Identification of significant amino acids in multiple transmembrane domains of human transient receptor potential ankyrin 1 (TRPA1) for activation by eudesmol, an oxygenized sesquiterpene in hop essential oil*

Kazuaki Ohara¹, Takafumi Fukuda¹, Hiroyuki Okada¹, Sayoko Kitao¹, Yuko Ishida¹, Kyoko Kato¹, Chika Takahashi¹, Mikio Katayama¹, Kunitoshi Uchida², and Makoto Tominaga²

¹Research Laboratories for Health Science and Food Technologies, Kirin Company, Limited. Yokohama, Kanagawa, 236-0004, Japan
²Division of Cell Signaling, Okazaki Institute for Integrative Bioscience (National Institute for Physiological Sciences), National Institute of Natural Sciences, Okazaki, Aichi, 444-8787, Japan

*Running title: Hop derived sesquiterpene eudesmol activates human TRPA1

To whom correspondence should be addressed: Kazuaki Ohara, Kirin Company, Limited, Research Laboratories for Health Science and Food Technologies, 1-13-5, Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan; Tel.:+81-45-330-9006; Fax.:+81-45-788-4047; e-mail: Kazuaki_Ohara@kirin.co.jp

Key words: calcium channel, mutagenesis, natural product, terpenoid, transient receptor potential channels (TRP channels)

Capsule
Background: Transient receptor potential ankyrin 1 (TRPA1) is activated by many spicy compounds by unknown mechanisms.
Results: The amino acids critical for the activation of TRPA1 by the hop derived sesquiterpene β-eudesmol, were identified.
Conclusion: Multiple transmembrane domains are crucial for β-eudesmol derived TRPA1 activation.
Significance: New insight for activation mechanism of TRPA1 is revealed by our study.

ABSTRACT

Transient receptor potential ankyrin 1 (TRPA1) is a calcium-permeable non-selective cation channel, which is activated by various noxious or irritant substances in nature, including spicy compounds. Many TRPA1 chemical activators have been reported, however, only limited information is available regarding the amino acid residues that contribute to the activation by non-electrophilic activators, while activation mechanisms by electrophilic ligands have been well characterized. We used intracellular Ca²⁺ measurements and whole-cell patch-clamp recordings to show that eudesmol, an oxygenated sesquiterpene present at high concentrations in the essential oil of hop cultivar Haratau Hersbrucker, could activate human TRPA1. Gradual activation of inward currents with outward rectification by eudesmol was observed in human embryonic kidney-derived 293 cells expressing human TRPA1. This activation was completely blocked by a TRPA1-specific inhibitor, HC03-0031. We identified three critical amino acid residues in human TRPA1 in putative transmembrane domains 3, 4, and 5, namely serine at 813, tyrosine at 840, and serine at 873, for activation by β-eudesmol in a systematic mutational study. Our results revealed a new TRPA1 activator in hop essential oil, and provides a novel insight into mechanisms of human TRPA1 activation by non-electrophilic chemicals.

The transient receptor potential (TRP) channel family is known to be involved in detection of various noxious stimuli, such as thermal, chemical and mechanical stimulus (1). Among them, transient receptor potential ankyrin 1 (TRPA1) channel is a nonselective cation channel mainly expressed in primary sensory neurons (2). It responds to a wide variety of irritant sensory stimuli, including pungent ingredients of various...
spices and herbal medicines (3) which indicates its involvement in pungent sensations. Many TRPA1 activators have a reactive electrophilic group, allowing covalent binding to cysteine residues in the cytosolic N-terminus (4,5). In addition, non-electrophilic TRPA1 activators also exist in nature (6-8). The activation mechanisms of these compounds are not fully understood, although important amino acids for activation of TRPA1 by non-electrophilic activators have been reported (9,10).

Plants have defense mechanisms such as chemical irritant accumulation to avoid predation and infection. In addition, plant-produced chemical compounds also contribute to human life as natural medicines, fragrances, seasoners, or spices. Essential oils whose major components are volatile terpenoids are used worldwide as flavorings; they have large structural variations and each compound possesses a characteristic flavor. Hop cones, the immature inflorescences of the female plant of *Humulus lupulus* L., are widely used in beer production to add flavor and bitterness. Essential oils in hop cones contain various volatile terpenoids which are important components, determining beer flavor. In the production of new flavor patterns in beer, many hop cultivars with differing volatile terpenoid patterns have been developed.

Previous research has focused on the olfactory sensation of hop essential oil (11-13), however, little is known about their effects on sensory receptors, yet sensory stimulation should be an important factor in beer development. A particular hop cultivar, Haratau Hersbrucker (HHE), is described as adding a “spicy” character to beer (14). A previous report showed that the oxygenated-sesquiterpenoid fraction from hops contributed to its spicy character (15) but mechanisms underlying this have not been well studied. Previous reports have implied that characteristic ingredients of HHE essential oil can modulate sensory receptors, such as TRP channels (14,15).

In this report, we provide evidence that the human TRPA1 (hTRPA1) channel is activated by eudesmol, a characteristic oxygenated-sesquiterpenoid of HHE. There are three eudesmol structural isomers, namely α-eudesmol, β-eudesmol and γ-eudesmol (Figure 1A inset), which do not have a reactive electrophilic group, suggesting they should be classified into a non-electrophilic type activator of TRPA1. Site-directed mutagenesis identified three critical amino acids in transmembrane domains 3, 4, and 5 of hTRPA1 required for activation by β-eudesmol. Our results provide further understanding of the activation mechanism of human TRPA1 by non-electrophilic activators.

**EXPERIMENTAL PROCEDURES**

**Materials** - β-Eudesmol was purchased from Wako Pure Chemical (Tokyo, Japan). Hops were a generous gift from Dr. Atsushi Murakami of Kirin Company (Tokyo, Japan). α-Eudesmol and γ-eudesmol were a generous gift from Takasago International Corporation (Tokyo, Japan).

**GC-MS analysis** - Hops were ground to a fine powder using a pestle and mortar and dichloromethane (50 mg dry weight/ml for 1 hour) was used in the extraction procedure. Borneol was used as an internal standard for quantification. Extracts were dehydrated using Na2SO4, and then 1 µl was used for GC-MS. GC–MS was performed using a QP2010 mass spectrometer (Shimadzu, Kyoto, Japan) coupled with a GC-2010 gas chromatograph (Shimadzu) in split mode (1:30). The ion source was operated at 70 eV. The gas chromatograph was equipped with a HP-INNOWAX capillary column (60 m x 0.25 mm, 0.25 µm film thickness; Agilent technologies, CA, USA). The oven temperature was programmed from 40°C (0.18 min. hold) to 240°C at a rate of 3°C / min, using helium as the carrier gas at 2.07 ml/min. Quantitative data is shown as the mean of two independent experiments.

**Plasmid Vector** - All human TRP channel expression vectors were purchased from OriGene Technologies (MD, USA). Nucleotide substitution was performed using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) with slight modification. Oligonucleotides used for nucleotide substitution are listed in Table 1. The coding regions were fully sequenced before they were used.

**Calcium-imaging** - HEK293 (human embryonic kidney-derived 293) cells were maintained in Dulbecco’s modified Eagle’s
Hop derived sesquiterpene eudesmol activates human TRPA1

medium (supplemented with 10% fetal bovine serum, penicillin, streptomycin and L-glutamine and transfected with expression vector using Lipofectamine™ LTX (Life Technologies). Cells were then incubated for 24 hours in phenol red free DMEM medium (supplemented with 1% fetal bovine serum), before use in calcium-imaging experiments. Fluo-4 Direct™ Calcium Assay Kits (Invitrogen) were used according to manufacturer’s instructions. Compound addition and fluorescence measurement was performed using the FDSS µCell system (Hamamatsu Photonics, Hamamatsu, Japan). The DMSO solution of eudesmol and the buffer used for dilution were kept at 40°C prior to the experiments to maximize solubility. Dilutions were carried out just before the experiments, and the diluted solution was used immediately in the experiments to avoid deposition of eudesmol. This procedure was also adopted for other cell-based assays.

Whole-cell patch-clamp experiment - HEK293 cells were transfected with human TRPA1 or human TRPV3 and 0.1 µg pGreen Lantern-1 using the same method as in the calcium-imaging experiment, and incubated with Dulbecco’s modified Eagle’s medium (supplemented with 10% fetal bovine serum, penicillin, streptomycin and L-glutamine) for 14 to 24 hours. The bath solutions for the patch-clamp experiments contained 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 10 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), pH 7.4 (with NaOH). Calcium-free bath solutions for patch-clamp experiments contained 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, 10 mM glucose, 5 mM ethylene glycol tetraacetic acid (EGTA), 10 mM HEPES, pH 7.4 (with NaOH). The pipette solution contained 140 mM KCl, 5 mM EGTA, and 10 mM HEPES, pH7.4 (with KOH). 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). The cell was voltage-clamped at -60 mV. The current-voltage relationship was obtained using 500 ms voltage-ramp pulses from -100 to +100 mV applied every 5 sec. All experiments were performed at room temperature, except for eudesmol solution preparation described above.

Statistical analysis - Mann-Whitney U test, Kruskal Wallis test following the Steel test, one-way ANOVA following the Bonferroni test, or one-way ANOVA following the Dunnett test were used for statistical evaluation. Significance was assumed if the p value was <0.05.

RESULTS

Eudesmol is a characteristic oxygenated sesquiterpenoid found in hop cones of cultivar Haratau Hersbrucker - The characteristic spicy taste from hop cones of cultivar Haratau Hersbrucker (HHE) is reported to be due to oxygenated sesquiterpenoid (15). Quantitative GC-MS data showed that HHE accumulated the highest amount of eudesmol among 44 hop cultivars; seven other cultivars also contained eudesmol but at a lower level (Figure 1A). Eudesmol has been reported to be one of the representative oxygenated sesquiterpenoids in hop (16) and there are three structural isomers, whose structural formulas are shown in insets of Figure 1A. Representative total ion chromatograms and mass spectrums of HHE extract and eudesmol standerd are shown in Figure 1B and 1C. α-Eudesmol and β-eudesmol accumulated in abundance in HHE in comparison with γ-eudesmol (Figure 1A, B). Other volatiles, such as sesquiterpene, β-caryophyllene and α-humulene, accumulated in almost all the cultivars tested (Figure 2). These results indicated that eudesmol is one of the characteristic compounds which accumulates in HHE.

Activation of human TRPA1 and TRPV3 by eudesmol - To investigate whether eudesmol could activate human sensory channels, we investigated six representative human TRP (hTRP) channels. HEK293 cells transiently expressing each hTRP channel were utilized to investigate changes in intracellular Ca2+ concentrations ([Ca2+]i) which were detected by fluorescence of Fluo-4. hTRPA1 was the most activated channel after eudesmol treatment among the hTRP channels tested. Although apparent activation of hTRPV3 was observed, distinct activation of other hTRP channels, hTRPV1, hTRPV2, hTRPV4, and hTRPM8 were not observed under our experimental conditions (Figure 3A).
calcium-imaging of hTRPA1 are shown in Figure 3B in which allyl isothiocyanate (AITC) was used as an agonist. Three eudesmol isomers increased [Ca$^{2+}$], in hTRPA1-expressing cells. [Ca$^{2+}$], increases by eudesmol isomers were dose-dependent between 12.5 to 100 µM (Figure 3D), and EC$_{50}$ values for activation of hTRPA1 by α-eudesmol, β-eudesmol and γ-eudesmol were calculated as 50.3 ± 2.01, 32.5 ± 0.38 and 31.0 ± 1.55 µM, respectively.

Representative traces of calcium-imaging of hTRPV3 are shown in Figure 3D in which carvacrol was used as an agonist. Three eudesmol isomers increased [Ca$^{2+}$], in hTRPV3-expressing cells. [Ca$^{2+}$], increases by eudesmol isomers were dose-dependent between 12.5 to 100 µM (Figure 3E), and EC$_{50}$ values for activation of hTRPV3 by α-eudesmol, β-eudesmol and γ-eudesmol were calculated as 16.4 ± 2.54, 26.7 ± 2.76 and 19.3 ± 3.88 µM, respectively.

Electrophysiological experiments were carried out to confirm hTRPA1 activation by eudesmol. Representative whole-cell patch-clamp current traces are shown in Figure 4A. Each eudesmol isomer (100 µM) gradually brought inward currents with an outwardly-rectifying current-voltage relationship, a characteristic property of the TRPA1 channel. Densities of the currents activated by α-eudesmol, β-eudesmol and γ-eudesmol at a holding potential of -60 mV were calculated as 62.4 ± 19.6, 35.2 ± 9.01 and 34.1 ± 17.5 pA/pF, respectively. In Figure 4A, we utilized a high concentration of AITC (100 µM) as a positive control. Currents activated by AITC (100 µM) after eudesmol treatment were relatively small because of desensitization. The current-voltage (I-V) relationship differed between eudesmol- and AITC-activated currents in that eudesmol-activated currents exhibited clear outward rectification while currents activated by a high concentration of AITC exhibited a more linear I-V relationship. It is well known that TRPA1-mediated currents show different I-V-relationships depending on the channel activation (Figure 4B) (17). The data also indicates that eudesmol is a relatively weak TRPA1 agonist. Inward currents evoked by eudesmol showed significant differences in densities between hTRPA1-expressing cells and vector control cells (Figure 4C). No significant differences were observed in the densities of currents activated by isomers of eudesmol in hTRPA1-expressing cells, suggesting the similar abilities of the isomers of eudesmol to activate hTRPA1.

Small but significant activation of hTRPV3 by eudesmol (100 µM) was also confirmed using a patch-clamp method. Each eudesmol isomer (100 µM) caused hTRPV3-mediated current activation with outward-rectification. A representative trace of the β-eudesmol-induced hTRPV3-mediated current is shown in Figure 5A. Densities of the outward currents activated by eudesmol at +100 mV (268.9 ± 64.4, 172.4 ± 100.5, 106.0 ± 57.0 pA/pF, for α-eudesmol, β-eudesmol and γ-eudesmol, respectively) were significantly larger than those for vector control cells (Figure 5B), while current densities at -60 mV did not differ (19.1 ± 7.48, 19.9 ± 17.5 and 14.9 ± 12.4 pA/pF for α-eudesmol, β-eudesmol and γ-eudesmol, respectively). Among the isomers of eudesmol, no significant differences in both the inward current densities at -60 mV and outward current densities at +100 mV were observed, as in hTRPA1 activation.

We utilized β-eudesmol as the representative in subsequent experiments because almost the same results were obtained for the structural isomers and because α-eudesmol and γ-eudesmol were not commercially available. To verify that a response by β-eudesmol was elicited via hTRPA1 channel activation, the effect of an inhibitor was examined. HC03-0031, a TRPA1-specific antagonist, completely suppressed both inward and outward currents induced by β-eudesmol (Figure 6A, B), supporting our findings that β-eudesmol activates hTRPA1. It is well known that Ca$^{2+}$ ions entering the cells through TRPA1 enhance TRPA1 channel activity, therefore we performed the experiments in the absence of extracellular Ca$^{2+}$. Currents activated by β-eudesmol in the absence of extracellular Ca$^{2+}$ were very small while much bigger currents were elicited by β-eudesmol and AITC in the presence of 2 mM extracellular Ca$^{2+}$ (Figure 6C). Inward currents showed significant difference in densities between absence and presence of 2 mM Ca$^{2+}$ (Figure 6D). The result indicated an extracellular
Hop derived sesquiterpene eudesmol activates human TRPA1

Ca$^{2+}$ dependency of the β-eudesmol-evoked current response, further supporting the activation of TRPA1 by β-eudesmol. In addition, β-eudesmol did not affect the inward currents evoked by the maximum dose of AITC (Figure 6E-G). These data revealed that hTRPA1 is activated by β-eudesmol. Three amino acids in human TRPA1 transmembrane domains 3, 4, and 5 are important for activation by β-eudesmol - Next, we attempted to identify the amino acid residues involved in β-eudesmol-induced activation of hTRPA1. β-Eudesmol is thought to be a non-electrophilic activator of hTRPA1 because it does not have the α,β-unsaturated carbonyl structure characteristic of electrophilic activators like AITC. Previous studies of ligand-gated TRP channels suggested that transmembrane domains contain important amino acids for hydrophobic activators, and these amino acids tend to possess hydroxyl groups in their side-chain which may contribute to hydroxyl-bonding with activator molecules (9,18). Since β-eudesmol is a hydrophobic compound and has a hydroxy group, we focused on amino acids possessing hydroxyl groups in the transmembrane domains of hTRPA1. Based on the prediction of transmembrane domains using the SOSUI program (http://harrier.nagahama-i-bio.ac.jp/sosui/), we prepared 21 hTRPA1 mutants in which serine, threonine or tyrosine in putative transmembrane domains were substituted with alanine. Four mutant channels (Y726A, T734A, S781A, and T869A) were eliminated from subsequent analyses because no activation by AITC was observed. In addition, these four mutant channels did not respond to carvacrol, a non-electrophilic TRPA1 activator. These four mutant channels may have lost channel function or may not have been expressed in the plasma membrane. Figure 7A shows a schematic diagram of the putative structure of hTRPA1, in which the remaining 17 amino acid positions are indicated by circles. The first screening results used a calcium-imaging method in which β-eudesmol-evoked responses were normalized to AITC-evoked responses in order to exclude any unexpected effects for protein expression levels or ion channel functions. Seventeen mutant channels were analyzed and five mutants (T813A, Y840A, Y849A, S873A, and T874A) showed significant reductions in response to β-eudesmol (Figure 7B). To confirm the importance of these five amino acids, electrophysiological experiments were carried out. All five mutant channels produced significantly smaller whole-cell currents compared with WT (Figure 7C, upper). To investigate the significance of these mutants in terms of responses to β-eudesmol, we normalized the currents by β-eudesmol to those by AITC because these five mutants also exhibited significantly small AITC-evoked currents (Figure 7C, middle). T813A, Y840A, and S873A could be more important amino acids for β-eudesmol-evoked hTRPA1 activation. Representative traces of these three mutants are shown in Figure 7D. These mutant channels almost completely lost the response to β-eudesmol while responses to AITC were observed. The data indicated that these three amino acids play a critical role in hTRPA1 activation by β-eudesmol.

DISCUSSION
Empirical sensory assessments have evaluated that HHE produces a spicy character in beer (14): our results of hTRPA1 activation by eudesmol could help explain these sensory phenomena. HHE-flavored beer contains eudesmol at about 1 µM which is lower than the effective concentrations activating hTRPA1 revealed in this study. This discrepancy could be explained by potential synergistic effects of other chemicals in beer. There are reports that combinations of TRPA1 activators at lower concentrations could evoke a large synergistic activation (19). Since many TRPA1 activators are found in natural compounds, beer may include other hTRPA1 activators, to produce synergistic TRPA1 activation with eudesmol in vivo.

Various stimulations have been found to evoke TRPA1 activation and in many cases, these stimulations have been viewed as noxious. Meanwhile, TRPA1 activators have been found in food ingredients, such as horseradish, garlic, cinnamon, and pepper. These ingredients could produce desired
Hop derived sesquiterpene eudesmol activates human TRPA1 sensory stimulations and health benefits under appropriate conditions (3). Eudesmol appears to be a weak activator of hTRPA1, indicative that this compound is unlikely to be a useful pharmacological tool, however, identification of a TRPA1 activator in hop cones provides the possibility of favorable control of the sensory stimulation of beer.

β-Eudesmol activated hTRPA1 under extracellular calcium-free conditions, as shown in Figure 6C, although activation was reduced compared with 2 mM Ca\(^{2+}\) extracellular conditions. This result, together with the fact that gradual current activation was observed upon β-eudesmol application (Figure 4A), suggests that calcium ions entering the cells through TRPA1 activated by β-eudesmol enhance the channel activity. Previous studies revealed that intracellular calcium ions directly activate TRPA1 via cytosolic N-terminal EF-hand calcium-binding domains (20).

We also found eudesmol produced weak but significant activation of hTRPV3, a warm-sensitive Ca\(^{2+}\) permeable cation channel. Carvacrol, a major component of essential oil from oregano, also activates both TRPA1 and TRPV3 (21). A previous report showed that carvacrol produced both a pungent and warm sensation on the tongue (21), whereas previous sensory assessments of HHE have not reported a warm sensation. The data that eudesmol-evoked hTRPV3-mediated currents at a holding potential of -60 mV were negligible, similar to the current in the vector control cells, could imply that eudesmol-evoked hTRPV3 activation may be too weak to contribute to the occurrence of a warm sensation.

Structural determinants for TRPA1 activation have been studied in other non-electrophilic TRPA1 activators. Oleocanthal, another non-electrophilic TRPA1 activator found in extra virgin olive oil, needs a particular double bond in the molecule for TRPA1 activation (6). Three eudesmol structural isomers possess double bonds, in different positions, while the ability of these isomers to activate hTRPA1 did not differ. These data indicate that the position of the double bond in eudesmol is not critical for TRPA1 activation, and suggests other structure determinants for TRPA1 activation in the eudesmol molecule.

To explore the critical amino acids for hTRPA1 activation by β-eudesmol, we screened for amino acids with hydroxyl groups in their side chains within the transmembrane domains because this has been reported in other TRP channels. For example, tyrosine and serine in transmembrane domains 3 and 4 are known to contribute to capsaicin binding in TRPV1 (22). A menthol-binding site was identified as tyrosine in transmembrane domain 3 of TRPM8 (18). In addition, a menthol-binding site in TRPA1 was reported as threonine in transmembrane domain 5 (9). Recently, a tyrosine residue in hTRPA1 transmembrane domain 3 was shown to be essential for inhibition by borneol, a monoterpane alcohol (23). Systematic screening identified three critical amino acids in transmembrane domains 3, 4, and 5 which were required for hTRPA1 activation by β-eudesmol. Xiao et al. presented a model of hydrogen bonding between the hydroxyl-groups of threonine and menthol in transmembrane domain 5 of TRPA1 (9). Our results may also suggest that hydrogen bonding occurs between β-eudesmol and the identified amino acids of hTRPA1 and this might contribute to channel activation. The published model shows a sole menthol binding-site in TRPA1, whereas the three amino acids in different transmembrane domains, shown in the current study, may contribute to TRPA1 activation in different ways. To reveal the roles of the identified amino acids in the β-eudesmol-evoked activation of hTRPA1, experiments examining the shift of EC\(_{50}\) values and differences in maximal potentiation would provide valuable information (24). It has been reported that multiple tyrosine residues play distinct roles in ligand-activated channels, such as the GABAc receptor and 5HT\(_3\) receptor (25,26).

Ligand binding sites in TRP channels were studied using site-directed mutagenesis, chimeric analysis between different TRP channels, and structural data (27). The 3D structure of TRPA1 was revealed by electron microscopy (28), in which TRPA1 was shown to form tetramer. Multiple cytosolic AITC binding sites in the N-terminus were shown to locate at overlapped regions of each monomer, and ligand binding was thought to be affected by either monomer or tetramer interaction to

To explore the critical amino acids for hTRPA1 activation by β-eudesmol, we screened for amino acids with hydroxyl groups in their side chains within the transmembrane domains because this has been reported in other TRP channels. For example, tyrosine and serine in transmembrane domains 3 and 4 are known to contribute to capsaicin binding in TRPV1 (22). A menthol-binding site was identified as tyrosine in transmembrane domain 3 of TRPM8 (18). In addition, a menthol-binding site in TRPA1 was reported as threonine in transmembrane domain 5 (9). Recently, a tyrosine residue in hTRPA1 transmembrane domain 3 was shown to be essential for inhibition by borneol, a monoterpane alcohol (23). Systematic screening identified three critical amino acids in transmembrane domains 3, 4, and 5 which were required for hTRPA1 activation by β-eudesmol. Xiao et al. presented a model of hydrogen bonding between the hydroxyl-groups of threonine and menthol in transmembrane domain 5 of TRPA1 (9). Our results may also suggest that hydrogen bonding occurs between β-eudesmol and the identified amino acids of hTRPA1 and this might contribute to channel activation. The published model shows a sole menthol binding-site in TRPA1, whereas the three amino acids in different transmembrane domains, shown in the current study, may contribute to TRPA1 activation in different ways. To reveal the roles of the identified amino acids in the β-eudesmol-evoked activation of hTRPA1, experiments examining the shift of EC\(_{50}\) values and differences in maximal potentiation would provide valuable information (24). It has been reported that multiple tyrosine residues play distinct roles in ligand-activated channels, such as the GABAc receptor and 5HT\(_3\) receptor (25,26).

Ligand binding sites in TRP channels were studied using site-directed mutagenesis, chimeric analysis between different TRP channels, and structural data (27). The 3D structure of TRPA1 was revealed by electron microscopy (28), in which TRPA1 was shown to form tetramer. Multiple cytosolic AITC binding sites in the N-terminus were shown to locate at overlapped regions of each monomer, and ligand binding was thought to be affected by either monomer or tetramer interaction to
gate the channel open. Our results demonstrated that important identified amino acids were distributed in multiple transmembrane domains, suggesting that multiple binding sites of β-eudesmol exist in hTRPA1, as shown by covalent-modification by electrophilic activators such as AITC. It is also conceivable that a single well-organized binding site consists of multiple transmembrane α-helices. Either way, hydroxylated amino acids, threonine, serine and tyrosine are known to break the α-helical symmetry (29). This property may lead to speculation that transmembrane domains 3, 4, and 5 form hinges at identified hydroxylated amino acids. In the case of TRPA1 activation by menthol, hydroxylated amino acids serine and threonine in transmembrane domain 5 are shown to be important, and it is suggested that these amino acids may also act as the hinge (9). Two hydroxylated-terpenes, menthol and β-eudesmol might share the TRPA1 activation mechanism to elicit conformational changes through hydroxylated amino acids in transmembrane domains, although the distribution patterns of important amino acids differed. Different regions have been reported to be crucial for activation among non-electrophilic TRPA1 activators. A recent report revealed that amino acids critical for TRPA1 activation by a non-electrophilic TRPA1 activator, methyl anthranilate, were not in the transmembrane domains, but in the N-terminal cytosolic region (30). Methyl anthranilate may affect either monomer or tetramer structures by changing the N-terminus environments to gate the channel open without covalent modification. This difference in important regions for activation among non-electrophilic activators implies that different activation mechanisms may exist among non-electrophilic TRPA1 activators. However, it is important to note that our results do not indicate the direct binding of β-eudesmol to hTRPA1. It is possible that indirect effects of β-eudesmol occur through the modification of the lipid bilayer. Thus, either direct interactions or indirect effects may be detected by these hydroxylated amino acid residues, and this could contribute to conformational changes leading to the channel opening. Our study revealed important amino acids required for activation of hTRPA1 by β-eudesmol located in multiple transmembrane domains, as observed in other ligand-gated TRP channels (31,32). However, we cannot exclude the involvement of other amino acids. More intensive studies are required for accurate understanding of the TRPA1 activation mechanisms by non-electrophilic activators and the roles of the amino acids identified in this study.
REFERENCES

1. Julius, D. (2013) TRP channels and pain. *Annu Rev Cell Dev Biol* **29**, 355-384
2. Story, G. M., Peier, A. M., Reeve, A. J., Eid, S. R., Mosbacher, J., Hricik, T. R., Earley, T. J., Hergarden, A. C., Andersson, D. A., Hwang, S. W., McIntyre, P., Jegla, T., Bevan, S., and Patapoutian, A. (2003) ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* **112**, 819-829
3. Nilius, B., and Appendino, G. (2012) Spices: the savory and beneficial science of pungency. *Rev Physiol Biochem Pharmacol* **164**, 1-76
4. Hinman, A., Chuang, H. H., Bautista, D. M., and Julius, D. (2006) TRP channel activation by reversible covalent modification. *Proc Natl Acad Sci U S A* **103**, 19564-19568
5. Macpherson, L. J., Dubin, A. E., Evans, M. J., Marr, F., Schultz, P. G., Cravatt, B. F., and Patapoutian, A. (2007) Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. *Nature* **445**, 541-545
6. Peyrot des Gachons, C., Uchida, K., Bryant, B., Shima, A., Sperry, J. B., Dankulich-Nagrudny, L., Tominaga, M., Smith, A. B., 3rd, Beauchamp, G. K., and Breslin, P. A. (2011) Unusual pungency from extra-virgin olive oil is attributable to restricted spatial expression of the receptor of oleocanthal. *J Neurosci* **31**, 999-1009
7. Karashima, Y., Damann, N., Prehen, J., Talavera, K., Segal, A., Voets, T., and Nilius, B. (2007) Bimodal action of menthol on the transient receptor potential channel TRPA1. *J Neurosci* **27**, 9874-9884
8. Nagatomo, K., and Kubo, Y. (2008) Caffeine activates mouse TRPA1 channels but suppresses human TRPA1 channels. *Proc Natl Acad Sci U S A* **105**, 17373-17378
9. Xiao, B., Dubin, A. E., Bursulaya, B., Viswanath, V., Jegla, T. J., and Patapoutian, A. (2008) Identification of transmembrane domain 5 as a critical molecular determinant of menthol sensitivity in mammalian TRPA1 channels. *J Neurosci* **28**, 9640-9651
10. Nagatomo, K., Ishii, H., Yamamoto, T., Nakajo, K., and Kubo, Y. (2010) The Met268Pro mutation of mouse TRPA1 changes the effect of caffeine from activation to suppression. *Biophys J* **99**, 3609-3618
11. Gros, J., Peeters, F., and Collin, S. (2012) Occurrence of odorant polyfunctional thiols in beers hopped with different cultivars. First evidence of an S-cysteine conjugate in hop (*Humulus lupulus* L.). *J Agric Food Chem* **60**, 7805-7816
12. Takoi, K., Degueil, M., Shinkaruk, S., Thibon, C., Maeda, K., Ito, K., Bennetau, B., Dubourdieu, D., and Tominaga, T. (2009) Identification and characteristics of new volatile thiols derived from the hop (*Humulus lupulus* L.) cultivar Nelson Sauvin (dagger). *J Agric Food Chem* **57**, 2493-2502
13. Eyres, G. T., Marriott, P. J., and Dufour, J. P. (2007) Comparison of odor-active compounds in the spicy fraction of hop (Humulus lupulus L.) essential oil from four different varieties. *J Agric Food Chem* **55**, 6252-6261

14. Kishimoto, T., Wanikawa, A., Kono, K., and Shibata, K. (2006) Comparison of the odor-active compounds in unhopped beer and beers hopped with different hop varieties. *J Agric Food Chem* **54**, 8855-8861

15. Goiris, K., Ridder, M., Rouck, G., Boeykens, A., Opstaele, F., Aerts, G., Cooman, L., and Keukeleire, D. (2002) The oxygenated sesquiterpenoid fraction of hops in relation to the spicy hop character of beer. *Journal of the Institute of Brewing* **108**, 86-93

16. Kishimoto, T., Wanikawa, A., Kagami, N., and Kawatsura, K. (2005) Analysis of hop-derived terpenoids in beer and evaluation of their behavior using the stir bar-sorptive extraction method with GC-MS. *J Agric Food Chem* **53**, 4701-4707

17. Nilius, B., Prenen, J., and Owssianik, G. (2011) Irritating channels: the case of TRPA1. *J Physiol* **589**, 1543-1549

18. Bandell, M., Dubin, A. E., Petrus, M. J., Orth, A., Mathur, J., Hwang, S. W., and Patapoutian, A. (2006) High-throughput random mutagenesis screen reveals TRPM8 residues specifically required for activation by menthol. *Nat Neurosci* **9**, 493-500

19. Komatsu, T., Uchida, K., Fujita, F., Zhou, Y., and Tominaga, M. (2012) Primary alcohols activate human TRPA1 channel in a carbon chain length-dependent manner. *Pflugers Arch* **463**, 549-559

20. Doerner, J. F., Gisselmann, G., Hatt, H., and Wetzel, C. H. (2007) Transient receptor potential channel A1 is directly gated by calcium ions. *J Biol Chem* **282**, 13180-13189

21. Xu, H., Delling, M., Jun, J. C., and Clapham, D. E. (2006) Oregano, thyme and clove-derived flavors and skin sensitizers activate specific TRP channels. *Nat Neurosci* **9**, 628-635

22. Jordt, S. E., and Julius, D. (2002) Molecular basis for species-specific sensitivity to "hot" chili peppers. *Cell* **108**, 421-430

23. Takaishi, M., Uchida, K., Fujita, F., and Tominaga, M. (2014) Inhibitory effects of monoterpenes on human TRPA1 and the structural basis of their activity. *J Physiol Sci* **64**, 47-57

24. Amin, J., Brooks-Kayal, A., and Weiss, D. S. (1997) Two tyrosine residues on the alpha subunit are crucial for benzodiazepine binding and allosteric modulation of gamma-aminobutyric acidA receptors. *Mol Pharmacol* **51**, 833-841

25. Price, K. L., and Lummis, S. C. (2004) The role of tyrosine residues in the extracellular domain of the 5-hydroxytryptamine3 receptor. *J Biol Chem* **279**, 23294-23301

26. Lummis, S. C., Harrison, N. J., Wang, J., Ashby, J. A., Millen, K. S., Beene, D. L., and
Dougherty, D. A. (2012) Multiple Tyrosine Residues Contribute to GABA Binding in the GABA(C) Receptor Binding Pocket. ACS Chem Neurosci 3, 186-192

27. Steinberg, X., Lespay-Rebolledo, C., and Brauchi, S. (2014) A structural view of ligand-dependent activation in thermoTRP channels. Front Physiol 5, 171

28. Cvetkov, T. L., Huynh, K. W., Cohen, M. R., and Moiseenkova-Bell, V. Y. (2011) Molecular architecture and subunit organization of TRPA1 ion channel revealed by electron microscopy. J Biol Chem 286, 38168-38176

29. Levitt, M. (1978) Conformational preferences of amino acids in globular proteins. Biochemistry 17, 4277-4285

30. Saito, S., Banzawa, N., Fukuta, N., Saito, C. T., Takahashi, K., Imagawa, T., Ohta, T., and Tominaga, M. (2014) Heat and Noxious Chemical Sensor, Chicken TRPA1, as a Target of Bird Repellents and Identification of Its Structural Determinants by Multispecies Functional Comparison. Mol Biol Evol 31, 708-722

31. Vriens, J., Owsianik, G., Janssens, A., Voets, T., and Nilius, B. (2007) Determinants of 4 alpha-phorbol sensitivity in transmembrane domains 3 and 4 of the cation channel TRPV4. J Biol Chem 282, 12796-12803

32. Cao, E., Liao, M., Cheng, Y., and Julius, D. (2013) TRPV1 structures in distinct conformations reveal activation mechanisms. Nature 504, 113-118
Acknowledgements- We thank Dr. Y. Suzuki and Ms. N. Fukuta of the National Institute for Physiological Sciences, and Mr. F. Manabe and Mr. Y. Kaneko of Kirin Company, Mr. J. Ouchi and Dr. K. Sasaki of Kyowa Hakko Kirin Company for their excellent technical support and valuable discussions.

FOOTNOTES
The abbreviations used are: AITC, allyl isothiocyanate; HEK293, human embryonic kidney-derived 293; HHE, Haratau Hersbrucker; [Ca^{2+}], intracellular Ca^{2+} concentrations; TRPA1, Transient receptor potential ankyrin 1

FIGURE LEGENDS

FIGURE 1. GC-MS analyses of hop extract. A, Eudesmol contents in hop dichloromethane extract from various hop cultivars. Structures of three structural isomers are shown in the inset. B, Representative total ion chromatograms of HHE extract (upper) and eudesmol standards (lower). C, Mass spectrum at retention time of each eudesmol isomer in HHE extract and eudesmol standards (STD).

FIGURE 2. Quantification of other identified sesquiterpenes in hop by GC-MS. β-Caryophyllene (A) and α-humulene (B) contents in hop dichloromethane extract from various hop cultivars. Structural formulas are shown in the insets.

FIGURE 3. Effects of eudesmol on human TRP channels. A, Effects of eudesmol (100 µM) on different hTRP channels transiently expressed in HEK293 cells using a calcium-imaging method. Values are presented as the mean ± s.e.m. (n=4-11). Kruskal Wallis test following the Steel test was used for statistical evaluation between each vector control and hTRP channel. Significance was assumed if the p value was <0.05. **, ##, $$; p<0.01 vs each vector control. B, Representative traces of the calcium-images in HEK293 cells transiently expressing hTRPA1. AITC (50 µM) was used as an agonist of hTRPA1. C, Dose dependency of hTRPA1 activation by eudesmol. Values are presented as the mean ± s.e.m. (n=5-8). D, Representative traces of the calcium-imagings in HEK293 cells transiently expressing hTRPV3. Carvacrol (50 µM) was used as an agonist of hTRPV3. E, Dose dependency of hTRPV3 activation by eudesmol.

FIGURE 4. Eudesmol activates human TRPA1. A, Representative traces of whole-cell patch-clamp currents activated by eudesmol (100 µM) in HEK293 cells expressing hTRPA1. Arrowheads indicate the points of ramp pulse application to generate I-V, shown to the right. AITC (100 µM) was used as a TRPA1 stimulant. B, Representative I-V curves by AITC (3 µM or 100 µM). C, Comparison of the inward currents evoked by eudesmol isomers (100 µM) between hTRPA1-expressing cells and vector control cells. All values are presented as the mean ± s.e.m. Mann-Whitney U test was adopted for comparison of the currents between hTRPA1-expressing cells and vector control cells. One-way ANOVA following Bonferroni test was used for the statistical evaluation among isomers. Significance was assumed if the p value was <0.05. *, p<0.05; ** p<0.01.

FIGURE 5. Eudesmol activates human TRPV3. A, A representative whole-cell current trace in HEK293 cells transiently expressing hTRPV3; (a) indicates the point of ramp pulse application to generate an I-V curve shown to the right. A mixture of 2-APB and carvacrol was used to activate TRPV3. B, Comparison of densities of the currents activated by eudesmol isomers (100 µM) at ±100 mV between hTRPV3-expressing cells and vector control cells. All values are presented as the mean ± s.e.m. (n=5-6). Mann-Whitney U test was adopted. One-way ANOVA following Bonferroni test was used for the statistical evaluation among isomers. Significance was assumed if the p value was <0.05. *, p<0.05; ** p<0.01.
Hop derived sesquiterpene eudesmol activates human TRPA1

FIGURE 6. Effect of an inhibitor, extracellular calcium or activator on β-eudesmol evoked human TRPA1 activation. A, A representative trace of the whole-cell current in the presence of a TRPA1 inhibitor, HC03-0031 (30 µM). β-Eudesmol and AITC were used at 100 µM. B, Comparison of the inward currents evoked by β-eudesmol (100 µM) in hTRPA1-expressing cells between absence and presence of HC03-0031 (30 µM). C, Effect of extracellular calcium. Two bath solutions were prepared: a calcium-free bath solution which was then converted to a 2 mM Ca²⁺ bath solution. β-Eudesmol and AITC were used at 100 µM. D, Comparison of the inward currents evoked by β-eudesmol (100 µM) in hTRPA1-expressing cells between absence and presence of extracellular calcium. E, A representative trace of the whole-cell current by AITC (100 µM). F, Effect of β-eudesmol (100 µM) on the inward currents evoked by AITC (100 µM). G, Comparison of the inward currents evoked by AITC (100 µM) in hTRPA1-expressing cells between absence and presence of β-eudesmol (100 µM). All values are presented as the mean ± s.e.m. (n=5-20). Mann-Whitney U test was adopted for comparison. Significance was assumed if the p value was <0.05. *, p<0.05; **, p<0.01; n. s., not significant.

FIGURE 7. Critical amino acid residues in human TRPA1 required for activation by β-eudesmol. A, Schematic drawing of a putative hTRPA1 structure. Amino acid positions for analyses are indicated by circles. Open circles indicated three crucial amino acids for β-eudesmol-evoked activation. B, Relative activity of each mutated hTRPA1 channel with β-eudesmol (50 µM), using a calcium-imaging method. Values are presented as the mean ± s.e.m (n=4-5). Data were normalized to the responses by AITC (5 µM). C, Densities of the inward currents at -60 mV activated by β-eudesmol (100 µM) (upper) or AITC (100 µM) (middle), and ratio of the β-eudesmol-activated currents divided by AITC-activated ones (lower). All values are presented as the mean ± s.e.m. (n=5-14). One-way ANOVA, following the Dunnett test, was used for statistical evaluation. Significance was assumed if the p value was <0.05. *, p<0.05; ** p<0.01. D, Representative traces of the whole-cell currents treated with β-eudesmol (100 µM) in HEK293 cells expressing mutated human TRPA1 channels. AITC (100 µM) was used as a TRPA1 agonist.
Hop derived sesquiterpene eudesmol activates human TRPA1

TABLE 1. Oligonucleotides used for site-directed mutagenesis of human TRPA1 in this study. Underlining indicates changed nucleotides for mutagenesis.

| Name | Forward | Reverse |
|------|---------|---------|
| Y726A | GAA TTAGGATCGC CTGGT TGGTCT | GACCA AGA AGG GAGA TCTAAATC |
| T734A | GCTACACCTACG CGATCTCTGTTGTC | GACAACGGAGAATGC CATGATATGAG |
| S748A | GGAATGGCTTCCA ACGGCACTGCT | GTAAGCGGATGTC GTTGAAGCGCATTG |
| S767A | CTAAGA CCGAATG ATCTA ATAAAAAC | GTT TTAATGATATG ATCTGGAATCTG |
| Y768A | GATACCA CGAATTCGCTTC AAAAAACTT | CAAGTTTTATATGA TATGGAATGTA |
| T772A | CGAATCGAATCATA ATTGGATTGGTATGTG | CACTAAAATCAACAA GTTTATAGA ATGAAATTCG |
| S780A | GATTITATGTITTTT TACAGT AATTGGG | CAAAATATCTTTC TAAAACACTAAAAATC |
| S781A | GTTTTATACGCTTAA TTTGGGTGAT | CAAATCCAAATATG CTAAAAAATC |
| Y785A | CAATATTTTAAAGATATG GAAATGAGC | CGTTTTTTGAAGC CCAAATATCTT |
| S804A | GAATATTTTAAAGATATG GAAATGAGC | CAAATCCAAATATG CTAAAAAATC |
| Y812A | GAATGGAATACCTA ACGGACAGCACC | GATGCCCCGTGTCG CTAATCCTATCC |
| T813A | GAATGGAATACCTA ACGGACAGCACC | CAAAATATCGTCCCTGCTG TGTGATATCCTATCC |
| T814A | GGAATACCTACAGCCGATCCATTTTG | CAAAATATCGTCCCTGCTG TGTGATATCCTATCC |
| Y840A | GCAATGCTTGGATC TCCTAATGGAAG | CATCAATAAGAC CACACGAAATG |
| Y842A | GCTGGTACCTCTTGGAAATTT | GAAATCTCCAAACG GAATTCAGC |
| Y849A | GAATCTCTTATTTGT TCTTGGAAATTT | GAAATCTTGAAGC CAAATGAAATTC |
| T869A | GGATATTTTAAAGATATG GAAATGAGC | CAAATCCAAATATG CTAAAAAATC |
| S873A | GAAAACCTTGGATGACGCTACAGTTG | CAATGCTGACTCCACAAAGT TTTTC |
| T874A | GGTGGATGCTCAGT GTTGATATTC | GAAATATCTACGCA CAGCCCTT |
| S943A | CTTTGCACAACTGCTTGCCTC ACAAATTG | CAAATATCGTCCCTGCTG TGTGATATCCTATCC |
| T945A | CTTGTCTCTTCG AAATTTGTGCA CCTCATAATG | CAAATGCGAATATG CAAAGGAAACAG |
Hop derived sesquiterpene eudesmol activates human TRPA1

Figure 1, Ohara et al.
Hop derived sesquiterpene eudesmol activates human TRPA1

Figure 2, Ohara et al.
A

Bar graph showing relative ΔRatio (Max-Min) for various treatments:
- Vector control
- hTRPA1
- hTRPV1
- hTRPV2
- hTRPV3
- hTRPV4
- hTRPM8

Treatments:
- DMSO
- α-Eudesmol
- β-Eudesmol
- γ-Eudesmol

B

Time (sec.) vs. ΔRatio graph for hTRPA1:
- DMSO
- α-Eudesmol
- β-Eudesmol
- γ-Eudesmol

C

Time (sec.) vs. ΔRatio graph for hTRPA1 with AITC:
- α-Eudesmol
- β-Eudesmol
- γ-Eudesmol

D

Time (sec.) vs. ΔRatio graph for hTRPV3:
- DMSO
- α-Eudesmol
- β-Eudesmol
- γ-Eudesmol

E

Time (sec.) vs. ΔRatio graph for hTRPV3 with Carvacrol:
- α-Eudesmol
- β-Eudesmol
- γ-Eudesmol

Figure 3, Ohara et al.
Hop derived sesquiterpene eudesmol activates human TRPA1

Figure 4, Ohara et al.
Hop derived sesquiterpene eudesmol activates human TRPA1

Figure 5, Ohara et al.
Hop derived sesquiterpene eudesmol activates human TRPA1

Figure 6, Ohara et al.
Hop derived sesquiterpene eudesmol activates human TRPA1

Figure 7, Ohara et al.

A

B

C

D

hTRPA1-T813A

β-Eudesmol

1 nA

20 sec

hTRPA1-Y840A

β-Eudesmol

1 nA

20 sec

hTRPA1-S873A

β-Eudesmol

1 nA

20 sec

Figure 7, Ohara et al.
Identification of Significant Amino Acids in Multiple Transmembrane Domains of Human Transient Receptor Potential Ankyrin 1 (TRPA1) for Activation by Eudesmol, an Oxygenized Sesquiterpene in Hop Essential Oil

Kazuaki Ohara, Takafumi Fukuda, Hiroyuki Okada, Sayoko Kitao, Yuko Ishida, Kyoko Kato, Chika Takahashi, Mikio Katayama, Kunitoshi Uchida and Makoto Tominaga

*J. Biol. Chem. published online December 18, 2014*

Access the most updated version of this article at doi: 10.1074/jbc.M114.600932

Alerts:

- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts