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ION CHANNELS, RECEPTORS AND TRANSPORTERS

Primary alcohols activate human TRPA1 channel in a carbon chain length-dependent manner

Tomoko Komatsu · Kunitoshi Uchida · Fumitaka Fujita · Yiming Zhou · Makoto Tominaga

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Abstract Transient receptor potential ankyrin 1 (TRPA1) is a calcium-permeable non-selective cation channel that is mainly expressed in primary nociceptive neurons. TRPA1 is activated by a variety of noxious stimuli, including cold temperatures, pungent compounds such as mustard oil and cinnamaldehyde, and intracellular alkalization. Here, we show that primary alcohols, which have been reported to cause skin, eye or nasal irritation, activate human TRPA1 (hTRPA1). We measured intracellular Ca^{2+} changes in HEK293 cells expressing hTRPA1 induced by 1 mM primary alcohols. Higher alcohols (1-butanol to 1-octanol) showed Ca^{2+} increases proportional to the carbon chain length. In whole-cell patch-clamp recordings, higher alcohols (1-hexanol to 1-octanol) activated hTRPA1 and the potency increased with the carbon chain length. Higher alcohols evoked single-channel opening of hTRPA1 in an inside-out configuration. In addition, cysteine at 665 in the N terminus and histidine at 983 in the C terminus were important for hTRPA1 activation by primary alcohols. Furthermore, straight-chain secondary alcohols increased intracellular Ca^{2+} concentrations in HEK293 cells expressing hTRPA1, and both primary and secondary alcohols showed hTRPA1 activation activities that correlated highly with their octanol/water partition coefficients. On the other hand, mouse TRPA1 did not show a strong response to 1-hexanol or 1-octanol, nor did these alcohols evoke significant pain in mice. We conclude that primary and secondary alcohols activate hTRPA1 in a carbon chain length-dependent manner. TRPA1 could be a sensor of alcohols inducing skin, eye and nasal irritation in human.

Keywords Alcohol · Transient receptor potential · Pain · Sensory neurons · Calcium imaging · Patch clamp

Abbreviations

TRPA1	Transient receptor potential ankyrin 1
AITC	Allyl isothiocyanate
SEM	Standard error of the mean
ANOVA	Analysis of variance
C1OH	Methanol
C2OH	Ethanol
1-C3OH	1-Propanol
1-C4OH	1-Butanol
1-C5OH	1-Pentanol
1-C6OH	1-Hexanol
1-C7OH	1-Heptanol
1-C8OH	1-Octanol
2-APB	2-Aminoethoxydiphenyl borate
NP_o	Channel number × open probability
TPEN	*N*,*N*,*N*′,*N*′-Tetrakis (2-pyridylmethyl) ethylenediamine

T. Komatsu and K. Uchida contributed equally to this work.

T. Komatsu · K. Uchida (✉) · Y. Zhou · M. Tominaga (✉)
Division of Cell Signaling, Okazaki Institute for Integrative Bioscience (National Institute for Physiological Sciences), National Institutes of Natural Sciences,
Higashiyama 5-1, Myodaiji,
Okazaki, Aichi 444-8787, Japan
e-mail: kuchida@nips.ac.jp
e-mail: tominaga@nips.ac.jp

T. Komatsu
Japan Society for the Promotion of Science,
Tokyo, Japan

K. Uchida · Y. Zhou · M. Tominaga
Department of Physiological Sciences,
Graduate University for Advanced Studies,
Okazaki, Japan

F. Fujita
Central Research Laboratories, Mandam Corporation,
Osaka, Japan

DTPA	Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid
IOB	Inorganic organic balance
GABA	γ-Aminobutyric acid

Introduction

Transient receptor potential ankyrin 1 (TRPA1) channel is a non-selective cation channel that is mainly expressed in primary sensory neurons [32]. TRPA1 is activated by various stimuli, including mechanical stimuli [21], cold temperatures [32], intracellular alkalization [12], intracellular Ca^{2+} and Zn^{2+} ions [15, 35], and pungent and/or noxious chemical compounds [2, 18]. Although responsiveness to noxious cold and mechanical stimuli is still controversial in mammals [4, 20, 21], TRPA1 has attracted attention for its potential role in nociception [3, 21, 32]. Many TRPA1 agonists, including isothiocyanates such as allyl isothiocyanate (AITC) and reactive aldehydes such as cinnamaldehyde, have a reactive electrophilic group that activates TRPA1 through covalent binding to cysteine residues in the cytosolic N-terminus of the channel [14, 22]. Other non-electrophilic agonists of the channel include icilin, tetrahydrocannabinol, several NSAIDs, 2-aminoethoxydiphenyl borate (2-APB), menthol, and general anesthetics such as propofol [6, 8, 16, 18, 19, 23, 28], but their activation mechanisms have not been well characterized.

While primary alcohols are added to cosmetics as antiseptic agents and are used to make some compounds such as detergents, some reports have shown that primary alcohols cause skin, eye, and nasal irritation in rabbits and humans [5, 17, 30]. In particular, 1-octanol shows the strongest irritation among primary alcohols (from ethanol to 1-hexadecanol) [5, 30]. The molecular mechanisms for this irritation are little known, although some reports have shown that primary alcohols activate $GABA_A$ receptors, inhibit nicotinic ACh receptors and modulate $5\text{-}HT_3$ receptor activity, and high concentrations of ethanol activate TRPV1 [7, 9, 26, 33].

In this study, we tested the hypothesis that primary alcohol-evoked irritation involves the activation of TRPA1 channels. We found that primary alcohols activate human TRPA1 (hTRPA1) well, but show significantly less activation of mouse TRPA1 (mTRPA1). Furthermore, we observed that primary alcohol-evoked hTRPA1 activation depends on carbon chain length, and cysteine and histidine residues in the cytosolic termini were important for activation.

Experimental procedures

Animals

Male C57BL/6NCr mice (4–6 weeks old; SLC, Japan) were used as controls. TRPA1-deficient mice (generously provided by Dr. David Julius, UCSF, USA) [3] were backcrossed on a C57BL/6NCr background. Mice were housed in a controlled environment (12-h light/12-h dark cycle; room temperature, 22–24°C; 50–60% relative humidity) with free access to food and water. All procedures involving the care and use of animals were approved by the Institutional Animal Care and Use Committee of National Institutes of Natural Sciences and carried out in accordance with the NIH Guide for the care and use of laboratory animals (NIH publication no. 85-23. Revised 1985).

Cell culture

Human embryonic kidney-derived 293 (HEK293) cells were maintained in DMEM (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% FBS (Biowest SAS, Caille, France), 100 units/ml penicillin (Invitrogen Corp., Carlsbad, CA, USA), 100 mg/ml streptomycin (Invitrogen Corp.), and 2 mM L-glutamine (GlutaMAX; Invitrogen Corp.) at 37°C in 5% CO_2. For Ca^{2+} imaging, 1 μg plasmid DNAs of human TRPA1 (hTRPA1), mouse TRPA1 (mTRPA1, gift from Ardem Patapoutian), human TRPV1 (hTRPV1), human TRPV2 (hTRPV2) and human TRPM8 (hTRPM8) in pcDNA3 and 0.1 μg pCMV-DsRed-expression cDNAs in OPTI-MEM medium (Invitrogen Corp.) were transfected to HEK293 cells using Lipofectamine Plus Reagent (Invitrogen Corp.). For patch-clamp recordings, 0.1 μg pGreen Lantern 1 cDNA was used instead of DsRed cDNAs. After incubating for 3–4 h, cells were reseeded on coverslips and further incubated at 37°C in 5% CO_2. Ca^{2+} imaging and whole-cell patch-clamp recordings were performed 1 day after transfection.

Construction of TRPA1 mutants

Three types of hTRPA1 cysteine mutants were generated by cysteine–serine substitution at C621, C641 and/or C665 that are thought to be covalently modified by several electrophilic TRPA1 agonists. A histidine mutant of hTRPA1 was generated by histidine–alanine substitution at H983, and a cysteine mutant was generated by cysteine–serine substitution at C641 and C1021 that are thought to be sites for activation by a zinc ion. These mutants were made using a modified QuickChange Site-Directed Mutagenesis method (Stratagene Corp., La Jolla, CA, USA). In detail, polymerase chain reaction (PCR) was performed using hTRPA1 expression vector as templates, two synthetic oligonucleotide primers containing specific mutations (Table 1), and primeSTAR HS DNA polymerase (Takara Bio Inc., Shiga, Japan). The PCR products were digested with Dpn1 at 37°C for 1 h and transformed into DH5α competent cells. The entire sequence including desired substitution in the mutants was confirmed.

Table 1 Sequences of the primers used for construction of TRPA1 mutants

Mutation	Sense primer (5′→3′)	Antisense primer (5′→3′)
C621S	CTCCAGGCAATAAATCTCCAATTACAGAAATGATAG	CTATCATTTCTGTAATTGGAGATTTATTGCCTGGAG
C641S	GGTACTTTTAGATTTCAGCATGTTGCATTCCACAG	CTGTGGAATGCAACATGCTGAAATCTAAAAGTACC
C665S	GTATAATTTCAAATATCTTCAATCTCCATTAGAATTCACC	GGTGAATTCTAATGGAGATTGAAGATATTTGAAATTATAC
C1021S	GGGATGTTATTCCATATATTCTCTTTTTTATTTTGCACTGGG	CCCAGTGCAAAATAAAAAAGAGAATATATGGAATAACATCCC
H983A	GCAGGTGGAACTTGCTACCAGCTTAGAGAAG	CTTCTCTAAGCTGGTAGCAAGTTCCACCTGC

Ca^{2+}-imaging

HEK293 cells on the coverslips were mounted in an open chamber and superfused with standard bath solution (140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.4). Cytosolic-free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in HEK293 cells were measured by dual-wavelength fura-2 (Molecular Probes, Invitrogen Corp.) microfluorometry with excitation at 340/380 nm and emission at 510 nm. The ratio image was calculated and acquired using an imaging processing system (IP-Lab, Scanalytic Inc., Virginia, USA). The ratio value was normalized to the peak response by 5 μM ionomycin (Dojindo Laboratories, Kumamoto, Japan).

Electrophysiology

HEK293 cells on coverslips were mounted in an open chamber and superfused with standard bath solution as for the Ca^{2+}-imaging experiments. The pipette solution contained (mM) 140 KCl, 5 EGTA, 10 HEPES, pH 7.4 (with KOH). For inside-out single-channel recordings, the bath solution was the pipette solution for whole-cell patch-clamp recordings, and the pipette solution was the standard bath solution. Data from whole-cell voltage-clamp recordings were sampled at 10 kHz and filtered at 5 kHz for analysis (Axon 200B amplifier with pCLAMP software; Axon Instruments, Foster City, CA, USA). Membrane potential was clamped at −60 mV and voltage ramp pulses from −100 to +100 mV (300 ms) were applied every 3 or 5 s. Inside-out single-channel recording data were sampled at 10 kHz and filtered at 2 kHz for analysis. All experiments were performed at room temperature.

Pain-related behavioral test

Wild-type (WT) and TRPA1-deficient (TRPA1-KO) mice were placed individually in transparent cages (20×12×12 cm) for 1 h before experiments. An intraplantar injection of 20 μl 1-octanol (100 mM, solvent: 10% DMSO containing saline) was then made into the left hind paw. The time spent licking and biting the injected paw was measured for 10 min after injection.

Statistical analysis

Data are expressed as mean ± SEM. Octanol/water partition coefficient was calculated using ChemDraw software (CambridgeSoft Corporation, Cambridge, MA, USA). Statistical analysis was performed by Student's *t*-test, or one-way analysis of variance (ANOVA) followed by a two-tailed multiple *t*-test with Bonferroni correction. *P* values less than 0.05 were considered significant.

Results

Primary alcohols activate human TRPA1

To clarify whether primary alcohols activate TRPA1, we measured $[Ca^{2+}]_i$ changes using fura-2 microfluorometry in HEK293 cells expressing hTRPA1. $[Ca^{2+}]_i$ increases were observed during application of 1 mM of 1-octanol (1-C8OH) (Fig. 1a). Such $[Ca^{2+}]_i$ increases were not observed in the vector-transfected cells. When 1 mM of each primary alcohol (methanol, C1OH; ethanol, C2OH; 1-propanol, 1-C3OH; 1-butanol, 1-C4OH; 1-pentanol, 1-C5OH; 1-hexanol, 1-C6OH; 1-heptanol, 1-C7OH; 1-octanol, 1-C8OH) was applied to HEK293 cells expressing hTRPA1, $[Ca^{2+}]_i$ increases were observed for all the examined alcohols. Furthermore, $[Ca^{2+}]_i$ increases became larger when the carbon chain-length became longer from 1-C5OH to 1-C8OH (Fig. 1b). This carbon chain length-dependent activation of hTRPA1 was also observed for aldehydes and thiols (Fig. 1c). We then checked the effects of primary alcohols on other TRP channels which are expressed in primary sensory neurons. Although hTRPV1 responded weakly, hTRPV2 or hTRPM8 did not respond at all to 1 mM 1-C8OH (Fig. 1d).

To confirm that primary alcohols can activate TRPA1 channels, we performed whole-cell patch-clamp experiments in HEK293 cells expressing hTRPA1. Figure 2a shows a representative whole-cell current evoked by 0.6 mM 1-C8OH in HEK293 cells expressing hTRPA1. An inward current (at −60 mV) with an outwardly rectifying current–voltage (I–V) relationship by ramp pulses from −100 to +100 mV every 3 s was observed. Functional hTRPA1 expression was confirmed by the response to 20 μM AITC.

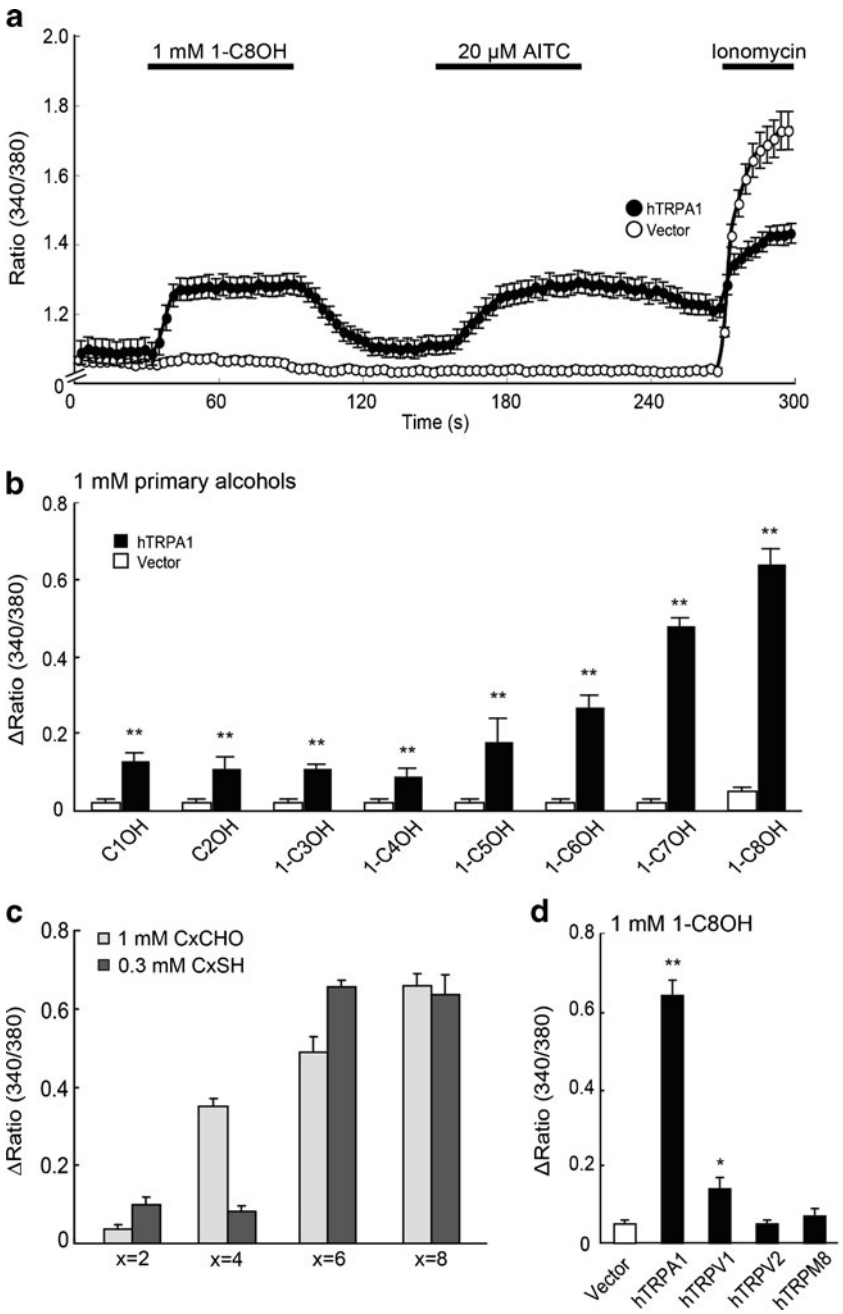

Fig. 1 TRPA1-mediated $[Ca^{2+}]_i$ increases induced by primary alcohols in HEK293 cells expressing human TRPA1. **a** $[Ca^{2+}]_i$ changes stimulated by 1 mM 1-octanol (*1-C8OH*) in HEK293 cells (hTRPA1), but not in non-transfected HEK293 cells (Vector). *Ordinates*: fura-2 ratio (340 nm/360 nm). *Abscissae*: time (s). Each symbol represents mean ± SEM from 34 (hTRPA1) or 55 (Vector) cells. **b** $[Ca^{2+}]_i$ increases stimulated by 1 mM primary alcohols (methanol [*C1OH*], ethanol [*C2OH*], 1-propanol [*C3OH*], 1-butanol [*C4OH*], 1-pentanol [*C5OH*], 1-hexanol [*C6OH*], 1-heptanol [*C7OH*], 1-octanol [*C8OH*]). *Ordinates*: Δratio (340/360 nm). Each column represents the mean ± SEM from 13 to 107 cells. Statistical significance was assessed using Student's *t*-test. $^{**}P<0.01$ vs. Vector. **c** TRPA1-mediated $[Ca^{2+}]_i$ increases by 1 mM of aldehydes (CxCHO) and 0.3 mM of thiols (CxSH) in HEK293 cells expressing human TRPA1. X indicates carbon chain length. Ordinates: Δratio (340/360 nm). Each column represents the mean ± SEM from 13 to 86 cells. **d** The effect of 1 mM 1-octanol on other TRP channels (hTRPV1, hTRPV2 and hTRPM8). *Ordinates*: Δratio (340/360 nm). Each column represents the mean ± SEM from 34 to 55 cells. Statistical significance was assessed using ANOVA followed by the two-tailed multiple *t*-test with Bonferroni correction. $^{*}P<0.05$, $^{**}P<0.01$ vs. Vector

Moreover, the currents were completely blocked by 10 μM HC030031, a specific TRPA1 antagonist (Fig. 2b) either during or prior to 1-C8OH application, further indicating that TRPA1 is activated by 1-C8OH. We next evaluated the dose–response profiles of the currents activated by C2OH, 1-C4OH, 1-C6OH, 1-C7OH and 1-C8OH. As shown in Fig. 2c, although significant $[Ca^{2+}]_i$ increases were observed upon application of C1OH to 1-C4OH in the Ca^{2+}-imaging experiments (Fig. 1b), C2OH and 1-C4OH did not evoke current responses even at a high concentration (100 mM), suggesting that the observed $[Ca^{2+}]_i$ increases seemed to be non-specific. On the other hand, not only 1-C8OH, but also 1-C6OH and 1-C7OH evoked TRPA1 activation while no TRPA1 activation was evoked in vector-transfected cells. Their potencies became higher as the carbon chain length became longer (EC_{50}: 7.94±0.93 mM for 1-C6OH, 2.72±0.42 mM for 1-C7OH, and 0.81±0.02 mM for 1-C8OH), while efficacies were lower in response to 1-C8OH (V_{max}: 633.2±58.1 pA/pF for 1-C6OH, 507.9±61.8 pA/pF for 1-C7OH, and 353.1±16.0 pA/pF for 1-C8OH). We could not observe the response to 1-C6OH in excess of 30 mM because of poor solubility (Fig. 2c).

To examine whether primary alcohols activate TRPA1 in a membrane-delimited fashion, we performed single-channel recordings with an inside-out configuration in HEK293 cells expressing hTRPA1. Basal TRPA1 activity

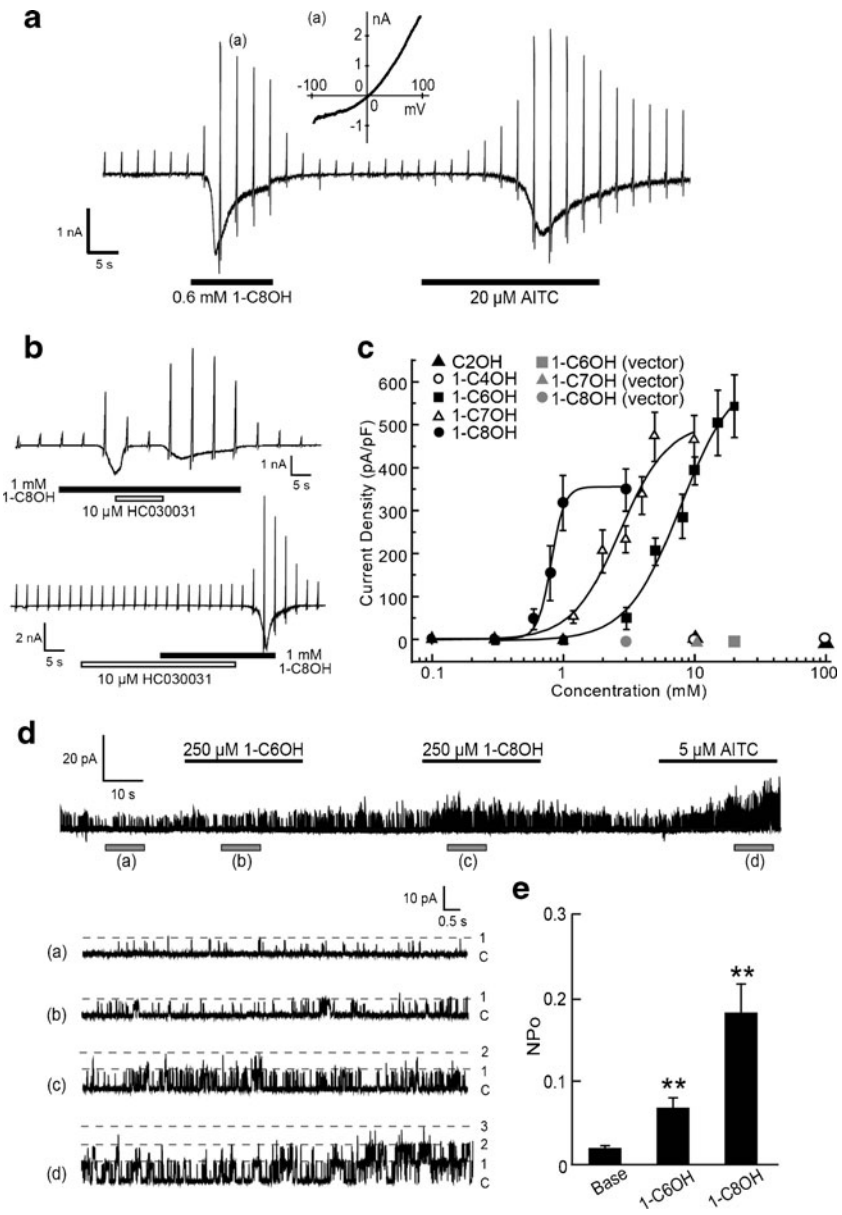

Fig. 2 Primary alcohols activate human TRPA1 in HEK293 cells. **a** A representative trace of the whole-cell current activated by 0.6 mM 1-octanol (*1-C8OH*) and AITC in HEK293 expressing human TRPA1 (hTRPA1). Holding potential (V_h), −60 mV. *Horizontal bars* indicate the duration of compound application. The inset indicates a representative *I–V* curve of the 1 mM 1-C8OH-activated current showing an outward rectification. **b** 10 μM HC030031, a TRPA1 antagonist, either during or prior to 1-C8OH application inhibits hTRPA1 activation by 1 mM 1-C8OH in HEK293 cells. V_h, −60 mV. *Horizontal bars* indicate the duration of compound application. **c** Dose–response profiles of primary alcohol (1-hexanol [*1-C6OH*], 1-heptanol [*1-C7OH*], and 1-octanol [*1-C8OH*])-mediated currents fitted with a Hill equation (n=4–6). *Gray symbols* indicate the current data in the vector-transfected cells. *Ordinates*: current density (pA/pF). *Abscissae*: concentration (mM). Each symbol represents the mean ± SEM. **d** A representative primary alcohol-activated single-channel current trace in a patch excised from a HEK293 cell expressing human TRPA1. V_h, −60 mV. *Horizontal black bars* indicate the duration of compound application. Single-channel currents for the *gray bars* ((a) baseline, (b) 250 μM 1-hexanol [*1-C6OH*], (c) 250 μM 1-octanol [*1-C8OH*] and (d) 5 μM AITC) were expanded. **e** NP_o values were calculated in the single-channel currents. *Ordinates*: NP_o (channel number × open probability). Each column represents the mean ± SEM from four cells. Statistical significance was assessed using ANOVA followed by the two-tailed multiple *t*-test with Bonferroni correction. $^{**}P<0.01$ vs. baseline (Base)

was observed when an excised inside-out patch membrane was exposed to a bath solution at the holding membrane potential of −60 mV as previously reported [12]. Application of 250 μM of 1-C6OH and 1-C8OH increased channel openings in a carbon chain length-dependent manner (Fig. 2d) similar to the dose–response profiles in the whole-cell current recordings (Fig. 2c). Opening of TRPA1 channels was confirmed by the similar observations of channel opening by

5 μM of AITC. The calculated unitary conductance in the KCl bath and NaCl-based pipette solutions was 98±2 picosiemens (pS, n=5), and a similar value was obtained for the AITC-activated currents (100±2 pS, n=5). These properties at the single-channel level support the idea that TRPA1 can be activated by primary alcohols directly or through mechanisms retained even in a small excised patch membrane. TRPA1 activation by primary alcohols was more clearly recognized when NP_o (channel number × open probability) values were plotted, where the NP_o values significantly increased upon increases in carbon chain length (Fig. 2e).

Structural requirements for the activation of TRPA1 by primary alcohols

It has been recently reported that TRPA1 is activated by reversible covalent modification of N-terminal cysteine residues by structurally unrelated compounds [14, 22]. To examine whether TRPA1 activation by primary alcohols requires its N-terminal cysteine residues, we constructed three types of mutants in which cysteines at C621, C641 and C665 were replaced with serines in hTRPA1 (C665S single, C621S/C641S double and C621S/C641S/C665S triple mutants). Figure 3a and b shows the representative traces of the whole-cell currents activated by 20 μM AITC (an electrophilic agonist), 500 μM 2-APB (a non-electrophilic agonist) or 1 mM 1-C8OH. Although TRPA1 activation by AITC or 2-APB was not abolished, TRPA1 activation by 1-C8OH was almost completely abolished in C665S mutant. We then analyzed the responses of these mutants to 500 μM 2-APB or 20 μM AITC. These mutants retained the ability to respond to 500 μM 2-APB, whereas they showed little or reduced responses to 20 μM AITC as expected (Fig. 3c). Currents activated by 1 mM 1-C8OH or 8 mM 1-C6OH were significantly reduced in the C665S mutant. Furthermore, currents activated by 1 mM 1-C8OH or 8 mM 1-C6OH were also significantly reduced in the C621S/C641S/C665S mutant. On the other hand, currents activated by 1-C8OH (1 mM) were not reduced in the C621S/C641S double mutant (Fig. 3c), indicating that C665 is critically involved in hTRPA1 activation by primary alcohols. It has also been reported that histidine and cysteine residues are needed for TRPA1 activation by zinc [15]. Therefore, we examined the activation of the histidine mutant (H983A) and cysteine mutant (C641S/C1021S double) by primary alcohols. Figure 3d and e shows the representative traces of whole-cell currents activated by 20 μM $ZnCl_2$, 500 μM 2-APB or 1 mM 1-C8OH. Although TRPA1 activation by 2-APB was intact, TRPA1 activation by $ZnCl_2$ or 1-C8OH was completely abolished in the H983A mutant. We then analyzed the H983A and C641S/C1021S mutant activities using 500 μM 2-APB, 20 μM AITC, and 20 μM $ZnCl_2$ (Fig. 3f). These mutants responded to 2-APB and AITC, but not to $ZnCl_2$ as shown in the previous study, although the responses to 2-APB were significantly reduced in these mutants. These mutants almost entirely lost sensitivity to 1 mM 1-C8OH or 8 mM 1-C6OH (Fig. 3f). These results suggest that the cysteine and histidine residues are important for the activation of TRPA1 by primary alcohols, and there are possible convergent activation mechanisms by electrophilic compounds, $ZnCl_2$ and primary alcohols. Therefore, we examined the effects of concomitant application of 1-C8OH (0.3 mM) with AITC (1 μM) or $ZnCl_2$ (1 μM) whose concentrations are reported to cause minimal TRPA1 activation. As expected, neither agonist could activate TRPA1 alone (Fig. 4a–e). However, any combination produced large TRPA1 currents, suggesting synergistic effects of the three agonists.

There is a report regarding an interaction between alcohol and alcohol dehydrogenase which involves zinc [13]. To elucidate whether the zinc ion is similarly needed for binding of primary alcohols with TRPA1, we examined the effects of a membrane-permeable zinc chelator (*N*,*N*,*N*′,*N*′-tetrakis (2-pyridylmethyl) ethylenediamine [TPEN]) and a membrane-impermeable zinc chelator (diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid [DTPA]) on TRPA1 activation by primary alcohols. As shown in Fig. 5a and b, pre- and co-application of 100 μM TPEN significantly reduced TRPA1 activation by 1 mM 1-C8OH. Furthermore, intracellular application of 30 μM DTPA also reduced TRPA1 activation by 1 mM 1-C8OH, while intracellular application of 30 μM DTPA did not significantly reduce TRPA1 activation by 100 μM AITC (Fig. 5b). These results suggest that intracellular zinc is important for the interaction between alcohols and TRPA1.

TRPA1-activating ability depends on alcohol lipophilicity, not the position of the hydroxy group

Having identified the amino acid residues involved in the TRPA1 activation by primary alcohols, we next wanted to find the structural basis for TRPA1 activation with regard to the alcohols. We examined the effects of primary and secondary alcohols on TRPA1-activating ability. As shown in Fig. 6a, there were no significant differences in the TRPA1-mediated $[Ca^{2+}]_i$ increases in HEK293 cells expressing hTRPA1 among the examined primary and secondary alcohols (1 mM of 1-C6OH, 3-C6OH, 1-C7OH, 2-C7OH, 3-C7OH, 4-C7OH, 1-C8OH, 2-C8OH and 3-C8OH), suggesting that the position of the hydroxy group does not affect the TRPA1-activating ability. In order to understand the mechanisms causing carbon chain length-dependent activation of TRPA1 by primary alcohols, we compared the TRPA1-activating ability (extent of $[Ca^{2+}]_i$ increase) by primary and secondary alcohols with alcohol lipophilicity (the calculated octanol/water partition coefficient). Interestingly, there was a good correlation between the TRPA1-activating ability and alcohol lipophilicity (R^2=0.965, Fig. 6b).

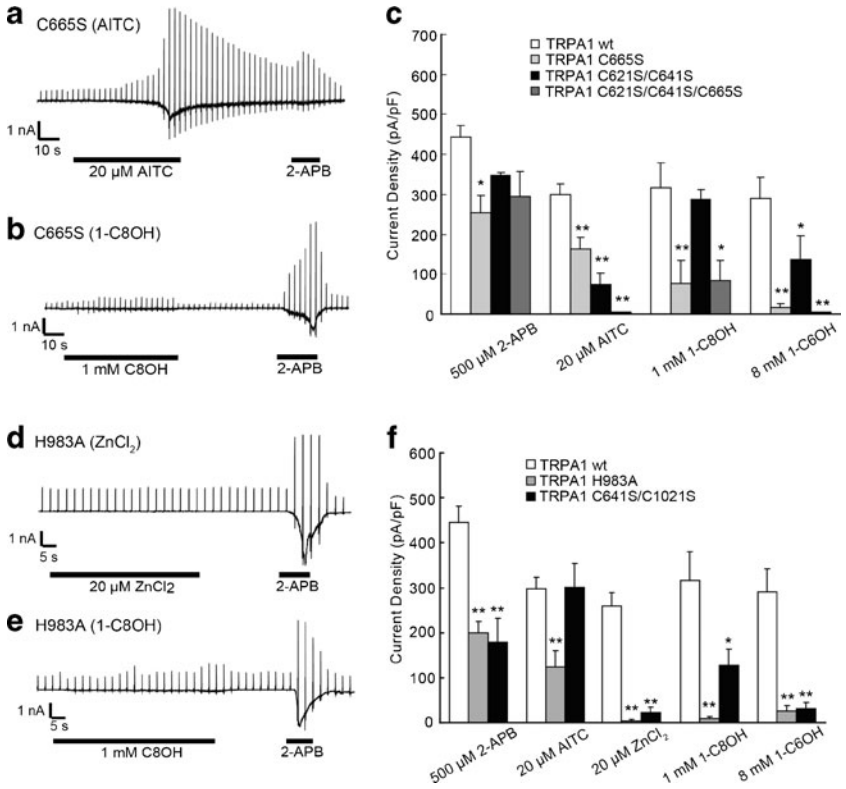

Fig. 3 Involvement of the cysteine and histidine residues of human TRPA1 in activation by primary alcohols. Representative traces of the whole-cell currents activated by 20 μM AITC (**a**) and 1 mM of 1-C8OH (**b**), followed by 500 μM of 2-APB in HEK293 cell expressing C665S mutant of hTRPA1. **c** Quantification of the current densities activated by 500 μM of 2-APB, 20 μM of AITC, 1 mM of 1-octanol (*1-C8OH*), and 8 mM of 1-hexanol (*1-C6OH*) in HEK293 cells expressing human TRPA1; wild-type (*wt*) and mutants (C665S, C621S/C641S, and C621S/C641S/C665S). Representative traces of the whole-cell currents activated by 20 μM of $ZnCl_2$ (**d**) and 1 mM of 1-C8OH (**e**), followed by 500 μM of 2-APB in HEK293 cell expressing H983A mutant of hTRPA1. **f** Quantification of the current densities activated by 500 μM of 2-APB, 20 μM of AITC, 20 μM of $ZnCl_2$, 1 mM of 1-C8OH, and 8 mM of 1-C6OH in HEK293 cells expressing human TRPA1; wild-type (*wt*), a H983A mutant, and C641S/C1021S mutant. *Ordinates*: current density (pA/pF). Each column represents the mean ± SEM from six to eight cells. Statistical significance was assessed using ANOVA followed by the two-tailed multiple *t*-test with Bonferroni correction. $*P<0.05$, $**P<0.01$ vs. TRPA1 wt

Primary alcohols have low activating ability on mouse TRPA1

There are few reports regarding irritation by primary alcohols in animals despite their well-known irritating effects in humans [5, 30]. Therefore, we checked whether primary alcohols activate mTRPA1. As shown in Fig. 7a and b, 8 mM 1-C6OH or 1 mM 1-C8OH caused low mTRPA1 activation in HEK293 cells compared with hTRPA1, although both hTRPA1 and mTRPA1 responded well to 100 μM AITC. In addition, intraplantar injection of 100 mM 1-C8OH to the hind paw caused licking or biting behaviors in mice to a lesser extent than another TRPA1 agonist AITC (10 mM) (Fig. 7c). Furthermore, 1-C8OH-induced behaviors were not abolished in TRPA1-KO mice, suggesting that the 1-C8OH-induced behaviors were non-specific. These results indicate that the ability of primary alcohols to activate TRPA1 depends on the species.

Discussion

TRPA1 is a calcium-permeable non-selective cation channel that is mainly expressed in primary sensory neurons. This channel represents a polymodal nocisensor for irritative agents and physical stimuli. In this study, we found that primary alcohols activate TRPA1, but not other TRP channels expressed in primary sensory neurons (Fig. 1). It is well known that primary alcohols induce skin, eye and nasal irritation in humans [5, 30], and one report shows that alcohols with longer carbon chain length (from C2OH to 1-C8OH) caused stronger eye and nose irritation with lower thresholds in humans [5]. From this report together with our results, we hypothesize that irritation by primary alcohols could be caused by activation of TRPA1. Interestingly, parabens were shown to cause TRPA1 activation in a carbon chain length-dependent manner [11], and a relationship between skin irritation and carbon chain length was also observed

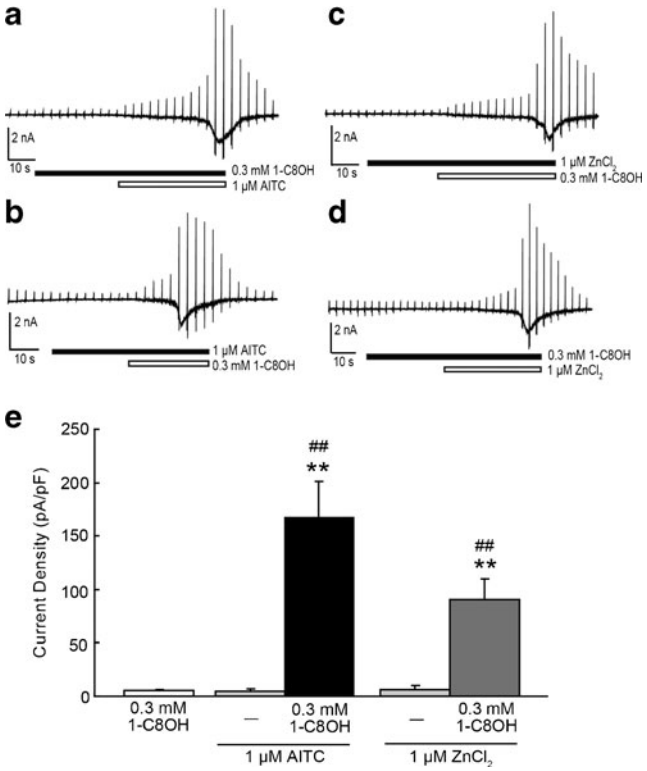

Fig. 4 The interaction between primary alcohol and other TRPA1 agonists for TRPA1 activation. **a** and **b** Synergistic effects of 0.3 mM of 1-octanol (1-C8OH) and 1 μM of AITC with different application order. **c** and **d** Synergistic effects of 0.3 mM of 1-C8OH and 1 μM of $ZnCl_2$ with different application order. V_h, −60 mV. *Horizontal bars* indicate the duration of compound application. **e** Quantification of the current densities activated by 0.3 mM of 1-C8OH, 1 μM of AITC with or without 0.3 mM of 1-C8OH and 1 μM of $ZnCl_2$ with or without 0.3 mM of 1-C8OH in HEK293 cells expressing hTRPA1. Ordinates: current density (pA/pF). Each column represents the mean ± SEM from four to eight cells. Statistical significance was assessed using ANOVA followed by the two-tailed multiple *t*-test with Bonferroni correction. $^{**}P<0.01$ vs. 0.3 mM 1-C8OH. $^{\#\#}P<0.01$ vs. 1 μM AITC or 1 μM $ZnCl_2$

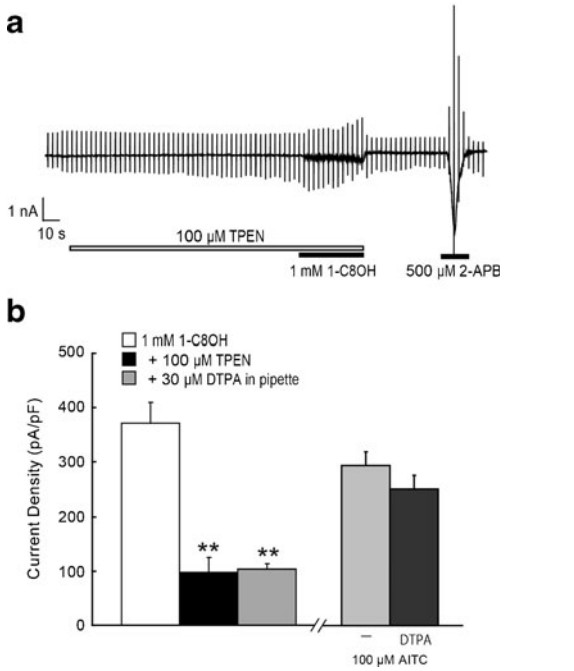

Fig. 5 A treatment with zinc chelators reduced TRPA1 activation by 1-C8OH. **a** A representative trace of the whole-cell current activated by 1 mM of 1-octanol (*1-C8OH*) with 100 μM of TPEN, a membrane-permeable zinc chelator. **b** The effect of 100 μM of TPEN or 30 μM of DTPA, a membrane-impermeable zinc chelator, in pipette solution on TRPA1 activation by 1 mM of 1-C8OH and 100 μM of AITC. Each column represents the mean ± SEM from five to eight cells. Statistical significance was assessed using ANOVA followed by a two-tailed multiple *t*-test with Bonferroni correction. $^{**}P<0.01$ vs. 1 mM 1-C8OH

[10], supporting our idea. Furthermore, we also found that TRPA1 activation by aldehydes and thiols also depends on carbon chain length (Fig. 1c), suggesting that there is a certain relationship between carbon chain length and the extent of TRPA1 activation.

What factor causes this relationship between carbon chain length and TRPA1-activating ability? One report has shown that 1-octanol is the most irritative compound among the primary alcohols (from C2OH to C18OH; 1-octadecanol) and that there was a high correlation between inorganic organic balance (IOB), an index of lipophilicity, and the extent of irritation [10]. This report is consistent with our results, explaining the extent of TRPA1 activation by primary alcohols. Regarding the TRPA1-activating ability between primary and secondary alcohols, the ability turned out not to depend on the position of the hydroxy group, but on carbon chain length (Fig. 6a). Furthermore, the activity correlated with the octanol/water partition coefficient (Fig. 6b), suggesting that lipophilicity could be a critical factor determining TRPA1-activating ability. This correlation has also been reported in $GABA_A$ receptor activation by alcohols, and it was shown that alcohols bind to a small pocket in the transmembrane domain of the $GABA_A$ receptor [24, 26]. Therefore, it is possible that primary alcohols activate hTRPA1 through the same mechanism as $GABA_A$ receptor. Such lipophilic-dependent activation was reported for another TRP channel, TRPV1 [25, 34], suggesting that lipophilicity could be an important factor determining the extent of TRP channel activity generally.

Structurally, alcohols are not electrophiles, suggesting that alcohols activate TRPA1 not through binding to cysteine residues. However, primary alcohol-evoked current responses were significantly reduced in the TRPA1 mutant (C665S) which was reported to be involved in covalent modification [14]. Cysteine 665 in the N terminus may have other important roles in activation of TRPA1 distinct from other cysteine residues, or be needed for agonist binding to TRPA1. We previously reported that intracellular alkalization activates TRPA1 without involvement of any cytosolic component [12]. In the report, we suggested the existence of endogenous activators that can be retained even in the excised patch

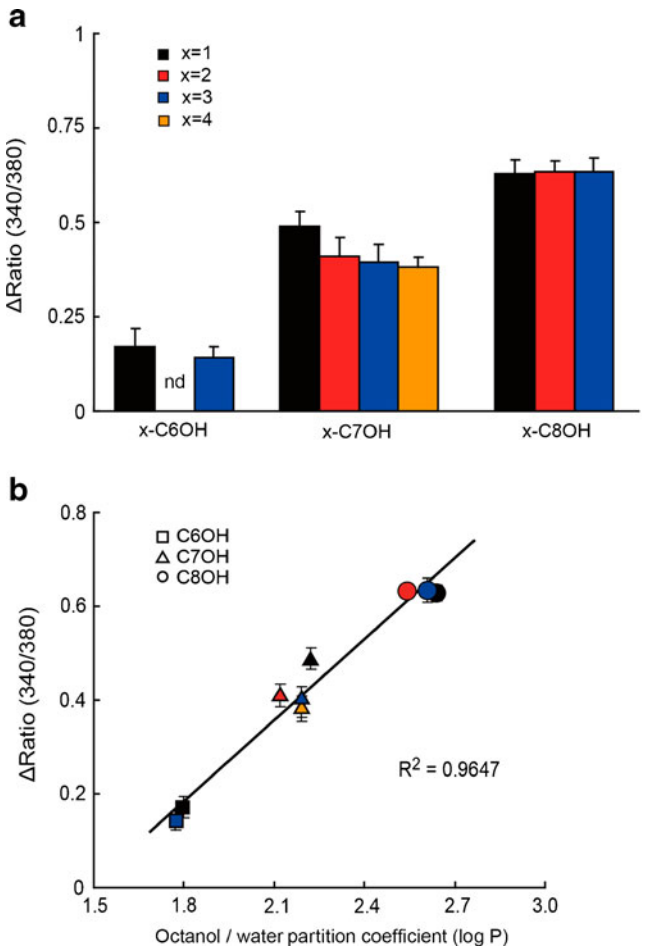

Fig. 6 TRPA1-activating ability depends on lipophilicity, but not position of hydroxy group. **a** $[Ca^{2+}]_i$ increases by 1 mM primary (x=1) and secondary (x=2–4) alcohols in HEK293 cells expressing human TRPA1. *X* indicates position of hydroxy group. *nd* no data. *Ordinates*: Δratio (340/360 nm). **b** Correlation between octanol/water partition coefficient and $[Ca^{2+}]_i$ increases by primary and secondary alcohols in TRPA1 expressing human TRPA1 (plotted using the data shown in **a**). *Ordinates*: Δratio (340/360 nm). *Abscissae*: octanol/water partition coefficient (log*P* values). Each *column* and *symbol* represents mean ± SEM from 28 to 79 cells

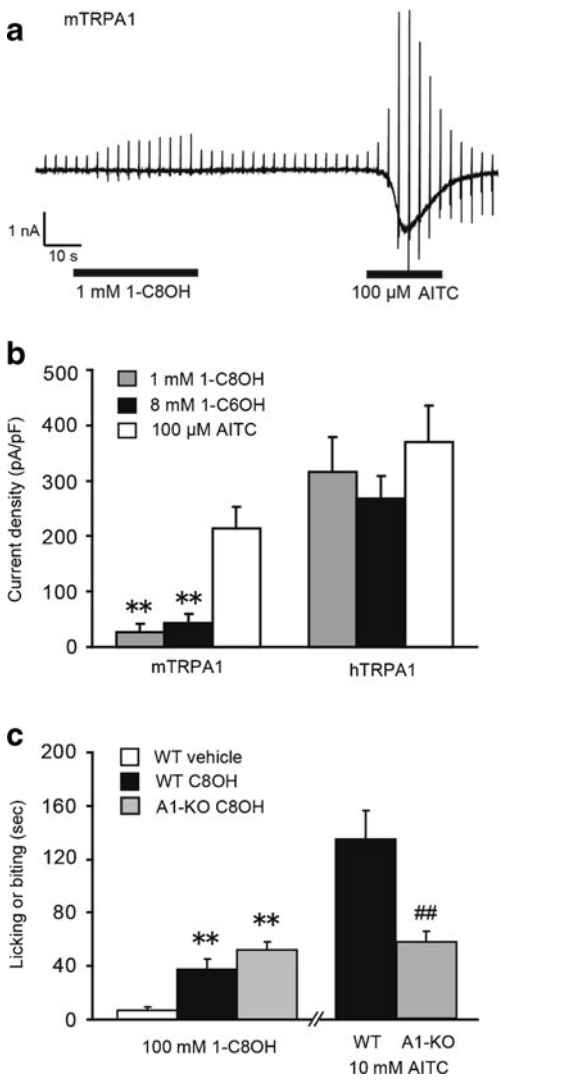

Fig. 7 Primary alcohols caused a modest activation of mouse TRPA1. **a** A representative trace of the whole-cell current activated by 1 mM 1-octanol (*1-C8OH*) in HEK293 expressing mouse TRPA1 (*mTRPA1*). Holding potential (V_h), −60 mV. *Horizontal bars* indicate the duration of compound application. **b** Quantification of TRPA1 current densities activated by 1 mM of 1-C8OH, 8 mM of 1-hexanol (1-C6OH) and 100 μM of AITC in HEK293 cells expressing mTRPA1 or hTRPA1. *Ordinates*: current density (pA/pF). Each column represents the mean ± SEM from four to eight cells. Statistical significance was assessed using ANOVA followed by a two-tailed multiple *t*-test with Bonferroni correction. ***P*<0.01 vs. 100 μM AITC. **c** Pain-related behaviors induced by intraplantar injection of 1-C8OH in WT or TRPA1-KO mice. The *right columns* show the time spent licking or biting induced by injection of 10 mM of AITC for 10 min in WT and TRPA1-KO (A1-KO) mice. *Ordinates*: the time spent licking or biting for 10 min (s). Each column represents the mean ± SEM from four to eight mice. Statistical significance was assessed using ANOVA followed by the two-tailed multiple *t*-test with Bonferroni correction or Student's*t*-test. ***P*<0.01 vs. WT vehicle. ##*P*<0.01 vs. AITC-treated WT

membrane because AITC- and 4-HNE (an endogenous TRPA1 agonist)-evoked currents were also enhanced by intracellular alkalization. Primary alcohols could similarly enhance AITC activity because synergistic effects were observed between 1-C8OH and AITC (Fig. 4a and b). Furthermore, not only the cysteine residue at 665 but also other cysteine residues at 641 and 1021 and a histidine residue at 983, which are involved in zinc activation, were found to be important for the TRPA1 activation by primary alcohols (Fig. 3b). In addition, 1-C8OH and $ZnCl_2$ exhibited synergistic activation (Fig. 4c and d). Alcohol–protein interaction has been studied in detail in alcohol dehydrogenase mechanism. It was suggested that alcohol dehydrogenase binds to alcohol (substrate) using zinc as a cofactor coordinated to two cysteine and one histidine residue [29]. In this study, we found that TRPA1 activation by primary alcohols was reduced in C665 single and C641/C1021 double mutants, suggesting that these cysteines may be important in binding zinc. Taken together, zinc coordinated to the specific histidine and cysteine residues

could be needed for binding of primary alcohols to TRPA1 which is supported by the fact that zinc chelators reduced TRPA1 activation by 1-C8OH (Fig. 5). The fact that aldehydes, TRPA1 agonists, can act as substrates for alcohol dehydrogenase [31] could support this concept. In addition, there is a report showing that clioquinol and pyrithione activate TRPA1 and their activation requires intracellular zinc [1]. Interestingly, clioquinol has a hydroxy group and pyrithione itself already coordinates zinc, suggesting that these TRPA1 agonists could activate TRPA1 by the same mechanisms as primary alcohols in this study. However, intracellular 30 μM DTPA did not affect TRPA1 currents activated by 100 μM AITC (Fig. 5b). This result suggests that zinc requirement for TRPA1 activation is agonist-dependent.

Although 1-C8OH elicited modest pain-related behavior in mice, primary alcohols caused low mTRPA1 activation (Fig. 7). TRPV1 also responded to 1-C8OH (Fig. 1d), suggesting that pain-related behaviors caused by primary alcohols in mice could involve activation of TRPV1 by primary alcohols to some extent. Such differences in compound sensitivity in TRP channels among mammalian species are well known. For example, mouse and rat TRPV2 are sensitive to 2-APB while hTRPV2 is hardly activated by 2-APB [27]. Inhibition of TRPA1 by menthol is observed in mouse clones but not in human clones, whereas both mouse and human TRPA1 can be activated by menthol [19]. One report showed that 1-C8OH and 1-C6OH activate rat TRPA1 (rTRPA1) [23]. 1-C8OH and 1-C6OH caused rTRPA1-activating current at highly positive holding potentials, but were hardly observed at negative holding potentials, suggesting that primary alcohols clearly activate TRPA1 only in humans under normal physiological conditions. While hTRPA1 activation by 1-C8OH was found to be likely caused by zinc coordination as shown in our study, TRPA1 activation by zinc was observed for both human and mouse TRPA1 [15]. Furthermore, histidine and cysteine residues are conserved between mouse and human TRPA1, suggesting the existence of unknown sites for TRPA1 activation by primary alcohols.

In conclusion, primary alcohols activate hTRPA1, and activation depends on alcohol carbon chain length, which may correlate with lipophilicity. Furthermore, cysteine and histidine residues are important for the activation of TRPA1 by primary alcohols. TRPA1 may be a critical component of the human response to alcohol-induced skin, eye and nasal irritation.

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