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Crotamiton, an Anti-Scabies Agent, Suppresses Histamine- and Chloroquine-Induced Itch Pathways in Sensory Neurons and Alleviates Scratching in Mice

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Abstract

Crotamiton is an anti-scabies drug, but it was recently found that crotamiton also suppresses non-scabietic itching in mice. However, the underlying mechanism is largely unclear. Therefore, aim of the study is to investigate mechanisms of the anti-pruritic effect of crotamiton for non-scabietic itching. Histamine and chloroquine are used as non-scabietic pruritogens. The effect of crotamiton was identified using fluorometric intracellular calcium assays in HEK293T cells and primary cultured dorsal root ganglion (DRG) neurons. Further *in vivo* effect was evaluated by scratching behavior tests. Crotamiton strongly inhibited histamine-induced calcium influx in HEK293T cells, expressing both histamine receptor 1 (H1R) and transient receptor potential vanilloid 1 (TRPV1), as a model of histamine-induced itching. Similarly, it also blocked chloroquine-induced calcium influx in HEK293T cells, expressing both Mas-related G-protein-coupled receptor A3 (MRGPRA3) and transient receptor potential A1 (TRPA1), as a model of histamine-independent itching. Furthermore, crotamiton also suppressed both histamine- and chloroquine-induced calcium influx in primary cultures of mouse DRG. Additionally, crotamiton strongly suppressed histamine- and chloroquine-induced scratching in mice. Overall, it was found that crotamiton has an anti-pruritic effect against non-scabietic itching by histamine and chloroquine. Therefore, crotamiton may be used as a general anti-pruritic agent, irrespective of the presence of scabies.

Key Words: Crotamiton, Itch, Histamine, Chloroquine, TRPV1, TRPA1

INTRODUCTION

Crotamiton (*N*-ethyl-*o*-methylphenyl-2-butenamide) has long been used as a topical drug for alleviating pruritus induced by scabies (Couperus, 1949; Smith *et al*., 1984; Sekine *et al*., 2012). Scabies is a contagious skin condition caused by infestation of the microscopic mite *Sarcoptes scabiei*, leading to relentless and severe itching. Interestingly, crotamiton also exerts an anti-pruritic effect against non-scabietic, pruritogen-induced itch, which is pathogenically different from the pruritus associated with scabies (Sekine *et al*., 2012; Kittaka *et al*., 2017). Although crotamiton ointment alleviated non-scabietic pruritogen-induced scratching in mice (Sekine *et al*., 2012), the underlying mechanisms remain largely unclear.

Itch is a sensation felt on the skin that causes a desire to scratch. Previous studies have revealed that the sensation is transmitted via sensory peripheral neurons by the activation of certain molecular receptors and/or ion channels (Kittaka and Tominaga, 2017). One of the most popular pruritogen (itch-inducing agent) is histamine (Ohsawa and Hirasawa, 2014). It is an endogenous pruritogen that is mostly released from mast cells (Cho *et al*., 2010). Once released, it binds to its own specific histamine receptor, mainly histamine receptor subtype 1 (H1R) in the peripheral sensory neurons, causing itching. H1R stimulation then leads to the activation of phospholipase A_2/12-lipoxygenase pathway, followed by activation of transient receptor potential vanilloid 1 (TRPV1) (Shim *et al*., 2007). TRPV1 is a non-selective cation channel present in the

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peripheral neurons and is involved in inducing various sensations, including itching (White *et al*., 2011). Thus, activation of H1R and TRPV1 is regarded as the molecular underpinning for histamine-induced itching in sensory neurons.

Moreover, chloroquine (an anti-malarial agent) causes a severe itching that is not related to histamine receptor activation (Olatunde and Obih, 1981). The molecular entities for chloroquine-induced itching have been identified to be Mas-related G-protein-coupled receptor A3 (MRGPRA3) and transient receptor potential A1 (TRPA1) ion channel (Wilson *et al*., 2011). Being unrelated to histamine, the chloroquine-induced itching is often regarded as a characteristic 'histamine-independent' itch signaling pathway.

Recently, Kittaka *et al*. (2017) reported that crotamiton inhibits activation of TRPV4 induced by GSK1016790A, a specific agonist for the TRPV4 ion channel. The anti-pruritic effect of crotamiton via the serotonin-mediated itch pathway may be further explained by the relationship between TRPV4 and serotonin-induced scratching behavior (Akiyama *et al*., 2016). However, crotamiton more readily suppresses histamine-induced scratching than that induced by serotonin (Sekine *et al*., 2012). Nevertheless, it is still unclear, from a neuromolecular perspective, how crotamiton inhibits histamine-induced itch. Further, its action on chloroquine-induced itching is unknown. Therefore, the present study sought to investigate the possible neuromolecular signaling pathways of crotamiton associated with non-scabietic itching induced by histamine and chloroquine.

MATERIALS AND METHODS

Reagents

All reagents including crotamiton, histamine, chloroquine, capsaicin, allyl isothiocyanate (AITC), and permethrin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Crotamiton, capsaicin, and AITC were dissolved in ethanol, histamine and chloroquine were dissolved in water, and permethrin was dissolved in dimethyl sulfoxide.

Primary culture of dorsal root ganglia (DRG) neurons

Lumbar and thoracic DRG neurons were isolated from 10-week-old mice and cultured as described previously (Malin *et al*., 2007). Neurobasal® medium (Gibco, Life Technologies, Greenland, NY, USA), containing 10% fetal bovine serum (FBS), 50–100 ng/mL nerve growth factor (Invitrogen, Gaithersburg, MD, USA), and 100 U/mL penicillin–streptomycin solution (Hyclone, Thermo Scientific, South Logan, UT, USA), was used for culturing DRG neurons. Isolated DRG neurons were incubated with 1 mg/mL collagenase (Worthington Biochemical, Lakewood, NJ, USA) for 30 min at 37°C, followed by incubation for an additional 30 min at 37°C in presence of 2.5 mg/mL trypsin (Gibco). Cells were then plated on poly-L-lysine-coated 8-well chambers (Lab-Tek™, Thermo Scientific) and incubated for 3 days at 37°C in a humidified atmosphere of 5% CO_2.

Cell culture and gene transfection

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, St. Louis, MO, USA), supplemented with 10% FBS and 100 U/mL penicillin–streptomycin solution (Hyclone), at 37°C in a humidified atmosphere of 5% CO_2-95% air. The cells were sub-cultured every 3 or 4 days with fresh DMEM medium for maintenance. Further, the cells were transfected with selected genes using a FuGENE® HD transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol. Each gene was transfected to give a final amount of 1 μg and all experiments were performed one day after transfection. The genes used in this study—mouse H1R (NM_001252643), mouse TRPV1 (NM_001001445), mouse MRGPRA3 (NM_153067), and mouse TRPA1 (NM_177781)—were cloned from mouse dorsal root ganglia and matched 100% for sequences with those in the NCBI GenBank database. All the genes were subcloned into pcDNA3.1 (Invitrogen).

Measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} was measured using an inverted microscope (Nikon, Eclipse Ti-U, Kanagawa, Japan) imaging technique ("calcium imaging"), following a previously described method (Pradhananga and Shim, 2015). Briefly, HEK293T cells or primary culture of DRG neurons were plated on poly-lysine-coated 8-well chambers (Lab-Tek™), and the incubated cells were transfected with H1R and/or TRPV1 cDNA. One day after transfection, the culture media were replaced with normal bath solution [140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose, and 5.5 mM HEPES (pH 7.4)]. For intracellular calcium detection, the cultures were incubated with the calcium-specific fluorescent dye Fluo-3 AM (5 μM, Invitrogen) for 1 h in the presence of 0.1% Pluronic F-127 (Invitrogen) at 37°C. Following incubation, the media were washed out, and histamine or chloroquine were applied to induce calcium influx. The calcium-specific fluorescence was observed at 488/515 nm (excitation/emission wavelengths). Microscopic images were captured using Nikon NIS Elements software (Nikon) at 1.5 s intervals. Changes in intracellular calcium levels were expressed as F/F_0 ratio, where F_0 is the initial fluorescence intensity. The ratio was calculated using the ImageJ program (Schindelin *et al*., 2015).

In vivo scratching behavior experiments

All animal experimental procedures were performed in accordance with the animal protocol approved by the Institutional Animal Care and Use Committee at Lee Gil Ya Cancer and Diabetes Institute (LCDI), Gachon University, Korea (Approved animal protocol number: LCDI-2014-0068). Male ICR mice (aged 10–11 weeks) were purchased from Orient Laboratory Animals (Seoul, Korea). The animals were allowed free access to food and water under a 12:12 h light:dark cycle. The scratching behavior test was performed as previously described (Jang *et al*., 2015). Briefly, 100 μL of histamine (100 μg/site) or chloroquine (200 μg/site) was administered via an intradermal injection into the nape to elicit scratching behavior. For evaluation of the anti-pruritic effect of crotamiton, a dose of 125 mg/kg was administered intraperitoneally 30 min before either histamine or chloroquine injection. All experiments were video-recorded, and bouts of scratching using the hind limbs were counted immediately after the intradermal injection for up to 1 h.

Statistics

All data are presented as mean ± SEM. For comparison between two groups, the unpaired student's *t*-test was applied. For multiple comparisons, one-way ANOVA with Tukey's *post*

hoc test was applied. Fisher's exact test was used to compare the cell responsiveness of the primarily cultured DRG neurons. Results of all measurements were reported as mean of 3 independent experiments. For calcium imaging data, total cell numbers indicate number of digitally-recognized "regions of interest" from at least 3 separate experiments. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). *p* values less than 0.05 indicated statistical significance.

RESULTS

Crotamiton inhibits histamine-induced calcium influx in the H1R/TRPV1 pathway

We first investigated whether crotamiton has the potential to inhibit signals induced by histamine. HEK293T cells that transiently express both H1R and TRPV1 (H1R/TRPV1), were used to develop an experimental model for calcium imaging, because histamine-induced itching is mostly triggered by calcium influx after coordinated activation of H1R and TRPV1 (Shim *et al*., 2007).

As shown in Fig. 1A and 1B, intracellular calcium levels were significantly increased upon histamine (10 μM) stimulation in H1R/TRPV1 cells ("Con", 874 cells), suggesting that the experimental model was successfully developed. However, upon pretreatment with crotamiton (1 mM) for 5 min, a negligible increase in intracellular calcium levels ("+crotamiton", 838 cells, Fig. 1A-1C) was observed after subsequent histamine treatment, suggesting that crotamiton inhibits histamine-induced calcium influx in H1R/TRPV1 cells. Moreover, the inhibitory effect of crotamiton was concentration-dependent (Fig. 1D, IC_{50}=101.2 μM), indicating a receptor-mediated inhibitory effect, probably via the H1R/TRPV1 pathway. To further investigate the effect of crotamiton, cells expressing only TRPV1 were used. As shown in Fig. 1E, pretreatment with crotamiton (1 mM) barely decreased the calcium influx induced by capsaicin (a TRPV1 agonist, 1 μM, 543 cells) compared to control (704 cells). Overall, it was found that crotamiton inhibits histamine-induced calcium influx via the H1R/TRPV1 pathway; however, it does not directly involve TRPV1.

Crotamiton inhibits chloroquine-induced calcium influx in the MRGPRA3/TRPA1 pathway

To further verify whether crotamiton also inhibits chloroquine-induced itching, a similar experimental approach was followed using HEK293T cells transiently expressing both MRGPRA3 and TRPA1 (MRGPRA3/TRPA1).

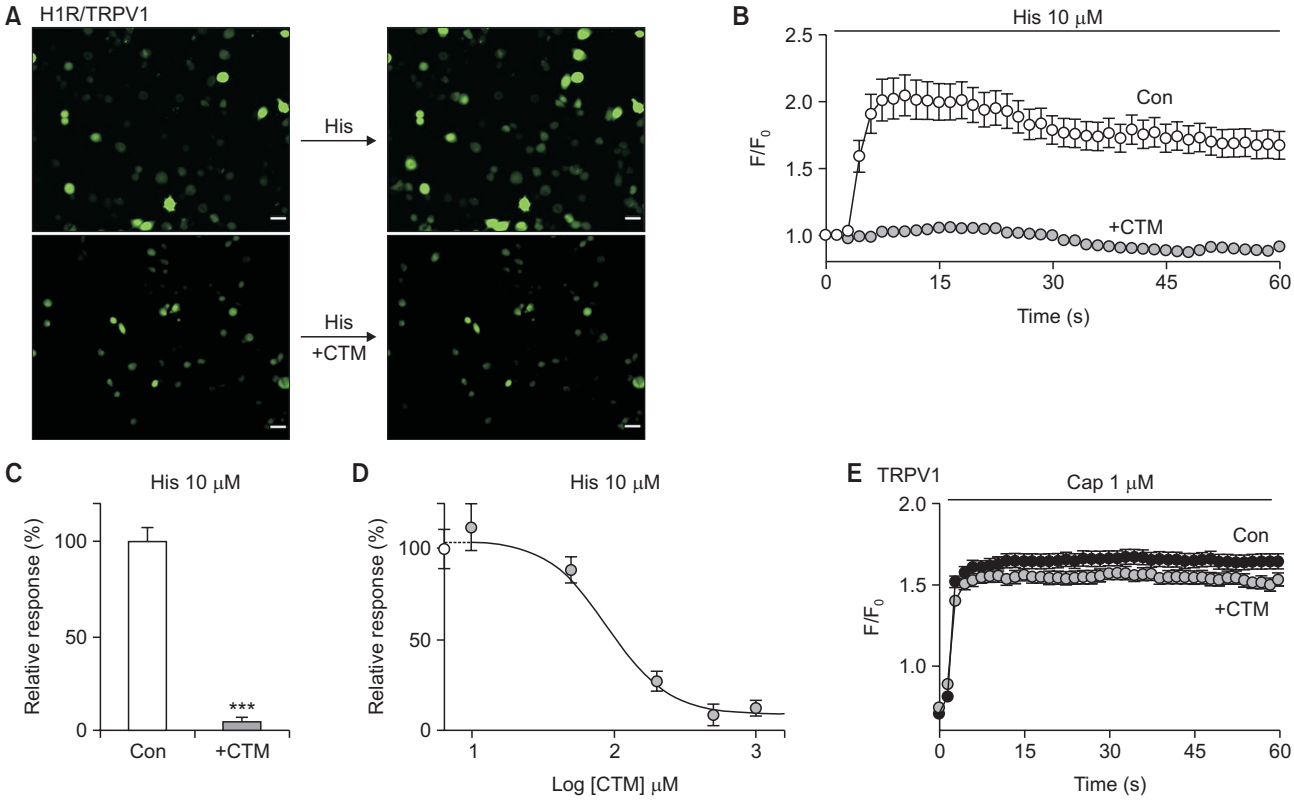

Fig. 1. Crotamiton inhibits histamine-induced calcium influx in the cells expressing H1R. (A) Representative images of calcium imaging experiments with in HEK293T cells that transiently expressed both H1R and TRPV1 (H1R/TRPV1). Bars indicate 50 μM. (B) Intracellular calcium changes reflected by changes in the fluorescence intensity (F) of the calcium-specific dye were measured after treatment with histamine (His, 10 μM) in the absence ("Con", 874 cells) or presence of 1 mM crotamiton ("+crotamiton", 838 cells) in H1R/TRPV1. (C) Relative response percentage of peak F/F_0 intensities after 10 μM His treatment in crotamiton pre-treated H1R/TRPV1. (D) A concentration-response curve obtained for peak F/F_0 intensities for a range of crotamiton concentrations against His-induced responses in H1R/TRPV1; IC_{50}=101.2 μM. (E) Crotamiton pre-treatment ("+crotamiton", 543 cells) barely reduced TRPV1 activation induced by 1 μM capsaicin (CAP) compared to that of the control ("Con", 704 cells). F_0 denotes initial F at 0 s. ***p<0.001.

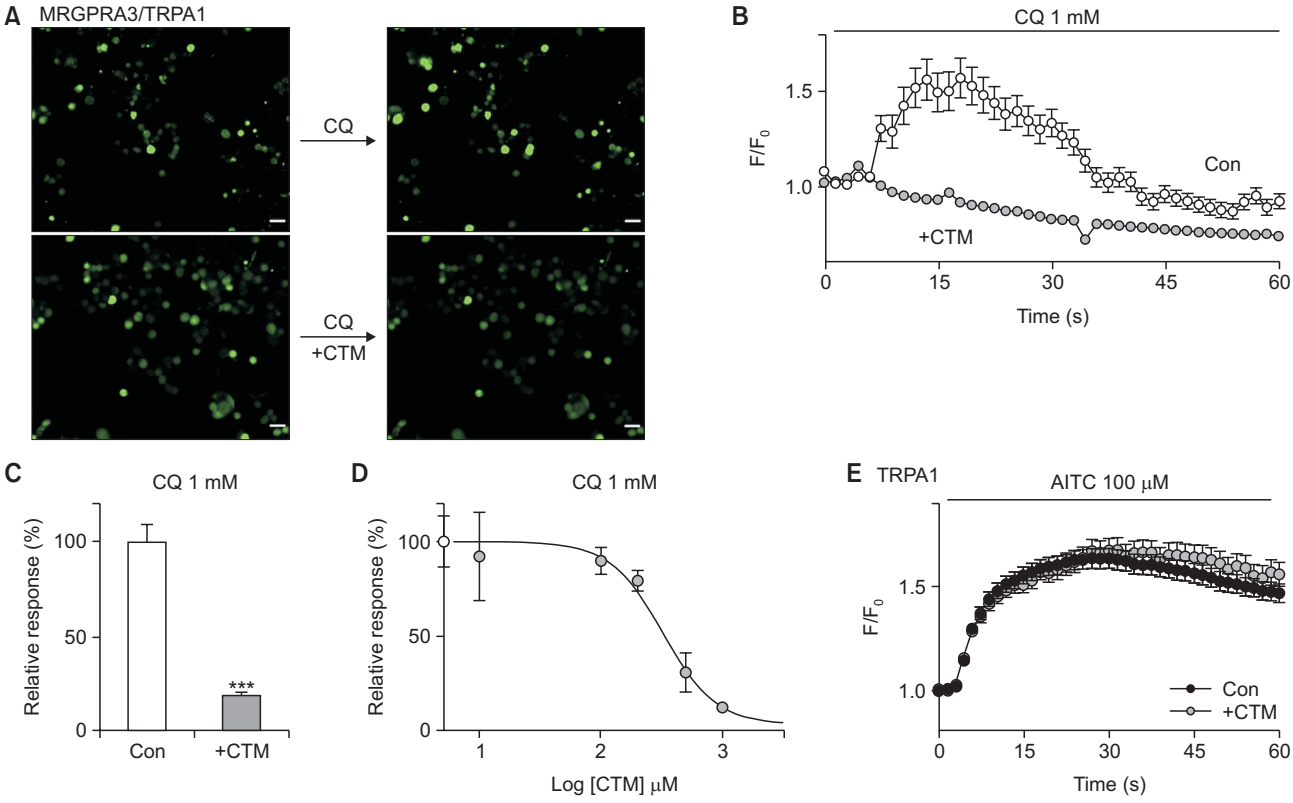

Fig. 2. Crotamiton inhibits chloroquine-induced calcium influx in the cells expressing MRGPRA3. (A) Representative images of calcium imaging experiments in HEK293T cells that transiently expressed both MRGPRA3 and TRPA1 (MRGPRA3/TRPA1). Bars indicate 50 μM. (B) Intracellular calcium changes reflected by the changes in the fluorescence intensity (F) of the calcium-specific dye were measured after treatment with 1 mM chloroquine in the absence ("Con", 1065 cells) or presence of 1 mM crotamiton ("+CQ" , 556 cells) in MRGPRA3/TRPA1. (C) Relative response percentage of peak F/F_0 intensities after 1 mM chloroquine treatment in crotamiton pre-treated MRGPRA3/TRPA1. (D) A concentration-response curve obtained for peak F/F_0 intensities for a range of crotamiton concentrations against chloroquine-induced responses in MRGPRA3/TRPA1; IC_{50}=326.2 μM. (D) Crotamiton pre-treatment did not reduce TRPA1 activation induced by 100 μM AITC ("Con", 1374 cells *vs.* "+crotamiton", 1168 cells). F_0 denotes initial F at 0 s. ***p<0.001.

The calcium influx induced by chloroquine (1 mM) significantly reduced upon pretreatment with crotamiton (1 mM) for 5 min ("Con": 1065 cells *vs.* "+crotamiton": 556 cells, Fig. 2A-2C), indicating that crotamiton also inhibits chloroquine-induced calcium influx via the MRGPRA3/TRPA1 pathway. Similar to the observations with histamine, the chloroquine-induced calcium influx was also inhibited in a concentration-dependent manner (Fig. 2D, IC_{50}=326.2 μM). To further pinpoint the target molecule, cells expressing only TRPA1 were used, and it was found that crotamiton did not block TRPA1 activation induced by AITC, a specific agonist for TRPA1 ("Con": 1374 cells *vs.* "+crotamiton": 1168 cells, Fig. 2E). This indicated that TRPA1 is not a direct target of crotamiton for the observed inhibition via the histamine-independent MRGPRA3/TRPA1 itch pathway.

Inhibitory effects of crotamiton on histamine- and chloroquine-induced calcium influx are specific responses

It was further investigated whether all the effects of crotamiton were due to non-specific reasons, such as cytotoxicity. The MTT assay revealed that incubation of HEK293T cells with crotamiton (1 mM) for 30 min did not cause any significant changes in cell viability (Control: 103.5 ± 2.334% *vs.* crotamiton: 94.39 ± 3.835%), as shown in Fig. 3A.

Scabies is accompanied by severe itching. Therefore, it is plausible that most anti-scabies agents also have similar anti-pruritic effects. For this purpose, the effects of permethrin, an anti-scabies agent with a different molecular structure, were compared with those of crotamiton by calcium imaging. However, pretreatment with permethrin (1 mM) did not exert any inhibitory effect on the calcium influx induced by either histamine- (103.2 ± 3.947%, 325 cells) or chloroquine (125.9 ± 7.323%, 193 cells), as shown in Fig. 3B. However, pretreatment with crotamiton (1 mM) significantly inhibited both histamine- (12.43 ± 4.026%, 441 cells) and chloroquine-induced calcium influx (12.89 ± 2.374%, 189 cells). Thus, this result strongly indicated that the inhibitory effects of crotamiton on calcium influx induced by histamine or chloroquine are intrinsic and specific because permethrin failed to inhibit histamine- and chloroquine-induced calcium influx.

Crotamiton inhibits histamine- and chloroquine-induced calcium influx in sensory neurons

To further verify the inhibitory effect of crotamiton on histamine- and chloroquine-induced responses in sensory neurons, primary cultures of mouse DRG neurons were used, and calcium influx was measured by using calcium imaging techniques. It was found that crotamiton (1 mM) significantly

reduced the number of histamine (100 μM)-responsive DRG neurons (Fig. 4A). However, the calcium influx induced by capsaicin (1 μM) was not altered by crotamiton pretreatment (Fig. 4B), which is in agreement with the findings shown in Fig. 1.

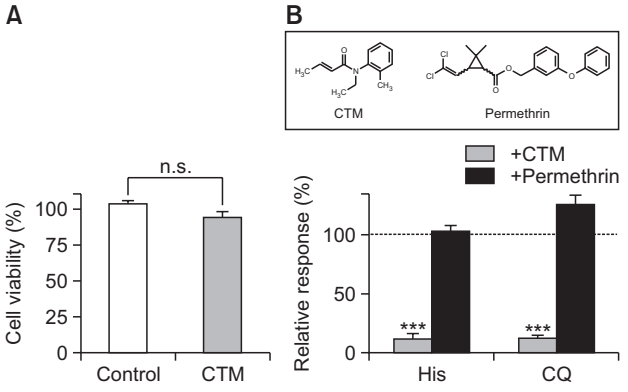

Fig. 3. Inhibitory effect of crotamiton is specific. (A) Results of cell viability test after 30 min of incubation with crotamiton (1 mM) showed no changes when compared to that in the control. (B) Pretreatment with permethrin (1 mM), an anti-scabies agent of a different class, did not show any inhibitory effect on both histamine- (325 cells) and chloroquine-induced calcium influx (195 cells) compared to pretreatment with crotamiton (histamine [His]: 441 cells, chloroquine [CQ]: 189 cells). n.s., not significant; ***p<0.001.

Similar results were obtained in chloroquine (1 mM)-stimulated DRG neurons. Crotamiton (1 mM) significantly reduced the chloroquine-induced calcium influx in DRG neurons and the number of chloroquine-responsive neurons (Fig. 4C). However, the calcium influx induced by AITC (100 μM) was not affected by crotamiton pretreatment (Fig. 4D), consistently with the findings shown in Fig. 2.

Therefore, these data strongly suggest that crotamiton inhibits histamine- and chloroquine-induced calcium influx in sensory neurons.

Crotamiton shows anti-pruritic effects on histamine- and chloroquine-induced scratching in mice

Finally, the *in vivo* anti-pruritic effects of crotamiton were evaluated in mice injected with either histamine or chloroquine. When crotamiton was administered (125 mg/kg, *i.p.*) before histamine injection, a significant reduction in the total number of scratching bouts was observed ("His," 111.0 ± 32.82, n=12 *vs.* "+crotamiton," 29.82 ± 8.370, n=11, Fig. 5A, 5B). Likewise, the total number of scratching bouts induced by chloroquine was also significantly reduced when crotamiton was intraperitoneally administered ("CQ," 286.0 ± 58.98, n=6 *vs.* "+crotamiton," 82.20 ± 22.76, n=5, Fig. 5C, 5D). Thus, it was found that intraperitoneal administration of crotamiton exhibited potent anti-pruritic effects on both histamine- and chloroquine-induced scratching in mice.

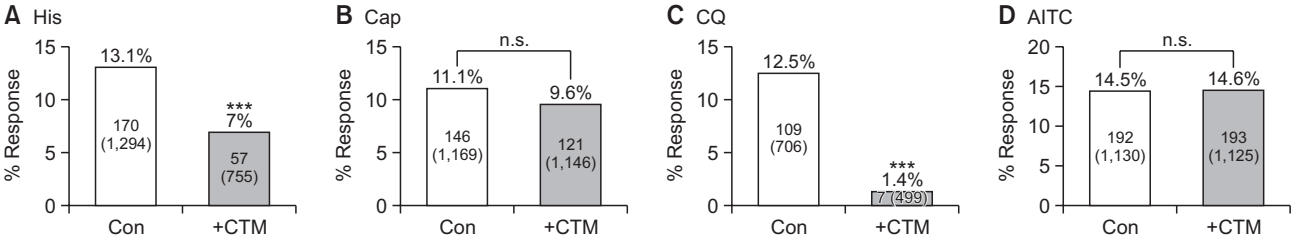

Fig. 4. Crotamiton suppresses histamine- and chloroquine-induced calcium influx in primary cultures of mouse dorsal root ganglia (DRG). Responsive DRG cells in the control (Con) were counted and compared with those in the crotamiton-pretreated group ("+crotamiton"). Numbers in the bar indicate the total number of responsive cells, whereas numbers in the parentheses show the total number of cells counted. Responses were induced by (A) 100 μM histamine (His), (B) 1 μM capsaicin (Cap), (C) 1 mM chloroquine (CQ), and (D) 100 μM AITC. n.s., not significant; ***p<0.001.

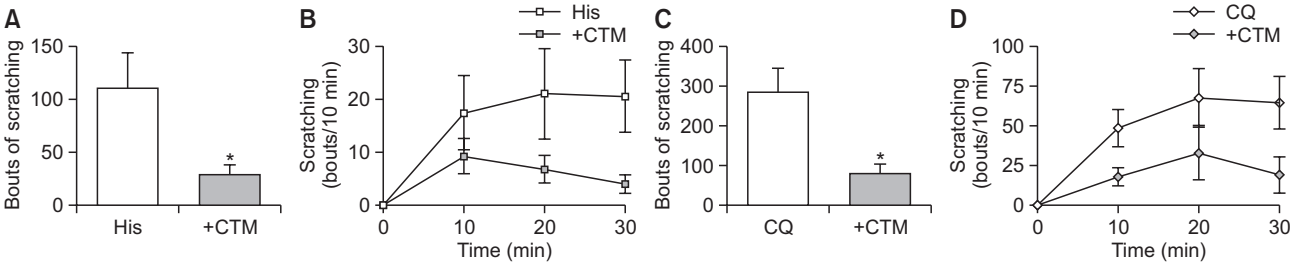

Fig. 5. Intraperitoneal injection of crotamiton successfully inhibited scratching behavior induced by histamine or chloroquine in mice. Crotamiton (125 mg/kg) was injected intraperitoneally before an intradermal injection of pruritogen into the nape of the mice, and bouts of scratching were counted. (A) Crotamiton ("+crotamiton," 11 mice) significantly decreased the total bouts of scratching induced by histamine ("His," 100 μg/site, 12 mice). (B) Bouts of scratching data of (A) separated by 10-min intervals. (C) Crotamiton significantly reduced the total bouts of scratching induced by chloroquine ("CQ," 200 μg/site, 6 mice *vs.* "+crotamiton," 5 mice). (D) Bouts of scratching data of (C) separated by 10-min intervals. *p<0.05.

DISCUSSION

Scabies is a contagious skin infection caused by the scabies mite, and its hallmark clinical symptom is a severe itching. Although crotamiton has long been used for the treatment of scabies, other drugs such as permethrin and ivermectin are preferred (Larson, 2008; Goldust *et al*., 2014). However, the present study suggests that crotamiton can be used as a general anti-pruritic drug, irrespective of the presence of scabies. Surprisingly, the anti-pruritic effect of crotamiton on non-scabietic pruritus-induced itching is known, but the exact underlying molecular mechanisms is largely elusive. The present study showed that crotamiton inhibits both histamine- and chloroquine-dependent itch pathways and suppresses scratching behavior induced by both the compounds in mice.

It had been vaguely predicted that crotamiton might inhibit histamine-induced itching by inhibiting H1R (Sekine *et al*., 2012). Consistent with this prediction by Sekine *et al*. (2012), the present study showed that crotamiton inhibits histamine-induced calcium influx in H1R/TRPV1 HEK293T cells and mouse DRG neurons. Moreover, crotamiton inhibited chloroquine-induced calcium influx in MRGPRA3/TRPA1 HEK293T cells and mouse DRG neurons (Fig. 2, 3). Therefore, these findings provide experimental evidence, for the first time, that crotamiton inhibits both histamine- and chloroquine-induced itching.

A recent study showed that crotamiton also inhibits the activation of TRPV4 by a specific TRPV4 agonist, GSK1016790A (Kittaka *et al*., 2017). TRPV4 was initially known as a temperature-sensitive ion channel activated by moderate heat (25–40°C) and expressed in both small and large DRG neurons in adult mice (Guler *et al*., 2002; Jang *et al*., 2012). However, TRPV4 mediates itching induced by serotonin but not that induced by histamine or chloroquine in sensory neurons (Akiyama *et al*., 2016). Combining with the current results, these reports imply that crotamiton may be used to suppress not only serotonin- but also histamine- and even chloroquine-induced itch pathways at sensory neuronal level.

Researchers are beginning to embrace the concept of G-protein-coupled receptor (GPCR)–TRP axis, which explains the common molecular mechanisms underlying transmission of various sensory signals, including itching (Veldhuis *et al*., 2015). Briefly, the concept suggests that sensory transmission is triggered by a ligand or agonist that initially binds to the GPCR, which then leads to activation of corresponding TRP ion channels with the aid of various signal cascades. The present study was also based on this concept, and suggests that crotamiton inhibits both H1R/TRPV1 and MRGPRA3/TRPA1 pathways. However, molecules that are being targeted by crotamiton for the inhibition of these pathways remain unidentified.

Our results showed that crotamiton does not directly inhibit TRP ion channels. Specifically, it was found that it does not significantly inhibit capsaicin-induced TRPV1 activation (Fig.1D). Likewise, crotamiton failed to inhibit chloroquine-induced TRPA1 activation (Fig. 2D). Kittaka *et al*. (2017) also reported that crotamiton had no effect on TRPV1 and TRPA1. Therefore, it could be speculated that crotamiton blocks these itch pathways by blocking either the GPCR or signal cascades that link the GPCR and TRP ion channels. Further studies are warranted to unravel the detailed inhibitory molecular mechanisms of crotamiton on these itch pathways.

Although TRPV1 and TRPA1 contribute to itch transmission in sensory neurons, the major function of these ion channels is sensing nociceptive stimuli, such as pain and temperature (Vay *et al*., 2012). In other words, blocking of these TRP ion channels may result in unwanted side effects because they are also involved in other sensory pathways. In fact, many TRPV1 antagonists cause serious side effects that induce burning sensations or hyperthermia (Lee *et al*., 2015). In this regard, failure to inhibit TRPV1 and TRPA1 by crotamiton could favor its use as a general anti-pruritic agent.

Overall, it was found that crotamiton, an anti-scabies agent, has novel inhibitory effects on both histamine- and chloroquine-dependent itch pathways in sensory neurons. In addition, it suppressed scratching behaviors induced by both histamine and chloroquine in mice. Therefore, the present study suggests that crotamiton can be used as a novel anti-pruritic agent in addition to being a scabicidal agent.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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