The role of TRPM8 in pelvic afferent activity from rat urinary bladder

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Abstract

The aim of this study was to explore the potential role of the transient receptor potential cation channel subfamily M member 8 (TRPM8) in the transmission of cool sensations and the regulation of the micturition reflex. The findings of the present study indicate that the TRPM8 protein is expressed in a highly specific subset of small-diameter C-fiber neurons within the dorsal root ganglia (DRG). Notably, the TRPM8 protein was not detected in hydroxystilbamidine (Fluoro-Gold, FG) -labelled bladder-projecting afferent neurons. In functional experiments, pelvic afferent nerve activity showed no significant differences in the responses to intravesical saline infusion at 37℃ and 25℃. Moreover, there were no significant differences in pelvic afferent nerve activity between intravesical infusions of saline and menthol (0.3-1.0 mM). These results suggest that TRPM8 contributes minimally, if at all, to the modulation of the bladder afferent nerve pathway activity involved in the control of the micturition and bladder-cooling reflexes in normal rats.

Keywords
Transient receptor potential cation channel subfamily M member 8
Dorsal root ganglion
Urinary bladder

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1. Introduction

Mammals rely on thermosensation and temperature regulation for survival; however, the cellular and molecular mechanisms that enable the detection of low temperatures remain incompletely understood. Insights into cold perception have been gained through the use of cooling agents such as menthol and icilin, which elicit a distinct sensation of cold (Bodding et al., 2007). The transient receptor potential (TRP) channel family plays a pivotal role in sensory transduction, including the detection of temperature. Several TRP channels with thermosensitive properties have been identified. Among them, TRPM8 is a non-selective, outwardly rectifying cation channel activated by temperatures at or below 28℃ and by cooling compounds such as menthol and icilin (McKemy et al., 2002, Peier et al., 2002).

Clinically, cooling stimuli are known to induce uninhibited bladder contractions in patients with overactive bladder, a phenomenon that has historically been used as a diagnostic indicator (Bors and Blinn, 1957; Al-Hayek and Abrams, 2010). The afferent nerve pathways innervating the urinary bladder comprise myelinated Aδ-fibers and unmyelinated C-fibers, which originate from small- to medium-diameter dorsal root ganglion (DRG) neurons located primarily in the L6 and S1 segments in rats (de Groat et al., 1981; Mallory et al., 1989; Vera and Nadelhaft, 1990, 1992). The bladder-cooling reflex is exaggerated in animals and humans with overactive bladder, reflecting the hyperexcitability of C-fiber afferent pathways (Mazières et al., 1998).

TRPM8, a member of the TRP channel superfamily, has been identified as a molecular sensor of environmental cold and cooling compounds such as menthol and icilin (McKemy et al., 2002; Tsukimi et al., 2005; Dhaka et al., 2007; Nomoto et al., 2008). Regarding urinary function, there have also been reports that TRPM8 channel activates bladder afferent fibers and induces bladder overactivity (Aizawa and Fujita, 2022); however, the underlying mechanisms remain to be fully elucidated.

The present study aimed to elucidate the potential role of TRPM8 in the transmission of cool sensation and in the modulation of the micturition reflex. Specifically, we investigated whether TRPM8 is genuinely expressed in DRG neurons innervating the urinary bladder, and whether pelvic afferent nerve activity from the bladder is influenced by cold stimuli, including intravesical infusion of chilled saline or menthol.

2. Results

2.1. Expression of TRPM8 in dorsal root ganglia (DRG)

Afferent pathways projecting to the urinary bladder consist of myelinated Aδ- and unmyelinated C-fibers, which originate from medium- and small-diameter neuronal populations in the dorsal root ganglia (DRG), respectively (Figs. 2 and 3). TRPM8 mRNA was detected in a small subset of small-diameter DRG neurons. More than 90% (198/202) of TRPM8 mRNA-positive neurons had somatic diameters of less than 30 μm (Fig. 1A, B).

In immunohistochemical analyses, TRPM8-immunoreactivity was also predominantly observed in small-diameter DRG neurons. Moreover, TRPM8 mRNA and protein were co-localized within individual small-diameter DRG neurons (Fig. 1C). This consistent expression pattern of TRPM8 channels in DRG neurons suggests that TRPM8 may participate in the transmission of sensory information mediated by small-diameter afferent pathways.

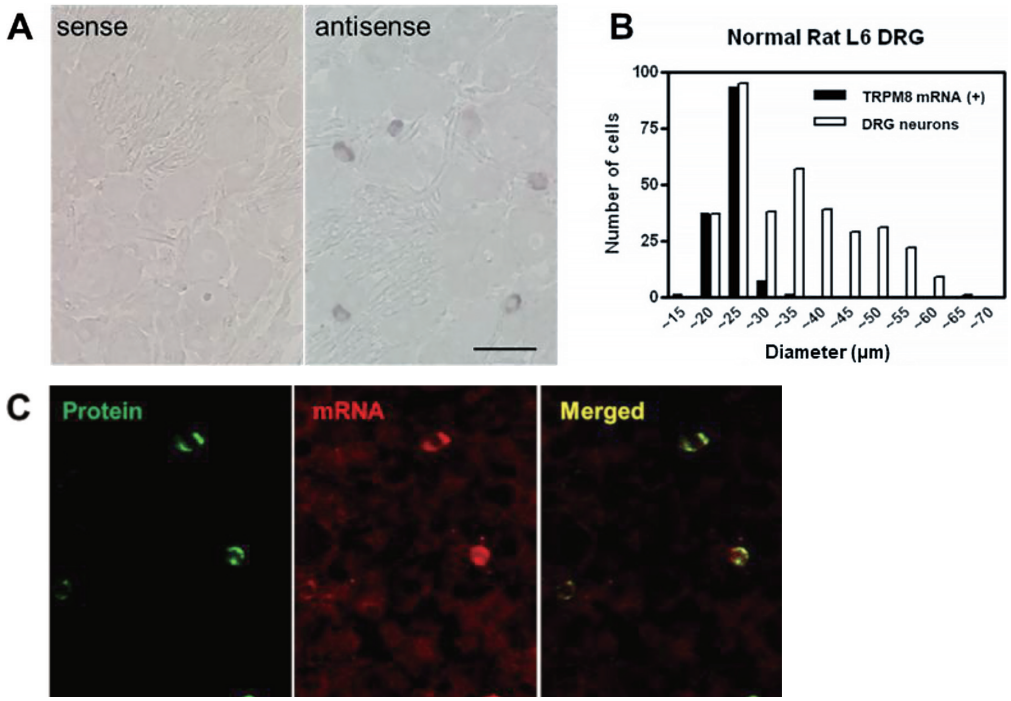

Figure 1 Expression of TRPM8 in dorsal root ganglion (DRG) neurons

(A) Rat DRG sections hybridized with TRPM8 sense and antisense RNA probes.

(B) Cell size distributions of DRG neurons, with filled and open bars representing TRPM8 mRNA–positive cells and total neurons, respectively.

(C) Co-expression of TRPM8 mRNA and protein in rat DRG neurons. Scale bars, 50 μm.

2.2. Expression of TRPM8 and other neuronal markers in the DRG

Co-expression of TRPM8 protein was not detected with the peptidergic neuronal marker calcitonin gene-related peptide (CGRP) (Figs. 2 and 4A) or with the non-peptidergic C-fiber marker isolectine B4 (IB4) (Fig. 3). Furthermore, TRPM8 was not co-expressed with the transient receptor potential cation channel subfamily V member 1 (TRPV1), another TRP family channel that is expressed in small- to medium-diameter DRG neurons and mediates warm and noxious heat sensations (Fig. 4B). These findings indicate that TRPM8 is expressed in a distinct subpopulation of small- diameter C-fiber neurons within the dorsal root ganglion.

2.3. Expression of TRPM8 in DRG neurons innervating the urinary bladder

TRPM8 immunoreactivity was not observed in bladder-projecting afferent neurons that were retrogradely labelled with Fluoro-Gold (FG) (Figs. 2 and 3; n = 7 rats, 42 sections from 7 DRGs). These morphological observations suggest that TRPM8 contributes minimally, if at all, to the modulation of bladder afferent activity during the micturition reflex or the bladder-cooling reflex.

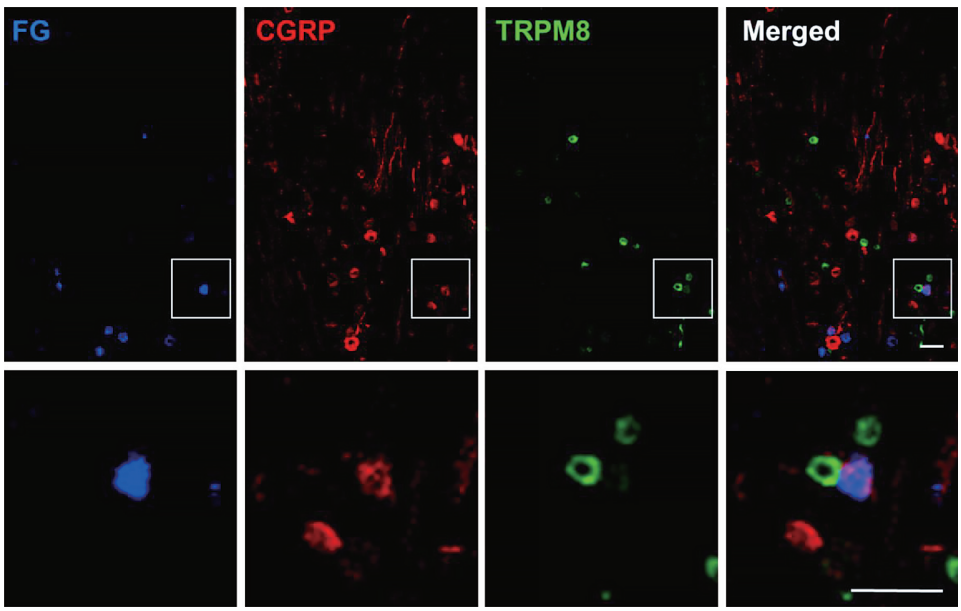

Figure 2　Expression of TRPM8 protein in Fluoro-Gold (FG)-labelled dorsal root ganglion (DRG) neurons innervating the urinary bladder

TRPM8 protein was rarely expressed in FG-labeled bladder afferent neurons. Calcitonin gene-related peptide (CGRP) was detected in some FG-labeled neurons; however, it was not co-expressed with TRPM8. Each lower panel shows an enlarged image of the area enclosed by the square in the corresponding upper panel. Scale bars, 50 μm.

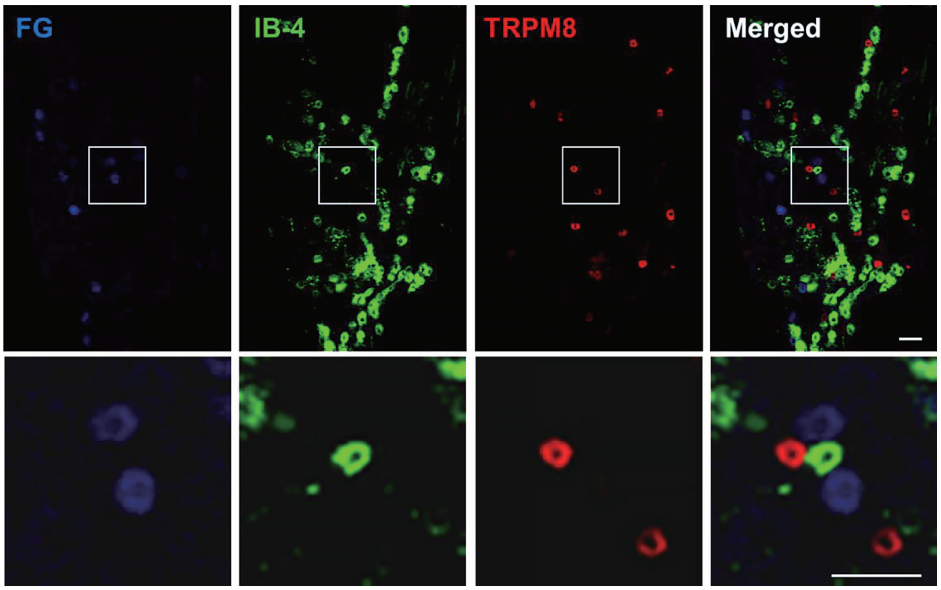

Figure 3 Expression of TRPM8 protein in dorsal root ganglion (DRG) neurons innervating the urinary bladder

TRPM8 protein was rarely expressed in Fluoro-Gold (FG) –labeled bladder afferent neurons. The non-peptidergic C-fiber marker isolectin B4 (IB4) was not expressed in FG-labeled bladder afferent neurons, nor was it co-expressed with TRPM8. Each lower panel shows an enlarged image of the area enclosed by the square in the corresponding upper panel. Scale bars, 50 μm.

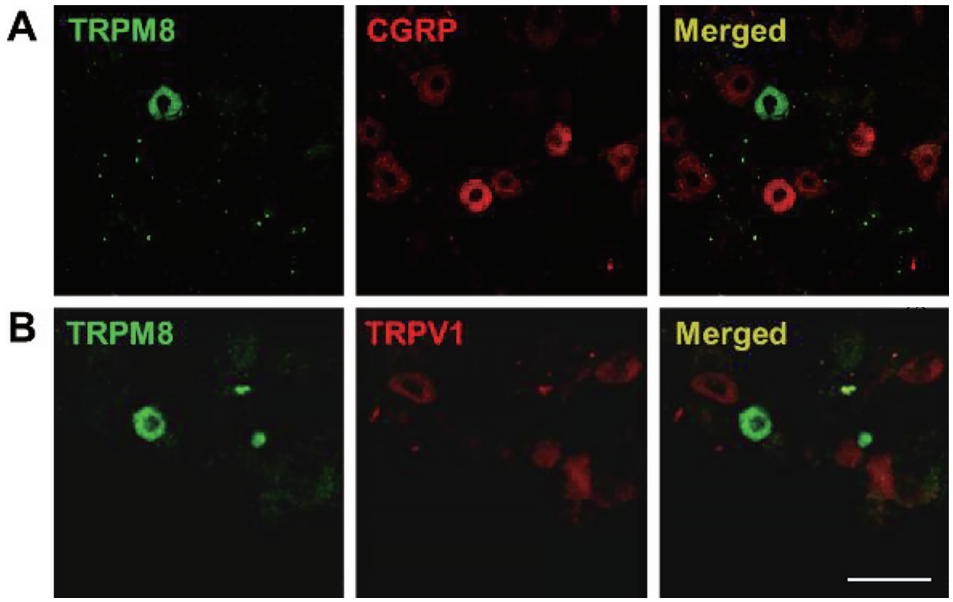

Figure 4 Expression of TRPM8, CGRP and TRPV1 in dorsal root ganglion (DRG) neurons

(A) TRPM8 was rarely co-expressed with calcitonin gene-related peptide (CGRP).

(B) TRPM8 was rarely co-expressed with transient receptor potential vanilloid 1 (TRPV1). Scale bar, 50 μm.

2.4. Pelvic afferent nerve activity from the urinary bladder

2.4.1. Effect of temperature on pelvic afferent nerve activity responding to intravesical infusion of saline

Two types of pelvic afferent nerve activity were observed in the urinary bladder: one showing a tonic discharge (Fig. 7) and the other exhibiting a phasic discharge in response to intravesical saline infusion (Fig. 6). At the onset of intravesical saline infusion at 37℃, intravesical pressure gradually increased and was maintained at approximately 20 cmH_2O. After termination of the infusion, intravesical pressure gradually decreased but remained at around 10 cmH_2O. During the infusion at 37℃, pelvic afferent nerve activity, which exhibited a mixed pattern of tonic and phasic discharges, increased and was sustained during the period of constant intravesical volume (Fig. 5). Following drainage, pelvic afferent nerve activity returned to the pre-infusion level. When the same protocol was repeated at 25℃, pelvic afferent nerve activity again increased and was sustained during constant intravesical volume (Fig. 5), and returned to the baseline level after drainage. No significant differences were detected in pelvic afferent nerve responses to intravesical saline infusion at 37℃ compared with those at 25℃. (Fig. 5).

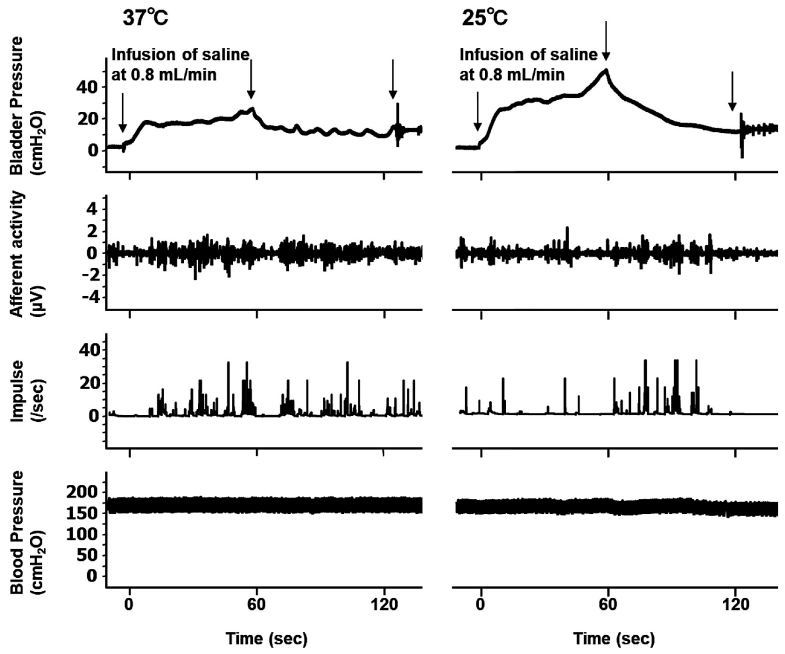

Figure 5 Representative responses of pelvic afferent nerve activity from the bladder to intravesical infusions of saline at 37℃ and 25℃

The first arrow indicates the onset of infusion, the second arrow indicates the termination of infusion, and the third arrow indicates drainage. The onset of infusion is defined as time 0 s.

2.4.2. Effect of menthol on pelvic afferent nerve activity responding to intravesical infusion of saline

At the onset of intravesical saline infusion, intravesical pressure progressively increased, whereas after termination of the infusion, it gradually decreased; however, a certain pressure level was maintained during the period of constant intravesical volume. Concurrently, pelvic afferent nerve activity exhibited both phasic (Fig. 6) and tonic (Fig. 7) elevations during intravesical saline infusion and remained elevated during the period of constant intravesical volume. The responses observed during intravesical infusion of saline containing menthol at concentrations of 0.3 to 1.0 mM were comparable to those observed during saline infusion without menthol. Similar results were consistently obtained in all six rats tested, indicating that there was no significant difference in pelvic afferent nerve activity between the absence and presence of menthol (0.3–1.0 mM).

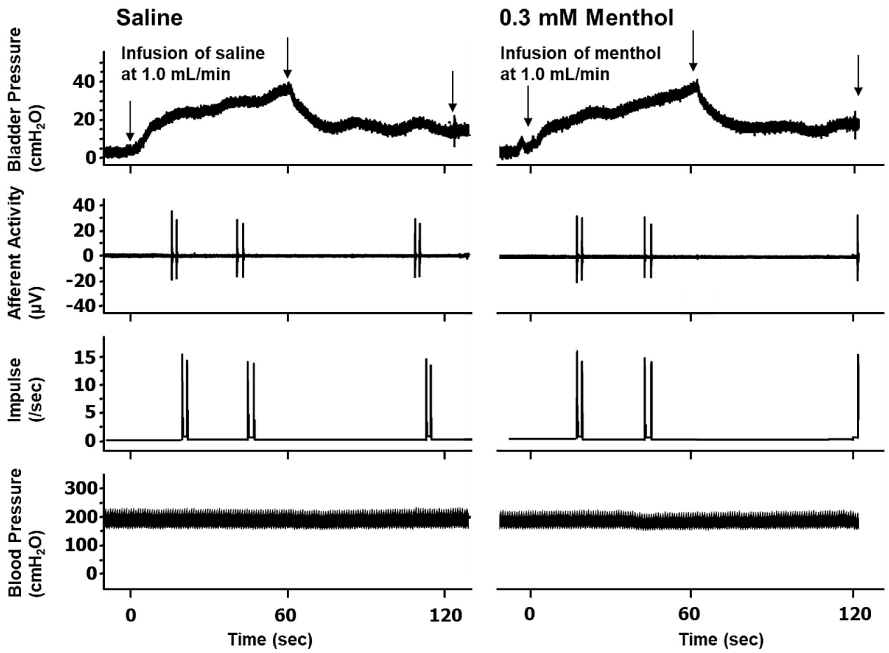

Figure 6 Representative response of pelvic afferent nerve activity to intravesical infusion of 0.3 mM menthol compared with saline

The phasic activity of afferent nerves from the urinary bladder in response to intravesical infusion of 0.3 mM menthol did not differ from that observed during saline infusion. The first arrow indicates the onset of infusion, the second arrow indicates the termination of infusion, and the third arrow indicates drainage. The onset of infusion is defined as time 0 s.

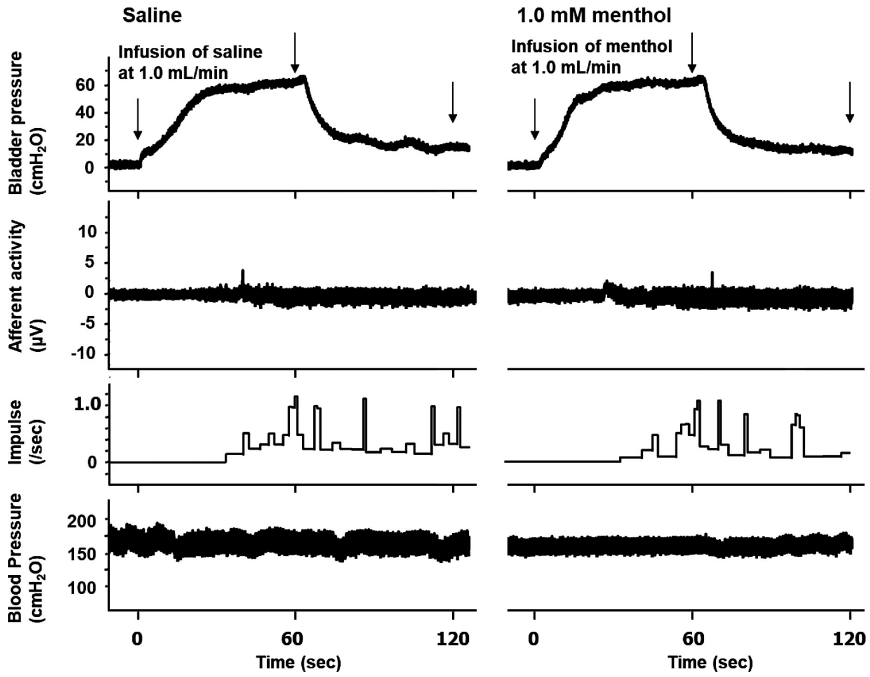

Figure 7　Representative response of pelvic afferent nerve activity to intravesical infusion of 1.0 mM menthol

The tonic activity of afferent nerves from the urinary bladder in response to intravesical infusion of 1.0 mM menthol did not differ from that observed during saline infusion. The first arrow indicates the onset of infusion, the second arrow indicates the termination of infusion, and the third arrow indicates drainage. The onset of infusion is defined as time 0 s.

3. Discussion

The present results indicate that TRPM8 is expressed in a specific subpopulation of small-diameter C-fiber neurons in the dorsal root ganglia (DRG) that do not project to the urinary bladder. Hayashi et al. (2009) reported that TRPM8 immunoreactivity was detected in a small proportion of wheat germ agglutinin–horseradish peroxidase (WGA-HRP) -labeled, small-diameter bladder afferent neurons. In the present study, TRPM8 expression was only minimally detected in bladder sensory neurons, even though the anti-rat TRPM8 antibody used in our experiments exhibited slightly higher specificity. The discrepancy between their results and ours may be attributed to differences in the anti-rat TRPM8 antibodies used, which were generated in rabbits (Abe et al., 2005).

To validate the histological findings, we further investigated whether TRPM8 contributes to the modulation of bladder afferent nerve activity within the pathways controlling the bladder-cooling reflex. At 25℃, intravesical saline infusion induced continuous contractions of

the bladder smooth muscle, resulting in higher vesical pressure than that observed at 37℃, consistent with a previous report by Mustafa and Thulesius (1999). These authors demonstrated that cooling of isolated rat urinary bladder preparations rapidly induced contractile responses of myogenic origin. Nevertheless, in the present study, no differences were observed in the temperature-dependent increases of pelvic afferent nerve activity between 37℃ and 25℃.

Intravesical infusion of the cooling compound menthol has been reported to facilitate the micturition reflex, whereas capsaicin pretreatment had no effect on this response, although menthol inhibited carbachol-induced contractions in isolated detrusor smooth muscle (Nomoto et al., 2008). These findings suggest that intravesically administered menthol cannot penetrate the muscle layer but instead acts on capsaicin-resistant afferents, likely through TRPM8 channels in the urothelium or sensory nerve endings, to facilitate the micturition reflex (Nomoto et al., 2008). Furthermore, another study demonstrated that TRPA1 and TRPM8 channels are expressed in cultured urothelial cells from rats, and activation of these channels elicits ionic current and Ca^{2+} influx (Kullmann et al., 2009). Recent studies have demonstrated that TRPM8 expression has been detected in the urothelial cells of patients with interstitial cystitis/bladder pain syndrome (IC/BPS), where it is thought to contribute to mechanosensation and to the modulation of reflex pathways under cold or inflammatory conditions (Wu et al., 2020). Moreover, pharmacological modulation of TRPM8 has been shown to alter bladder activity in animal models, suggesting its potential role in pathological hypersensitivity (Luyts et al., 2023).

In contrast to these previous reports, our findings revealed no significant differences in pelvic afferent nerve responses to increased intravesical pressure between saline and menthol (0.3-1.0 mM). Hayashi et al. (2009) detected TRPM8 immunoreactivity in only in a small proportion of small-diameter bladder afferent neurons. Thus, TRPM8 expression may be insufficient to exert a measurable effect on pelvic afferent nerve activity in response to increased intravesical pressure. It is also possible that TRPM8 contributes only to cutaneous acute cold-induced urgency, as suggested by previous studies (Chen et al., 2010; Uvin et al., 2015). In models of damage-induced cold allodynia associated with orofacial neuropathic pain, neuronal activity is modulated by the functional interplay between TRPM8 and voltage-gated potassium (Kv) channels (Piña et al., 2024). This observation suggests that TRPM8 may play a role in mediating cold sensation in the urinary bladder primarily under pathological conditions. We therefore speculate that TRPM8 does not function on capsaicin-resistant afferents in the

urothelium or sensory nerve endings to facilitate the micturition reflex. This interpretation is supported by a study showing that cold sensitivity in a substantial proportion of sensory neurons can occur in the absence of TRPM8 and TRPA1, albeit in acutely dissociated dorsal root ganglion neurons (Munns et al., 2007).

In conclusion, TRPM8 does not appear to contribute to the modulation of activity of bladder afferent nerve pathways controlling the micturition reflex or the bladder-cooling reflex in normal rats.

4. Experimental Procedure

4.1. Histological study

Experiments were conducted using female Sprague–Dawley and Wistar rats weighing 240–310 g. All procedures for the care and handling of animals were performed in accordance with institutional guidelines and were approved by the Animal Care and Use Committee of Nara Medical University. Afferent neurons projecting to the urinary bladder were identified by retrograde labeling with the fluorescent dye tracer hydroxystilbamidine (Fluoro-Gold; 4% w/v; Molecular Probes Inc., Eugene, OR, USA). The tracer was injected into the bladder wall at six to eight sites using a 30-gauge needle, with a total injection volume of 20 μL. Seven days after Fluoro-Gold injection, rats were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The L6 and S1 dorsal root ganglia (DRGs) were then removed, post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, and cryoprotected in graded sucrose solutions (10%, 20%, and 30%) prepared in 0.01 M phosphate-buffered saline. Both tissues were sectioned at a thickness of 10 μm for subsequent staining.

TRPM8 mRNA expression was examined using an in situ hybridization technique. Digoxigenin (Dig)-labeled RNA probes were transcribed from cDNA encoding rat TRPM8 (NM_134371, 2619–3320 bp) with T3 (sense) and T7 (antisense) RNA polymerases in the presence of a Dig labeling mix (Roche, Basel, Switzerland). Hybridization was performed as previously described (Tatsumi et al., 2005). Dig-labeled probes were visualized using alkaline phosphatase–conjugated anti-Dig antibodies and NBT/BCIP (Roche, Mannheim, Germany), or sequentially detected with anti-Dig sheep IgG (1:100, Roche), biotinylated anti-sheep IgG (1:100, Vector Laboratories, Burlingame, CA, USA), horseradish peroxidase–conjugated streptavidin (1:100, Molecular Probes Inc.), biotin-tyramide reagent (1:50, Molecular Probes Inc.), and Alexa Fluor 488–conjugated streptavidin (1: 100, Molecular Probes Inc.). Sections were observed under an Olympus BX51 microscope (Olympus Corp., Tokyo, Japan) in bright field and under

a Bio-Rad MRC 600 laser-scanning confocal microscope (Bio-Rad Laboratories, Hercules, CA, USA) for fluorescence imaging. The cross-sectional areas of TRPM8 mRNA–positive cells in DRG sections were measured using Scion Image software (Scion Corp., Frederick, MD, USA).

The anti-rat TRPM8 antibody was generated in rabbits. A fragment of the rat TRPM8 gene (NM_134371, bp 702–986) amplified by reverse transcription–polymerase chain reaction was inserted into the pGEX-4T-1 vector (Amersham Biosciences, Piscataway, NJ, USA). The construct was transformed into competent *Escherichia coli* to express recombinant TRPM8 protein fused with glutathione S-transferase (GST). The fusion protein was induced with 0.1 mM isopropylthiogalactoside and purified using a glutathione Sepharose 4B column. The purified TRPM8-GST fusion protein emulsified with adjuvant (TiterMax Gold, TiterMax USA Inc., Norcross, GA, USA) was administered subcutaneously to rabbits twice at 10-day intervals. Serum containing anti-TRPM8 antibodies was subsequently collected. The specificity of the antibody was verified by antigen absorption tests.

For immunofluorescence, sections of L6 and S1 DRG were incubated with the anti-TRPM8 antibody or with cocktails containing anti-TRPM8 and anti-calcitonin gene-related peptide (CGRP) antibodies (1 μg/mL; 1: 100; guinea pig anti-human CGRP, BIOMOL International, Plymouth Meeting, PA, USA) in 5% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and 0.3% Triton X-100 in 0.01 M phosphate-buffered saline (PBS). The sections were then incubated with Alexa Fluor 546–conjugated goat anti-rabbit IgG (1:1000, Molecular Probes Inc.) and Alexa Fluor 488–conjugated isolectin B4 (IB4) or with Alexa Fluor 488–conjugated goat anti-rabbit IgG (1:1000) and Alexa Fluor 546–conjugated goat anti-guinea pig IgG (1:1000, Molecular Probes Inc.), respectively. Additional DRG sections were incubated with a combination of anti-TRPM8 and anti-TRPV1 antibodies (VR1, 1:50, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in 5% bovine serum albumin and 0.3% Triton X-100 in 0.01 M PBS, followed by Alexa Fluor 488–conjugated chicken anti-rabbit IgG (1:500, Molecular Probes Inc.) and Alexa Fluor 546–conjugated donkey anti-goat IgG (1:500, Molecular Probes Inc.). Sections processed in parallel without primary antibodies served as negative controls. All fluorescently labeled sections were visualized using an Olympus FV1000 confocal microscope (Olympus Corp., Tokyo, Japan), and images were analyzed with FV10-ASW software (ver. 1.6, Olympus Corp.).

4.2. Physiological study

Experiments were conducted on rats anesthetized with urethane (1. 2 g/kg,

intraperitoneally) and artificially ventilated. Animals were immobilized with pancuronium bromide (1.0 mg/kg, intravenously). The left carotid artery and right external jugular vein were cannulated for arterial pressure monitoring and intravenous administration, respectively. Systemic arterial blood pressure was continuously recorded via a pressure transducer (Life Kit DX-312, NIHON KOHDEN, Tokyo, Japan). Following laparotomy, a catheter was inserted into the bladder through the bladder dome. Intravesical pressure was monitored through the catheter connected to a pressure transducer (Life Kit DX-312, NIHON KOHDEN) and an infusion pump via a three-way stopcock. Either warm (36–38℃) or chilled (23–25℃) saline, or saline at room temperature containing 0.3–1.0 mM menthol dissolved in 0.1% dimethyl sulfoxide, was infused into the bladder at a rate of 0.8–1.0 mL/min for 1.0–2.0 min, producing vesical distension that was sustained for an additional minute.

The bilateral vesical branches of pelvic nerves were exposed and transected near pelvic ganglia to avoid any influence of the micturition reflex. The electrode was placed on the left vesical branch of pelvic nerves from the bladder. Pelvic afferent nerve activities mediated through the electrode developed by Miki et al. (2002) were recorded with bioelectric amplifier (MEG-1200, NIHON KOHDEN) under filtration at LO CUT (0.08 Hz) and HI CUT (10 K).

All data were digitized and stored on a personal computer (Dell Inc., Round Rock, TX, USA) using a data acquisition system (PowerLab) and analyzed with LabChart software (version 5; AD Instruments, Bella Vista, NSW, Australia).

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