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RQ-00434739, a novel TRPM8 antagonist, inhibits prostaglandin E2-induced hyperactivity of the primary bladder afferent nerves in rats

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ABSTRACT

Aims: To examine the effects of RQ-00434739, a novel selective TRPM8 antagonist, on deep body temperature (DBT) and normal bladder sensory function and overactivity and its associated facilitation of mechanosensitive primary bladder single-unit afferent activities (SAAs) induced by intravesical L-menthol or prostaglandin E2 (PGE2) instillation in rats.

Main methods: The effect of RQ-00434739 on DBT was evaluated using intravenous administration of RQ-00434739 (1 mg/kg) or its vehicle under urethane anaesthesia. Cystometry (CMG) was performed on conscious and freely moving rats. SAAs were measured from the left L6 dorsal root under urethane anaesthesia, and the fibers were grouped as Aδ- or C-fiber based on their conduction velocity. For both CMG and SAA measurements, after baseline recording with saline instillation, further recording was performed with intravesical L-menthol (6 mM) or PGE2 (60 μM) instillation after pretreatment with intravenous RQ-00434739 (1 mg/kg) or its vehicle.

Key findings: RQ-00434739 did not significantly affect DBT. In CMG measurements, RQ-00434739 administration increased mean voided volume. Both L-menthol and PGE2 instillation decreased mean voided volume following vehicle pretreatment, whereas such effects were not observed following RQ-00434739 pretreatment. In SAA measurements, either L-menthol or PGE2 instillations increased SAAs of C-fibers, but not SAAs of Aδ-fibers, in the presence of vehicle. RQ-00434739 pretreatment significantly inhibited the L-menthol- and PGE2-induced activation of C-fiber SAAs.

Significance: The present results demonstrate that blockade of TRPM8 channels can inhibit the pathological activation of mechanosensitive C-fibers and suggest that RQ-00434739 may be a promising therapeutic drug candidate for bladder hypersensitive disorders without affecting DBT.

1. Introduction

Transient receptor potential melastatin 8 (TRPM8) channels respond to temperature changes ranging from innocuous to noxious cold (< 28 °C) as well as to chemical agents such as menthol and icilin [1,2]. TRPM8 channels are mainly expressed in primary sensory neurons (Aδ- and C-fibers in the dorsal root ganglion) [3], and TRPM8-knockout mice were lacking in temperature discrimination, noxious cold temperature sensation, injury-evoked hypersensitivity to cold after nerve injury and/or inflammation [4,5]. Therefore, TRPM8 channels may provide abnormal cold sensitivity under pathological conditions, resulting in neuropathic pain such as cold allodynia. We previously reported that TRPM8 channels may have physiological and pathophysiological roles in activating bladder afferent pathways mediated via mechanosensitive C-fibers [6,7], and it has been suggested that C-fibers fulfil a potentially important role in bladder sensory function in response to abnormal stimuli as 'silent' fibers [8–10]. In addition, the pathophysiological contribution of TRPM8 channels to bladder sensory (afferent) hyperactivity has also been proposed in rats and humans [11–14]. Thus, TRPM8 channels may be involved in the normal and abnormal sensory transduction of the bladder and would be a promising therapeutic target for hypersensitive bladder disorders. However, considering that TRPM8 channels are thermosensitive, it is possible that drugs acting on TRPM8 can influence body temperature [7], which may

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be unfavourable for clinical drug development. We recently developed a potent and selective TRPM8 antagonist, RQ-00434739 (RQ) [15]. This compound showed complete inhibition of icilin-induced wet-dog shaking in rats and robust efficacy in rat and monkey models of cold allodynia induced by the administration of oxaliplatin, a platinum-based chemotherapeutic agent [15].

Previous studies suggested that intravesical administration of prostaglandin E2 (PGE2) introduces the bladder hyperactivity via activation of EP1 receptors in human and rodents [16–18], and we previously demonstrated that intravesically administrated PGE2 significantly increased the bladder mechanosensitive afferent activity of C-fiber, but not Aδ-fiber, in the rat [19,20].

In the present study, to assess the pathophysiological roles of TRPM8 channels in hypersensitive bladder disorders and to determine whether RQ could be a therapeutic candidate for such disorders, we examined the effects of RQ on deep body temperature and normal bladder sensory function and bladder overactivity and its associated facilitation of mechanosensitive primary bladder afferent activities provoked by intravesical L-menthol and prostaglandin E2 (PGE2) instillation in rats.

2. Materials and methods

2.1. Animals

We used female Sprague Dawley rats (10–12 weeks old, 189–250 g, $N = 128$, Japan SLC, Inc. Shizuoka, Japan) in the present study. Rats were maintained under standard laboratory conditions with a 12/12 h light/dark cycle and free access to food and water. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Tokyo.

2.2. Deep body temperature measurements

Rats were anaesthetised with urethane (1.2 g/kg intraperitoneally) and placed on a heated blanket at 38 °C. Body temperature was measured using a temperature recorder (AX-KO4747-100, A&D Company Limited, Tokyo, Japan) by placing the thermal probe in the rectum. A PE-50 polyethylene catheter (Clay Adams, Parsippany, NJ, USA) was placed in the left jugular vein for intravenous (i.v.) drug administration. The measurements were repeated every minute before and after i.v. administration of RQ (1 mg/kg) or its vehicle for 60 min.

2.3. Cystometry measurements

Under anaesthesia with pentobarbital (40 mg/kg intraperitoneally), the bladder was exposed through a lower abdominal midline incision. A PE-50 catheter with a cuff was implanted into the bladder through the bladder dome and secured by a purse-string suture with a 5–0 silk thread. Another PE-50 catheter was placed in the left jugular vein for i.v. drug administration. Four days after the surgery, each rat was placed without restraint in a metabolic cage (3701 M081, Tecniplast, Varese, Italy) and allowed to adapt for 2 h prior to performing cystometry (CMG) at room temperature (22–24 °C).

To record intravesical pressure, the intravesical catheter was connected via a 3-way stopcock to a pressure transducer (DX-100, Nihon Kohden, Tokyo, Japan) and a syringe pump (KDS 200, Muromachi Kikai Co. Ltd., Tokyo, Japan). Intravesical pressure was measured using data acquisition software (PowerLab, ADInstruments, Sydney, Australia) at a sampling rate of 40 Hz. In addition, voided volume was measured using electric balance (GX-200, A&D Company Limited, Tokyo, Japan).

After 1 h of baseline recordings with intravesical saline instillation, i.v. RQ (1 mg/kg) or its vehicle was administered. After 5 min, saline or L-menthol (6 mM) or PGE2 (60 μM) was continuously instilled into the bladder for 1 h at a rate of 6 mL/h. We analysed the following CMG parameters: basal pressure, threshold pressure, maximum pressure and mean voided volume.

2.4. Afferent measurements

The rats were anaesthetised with urethane (1.2 g/kg intraperitoneally). Body temperature was maintained by a heated blanket at 38 °C. Single-unit afferent activity (SAA) measurements were performed as previously described [21,22]. In brief, after a pair of silver stimulation electrodes was placed around the left pelvic nerve, both L6 dorsal roots were cut near their entrance into the spinal cord. Unitary action potentials of mechanosensitive bladder afferent nerve fibers isolated from left L6 dorsal roots were identified as SAAs by electrical stimulation of the pelvic nerve and by bladder distension with saline instillation via a bladder catheter (PE-50). The SAAs were grouped based on their conduction velocity (CV), i.e. those with a CV of < 2.5 m/s were considered to correspond to C-fibers, whereas those with a CV of ≥ 2.5 m/s were considered to correspond to Aδ-fibers. SAAs and intravesical pressure were recorded during constant instillation of saline (6 mL/h) into the bladder. The filling continued until an intravesical pressure of 30 cmH_2O was achieved. The SAAs were expressed as firing rates (Hz) and evaluated in relation to intravesical pressure at each 5-cmH_2O interval.

At the beginning of the experiments, recording was repeated 2 or 3 consecutive times at 5-min intervals to evaluate reproducibility. Subsequently, i.v. RQ (1 mg/kg) or its vehicle was administered. Five minutes after administration, the recording was repeated 3 times during saline or L-menthol (6 mM) or PGE2 (60 μM) instillation (at a rate of 6 mL/h).

2.5. Drugs

For this study, we used RQ (synthesised by RaQualia Pharma Inc., Nagoya, Japan), 2-hydroxypropyl-beta-cyclodextrin (HP-β-CD, CycloChem Co., Ltd., Tokyo, Japan), NaOH (Wako Pure Chemical Industries, Ltd., Tokyo, Japan), L-menthol (Wako Pure Chemical Industries, Ltd.) and PGE2 (Cayman Chemical, Ann Arbor, MI, USA). RQ was dissolved in a solution of 0.1 mol/l NaOH:10 w/v% HP-β-CD in PBS (pH 7.4) = 2:98, and L-menthol was dissolved in saline. The stock solution of PGE2 (10^{-2} M) was prepared in absolute ethanol (stored at −80 °C) and diluted in saline just before use. The dose of RQ evaluated was selected based on our previous [15] and pilot studies, in which an antagonistic effect on icilin-induced wet-dog shaking behaviour with > 200 ng/mL plasma concentration of RQ was shown at 1 h after intravenous administration of 1 mg/kg.

2.6. Statistical analysis

All data are expressed as mean ± standard error of mean. Results were analysed using unpaired *t*-tests for comparisons between groups or paired *t*-tests or repeated measures one-way analysis of variance following Dunnett's test for comparison before and after drug administration in each group. *P* values of < 0.05 were considered statistically significant.

3. Results

3.1. Deep body temperature measurements

We used 14 rats for deep body temperature measurements in this study ($N = 7$ in each group). Mean deep body temperature of the rats was approximately 37.5 °C. Neither RQ nor its vehicle affected deep body temperature (Fig. 1).

3.2. CMG measurements

We used 36 rats for CMG measurements in this study ($N = 6$ in each

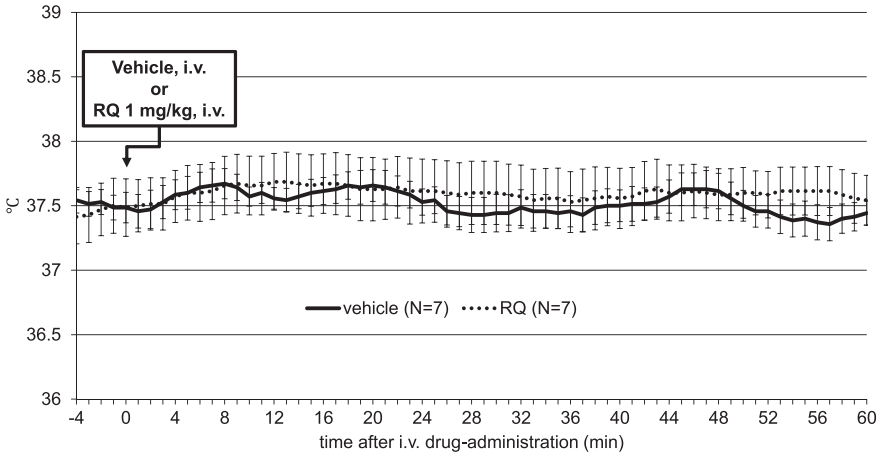

Fig. 1. Effects of i.v. administration of vehicle or RQ (1 mg/kg) on deep body temperature (intrarectal temperature) under urethane anaesthesia.
No significant differences were found before or after vehicle or RQ administration (repeated measures analysis of variance followed by Dunnett's test).
No significant differences were found between vehicle and RQ administration at each time-point (unpaired Student's *t*-test).

Table 1
Cystometric parameters before and after drug-administration (i.v.) during saline-(A), or L-menthol 6 mM (B), or PGE2 60 μM (C)-instillation under conscious free-moving conditions.

A	Groups (N = 6 in each group)	Saline-instillation (baseline)	Saline-instillation 5 min after vehicle or RQ i.v.-administration	(vs. baseline)
Basal pressure (cmH_2O)	Vehicle	2.37 ± 0.44	3.00 ± 0.56	(N.S.)
	RQ	3.32 ± 0.94	2.71 ± 0.46	(N.S.)
Threshold pressure (cmH_2O)	Vehicle	13.28 ± 1.76	11.89 ± 2.20	(N.S.)
	RQ	11.70 ± 1.25	12.56 ± 0.89	(N.S.)
Peak pressure (cmH_2O)	Vehicle	50.52 ± 4.41	46.73 ± 3.56	(N.S.)
	RQ	56.40 ± 3.03	55.00 ± 1.84	(N.S.)
Mean voided volume (mL)	Vehicle	1.58 ± 0.24	1.47 ± 0.23	(N.S.)
	RQ	1.39 ± 0.16	1.69 ± 0.10	(†)

B	Groups (N = 6 in each group)	Saline-instillation (baseline)	L-Menthol (6 mM)-instillation 5 min after vehicle or RQ i.v.-administration	(vs. baseline)
Basal pressure (cmH_2O)	Vehicle	2.76 ± 0.49	3.46 ± 0.65	(N.S.)
	RQ	3.31 ± 0.53	3.68 ± 0.61	(N.S.)
Threshold pressure (cmH_2O)	Vehicle	10.32 ± 1.30	7.39 ± 0.82	(†)
	RQ	11.28 ± 1.30	8.48 ± 1.45	(N.S.)
Peak pressure (cmH_2O)	Vehicle	39.03 ± 1.98	40.10 ± 2.57	(N.S.)
	RQ	37.15 ± 2.98	34.89 ± 5.22	(N.S.)
Mean voided volume (mL)	Vehicle	1.34 ± 0.06	0.86 ± 0.05	(‡)
	RQ	1.29 ± 0.25	1.19 ± 0.17##	(N.S.)

C	Groups (N = 6 in each group)	Saline-instillation (baseline)	PGE2 (60 μM)-instillation 5 min after vehicle or RQ i.v.-administration	(vs. baseline)
Basal pressure (cmH_2O)	Vehicle	2.78 ± 0.68	3.78 ± 0.81	(†)
	RQ	3.31 ± 0.80	2.86 ± 0.64#	(N.S.)
Threshold pressure (cmH_2O)	Vehicle	9.56 ± 1.03	7.99 ± 1.42	(N.S.)
	RQ	13.12 ± 0.79	7.87 ± 0.56	(‡)
Peak pressure (cmH_2O)	Vehicle	47.63 ± 4.89	50.72 ± 5.51	(N.S.)
	RQ	45.34 ± 7.07	55.05 ± 5.85	(†)
Mean voided volume (mL)	Vehicle	1.68 ± 0.15	0.81 ± 0.08	(‡)
	RQ	1.77 ± 0.18	1.36 ± 0.11###	(†)

Values are expressed as mean ± SEM.
N.S.: not significant difference.
† $P < 0.05$: from baseline in same group (paired Student's *t*-test).
‡ $P < 0.01$: from baseline in same group (paired Student's *t*-test).
# $P < 0.05$: from vehicle-treated group at each time point (unpaired Student's *t*-test).
$P < 0.01$: from vehicle-treated group at each time point (unpaired Student's *t*-test).
$P < 0.001$: from vehicle-treated group at each time point (unpaired Student's *t*-test).

group). During saline instillation, mean voided volume was significantly increased after RQ administration, but an increased response was not observed with vehicle administration (Table 1A). L-Menthol instillation, given after pretreatment with vehicle, significantly decreased threshold pressure and mean voided volume. In contrast, such decreased responses were not observed in rats pretreated with RQ, and change in mean voided volume was significant compared with those pretreated with vehicle (Table 1B and Fig. 2A). PGE2 instillation,

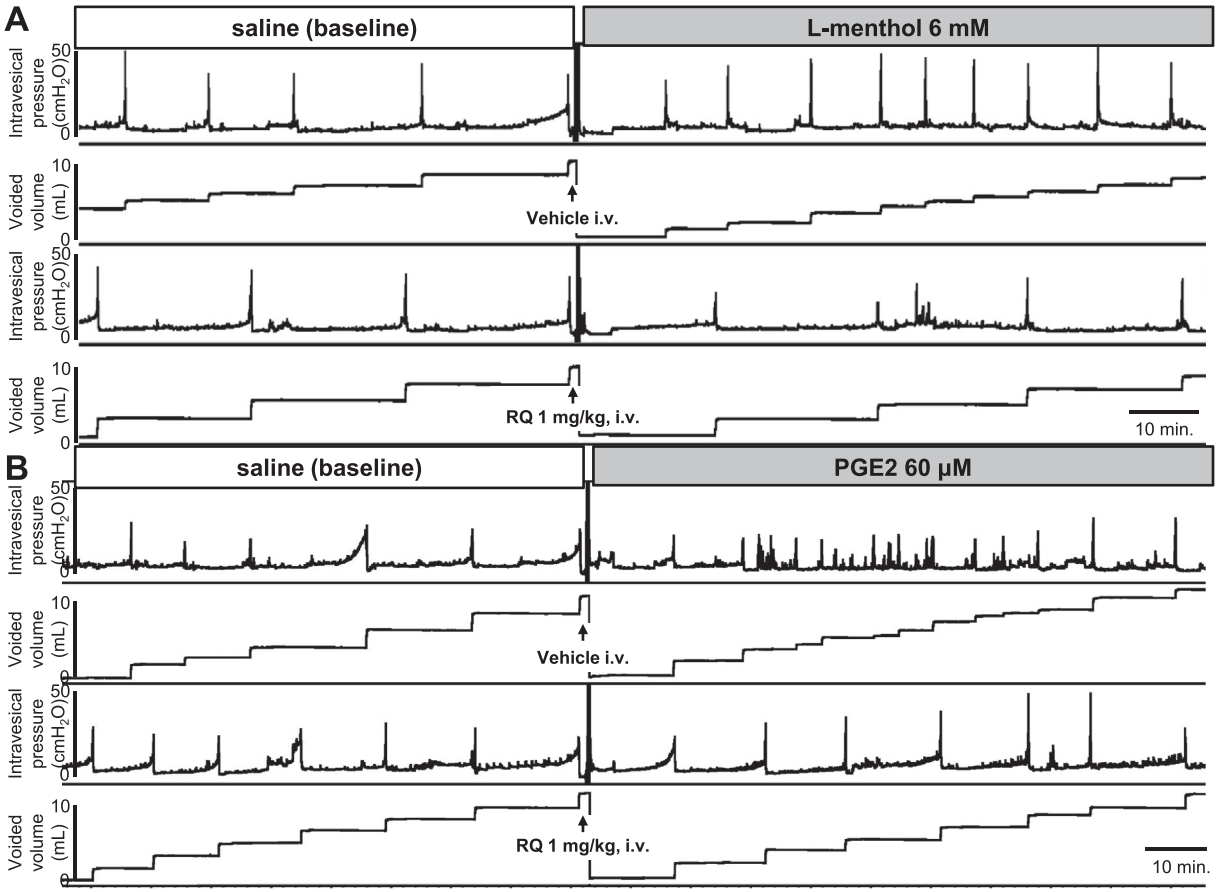

Fig. 2. Representative traces of CMG measurements before and after i.v. administration of vehicle or RQ (1 mg/kg) followed by L-menthol (6 mM) instillation (A) or PGE2 (60 μM) instillation (B).

given after pretreatment with vehicle, significantly increased basal pressure and decreased mean voided volume, whereas such increased and decreased responses were significantly inhibited by RQ pretreatment (Table 1C and Fig. 2B). In addition, rats pretreated with RQ had decreased threshold pressure and increased peak pressure during PGE2 instillation, but these changes were not significantly different from the vehicle-pretreated group (Table 1C).

3.3. Afferent measurements

Ninety-six single afferent fibers (n = 48 in each fiber, CVs: Aδ-fibers: 4.73 ± 0.43 m/s, C-fibers: 1.65 ± 0.05 m/s) were isolated from 78 rats. During saline instillation, SAAs of C-fibers, but not SAAs of Aδ-fibers, were significantly decreased following RQ administration, which was significant compared with those following vehicle administration (Figures 4AB). In the presence of vehicle, L-menthol instillation significantly increased the SAAs of C-fibers, whereas SAAs of Aδ-fibers were not significantly changed. Pretreatment with RQ significantly inhibited the L-menthol-induced activation of C-fiber SAAs (Figs. 3A and 4C, D). PGE2 instillation given after pretreatment with vehicle also significantly increased the SAAs of C-fibers, but not SAAs of Aδ-fibers. Pretreatment with RQ significantly inhibited such increased responses of SAAs of C-fibers (Figs. 3B and 4E, F).

4. Discussion

The present study demonstrated that i.v. administration of RQ (1 mg/kg) significantly increased mean voided volume and inhibited SAAs of C-fibers during saline instillation, but this dose of RQ (1 mg/kg) did not influence body temperature, and this point may be an advantage for clinical development. Unlike those in cats [8,9], C-fibers in rats respond to normal bladder distension similar to Aδ-fibers [10]. Thus, these results suggest that RQ has an inhibitory effect on physiological bladder sensory activation via suppression of mechanosensitive C-fibers in rats. We also used CMG measurements to confirm that L-menthol instillation showed decreased mean voided volume, suggesting that L-menthol induced frequent voiding in rats. In our previous study [7], we reported similar results that intravesical instillation of L-menthol at 3 mM, which was lower than that in the present study (6 mM), transiently decreased mean voided volume. Previous reports have indicated that L-menthol has agonistic effects not only for TRPM8 channels but also for TRPA1 and TRPV3 channels [23,24]. Although L-menthol is still categorised as a TRPM8 agonist, several reports have demonstrated that frequent voiding was induced by L-menthol instillation in rodents [25,26]. The present study showed that RQ counteracted L-menthol-induced frequent voiding. RQ was a potent antagonist of TRPM8 channel (IC_{50} = 14 nM) and is at least > 100-fold selective over other ion channels (TRPA1, TRPV1, TRPM2, Nav1.3, Nav1.5, Nav1.7, Cav2.2 and Cav3.2) [15]. In addition, this RQ exhibited antagonistic effects for icilin-induced wet-dog shaking in rats and for oxaliplatin-induced cold hypersensitivity in rats and monkeys [15]. Therefore, it is conceivable that L-menthol-induced frequent voiding, which was counteracted by RQ, was mediated via TRPM8 channels. Moreover, the present results of SAA measurements revealed that intravesical L-menthol instillation after pretreatment with vehicle showed SAA hyperactivity of C-fibers, but not those of Aδ-fibers. In addition, RQ inhibited the hyperactivity of C-fiber SAAs induced by L-menthol instillation. The present results suggest that TRPM8 channels play a role in activating bladder afferent pathways via mechanosensitive C-fibers; this suggestion is clearly consistent with the first

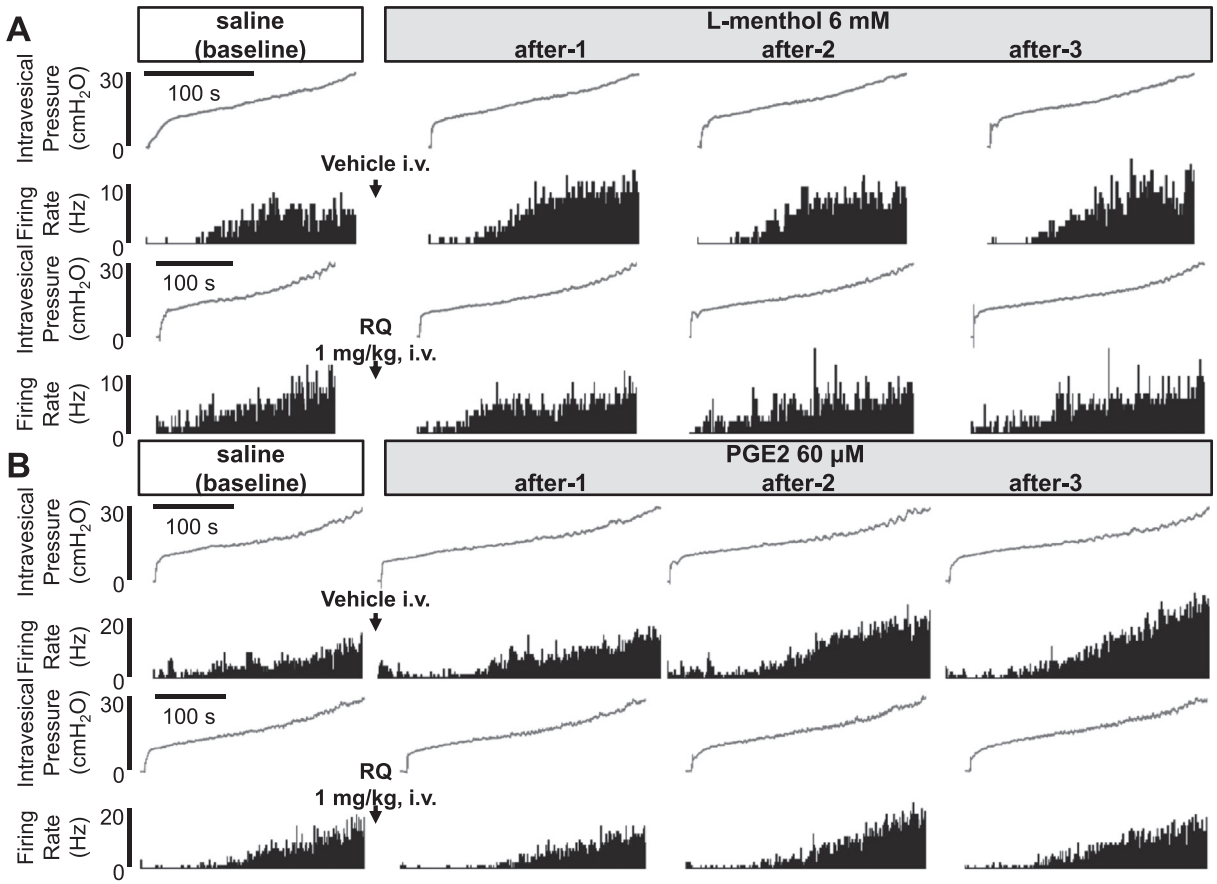

Fig. 3. Representative traces of the intravesical pressure and firing rate of C-fiber SAAs before and after i.v. administration of vehicle or RQ (1 mg/kg) followed by L-menthol (6 mM) instillation (A) or PGE2 (60 μM) instillation (B).

results of the CMG and SAA measurements with saline instillation in our study. In addition, we previously reported by measuring in vivo and ex vivo afferent activities that other TRPM8 antagonists inhibited activations of C-fiber SAAs [6,7], and the present results with RQ are in line with these previous results.

Compared with Aδ-fibers, C-fibers may fulfil a potentially different role in bladder sensory function in response to abnormal stimuli as 'silent' fibers [8–10]. We previously showed that intravesical instillation of PGE2 selectively exaggerated mechanosensitive C-fiber SAAs in rats [19,20]. Consistent with these previous studies, our study results demonstrated that intravesical PGE2 instillation selectively exaggerated mechanosensitive C-fiber SAAs. Interestingly, RQ inhibited hyperactivity of C-fiber SAAs induced by PGE2 instillation. Furthermore, in the CMG measurements, we showed that PGE2 instillation demonstrated frequent voiding, which was inhibited by pretreatment with RQ. To our knowledge, these results demonstrate for the first time that TRPM8 channel blockade can inhibit bladder sensory hyperactivity induced by PGE2 instillation via mechanosensitive C-fiber in rats. In our recent study, another selective TRPM8 antagonist, KPR-2579, inhibited acetic acid-induced bladder hyperactivity in the CMG measurements and inhibited the hyperactivity of C-fiber SAAs induced by acetic acid instillation, suggesting that antagonising TRPM8 channels can ameliorate abnormally exaggerated bladder mechanoafferent transduction [6]. Our results further support the possibility of TRPM8 channel antagonists to use for C-fiber mediated bladder sensory hyperactivity.

The present study has several limitations regarding the action sites of TRPM8 channel antagonists and mechanisms of interaction with PGE2-induced bladder hyperactivity. Although it is reasonable to consider that RQ counteracted L-menthol-induced bladder hyperactivity via blocking TRPM8 channel, we cannot determine whether the pathway of the inhibitory effects of RQ on PGE2-induced bladder hyperactivity is identical to that on L-menthol. A previous study by using immunohistochemistry demonstrated that the expressions for all EPs receptors (EP1–4) were intense in the urothelium, and intermediate to low in the muscle and the suburothelial layers regardless of gonadal status or gender in canine bladder [27], and several studies suggest that complex signal interactions occur within the urothelial and suburothelial layers in the bladder involving tachykinins, ATP, acetylcholine, nitric oxide, and PGE2 [16,18,28]. Further investigations are needed as TRPM8 channels are expressed on the urothelium in the bladder [29] and dorsal root ganglion neurons of the rat [11,29,30]. In addition, we used female rats, but did not check the estrous cycle in the present study. In a recent study, under normal physiological conditions, concentrations of regulatory neuropeptides related to afferent function (calcitonin gene related peptide, substance P, nerve growth factor, and brain derived neurotrophic factor) were similar in the urinary bladder and its afferent pathways (dorsal root ganglion and spinal cord) during all phases of the estrous cycle [31]. To our knowledge, there has been no study on the relationship between the estrous cycle and TRPM8 channel and/or intravesical PGE2 instillation in rats. These points may need to be addressed as further investigations.

5. Conclusions

The present results demonstrate that TRPM8 channels play a role in physiological activation of mechanosensitive C-fibers in rats, and the present results also support a pathophysiological role for bladder TRPM8-mediated pathways in experimental bladder hypersensitivity provoked by PGE2. These findings and suggest that RQ, a novel TRPM8 antagonist, may be a promising drug for treating bladder sensory

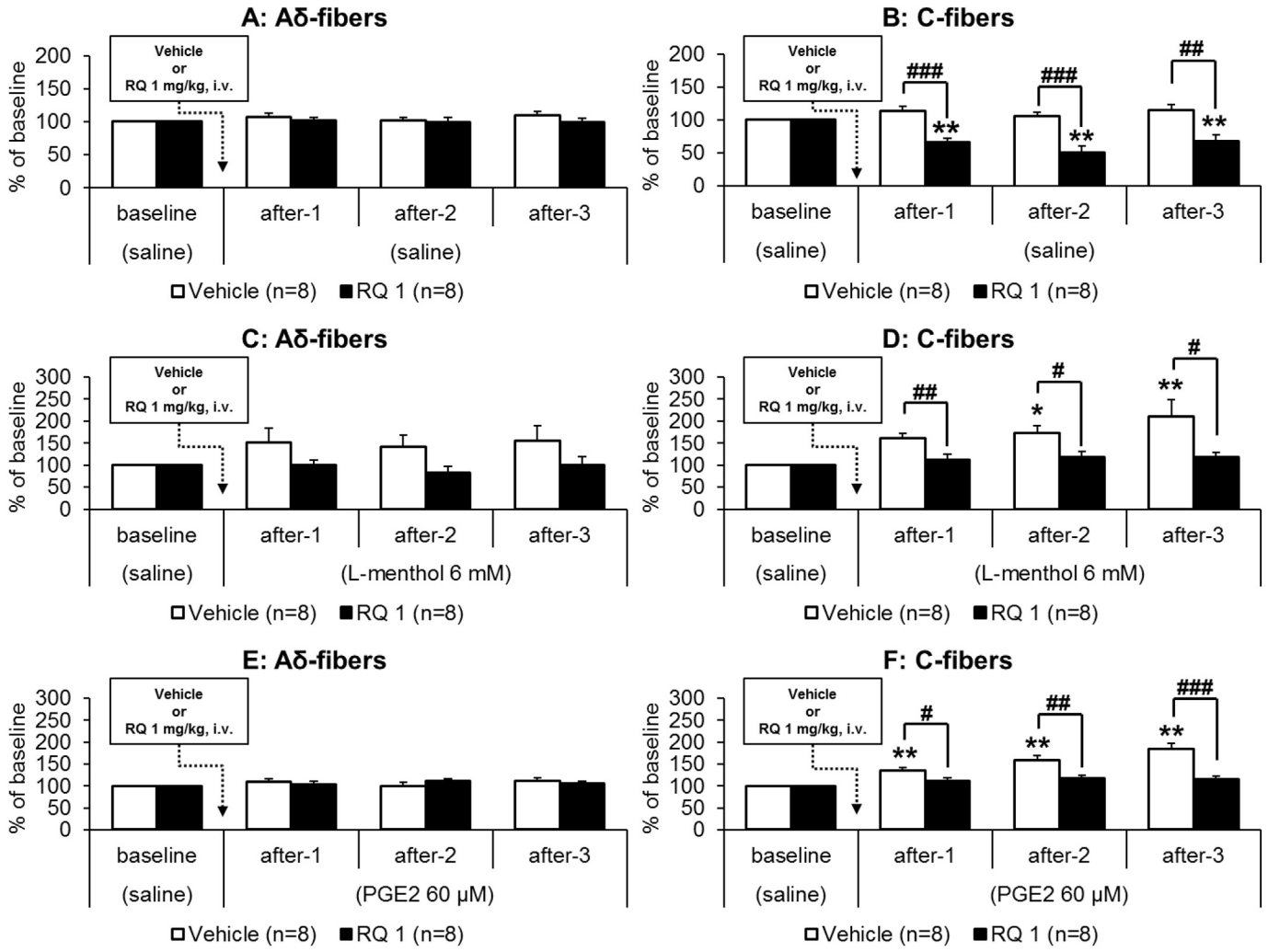

Fig. 4. Effects of RQ (1 mg/kg) on SAAs of Aδ-fibers (left) and those of C-fibers (right) during the instillation of saline (A and B), L-menthol (6 mM) (C and D), or PGE2 (60 μM) (E and F).
Values are expressed as mean ± standard error of mean.
* $P < 0.05$, ** $P < 0.01$ from baseline in the same group (repeated measures analysis of variance followed by Dunnett's test).
# P < 0.05, ## P < 0.01, ### $P < 0.001$ between groups (unpaired Student's *t*-test).

disorders without affecting body temperature.

Conflict of interest

NA, HO, and SW report no conflict of interest. HK has received consultancy honoraria and/or research support from Astellas, Bristol-Myers Squibb, Ono, Pfizer, Sanofi-Aventis, and Takeda. YH has received consultancy honoraria from Astellas, Daiichi-Sankyo, Integral, Nippon Shinyaku, and Takeda. YI has received consultancy honoraria and/or research support from Astellas, Kissei, and Kyorin.

Author contributions

NA and YI conceived and designed research; NA performed experiments, analysed data, and drafted the manuscript; HO, SW, KH, HY and YI revised and edited the manuscript.

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