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ORIGINAL ARTICLE

Therapeutic effects of the putative $P2X_3/P2X_{2/3}$ antagonist A-317491 on cyclophosphamide-induced cystitis in rats

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Abstract It is suggested that ATP and purinergic P2X receptors are involved in overactive bladder. In this study, we investigated the effect of the recently developed $P2X_3$ and $P2X_{2/3}$ receptor antagonist A-317491 on cyclophosphamide (CYP)-induced cystitis to determine whether a P2X receptor antagonist could be beneficial for the treatment of bladder overactivity induced by CYP. Female Sprague–Dawley (SD) rats were given 150 mg/kg CYP (i.p.). When the micturition activity was observed for 24 h in a conscious and unrestrained condition, CYP-treated rats exhibited increased urinary frequency. Two days after CYP injection, cystometry was performed in conscious rats, in which the bladder was continuously infused with saline (5 ml/h). In CYP-treated rats, non-voiding contractions were interposed between micturitions, suggestive of hyper-reflexia. Intravenous administration of A-317491 (20 or 50 mg/kg) or pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) tetrasodium (PPADS; a nonselective purinergic receptor antagonist, 10 mg/kg) prolonged the interval of voiding contraction and reduced the non-voiding contractions. On the other hand, oxybutynin (1 mg/kg), a muscarinic receptor antagonist, did not affect the frequency of non-voiding or voiding contractions in CYP-treated rats. A-317491 at the higher dose decreased the amplitude of voiding contractions, but increased the micturition volume. The residual urine in the bladder increased after treatment with CYP; A-317491 and PPADS reduced this, whereas oxybutynin had no effect. These data suggest that A-317491 is effective at improving the signs of CYP-induced cystitis and that the $P2X_3$ or $P2X_{2/3}$ receptor pathway is involved in bladder overactivity observed during CYP-induced cystitis.

Keywords Bladder · Cyclophosphamide · Cystitis · A-317391 · P2X receptor · Cystometry · Oxybutynin · Urinary frequency

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Introduction

Chronic bladder inflammatory conditions, such as interstitial cystitis, cause pelvic pain, increased urinary frequency, and urgency. During cystitis mechano-sensitive afferent nerves are sensitized, resulting in hyper-reflexia (Andersson and Hedlund 2002; Birder et al. 2004). It is thought that purinergic P2X receptors, especially $P2X_3$ or $P2X_{2/3}$ receptors, mediate sensory functions in lower urinary tracts (Rapp et al. 2005; Ford et al. 2006). It has been reported that the release of ATP from the bladder urothelium is increased (Smith et al. 2005), and the expression of $P2X_3$ receptor in the bladder is up-regulated (Brady et al. 2004) in some types of neurogenic bladder overactivity; moreover, these changes are suggested to be responsible for unstable bladder activity (Rapp et al. 2005; Ruggieri 2006). However, there has been no concrete evidence for the involvement of ATP and P2X receptor in cystitis-related hyper-reflexia. One reason for such ambiguity is the lack of a specific P2X receptor antagonist, especially a $P2X_3$ or $P2X_{2/3}$ receptor antagonist. A-317491 (5-((3-Phenoxybenzyl)[(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino carbonyl)-1,2,4- benzenetricarboxylic acid), a first non-nucleotide P2X receptor antagonist, was recently introduced (Jarvis et al. 2002). Jarvis et al. (2004) showed that A-317491 bound most strongly to the membranes of cells expressing $P2X_{2/3}$

receptor, then to those of $P2X_3$-expressing cells; thus, this compound was defined as a $P2X_{2/3}$ and $P2X_3$ receptor antagonist. If these receptors are up-regulated in a class of overactive bladders, A-317491 may be able to inhibit their activity and exert a therapeutic effect. In this regard, this drug has to be tested on the bladder overactivity that occurs during bladder inflammation.

Cyclophosphamide (CYP), an antitumor agent, is metabolized to acrolein in the liver, and acrolein injures the urothelium causing interstitial cystitis (Cruz 2004; Nazif et al. 2007). Rats inflamed with CYP exhibit bladder hyper-reflexia (Borvendeg et al. 2003; Hu et al. 2003). Although it has been reported that the urothelial ATP release is enhanced in CYP-treated rats (Chopra et al. 2005; Smith et al. 2005), it has not been demonstrated that $P2X_3$ or $P2X_{2/3}$ receptor is involved in the hyper-reflexia during CYP-induced cystitis. To explore this point, A-317491 could be a useful tool. In this study, we examined the effect of A-317491 on cystitis acutely induced by CYP in rats. If A-317491 was effective at treating signs occurring during CYP-induced cystitis, we thought this would indicate that ATP and P2X receptors play significant roles in bladder overactivity and that a P2X receptor antagonist could be a therapeutic agent for the treatment of cystitis.

Materials and methods

Animals

All animal experiments were carried out according to the guideline of the Committee for the Care and Use of Laboratory Animals, University of Miyazaki, which is based on the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology. Female Sprague–Dawley (SD) rats at 32–35 weeks age, which had given birth five times, received intraperitoneal injections of CYP (150 mg/kg) or the same volume of saline to elicit acute cystitis.

Micturition patterns over 24 h

At 48 h after injection, a rat from the control or CYP-treated group was put in a metabolic cage to observe micturition without any restraint. Rats were given water and diet ad libitum and left in the cage for 24 h. Illumination was turned on between 7:00 A.M. and 7:00 P.M. and turned off between 7:00 P.M. and 7:00 A.M. the next day. Urine dropped on a balance (A&D GF-200, Tokyo, Japan) was weighed, and the weight data were digitized by a PowerLab system (AD Instruments, Colorado Springs, CO, USA) and a software (Chart, AD Instruments).

Cystometry

Rats were anesthetized with ketamine (30 mg/kg i.p.) and medetomidine (300 μg/kg i.p.) 2 days before the cystometry experiment. A polyethylene cannula (Clay Adams PE50) was inserted into the lumen through a small incision at the dome of the bladder for infusion of saline and recording of intravesicular pressure, and another cannula (PE10) was inserted into the left femoral vein for drug injection. The other end of each cannula was exteriorized from the back skin and sutured. After surgery, atipamezole (300 μg/kg i.p.) was injected to hasten arousal. On the day of the experiment, the rat was put in a Ballman cage and allowed to adapt to the situation for 30 min. Then, the bladder was infused with saline (5 ml/h) using a syringe pump (Harvard Pump 11, Holliston, MA, USA). Intravesicular pressure and micturition were measured in the conscious condition. After observing stable intravesicular pressure and micturition for 1 h, a drug was injected via an intravenous route; then, these parameters were measured for 1 h. The data were digitized using the PowerLab and the software Chart. When the frequency of non-voiding contraction was calculated, non-voiding contractions with an amplitude of >5 mmHg were collected.

Residual urine volume and bladder weight

When micturition occurred after observing intravesicular pressure and micturition for 1 h after treatment with a drug, an excess dose of pentobarbital was intravenously injected into rats within 30 s, and the bladders were quickly removed. Urine volume in the bladder was measured.

Drugs

The following drugs were used: A-317491, pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) tetrasodium (PPADS), oxybutynin (Sigma, St. Louis, MO, USA), ketamine (Sankyo, Tokyo, Japan), medetomidine, atipamezole (Farmos, Turku, Finland). A-317491, PPADS, and oxybutynin were dissolved in saline.

Statistics

Data are expressed as means ± SEM. Statistical significance was tested by a Student's t test for non-paired samples or a paired t test for paired samples. For multiple comparisons, analysis of variance (ANOVA) was performed followed by a post hoc test (Dunnett's test). Significance was considered at $P < 0.05$.

Table 1 Micturition for 24 hours in saline- or CYP-treated rats in an unrestrained condition

Group	Voiding frequency			Total voided volume (ml)			Mean voided volume (ml)	Number of rats (*n*)
	Daytime	Nighttime	Total	Daytime	Nighttime	Total		
Control	4.8±0.9	9.0±1.3	13.8±2.1	8.3±1.9	13.9±2.3	22.2±4.1	1.61±0.17	8
CYP	17.3±4.1*	20.1±3.5*	37.4±7.0*	7.1±1.9	9.3±0.8	17.5±2.5	0.56±0.09*	8

Control rats were treated with saline at the same volume as CYP. Data are mean ± SEM. Daytime is from 7:00 A.M. to 7:00 P.M. Nighttime is from 7:00 PM to 7:00 A.M. on the next day.
* Significantly different from control ($P<0.05$).

Results

Micturition activity in control and CYP-treated rats

Table 1 shows the micturition activity over 24 h in control and CYP-treated rats measured in unrestrained condition. Water intake for 24 h was not different between the two groups (25.0±5.2 ml, $n=8$, and 23.1±1.9 ml, $n=8$, for control and CYP-treated rats, respectively). Micturition frequency was significantly higher, and mean voided volume was significantly lower in the CYP-treated group compared with the control group ($P<0.05$). These data indicate that CYP increased urinary frequency. The disparity between water intake and total voided volume was probably due to evaporation of urine; especially in CYP-treated rats, urine might easily evaporate due to a small amount of excretion.

Cystometry

Cystometry was performed in conscious rats, which received saline or CYP 48 h before the experiment. Bladder was continuously infused with saline (5 ml/h) via an intrabladder catheter. Fig. 1 shows representative records of a change in intravesicular pressure and micturition in control and CYP-treated rats. The frequency of micturition in CYP-treated rats was significantly higher (13.0±1.8 events/h, $n=31$) than that in control group (8.4±0.3 events/h, $n=21$). Mean voided volume was significantly less in CYP-treated rats (0.53±0.06 ml, $n=31$) than in control rats (0.79±0.13 ml, $n=21$). Between voiding contractions, small non-voiding contractions were seen in CYP-treated rats (29 of 31 CYP-treated rats). The amplitude and frequency of non-voiding contraction were variable among rats. Such non-voiding contractions were also observed in control rats, but the frequency of non-voiding contractions was less in control rats (3.5±0.6 events/h, $n=16$) than in CYP-treated rats (7.5±1.1 events/h, $n=29$).

After the steady bladder activity had been observed for 60 min, a drug was injected into the femoral vein. Representative records for the effects of A-317491 (50 mg/kg) and oxybutynin (1 mg/kg) are shown in Fig. 2. In control rats, A-317491 reduced the frequency of voiding contractions, without any effects on other parameters. In CYP-treated rats, A-317491 (20 or 50 mg/kg i.v.) decreased the frequency of non-voiding contraction or sometimes abolished it and also decreased the voiding contractions, prolonging the interval of micturition (Fig. 3). Similarly, PPADS (a nonselective purinergic receptor antagonist, 10 mg/kg) decreased the non-voiding and voiding contractions, whereas it decreased only the non-voiding contractions in control rats (Fig. 4). Thus, the effects of A-317491 and PPADS were more evident in CYP-treated rats than in control rats. On the other hand, oxybutynin (1 mg/kg i.v.), a muscarinic receptor antagonist, did not significantly affect the non-voiding contractions or

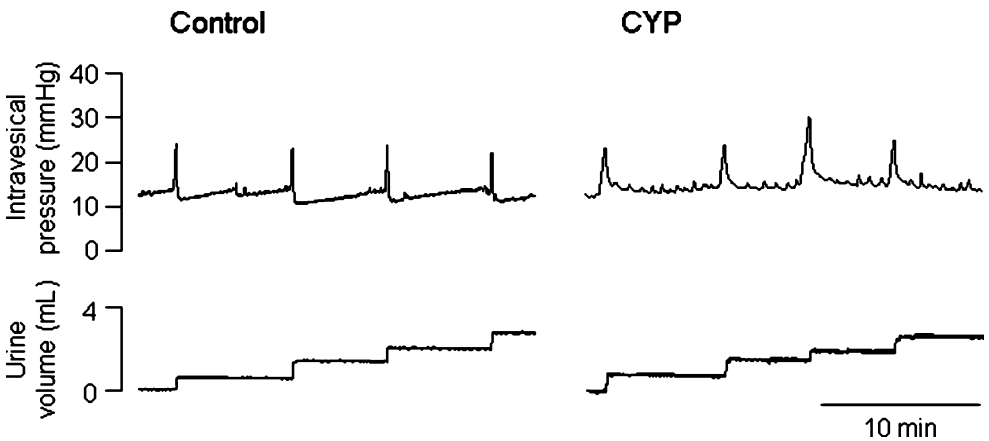

Fig. 1 Representative cystometry traces from control and CYP-treated rats. The records were taken 2 days after injection of saline (control) or CYP (150 mg/kg)

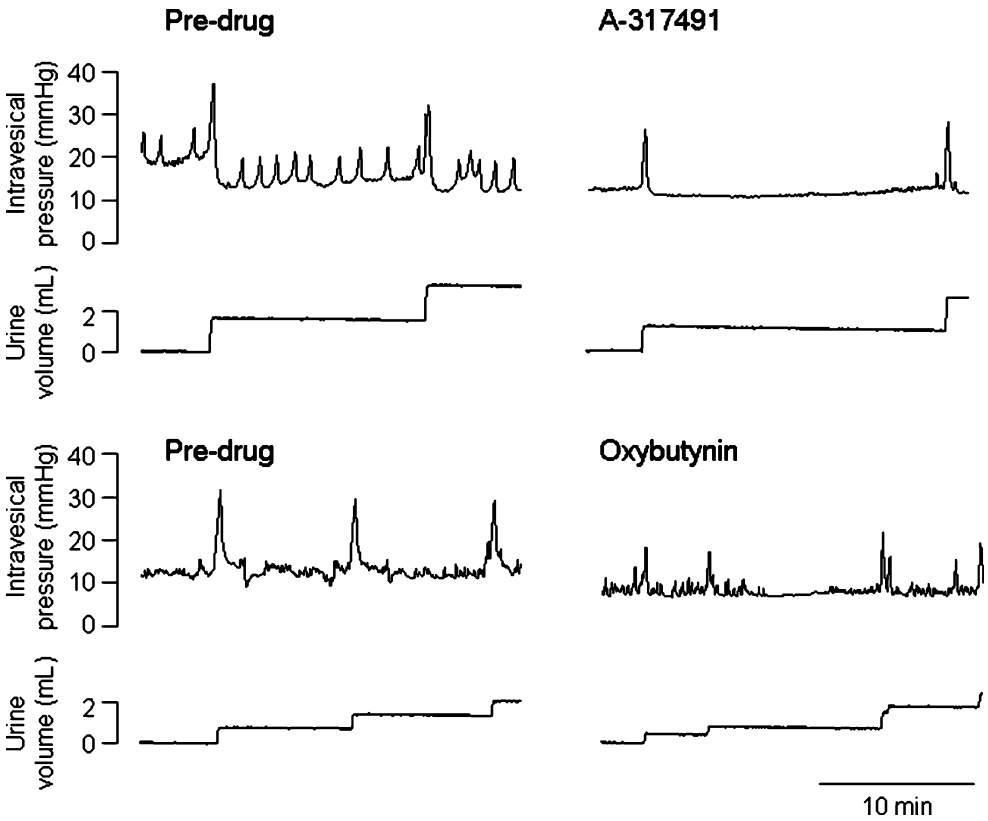

Fig. 2 Representative traces of the effects of A-317491 (50 mg/kg) and oxybutynin (1 mg/kg) on intravesical pressure and micturition in CYP-treated rats. Traces are from before (*left*) and after (*right*) administration of a drug

alter the interval of voiding contractions, but decreased the amplitude of voiding contractions in CYP-treated rats (Fig. 5).

CYP treatment increased the residual urine volume, which was measured just after the last micturition. A-317491 (20 and 50 mg/kg) and PPADS decreased the volume to control levels, while oxybutynin did not affect the residual urine volume (Fig. 6).

Discussion

In this study, we first tested the effect of A-317491, a putative $P2X_{2/3}$/$P2X_3$ antagonist, on bladder activity. Several types of P2X and P2Y receptor are found in the micturition pathway including the detrusor muscle, the urothelium, the afferent and efferent nerves, the spinal cord, and the pontine micturition center (Ruggieri 2006). Although the study using the cell expression system showed that A-317491 was highly selective to $P2X_{2/3}$ receptor and next to $P2X_3$ receptor (Jarvis et al. 2004), it has not been demonstrated that this compound specifically blocks $P2X_3$ or $P2X_{2/3}$ receptors when the dose used in this study is given to an intact animal. Therefore, we cannot exclude the possibility that this compound binds to other purinergic receptors and the blockade of these other receptors is at least partly responsible for the inhibitory effects observed in this study. As A-317491 prolonged the interval of micturition in CYP-treated rats, however, it is undoubtful that this compound acted on the micturition reflex pathway even if an action other than blockade of $P2X_3$ or $P2X_{2/3}$ receptors was also involved in the effects observed here.

P2X receptors are suggested to be involved in some kinds of nociception (Lewis et al. 1995; Cockayne et al. 2000; Souslova et al. 2000). A-317491 has been tested on inflammation-related hyperalgesia. Studies have shown that it inhibits Freund's complete adjuvant-induced pain at a dose of 10 or 30 mg/kg s.c. (Wu et al. 2004) and that it also attenuates thermal hyperalgesia and mechanical allodynia at 10–100 μmol/kg (5.67–56.7 mg/kg s.c., Jarvis et al. 2002). Thus, the dose of A-317491 used as an analgesic is close to the dose used in this study (20 or 50 mg/kg). Changes in sensory nerves are presumably involved in both hyperalgesia and bladder overactivity (Cockayne et al. 2000; Cruz 2004). The closeness of doses of A-317491 between two types of study suggests a possibility that this drug targets a similar site (sensory nerve) when it works as an analgesic or an anti-cystitic. In this respect, it will be interesting to test if A-317491 alleviates pelvic pain associated with cystitis.

CYP-treated rats exhibited increased urinary frequency when measured 2 days after injection. However, an increase in urinary frequency in CYP-treated rats was greater in the experiment that the micturition was measured in a conscious and unrestraint condition for 24 h than that measured for 1 h in the cystometry experiment. This may reflect the

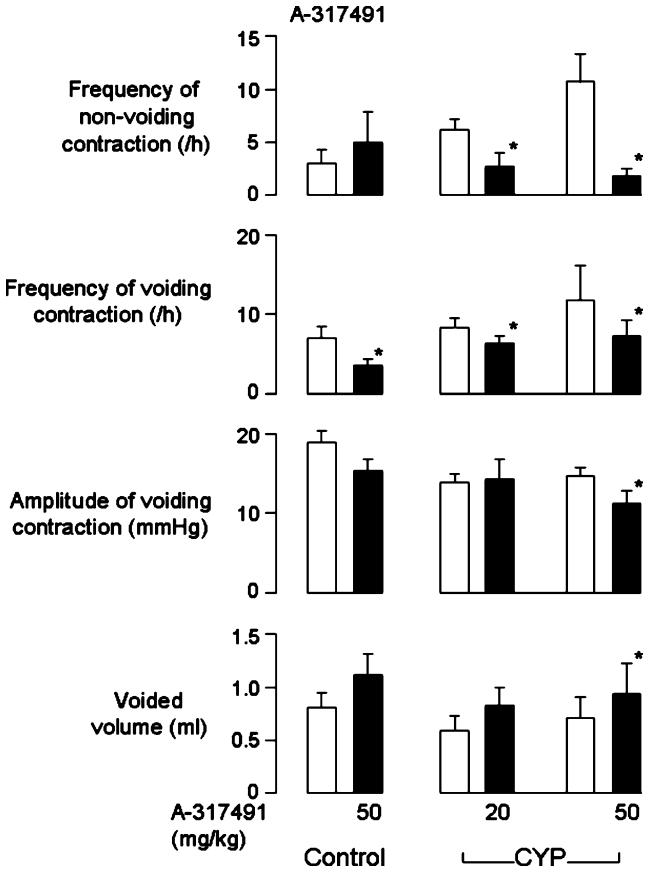

Fig. 3 Effects of A-317491 on cystometrogram parameters in control and CYP-treated rats. *Open columns* are before the administration of A-317491, and *filled columns* are after its administration. A-317491 (20 or 50 mg/kg) was administered to CYP-treated rats while only 50 mg/kg to control rats. Data are means ± SEM. *Significantly different from the pre-drug data (paired *t* test, $P < 0.05$). n=5–7. The data for non-voiding contractions were collected from animals that exhibited non-voiding contractions (n=4–6)

difference of experimental conditions; invasion from surgery, restraint stress, and restriction of water drinking in the latter experiment may influence the voiding behavior as suggested in some papers (Angelico et al. 2005; Million et al. 2007). In the cystometry experiment, rats treated with CYP exhibited signs of bladder overactivity; non-voiding contractions, and an increase in residual urine volume as well as increased urinary frequency. The effects of A-317491 on these parameters were similar to those of PPADS but were in contrast to those of oxybutynin. A-317491 and PPADS inhibited non-voiding contractions, increased the interval of micturition, and reduced the residual urine volume in CYP-treated rats, whereas oxybutynin did not affect these parameters except that it decreased the amplitudes of voiding contractions. Thus, P2X receptor antagonists seem to be more effective for bladder overactivity associated with CYP-induced cystitis than a muscarinic receptor antagonist. Non-voiding contractions are a characteristic of unstable bladder activity and may reflect the nerve activity underlying hyper-reflexia. Residual urine brings about an unpleasant sensation in patients with cystitis. Inhibition of non-voiding contractions, a prolongation of micturition interval, and a decrease in residual urine by a drug are clinically valuable for the alleviation of cystitis-related signs. Improvement of these signs by A-317491 confers benefits for the treatment of cystitis. The dose of oxybutynin used in this study was high enough to block the muscarinic receptor in the bladder on the basis of other papers in which oxybutynin decreased the amplitude of voiding contraction with a 50% inhibitory dose of 175 nmol/kg (0.69 mg/kg i.v.) in normal rats (Modiri et al. 2002) or inhibited the micturition reflex at 100 nmol/kg (0.39 mg/kg i.v.) in bladder outlet-obstructed rats (Pinna et al. 2005). Although there have been papers

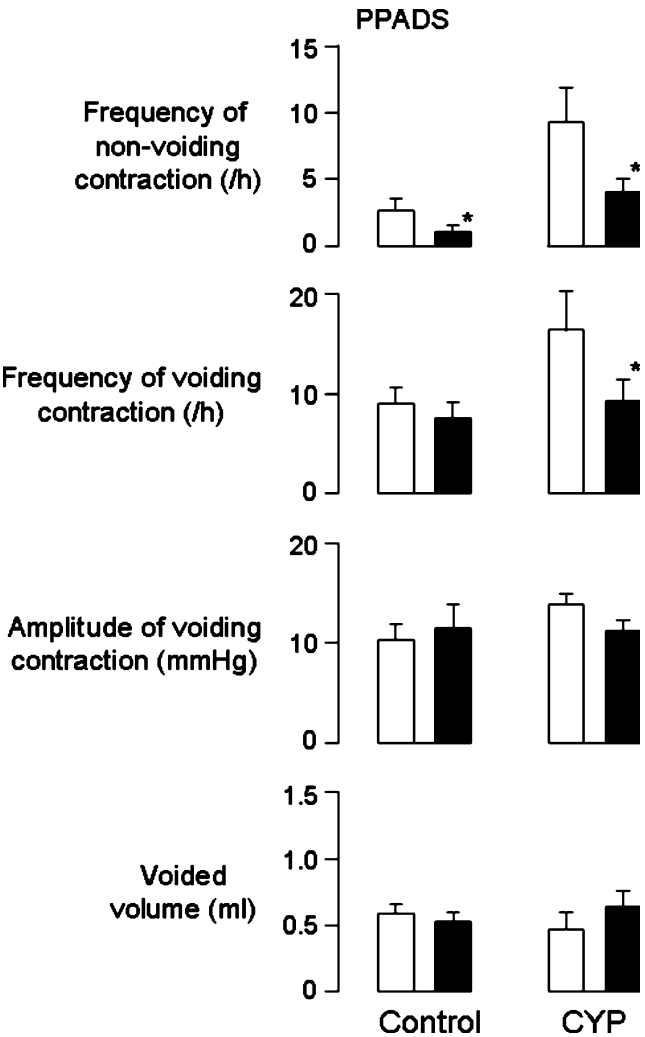

Fig. 4 Effects of PPADS (10 mg/kg) on cystometrogram parameters in control and CYP-treated rats. *Open columns* are before administration of PPADS, and *filled columns* are after its administration. Data are means ± SEM. *Significantly different from the pre-drug data (paired *t* test, $P < 0.05$). n=7–9 for control and CYP-treated groups, respectively. The data for non-voiding contractions were collected from animals that exhibited non-voiding contractions (n=6–7)

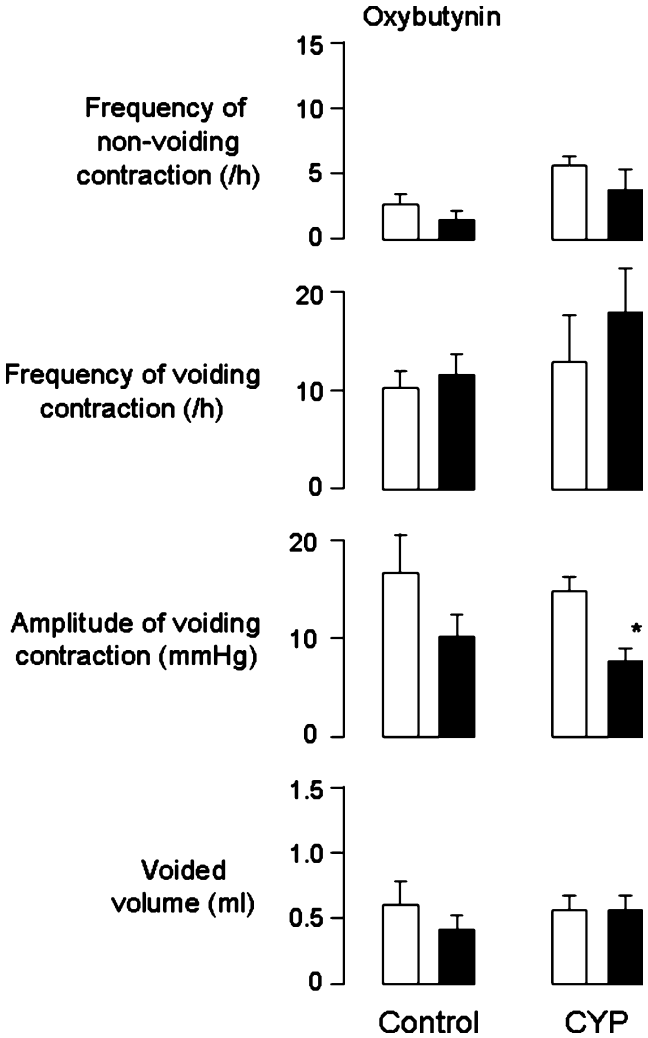

Fig. 5 Effects of oxybutynin (1 mg/kg) on cystometrogram parameters in control and CYP-treated rats. *Open columns* are before administration of oxybutynin, and *filled columns* are after its administration. Data are mean ± SEM. *Significantly different from the pre-drug data (paired *t* test, $P<0.05$). $n=6$–8 for control and CYP-treated group, respectively. The data for non-voiding contractions were collected from animals that exhibited non-voiding contractions ($n=6$ in each group)

showing that an anti-muscarinic drug increases the bladder capacity in rats with cerebral infarction (Suzuki et al. 2005) or resiniferatoxin-treated rats (Hedlund et al. 2007), a paper showed that the drug had no effect on the capacity (Angelico et al. 2005). Thus, the effect of anti-muscarinic drugs was variable depending on drug and type of disease. Oxybutynin has not been shown to be effective on CYP-induced bladder overactivity. The present data suggest that a muscarinic receptor antagonist does not substantially improve the signs observed in the CYP-induced cystitis. This study clearly shows that a selective P2X receptor antagonist has a therapeutic advantage over muscarinic receptor antagonists for the treatment of a CYP-induced bladder disorder.

In spite of similar effects on cystometric parameters produced by A-317491 and PPADS, there was a little difference between the effects of these drugs. A-317491 decreased the frequency of voiding contraction in control rats and reduced the amplitude of voiding contraction in CYP-treated rats, while PPADS did not exhibit such effects but decreased the frequency of non-voiding contraction in control rats. Based on the limited present data, it is difficult to discuss whether these differences are related to the receptor selectivity of these drugs. Furthermore, a higher dose of A-317491 (50 mg/kg) decreased the amplitude of voiding contractions. A possible mechanism for this inhibition is that A-317491 inhibited contraction of the detrusor muscle by antagonizing a P2X receptor in the muscle. However, this is unlikely because the main P2X receptor type responsible for detrusor smooth muscle contraction is $P2X_1$ (Rapp et al. 2005), and the affinity of A-317491 to $P2X_1$ receptor is much less than that to $P2X_3$ or $P2X_{2/3}$ receptor (Jarvis et al. 2002, 2004). Another possibility is that A-317491 inhibits release of transmitters that cause smooth muscle contraction by acting on afferent or efferent nerves. Undoubtedly, some points should be examined as to how differently A-317491 and PPADS affects the micturition reflex and detrusor activity, whether A-317491 directly inhibits contractions of detrusor muscle, and whether A-317491 affects release of ATP or acetylcholine. It is interesting that A-317491 and PPADS reduced the residual urine volume in CYP-treated rats, even when a higher dose of A-317491 (50 mg/kg) decreased the amplitude of voiding contraction. The data suggest that a P2X antagonist improves the voiding function of the bladder. Therefore, it should be examined whether A-317491 affects the outlet resistance of urethra.

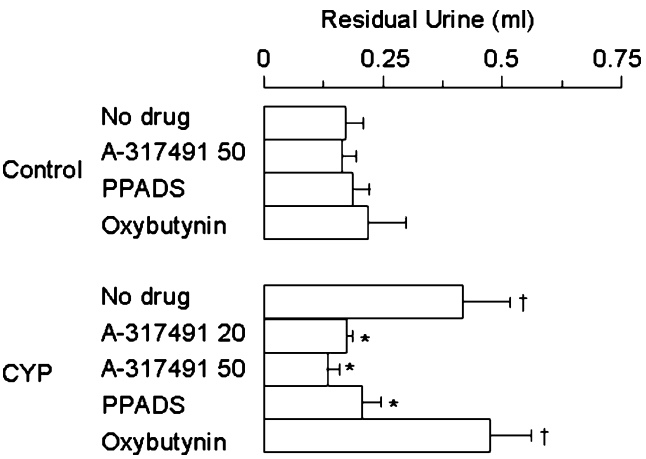

Fig. 6 Effects of A-317491, PPADS, and oxybutynin on the residual urine volume in control and CYP-treated rats. To CYP-treated rats A-317491 at 20 or 50 mg/kg was administered, while 50 mg/kg to control rats was administered. Data are mean ± SEM. *Significantly different from the data in control rats. *Dagger*: significantly different from the pre-drug data. $n=5$–10 for control group. $n=6$–7 for CYP-treated group

An important finding in this study is that A-317491 decreased the non-voiding contractions and the residual urine volume in CYP-treated rats, whereas it did not affect these parameters in control rats. These results suggest that an A-317491-sensitive mechanism is enhanced in CYP-induced cystitis, but that it does not function so much in noninflamed animals. One possibility inferred from the differential effects is that afferent C-fibers are activated during CYP-induced inflammation, and these are responsible for the overactivity. In the healthy condition, C-fibers have a high threshold for intravesical pressure and do not play an important role in the micturition reflex. Usually Aδ fibers work as a main mechano-sensitive afferent nerve that mediates the micturition reflex (de Groat 2006). When inflammation is present, however, C-fibers can be activated and become more sensitive to pressure (Yoshimura and de Groat 1999; Cruz 2004).

It has been shown that intravesically applied ATP enhances the micturition reflex (Pandita and Andersson 2002; Nishiguchi et al. 2005). Furthermore, ATP is suggested to stimulate C-fibers (Pandita and Andersson 2002; Rong et al. 2002), probably by acting on the $P2X_3$ or $P2X_{2/3}$ receptor (Cockayne et al. 2000; Zhong et al. 2003). Interestingly, $P2X_3$-null mice exhibit a bladder hyporeflexia, characterized by decreased voiding frequency and increased bladder capacity (Cockayne et al. 2000). Hence, it is possible that the $P2X_3$ or $P2X_{2/3}$ receptor is involved in the hyper-reflexia when C-fibers are activated. Although there has been no direct evidence that C-fibers are activated during CYP-induced cystitis, some papers have postulated that C-fiber sensitization is involved in CYP-induced bladder overactivity Yoshimura and de Groat (1999) reported that treatment of rats with CYP increased the excitability of C-fiber afferent nerves by suppressing A-type K channels. A paper showing that treatment of rats with capsaicin, which desensitizes afferent C-fibers, inhibited the overactivity during CYP-induced cystitis (Tsukimi et al. 2004) supports the possibility that the activation of C-fiber is involved in this model. Moreover, Smith et al. (2005) demonstrated that the release of ATP from the CYP-inflamed urothelium was increased. They also showed that inhibition of ATP release by botulinum toxin decreased non-voiding contractions in CYP-treated rats. These papers suggest that C-fibers are activated and that ATP plays a significant role in bladder overactivity induced by CYP. These form the rationale for the A-317491-induced improvements of the signs that appeared during CYP-induced cystitis. The present findings add more confirming evidence that P2X receptors are important for the enhanced micturition reflex.

It is still necessary to clarify where the expression of $P2X_3$ or $P2X_{2/3}$ receptor is increased and how the micturition reflex pathway is altered during CYP-induced cystitis. The present data obtained showed clear effects of A-317491 on cystitis-associated signs. To date, therapies aimed at blocking the function of the P2X receptor in the micturition pathway is limited to intravesical application of capsaicin or resiniferatoxin, which desensitizes the afferent C-fiber, or the same application of botulinum toxin, which inhibits the release of ATP (Cruz 2004; Smith et al. 2005). Application of these substances is neither easy nor prevalent because they are toxic and difficult to perform. If a selective P2X receptor antagonist is available, it may carve out a new strategy for the control of overactive bladder.

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