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Specific Hydroxy Fatty Acids in Royal Jelly Activate TRPA1

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ABSTRACT: This is the first report of TRPA1 activation by fatty acids. Activation of TRPA1 and TRPV1 induces thermogenesis and energy expenditure enhancement. In this study, we searched for novel agonists of TRPA1 and TRPV1 from a nonpungent food, royal jelly (RJ). We measured the activation of human TRPA1 and TRPV1 by RJ extracts and found that the hexane extract contains TRPA1 agonists. The main functional compounds in the hexane extract were *trans*-10-hydroxy-2-decenoic acid (HDEA) and 10-hydroxydecanoic acid (HDAA). These are characteristic fatty acids of RJ. Their EC_{50} values were about 1,000 times larger than that of AITC, and their maximal responses were equal. They activated TRPA1 more strongly than TRPV1. Their EC_{50} values for TRPV1 were 2 times larger, and the maximal response was less than half of that for TRPA1. Next, we studied the potencies of other lipid components for both receptors. Most of them have higher affinity to TRPA1 than TRPV1. Among them, dicarboxylic acids showed equal efficacy for both receptors, but those are present in only small amounts in RJ. We concluded that the main function of RJ is TRPA1 activation by HDEA and HDAA, the major components of the RJ lipid fraction.

KEYWORDS: Royal jelly, fatty acid, *trans*-10-hydroxy-2-decenoic acid, 10-hydroxydecanoic acid, TRPA1, TRPV1, intracellular calcium concentration

INTRODUCTION

Transient receptor potential ankyrin 1 (TRPA1) and vanilloid 1 (TRPV1), both of which belong to the TRP family, are Ca^{2+}-permeable nonselective cation channels.[1,2] TRPA1 and TRPV1 are expressed in sensory neurons in the whole body[3,4] including both dosal root ganglion and nodose ganglion neurons innervating stomach and in nerve fibers in the gastric wall.[5−7] These 2 channels function as alarm molecules.[8−11] TRPA1 is activated by cold temperatures[3] and inflammatory signals,[11−13] while TRPV1 is activated by noxious heat[2] and protons.[4,10] Furthermore, TRPA1 and TRPV1 are also activated by food components. For example, allyl isothiocyanate (AITC)[14] and cinnamaldehyde (CNA)[11] act on TRPA1, and capsaicin (CAP) on TRPV1.[2] Interestingly, it has been reported that TRPA1 agonists, such as AITC, elevate the temperature of brown adipose tissue.[15] Recently, we showed that the TRPA1 agonists, AITC and CNA, induce adrenaline secretion in anesthetized rats.[16] Furthermore, CAP enhances energy expenditure and thermogenesis in rodents[17] and humans.[18,19] Therefore, ingestion of TRPA1- and TRPV1-active food components can be effective against obesity through energy expenditure enhancement. Many of the known agonists for TRPA1 and TRPV1 are pungent compounds. Nevertheless, some nonpungent compounds also activate TRPV1, e.g., capsiate (from nonpungent chili pepper, CH-19 Sweet)[20] and monoacylglycerols (found in wheat flour).[21] In order to find new nonpungent agonists, we focused our attention on royal jelly (RJ), a multifunctional food.

Royal jelly (RJ) is a viscous substance secreted from the hypopharyngeal and mandibular glands of worker honeybees (*Apis mellifera* Linne)[22,23] and is the exclusive food of a queen honeybee. A queen honeybee has a long life and lays 2000 eggs every day.[22,23] The tremendous vital energy of queen bees is attributed to RJ, which led to its wide use in commercially available drugs, foods, and cosmetics in many countries. RJ consists of 50−60% water, 18% proteins, 15% carbohydrates, 3−6% lipids, and 1.5% other compounds, such as minerals and vitamins.[23,24] The lipid composition is reported as 80−85% fatty acids, 4−10% phenols, 5−6% waxes, 3−4% steroids, and 0.4−0.8% phospholipids. The fatty acid fraction consists of 32% *trans*-10-hydroxy-2-decenoic acid (HDEA), 24% gluconic acid, 22% 10-hydroxydecanoic acid (HDAA), 5% dicarboxylic acids, and several other acids. HDEA and HDAA are specific components of RJ.[25−27]

RJ shows numerous biological effects such as antioxidant,[28,29] antitumor,[30] cell-proliferative,[31] and antihypertensive[32] activities. Thus, RJ has diverse physiological functions, which led us to expect that it may contain agonists for TRPA1 and TRPV1. In this article, we searched for agonists for TRPA1 and TRPV1 in RJ and found that hydroxy fatty acids more strongly activate TRPA1 than TRPV1. Furthermore, some structure−activity relationships were compared using a small number of compounds.

MATERIALS AND METHODS

Materials. CAP, 10-hydroxydecanoic acid (HDAA), and 1,6-hexanedicarboxylic acid were purchased from Sigma (St. Luis, MO, USA). Allyl isothiocyanate (AITC), *trans*-10-hydroxy-2-decenoic acid (HDEA), D-gluconic acid, D-glucono-1,5-lactone, 8-hydroxyoctanoic acid, *p*-hydroxybenzonic acid, and phenol were obtained from Wako Pure Chem. Ind. (Osaka, Japan). HC-030031 was obtained from ChemBridge (San Diego, CA, USA). Octanoic acid was purchased from Nacalai Tesque (Kyoto, Japan). 1,8-Octanedicarboxylic acid was obtained from Kisida Chemical (Osaka, Japan). 9-Oxo-2-decenoic acid was purchased from SPECS (HT Delft, Netherlands). The chemical structures of the 11 RJ

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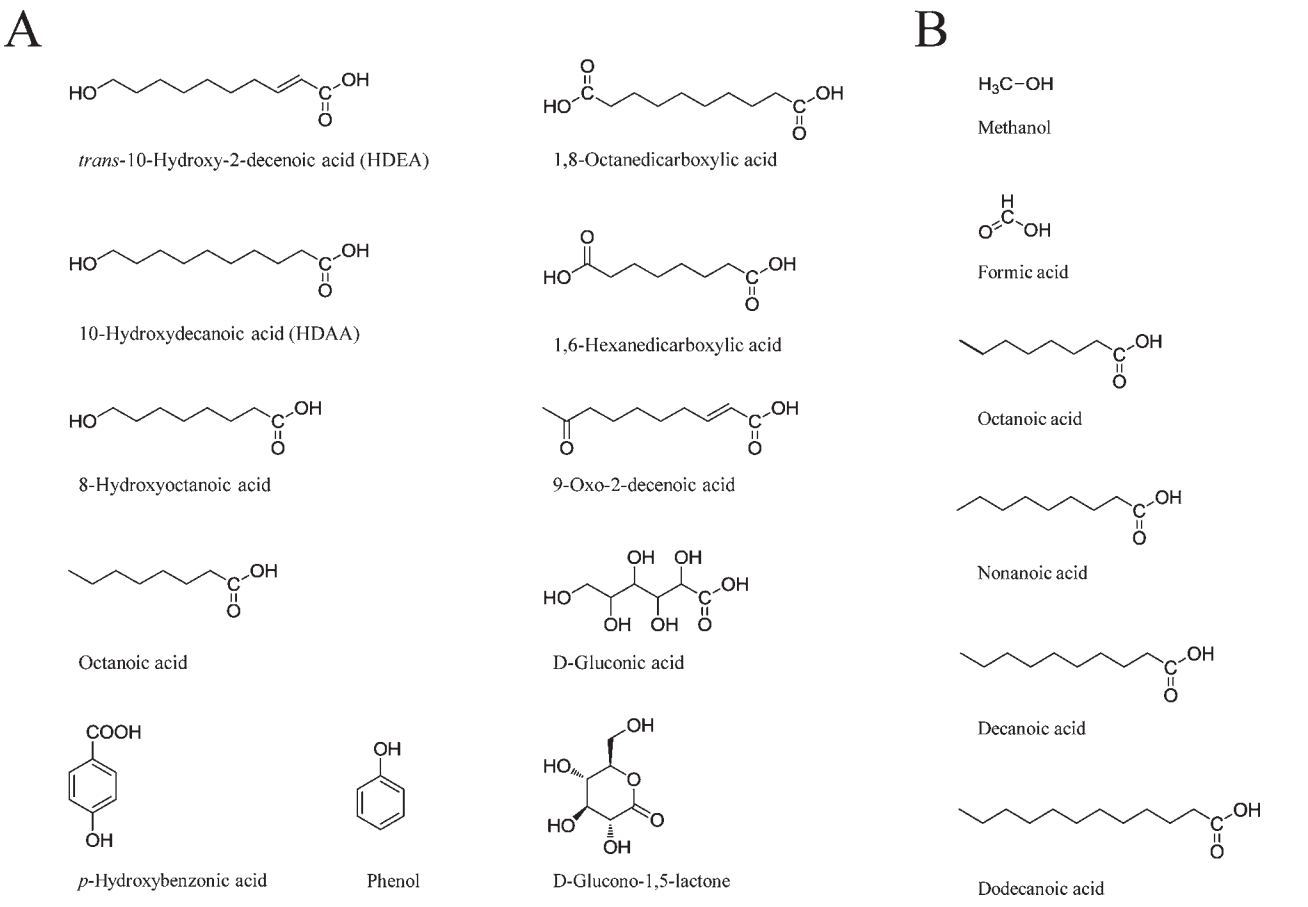

Figure 1. Chemical structures of the 11 RJ Components and the 6 substances tested in the SAR studies. (A) Chemical structures of the 11 RJ components. (B) Chemical structures of the 6 substances for the SAR studies.

components and related compounds used for structure−activity relationship (SAR) studies are shown in Figure 1A and B, respectively. Lyophilized RJ was supplied from the Japan Royal Jelly Fair Trade Council (Tokyo, Japan). A part of the composition of the raw RJ used for freeze-drying was shown as follows: 64.1% of water, 14.4% of proteins, and 2.05% of HDEA.

Extraction and Purification of RJ. Lyophilized RJ (20 g) was extracted successively with 400 mL of hexane, ethyl acetate, and methanol each, and the TRPA1 and TRPV1 activity of each extract was measured. Then the TRPA1-active hexane extract was separated into 3 fractions (neutral lipids, free fatty acids, and phospholipids) by solid-phase extraction. We examined whether the free fatty acid fraction contained HDEA or HDAA by thin-layer chromatography along with the development of sulfuric acid. HDEA, HDAA, and a fraction of free fatty acids gave a single spot, and their rates of flow values were equal.

Identification and Quantification of HDEA and HDAA in the Hexane Extract. In order to measure the HDEA and HDAA content of the hexane extract, the free fatty acid fraction was analyzed by gas chromatography (GC) using GC-14B gas chromatography (Shimazu, Kyoto Japan). A WCOT-fused silica CP-Sil 88 column (0.25 mm × 50 m; Varian, Inc., Palo Alto, CA USA) was used with helium at a flow rate of 2 mL/min. The injector temperature was set at 260 °C, and the detector temperature was set at 280 °C. The column oven temperature was changed from 160 to 190 °C (3 °C/min). Trimethylsilyl (TMS) fatty acids from the free fatty acid fraction were obtained by reaction with *N*,*O*-bis (trimethylsilyl) trifluoroacetamide (GL Science, Tokyo Japan) and boiling for 1 h. After cooling, 30 μL of the reaction mixture was diluted with 270 μL of hexane and submitted to GC analysis.

Cloning and Expression of Human TRPA1 and Human TRPV1. Human TRPA1 and TRPV1 cDNAs were amplified by RT-PCR, using mRNA obtained from human brain first-strand cDNA (Agilent Technologies, Santa Clara, CA, USA) and human WI38 cells, respectively. The expression of full-length human TRPA1 in stable HEK cells was induced using the tetracycline-inducible T-REx expression system from Invitrogen. The hTRPA1 cDNA was subcloned into pcDNA4/TO (Invitrogen) and then transfected into HEK T-REx cells by using the Lipofectamine 2000 reagent (Invitrogen). HEK T-REx cells that stably maintained the hTRPA1 gene were selected using 500 μg/mL zeocin and 10 μg/mL blasticidin and grown according to the manufacturer's instructions.

Human TRPV1 cDNA was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA, USA) and then transfected into HEK293T cells by using the SuperFect transfection reagent (Qiagen, Hilden, Germany). After culturing cells in the presence of 750 μg/mL G418, we obtained a stable HEK293T cell line that expresses human TRPV1.

The following primers were used for cloning: human TRPA1 forward primer 5′-TGGGTCAATGAAGTGCAG-3′ and reverse primer 5′-GAAGGTCTGAGGAGCTAAGGC-3′; human TRPV1 forward primer 5′-GCAAGGATGAAGAAGAAATGGA-3′ and reverse primer 5′-TCACTTCTCCCCGGAAGCGC-3′.

Measurement of Intracellular Ca^{2+} Concentration. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured at 25 °C by using a FlexStation II system (Molecular Devices, Sunnyvale, CA, USA). The cells were seeded in 96-well plates 24 h before the assay. To obtain TRPA1-expressing HEK cells, 1 μg/mL tetracycline was added to induce the expression of TRPA1 protein. The cells were subcultured every week, and the highest passage number was 40. The cells were loaded

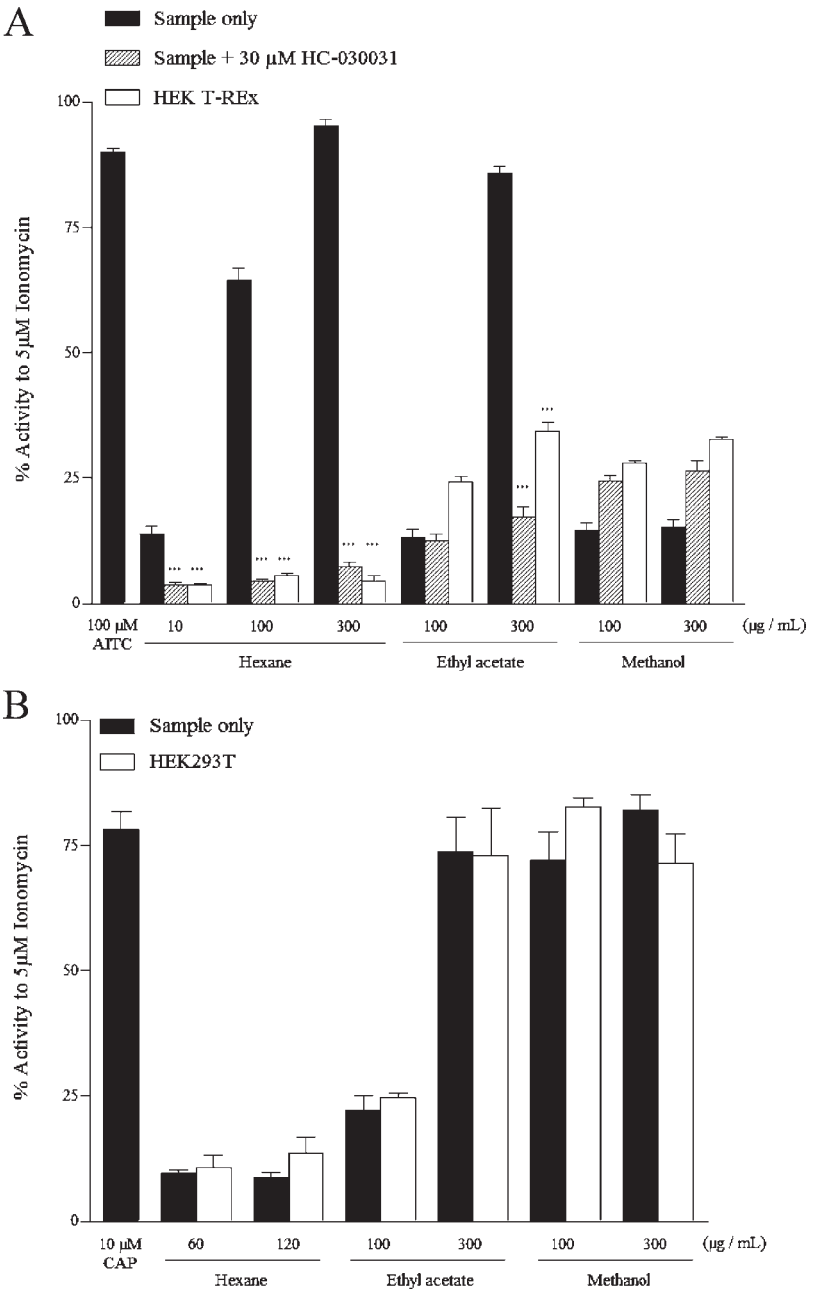

Figure 2. Activation of TRPA1 and TRPV1 by the RJ extracts. Fluo-4 Ca^{2+} responses of HEK T-REx cells expressing human TRPA1 and HEK293T cells expressing human TRPV1 are shown. Data values for each of these extracts are expressed as a percentage response to that of 5 μM ionomycin. Each data point represents the mean ± SEM (n = 4−6). (A) Ca^{2+} responses to extracts of RJ in TRPA1-expressing cells. Filled columns show TRPA1 activation by 100 or 300 μg/mL extracts except for the hexane extract when 10, 100, or 300 μg/mL was used. Striped columns indicate TRPA1 activation by these extracts with the addition of 30 μM HC-030031. Unfilled columns indicate the responses to those extracts of HEK T-REx cells not expressing TRPA1. *** indicates $p < 0.0005$ (unpaired t-test). (B) Ca^{2+} responses to extracts of RJ in TRPV1-expressing cells. Filled columns show TRPV1 activation by 100 or 300 μg/mL of extracts except for the hexane extract, of which 60 or 120 μg/mL were administered. Unfilled columns indicate the responses to these extracts of HEK293T cells not expressing TRPV1.

with 3 μM Fluo-4-AM (Molecular Probes, Eugene, OR, USA) for 1 h at 37 °C in a loading buffer (5.37 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 0.34 mM $Na_2HPO_4 \cdot 7H_2O$, 5.56 mM D-glucose, 20 mM HEPES, 1 mM $CaCl_2$, 0.1% BSA, and 250 mM probenecid at pH 7.4). To study the inhibitory activity of the respective antagonist, 30 μM CPZ for TRPV1 or 30 μM HC-030031 for TRPA1 was added to 10−300 μg/mL of extracts of RJ, 500 or 1,000 μM of the RJ compounds and related substances for the SAR studies. To obtain the dose−response curves of TRPV1, we used 100 μM-1.2 mM 8-hydroxyoctanoic acid, 1,8-octanedicarboxylic acid, and 1,6-hexanedicarboxylic acid, and 0.1 nM-10 μM CAP.

For TRPA1, we used 1−500 μg/mL hexane extract; 10 μM−1 mM HDEA and HDAA; 1 μM−1 mM 8-hydroxyoctanoic acid; 100 μM−1 mM 1,8-octanedicarboxylic acid and 1,6-hexanedicarboxylic acid; 100 μM−1.5 mM octanoic acid, 9-oxo-2-decenoic acid, and phenol; 10−500 μM decanoic acid; 30 μM−500 μM nonanoic acid; and 0.01−100 μM AITC. In some experiments, the TRPA1 antagonist HC-030031 (30 μM) was added along with these compounds.

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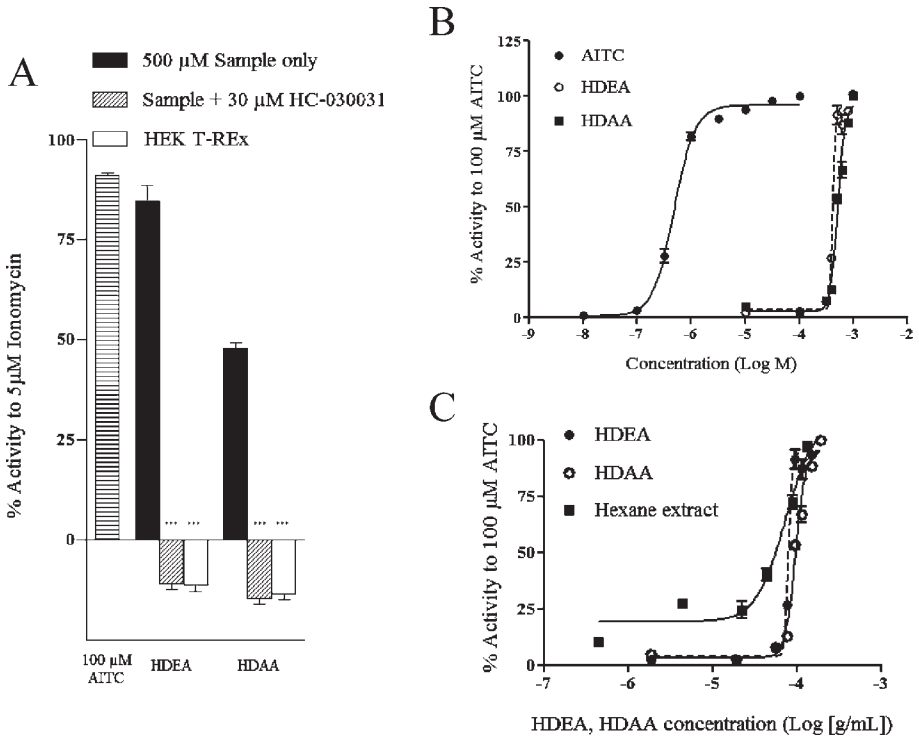

Figure 3. Activation of TRPA1 by HDEA, HDAA, and hexane extract. Fluo-4 Ca^{2+} responses of HEK T-REx cells expressing human TRPA1 are shown. The data values for each of these compounds are expressed as a percentage response to those of 5 μM ionomycin (A) and 100 μM AITC (B, C). Each data point represents the mean ± SEM ($n = 6$). (A) Ca^{2+} responses to HDEA and HDAA. Filled columns show TRPA1 activation by 500 μM HDEA and HDAA. Striped columns indicate TRPA1 activation by these compounds with the addition of 30 μM HC-030031. Unfilled columns indicate the responses to these compounds of HEK T-REx cells not expressing TRPA1. *** indicates $p < 0.0005$ (unpaired t-test). (B) Concentration−response curves for HDEA and HDAA. (C) Concentration−response curves for HDEA, HDAA, and hexane extract. The x axis indicates the concentration (log [g/mL]) of HDEA or HDAA.

Table 1. TRPA1 and TRPV1 Activation Potency of the RJ Compounds and the Carboxylic Acids

	TRPA1		TRPV1	
	EC_{50} (μM)	max (%)[a]	EC_{50} (μM)	max (%)[a]
CAP			0.0024	60.3
AITC	0.51	88.7		
HDEA	422	84.1	850	27.9
HDAA	510	85.9	893	37.3
8-hydroxyoctanoic acid	220	104.9	760	53.9
octanoic acid	801	93.7		
1,8-octanedicarboxylic acid	401	87.1	535	65.9
1,6-hexanedicarboxylic acid	343	86.3	569	76.2
9-oxo-2-decenoic acid	596	88.5		
phenol	620	80.4		
nonanoic acid	216	101.8		
decanoic acid	67	100		

[a] Percent value to the response to 5 μM Ionomycin.

The test compounds were prepared in DMSO and added to the loading solution (final DMSO concentration: 0.1−0.5%). To each well, 5 μM ionomycin was added to elicit maximum fluorescence intensity. The data values for the test compounds were expressed as a percent response to 5 μM ionomycin. Curve fitting and parameter estimation were performed using Prism 5a software (Graph Pad Software, San Diego, CA, USA).

RESULTS

Effect of RJ Extracts on Human TRPA1 and TRPV1. We extracted lyophilized RJ successively with hexane, ethyl acetate, and methanol, and measured their efficacies for TRPA1 and TRPV1. Figure 2 (A and B) shows the Ca^{2+} response induced by each extract of RJ in TRPA1- or TRPV1-expressing cells.

Both the hexane extract and the ethyl acetate extract increased $[Ca^{2+}]_i$ in TRPA1-expressing HEK T-REx cells. The hexane extract acted on TRPA1-expressing cells at a low concentration (10 μg/mL) and in a concentration-dependent manner. The ethyl acetate extract acted at a high concentration (300 μg/mL). The addition of the TRPA1 antagonist HC-030031 (30 μM) significantly decreased the Ca^{2+} response induced by these 2 extracts (Figure 2A). Moreover, these extracts hardly increased $[Ca^{2+}]_i$ in HEK T-REx cells not expressing TRPA1 (Figure 2A).

These results indicated that the hexane extract contains a TRPA1 agonist. Thereupon, we focused on the hexane extract and tried to identify its functional compounds.

However, 300 μg/mL ethyl acetate extract and 100 and 300 μg/mL methanol extract showed $[Ca^{2+}]_i$ responses; but these responses were not due to TRPV1 activation because the same samples showed similar responses in the HEK cells without TRPV1 (Figure 2B).

Effect of HDEA and HDAA on Human TRPA1. We selected HDEA and HDAA as candidates for TRPA1 agonists and measured their activities for TRPA1 because HDEA and HDAA are the main components of the RJ lipid fraction.

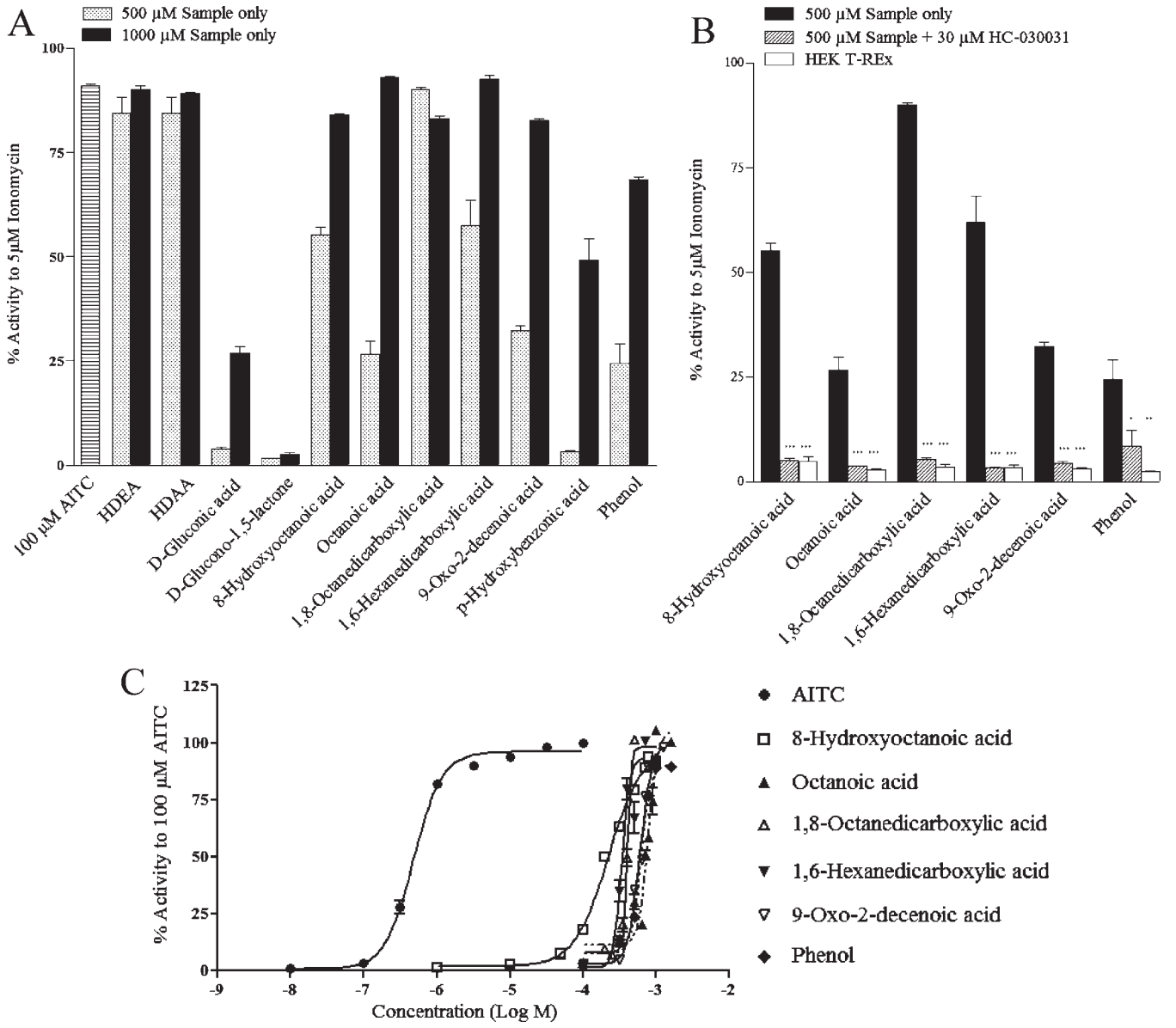

Figure 4. Activation of TRPA1 by the different RJ components. Fluo-4 Ca^{2+} responses of HEK T-REx cells expressing human TRPA1 are shown. Data values for these compounds are each expressed as a percentage response to that of 5 μM ionomycin (A, B) and 100 μM AITC (C). Each data point represents the mean ± SEM ($n = 4$). (A) Ca^{2+} responses to 9 RJ components. Dotted columns show TRPA1 activation by 500 μM of the components. Filled columns show TRPA1 activation by 1,000 μM of the components. (B) Effect of TRPA1 antagonist on RJ component-induced Ca^{2+} responses. Filled columns show TRPA1 activation by 500 μM of the components. Striped columns indicate TRPA1 activation by these compounds with the addition of 30 μM HC-030031. Unfilled columns indicate the responses to those compounds of HEK T-REx cells not expressing TRPA1. *, **, and *** indicate $p < 0.05$, $p < 0.005$, and $p < 0.0005$, respectively (unpaired t-test). (C) Concentration−response curves for 6 RJ components.

We measured the contents of HDEA and HDAA of the hexane extract by GC. After separating the extract into 3 fractions-neutral lipids, free fatty acids, and phospholipids-by using solid-phase extraction, the trimethylsilylated free fatty acid fraction was submitted for GC analysis. Under the GC conditions used, the separation of HDEA (rt 6.34 min) and HDAA (rt 6.21 min) was not satisfactory. However, the peak areas for the same quantity of both fatty acids were equal, and these fatty acids could be quantified. The GC spectrum of the TMS fatty acid fraction showed a peak at 6.35 min, and the sum of HDEA and HDAA was almost equal to the total fatty acid fraction of RJ.

Next, the Ca^{2+} response induced by these 2 compounds in TRPA1-expressing cells was evaluated. Both compounds increased $[Ca^{2+}]_i$ in TRPA1-expressing HEK T-REx cells. The addition of the TRPA1 antagonist HC-030031 (30 μM) significantly decreased the Ca^{2+} response induced by these extracts. Moreover, these extracts hardly increased $[Ca^{2+}]_i$ in HEK T-REx cells that did not express TRPA1. The dose−response curve for these 2 compounds is shown in Figure 3B. The EC_{50} values for these compounds were 1,000 times higher than that of the representative TRPA1 agonist AITC (0.5 μM). The relative maximum activity of HDEA and HDAA was nearly equal to that of AITC (Table 1).

On a weight basis, the EC_{50} value of the hexane extract was 73 μg/mL, and those of HDEA and HDAA were 415 μM (77 μg/mL) and 557 μM (101 μg/mL), respectively. In fact, the EC_{50}

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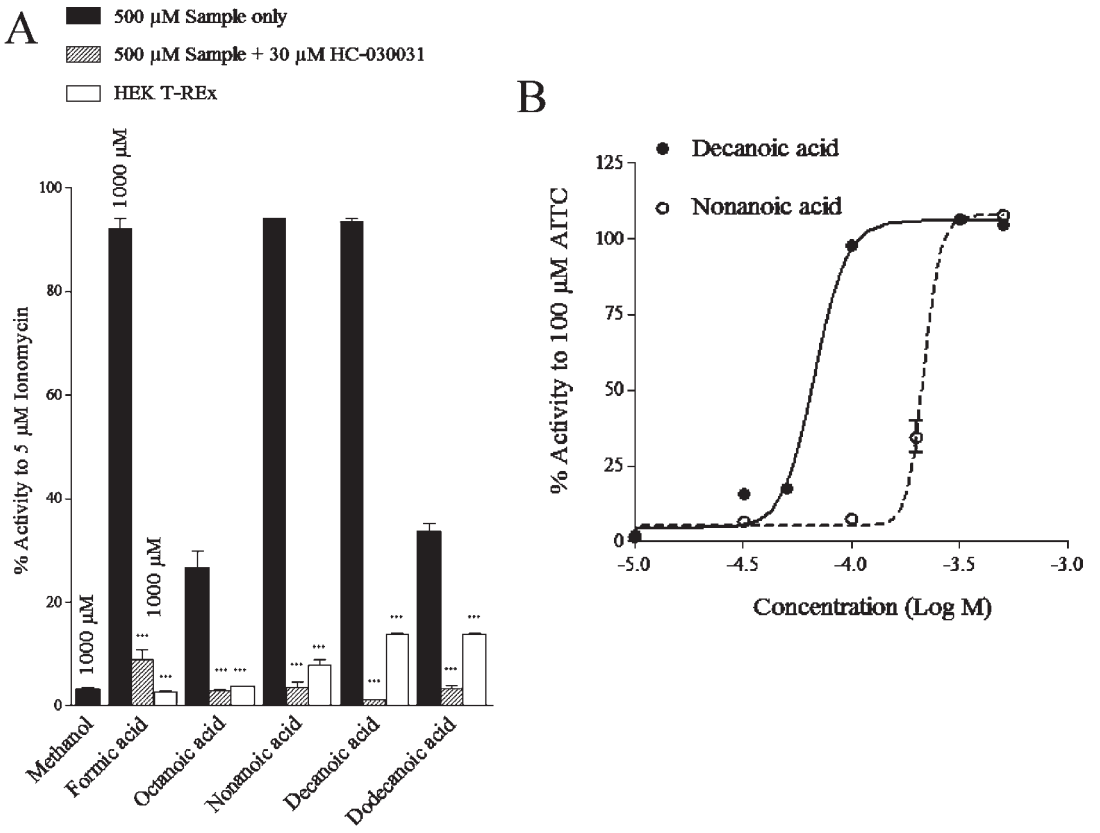

Figure 5. Activation of TRPA1 by the chemical substances used for SAR studies. Fluo-4 Ca^{2+} responses of HEK T-REx cells expressing human TRPA1 are shown. Data values for these compounds are each expressed as a percentage response to that of 5 μM ionomycin (A) and 100 μM AITC (B). Each data point represents the mean ± SEM (n = 4). (A) Ca^{2+} responses to formic acid, methanol, and 4 carboxylic acids. Filled columns show TRPA1 activation by 1,000 μM methanol and formic acid and 500 μM of 4 carboxylic acids. Striped columns indicate TRPA1 activation by these components with the addition of 30 μM HC-030031. Unfilled columns indicate the responses to these components of HEK T-REx not cells expressing TRPA1. *** indicates $p < 0.0005$ (unpaired t-test). (B) Concentration−response curves for nonanoic acid and decanoic acid.

value of the hexane extract was nearly equal to that of HDEA and HDAA. This means that the major agonists in the hexane extract are HDEA and HDAA.

Effect of 9 RJ Compounds on Human TRPA1. We evaluated lipid components in RJ other than HDEA and HDAA for TRPA1 activity, including phenol because it is the second most abundant RJ component after carboxylic acids.[22,26] We measured the activation of TRPA1 by 8 fatty acid analogues and phenol. Furthermore, we performed SAR studies with these compounds. The activity of TRPA1 induced by the 9 RJ components and 6 chemical substances for SAR analysis was compared to that induced by the TRPA1 agonist AITC.

Figure 4A shows the Ca^{2+} response induced by each of the 9 RJ components in TRPA1-expressing cells. Among these components, 8-hydroxyoctanoic acid, octanoic acid, 1,8-octanedicarboxylic acid, 1,6-hexanedicarboxylic acid, 9-oxo-2-decenoic acid, and phenol increased $[Ca^{2+}]_i$ in TRPA1-expressing HEK T-REx cells (Figure 4A) at 500 μM. The dose−response curve for these compounds is shown in Figure 4C. Judging from the EC_{50} values, we can state that 8-hydroxyoctanoic acid, 1,8-octanedicarboxylic acid, and 1,6-hexanedicarboxylic acid have relatively strong efficacy for TRPA1. The EC_{50} values for 3 compounds were 440−1,000 times higher than that of the representative TRPA1 agonist AITC (0.5 μM). The relative maximum activities of these 3 compounds were nearly equal to that of AITC (Table 1). The addition of the TRPA1 antagonist HC-030031 (30 μM) significantly decreased the Ca^{2+} response induced by these compounds (Figure 4B). In addition, these compounds barely increased $[Ca^{2+}]_i$ in HEK T-REx cells not expressing TRPA1 (Figure 4B). These results indicate that the analogues and phenol activate TRPA1 as well.

SAR Studies of Fatty Acids on Human TRPA1. Figure 5A shows the Ca^{2+} response induced by formic acid and methanol in TRPA1-expressing cells. Among them, formic acid strongly increased $[Ca^{2+}]_i$ in TRPA1-expressing HEK T-REx cells (Figure 5A), but methanol did not activate TRPA1. The addition of the TRPA1 antagonist HC-030031 (30 μM) significantly decreased the Ca^{2+} response induced by formic acid (Figure 5A). In addition, formic acid hardly increased $[Ca^{2+}]_i$ in HEK T-REx cells not expressing TRPA1 (Figure 5A). We concluded from this result that the carboxyl groups might be important for activating TRPA1.

Figure 5A shows the Ca^{2+} response induced by each of the 4 carboxylic acids in TRPA1-expressing cells. Among these components, nonanoic acid and decanoic acid strongly increased $[Ca^{2+}]_i$ in TRPA1-expressing HEK T-REx cells (Figure 5A). The dose−response curve for the 2 compounds is shown in Figure 5B. The EC_{50} values for the 2 compounds were 130−400 times higher than that of the representative TRPA1 agonist AITC (0.5 μM). The relative maximum activity of nonanoic acid and decanoic acid was nearly equal to that of AITC (Table 1). The addition of the TRPA1 antagonist HC-030031 (30 μM)

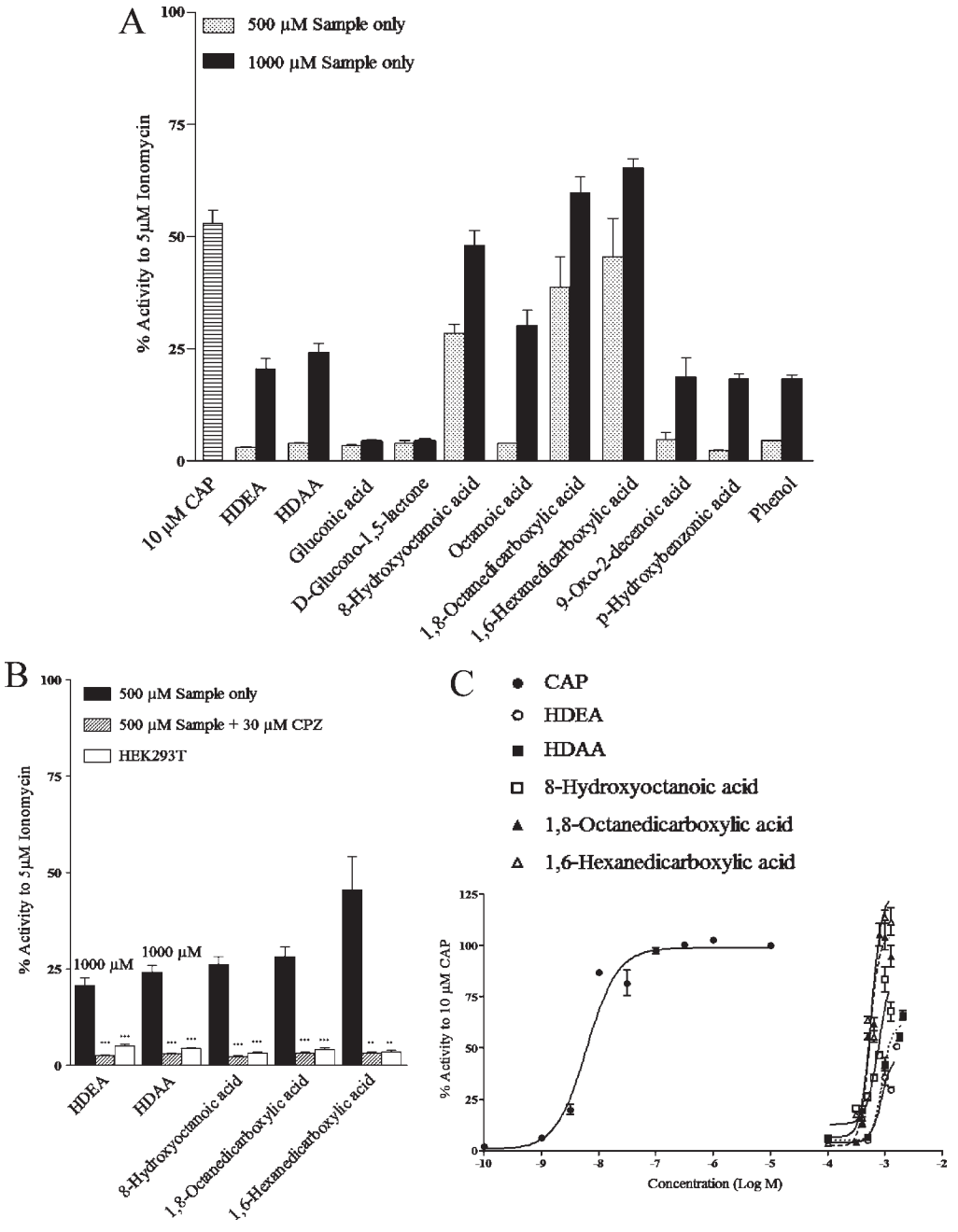

Figure 6. Activation of TRPV1 by the different RJ components. Fluo-4 Ca^{2+} responses of HEK293T cells expressing human TRP V1 are shown. Data values for these compounds are each expressed as a percentage response to that of 5 μM ionomycin (A, B) and 10 μM CAP (C). Each data point represents the mean ± SEM ($n = 4$). (A) Ca^{2+} responses to 11 RJ components. Dotted columns show TRPV1 activation by 500 μM of the components. Filled columns show TRPV1 activation by 1,000 μM of the components. (B) Effect of TRPV1 antagonist on RJ component-induced Ca^{2+} responses. Filled columns show TRPV1 activation by 500 μM of the components except for HDEA and HDAA when 1000 μM was used. Striped columns indicate TRPV1 activation by these compounds with the addition of 30 μM CPZ. Unfilled columns indicate the responses to those compounds of HEK293T cells not expressing TRPV1. ** and *** indicate $p < 0.005$ and $p < 0.0005$, respectively (unpaired t-test). (C) Concentration−response curves for 5 RJ components.

significantly decreased the Ca^{2+} response induced by these compounds (Figure 5A). In addition, these compounds hardly increased $[Ca^{2+}]_i$ in HEK T-REx cells not expressing TRPA1 (Figure 5A). The carboxylic acid with a 10-carbon chain showed the strongest efficacy on TRPA1.

Effect of 11 RJ Components on Human TRPV1. We measured the potency of lipid components for TRPV1. Figure 6A shows the Ca^{2+} response induced by each of the 11 RJ components in TRPV1-expressing cells. Among these compounds, 8-hydroxyoctanoic acid, 1,8-octanedicarboxylic acid, and 1,6-hexanedicarboxylic acid strongly increased $[Ca^{2+}]_i$ in TRPV1-expressing HEK293T cells. A dose−response curve was drawn for these compounds and for HDEA and HDAA (Figure 6C). Their EC_{50} values were 200,000−370,000 times larger than that of the representative TRPV1 agonist CAP (2.4 nM). The maximum activities of these compounds were half to

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equal to that of CAP (60.3%) (Table 1). The TRPV1 antagonist CPZ significantly decreased the Ca^{2+} response induced by these compounds (Figure 6B). In addition, these compounds hardly increased $[Ca^{2+}]_i$ in HEK293T cells not expressing TRPV1 (Figure 6B). These results indicate that RJ contains TRPV1 agonists and that dicarboxylic acids, 1,8-octanedicarboxylic acid, and 1,6-hexanedicarboxylic acid have relatively strong agonist activities. Their EC_{50} values were equal to that for TRPA1, and the maximal responses were equal to that of CAP.

DISCUSSION

We searched for novel agonists of TRPA1 and TRPV1 in RJ and found that hexane extract activates TRPA1. From GC analysis, the main functional compounds were identified to be HDEA and HDAA, characteristic fatty acids of RJ. Next, we measured the TRPA1 activity of the other carboxylic acids and phenol. We also measured the activity of all the lipid components for TRPV1. Most of the fatty acids had higher affinity for TRPA1 than TRPV1. Their EC_{50} values for TRPV1 were 2 to 3 times larger, and the maximal responses were less than half compared to that for TRPA1. Among them, 1,8-octanedicarboxylic acid and 1,6-hexanedicarboxylic acid showed relatively strong effects on both receptors. Their potencies for TRPA1 and TRPV1 were nearly equal, but they only constitute a small percentage of RJ. Their amount was estimated to be <1.5% of lyophilized RJ analyzed in previous reports.[26] On the contrary, the RJ content of HDEA and HDAA was estimated to be approximately 10%.[26] Therefore, we concluded that the main function of RJ is TRPA1 activation and that this is brought about by HDEA and HDAA, the main components of the RJ lipid fraction.

This is the first report on the effect of fatty acids on TRPA1 activity. The numbness of the tongue observed after RJ consumption is a phenomenon caused by the astringency of fatty acids.[33] There is a possibility that the numb sensation is triggered by the activation of TRPA1.

It was reported that not just TPRV1 but TRPA1 is also activated by protons. The EC_{50} values of acids are pH 5.4 for TRPV1 (in vitro) and pH 6.5 for TRPA1 (in vitro) at room temperature.[34] Therefore, we measured the pH of a solution of each RJ component in loading buffer, and we found that the pH was kept in the range of 7.3−7.7. Therefore, we concluded that the activation of TRPA1 and TRPV1 by fatty acids in RJ is not caused by protons.

In our SAR studies of fatty acids on human TRPA1, formic acid strongly acted on TRPA1, but methanol had no influence. These results indicated that carboxy groups could be important for an effect on TRPA1. Noteworthy, activation of TRPA1 by several reactive compounds has been demonstrated to be mediated through covalent modification of cytoplasmic cysteines located in the *N*-terminus of the channel.[35,36] In addition, carboxylic acids release protons in aqueous conditions and give rise to electrophilic carbonyl carbons.[37]Consequently, we thought that the carbonyl group acts as an electrophilic agent and forms covalent modifications with cysteine residues located in the *N*-terminus. Nonetheless, TRPA1 activity is also controlled by some nonreactive chemicals such as icilin,[3] trinitrophenol,[38] and 5-nitro-2-(3-phenylpropylamino) benzonic acid (NPPB).[39] They activate TRPA1 by acting on some regions except for the cysteine residues located in the *N*-terminus or affect it mechanically.[3,38,39] The activation mechanisms mediated by most nonreactive chemicals are yet unknown. In our study, we also found that phenol does not have an electrophilic region. We therefore hypothesized that phenol acts on regions other than cysteine residues located in the *N*-terminal ankyrin repeat.

Subsequently, we compared the activities of 4 medium chain carboxylic acids for their ability to induce TRPA1 activity. Carboxylic acid with a 10-carbon chain (decanoic acid) showed the strongest efficacy on TRPA1. Judging from this result, we considered that the carboxylic acid of the C10 carbon chain easily passes through the cell membrane owing to its moderate hydrophobicity and likely reacts with cytoplasmic cysteine residues. HDEA and HDAA are characteristic hydroxy carboxylic acids of RJ. Hydroxy carboxylic acids are not unusual compounds in nature, but those of the long carbon chain are found in lipids of vegetables.[40] We can therefore conclude that HDEA and HDAA can activate TRPA1 because they have a 10-carbon chain that renders them moderately hydrophobic.

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ABBREVIATIONS USED

AITC, allyl isothiocyanate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CAP, capsaicin; CPZ, capsazepine; HDEA, *trans*-10-hydroxy-2-decenoic acid; HDAA, 10-hydroxydecanoic acid; RJ, royal jelly; SAR, structure-activity relationship; TRP, transient receptor potential; TRPA1, transient receptor potential cation channel, subfamily A, member 1; TRPV1, transient receptor potential cation channel, subfamily V, member 1.

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