or functional bladder disorders such as PBS/IC, findings in both rats and mice suggest that selective P2X2/P2X3 antagonists may have therapeutic utility in the treatment of these conditions.

Summary

Bladder inflammation increases bladder afferent excitability and afferent purinergic signaling; P2X2 and P2X3 receptor signaling change, suggesting important roles in painful bladder syndrome/interstitial cystitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Examples of principal purinergic currents in LS and TL bladder sensory neurons in response to ATP (30 μM) and αβ-Met ATP (30 μM). Based on current inactivation kinetics and agonist responses, three distinct current types were identified: slow desensitizing currents predominated in LS (~90%, A) and TL (~75%, C) neurons; (B) sustained currents without an obvious inactivation phase were found only in LS neurons (~10%); and (D) fast desensitizing currents were found only in TL neurons (~25%). The duration of agonist application (4 sec) is denoted by the horizontal bar.

Figure 2.

Examples of antagonism of purinergic agonist-evoked currents in LS and TL bladder sensory neurons from naïve mice. Slow desensitizing currents in LS neurons were attenuated by the P2X3 receptor-selective antagonist TNP-ATP (A) and by the non selective purinergic antagonist PPADS (B). Sustained currents in LS neurons were not inhibited by TNP-ATP (C), but were inhibited by PPADS (D). Fast desensitizing currents in TL neurons were inhibited by TNP-ATP (E). The duration of agonist application (4 sec) is denoted by the horizontal bar.

Figure 3.

Examples of purinergic currents in P2X3^{-/-} mice. Only a sustained current was evoked by ATP in LS bladder neurons (A), which was inhibited by PPADS (C) but not by TNP-ATP (D). No obvious response to ATP or αβ-Met ATP was observed in TL bladder neurons (B). The duration of agonist application (4 sec) is denoted by the horizontal bar.

Figure 4.

Examples of responses to current injection and agonist application. A series of current pluses (20 pA increments, 1 s interval) was injected, and rheobase determined. Relative to bladder sensory neurons taken from saline-treated mice (A,C), the injected current required to evoke an action potential in LS and TL bladder neurons was significantly reduced after CYP treatment (B,D). The magnitude of membrane depolarization in LS and TL bladder neurons was increased after CYP-treatment compared with saline-treated controls in response to $\alpha\beta$ -met ATP (F, H) but not to ATP (E,G).

Figure 5. Single cell nested RT-PCR of P2X2 and P2X3 receptor subunits in LS and TL bladder neurons from saline- and CYP-treated mice. (A) An example of positive nested single cell PCR amplification of P2X2 and P2X3 mRNA. Product length corresponded with expected size. The frequency of P2X2 and P2X3 transcription in bladder neurons is summarized in (B). Percentage of cells only expressing P2X2, P2X3, or both P2X2 and P2X3 is illustrated in (C). (* TL vs. LS, $P < 0.05$; ** $P < 0.01$; † saline vs. CYP, $P < 0.05$)

Table 1

External and internal primers for mouse P2X2, P2X3 and GAPDH cDNA

P2X2 (NM_153400)	External Forward	CTCTTCAGTAACCATGTCCACG
	External Reverse	CCGGAAGACAGCTCTAATTGG
	Internal Forward	GAAGATAGGCATCTTGCTCTGG
	Internal Reverse	GGGATCCTATGAGGAGTTCTGT
P2X3 (NM_145526)	External Forward	GCTCCCTAGAAGAAGATGGAGA
	External Reverse	CTGTGTGACCATGTTAGGGATG
	Internal Forward	TGTCCTAAGAGGATCCTGTACC
	Internal Reverse	GGCATCTAGCACATAGAAGTGG
GAPDH (NM_008084)	External Forward	GCTGAGTATGTCGTGGAGTCTA
	External Reverse	CATACTTGGCAGGTTTCTCCAG

Table 2

Purineric currents in LS and TL bladder sensory neurons from naïve, saline-treated and CYP-treated mice.

		ATP			$\alpha\beta$ -metATP		
		naïve	saline-treated	CYP-treated	naïve	saline-treated	CYP-treated
LS	% responding Current type	93.0% (106/114)	96.9% (31/32)	93.3% (42/45)	83.3% (95/114)	87.5% (28/32)	53.3% $\ddagger\ddagger$ (24/45)
	sustained	10.4%	9.7% (3/31)	42.9% $\ddagger\ddagger$ (18/42)	na	na	na
	slow	89.6% *	90.3% (28/31)	57.1% $\ddagger\ddagger$ (24/42)	100% (95/95)	100% (28/28)	100% (24/24)
TL	fast	na	na	na	na	na	na
	% responding Current type	76.9% (70/91)	78.6% (22/28)	85.3% (29/34)	76.9% (70/91)	78.6% (22/28)	85.3% (29/34)
	sustained	na	na	na	na	na	na
	slow	74.3% (52/70)	72.7% (16/22)	79.3% (23/29)	74.3% (52/70)	72.7% (16/22)	79.3% (23/29)
	fast	25.7% (28/70)	27.3% (6/22)	20.7% (6/29)	25.7% (28/70)	27.3% (6/22)	20.7% (6/29)

na, not applicable

* P < 0.05 vs. the corresponding TL current type.

$\ddagger$ P < 0.05

$\ddagger\ddagger$ P < 0.01, vs. control (saline) treatment.

Table 3

Properties of purinergic currents in LS and TL bladder sensory neurons from naive mice.

current type	ATP % responding: LS-93.0%** (106/114)		αβ-metATP % responding: LS-83.3% (95/114)		TL-76.9% (70/91)	
	current density (pA/pF)	time to peak (ms)	current density (pA/pF)	time to peak (ms)	desensitizing tau (ms)	desensitizing tau (ms)
LS	sustained	15.5 ± 3.0	3898 ± 86	na	na	na
	slow	25.7 ± 1.9**	641 ± 34	2790 ± 210	1497 ± 124	4587 ± 582
	fast	na	na	na	na	na
TL	sustained	na	na	na	na	na
	slow	17.0 ± 2.2	638 ± 48	2475 ± 184	1458 ± 126	4280 ± 662
	fast	9.7 ± 2.3	253 ± 32	224 ± 18	272 ± 36	211 ± 25

Data are means ± SEM.

na, not applicable

* P < 0.05

** P < 0.01 vs. the corresponding TL current type.

Table 4

Passive and active electrical properties of LS and TL bladder neurons from naïve, saline-treated and CYP-treated mice.

		LS	TL
RMP (mV)	naïve	-70.4±1.7	-70.2±1.75
	saline	-70.5±1.6	-72.1±2.7
	CYP	-68.6±1.6	-66.5±2.1
input resistance(MΩ)	naïve	526.1±56.0	501.9±41.4
	saline	435.1 ± 37.3	474.4±45.1
	CYP	481.7 ± 30.7	419.4±29.9
rheobase (pA)	naïve	147.5 ± 12.6	160 ± 14.5
	saline	144.3±8.7	178±22.6
	CYP	110.9±9.5 [†]	128.2±8.5 [†]
AP threshold (mV)	naïve	-34.1±0.5 ^{**}	-28.5±0.8
	saline	-33.4±0.7	-30.2±1.7
	CYP	-35.6±0.8	-30.9±1.1
AP amplitude (mV)	naïve	117.1±4.5	115±2.3
	saline	121.1±2.5	116.4±4.2
	CYP	115±3	108.7±2.3
AP duration (ms)	naïve	3.6±0.3	4.5±0.3
	saline	4.3±0.3	4.3±1.7
	CYP	4.5±0.8	4.2±1.1
AP overshoot (ms)	naïve	51.4±2.6	44.8±1.4
	saline	50.6±1.7	44.3±2.3
	CYP	50.5±1.4	46.4±2.0
AP falling rate (mV/ms)	naïve	19.2±1.1	19.96±1.2
	saline	19.5±1.3	21.2±1.7
	CYP	21.0±1.9	20.7±1.3
depolarization (mV)	naïve	25.5±4.9	21.5±4.6
	saline	25.8±3.8	21.0±3.1
	CYP	32.8±5.4	25.0±2.4
number of APs	naïve	21/32 [*]	10/30
	saline	19/25 [*]	10/23
	CYP	28/33 ^{**}	10/28
αβ-metATP (30 μM) depolarization (mV)	naïve	15.9±2.9	10.1±2.2
	saline	15.0±3.0	11.5±1.8

		LS	TL
number of APs	CYP	26.5±3.1 [†]	17.2±1.7 [†]
	naïve	10/32	5/30
	saline	7/25	3/23
	CYP	8/33	5/28

Data are means ± SEM.

*
P < 0.05

**
P < 0.01 vs. TL counterparts

[†]
P < 0.05 vs. control (saline) treatment.

Table 5
Properties of purinergic currents in LS and TL bladder sensory neurons from saline-treated and CYP-treated mice.

current type	treatment	ATP			$\alpha\beta$ -metATP		
		current density (pA/pF)	time to peak (ms)	desensitizing g tau (ms)	current density (pA/pF)	time to peak (ms)	desensitizing tau (ms)
LS	sustained	15.5±5.2	311.4±75.9	n/a	n/a	n/a	n/a
	CYP	20.9±2.7	361.5±24.4	n/a	n/a	n/a	n/a
	slow	25.2±5.6	662.3± 33.4	2160±163	7.9±1.7	1493±144	5586±850
	CYP	31.6±4.0	771.4± 72.9	2710±402	10.7±1.7	1596±158	7873±1309
TL	saline	n/a	n/a	n/a	n/a	n/a	n/a
	CYP	n/a	n/a	n/a	n/a	n/a	n/a
	sustained	n/a	n/a	n/a	n/a	n/a	n/a
	CYP	n/a	n/a	n/a	n/a	n/a	n/a
	slow	17.4±4.0	642.3± 69.6	1840±270	7.4±1.6	1683±119	7543±4190
	CYP	10.9±1.6	633.9± 28.1	2440±173	3.7±0.9*	2202±199	4825±2499
	fast	9.1±1.7	261.3± 50.4	181.6± 7.5	4.5±1.1	268.9±56.9	202±4.1
	CYP	24.2± 5.0*	242.4± 15.3	128.9±6.5*	14.4±2.3*	258.0±25.6	157.9± 5.0

Data are means ± SEM.

na, not applicable

* P < 0.05 vs. the corresponding saline-treated group.