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Pharmacology

Canine, mouse, and human transient receptor potential ankyrin 1 (TRPA1) channels show different sensitivity to menthol or cold stimulation

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ABSTRACT. Transient receptor potential ankyrin 1 (TRPA1) is a nonselective cation channel that is activated by a variety of stimuli and acts as a nociceptor. Mouse and human TRPA1 exhibit different reactivity to some stimuli, including chemicals such as menthol as well as cold stimuli. The cold sensitivity of TRPA1 in mammalian species is controversial. Here, we analyzed the reactivity of heterologously expressed canine TRPA1 as well as the mouse and human orthologs to menthol or cold stimulation in Ca^{2+}-imaging experiments. Canine and human TRPA1 exhibited a similar response to menthol, that is, activation in a concentration-dependent manner, even at the high concentration range in contrast to the mouse ortholog, which did not respond to high concentration of menthol. In addition, the response during the removal of menthol was different; mouse TRPA1-expressing cells exhibited a typical response with a rapid and clear increase in $[Ca^{2+}]_i$ ("off-response"), whereas $[Ca^{2+}]_i$ in human TRPA1-expressing cells was dramatically decreased by the washout of menthol and $[Ca^{2+}]_i$ in canine TRPA1-expressing cells was slightly decreased. Finally, canine TRPA1 as well as mouse and human TRPA1 were activated by cold stimulation (below 19–20°C). The sensitivity to cold stimulation differed between these species, that is, human TRPA1 activated at higher temperatures compared with the canine and mouse orthologs. All of the above responses were suppressed by the selective TRPA1 inhibitor HC-030031. Because the concentration-dependency and "off-response" of menthol as well as the cold sensitivity were not uniform among these species, studies of canine TRPA1 might be useful for understanding the species-specific functional properties of mammalian TRPA1.

KEYWORDS: cold sensitivity, menthol, species difference, transient receptor potential ankyrin 1 (TRPA1)

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Transient receptor potential ankyrin 1 (TRPA1) is a Ca^{2+}-permeable non-selective cation channel [13] that is expressed mainly in primary sensory neurons [31] and is involved in the perception of painful stimuli. Mammalian TRPA1 is activated by a wide variety of reactive compounds, including pungent natural substances such as allyl isothiocyanate (AITC), cinnamaldehyde [1], and allicin [2]. In addition to these reactive chemicals, numerous nonreactive organic chemicals also activate TRPA1, including icilin [31] and farnesyl thiosalicylic acid [20]. The reactivity of TRPA1 to many of these stimuli is conserved among mammalian species because the binding sites of mammalian TRPA1 to these chemicals are conserved.

Interestingly, rodent and human TRPA1 exhibit different reactivity to some chemical ligands [5, 25, 34]. For instance, menthol, a stimulator of transient receptor potential channel subfamily M member 8, blocks TRPA1 in rodents at high concentrations [19], whereas it stimulates this channel at lower concentrations [14]. In contrast, TRPA1 in humans and some primates is activated by menthol in a concentration-dependent manner, even at high concentrations [3, 34], which is attributable to the three amino acid residues near the beginning of the fifth transmembrane (TM5) region of TRPA1 [34].

Mammalian TRPA1 is also activated by physiological stressors such as mechanical stimuli and noxious cold stimuli (reviewed in e.g., [12, 27, 32]). In the initial report, Story *et al*. found that noxious cold temperatures of less than approximately 17°C activated

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mouse TRPA1 [31]. The response of TRPA1 to cold temperature remains controversial, although many studies have been carried out *in vitro* (heterologous expression system or cultured nerve cells), *ex vivo* (primary nerve cells), and *in vivo* (TRPA1 knockout mice) (reviewed in e.g., [18, 35]). To date, several studies have provided evidence either for (e.g., [4, 7, 15, 22, 29]) or against (e.g., [4, 13, 17, 21, 24]) the activation of TRPA1 by cold temperature. Possible reasons for such discrepancies include species differences and/or differences in the methodologies or experimental conditions.

Given that the reactivity of mammalian TRPA1 to menthol and cold stimuli appears to differ among species, it might be helpful to investigate this in mammalian species other than rodents and primates in order to further clarify the species-specific differences in the properties of mammalian TRPA1. In dogs (*Canis lupus familiaris*), TRPA1 cDNA has been cloned and its reactivity to several typical TRPA1 stimulators has been shown by using a heterologous expression system [8]. In that study, the reactivity of canine TRPA1 to AITC, cinnamaldehyde, allicin, diallyl disulfide and icilin was found to be similar to that of human and rodent TRPA1. However, the reactivity of canine TRPA1 to menthol and cold temperature, in which discrepancies have been observed in other mammals, has yet to be investigated.

In the present study, the reactivity of canine TRPA1 to menthol and cold stimulation was clarified by performing Ca^{2+}-imaging using a heterologous expression system. Additionally, the sensitivity of human and mouse TRPA1 to low temperature was analyzed and compared among three different mammalian species to pave the way for a better understanding of TRPA1 cold sensitivity in mammals.

MATERIALS AND METHODS

Cloning of TRPA1 genes and plasmid construction

Canine TRPA1 was cloned from dog spinal cord cDNA (healthy adult Beagle donor; Zyagen, San Diego, CA, USA) by applying a PCR-based approach and was subcloned into the pIRES-hr-GFP-1a expression vector (Agilent Technologies, Santa Clara, CA, USA). A single nucleotide was modified to remove the original stop codon of the TRPA1 cDNA, and the sequence was added encoding three consecutive DYKDDDDK (FLAG TM-tag) to its 3′-ends. The translated canine TRPA1 amino acid sequence was identical to GenBank accession no. XP_038297051.1. Human TRPA1 (GenBank accession no. NM_007332.3) and mouse TRPA1 (GenBank accession no. NM_001348288.1) were also subcloned into pIRES-hr-GFP-1a for protein expression.

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells (American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics. For TRPA1 expression, X-tremeGENE™ HP DNA Transfection Reagent (Sigma-Aldrich, St. Louis, MO, USA) was used. The cells were seeded onto collagen-coated glass coverslips in a 35-mm dish at 1 day before transfection. Plasmids (2 µg) were mixed with 2 µL X-tremeGENE™ HP DNA reagent in 200 µL serum-free medium. The transfected cells were subjected to Ca^{2+}-imaging at 1 day after transfection. "Mock" indicates HEK293 cells transfected with empty vector.

Chemicals

Stock solutions of AITC (Sigma-Aldrich), DL-menthol (Fujifilm Wako Pure Chemical Corp., Osaka, Japan), and HC-030031 (Fujifilm Wako Pure Chemical Corp.) were dissolved in dimethyl sulfoxide, while ionomycin (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in 99.5% ethanol (final dimethyl sulfoxide/ethanol concentration was ≤0.1%).

Ratiometric Ca^{2+}-imaging

Ca^{2+}-imaging analysis was performed as described by Kita *et al*. with some modifications [16]. Cells plated on glass coverslips were incubated with 5 µM Fura-2 acetoxymethyl ester (Dojindo, Kumamoto, Japan) for more than 30 min at 37°C and transferred to a recording chamber (approximately 0.4 mL) mounted on the stage of an inverted fluorescence microscope (NIKON Corp., Tokyo, Japan) equipped with a cooled CMOS CoolSNAP camera (Teledyne Photometrics, Tucson, AZ, USA). The cells were superfused with HEPES-buffered physiological solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES (pH 7.4 with NaOH). The solution was perfused at approximately 2 mL/min. The experiments were performed at room temperature (23–25°C) unless stated otherwise when the temperature was regulated and monitored by a CL-100 bipolar temperature controller (Warner Instruments, Hamden, CT, USA) with an SC-20 dual in-line heater/cooler (Warner Instruments) and TA-29 bead thermistor (Warner Instruments). The temperature of the perfusion solution was lowered from 24°C to below 16°C, with a decrease of less than 0.2°C in 10 sec. To analyze intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), Fura-2-loaded HEK293 cells were excited alternatively at 340 and 380 nm, and images of the emitted fluorescence at 510 nm were collected every 10 sec, using NIS-Elements acquisition software (NIKON Corp.). The ratio of fluorescence intensity at both excitation wavelengths was calculated. For the illustration of the traces of average changes, the mean values of the ratio were averaged from more than 16 individual cells at each recording point in a single experiment. To calculate relative reactivity, the ratio values of five consecutive time points were averaged and the highest one in each stimulation was normalized with the peak response induced by the Ca^{2+} ionophore ionomycin (5 µM). The peak response to ionomycin was defined as the mean ratio value after stabilization (less than 0.3% increase in five consecutive time points) and this ratio value was used for normalization. For the Ca^{2+}-imaging experiments using AITC, the cells showing more than 10% in their relative reactivity in response to AITC were defined as TRPA1-expressing cells. In the case of the experiments without AITC, the cells showing more than 10% in their relative reactivity in response to menthol were defined as menthol-reactive TRPA1-expressing cells. For the analysis of cold sensitivity, cells with a >10% relative reactivity compared with AITC in response

to cold stimulation were defined as cold-sensitive cells. The threshold temperature of activation was defined as the temperature at which a 10% increase from baseline at the 340/380-nm ratio was observed. For each Ca^{2+}-imaging experiment, at least four individual experiments were conducted, unless stated otherwise.

Data analysis

Concentration-response data were fitted to sigmoidal curves to obtain EC_{50} value using Prism software ver. 9 (GraphPad Software Inc., San Diego, CA, USA). The statistical significance of the results was determined via an unpaired *t*-test with Welch's correction, Brown–Forsythe and Welch analysis of variance (ANOVA) tests with a Dunnett's T3 multiple comparisons test, and repeated one-way ANOVA with the Greenhouse–Geisser correction followed by Tukey's multiple comparisons test using Prism software ver. 9 (GraphPad Software Inc.).

RESULTS

Menthol activates canine TRPA1 in a heterologous expression system

Using canine TRPA1 channels expressed heterologously in HEK293 cells, Ca^{2+}-imaging experiments were performed to determine whether canine TRPA1 was activated by menthol. Expression on the plasma membrane was confirmed by western blotting (Supplementary Fig. 3). The application of menthol clearly increased $[Ca^{2+}]_i$ in canine TRPA1-transfected HEK293 cells (Fig. 1A). This reaction was suppressed by 30 µM HC-030031, a selective TRPA1 inhibitor (Fig. 1B and 1D). Such reactions were not observed in mock-transfected HEK293 cells (Fig. 1C). Menthol increased $[Ca^{2+}]_i$ in canine TRPA1-transfected HEK293 cells in a concentration-dependent manner up to 3 mM, and the EC_{50} value was calculated as 151.3 µM. HC-030031 significantly inhibited the increase in $[Ca^{2+}]_i$ in response to 0.3, 1, and 3 mM menthol (Fig. 1D). Next, the proportion of menthol-sensitive cells in canine TRPA1-expressing HEK293 cells was assessed. For this experiment, menthol was applied to canine TRPA1-transfected HEK293 cells followed by perfusion with 100 µM AITC, a selective TRPA1 stimulator, to confirm the expression of canine TRPA1. Most of the TRPA1-expressing (AITC-sensitive) cells were sensitive to menthol (colored dots), with a few exceptions (gray dots) (Fig. 1E). Among the 194 canine TRPA1-expressing cells from five individual experiments, 189 (97.4%) were sensitive to menthol (Fig. 1F).

Canine TRPA1-expressing HEK293 cells respond to cold stimulation

Next, the cold sensitivity of canine TRPA1 was analyzed by Ca^{2+}-imaging experiments using canine TRPA1-transfected HEK293 cells. The temperature of the perfusion solution was gradually lowered from 24°C to below 16°C. The cells that were activated by the

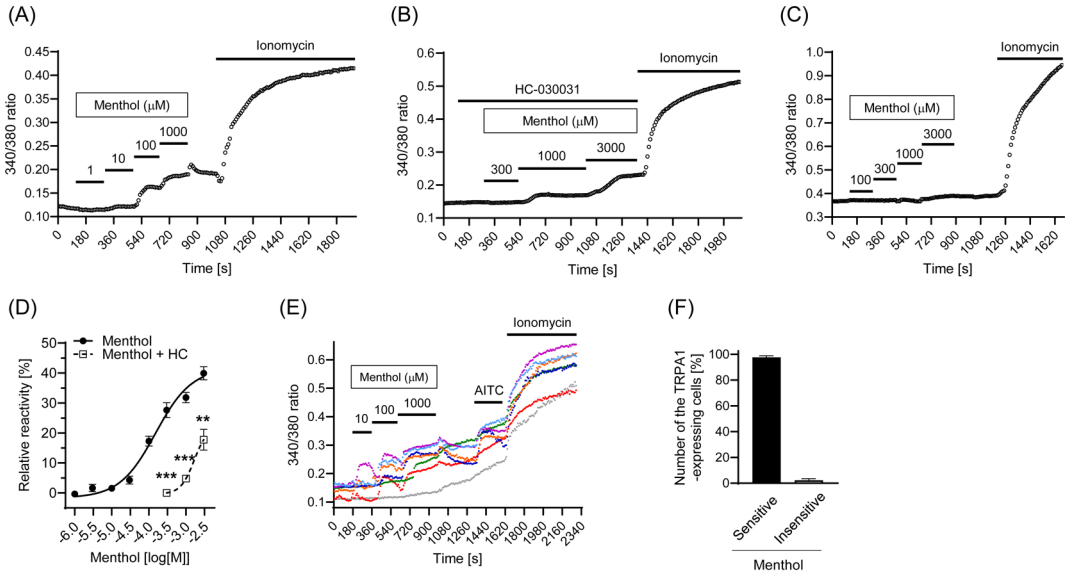

Fig. 1. Response of canine transient receptor potential ankyrin 1 (TRPA1) to menthol as assayed by ratiometric calcium imaging. (**A**–**C**) Dotted trace of the average changes in $[Ca^{2+}]_i$ induced by increasing the concentration of menthol in canine TRPA1-transfected human embryonic kidney (HEK) 293 cells in the absence (**A**) or presence (**B**) of 30 µM HC-030031, and mock-transfected HEK293 cells (**C**). A trace represents a typical result from at least four experiments, and each dot represents the mean value of 17–25 cells from one experiment. The cells were perfused with 5 µM ionomycin. (**D**) Concentration-response relationship of canine TRPA1 to menthol. Closed circles with a bold line represent menthol, and open squares with a dotted line represent menthol plus 30 µM HC-030031 (HC). The ordinate axis represents the relative reactivity of each concentration of menthol with or without HC-030031 to that of ionomycin. Mean ± SEM (n≥7). ***P<0.001 and **P<0.01 compared with the response without inhibitor by an unpaired *t*-test with Welch's correction. (**E**) Typical dotted traces in individual cells, $[Ca^{2+}]_i$ induced by menthol, and subsequent perfusion of 100 µM allyl isothiocyanate (AITC) to canine TRPA1-transfected HEK293 cells. (**F**) Percentage of menthol-sensitive (closed bar; left) and menthol-insensitive cells (open bar; right) in canine TRPA1-expressing cells. Mean + SEM (n=5).

subsequent perfusion of 100 μM AITC were regarded as TRPA1-expressing cells. Among canine TRPA1-expressing cells, not only cold-sensitive cells (colored dots) but also cold-insensitive cells (gray dots) were observed (Fig. 2A). In many cold-sensitive canine TRPA1-expressing cells, $[Ca^{2+}]_i$ was clearly increased when the temperature was lower than 18°C (Fig. 2A and Supplementary Fig. 1A). The ratio of low temperature-sensitive cells was calculated as 59.9% (94 out of 157 cells, from five individual experiments) (Fig. 2B). When 30 μM HC-030031 was applied during cold stimulation, the increase of $[Ca^{2+}]_i$ in canine TRPA1-expressing cells was hardly observed (Fig. 2C). HC-030031 decreased the reactivity of canine TRPA1-expressing cells to cold stimulation (Fig. 2D), to AITC (100 μM) at 25°C to similar extent and to that at lower temperature to a lesser extent (Supplementary Fig. 2). The increase of $[Ca^{2+}]_i$ was not observed in mock-transfected HEK293 cells during cold or AITC stimulation (Fig. 2E).

Human and mouse TRPA1 exhibit sensitivity to menthol as well as to cold stimulation

For comparison, human and mouse TRPA1 were subjected to similar analysis as canine TRPA1. Expression on the plasma membrane was confirmed by western blotting (Supplementary Fig. 3). First, to confirm the reactivity of human or mouse TRPA1-transfected cells to menthol, Ca^{2+}-imaging experiments were performed using heterologously expressed channels. As previously reported [34], human or mouse TRPA1-transfected cells were activated by menthol in a concentration-dependent manner, up to 1 mM for human TRPA1 (Fig. 3A) and 10 μM for mouse TRPA1 (Fig. 4A). Similar to the results in canine TRPA1-transfected cells, a large majority of human or mouse TRPA1-expressing cells reacted to menthol. From four individual experiments, there were 129 menthol-sensitive cells out of 134 (96.3%) human TRPA1-expressing (AITC-sensitive) cells (Fig. 3A and 3B) and 153 menthol-sensitive cells out of 165 (92.7%) mouse TRPA1-expressing cells (Fig. 4A and 4B).

Next, the cold sensitivity of human and mouse TRPA1 was analyzed using heterologously expressed channels in HEK293 cells. Among human or mouse TRPA1-expressing (AITC-sensitive) cells, similar to canine TRPA1-expressing cells, not only cold-sensitive cells (colored dots) but also cold-insensitive cells (gray dots) were observed (Figs. 3C and 4C). From four individual experiments, there were 85 cold-sensitive cells out of 130 (65.4%) human TRPA1-expressing cells (Fig. 3D) and 96 cold-sensitive cells out of 140 (68.6%) mouse TRPA1-expressing cells (Fig. 4D). The proportion of cold-sensitive cells in human or mouse TRPA1-expressing cells was similar to that of canine TRPA1-expressing cells. HC-030031 decreased the reactivity of mouse or human TRPA1-expressing cells to cold stimulation (Figs. 3E, 3F and 4E, 4F) and to AITC (100 μM) at lower temperature to a lesser extent (Supplementary Fig. 2). Notably, the ratio value in human TRPA1-expressing cells appeared to be decreased by HC-030031 perfusion (Fig. 3E).

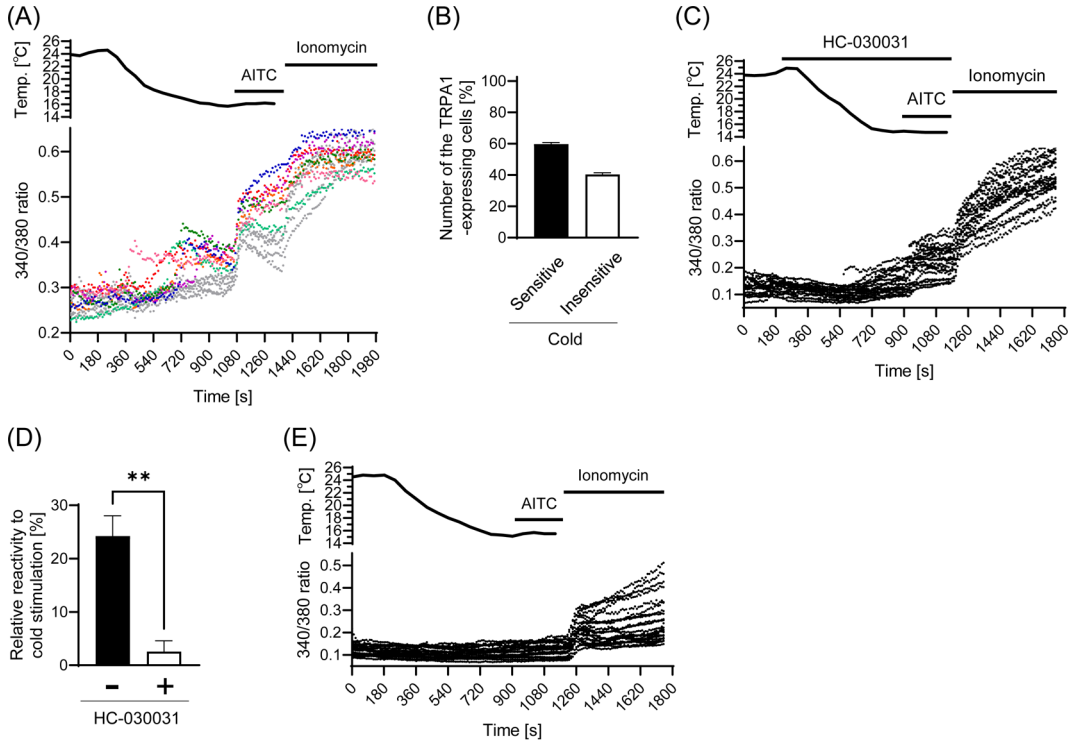

Fig. 2. Response of canine transient receptor potential ankyrin 1 (TRPA1) to cold stimulation as assayed by ratiometric calcium imaging. (**A**, **C**, **E**) Typical dotted traces in individual cells, $[Ca^{2+}]_i$ induced by lowering the temperature, and subsequent perfusion of 100 μM allyl isothiocyanate (AITC) to canine TRPA1-transfected human embryonic kidney (HEK) 293 cells in the absence (**A**) or presence (**C**) of 30 μM HC-030031, and mock-transfected HEK293 cells (**E**). The AITC-sensitive cells were considered as canine TRPA1-expressing cells. The cells were perfused with 5 μM ionomycin. (**B**) Percentage of cold-sensitive (closed bar; left) and cold-insensitive cells (open bar; right) in canine TRPA1-expressing cells. Mean + SEM (n=5). (**D**) Comparison of the relative reactivity to cold stimulation in the absence (−; closed bar) or presence (+; open bar) of HC-030031. Asterisks indicate a significant difference between the absence and presence of HC-030031 as determined using an unpaired *t*-test with Welch's correction (**P<0.01). Mean + SEM (n≥4).

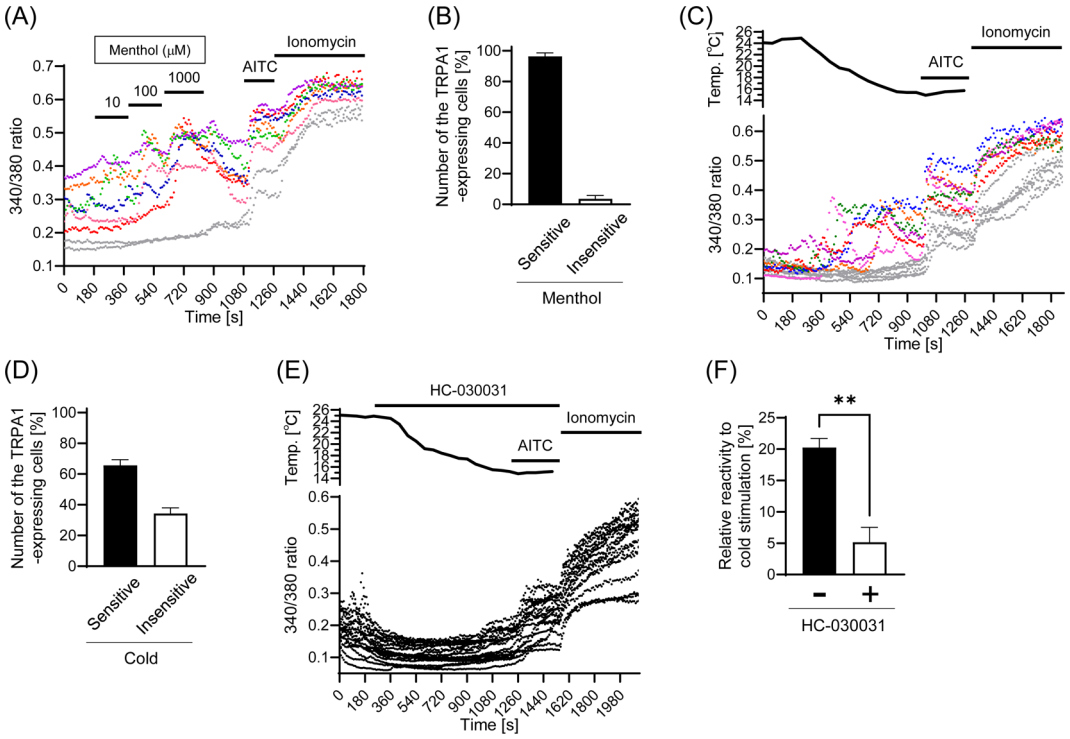

Fig. 3. Response of human transient receptor potential ankyrin 1 (TRPA1) to menthol- or cold-stimulation. (**A**) Typical dotted traces in individual cells, $[Ca^{2+}]_i$ induced by menthol, and subsequent perfusion of 100 μM allyl isothiocyanate (AITC) to human TRPA1-transfected human embryonic kidney (HEK) 293 cells. (**B**) Percentage of menthol-sensitive (closed bar; left) and menthol-insensitive cells (open bar; right) in human TRPA1-expressing cells. Mean + SEM (n=4). (**C**, **E**) Typical dotted traces in individual cells, $[Ca^{2+}]_i$ induced by lowering the temperature, and the subsequent perfusion of AITC to human TRPA1-transfected HEK293 cells in the absence (**C**) or presence (**E**) of 30 μM HC-030031. The AITC-sensitive cells were considered as canine TRPA1-expressing cells. The cells were perfused with 5 μM ionomycin. (**D**) Percentage of cold-sensitive (closed bar; left) and cold-insensitive cells (open bar; right) in human TRPA1-expressing cells. Mean + SEM (n=4). (**F**) Comparison of the relative reactivity to cold stimulation in the absence (−; closed bar) or presence (+; open bar) of HC-030031. Asterisks indicate a significant difference between the absence and presence of HC-030031 as determined using an unpaired *t*-test with Welch's correction (**P<0.01). Mean + SEM (n=4).

Comparison of the "off-response" and cold sensitivity in canine, mouse, and human TRPA1

The "off-response" phenomenon (an increase of $[Ca^{2+}]_i$ by the rapid washout of menthol) was observed in mouse TRPA1-expressing cells (Figs. 4A and 5B) but not in human or canine TRPA1-expressing cells (Figs. 1A, 1E, 3A, 5A and 5C). By analyzing the changes in relative reactivity before and during the washout of menthol, the reactivity of mouse TRPA1 was confirmed to be significantly enhanced (Fig. 5D). In contrast, the relative reactivity of canine TRPA1 tended to be slightly decreased during the washout period while that of human TRPA1 was dramatically and significantly decreased.

The threshold temperature of activation was compared among canine, mouse, or human TRPA1-expressing cold-sensitive cells. The average activation temperature was 19.2°C ± 0.2°C, 19.3°C ± 0.3°C, and 20.2°C ± 0.2°C for canine, mouse, and human TRPA1-expressing cells, respectively. The threshold temperatures of canine and mouse TRPA1-expressing cells were significantly lower than that of human TRPA1-expressing cells (Fig. 6A). Additionally, the relative reactivity of cold-sensitive cells was also compared at approximately 16°C, 18°C, 20°C, and 22–23°C. In all three species, the relative reactivity of TRPA1-expressing cells was increased as the temperature was lowered. Relative reactivity was significantly enhanced when the temperature was lowered to <18°C (Fig. 6B and Supplementary Fig. 1). Of note, the relative reactivity of human TRPA1-expressing cells tended to be enhanced at 20°C.

DISCUSSION

In this study, by analyzing heterologously expressed TRPA1 channels in Ca^{2+}-imaging experiments, we demonstrated some of the functional properties of canine TRPA1, specifically, its reactivity to menthol as well as to cold stimulation. Canine TRPA1 was activated by menthol in a concentration-dependent manner. Canine TRPA1 was also activated by cold stimulation, and its activation increased as the temperature decreased. In addition to canine TRPA1, we demonstrated the cold sensitivity of mouse and human TRPA1, which has been controversial.

Heterologously expressed canine TRPA1 was shown to respond to menthol, and this reaction was not observed in the presence of 30 μM HC-030031, a selective inhibitor of TRPA1 [10], or in mock-transfected cells (Fig. 1). Thus, we concluded that the increase

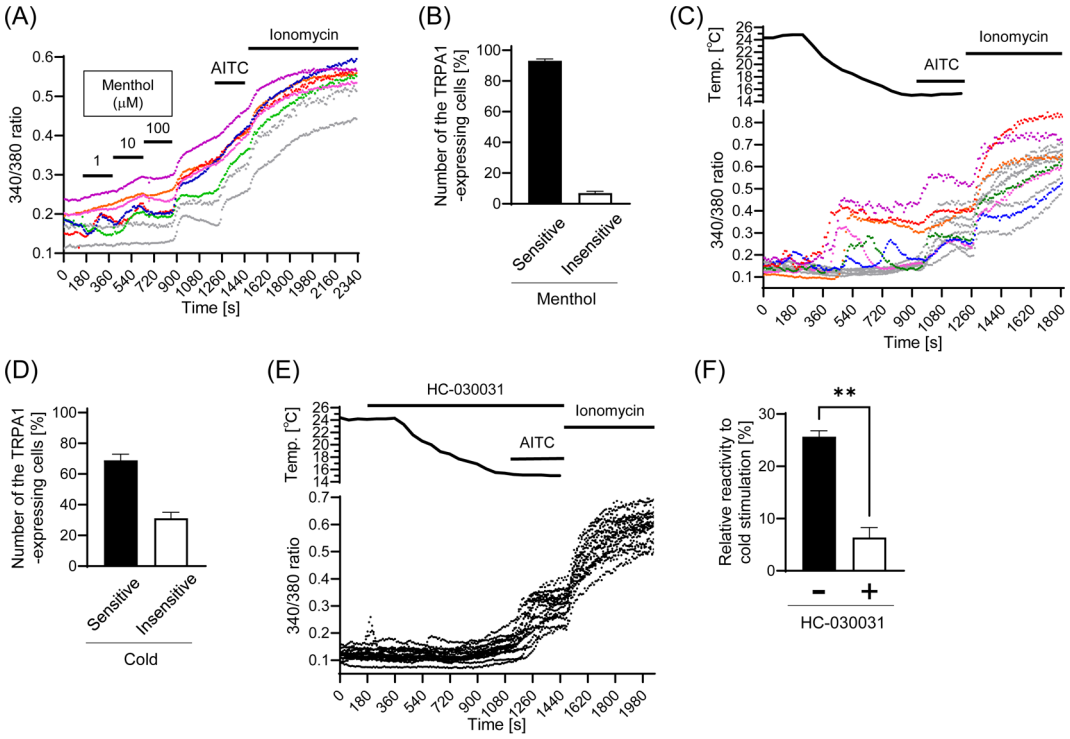

Fig. 4. Response of mouse transient receptor potential ankyrin 1 (TRPA1) to menthol- or cold-stimulation. (**A**) Typical dotted traces in individual cells, $[Ca^{2+}]_i$ induced by menthol, and subsequent perfusion of 100 μM allyl isothiocyanate (AITC) to mouse TRPA1-transfected human embryonic kidney (HEK) 293 cells. (**B**) Percentage of menthol-sensitive (closed bar; left) and menthol-insensitive cells (open bar; right) in mouse TRPA1-expressing cells. Mean + SEM (n=4). (**C**, **E**) Typical traces in individual cells, $[Ca^{2+}]_i$ induced by lowering the temperature, and subsequent perfusion of AITC to mouse TRPA1-transfected HEK293 cells in the absence (**C**) or presence (**E**) of 30 μM HC-030031. The AITC-sensitive cells were considered as mouse TRPA1-expressing cells. The cells were perfused with 5 μM ionomycin. (**D**) Percentage of cold-sensitive (closed bar; left) and cold-insensitive cells (open bar; right). Mean + SEM (n=4). (**F**) Comparison of the relative reactivity to cold stimulation in the absence (−; closed bar) or presence (+; open bar) of HC-030031. Asterisks indicate a significant difference between the absence and presence of HC-030031 as determined using an unpaired *t*-test with Welch's correction (**P<0.01). Mean + SEM (n=4).

of $[Ca^{2+}]_i$ induced by menthol was attributable to canine TRPA1. Until now, mammalian TRPA1 orthologs have been reported to be sensitive to menthol in humans, rhesus monkeys, rats, and mice. Interestingly, there is a clear difference in concentration-dependency between TRPA1 in primates and rodents. Human TRPA1 is activated in a concentration-dependent manner by menthol up to 1 mM, whereas mouse TRPA1 exhibits a bell-shaped concentration-response curve to menthol [34]. The sensitivity to menthol has been shown to be attributable to three amino acid residues in the TM5 region of TRPA1, namely, Ser-Thr-Val/Gly [34]. These Ser and Thr residues are essential for the reactivity to menthol, and they are well conserved in mammals. The subsequent residue, Val or Gly, determines the distinct dose dependency. Compared with previous studies, the reactivity of canine TRPA1 to menthol in the present study is similar to that of human TRPA1 but not to that of the mouse ortholog. Our observations are compatible with sequence information confirmed by us and others [8], in that canine TRPA1 contains Ser-Thr-Val residues in its TM5 region, which is the same as in human TRPA1 (Ser873, Thr874, and Val875) but not in the mouse ortholog (Ser876, Thr877, and Gly878).

Xiao *et al*. (2008) reported another interesting and distinctive property of mouse TRPA1, namely its "off-response" phenomenon. They showed that 250 μM menthol did not induce an increase of $[Ca^{2+}]_i$ in mouse TRPA1-expressing cells but that subsequent rapid washout of menthol led to an increase of $[Ca^{2+}]_i$ [14, 34]. In the present study, the "off-response" was analyzed after the perfusion of 1 mM menthol because we and others have shown that this concentration of menthol induces a nearly maximal reaction in these species [34]. A clear "off-response" was observed only in mouse TRPA1 (Fig. 5). In addition, an interesting difference was observed in the reaction of canine and human TRPA1 to the washout of menthol; that is, the reactivity of canine TRPA1 was only slightly decreased, whereas that of human TRPA1 was decreased to almost the quiescent level. We considered that the washout of menthol was sufficient given that the solution in the chamber was theoretically exchanged 20 times after perfusion for 4 min. Although the mechanisms underlying such an effect of menthol on mouse TRPA1 have not well been determined, Xiao *et al*. (2008) proposed that menthol may initially activate mouse TRPA1 at a single binding site but the channel switches rapidly into a non-conducting state, which has access to the open state after the removal of menthol. Others have proposed that menthol may have two separate binding sites on mouse TRPA1: one responsible for activation, the other for inhibition [14]. In any case, our observations with canine, human, and mouse TRPA1 together with the previous findings emphasize the importance of the residues in the TM5 region for TRPA1 responsiveness to menthol. For instance, canine and human TRPA1 share Ser-Thr-Val residues in the TM5 region (essential residues for menthol

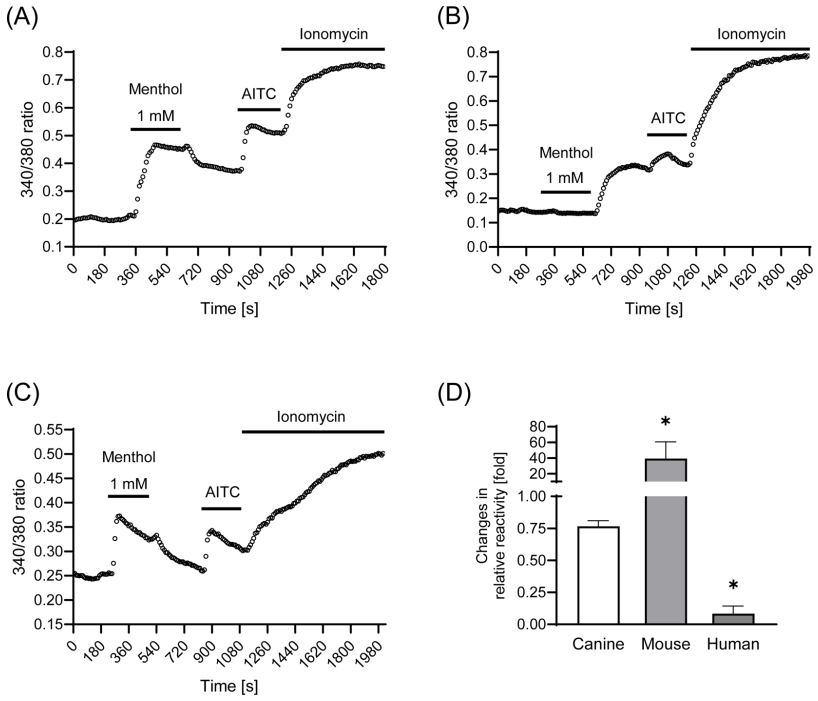

Fig. 5. Comparison of the "off-response" in canine, mouse, and human transient receptor potential ankyrin 1 (TRPA1). (**A**–**C**) Dotted traces of the average changes in $[Ca^{2+}]_i$ induced by perfusion of 1 mM menthol and subsequent allyl isothiocyanate (AITC) perfusion to human embryonic kidney (HEK) 293 cells that were transfected with canine (**A**), mouse (**B**), or human (**C**) TRPA1. For washout, the cells were perfused with a menthol-free solution for at least 4 min. A trace represents a typical result from at least four experiments, and each dot represents the mean value of 17–30 cells from one experiment. (**D**) Changes in relative reactivity by washout of menthol in canine, mouse, or human TRPA1-expressing HEK293 cells. Relative reactivity of the cells just before the washout step was set to 1 (serve as a baseline), and relative reactivity after 4-min perfusion of a menthol-free solution was compared. Asterisks indicate a significant difference between before and during washout of menthol in the different species as determined using an unpaired *t*-test with Welch's correction (**P*<0.05). Mean + SEM (n=4).

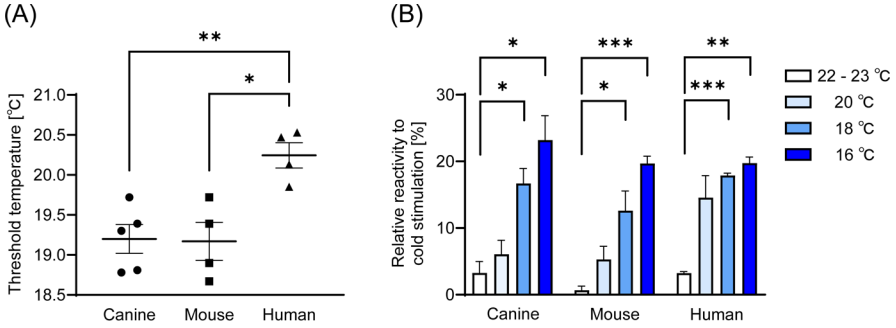

Fig. 6. Comparison of cold sensitivity in canine, mouse, and human transient receptor potential ankyrin 1 (TRPA1). (**A**) Threshold temperatures of activation for canine, mouse, and human TRPA1-expressing human embryonic kidney (HEK) 293 cells in Ca^{2+}-imaging experiments with cold stimulation. Each dot represents the mean value of 16–26 cells from one experiment. Cross-bars indicate the mean value of all experiments in each species. Asterisks indicate a significant difference, as determined by Brown–Forsythe and Welch analysis of variance (ANOVA) tests followed by a Dunnett's T3 multiple comparisons test (***P*<0.01, **P*<0.05). Mean ± SEM (n≥4). (**B**) Relative reactivity of canine, mouse, or human TRPA1-expressing HEK293 cells in Ca^{2+}-imaging experiments with cold stimulation was compared at approximately 16°C, 18°C, 20°C, and 22–23°C (with ± 0.5°C margin for all temperatures). The ordinate axis shows relative reactivity at these temperatures to that of 5 µM ionomycin. Asterisks indicate a significant difference as determined using repeated one-way ANOVA with the Greenhouse–Geisser correction followed by Tukey's multiple comparisons test (****P*<0.001, ***P*<0.01, **P*<0.05). Mean + SEM (n≥4).

sensitivity as discussed above), but several other residues in the TM5 and neighboring regions are not identical. Such differences may be responsible for the different reactivity of canine and human TRPA1 to menthol washout. Additionally, one of the possible factors could be the variation in TRPA1 expression level in the plasma membrane among the species observed in the present study. Further studies are required to clarify this point.

It is known that mammalian TRPA1 serves as an irritancy receptor for a wide variety of chemicals, but its role in cold sensation remains controversial. One of the possible reasons has been argued to be species differences. Nevertheless, most studies in mammals have been carried out in humans, mice, or rats. This situation prompted us to analyze the cold sensitivity of TRPA1 in non-primate and non-rodent species to further clarify the species differences in the cold sensitivity of mammalian TRPA1 based on comparative perspectives. In the present study, there was no increase in $[Ca^{2+}]_i$ during cold stimulation in the presence of HC-030031 or in mock-transfected cells (Figs. 2–4). Thus, we concluded that the observed increase of $[Ca^{2+}]_i$ during cold stimulation was attributable to TRPA1 in all three tested species.

During the cold stimulation of cells transfected with TRPA1 from all species tested in this study, not all cells were observed to be sensitive to cold stimulation (Figs. 2–4), whereas menthol-insensitive cells were hardly observed in the TRPA1-expressing cells (Figs. 1, 3, and 4). This observation might be explained by differences in the interaction between TRPA1 and the distinct types of stimuli. Chemicals such as menthol and AITC exhibit direct interactions with TRPA1 by binding to their binding site(s) in the channel [11]. However, although the mechanisms underlying the cold sensitivity of TRPA1 remain to be clarified, human TRPA1 is reported to function as a noxious cold sensor without direct cold sensing by transducing cold-induced reactive oxygen species signaling [21]. For that, the hydroxylation status of a proline residue (Pro394) in human TRPA1 plays an essential role. Cold triggers the generation of reactive oxygen species, which are unable to activate TRPA1 with a hydroxylated proline residue, whereas they can activate a non-hydroxylated proline, resulting in cold hypersensitivity. These findings might provide a further hint to explain our observations. The variation of cold sensitivity among cells expressing TRPA1 from the same species might be attributable to differences in the proline hydroxylation status (by unknown reason[s]) among the cells.

By comparing the cold sensitivity of canine, mouse, and human TRPA1, we found that TRPA1 in all three species exhibited a significant increase in their reactivity to cold stimulation when the temperature was lower than 18°C, whereas the threshold temperature of activation was different (Fig. 6). The threshold temperature of canine and mouse TRPA1 was lower than that of the human ortholog. In addition, considering that the ratio value in human TRPA1-expressing cells tended to be decreased by HC-030031 perfusion (Fig. 3E), human TRPA1 might be activated to some extent even at room temperature. The threshold temperature for TRPA1 activation is considered to vary among mammalian species. In several invertebrate and ancestral vertebrate species, TRPA1 is reported to be heat-sensitive (e.g., [9, 26, 28]), and the bimodal thermal properties of TRPA1 have been reported even in mammalian species [23, 30]. The pore-forming domain of TRPA1 is essential for its temperature sensitivity, and the ankyrin repeat domain in the N-terminal region is important for regulating the activating temperature or reactivity to cold [6, 33]. For example, human TRPA1 is sensitive to cold regardless of whether the N-terminal ankyrin repeat domain is retained or removed, but its reactivity to cold is different [22]. These observations might explain the differences in the threshold temperature of activation among canine, mouse, and human TRPA1 in the present study, in which the threshold temperature of activation and/or reactivity to cold might be differently affected according to their N-terminal ankyrin repeat domain. Further studies are required to fully identify the mechanisms of mammalian TRPA1 cold sensitivity.

The present study showed that human and mouse TRPA1 are sensitive to cold, but this is inconsistent with the findings of some other studies (e.g., [4, 21, 24]). Conceivable reasons for this inconsistency are differences in the methodologies and/or experimental conditions. Moparthi *et al*. (2016) argued that the regulation of TRPA1 is complex and that its sensitivity to ligands is dependent on cellular context such as the redox state and protein kinase/phosphatase enzymes. Many of these factors are probably dependent on the cell expression system and experimental conditions [23, 36]. These might also explain the intra-species difference in cold sensitivity found in the present study (discussed above). Another possible difference in the experimental conditions might be the speed at which the temperature was lowered. In the present study, the temperature was lowered gradually (a decrease of approximately 10°C in 12 min), whereas in the other studies, the temperature was lowered rapidly. Prolonged cold stimulation might enhance the cold sensitivity of TRPA1 via one or more unknown mechanisms.

In conclusion, we demonstrated the reactivity of canine TRPA1 to menthol as well as to cold stimulation. Because the responses of TRPA1 to menthol and cold differed among mammalian species, the information provided by the current study with canine TRPA1 might help to deepen our understanding of the species-specific characteristics of TRPA1, as well as lead to improvements in the current drug development process, in which rodent models are heavily relied upon, although many human TRPA1 stimulators react differently toward mouse or rat channels.

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