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TRPM8 mediates cold and menthol allergies associated with mast cell activation

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ABSTRACT

Exposure to low temperatures often causes allergic responses or urticaria. Similarly, menthol, a common food additive is also known to cause urticaria, asthma, and rhinitis. However, despite the obvious clinical implications, the molecular mechanisms responsible for inducing allergic responses to low temperatures and menthol have not been determined. Because a non-selective cation channel, transient receptor potential subtype M8 (TRPM8) is activated by cold and menthol, we hypothesized that this channel mediates cold- and menthol-induced histamine release in mast cells. Here, we report that TRPM8 is expressed in the basophilic leukemia mast cell line, RBL-2H3, and that exposure to menthol or low temperatures induced Ca^{2+} influx in RBL-2H3 cells, which was reversed by a TRPM8 blocker. Furthermore, menthol, a TRPM8 agonist, induced the dose-dependent release of histamine from RBL-2H3 cells. When TRPM8 transcripts were reduced by siRNA (small interfering RNA), menthol- and cold-induced Ca^{2+} influx and histamine release were significantly reduced. In addition, subcutaneous injection of menthol evoked scratching, a typical histamine-induced response which was reversed by a TRPM8 blocker. Thus, our findings indicate that TRPM8 mediates the menthol- and cold-induced allergic responses of mast cells, and suggest that TRPM8 antagonists be viewed as potential treatments for cold- and menthol-induced allergies.

1. Introduction

Cold-induced urticaria (CIU) develops when susceptible individuals are exposed to low temperatures, such as, simply holding a cold object, exposure to wind, walking in cold environments, or being immersed in cold water [1]. Localized or systematic exposure to cold can cause localized symptoms like a burning sensation, dermatographism, erythema, pruritus, urticaria, and edema, but can also induce anaphylactic reactions in susceptible individuals [1]. The incidence of CIU is comparatively high and has been estimated at 2–3% in the general population [2]. Furthermore, more than 90% of CIU patients have idiopathic cold urticaria [3], which can be diagnosed when erythema is produced by placing an ice cube on a patient's forearm [4].

Menthol (2-isopropyl-5-methyl cyclohexanol) is the active ingredient of peppermint and is widely used as flavoring in foods, cosmetics, mouth washes, and toothpastes. Contact sensitivity to menthol is rare, but menthol-induced urticaria has long been recognized [5,6], and several clinical reports have concluded that menthol-containing products like toothpaste, cigarettes, and ointments can cause asthma and rhinitis [6,7]. Usually these symptoms are treated by prescribing antihistamines or steroid hormones. Or, patients are simply advised to avoid menthol-containing products [1,7].

TRPM8 (transient receptor potential subfamily melastatin 8) is expressed in sensory neurons and activated at temperatures below 26 °C, and thus, it is referred to as a cold receptor [8,9]. Furthermore, TRPM8 is a non-selective cation channel permeable to monovalent cations and Ca^{2+} [8,9], and interestingly, it is also activated by menthol [8,9]. Physiological role in sensing cold was proven when mice lacking TRPM8 gene fail to sense cold [10,11].

Mast cells have an important role in the pathology of allergy, asthma, pulmonary fibrosis, and rheumatoid arthritis [12]. Their activations induce intracellular Ca^{2+} increases and the subsequent releases of allergic response mediators, such as, histamine, serotonin, and cytokines from their secretory granules [13]. When exposed to a cold environment, mast cells release histamine [3,14], and similarly, mast cells exposed to menthol also releases histamine [7]. Thus, these findings suggest the existence of a common link between CIU and menthol-induced urticaria. However, the molecular mechanisms underlying cold and menthol-induced histamine release from mast cells have not been determined, and therefore, we postulated that low temperatures and menthol exposure both activate TRPM8, and thereby, cause Ca^{2+} influx into mast cells and consequent histamine release.

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2. Materials and methods

2.1. Materials

A23187, l-menthol, *o*-phthaldialdehyde and compound 48/80 were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO (Gaithersburg, MD). Fluo3-AM, Pluronic F-127, Opti-MEM, Lipofectamine 2000, oligofectamine, and the SuperScript™ First-strand Synthesis System were purchased from Invitrogen (Carlsbad, CA). N-(4-t-Butylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carboxamide (BCTC) was from BIOMOL (Exeter, UK). All other reagents were purchased from Sigma.

2.2. Cell culture and TRPM8 transfection

The rat basophilic leukemia cell line, RBL-2H3 was kindly donated by Professor Sangsoo Shim (Chung-Ang University, Korea). Cells were maintained in DMEM supplemented with 4.5 g/l D-glucose, L-glutamine, 25 mM HEPES, 1% sodium pyruvate, 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. HEK-293T cells were maintained in DMEM supplemented with 10% FBS. Cells were seeded in Lab-Tek 8 chamber (Nunc Inc., Roskilde, Denmark). When cell densities in a well were 80–90% confluent, they were transfected with 0.32 μg of rat TRPM8 or rat TRPV1 cDNA using Lipofectamine 2000 (0.8 μl).

2.3. Immunohistochemistry

RBL-2H3 cells cultured on round coverslips were fixed with 4% paraformaldehyde for 30 min at 4 °C. The coverslips were then washed three times with phosphate-buffered saline (PBS), incubated with 0.5% Triton X-100 in PBS for 7 min, rewashed, and incubated in blocking buffer (0.5% BSA and 0.05% Triton X-100 in PBS) for 1 h at room temperature. Fixed cells in coverslip were incubated for overnight at 4 °C with primary rabbit polyclonal antibody raised against a rat TRPM8 (1:200 dilution; Abcam) and goat polyclonal antibody (1:100 dilution; Santa Cruz) raised against rat mast cell protease-1. The cells were washed and incubated with secondary antibodies Alexa Fluor 488-conjugated chick anti-rabbit IgG (1:200 dilution; Molecular Probes) and with Alexa Fluor 546-conjugated donkey anti-goat-IgG (dilution 1:2000; Molecular Probes) for 1 h at room temperature. After mounting, the cellular localizations of TRPM8 and mast cell protease-1 were observed in a confocal microscope (UltraView, Perkin Elmer, Cambridge, UK).

2.4. Measurement of intracellular calcium

RBL-2H3 cells plated on a 96 well culture plate were loaded with Fluo3-AM (Invitrogen) mixed with 0.1% Pluronic F-127 (Invitrogen) and diluted in Ringer solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose, 5.5 mM HEPES (pH 7.4) to a final concentration of 5 μM. Cells were then incubated at 37 °C for 40 min. The intensity of fluorescence was measured using a Flex Station II (Molecular Devices) at an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

2.5. Small interfering RNA (siRNA)

To knock-down endogenous TRPM8 in RBL-2H3 cells, siRNA was synthesized using the Silencer™ siRNA cocktail kit (AMbion, Austin, TX) as previously described [15]. Long double-stranded RNA was generated by *in vitro* transcription from T7 promoter sequence (underlined) containing PCR template corresponding to nucleotides 504–884 of rat TRPM8. The PCR template was amplified from cDNA of rat TRPM8 with 5′ TAA TAC GAC TCA CTA TAG GGT GAT CTA CAT CGC TCA GTC 3′ as a forward primer and 5′ TAA TAC GAC TCA CTA TAG GGT AGT TGG AAT CTT GAC TGG 3′ as a reverse primer. Double-stranded RNA was digested with RNase III for 1 h at 37 °C to generate siRNA cocktail. RBL-2H3 cells were treated twice at intervals of 48 h with TRPM8 siRNA cocktails at a final concentration of 100 nM using oligofectamin transfection reagent (Invitrogen) according to the manufacturer's protocol. Scrambled siRNA (*Silencer*® Negative Control #1 siRNA, Ambion) was used as a negative control. RT-PCR was performed to confirm the knockdown of TRPM8.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using easy-BLUE™ solution (iNtRON Biotech, Korea), and reverse transcribed with SuperScript III cDNA synthesis kit (Invitrogen). RT-PCR was performed using the primers mentioned above for siRNA synthesis. The PCR condition was 35 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min and followed by a further extension for 7 min at 72 °C.

2.7. Analysis of histamine release

The histamine contents both in the Ringer solution and residual histamine in the cells were determined using an *o*-phthaldialehyde fluorometric method [16]. Briefly, RBL-2H3 cells on six-well plates were stimulated by Ringer solution containing 5 mM menthol dissolved in 5% ethanol for 10 min at 37 °C. The histamine released into Ringer solution was collected by aspiration. To collect the residual histamine in RBL-2H3 cells, cells were scraped and disrupted by sonication. After disruption, the lysate was centrifuged at $30 \times g$ for 3 min and the supernatant was collected.

The histamine concentration in each supernatant was measured by adding 0.4 ml of 1 M NaOH and 0.1 ml of *o*-phthaldialdehyde reagent (1 mg/ml, complete). After 4 min of *o*-phthaldialdehyde reagent incubation, the reaction was terminated by adding 0.2 ml of 3 M HCl. Fluorescence intensity was measured at 355 and 455 nm (excitation and emission) using a Flex Station II (Molecular Devices). Histamine release percentage was calculated by following formula: (histamine in the supernatant/histamine in the pellet + histamine in the supernatant) × 100.

2.8. Behavioral test

Male 7-week-old ICR mice, weighing 33–35 g, were purchased from Samtako Bio Korea (Kyoung-Ki, Korea). Menthol (94 μg in 200 μl) was injected subcutaneously (s.c.) with a 26-gauge needle into the rostral portion of the back. Scratching behavior was observed over 10 min [17]. BCTC (3 mg/kg, 200 μl) or vehicles were administered (i.p.) 30 min before the menthol injection.

All the procedures used in these experiments have been approved by the Institute of Laboratory Animal Resources Seoul National University.

2.9. Data analysis

Data were analyzed using GraphPad Prism 4 software. Results are presented as means ± S.E.M. Statistical analysis was performed using the Student's *t*-test or one-way ANOVA. *p* values of <0.05 were considered significant.

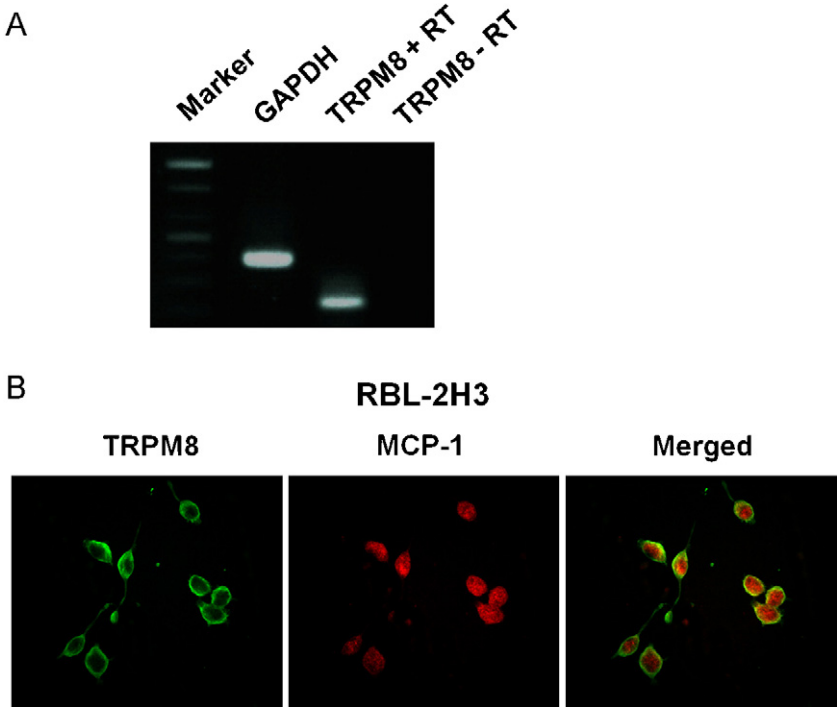

Fig. 1. RBL-2H3 cells and TRPM8 expression. (A) RT-PCR confirmed the expression of TRPM8 mRNA in RBL-2H3 cells (predicted size 212 bp). (B) Co-expression of TRPM8 and MCP-1 (mast cell marker) in RBL-2H3 cells. Confocal images of RBL-2H3 cells double-labeled with anti-TRPM8 antibody (visualized with Alexa 488-labeled chick anti-rabbit IgG, left panel) and MCP-1 (visualized with Alexa 546-labeled donkey anti-goat IgG, middle panel).

3. Results

3.1. Cold induces Ca^{2+} influx in RBL-2H3 cells

The presence of TRPM8 transcripts in RBL-2H3 cells was confirmed by RT-PCR (Fig. 1A). The immunofluorescence study also revealed that TRPM8 was co-expressed with mast cell protease-1 (MCP-1), a marker for mast cell in RBL-2H3 cells [18]. As shown in Fig. 1, TRPM8 was mainly expressed in the plasma membrane whereas MCP-1 was in the cytosol.

In order to test the effect of cold on Ca^{2+} influx, RBL-2H3 cells in confocal chamber were stimulated with chilled (10 °C) Ringer solution. In this cold condition, concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) was found to increase 3.5 folds ($n = 10$) from untreated controls (Fig. 2A, B). In contrast, when the cells were placed in a room at ambient temperature, $[Ca^{2+}]_i$ increases were minimal (1.1 ± 0.05 fold increase; $n = 4$). To determine whether this increase in $[Ca^{2+}]_i$ was mediated by TRPM8, a TRPM8 blocker, BCTC was treated for 10 min prior to cold application [19]. When cells were pretreated with 30 μM BCTC, the cold-induced Ca^{2+} influx increase was completely blocked ($p < 0.0001$, $n = 12$) (Fig. 2).

Cold-induced increases in $[Ca^{2+}]_i$ were paralleled by the expression of TRPM8 in human embryonic kidney HEK-293T cells. As shown in Fig. 2A, cold treatment increased in $[Ca^{2+}]_i$ in TRPM8-transfected HEK-293T cells, but not in mock-transfected cells (data not shown). Cold treatment failed to increase in $[Ca^{2+}]_i$ HEK-293T cells transiently transfected with rat TRPV1, a capsaicin and heat-activated channel [20], which was used as a negative control. These results suggest that cold exposure increases in $[Ca^{2+}]_i$ in RBL-2H3 cells via TRPM8.

3.2. Menthol induces $[Ca^{2+}]_i$ in RBL-2H3 cells

Because menthol is an agonist of TRPM8 [9], RBL-2H3 cells expressing TRPM8 were expected to respond to menthol. Indeed, menthol at 3 mM evoked an increase in $[Ca^{2+}]_i$ in RBL-2H3 cells (Fig. 3A), which was abolished by the pretreatment of 10 μM BCTC (Fig. 3A). Treatment with 3 mM menthol also increased $[Ca^{2+}]_i$ in HEK-293 cells transfected with rat TRPM8 (Fig. 3A), but failed to increase $[Ca^{2+}]_i$ in HEK-293T cells transfected with rat TRPV1 (Fig. 3A).

Furthermore, the response of RBL-2H3 cells to menthol was found to be dependent on menthol concentration (Fig. 3B), as $[Ca^{2+}]_i$ increased with menthol concentration with a minimal concentration threshold of 0.9 mM and a saturation concentration of 9.1 mM. The half maximal effective concentration (EC_{50}) of menthol in inducing a $[Ca^{2+}]_i$ increase in RBL-2H3 cells was 2.8 ± 0.03 mM ($n = 9$). Moreover, treating cells with the menthol vehicle (5% ethanol) failed to evoke any increase in $[Ca^{2+}]_i$ in RBL-2H3 cells (data not shown). These results suggest that menthol increases $[Ca^{2+}]_i$ in RBL-2H3 cells via TRPM8.

3.3. Menthol evokes histamine release from RBL-2H3 cells

Because the activations of mast cells by immunological inputs or physical stimuli induce histamine release [21], we sought to determine whether RBL-2H3 cells release histamine via TRPM8 when they are exposed to menthol. Histamine release to medium from RBL-2H3 cells was negligible in the basal condition. But when treated with 5 mM menthol, histamine release to medium amounted to $48.9 \pm 3.2\%$ ($n = 6$) of the total cellular histamine pool (Fig. 4A). As a positive control, RBL-2H3 cells were treated with a Ca^{2+} ionophore, A23187, which induced the release of $34.3 \pm 0.5\%$ ($n = 7$) of total cellular histamine content. Furthermore, menthol released histamine in a dose dependent manner ($n = 6$), with an EC_{50} of 1.29 mM (Fig. 4B). Histamine release induced by cold could not be determined because RBL-2H3 cells failed to exocytose under low temperature conditions [22].

3.4. TRPM8 siRNA reduces cold and menthol-induced effects

In order to determine further whether TRPM8 mediates cold- and menthol-induced effects in RBL-2H3 cells, we knocked-down

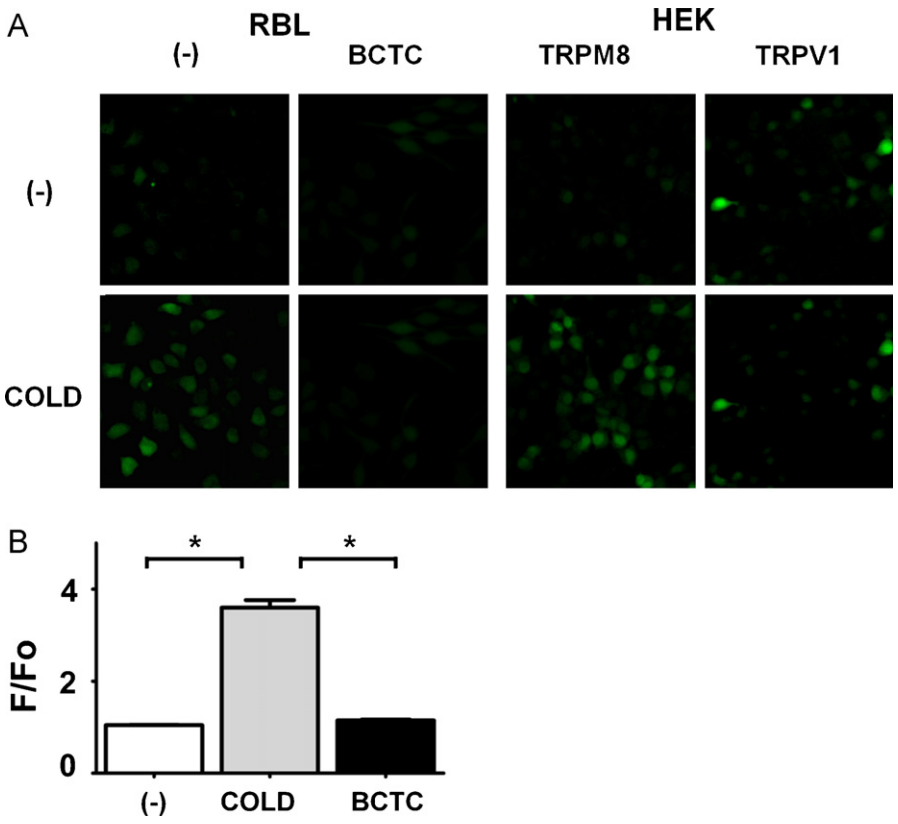

Fig. 2. Cold-induced calcium influx. (A) Fluo-3 AM-loaded RBL-2H3 cells and HEK-293T cells were stimulated with chilled Ringer solution, which drops the temperature to 10C ($n = 6$). (B) Summary of cold-induced calcium influx in RBL-2H3 cells. (−) represents experiments at room temperature. *$p < 0.0001$.

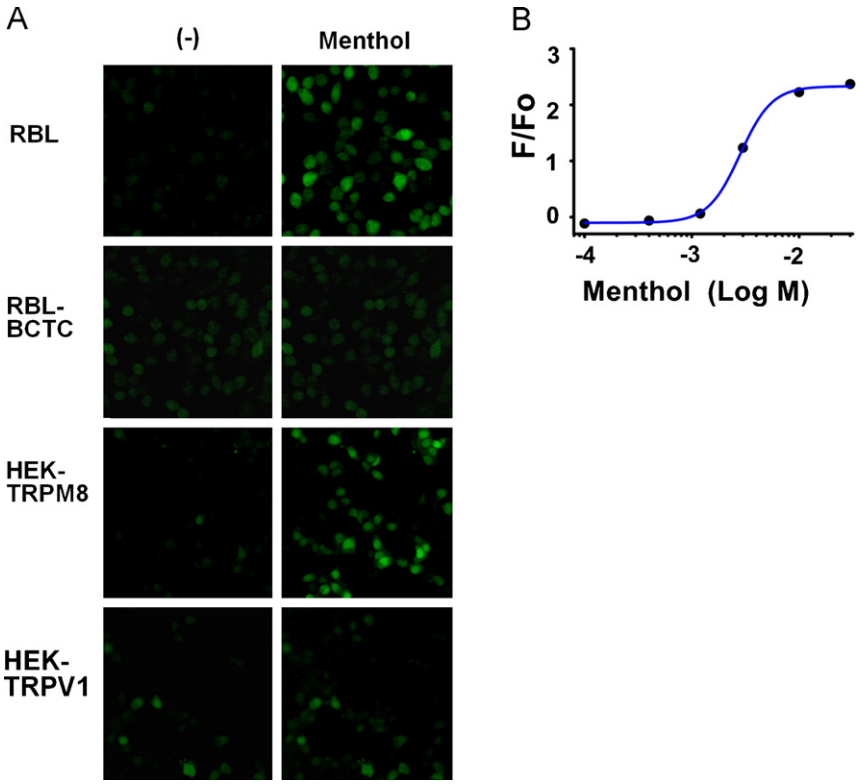

Fig. 3. Menthol-induced calcium influx. (A) Fluorescence images of fluo-3 AM-loaded RBL-2H3 cells and HEK-293T cells transfected with TRPM8 or TRPV1 channel genes. Cells were treated with menthol (3 mM) dissolved in 5% ethanol. BCTC reduced menthol-induced calcium influx. (B) Dose–response curve of menthol-induced calcium influx in RBL-2H3 cells ($n = 9$). Menthol was administered at the shown concentrations and fold increases in intracellular calcium levels (F/Fo) were monitored. The ratio (F/Fo) of the fluorescence (F) was obtained from intracellular calcium level change and the fluorescence (Fo) obtained from initial intracellular calcium level.

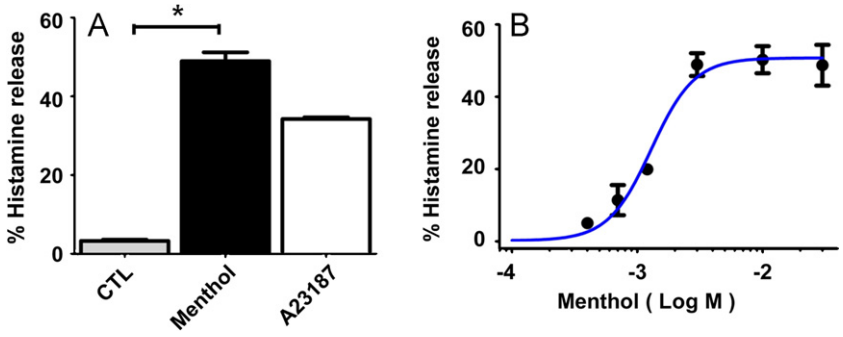

Fig. 4. Menthol-stimulated histamine release. (A) Menthol-induced histamine release to medium. CTL indicates the addition of control Ringer solution. A23187 (a calcium ionophore) was used as a histamine release control. Amounts of histamine released to media content and intracellular histamine were measured 10 min after menthol treatment. Estimation of % histamine release is explained in Section 2. (B) Histamine release as a function of menthol concentration. Cultured RBL-2H3 cells were treated with the indicated concentrations of menthol ($n = 6$).

TRPM8 by treating cells with small interfering RNA (siRNA). Three days after siRNA treatment, TRPM8 transcript levels had fallen to $24.8 \pm 0.9\%$ ($n = 5$, $p < 0.0001$) of those of untreated cells (Fig. 5A), whereas transcript levels in cells treated with scrambled siRNA had fallen to only $91.9 \pm 1.4\%$ ($n = 5$). When TRPM8 siRNA was applied to RBL-2H3 cells, 3 mM menthol increased $[Ca^{2+}]_i$ by only 0.9 ± 0.1 fold, whereas when scrambled siRNA was applied to RBL-2H3 cells, the menthol increased $[Ca^{2+}]_i$ by 2.2 ± 0.2 fold ($n = 9$), which was significantly greater than those observed in TRPM8 siRNA-treated cells ($p < 0.01$, $n = 9$) (Fig. 5B). Similarly, when 5 mM of menthol was added to scrambled siRNA treated cells, it caused a histamine release of $45.3 \pm 2.3\%$ of total cellular histamine ($n = 8$). On the other hand, when menthol was added to TRPM8 siRNA-transfected cells, a histamine release of total cellular histamine fell to $14.9 \pm 2.9\%$, significantly less than that observed in scrambled siRNA-treated cells ($p < 0.0001$, $n = 8$) (Fig. 5C).

Similarly, treating RBL-2H3 cells with TRPM8 siRNA reduced cold (10 °C)-induced increases in $[Ca^{2+}]_i$. As shown in Fig. 5, cold

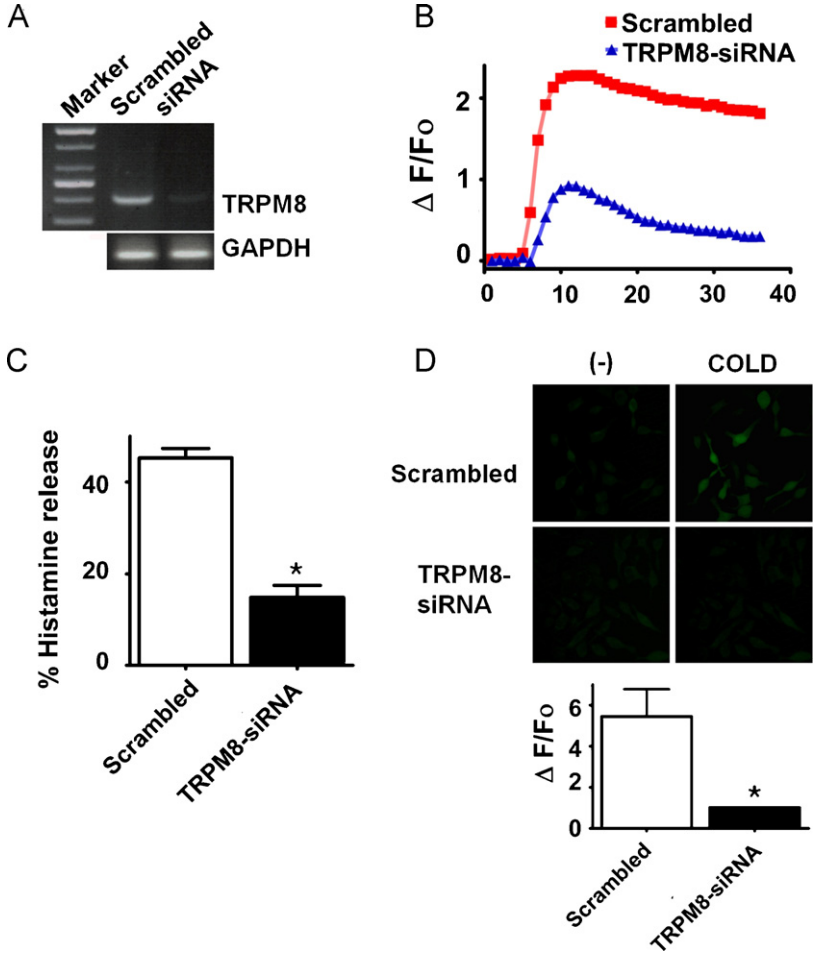

Fig. 5. Effect of TRPM8 gene knock-down on calcium influx and histamine release from RBL-2H3 cells. (A) siRNA treatment reduced TRPM8 gene expression. Scrambled refers to a non-specific siRNA mixture. (B) Menthol-induced calcium influx was reduced by TRPM8 siRNA. (C) Reduction of menthol-induced histamine release by TRPM8 siRNA ($n = 8$). (D) TRPM8 siRNA treatment reduced cold-induced calcium influx. TRPM8-siRNA transfected cells were stimulated in a confocal microscope chamber with chilled solution.

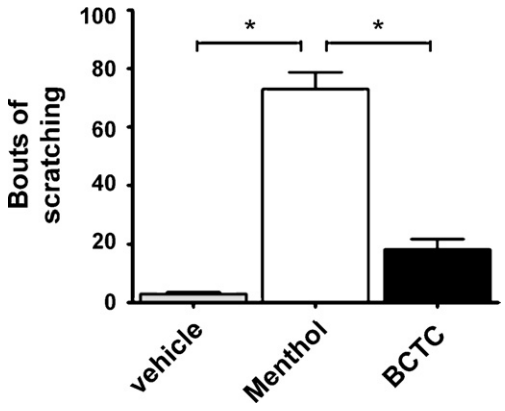

Fig. 6. Menthol evoked scratching behavior in ICR mice. Menthol was injected s.c. into the neck. Scratching bouts were counted over the following 10 min ($n = 10$). BCTC (3 mg/kg, i.p.) or vehicle (200 μl) was pretreated 30 min before menthol ($n = 6$).

exposure evoked a 5.5 ± 1.4 fold increase in $[Ca^{2+}]_i$ in scrambled siRNA-treated RBL-2H3 cells whereas only a 1.0 ± 0.02 fold increase in siRNA treated cells, significantly less than that in scrambled siRNA-treated cells ($p < 0.0001$, $n = 12$).

3.5. Menthol evokes scratching in mice

In order to observe menthol induced allergy-like responses *in vivo*, menthol was injected subcutaneously (s.c.) into the necks of male ICR mice. Numbers of scratching bouts were counted for 10 min after an injection of menthol (94 μg in 200 μl, s.c.). Vehicle injection alone (5% ethanol in phosphate-buffered solution, 200 μl, s.c.) only caused 3 ± 0.7 ($n = 7$) bouts of scratching, whereas menthol injection evoked 73 ± 6.2 bouts, significantly greater than those of the vehicle injection ($p < 0.0001$, ANOVA, $n = 10$). Furthermore, menthol-induced scratching was significantly ($p < 0.001$) reduced to 18 ± 3.9 ($n = 6$) bouts per 10 min when BCTC (3 mg/kg, i.p.) was administered 30 min before menthol. These results indicate that TRPM8 mediates menthol-induced itch (Fig. 6).

4. Discussion

The present study shows that TRPM8 mediates cold- and menthol-induced histamine-release from mast cells. Furthermore, cold and menthol were found to increase Ca^{2+} influx in RBL-2H3 cells, which was prevented by a TRPM8 antagonist. More importantly, menthol-induced histamine release from RBL-2H3 cells and scratching in mice were also markedly reduced by a TRPM8 antagonist. These results provide first insight of the molecular mechanisms underlying cold- and menthol-induced allergies or urticaria, and indicate that TRPM8 is a therapeutic target for the treatment cold- or menthol-induced allergies and urticaria.

Children with CIU are at high risk of having or developing asthma or allergic rhinitis [23]. Furthermore, one third of pediatric patients and one half of adult patients with CIU have anaphylaxis, which is sometimes life-threatening [3,23]. CIU has a poor prognosis because it is associated with the development of urticaria symptoms. Furthermore, the durations of its symptoms are longer than those of other forms of urticaria [24]. Clinical reports suggest that CIU is IgE-mediated because treatment with anti-IgE antibody alleviates symptoms [25,26]. However, the etiology of CIU has not been well defined [1], and in particular, it is not known how cold is sensed by mast cells. The present study strongly suggests that TRPM8, a cold receptor, detects cold and causes an influx of Ca^{2+} into mast cells, because blockade of TRPM8 or its knock-down reduced cold-induced Ca^{2+} influx. However, the present study does not rule out the possibility that IgE mediates some forms of CIU.

Menthol also induces urticaria, rhinitis or asthma [5–7]. When menthol is applied to the skin or mucosa at low concentration, a cooling sensation is produced or respiratory reflexes are reduced. However, at higher concentrations, menthol often elicits a pungent, irritating, or burning sensation [8,27]. Patients with menthol urticaria also often suffer from erythema, itching, stomatitis, eczema, sneezing, or asthma [5,7,28], and because menthol is commonly added to cosmetics, candy, cigarettes, and toothpaste, patients with menthol urticaria are at constant risk. However, despite the serious risk posed by menthol-induced urticaria, its etiology, in the majority of those affected, is unknown.

Mast cells play pivotal roles in the pathogeneses of urticaria and allergic immune responses [21]. Allergens or irritants stimulate mast cells, which then release histamine or cytokines. Histamine release is induced by specific antigens or non-immunological stimulators like compound 48/80, neuropeptide substance P, or ionophores [29]. Furthermore, because Ca^{2+} plays a key role in exocytosis, elevated $[Ca^{2+}]_i$ levels are a prerequisite of mast cell response [30,31]. In the present study, the observations that the blocking of TRPM8 or the knock-down of its transcripts markedly reduced $[Ca^{2+}]_i$ in response to cold or menthol, indicate that TRPM8 is a key mediator of $[Ca^{2+}]_i$ increases when mast cells are exposed to cold or menthol. However, it should be added that our findings do not exclude the possible release of Ca^{2+} from internal stores by menthol or cold exposure.

TRPM8 is one of eight temperature-sensing TRP channels (thermo TRP channels), which are activated at specific temperatures [8,9,32]. For example, channels TRPV1 to TRPV4 are activated by temperatures ranging from 25 to 52 °C [20,32–35]. On the other hand, TRPM8 is activated by cold at <26 °C [8,9], whereas TRPA1, which interestingly, is also activated by mustard oil [36,37], has been reported to be activated by cold in the noxious range (<18 °C) [36]. However, this activation of TRPA1 is not certain because another study failed to observe its activation [37]. In the present study, we investigated whether RBL-2H3 cells express the TRPA1 transcript, and RT-PCR showed that TRPA1 mRNA is barely expressed in RBL-2H3 cells. Furthermore, treating cells with mustard oil failed to increase $[Ca^{2+}]_i$ (data not shown). These results indicate that menthol and cold exposure evoked responses in RBL cells are mediated by TRPM8 and not by TRPA1.

Antihistamines and cromolyn or epinephrine (epinephrine stabilizes mast cells) are commonly used to treat CIU and menthol-induced urticaria [1,38]. The common mechanism involves the reduction of histamine release from mast cells [39,40]. However, these medications may induce side effects or fail to improve symptoms [41,42]. Our findings show that menthol-induced scratching was markedly attenuated by the TRPM8 antagonist, BCTC. Thus, based on our *in vivo* and *in vitro* findings, we conclude that TRPM antagonists should be viewed as candidate agents for the treatment of CIU and of menthol-induced urticaria.

Conflict of interest statement

We declare no competing interests.

Acknowledgements

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