TRPA1 and TRPV1 contribute to iodine antiseptics-associated pain and allergy

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Abstract

Iodine antiseptics exhibit superior antimicrobial efficacy and do not cause acquired microbial resistance. However, they are under-used in comparison with antibiotics in infection treatments, partly because of their adverse effects such as pain and allergy. The cause of these noxious effects is not fully understood, and no specific molecular targets or mechanisms have been discovered. In this study, we show that iodine antiseptics cause pain and promote allergic contact dermatitis in mouse models, and iodine stimulates a subset of sensory neurons that express TRPA1 and TRPV1 channels. *In vivo* pharmacological inhibition or genetic ablation of these channels indicates that TRPA1 plays a major role in iodine antiseptics-induced pain and the adjuvant effect of iodine antiseptics on allergic contact dermatitis and that TRPV1 is also involved. We further demonstrate that iodine activates TRPA1 through a redox mechanism but has no direct effects on TRPV1. Our study improves the understanding of the adverse effects of iodine antiseptics and suggests a means to minimize their side effects through local inhibition of TRPA1 and TRPV1 channels.

Keywords allergy; iodine; pain; TRPA1; TRPV1

Subject Categories Immunology; Neuroscience; Physiology

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Introduction

The therapeutic effects of iodine on wounds were discovered almost 2,000 years ago [1,2]. Elemental iodine was discovered in 1811. Shortly thereafter, iodine became widely used as an antiseptic and disinfectant worldwide, owing to its efficacy and low cost [1–4]. Iodine has an antimicrobial activity superior to that of other

antiseptics and disinfectants [1,4]. It is the only agent that is simultaneously active against Gram-positive and Gram-negative bacteria, spores, amebic cysts, virus, fungi, protozoa, and yeasts [1,2,4–6]. Importantly, despite more than 150 years of prolonged and extensive use, microbial resistance to iodine has not been observed in a clinical setting to date [1,2,5,6]. Antimicrobial resistance has become a serious threat to global public health. The overuse of antibiotics is a key factor contributing to antibiotic resistance [7]; thus, iodine antiseptics have regained attention [5,6].

As Alexander Fleming stated in 1919, in estimating the value of an antiseptic, it is necessary to study its effect on the tissues more than its effect on bacteria [8]. The adverse effects of iodine antiseptics are major factors limiting their clinical use, but their underlying mechanisms are largely unclear. Several forms of iodine antiseptics exist and have varying adverse effects. Lugol's solution and iodine tincture, which typically contain 2–7% iodine, cause substantial pain and irritation to wounds, skin, and mucosa [2–4,6]. To overcome this limitation, iodophors, the complexes of iodine and iodine-releasing agents, have been developed since the middle of the last century. Currently, povidone-iodine (PVP-I) is the most commonly used iodophor [1–4,6]. Iodophors release only very low concentrations of free iodine, thus dramatically reducing its noxious effects [1–4,6]. However, even iodophors are associated with burning or stinging sensations, local irritation, contact dermatitis, and deleterious effects on wound healing in patients [2,3,6,9–13]. These adverse effects are believed to result from iodine's chemical or immunogenic properties, but specific molecular targets and mechanisms are not known.

TRPA1 and TRPV1 are structurally related, non-selective, Ca²⁺-permeable cation channels that belong to the transient receptor potential (TRP) ion channel superfamily [14]. The majority of TRPA1 is co-expressed with TRPV1 in a subset of C-fiber nociceptors that express the neuropeptides substance P, neurokinin A, and CGRP [14,15]. Both channels are activated by a variety of noxious stimuli and play critical roles in pain and inflammatory disorders, making them key molecular targets for drug development [14,15].

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In this work, we demonstrate that iodine can cause pain and promote allergic contact hypersensitivity induced by an experimental allergen in mice. Both effects are largely dependent on the nociceptor ion channel TRPA1, which is directly activated by iodine. Another pain-sensing ion channel TRPV1 is not directly activated by iodine but plays a minor role in iodine-induced pain and iodine-enhanced allergy.

Results and Discussion

Iodine-induced pain in mice is mediated by TRPA1 and TRPV1

We first examined whether iodine could cause pain in mice as it does in humans. Intraplantar injection of 500 ppm (parts per

million) (500 ppm is equal to 0.05%) iodine in aqueous solution (25 μ l volume) into the mouse hindpaw produced substantial nociceptive behavior, including licking and lifting of the injected hindpaw (Fig 1A). The same dose of iodine in a smaller volume (1,250 ppm in 10 μ l) had a similar effect in mice (Appendix Fig S1). In addition, intraplantar injection of a 50% alcohol solution with 1,000 ppm iodine also produced a much stronger pain behavior than did the alcohol solution alone (Appendix Fig S2). These concentrations are far below that experienced by patients treated with local applications of Lugol's solution or iodine tincture, which contain 2–7% (i.e. 20,000–70,000 ppm) iodine [1,3]. Because pain is initially detected by sensory neurons, we tested the hypothesis that iodine directly stimulates pain-sensing neurons. Iodine at a concentration of 0.25 ppm induced Ca^{2+} influx in a subset of dorsal root ganglion (DRG) neurons in adult mice (Fig 1B). All

Figure 1. TRPA1 and TRPV1 mediate iodine-induced pain in mice.

- A Quantification of the nociceptive responses in mice within 5 min after intraplantar injection of control saline or 500 ppm iodine. In this and subsequent similar figures, the number of mice is indicated.
- B Averaged intracellular Ca^{2+} signals in cultured mouse DRG neurons in response to consecutive applications of 0.25 ppm iodine, 30 μ M AITC, and 2 μ M capsaicin. All of the iodine responsive neurons ($n = 332$) from 6 adult mice were included in the analysis.
- C, D Quantification of the nociceptive responses in mice within 5 min after intraplantar injection of control saline or 500 ppm iodine, following the intraperitoneal injection of HC030031 (HC) (C), AMG517 (D), or vehicle.
- E Quantification of the nociceptive responses within 5 min after intraplantar injection of control saline in WT mice or 500 ppm iodine in WT and TRPA1^{-/-} mice.
- F Quantification of the nociceptive responses in TRPA1^{-/-} mice within 5 min after intraplantar injection of control saline or 1,000 ppm iodine, following intraperitoneal injection of AMG517 or vehicle.

Data information: Data are presented as mean $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-tailed Student's t -test).

iodine-sensitive neurons responded to capsaicin, and most of them (93%) also responded to allyl isothiocyanate (AITC). Capsaicin is a specific agonist of TRPV1, and AITC is a potent agonist of TRPA1 but also activates TRPV1 with a low potency [16]. Therefore, we examined whether TRPV1 or TRPA1 is involved in iodine-induced pain in mice. We performed an intraperitoneal injection of the TRPA1-specific antagonist HC030031 or the TRPV1-specific antagonist AMG517 1 h prior to iodine injection. These agents substantially suppressed the nociceptive responses in mice, by ~93 and ~46%, respectively (Fig 1C and D). Moreover, in TRPA1^{-/-} mice, iodine-induced nociceptive responses were also substantially attenuated by ~78% (Fig 1E). These data indicate that TRPA1 is a major mediator of iodine-induced pain. The nociceptive reactions induced by iodine in TRPA1^{-/-} mice were almost completely inhibited by the TRPV1-specific antagonist AMG517 (Fig 1F). In contrast to the partial analgesic effect of AMG517 in wild-type (WT) mice (Fig 1D), this result suggests that TRPV1 accounts for the remainder of the iodine-induced pain. Thus, TRPA1 and TRPV1 are specifically responsible for the iodine-induced pain *in vivo*.

Iodine in PVP-I promotes contact hypersensitivity in mice through TRPA1 and TRPV1

PVP-I, a complex of povidone (polyvinylpyrrolidone) and iodine, is the most widely used iodophor and does not cause serious pain, owing to the low concentration of iodine (0.2–10 ppm free iodine) [3,4,11–13]. Indeed, intraplantar injection of 5% PVP-I caused much less nociceptive behavior in mice than did the injection of 500 ppm iodine (compare Fig 1A and Appendix Fig S3). However, numerous clinical case reports and studies from the past 30 years have shown that PVP-I is associated with allergic contact dermatitis [9–13]. The reported prevalence of PVP-I allergy in clinics is highly variable (between 0.7 and 41%) [2,9,17], and the effects of PVP-I on allergic contact dermatitis have never been experimentally verified and are mechanistically unclear. Furthermore, whether and how iodine plays a role in allergic reactions is controversial [18,19]. We therefore explored whether PVP-I could promote skin allergy in mice. Topical application of 5% clinical PVP-I solution on mouse skin twice a day for 2 days had no significant effects (Appendix Fig S4). However, the same treatment substantially promoted a delayed-type cutaneous allergy in a modified mouse model of allergic contact dermatitis (Fig 2A). In this model, an oxazolone derivative (Oxa) was used as an allergen to sensitize the mouse through topical application to the shaved abdomen, and 50% more amount of Oxa was used in TRPA1^{-/-} mice than in wild type in order to compensate the lower Oxa sensitivity of TRPA1^{-/-} mice [20]. Six days later, the mouse ear was challenged with relative low concentrations of Oxa to elicit delayed-type cutaneous allergies, as measured by the ear thickness change. In this model, Oxa elicits similar allergic responses in wild-type and TRPA1^{-/-} mice (Fig EV1). At a subthreshold concentration of 0.15%, Oxa did not elicit significant allergic reactions in Oxa-sensitized mice (Figs 2B and EV1). However, the concomitant application of 5% PVP-I with 0.15% Oxa induced marked ear swelling in Oxa-sensitized mice, and the reaction was substantially diminished in TRPA1^{-/-} mice (Fig 2A). In addition, 5% povidone, one of the major components of PVP-I, was co-applied with 0.15% Oxa. This combination had no significant effect on Oxa-sensitized mice (Fig 2A). In contrast, iodine alone

with 0.15% Oxa caused substantial delayed-type cutaneous reactions in a dose-dependent manner (Fig 2B). The iodine effect was observed only in Oxa-sensitized mice (Fig 2B), suggesting that the reaction is an Oxa allergy, and iodine acts as an adjuvant. In order to examine whether the adjuvant effect of iodine came from its nonspecific impairment of the skin, we showed in a control experiment that mechanical polish of the mouse ear skin with a fine abrasive paper did not cause a significant potentiation of the allergenic effect of Oxa (Appendix Fig S5). This result indicates that slight damages of the skin have no obvious effect on 0.15% Oxa-induced cutaneous allergy. However, the iodine effect was attenuated by more than 70% in TRPA1^{-/-} mice (Fig 2C), and this effect was anatomically evident (Fig 2D). Further pharmacological inhibition of TRPV1 by AMG 517 suppressed the retained allergic inflammation in TRPA1^{-/-} mice (Fig 2E). The effect of iodine in this animal model mimics a situation in which PVP-I triggers allergic contact dermatitis by aggravating pre-existing sensitization in patients with asymptomatic contact allergy [21]. Our study does not explain all types of allergy correlated with iodine antiseptics, but it demonstrates that iodine promotes cutaneous allergy in some conditions through a TRPA1- and TRPV1-dependent mechanism. These data may also explain why the prevalence of PVP-I-dependent allergy varies considerably in patients. If PVP-I acts as an adjuvant, the apparent prevalence of “PVP-I allergy” would be determined by multiple factors, including the concentrations of true allergens and patients’ sensitivity to the allergens. In the animal model described above, the true allergen is Oxa.

Activation of TRPA1 and TRPV1 causes release of neuropeptides, such as substance P (SP) and calcitonin gene-related peptide (CGRP), which are critical for allergic inflammation in the dermatitis, colitis, and asthma [15,20,22,23]. We thus examined the downstream signaling pathways by testing the effects of SP receptor NK1 or CGRP receptor antagonists on iodine-promoted cutaneous allergies. Intraperitoneal injection of NK1 antagonist RP67580 significantly suppressed the effects of iodine on Oxa-sensitized mice (Fig 2F). In contrast, the CGRP receptor antagonist BIBN4096 was ineffective (Fig EV2A), although in a positive control experiment, BIBN4096 was shown to effectively attenuate complete Freund’s adjuvant (CFA)-induced mechanical hypersensitivity in mice (Fig EV2B) [24]. These data suggest that only the SP signaling pathway is involved in the adjuvant effect of iodine on cutaneous allergy in mice.

Iodine directly activates TRPA1 but not TRPV1

We then sought to determine whether iodine directly acts on TRPA1 and TRPV1 to cause these adverse effects. We examined the effects of iodine on TRPA1 and TRPV1 activity. Iodine increased the intracellular Ca²⁺ concentration in HEK 293 cells expressing recombinant human TRPA1 (hTRPA1) in a dose-dependent manner, with an EC₅₀ of 0.25 ppm (Fig 3A and B). However, these effects did not occur in mock-transfected cells or in cells expressing TRPV1 (Fig EV3A and B). Iodine also elicited membrane currents in hTRPA1-expressing *Xenopus* oocytes with an EC₅₀ of 0.19 ppm at +80 mV (Fig 3C and D), and the currents were completely inhibited by the TRPA1 antagonist HC030031 or the non-specific TRP channel antagonist ruthenium red (Figs 3C and EV3C). In addition, iodine-induced HC030031-sensitive macroscopic currents in an inside-out

Figure 2. Iodine in PVP-I promotes contact hypersensitivity in mice mainly through TRPA1 and TRPV1.

A Left, time course of ear swelling elicited by topical application of 5% PVP-I solution in 0.15% Oxa-challenged WT and TRPA1^{-/-} mice, or 5% povidone solution in Oxa-challenged WT mice. In this and subsequent similar figures, the number of mice is indicated. Right, bar graph highlighting the response at 24 h shown at left.

B Time course of ear swelling elicited by topical application of the indicated concentrations of iodine in 0.15% Oxa-challenged mice and a bar graph highlighting the response at 24 h.

C Time course of ear swelling elicited by topical application of 3% iodine in 0.15% Oxa-challenged WT and TRPA1^{-/-} mice and bar graph highlighting the response at 24 h.

D Hematoxylin- and eosin-stained tissue sections of iodine-treated ear in 0.15% Oxa-challenged WT and TRPA1^{-/-} mice ($n \geq 3$).

E Time course of ear swelling elicited by topical application of 3% iodine in 0.15% Oxa-challenged TRPA1^{-/-} mice, following intraperitoneal injection of AMG517 or vehicle, and bar graph highlighting the response at 24 h.

F Time course of ear swelling elicited by topical application of 3% iodine in 0.15% Oxa-challenged mice, following intraperitoneal injection of RP67580 or vehicle, and bar graph highlighting the response at 24 h.

Data information: Data are presented as mean $\pm$ s.e.m. Statistical significance was evaluated using two-tailed Student's *t*-test (for all two-group comparisons) or one-way analysis of variance (ANOVA) followed by Tukey's test (for multi-group comparisons). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

membrane patch from hTRPA1-expressing HEK 293 cells (Fig 3E) or whole-cell currents in cells expressing mouse TRPA1 (Fig EV3D). In contrast, iodine had no effects on HEK 293 cells and *Xenopus* oocytes lacking TRPA1 expression (Fig EV3E and F). Notably, the EC₅₀ values of hTRPA1 activation by iodine derived from the calcium imaging or electrophysiological experiments were far below the concentrations of iodine in most iodine antiseptics, such as Lugol's solution (2–5%), iodine tincture (2–7%), and 10% PVP-I solution (~10 ppm free iodine) [1,3,11–13].

We then sought to understand how iodine activates TRPA1. Iodine's microbicidal effect is dependent on its reactions with amino acid residues such as cysteine and lysine, resulting in lethal changes

to bacterial protein structures (Appendix Fig S6A), and reactions with unsaturated fatty acids in the bacteria membrane (Appendix Fig S6B), changing the chemico-physical properties of the membrane [3,17]. Previous studies have demonstrated that electrophilic compounds activate TRPA1 through a mechanism of covalent modification of cysteine and lysine [25,26]. Therefore, we test the hypothesis that reactive amino acid residues may be involved in the activation of TRPA1 by iodine. Iodine-induced hTRPA1 currents were not reversible by washout (Fig 3C), but 3 mM dithiothreitol (DTT), a cell-permeable reducing agent, completely reversed the sustained hTRPA1 activation (Fig 3F). In contrast, DTT did not significantly affect TRPA1 currents elicited by a non-reactive TRPA1

Figure 3. Iodine activates TRPA1 but not TRPV1.

- A Representative intracellular Ca^{2+} signals in hTRPA1-expressing HEK 293 cells in response to different concentrations of iodine. 2-APB, a TRPA1 agonist, was subsequently applied to fully activate TRPA1. RFU: relative fluorescence unit.
- B Concentration–response relationships of iodine-induced intracellular Ca^{2+} increase in HEK 293 cells expressing WT or mutant TRPA1 channels. Data are presented as mean $\pm$ s.e.m. $n \geq 8$ for each construct at each concentration. The smooth curves are fits to the Hill equation.
- C Time course of iodine-induced currents in hTRPA1-expressing *Xenopus* oocytes. HC: HC030031.
- D Concentration–response relationship of iodine-induced currents in hTRPA1-expressing *Xenopus* oocytes. Data are presented as mean $\pm$ s.e.m. $n \geq 8$ for each concentration. The smooth curve is a fit to the Hill equation.
- E Time course of intracellular iodine-induced macroscopic currents in an inside-out patch from hTRPA1-expressing HEK 293 cell ($n = 3$).
- F Time course of iodine-induced currents in hTRPA1-expressing *Xenopus* oocytes, which presents the current reduction upon DTT treatment ($n = 3$).
- G, H Comparisons of the iodine-induced whole-cell current in HEK 293 cells expressing WT hTRPA1 (G) or the triple mutant channel (C421S/K710R/C856S) (H) ($n \geq 4$).
- I Representative whole-cell currents in HEK 293 cells expressing human TRPV1 in response to iodine and subsequently applied capsaicin ($n = 3$).

agonist, 2-APB (2-aminoethoxydiphenyl borate) (Appendix Fig S7) [26,27]. These data suggest that oxidation is required for iodine-induced TRPA1 activation. Indeed, TRPA1 is described as a neuronal sensor for oxygen and many oxidants [23,28–31]. Since single mutations of C421, K710, and C856 attenuate TRPA1 activation by electrophilic compounds or hyperoxia, respectively [25,26,31], we constructed double and triple mutations of these residues and found that a double (C421S/C856S) and a triple mutation (C421S/K710R/C856S) decreased both the potency and efficacy of

iodine (Figs 3B and EV4A and B). Whereas iodine (2 ppm) elicited robust currents in cells expressing WT TRPA1 (Fig 3G), it produced little currents in cells expressing the C421S/K710R/C856S mutant channel (Fig 3H). The mutant channels, however, responded normally to 2-APB (Fig EV4A and B). Taken together, these results suggest that iodine directly activates TRPA1 and that redox reactions between iodine and reactive residuals in TRPA1 play an important role in this activation. On the other hand, another triple mutation (C621S/C641S/C665S) known to specifically ablate TRPA1

sensitivity to electrophilic agonists [26] did not significantly affect the channel responses to iodine (Figs 3B and EV4C). This result indicates that these cysteines are not involved in iodine activation of TRPA1, suggesting a mechanistic difference in the activation of TRPA1 by iodine and electrophilic agonists.

In contrast to the robust activation of TRPA1, iodine did not elicit currents at concentrations of up to 300 ppm in HEK 293 cells expressing hTRPV1 (Fig 3I). TRPA1 is heavily co-expressed with TRPV1 in sensory neurons, and recent studies suggest that TRPA1 and TRPV1 can form heteromeric complexes and functionally inter-regulate each other [32–35]. Thus, it is possible that iodine may affect TRPV1 through TRPA1 activation. However, we found that the nociceptive behavior or allergic inflammation elicited by iodine in TRPA1^{−/−} mice could be diminished by the TRPV1-specific antagonist AMG517 (Figs 1F and 2E), which suggests that at least part of TRPV1's contribution to the noxious effects of iodine is independent of TRPA1. In addition, previous studies demonstrate that TRPV1 is sensitized by endogenous agents in an “inflammatory soup”, and this sensitization reduces the threshold of TRPV1 activation and increases the responsiveness of pain-sensing neurons [14,15]. Interestingly, we observed that intraplantar injection of iodine, in addition to eliciting nociceptive reactions, caused acute inflammation within 10 min as measured by paw swelling, but the inflammation was not affected by genetic ablation or pharmacological inhibition of TRPA1 and TRPV1 (Fig EV5). Therefore, it is conceivable that iodine may affect TRPV1 activity through secondary mediators of iodine-induced inflammation. We further tested the effects of iodine on DRG neurons by using whole-cell patch-clamp recording. About 1 ppm iodine elicited HC030031-sensitive currents in some neurons, and some capsaicin-sensitive neurons did not respond to 100 ppm iodine (Fig EV3G and H). These results suggest that iodine activates TRPA1 in native cells.

To our knowledge, TRPA1 is the first discovered endogenous target of iodine antiseptics in animals. Although iodine may also target other unknown endogenous molecules or pathways independently of TRPA1 and TRPV1, our *in vivo* studies demonstrate that both the iodine-induced pain behavior and its adjuvant effect on Oxa-induced allergy were attenuated by more than 70% in TRPA1^{−/−} mice and almost completely blocked by further pharmacological inhibition of TRPV1. Thus, other mechanisms do not significantly contribute to the noxious effects of iodine in our study.

The use of iodine antiseptics is limited due to their side effects [2,5,6]. Newer formulations of iodine antiseptics have undergone testing to minimize the adverse reactions, but all the testing has focused on the delivery systems associated with iodine release. Our study provides a rationale for a new approach to developing better iodine antiseptics through the local inhibition of TRPA1 and TRPV1 channels.

Materials and Methods

Chemicals

Iodine was purchased from Xilong Chemical Co. Povidone–iodine (PVP-I) was obtained from Caoshanhu Disinfection Supplies Co.; povidone (polyvinylpyrrolidone) was obtained from Adamas-beta. HC030031, 2-APB (2-aminoethoxydiphenyl borate), capsaicin,

4-ethoxymethylene-2-phenyloxazol-5-one (an oxazolone derivative used as the hapten and simply called Oxa in this study), and complete Freund's adjuvant (CFA) were obtained from Sigma-Aldrich. Allyl isothiocyanate was obtained from Ai Keda Chemical Technology Co. Ionomycin was obtained from Cayman Chemical. Ruthenium red was obtained from Santa Cruz. DTT (dithiothreitol) was obtained from Sangon Biotech. AMG517 was obtained from MedChem Express. RP67580 and BIBN4096 were from TOCRIS.

Mouse models

Mice used for this study were kept in the animal services facility of Kunming Institute of Zoology. TRPA1^{+/-} mice were inbred to produce TRPA1^{+/+} (C57BL/6 wild type), TRPA1^{-/-}, and TRPA1^{+/-} mice that were identified by genotyping PCR. Seven- to nine-week-old C57BL/6 wild-type and TRPA1^{-/-} mice were used, half male and half female.

Before the assessment of pain behavior, mice were acclimated to a Plexiglas chamber for 30–60 min. In addition, 500 ppm or 1,000 ppm iodine solution was prepared in a 0.9% saline solution in which 0.1% or 0.2% NaI was added to facilitate iodine solubility. Freshly prepared iodine solution or control saline in a 25-μl volume was injected into the plantar surface of mouse hindpaws, and the mice were immediately returned to the Plexiglas chamber and recorded by a digital video camera. The time mice spent licking or lifting the injected paw was counted during 5 min after iodine injection. The thickness of the paw before and at 10 min after iodine injection was measured by using a digital thickness gauge (Mitutoyo Corp.), which had a resolution of 10 μm. Paw swelling was calculated as: [(thickness at 10 min) – (thickness at 0 min)]. In some experiments, the HC030031 (300 mg/kg) or AMG517 (3 mg/kg) and their corresponding vehicles were intraperitoneally injected 1 h prior to the intraplantar injection of iodine.

For the mouse model of inflammatory pain, mice received an intraplantar injection of 1:1 saline/CFA emulsion (20 μl) into the right hindpaw. BIBN4096 (2 mg/kg) or vehicle was administered by intraperitoneal injection at −1, 8, and 16 h after CFA injection for a total of three injections. The CFA-induced mechanical hypersensitivity was studied at 24 h after CFA injection. Briefly, the mice in each group were placed in a plastic cage with a wire mesh bottom and were allowed to acclimate for 20–30 min until cage exploration and major grooming activities ceased. Subsequently, the 50% paw withdrawal thresholds were assessed with von Frey filaments (North Coast Medical, Inc.) using the up-down method [36].

In the mouse model of allergic contact dermatitis, wild-type mice were sensitized by painting 3% Oxa in ethanol (150 μl) onto the shaved abdomens. Six days later, the mice were challenged with a subthreshold concentration (0.15%) of Oxa (in 20 μl ethanol) on the right ear, and ethanol (20 μl) was applied on the left ear as the control. TRPA1^{-/-} mice were sensitized by 4% Oxa in ethanol (169 μl) and challenged with 0.15% of Oxa (in 20 μl ethanol). At 24 and 0 h prior to Oxa challenge, iodine was applied to both ears. The mice in the “W/O Oxa sensitization” group served as the negative control. These mice were not sensitized by Oxa but were challenged with 0.15% Oxa and were painted with 3% iodine on both ears. Ear thickness at 0, 24, 48, and 72 h after Oxa challenge was blindly measured using a digital thickness gauge (Mitutoyo Corp.). Ear swelling was calculated as: [(ear thickness of the right ear

at x hours) – (ear thickness of the right ear at 0 h)] – [(ear thickness of the left ear at x hours) – (ear thickness of the left ear at 0 h)]. For the *in vivo* pharmacological experiments, the HC030031 (300 mg/kg), AMG517 (3 mg/kg), neurokinin 1 (NK1) receptor antagonist RP67580 (3 mg/kg), CGRP receptor antagonist BIBN4096 (2 mg/kg), or vehicle were administered by intraperitoneal injection at –1, 8, and 16 h after Oxa challenge for a total of three injections.

The delivery methods and concentrations of HC030031, AMG517, RP67580, and BIBN4096 that were used in our study were chosen based on those used in other studies inhibiting TRPA1, TRPV1, neurokinin 1 receptor, or CGRP receptor in mice [20,37–42].

Histology

When iodine-induced ear swelling became most prominent (24 h after Oxa challenge), mice painted with 0% (control) or 3% iodine on the ears were euthanized. The ears were excised and fixed in Bouin solution (75 ml saturated picric acid aqueous solution, 25 ml 40% formalin aqueous solution, and 5 ml glacial acetic acid). The tissues were embedded in paraffin after dehydration and paraffin infiltration. Then, 5- μ m paraffin sections were cut by using a semi-automated rotary microtome (Leica Biosystems) and were stained with hematoxylin–eosin, according to standard procedures by using a Hematoxylin–eosin Staining Kit (Beyotime Institute of Biotechnology). Images were obtained using an Olympus cellSens platform (Olympus Corporation).

Primary DRG neuronal culture

TRPA1^{+/+} (C57BL/6 wild-type) mice (7–9-week-old) were euthanized, and the dorsal root ganglia were dissected out, rinsed with Hank's buffer (Gibco), and digested in the same solution containing 1 mg/ml collagenase P (Roche Diagnostics) for 30–40 min at 37°C. Partially digested tissues were centrifuged at 157 g for 3 min, and the pellets were resuspended in 0.25% trypsin–EDTA (Gibco) and digested for an additional 5 min at 37°C. The digested ganglia were spun down, resuspended, and triturated with plastic pipette tips to release neurons. The cells were filtered through a 70- μ m cell strainer (Biologix), plated into 96-well plates, and cultured in DMEM/F-12 supplemented with GlutaMAX (Gibco), 10% serum (Gibco), and penicillin (100 U/ml)/streptomycin (0.1 mg/ml) (Biological Industries). Twelve hours after plating, the culture medium was gradually replaced by Neurobasal-A (Gibco) supplemented with B27 (Gibco), penicillin (100 U/ml)/streptomycin (0.1 mg/ml) (Biological Industries), and GlutaMAX (Gibco). Calcium imaging experiment was performed after 48 h.

Xenopus oocyte preparation and injection

Xenopus laevis (African clawed) frogs were anesthetized in a 0.3% tricaine solution. Ovarian lobes were excised in OR2 solution (mM: NaCl 82.4, KCl 2.5, MgCl₂ 1, HEPES 5) and digested in OR2 solution supplemented with 0.2 mg/ml collagenase (Sigma-Aldrich) for 2–3 h at room temperature. Then, single defolliculated oocytes were individually selected and injected with ~50 ng synthesized cRNA of human TRPA1. Recordings were performed 2–3 days after injection.

All animal experiments described above were approved by the Animal Care and Use Committee at Kunming Institute of Zoology, Chinese Academy of Sciences.

Clones and mutagenesis

Human and mouse TRPA1 (GenBank accession number NM_007332 and NM_177781, respectively) and human TRPV1 (NM_080706) were all cloned in pcDNA3.1. Site-directed mutations were constructed by oligonucleotide-based mutagenesis using PCR with Q5 polymerase (New England Biolabs) following the instruction manual of QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) and were confirmed by DNA sequencing.

HEK 293 cell culture and transfection

HEK 293 cells were grown in DMEM (HyClone) plus 10% newborn calf serum (Gibco) and penicillin (100 U/ml)/streptomycin (0.1 mg/ml) (Biological Industries). HEK 293 cells were transiently transfected with wild-type or mutant TRP channels with or without enhanced GFP plasmids using X-tremeGENE HP (Roche Diagnostics) and used within 48 h.

Intracellular Ca²⁺ imaging

Intracellular calcium imaging of transiently transfected HEK 293 cells was performed by using the FlexStation 3 microplate reader (Molecular Devices). Cells were plated in 96-well plates and loaded with fluo-4 AM (10 μ M) and Pluronic F-127 (0.02%) (Molecular Probes) at 37°C for 1 h in a Ca²⁺-free imaging solution. Subsequently, real-time fluorescence changes in cells upon the addition of a test compound were measured.

For intracellular calcium imaging of DRG neurons, the neurons were loaded with Fura-2 AM (10 μ M) and Pluronic F-127 (0.02%) (Molecular Probes) at 37°C for 1 h in a Ca²⁺-free imaging solution. The fluorescence ratios of F340/F380 were measured by using a fluorescence microscopic system (Olympus Corporation, Sutter Instrument and Molecular Devices).

The standard imaging solution contained (in mM) 145 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, pH 7.4. The solutions containing iodine were prepared immediately before the experiment and used within 5 min.

Electrophysiology

All experiments were performed at room temperature (~22°C). For whole-oocyte recordings with a two-electrode voltage clamp, electrodes were filled with 3 M KCl and had a resistance of 2–10 M Ω . Currents were recorded with an OC-725C Oocyte Clamp (Warner Instrument Corp.) and digitized by Digidata 1440A (Molecular Devices). Oocytes were clamped at –60 mV, and currents were elicited by 200-ms voltage ramps from –100 to +100 mV at a frequency of 0.5 Hz. Currents were low-pass-filtered at 2 kHz and sampled at 10 kHz. pCLAMP software (Molecular Devices) was used for data acquisition and analysis. The recording solution contained (in mM) 100 NaCl, 1 MgCl₂, 2.5 KCl, and 5 HEPES, pH 7.6. Test solutions containing iodine were prepared immediately before the experiments and used within 5 min.

For patch-clamp recordings, pipettes were fabricated and fire-polished to resistances of 1–2 M Ω for inside-out patch recording and 2–3 M Ω for whole-cell recording. Currents were elicited in HEK 293 cells by 500-ms voltage ramps from –100 to +100 mV at a frequency of 0.5 Hz with a holding potential of 0 mV. Currents were amplified by Axopatch 200B and digitized by Digidata 1440A (Molecular Devices). Currents were low-pass-filtered at 2 kHz and sampled at 10 kHz. pCLAMP software (Molecular Devices) was used for data acquisition and analysis. The standard extracellular solution contained (in mM) 150 NaCl, 1 MgCl₂, and 10 HEPES, pH 7.4; the standard intracellular solution contained (in mM) 150 NaCl, 1 MgCl₂, 1 EGTA, and 10 HEPES, pH 7.4. For inside-out patch recording, 5 mM sodium triphosphate was included in the intracellular solution [43]. For the whole-cell patch-clamp recording of DRG neurons, the holding potential is –60 mV and the iodine or capsaicin-induced currents were recorded at –60 mV. The standard extracellular solution for neuronal recording contained (in mM) 150 NaCl, 1 MgCl₂, and 10 HEPES, pH 7.4; the intracellular solution contained (in mM) 140 CsCl, 2 Na₂-ATP, 1 MgCl₂, 5 EGTA, and 10 HEPES, pH 7.2. Test solutions containing iodine were prepared immediately before the experiment and used within 5 min.

Statistics

We used sample/animal sizes that were deemed suitable for statistics and were similar to other studies in the field. Animals were randomly selected and allocated to experimental groups. Data normality was assessed by the Shapiro–Wilk method, and the equality of variances for two or more data groups was determined by *F*-test or Levene's test. Statistical significance was evaluated using two-tailed *t*-test (for all two-group comparisons) or one-way analysis of variance (ANOVA) followed by Tukey's test (for multi-group comparisons). Data are presented as the mean $\pm$ standard error of the mean (s.e.m.), and a *P*-value < 0.05 is considered statistically significant. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. NS indicates no significant difference.

Expanded View for this article is available online.

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Author contributions

SW and JY conceived the project and designed and supervised the experiments, in discussion with JC and MZ. DS and HZ performed mutagenesis,

electrophysiology, and Ca²⁺ imaging. JH, HZ, and DT did animal experiments with assistance from DS. DS performed histology. SW, JY, and DS wrote the manuscript, with inputs from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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