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Application of Vapor Phase Stimulation Method for Screening of Human Odorant Receptors Responding to Cinnamaldehyde

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Flavors consist of a combination of various odor molecules. Animals sense flavor through the responses of multiple odorant receptors (ORs). Previously, we developed a vapor phase stimulation method for the functional analysis of ORs. In this method, odor molecules are supplied through the vapor phase to cells expressing ORs. In the mammalian odorant sensing system, odor components are transmitted through air, dissolve in nasal mucus covering olfactory sensory neuron (OSN) cells, and then stimulate ORs. Therefore, the vapor phase stimulation method mimics the authentic olfactory response. We tested this method to identify human ORs that respond to cinnamaldehyde, the main component of cinnamon flavor, as a practical model fragrance. Although there is a lag time required for the dissolution of odor in the buffer, the vapor phase activation method gave almost the same results as the liquid phase activation method. Interestingly, a human OR responded to cinnamaldehyde only in vapor phase activation. The method also worked well with the flavor from cinnamon powder.

1. Introduction

Flavors and fragrances, which are mixtures of various odorant molecules, significantly contribute to the quality of foods, cosmetics, and daily necessities. Therefore, it is possible to create the desired fragrance by a suitable combination of odorants. Although analytical instruments such as gas chromatography–mass spectrometry and gas chromatography–olfactometry systems are used to evaluate individual components of fragrances,[1] sensory evaluation by humans is still essential for the evaluation of flavors.[2]

Mammals detect odorants via odorant receptors (ORs). ORs from the largest G-protein-coupled receptor family with ~400 functional members in humans and ~1200 members in mice.[3,4] Activated ORs couple with G-proteins, increasing the amount of cyclic AMP (cAMP) and ultimately leading to olfactory sensory neuron (OSN) depolarization and action potential firing.[5] Mammalian OR genes are mutually exclusive and monoallelic in OSNs (one receptor–one neuron rule).[6] One OR responds to various odor molecules, and one odor molecule activates various ORs.[7,8] Thus, the response in the presence of multiple odor molecules is not a simple addition of responses to individual odor molecules.[9–11] Animals perceive odors around them

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through pattern recognition using multiple OR activities.[12] Therefore, animals can sensitively perceive changes in aroma when there is a slight difference in mixture ratio or when foreign substances are included in the fragrance.

Most ORs are not exported to the plasma membrane but are retained in the endoplasmic reticulum when expressed alone in non-olfactory cells, including neuronal cells. The Co-expression of receptor-transporting proteins RTP1S and RTP2, which have been proposed to act as chaperones in OSNs, enhances the cell surface expression of many ORs in heterologous cells.[13–16] Therefore, OR ligand assays are conducted using cells co-expressing ORs with RTP1S.[17] Compared with that of mouse ORs, the functional expression of human ORs is generally difficult even in the presence of RTP1S.

Previously, we established an odor detection method that mimics the sense of smell of mammals using ORs.[18,19] In the system, odors are provided through the vapor phase. Cells expressing ORs respond to the odors dissolved from the vapor phase into the solution phase in real time. This technique may be applied to analyze and quantify the odor responses of mixtures and odor differences by identifying functional groups of fragrances. This method can be used to monitor the changes in olfactory response to odorants in the presence of the metabolic enzyme secreted by Bowman's gland.[20,21] In this study, we used this method for the screening of human ORs responding to cinnamaldehyde, which is the main component of cinnamon flavor.

2. Materials and Methods

2.1 DNA and vector preparation

Open reading frames of OR genes were cloned into pCI (Promega) with a Rhodopsin tag at the N terminal. OR expression plasmids were amplified using *Escherichia coli* XL10 Gold (Agilent Technologies) and purified with NucleoSpin plasmid TF Grade (Marcherey Nagel Inc). The plasmids for the expressions of human RTP1S, pRTP1S, and pGloSensor F-22 (Promega) were amplified and purified by the same method. All plasmid sequences were verified by Sanger sequencing.

2.2 Cell culture

Hana3A cells[13] were grown in Eagle's Minimal Essential Medium (FUJIFILM Wako Chemicals) containing 10% fetal bovine serum (vol/vol) (HyClone, Cytiva), penicillin-streptomycin, and amphotericin B (FUJIFILM Wako Chemicals). All cells were cultured at 37 °C at the saturated humidity and 5% CO_2. No mycoplasma infection was detected in all cell cultures.

2.3 Vapor odor detection

In the vapor odorant detection test, GloSensor cAMP Assay (Promega) was used to measure the real-time changes in cAMP level caused by receptor activation upon ligand binding

[Fig. 1(a)]. Hana3A cells were plated on a 96-well plate. After incubation for 18–24 h, the cells were transfected with 80 ng/well of OR expression plasmids, 5 ng/well of pRTP1S, and 10 ng/well of pGloSensor F-22. After 20–24 h, the medium was replaced with 25 μL of HBSS (FUJIFILM Wako Chemicals) containing 10 mM HEPES and 1 mM glucose, followed by 25 μL of HBSS containing GloSensor cAMP reagent (Promega). Plates were kept in a dark place at room temperature for 2 h to equilibrate the cells with the reagent. We filled the gaps between the wells of the plate with mineral oil (Sigma-Aldrich) containing 1% (vol/vol) cinnamaldehyde without direct contact with the cells, then covered the plate lid again. Immediately, the test plate was inserted into a GloMax Multi plate reader (Promega). The luminescence in each well was measured at 120 s intervals for 10 cycles. Multiple comparisons were performed using one-way ANOVA followed by Dunnett's test.

2.4 Liquid phase stimulation assay

The GloSensor cAMP reagent substrate in the buffer was incorporated into the cells in the same manner as described in Sect. 2.3. After an equilibration step with the GloSensor cAMP reagent, the cells were stimulated with cinnamaldehyde solution diluted in HBSS buffer. Immediately, the test plate was inserted into the multiplate reader. The luminescence in each well was measured at 120 s intervals for 10 cycles.

2.5 Solid fragrance vapor detection assay

The GloSensor cAMP reagent substrate in the buffer was incorporated into the cells in the same manner as described in Sect. 2.3. One gram of pure cinnamon powder (Tomizawa Shoten)

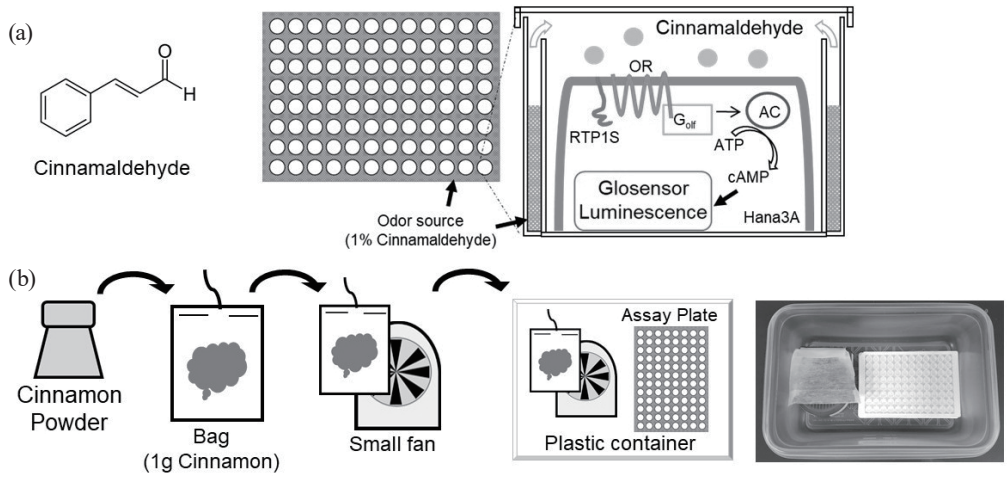

Fig. 1. (a) Screening of human ORs responding to cinnamaldehyde by vapor range stimulation assay: chemical structure of cinnamaldehyde and schematic representation of vapor phase stimulation assay with the OR signal transduction pathway. AC: adenylyl cyclase and ATP: adenosine triphosphate. (b) Schematic representing the solid fragrance assay. OR-expressing cells were stimulated in a plastic chamber including a nonwoven bag containing cinnamon powder.

was wrapped in a nonwoven fabric bag (empty teabag purchased from a grocery store) so that the powder would not leak, then the bag was attached to a small fan. The small fan and the cell culture 96-well plate were placed in a closed container [Fig. 1(b)]. The container was sealed with the fan, which was activated to fill it with the volatile components of the solid fragrance. After 10 min of stimulation, the cell culture 96-well plate was taken out and inserted into the GloMax multiplate reader (Promega). The luminescence value was standardized with that of vector control cells not expressing ORs and that before the odor stimulation of each well. The odor responses of ORs were quantified and compared.

3. Results and Discussion

3.1 Screening of human ORs responding to cinnamaldehyde

As a model odorant, we selected cinnamaldehyde, the main component of cinnamon fragrance [Fig. 1(a)]. First, Hana3A cells expressing human ORs were cultured on a 96-well plate. To stimulate them with cinnamaldehyde through the vapor phase, 1% (vol/vol) cinnamaldehyde solution was poured into the gaps between the cell-culturing wells in the 96-well plate. After incubation, the plate was inserted into the microplate reader to measure responses to cinnamaldehyde [Fig. 1(a)]. Among the 394 human ORs, four (OR2W1, OR10A5, OR2J2, and OR2J3) exhibited significant responses to cinnamaldehyde through the vapor phase (Fig. 2). Since OR2W1 has a broad substrate specificity range including that for aldehyde molecules,[7] we selected OR2W1 as the positive control for normalizing the data in this screening. A previous report has shown that OR2J2 and OR2J3 respond to cinnamaldehyde.[22] Other human ORs previously reported to be cinnamaldehyde receptors[23–25] did not show a significant response in this screening.

3.2 Comparison of cinnamaldehyde responses of human OR panel between vapor phase and liquid phase stimulations

Next, we compared the responses of human ORs to cinnamaldehyde between the vapor phase and liquid phase stimulation methods. We selected 30 human ORs from the screening results

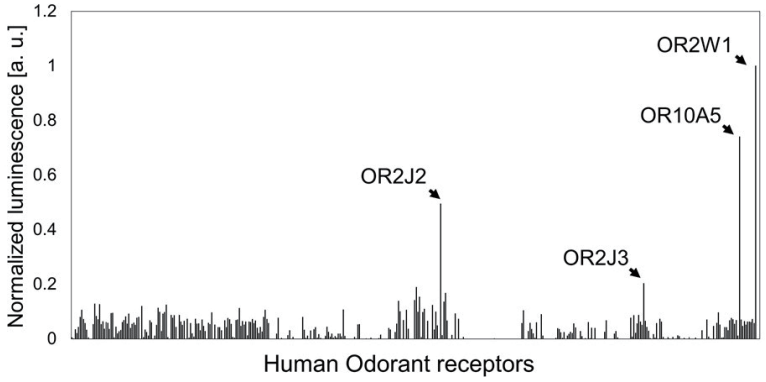

Fig. 2. Response of human ORs to vapor phase of 1% (vol/vol) cinnamaldehyde solution.

(Fig. 2). On the basis of comparison results of a liquid phase stimulation in a previous paper,[18] we decided to set the concentration of cinnamaldehyde to 100 μM for the liquid phase stimulation and 1% (vol/vol) for the vapor phase stimulation. Figure 3 shows the time courses of the responses of six selected ORs. There is a time lag for the response in the vapor phase stimulation for four ORs (OR2W1, OR10A5, OR2J2, and OR2J3), which should correspond to the time required for the component to dissolve in the buffer. On the other hand, in the liquid phase stimulation, ORs responded immediately. The responses were weaker in the vapor phase stimulation than in the liquid phase stimulation. Interestingly, some ORs (OR1L1 and OR10J5) showed different responses to the stimulation methods (Fig. 3). OR1L1 responded to the vapor

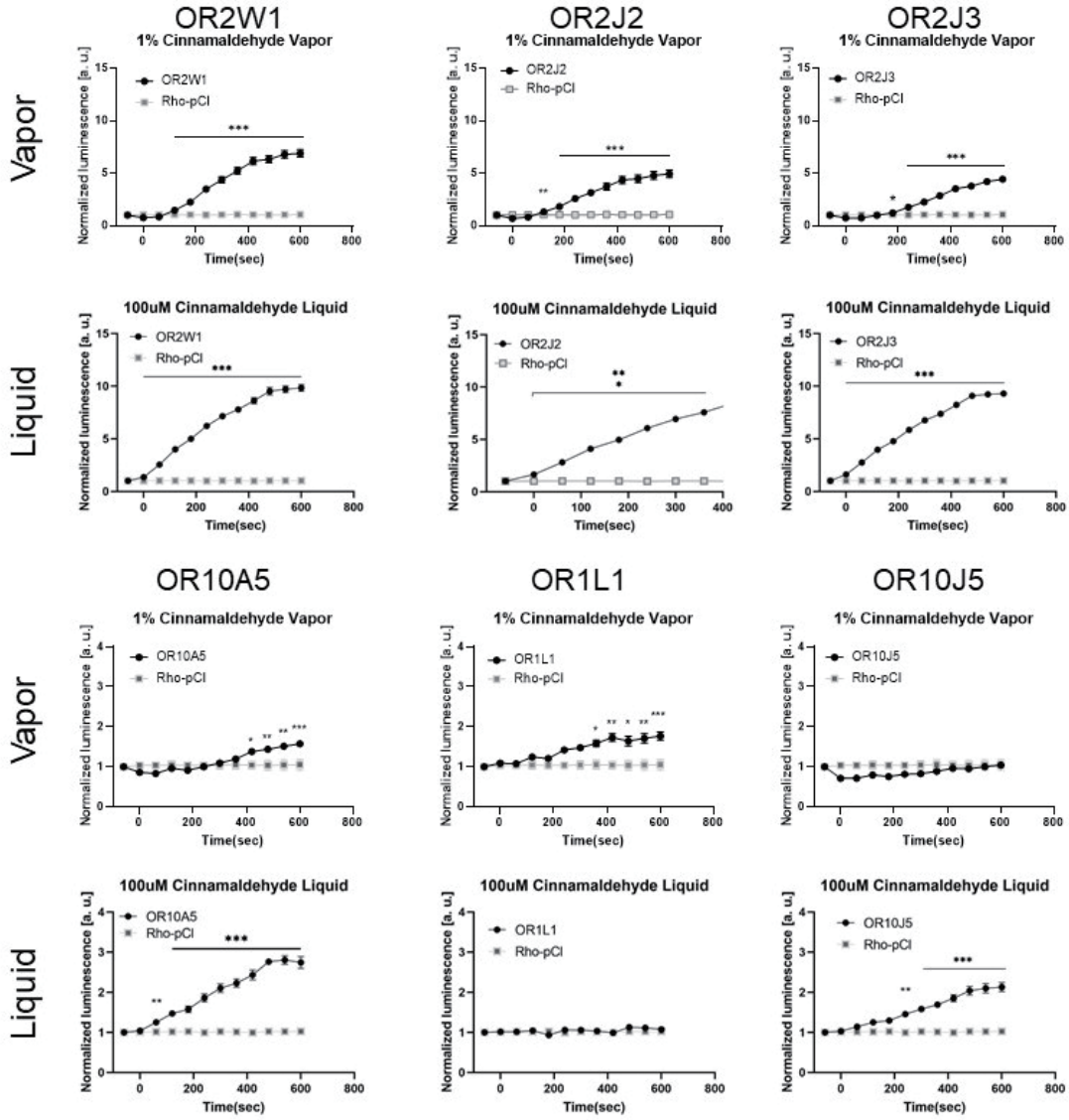

Fig. 3. Activation of ORs that responded differently to vapor phase stimulation of 1% cinnamaldehyde and liquid phase stimulation of 100 μM cinnamaldehyde. Error bars indicate s.e.m. (n = 3). Multiple comparisons were performed using one-way ANOVA followed by Dunnett's test (*p<0.05. **p<0.01, ***p<0.001).

phase stimulation, but no response to the liquid phase stimulation was observed. In contrast, OR10J5 was not stimulated by vapor phase stimulation. In the mammalian odorant sensing system, the odor components are transmitted through air, dissolve in the nasal mucus covering OSN cells, and stimulate ORs. Mammalian olfaction might be affected by odorant application.

3.3 Vapor phase stimulation assay for solid fragrance: cinnamon

Cinnamon is a natural product obtained from the inner bark of trees of the Cinnamomum genus. In addition to cinnamaldehyde, cinnamon contains various odors such as eugenol and safrole.[26–28] Generally, cinnamon is used as a powder or stick. Using the vapor phase stimulation method, we examined the responses of ORs to cinnamon. The cinnamon powder was wrapped in a nonwoven fabric and placed in a closed container with a 96-well plate containing Hana3A cells expressing human ORs. To make the concentrations of the odors uniform, air was circulated by a small air circulation fan [Fig. 1(b)]. After incubation, the plate was placed in the container and response analysis was performed.

Some human ORs, including those that responded to cinnamaldehyde, and mouse Olfr746, a positive control cinnamaldehyde receptor, showed significant responses with high reproducibility (Fig. 4). Figure 5 shows a comparison of the responses to cinnamaldehyde and cinnamon powder. Among the tested ORs, OR10A5 and OR10J5 exhibited relatively weak responses to the cinnamon powder vapor, correlating well with the weak responses to cinnamaldehyde in both the liquid phase and vapor phase stimulations. Similar to the response to cinnamaldehyde, OR1L1 responded to cinnamon in the vapor phase stimulation. The cause of the difference in responsiveness with the stimulation method cannot yet be elucidated owing to insufficient information about the agonist recognition mechanism of mammalian ORs. The properties of an odor molecule may depend on whether it dissolves naturally from the vapor phase or forcibly using dimethyl sulfoxide (DMSO) as a solvent. Altogether, our vapor phase stimulation assay using human-OR-expressing cells is applicable to solid fragrances.

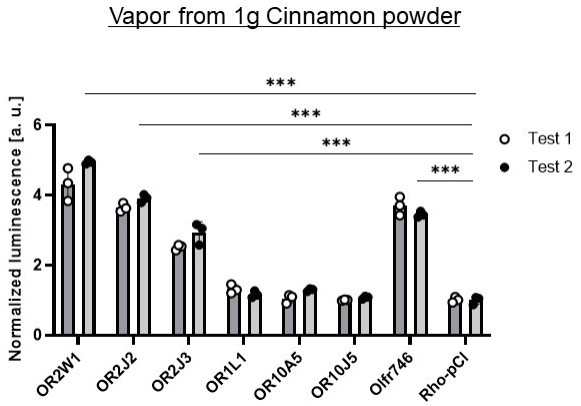

Fig. 4. Responses of ORs against the vapor phase from solid cinnamon powder. OR responses in two different assay tests were determined to show the reproducibility of results. Error bars indicate s.e.m (n = 3). Multiple comparisons were performed using one-way ANOVA followed by Dunnett's test (*p<0.05. **p<0.01, ***p<0.001).

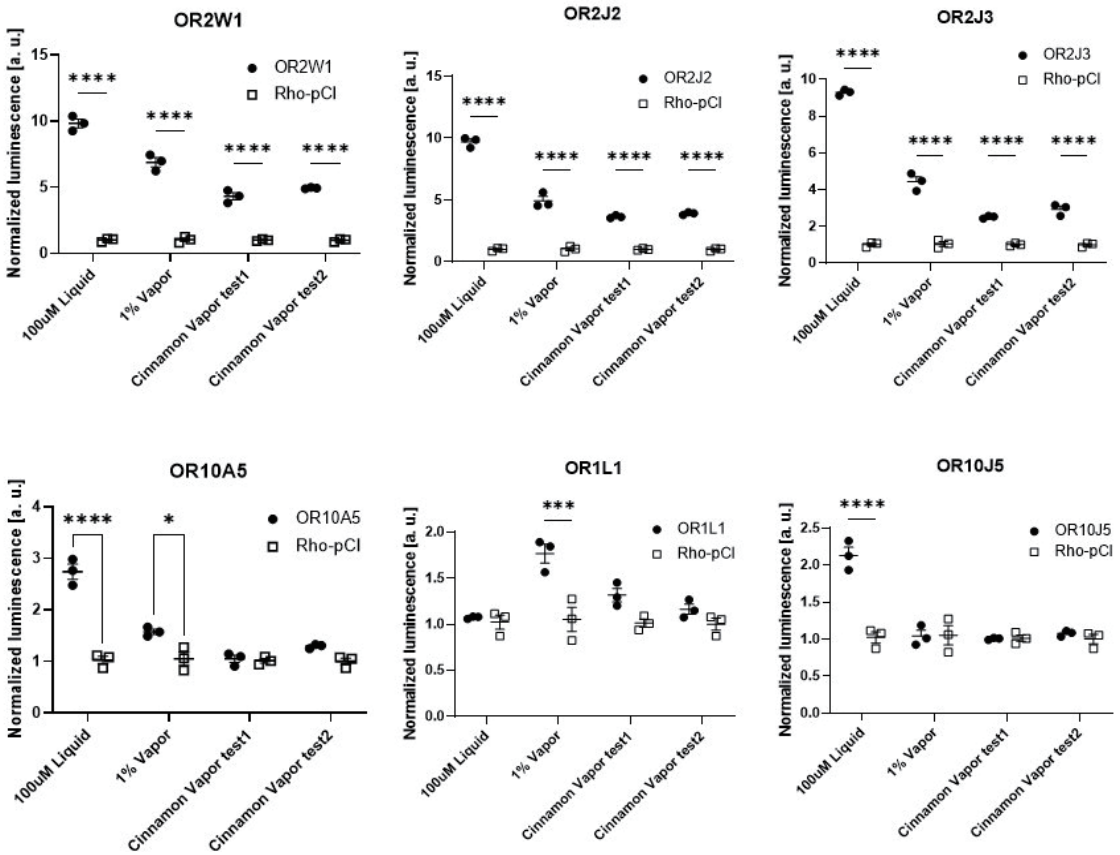

Fig. 5. Summary of the comparison of human ORs for three types of stimulation with the cinnamon-related odor. Multiple comparisons were performed using two-way ANOVA followed by Dunnett's multiple comparison test (*p<0.05. **p<0.01, ***p<0.001, ****p<0.0001).

4. Conclusions

In this study, we examined the responses of human ORs to cinnamon and its main component, cinnamaldehyde. We identified multiple human ORs responding to cinnamaldehyde by both vapor phase and liquid phase activation methods. Interestingly, some ORs responded differently for different activation methods. Mammalian olfaction might be affected by odorant application. The advantage of the vapor phase stimulation method is that it is applicable to evaluating solid flavors such as cinnamon. Although the heterologous expression of human ORs is difficult compared with that of mouse ORs, the present study shows that it is possible to screen human ORs by the vapor phase activation method. We hope that this method will be used as a practical evaluation method for flavors and fragrances.

Acknowledgments

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Competing Financial Interests

The authors declare no competing financial interests.

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