Brief Communications

Endogenous Purinergic Control of Bladder Activity via Presynaptic $P2X_3$ and $P2X_{2/3}$ Receptors in the Spinal Cord

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$P2X_3$ and $P2X_{2/3}$ receptors are localized on sensory afferents both peripherally and centrally and have been implicated in various sensory functions. However, the physiological role of these receptors expressed presynaptically in the spinal cord in regulating sensory transmission remains to be elucidated. Here, a novel selective $P2X_3$ and $P2X_{2/3}$ antagonist, AF-792 [5-(5-ethynyl-2-isopropyl-4-methoxy-phenoxy)-pyrimidine-2,4-diamine, previously known as RO-5], in addition to less selective purinoceptor ligands, was applied intrathecally *in vivo*. Cystometry recordings were made to assess changes in the micturition reflex contractions after drug treatments. We found that AF-792 inhibited micturition reflex activity significantly (300 nmol; from baseline contraction intervals of 1.18 ± 0.07 to 9.33 ± 2.50 min). Furthermore, inhibition of $P2X_3$ and $P2X_{2/3}$ receptors in the spinal cord significantly attenuated spinal activation of extracellular-signal regulated kinases induced by acute peripheral stimulation of the bladder with 1% acetic acid by 46.4 ± 12.0% on average. Hence, the data suggest that afferent signals originating from the bladder are regulated by spinal $P2X_3$ and $P2X_{2/3}$ receptors and establish directly an endogenous central presynaptic purinergic mechanism to regulate visceral sensory transmission. Identification of this spinal purinergic control in visceral activities may help the development of $P2X_3$ and $P2X_{2/3}$ antagonist to treat urological dysfunction, such as overactive bladder, and possibly other debilitating sensory disorders, including chronic pain states.

Introduction

Presynaptic regulation of neurotransmission by a variety of receptors, including nicotinic, ionotropic glutamate, P2X, and $5\text{-}HT_3$ receptors, is important for various nervous system functions such as learning, memory, and spinal reflexes (for review, see Engelman and MacDermott, 2004). In particular for sensory neurotransmission gating, P2X receptors have been characterized extensively based on electrophysiological evidence using spinal cord slices (Nakatsuka and Gu, 2001; Nakatsuka et al., 2002, 2003; Chen and Gu, 2005). However, no *in vivo* studies have been undertaken to determine the functional significance of central presynaptic P2X receptors under normal physiological condition.

Previous studies have established the role of peripheral $P2X_3$ and $P2X_{2/3}$ receptors in facilitating afferent transmission of the micturition reflex pharmacologically (King et al., 2004; Nishiguchi et al., 2005) in addition to using knock-out mice (Cockayne et al., 2000, 2005). Importantly in the spinal cord, expression of $P2X_3$ and $P2X_{2/3}$ receptors appears to be mostly restricted to the presynaptic terminals of nonpeptidergic afferents terminating in lamina IIi (Vulchanova et al., 1997; Bradbury et al., 1998; Guo et al., 1999). In this study, the micturition reflex contraction intervals were chosen to assess the functional sensory modulation (Cefalu et al., 2007b) by spinal presynaptic $P2X_3$ and $P2X_{2/3}$ receptors. A paucity of selective pharmacological tools has hindered the efforts to study the contribution the receptors in sensory functions *in vivo*. However, recent advances in medicinal chemistry have been made, and AF-792 [5-(5-ethynyl-2-isopropyl-4-methoxy-phenoxy)-pyrimidine-2,4-diamine, previously known as RO-5], which is one of a new series of metabolically stable selective and the most potent drug-like $P2X_3$ and $P2X_{2/3}$ antagonists identified to date (Gever et al., 2006; Carter et al., 2009; Jahangir et al., 2009), is used here. The structure has already been published and described as Compound 28 by Carter et al. (2009).

We present data demonstrating the role of presynaptic $P2X_3$ and $P2X_{2/3}$ receptors in the spinal cord in regulating bladder micturition reflex neurotransmission and, in particular, via the extracellular-regulated signal kinase 1 (ERK1) and ERK2 pathway after acute noxious bladder stimulation. By using a novel and selective $P2X_3$ and $P2X_{2/3}$ antagonist, we reveal an endogenous presynaptic purinergic sensory regulation of visceral neurotransmission in the spinal cord *in vivo*.

Materials and Methods

All procedures were performed in accordance with United Kingdom Home Office regulations (Animals Scientific Procedures Act, 1986). Adult female Sprague Dawley rats (200–280 g; Harlan) were used and housed with access to food and water *ad libitum* at 25°C with an alternating 12 h light/dark cycle.

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Surgical procedures. For intrathecal delivery, catheters were implanted into the lumbar subarachnoid space at least 1 d before cystometry and phosphorylated-ERK (pERK) immunohistochemical studies based on methods described previously (Lever et al., 2003). In brief, rats were anesthetized with medetomidine (0.25 mg/kg, i.p.) and ketamine (60 mg/kg, i.p.). A small laminectomy was made at the sixth or seventh thoracic vertebra, and a fine cannula was inserted under the dura mater such that the tip was located at L1 level. Anesthesia was reversed by atipamezole hydrochloride (1 mg/kg, s.c.) after surgery. On the study day, the urinary bladder was cannulated according to procedures described by Cefalu et al. (2007b). Briefly, animals were anesthetized with urethane (1.2 g/kg, i.p.). An abdominal incision was made along the linea alba to expose the bladder and ureters for cannulation and ligation, respectively. The external urethral orifice was ligated to create an isovolumetric system.

Cystometry studies. The bladder cannula was connected to a pressure transducer for measurement of intravesicular bladder pressure and to a saline infusion pump. The bladder was infused with saline at 100 μl/min until the threshold was reached to elicit micturition reflex contractions. Then the rate was lowered to 3–5 μl/min to maintain stable isovolumetric bladder contractions. After observations of stable baseline contractions (a minimum of 10 min), vehicle was administered. This was followed by various doses of α,β-methylene-ATP ($\alpha\beta$meATP) (0.1–100 nmol), pyridoxal-phosphate-6-azophenyl-2′,4′,-disulphonic acid (PPADs) (1–400 nmol), 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate monolithium trisodium salt (TNP–ATP) (0.1–100 nmol), and AF-792 (1–300 nmol). Each dosing (10 μl; with a minimal separation interval of 8 min or when the contraction frequency returned to baseline; $n = 5$–15) was administered via the implanted intrathecal cannula slowly using a Hamilton syringe, followed by 10 μl of flush of sterile saline. $\alpha\beta$meATP, PPADs, and TNP–ATP (Sigma-Aldrich) were dissolved in sterile saline; AF-792 (Afferent Pharmaceuticals) was dissolved in 5% DMSO (in saline). Recordings and analysis were made using PowerLab and Chart 5 (ADInstruments).

ERK activation immunohistochemical studies. In separate experiments, ERK activation was studied in the spinal cord after acute noxious stimulation in the bladder of naive rats. At least 1 h after bladder cannulation, either AF-792 (300 nmol) or its vehicle (5% DMSO) was administered via the implanted intrathecal cannula. Ten minutes later, 700 μl of 1% acetic acid was instilled intravesically into the bladder for 2 min. For the sham group, animals were administered with vehicle (5% DMSO, i.t.) without acetic acid instillation and left untouched for 12 min. Animals were then transcardially perfused with heparinized saline (5 IU/ml in 0.9% saline), followed by 4% paraformaldehyde (in 0.1 M phosphate buffer; VWR) with 15% saturated picric acid (Sigma-Aldrich). Lumbar spinal cord was dissected out of the animals, postfixed overnight, and transferred into 20% sucrose before being embedded and frozen in OCT compound (BDH). Transverse L6 spinal cord segment sections (20 μm) were cut with a cryostat and mounted onto Superfrost slides. Immunohistochemical detection of pERK staining was determined using tyramide signal amplification protocol detailed by Wong et al. (2006). Slides were incubated in the following (with three 5 min PBS washes between each step): polyclonal rabbit anti-phospho-p44/42 mitogen-activated protein kinase (Thr202/Tyr204) (pERK) antibody (1:400, overnight; New England Biolabs), biotinylated donkey anti-rabbit secondary antibody (1:400, 1.5 h; Jackson ImmunoResearch), avidin–biotin peroxidase complex (30 min, Vectastain ABC Elite kit; Vector Laboratories), biotinyl tyramide (1:75, 10 min; PerkinElmer Life and Analytical Sciences), and extra-avidin FITC (1:500, 2 h; Sigma-Aldrich). Last, the slides were washed and coverslipped with Vectashield mounting medium (Vector Laboratories). Images were taken with a fluorescence Carl Zeiss microscope and AxioVision 4.6 at 20× objective magnification. Each treatment group consisted of four animals, and three sections at the L6 level were randomly chosen from each animal for analysis. Counting of pERK-positive cells was done blindly to the experimental conditions.

Statistical analysis. Data for cystometry studies were statistically compared using one-way repeated-measures ANOVA, followed by the

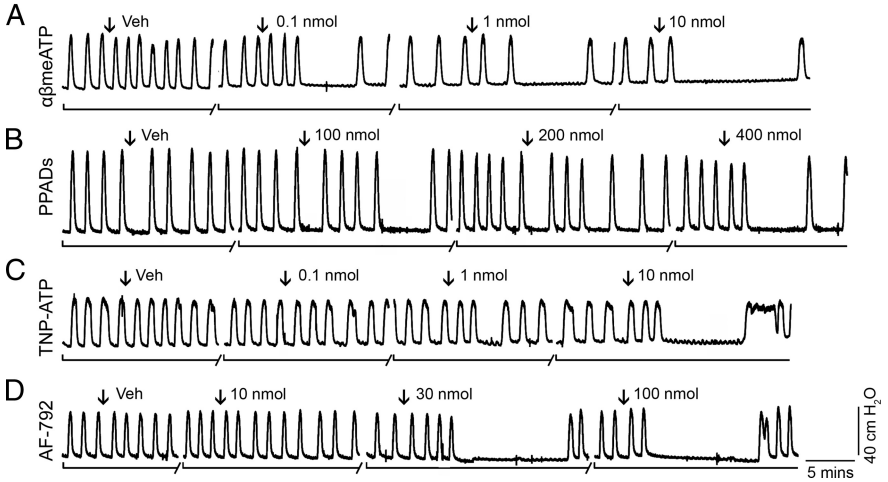

Figure 1. Sample traces of cystometry recording after intrathecal application of $\alpha\beta$meATP (***A***), PPADs (***B***), TNP–ATP (***C***), and AF-792 (***D***) and their respective vehicles. Calibration: 5 min, 40 cm H_2O.

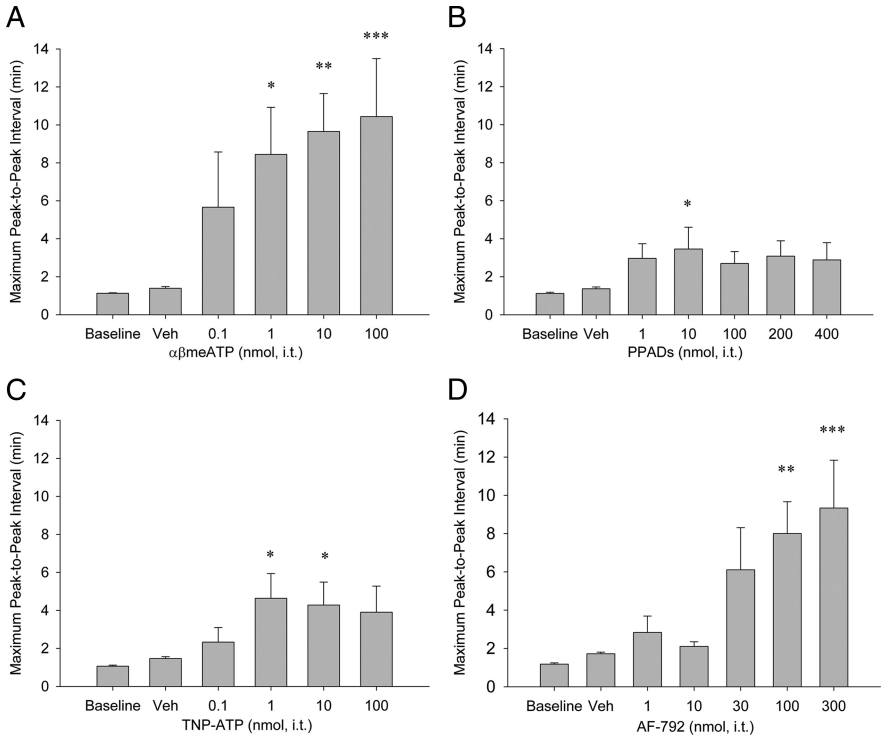

Figure 2. Intrathecal application of $\alpha\beta$meATP (***A***), PPADs (***B***), TNP–ATP (***C***), and AF-792 (***D***) inhibits isovolumetric bladder contractions in naive anesthetized animals *in vivo* ($n = 5$–12 for each dose). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with vehicle.

Table 1. Pharmacological selectivity of AF-792

Receptor	*n*	$pIC_{50} \pm SEM$
Rat $P2X_3$	13	8.20 ± 0.06
Human $P2X_{2/3}$	15	7.94 ± 0.08
Human $P2X_1$	3	<5
Human $P2X_2$	3	<5
Human $P2X_4$	3	<5
Rat $P2X_5$	3	<5
Human $P2X_7$	3	<5

pIC_{50} values for all of the P2X channels were obtained from $\alpha\beta$meATP or ATP-evoked intracellular calcium flux in recombinant cell lines in the presence of AF-792.

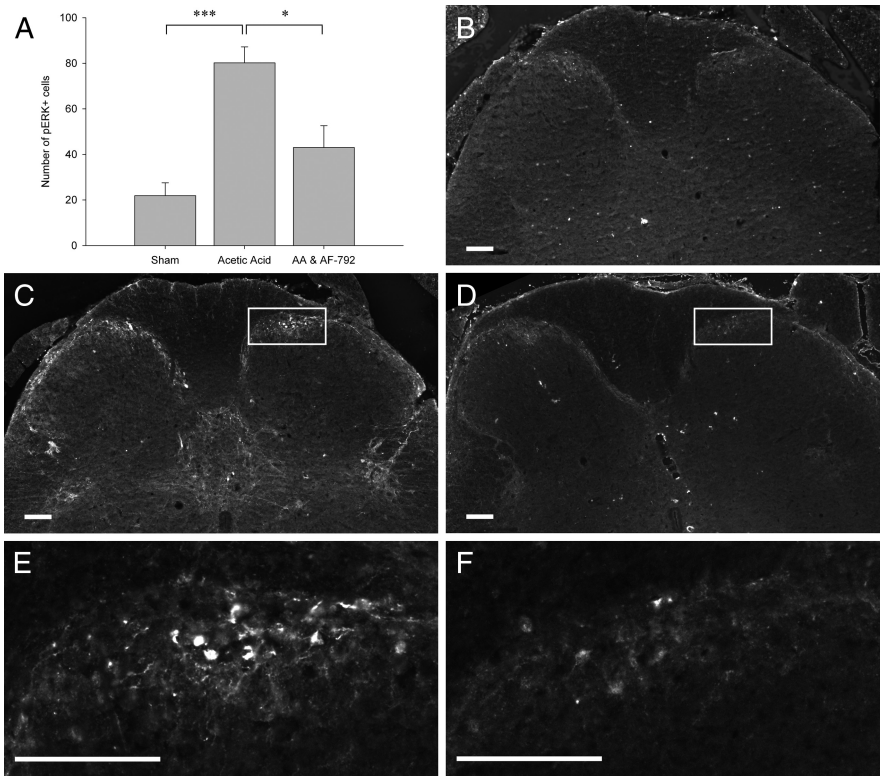

Figure 3. ***A***, AF-792 (300 nmol, i.t.) significantly reduces pERK-positive cells in the spinal cord after 1% acetic acid stimulation of the bladder ($n = 4$ for each group). $^*p < 0.05$, $^{***}p < 0.001$ compared with 1% acetic acid-stimulated group. Representative photomicrographs of sham group with vehicle (intrathecally) treatment (***B***), 1% acetic acid-stimulated bladder group with vehicle (intrathecally) treatment (***C***), and 1% acetic acid-stimulated bladder group with AF-792 (300 nmol, i.t.) treatment (***D***) with high-power magnification in ***E*** and ***F*** for the latter two groups, respectively, are shown. White boxes indicate the area of high-power magnification. Scale bars, 100 μm.

Bonferroni's *post hoc* test (SigmaStat 3.5 software). The number of pERK-positive cells was analyzed using one-way ANOVA, followed by the Tukey's *post hoc* test. All data are presented as means ± SEM, and statistical significance was set at $p < 0.05$.

Results

We investigated the role of presynaptic spinal $P2X_3$ and $P2X_{2/3}$ receptors in controlling micturition reflexes by intrathecal administration of purinergic compounds, using *in vivo* cystometry and comparing the activation of ERK in spinal cord neurons after noxious stimulation of the bladder.

Under isovolumetric conditions, the urinary bladder of anesthetized rats displayed stable rhythmic contractions that did not alter significantly after vehicle injections ($\alpha\beta$meATP: baseline, 1.13 ± 0.03 min; vehicle, 1.39 ± 0.09 min, $n = 9$; PPADs: baseline, 1.12 ± 0.07 min; vehicle, 1.37 ± 0.09 min, $n = 13$; TNP–ATP: baseline, 1.06 ± 0.06 min; vehicle, 1.47 ± 0.10 min, $n = 15$; AF-792: baseline, 1.18 ± 0.07 min; vehicle: 1.72 ± 0.08 min, $n = 8$). All purinoceptor compounds tested reduced the frequency of micturition reflex contractions indicated by an increase in intercontraction intervals (ICIs) (Figs. 1, 2). $\alpha\beta$meATP, a desensitizing purinergic agonist/antagonist, dose dependently inhibited the contractions significantly by prolonging the ICI up to 10 min, with the lowest significant dose started at 1 nmol (intrathecally) (8.45 ± 2.48 min; $p = 0.026$, $n = 6$) (Figs. 1*A*, 2*A*). PPADs (pIC_{50} at $P2X_3$, 6; at $P2X_{2/3}$, 6) increased the ICI to >2 min but only with significance at 10 nmol (intrathecally) (3.50 ± 1.15 min; $p = 0.042$, $n = 9$). Its variable effects were likely attributable to its weak antagonism (Figs. 1*B*, 2*B*). TNP–ATP (pIC_{50} at $P2X_3$, 9; at $P2X_{2/3}$, 8.4; at $P2X_1$, 8.22), a more selective antagonist at $P2X_3$ and $P2X_{2/3}$ but also with activity on P2X1 receptor, was next tested. TNP–ATP caused a more potent inhibition on contractions than PPADs by increasing the ICI significantly at 1 nmol (intrathecally) (4.64 ± 1.30 min; $p = 0.015$, $n = 11$) and 10 nmol (intrathecally) (4.28 ± 1.21 min; $p = 0.037$, $n = 11$) (intrathecally) (Figs. 1*C*, 2*C*). AF-792 is a novel selective $P2X_3$ and $P2X_{2/3}$ antagonist (pIC_{50} at $P2X_3$, 8.2; at $P2X_{2/3}$, 7.9) from the same diaminopyrimidine series as RO-3 [5-(2-isopropyl-4,5-dimethoxybenzyl)-pyrimidine-2,4-diamine] and RO-4 [5-(5-iodo-2-isopropyl-4-methoxy-phenoxy)-pyrimidine-2,4-diamine] [Gever et al., 2006; Carter et al., 2009 (pIC_{50} values for all the antagonists mentioned above are cited from these two studies)]. The selectivity of AF-792 (previously known as RO-5) for $P2X_3$ and $P2X_{2/3}$ receptors over other P2X receptors was established by testing the ability of AF-792 to block agonist-evoked intracellular calcium flux in cell lines expressing recombinant P2X receptors. pIC_{50} values and the selectivity profile for AF-792 were determined according to methods previously described for RO-3 by Ford et al. (2006). In brief, pIC_{50} values were determined by measuring cytosolic calcium flux evoked by $\alpha\beta$meATP or ATP (300 nM to 10 μM depending on receptor subtype) in Fluo-3-loaded CHOK1 (transfected with recombinant human $P2X_1$, rat $P2X_3$, human $P2X_4$, rat $P2X_5$, or human $P2X_7$ receptors) and 1321N1 astrocytoma (transfected with cloned human $P2X_2$ or human $P2X_{2/3}$ receptors) cells (Table 1). In summary, AF-792 was found to be very selective with actions on $P2X_3$ and $P2X_{2/3}$ receptors only with no inhibition at other P2X receptors up to a concentration of 10 μM. In addition, AF-792 has been profiled extensively in two commercially available screens, one covering 75 receptors, channels, enzymes, and transporters (Cerep) and a second one covering >100 kinases (Ambit), and the results demonstrated little or no inhibition of radioligand binding or function in the presence 10 μM AF-792. In the current study, AF-792 caused significant inhibition of contractions at 100 nmol (intrathecally) (8.00 ± 1.67 min; $p = 0.002$, $n = 8$) and 300 nmol (intrathecally) (9.33 ± 2.50 min; $p < 0.001$, $n = 6$) (Figs. 1*D*, 2*D*). The maximum effect of AF-792 in prolonging the ICI was longer compared with those after TNP–ATP and PPADs administration. The amplitudes of bladder contractions before

and after every dose of all four purinergic ligands were analyzed and found not to be significantly affected (data not shown).

ERK activation in the spinal cord is correlated with bladder hyperactivity, which may be associated with bladder inflammation caused by agents including acetic acid (Cruz et al., 2005, 2007). Therefore, we compared the level of pERK in the spinal cord after acute acetic acid stimulation of the bladder with and without spinal $P2X_3$ and $P2X_{2/3}$ receptor blockade to further investigate the possible receptor signaling cascade. Instillation of 1% acetic acid into the bladder after vehicle injection intrathecally significantly ($p = 0.001$) increased the number of pERK-positive cells in the L6 spinal cord segment (80.2 ± 7.0 cells per section) compared with sham-vehicle control animals (21.9 ± 5.7 cells per section) (Fig. 3*A–C*). Activation of ERK by 1% acetic acid was seen throughout the spinal cord in the regions of the superficial dorsal horn, dorsal commissure, and sacral parasympathetic nucleus (Fig. 3*C*,*E*). The injection of AF-792 (300 nmol, i.t.) significantly reduced the spinal expression of pERK-positive cells (43.0 ± 9.6 cells per section; $p = 0.018$) induced by acetic acid stimulation in the bladder and to a level that is not significantly different from sham-vehicle control animals (Fig. 3*A*,*D*,*F*). On average, the number of pERK-positive cells throughout the spinal cord after AF-792 intervention represented a significant reduction of 46.4 ± 12.0% compared with the number in vehicle-treated animals after acetic acid stimulation in the bladder. In summary, the increases in micturition reflex ICI by $P2X_3$ and $P2X_{2/3}$ inhibitors intrathecally and the reduced ERK activation after intrathecal application of AF-792 under acute noxious stimulation of the bladder provided evidence of a spinal endogenous presynaptic purinergic regulation of sensory neurotransmission originating from the bladder.

Discussion

Multiple lines of evidence have identified $P2X_3$ and $P2X_{2/3}$ receptors to be involved in sensory processing (Khakh and North, 2006), including mediating partly the afferent transmission of bladder reflexes (Cockayne et al., 2000, 2005; King et al., 2004). More recently, spinal $P2X_3$ and $P2X_{2/3}$ receptors have been implicated in chronic neuropathic and inflammatory pain conditions (McGaraughty et al., 2003; Sharp et al., 2006), and electrophysiological studies added support to their role in sensory neurotransmission (Nakatsuka and Gu, 2006). We had reported previously preliminary data of the involvement of both peripheral and central $P2X_3$ and $P2X_{2/3}$ receptors in micturition reflex (Cefalu et al., 2007a). In this study, our data reveal that peripheral afferent inputs from the urinary bladder are under tonic physiological control of an endogenous purinergic system via presynaptic spinal $P2X_3$ and $P2X_{2/3}$ receptors.

We demonstrated directly the functional significance of spinal $P2X_3$ and $P2X_{2/3}$ receptors by applying intrathecally $P2X_3$ and $P2X_{2/3}$ inhibitors (in the form of desensitizing agonist or antagonists) *in vivo*. Inhibition of micturition reflex contractions by $\alpha\beta$meATP correlated with its previous pharmacological characterization as a desensitizing purinergic agonist/antagonist (Kasakov and Burnstock, 1982; Tsuda et al., 1999; Nakatsuka et al., 2003; King et al., 2004). Similarly, commercially available purinoceptor antagonists, PPADs and TNP–ATP, reduced micturition reflex contraction frequency. In the past, lack of suitable drugs with receptor subtype selectivity has hindered additional direct proof of the role of $P2X_3$ and $P2X_{2/3}$ receptors in sensory functions until recently when two novel non-nucleotide small molecule antagonists, A-317491 (5-[[[(3-phenoxyphenyl)methyl][(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]carbonyl]-1,2,4-benzenetricarboxylic acid) and RO-3, were reported to alleviate pain behavior in animal models (Jarvis et al., 2002; Ford et al., 2006). A-317491 was found to improve urodynamic parameters in animals with spinal cord injury or cyclophosphamide cystitis when administered intravenously (Lu et al., 2007; Ito et al., 2008) with presumed peripheral site of action because A-317491 has negligible CNS permeability (Wu et al., 2004). Here, we intrathecally applied AF-792 (previously known as RO-5), a novel and potent selective antagonist that belongs to the same chemical series as RO-3, to clarify the role of spinal $P2X_3$ and $P2X_{2/3}$ receptors in micturition reflex pharmacologically. AF-792 produced longer-lasting inhibition of the micturition reflex contractions than PPADs and TNP–ATP. This may be because AF-792 is metabolically stable and much more selective for $P2X_3$ and $P2X_{2/3}$ receptors than other purinergic receptors. In contrast, PPADs is a nonselective purinergic antagonist, and TNP–ATP is susceptible to rapid hydrolysis and breakdown by ectonucleotidases (Lambrecht, 2000). Thus, presynaptic $P2X_3$ and $P2X_{2/3}$ receptors are involved in facilitating the micturition reflex contraction via direct actions at the spinal cord level.

C-fibers that express $P2X_3$ and $P2X_{2/3}$ receptors mediate at least in part acetic acid-induced bladder hyperactivity (Avelino et al., 1999; Zhang et al., 2003) that may be contributed by increased urothelial release of ATP (Sugaya et al., 2007). Phosphorylation of ERK is accepted as a nociceptive signaling marker (Obata and Noguchi, 2004; Cruz and Cruz, 2007), and inhibition of spinal ERK reduces inflammatory bladder hyperactivity (Cruz et al., 2005). Therefore, spinal ERK activation was used as another measure of the degree of afferent input into the spinal cord after acute bladder stimulation. Here we found that noxious stimulation of the bladder by acetic acid led to significant ERK activation in the spinal cord in regions that are known to contain sensory afferent inputs from the bladder and also where further central projections are made, confirming observations made by Cruz et al. (2005, 2007). Intrathecal application of AF-792 reduced significantly the number of pERK-positive cells throughout all these regions. Our results are consistent with the previous suggested role of spinal P2X receptors in mediating bladder overactivity (Masuda et al., 2005). Hence, in the spinal cord, inhibition of presynaptic $P2X_3$ and $P2X_{2/3}$ receptors likely dampened the afferent hyperexcitability originating from stimulation of the primary afferents by acetic acid in the bladder, as reflected by the overall decrease in ERK activation. Because $P2X_3$ and $P2X_{2/3}$ receptors are also expressed on afferents peripherally, AF-792 would be expected to cause similar inhibitory actions if administered peripherally, as demonstrated with other less selective purinergic compounds (King et al., 2004).

Here, we showed the novel endogenous role of presynaptic $P2X_3$ and $P2X_{2/3}$ receptors in the spinal cord to facilitate the sensory input of micturition reflex by applying a novel selective antagonist *in vivo* and activate the ERK signaling pathway after peripheral noxious stimulation. Hyperactivity in the spinal cord is common in diseased chronic pain states (D'Mello and Dickenson, 2008), and multiple pain mediators can sensitize and affect the expression of $P2X_3$ receptors peripherally and centrally (Paukert et al., 2001; Ramer et al., 2001). Because development of $P2X_3$ and $P2X_{2/3}$ receptor antagonists to treat various sensory dysfunctions including chronic pain states and overactive bladder is currently underway, our novel data further strengthen its indication and make aware the importance of not overlooking the critical contribution of the receptors located presynaptically in the spinal cord.

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