Divergent effects of olfactory receptors on transient receptor potential vanilloid 1 activation by capsaicin and eugenol

Running title: OR's modulation of TRPV1 activation

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ABSTRACT

We analyzed the effects of olfactory receptors (ORs) on transient receptor potential vanilloid 1 (TRPV1) activation using HEK293T cells co-expressing TRPV1 and OR51E1. We demonstrate here that the effect of OR51E1 on TRPV1 activation varies depending on the two TRPV1 ligands: capsaicin and eugenol. Notably, both of these ligands are vanilloid analogs. OR51E1 enhanced the response of TRPV1 to capsaicin but diminished that to eugenol. OR51E2 also showed similar effects. Based on the susceptibility to the OR's modulatory effects, various TRPV1 ligands could be classified into capsaicin and eugenol types. Activation of OR51E1 enhanced cAMP production. In addition, forskolin (FSK) exhibited almost identical effects as ORs on TRPV1 responses to both types of ligands. These results suggest that OR51E1-induced cAMP elevation leads to a modification of TRPV1, presumably phosphorylation of TRPV1, which amplifies the susceptibility of TRPV1 to the two types of ligands differently.

Keywords: olfactory receptors, transient receptor potential vanilloid 1, capsaicin, eugenol, cAMP.

Introduction

Human olfactory receptors (ORs) belong to the G protein-coupled receptor (GPCR) family, characterized by seven transmembrane structures. The human genome contains nearly 400 OR genes (Jimenez *et al.* 2021). These ORs are primarily expressed in olfactory sensory neurons (OSNs) and play a crucial role in odorant recognition (Lankford *et al.* 2020). In the process of olfaction, many odors and their combinations are believed to be distinguished through the pattern recognition of ORs (Malnic *et al*. 1999). When odors stimulate ORs, it triggers a signal transduction cascade (Sklar *et al*. 1986; Mombaerts *et al.* 1996; Nakamura and Geoffrey 1987). Odor molecules bind to ORs, which activate G protein heterotrimers composed of GNAL (G_{α}olf), G_{β}, and G_{γ}. Resultant GNAL-GTP then stimulates adenylate cyclase (AC), leading to cAMP synthesis. The increase in intracellular cAMP in OSNs opens cyclic nucleotide-gated (CNG) channels, mediating the influx of Ca^{2+} and Na^{+} ions. The cationic ion influx subsequently activates Cl^{-} channels, causing Cl^{-} efflux and amplifying depolarization from the cilia to the axon of an OSN. Finally, these signals are integrated and processed in the brain to generate the sensation of smell.

OR51E1 and OR51E2 exhibit about 60% amino acid identity, which surpasses that of other ORs, where the identity is around 30%. OR51E1 responds to short-chain to medium-chain carboxylic acids (C3-C9) but it does not react to acetic acid. While OR51E2 responds to acetic and propionic acids, but it does not react to longer-chain carboxylic acids. The stimulation of OR51E1 and OR51E2 by these ligands leads to an increase in intracellular cAMP levels (Mainland *et al*. 2014; Pronin *et al*. 2021). Both receptors are expressed not only in OSNs but also in ectopic locations, such as prostate cancer cells (Pronin *et al*. 2021).

On the other hand, the transient receptor potential (TRP) family comprises 28 members, all of which are nonselective cation channels. This family is further divided into subfamilies, one of which is the transient receptor potential vanilloid (TRPV) subfamily. The TRPV subfamily includes TRPV1 through TRPV6, which are characterized by their six transmembrane structures and can be activated by vanilloid compounds (Samanta *et al.* 2018). TRPV1 was discovered as a receptor for capsaicin, a pungent ingredient of hot chili peppers. TRPV1 is highly expressed in the nervous system, and it plays a vital role in pain perception (Koivisto *et al.* 2022). TRPV1 expression is also detected in non-neuronal tissues, suggesting that it plays regulatory roles in non-neuronal cells and neurons, including immune cells (Bujak *et al.* 2019). The TRPV1 channel, which allows Ca^{2+} to pass through its pore, is formed by a homologous tetramer of TRPV1 molecules. Various factors can affect the structure and functions of TRPV1, including phosphorylation. TRPV1 can be activated by binding ligands including capsaicin, eugenol, camphor, and resiniferatoxin (Xu *et al.* 2005; Yang *et al.* 2003; Yelshanskaya *et al.* 2022). Activation of the TRPV1 channel induces Ca^{2+} influx, which could trigger various cellular events (Peyravian *et al.* 2020).

Both ORs and TRPV1 are expressed in OSNs (Ahmed *et al.* 2009; Nakashimo *et al.* 2010; O'Hanlon *et al.* 2007; Olender *et al.* 2016; Seki *et al.* 2006). Furthermore, previous studies have shown that TRPV1 plays an important role in the regeneration of OSNs (Sakatani *et al.* 2023). Notably, ORs and TRPV1 share common properties: (1) ligand stimulation of both receptors results in a Ca^{2+} influx in neurons; (2) some ligands, such as eugenol and camphor, can stimulate both receptors. At least several ORs, i.e., OR5D18 (OR73), OR4Q3, and OR10G7, have been reported to respond to eugenol (Malnic B *et al.* 2003; Mainland *et al*. 2014). On the other hand, the modulation of TRPV1 activation by some GPCRs has been studied, particularly in nociceptive regulation. The following processes have been elucidated: prostaglandin E2-stimulated EP3C and EP4 receptors coupling to $G_{\alpha}s$ activate a cAMP production pathway and protein kinase A (PKA) phosphorylates TRPV1, which is implicated in the regulation of TRPV1 desensitization and the development of thermal hyperplasia (Moriyama *et al.* 2005). An AC activator, FSK, can replace the prostaglandin receptor activation to initiate the processes of TRPV1 phosphorylation. In contrast, stimulation of the μ opioid receptor (MOR) coupling to $G_{\alpha}i$ suppresses FSK-induced potentiation of TRPV1-mediated Ca^{2+} influx (Vetter I *et al.* 2008; Melkes *et al.* 2020). In addition, previous reports indicate that various other GPCRs, i.e., receptors coupling with $G_{\alpha}q/11$, $G_{\alpha}s$, and $G_{\alpha}i/o$, influence TRPV1 activation through regulation of its phosphorylation (Salzer I *et al.* 2019). However, the molecular mechanisms of the interactions between ORs and TRPV1 remain largely unknown. In this study, we examined using HEK293T cells co-expressing OR and TRPV1 whether ORs exhibit modulatory activities for TRPV1 activation similarly to the previously reported other GPCRs.

Materials and methods

Plasmids

A plasmid to express human TRPV1 tagged with a C-terminal flag (DYKDDDK) (catalog number: OHu22257D) was purchased from Genscript (Piscataway, NJ, USA). We obtained a human sodium taurocholate cotransporting peptide (NTCP) expression plasmid (catalog number: HG16027-UT) from Sino Biological Inc. (Kawasaki, Kanagawa, Japan). An empty vector plasmid (catalog number: 3240) was purchased from Takara Bio Inc. (Kusatsu, Shiga, Japan). We acquired OR51E1 and OR51E2 expression plasmids from Thermo Fisher Scientific (Waltham, MA, USA). Synthetic DNA fragments encoding OR51E1 (NCBI accession No. NM_152430.4) and OR51E2 (NCBI accession No. NM_030774.3), along with DNA encoding the N-terminal Lucy and Rho tag, were inserted downstream of the CMV promoter in the pcDNA5 vector. The green fluorescence protein (GFP) expression plasmid was prepared as previously described (Fujita *et al*. in 2021). We purchased pGloSensor 22F cAMP plasmid (catalog number: E2301) from Promega (Madison, WI, USA).

Cell Culture

HEK293T cells were obtained from RIKEN BRC (Tsukuba, Ibaraki, Japan). They were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and antibiotics and then used for experiments as described previously (Fujita *et al.* 2019).

Reagents

Eugenol (catalog number: A0232; TCI chemicals, Tokyo, Japan), N-arachidonoyl dopamine (NADA; catalog number: AB120099-5; Abcam, Cambridge, United Kingdom), capsaicin (catalog number: 034-11351; Fujifilm Wako Pure Chemical Corp., Osaka, Japan), piperine (catalog number: 162-17241; Fujifilm Wako Pure Chemical Corp.), and FSK (catalog number: 067-02191; Fujifilm Wako Pure Chemical Corp.) were initially dissolved in ethanol and subsequently diluted to the necessary concentrations with Hanks' Balanced Salt Solution (HBSS; catalog number: 084-08965; Fujifilm Wako Pure Chemical Co.). Isovaleric acid (catalog number: M0182; TCI chemicals, Tokyo, Japan) and propionic acid (catalog number: 194-03012; Fujifilm Wako Pure Chemical Corp.) were dissolved in HBSS while 3-isobutyl-1-methylxanthine (IBMX; catalog number: I5879; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (catalog number: 046-21981; Fujifilm Wako Pure Chemical Corp.). Then, they were diluted to appropriate concentrations with HBSS.

Flow cytometric analysis

Transfection of plasmids into HEK293T cells was performed as described previously (Hinuma *et al.* 2022). After being cultured for 24 h, cells were harvested with trypsinization. They were washed twice with phosphate-buffered saline (PBS) at 4 ℃ by centrifugation and then pelleted in a tube. Fixation

and permeabilization of cells were performed using Leucoperm (catalog number: BFU09; Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. To detect OR51E1, we used mouse monoclonal anti-Rho antibody (catalog number: 200-301-G36; Rockland, Limerick, PA, USA) as a 1st antibody in a dilution of 1:200 and Alexa Fluor 647-conjugated goat polyclonal anti-mouse IgG (H+L) $F(ab')_2$ fragment (catalog number: 4410; Cell Signaling Technology, Carlsbad, CA, USA) as a 2nd antibody in a dilution of 1:500. To detect TRPV1, we used rabbit polyclonal anti-Flag antibody (catalog number: 20543-1-AP; Proteintech, Tokyo, Japan) as a 1st antibody in a dilution of 1:200 and Alexa Fluor 488-conjugated goat polyclonal anti-rabbit IgG (H+L) $F(ab')_2$ fragment (catalog number: 20543-1-AP-150; Cell Signaling Technology) as a 2nd antibody in a dilution of 1:500. Cells were incubated with these antibodies at 4 ℃ for 1 h. After the incubation with antibodies, they were washed twice with PBS. Then, they were subjected to flow cytometry. The fluorescence intensities (FIs) of 1×10^4 cells were analyzed using the FITC and APC channels of a FACSCant™ II (BD Biosciences, Franklin Lakes, NJ, USA). Geometric mean values were analyzed using software (FlowJo™ 7.6.5, BD Biosciences). To obtain histograms, measurements were taken from 3×10^4 cells.

Detection of cAMP production

HEK293T cells were cultured in a 96-well white plate (catalog number: 236105; Thermo Fisher Scientific) coated with poly-L-lysine (catalog number: VBH- SPL01; Cosmo Bio, Tokyo, Japan). We then seeded cells at a density of 4×10^5 cells/mL in 50 μL/well of culture medium. These cells were cultured for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. We transfected the cells with expression plasmids, i.e., GloSensor 22F, GFP, empty vector, and OR51E1 to be 300 ng in a serum-free Opti-MEM (catalog number: 31985062; Thermo Fisher) per well in total using PolyMagNeo, and then fresh medium (100 μL) was added to each well. Following a 24-h culture period, we used a Synergy 2 plate reader (BioTek, Winooski, VT, USA) to determine the FI of GFP in each well. We then replaced the supernatant with 75 μL/well of HBSS containing a 2% GloSensor cAMP reagent (catalog number: E1290; Promega) and allowed the cells to incubate for 2 h at room temperature. Afterward, we added an aliquot (25 μL) of HBSS containing both ligands and IBMX (with a final concentration of 0.5 mM) to each well. Immediately following this, we measured the change in luminescence at 2-min intervals for 20 min. We obtained normalized relative luminescence unit (RLU) using the following calculations. (1) The FI of GFP was calculated in each well using the formula: (FI in a well with GFP expression plasmid transfection) - (that without GFP expression plasmid transfection). (2) A coefficient value (Co) to normalize RLU in each well based on GFP plasmid transfection efficacy using the formula: (FI of GFP in a well with receptor expression plasmid transfection) / (that with empty vector plasmid transfection). (3) RLU using the formula: (maximal luminescence in a well with receptor expression plasmid transfection after addition of HBSS with or without a ligand) / (that with empty vector plasmid transfection after addition of HBSS without a

ligand). (4) We obtained normalized RLU from the formula: RLU/Co. We performed assays in triplicate and expressed data as mean values and SD.

Detection of intracellular Ca^{2+} influx

We initiated our experiment by coating a 96-well black/clear bottom Plate (catalog number: 165305; Thermo Fisher Scientific) with poly-L-lysine. We then seeded cells at a density of 4×10^5 cells/mL in 50 μL/well of culture medium. These cells were cultured for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. We transfected the cells with plasmids (300 ng/well in total) using PolyMagNeo as described above. Then fresh medium (100 μL) was added to each well. The transfected cells were cultured for an additional 24 h. After removing the culture medium, we incubated the cells in HBSS (50 μL/well) containing a dye loading buffer supplied with a Calcium 6 Assay Explorer Kit (catalog number: OZB-PG60200-200-200; OZ Biosciences, Marseille, France). The incubation was carried out for 2 h at 37°C in a humidified atmosphere containing 5% CO_2. Subsequently, we added HBSS with or without a ligand (50 μL/well) and measured changes in FI using a FLIPR tetra (Molecular Devices; San Jose, CA, USA). When treatment with FSK (20 μM) was required, it was added to the cells 15 min before measuring changes in FI by ligand stimulations. Finally, we measured FI changes (%) induced by the addition of ligands every second for 3 min. Specific responses of cells to ligands, which were caused by the transfection of TRPV1 expression plasmid, were calculated by subtracting the FI changes in control cells, which were not transfected with the TRPV1 expression plasmid, from the FI changes in cells that were transfected with the TRPV1 expression plasmid.

Results

Functional expression of OR51E1 and TRPV1 in HEK293T cells. In this study, we utilized ORs tagged with a secretory peptide tag, Lucy-Rho, at their N-termini. This tag is reported to enhance the functional expression of ORs when they are introduced into heterologous cells, such as HEK293T cells (Shepard *et al.* 2013). To express TRPV1 in HEK293T cells, we used an expression plasmid for TRPV1 with a C-terminal Flag tag. The protein expressions of OR51E1 and TRPV1 were confirmed using flow cytometric analyses when plasmids to express the two receptors were transfected into HEK293T cells. For flow cytometry, transfected cells were fixed and permeabilized, and then they were stained using 1st and 2nd antibodies. To detect OR51E1, we used 1st antibody against Rho. While TRPV1 was detected using 1st antibody against the Flag. As shown in Figure 1a (histogram) and Figure 1b (geometric values of FI), OR51E1 protein expression was detected in HEK293T cells, both when transfected with OR51E1 alone and when co-transfected with TRPV1 expression plasmids. Although OR51E1 protein expression patterns in cells appeared to differ between the transfection of OR51E1 alone (as depicted – in Figure 1a) and the co-transfection of OR51E1 and TRPV1 (as depicted – in Figure 1a), OR51E1 expression in cells co-transfected with the two receptors increased 1.1 times more

than that in cells transfected OR51E1 alone (Figure 1b). On the other hand, evident TRPV1 protein expression was detected in cells transfected with TRPV1 alone and in those co-transfected with OR51E1 and TRPV1 expression plasmids (Figure 1c and Figure 1d). In histograms, TRPV1 protein expression patterns in cells were similar between the transfection of TRPV1 alone (as depicted – in Figure 1c) and the co-transfection of OR51E1 and TRPV1 (as depicted – in Figure 1c), TRPV1 expression in cells co-transfected with the two receptors increased 1.1 times more than that in cells transfected TRPV1 alone (Figure 1d). These results demonstrate that OR51E1 and TRPV1 proteins are produced when these expression plasmids are transfected into HEK293T cells. In addition, the protein expression levels of TRPV1 were comparable between the transfection of TRPV1 expression plasmid alone and the co-transfection of TRPV1 and OR51E1 expression plasmids. These results indicate that OR51E1 and TRPV1 do not drastically affect each other regarding their protein expression levels in HEK293T cells.

(Figure 1)

It is well-established that ORs activated by their ligands enhance intracellular cAMP production (Sklar *et al*. 1986; Mombaerts *et al.* 1996; Nakamura and Geoffrey 1987). We examined the responses of cells expressing OR51E1 to isovaleric and propionic acids in cAMP production assays. HEK293T cells expressing OR51E1 dose-dependently responded to isovaleric acid (Figure 2a). While OR51E1's response to propionic acid was considerably lower than that to isovaleric acid (Figure 2b). Under the conditions employed here, increasing amounts of OR expression plasmids in transfection strengthened the responses of OR51E1 to isovaleric acid. The ligand specificity of OR51E1 was well consistent with that reported elsewhere (Pronin *et al*. 2021). Notably, under the conditions without ligand stimulation, the intracellular cAMP level of cells transfected with OR51E1 expression plasmid was greater than that of cells transfected with empty vector plasmid (Figure 2c). In the absence of ligands, transfection of OR51E1 dose-dependently enhanced cAMP production. These findings indicate that the expression of OR51E1 in HEK293T cells may inherently trigger the cAMP production pathway to some degree, even without ligand stimulation. However, the activation level was significantly lower compared to when the ligands stimulated it. Furthermore, we investigated how OR51E1 responds to capsaicin and eugenol, which were utilized as ligands to activate TRPV1 in subsequent experiments. Figure 2d indicates that neither capsaicin nor eugenol induced cAMP in cells. This was observed in both the cells transfected with OR51E1 (as depicted in the right section of Figure 1d) and those without the transfection (shown in the left section of Figure 1d). In contrast, isovaleric acid induced cAMP production in cells transfected with OR51E1 as depicted in the right section of Figure 1d. These results demonstrate that capsaicin and eugenol do not activate OR51E1 as agonistic ligands.

(Figure 2)

To examine the responsiveness of TRPV1, we measured Ca^{2+} influx, which was detected as changes in FI, in HEK293T cells transfected with empty vector plasmid or TRPV1 expression plasmid after stimulation with TRPV1 ligands. As these ligands, we chose capsaicin and eugenol because capsaicin is a typical TRPV1 agonist while eugenol, an analog of capsaicin, is known not only as a TRPV1 ligand but also as a low molecular weight odorant molecule. As shown in Figure 3a and Figure 3c, capsaicin or eugenol caused greater dose-dependent responses in cells transfected with TRPV1 expression plasmid than those transfected with empty vector plasmid. However, control cells that were transfected with the empty vector plasmid also exhibited responses to capsaicin or eugenol, although these were significantly less pronounced compared to the responses of the TRPV1 expression plasmid-transfected cells. There is a possibility that the low levels of responses in control cells to these ligands are due to the expression of endogenous TRPV1. Publicly available RNA-seq data, ARCHS4, indicates that a certain level of TRPV1 is expressed in a variety of cells including HEK293T cells (https://maayanlab.cloud/archs4/gene/TRPV1). However, we could not definitively exclude the possibility that the presence of other receptors for these ligands or a combination of endogenous TRPV1 and other receptors contributes to the response of control cells to these ligands. Therefore, in this study, we defined specific TRPV1 responses to capsaicin or eugenol, which were attributable to the transfected TRPV1 as IF changes, i.e., response/baseline (%), in TRPV1-transfected cells subtracting those in control cells (Figure 3b and Figure 3d). The dose-response curve of eugenol differed from that of capsaicin, particularly in terms of effective doses, maximal responses, and curve patterns. Under the experimental conditions employed in this study, cell viability remained relatively stable even at high concentrations of capsaicin and eugenol during short-term measurements. These results suggest that the way, in which these two ligands interact with TRPV1, is not the same. However, both capsaicin and eugenol are vanilloid compounds and a previous report indicates that they bind to a similar pocket on TRPV1 (Harb *et al*. 2019). Considering them, it's likely that there are both common and unique aspects in the interactions between TRPV1 and these two ligands. Similar to the case with capsaicin, control cells exhibited a response to eugenol. However, these response levels were significantly lower compared to those observed in TRPV1-transfected cells. Therefore, the specific response of TRPV1 to eugenol was also determined by subtracting the responses of control cells from those of TRPV1-transfected cells (Figure 3d).

(Figure 3)

Modulatory effects of OR51E1 on TRPV1 responses to capsaicin and eugenol. We investigated whether ORs could alter TRPV1 responses to capsaicin or eugenol, using HEK293T cells co-

transfected with OR51E1 and TRPV1 expression plasmids. As shown in Figure 4a, the addition of HBSS without TRPV1 ligands did not induce Ca^{2+} influx in HEK293T cells transfected with any plasmids. On the other hand, the addition of capsaicin extensively induced Ca^{2+} influx in cells transfected with TRPV1 (Figure 4b). Co-expression of OR51E1 with TRPV1 enhanced capsaicin-induced Ca^{2+} influx. As explained in Figure 3, capsaicin could induce a low level of Ca^{2+} influx in cells transfected with empty vector plasmid (control). To obtain a transfected TRPV1-dependent response, we calculated a specific TRPV1 response to capsaicin (Figure 4c). When the dose of OR51E1 expression plasmid in transfection was doubled from 15 to 30 ng per well, the capsaicin-induced specific response of TRPV1 was enhanced by 1.3 and 1.8 times in maximal responses, respectively. In these experiments, we refrained from stimulating OR51E1 with its ligand. Despite this, the co-expression of OR51E1 modified the TRPV1 response to capsaicin. As shown in Figure 2c, the transfection of OR51E1 could trigger the cAMP production pathway, even in the absence of ligand stimulation. Taking all these results into account, our results suggest that the signaling dependent on OR51E1, i.e., eliciting cAMP production, amplified the TRPV1 response to capsaicin. In contrast to the effect of OR51E1 on TRPV1 response to capsaicin, TRPV1-mediated Ca^{2+} influx by stimulation with eugenol was decreased by the co-expression of OR51E1 (Figure 4d). When the OR51E1 expression plasmid was co-transfected at doses of 15 to 30 ng per well, the specific response of TRPV1 to eugenol was respectively reduced to 0.4 and 0.3 times its original level in maximal responses (Figure 4e). Our results indicate that OR51E1 can differentially modulate TRPV1 activation in response to stimulation by capsaicin and eugenol.

(Figure 4)

Amplification of modulatory activities of OR51E1 by stimulation with isovaleric acid. We verified if stimulation of OR51E1 with its ligand, i.e., isovaleric acid, could intensify its modulatory activities for the responsiveness of TRPV1 to capsaicin and eugenol. The addition of isovaleric acid to cells transfected with the TRPV1 expression plasmid, without the co-transfection of OR51E1 expression plasmid, did not affect the Ca^{2+} influx via TRPV1 in response to either capsaicin or eugenol as indicated by ■ and □ in Figure 5a and Figure 5b. Conversely, the same treatment with isovaleric acid intensified the enhancing effect of OR51E1 on the TRPV1 response to capsaicin, as well as the inhibitory effect of OR51E1 on the TRPV1 response to eugenol (■ and □ in Figure 5a and Figure 5b). These results suggest that the cAMP production pathway triggered by activated OR51E1 plays a vital role in the modulatory effects of ORs on TRPV1 in responses to both capsaicin and eugenol, despite the effects appearing to be divergent between TRPV1 responses to these two ligands.

(Figure 5)

Modulatory effects of OR51E2 but not NTCP on TRPV1 responses. As shown in Figure 4 and Figure 5, the co-expression of OR51E1 influenced TRPV1 responses to ligands, although its effects differed between eugenol and capsaicin. We further examined whether another OR, i.e., OR51E2, could exhibit similar modulatory effects. The co-expression of OR51E2 enhanced the specific response of TRPV1 to capsaicin while suppressing that to eugenol (Figure 6a). However, the co-expression of NTCP, which is not a GPCR, did not exhibit such modulatory effects on TRPV1 responses (Figure 6b). These results suggest that the modulatory effects of OR51E1 and OR51E2 on the ligand responses of TPPV1 are closely tied to OR functions.

(Figure 6)

Modulatory effects of FSK on TRPV1 responses to various TRPV1 ligands. Considering that stimulation of ORs promotes cAMP production, we hypothesized that the activation of AC, which catalyzes the conversion of ATP to cAMP, could be involved in this process. We examined whether FSK, an AC activator, could mimic the effects of OR on the ligand responses of TRPV1. As shown in Figure 7a, compared to untreated (■), FSK treatment (■) significantly enhanced the capsaicin-induced TRPV1 response. Conversely, the eugenol-induced TRPV1 response was reduced by FSK (Figure 7b). These results indicate that FSK can replicate the effects of ORs on the ligand responses of TRPV1 and that AC activation plays a crucial role in OR-dependent modulations. Furthermore, we evaluated the effects of FSK on the responsiveness of TRPV1 to other compounds, NADA and piperine, which are known as TRPV1 ligands (Ferreira *et al.*2009; McNamara *et al.* 2005). FSK increased the TRPV1 response to NADA (Figure 7a), whereas it diminished the response to piperine (Figure 7b). It has been reported that FSK treatment augments the TRPV1 response to capsaicin (Melkes *et al.* 2020). Regarding the effect of FSK on capsaicin-induced TRPV1 response, these results were consistent with the previous report. However, our findings indicate that FSK or ORs can modulate TRPV1 responses in different manners that are dependent on the TRPV1 ligands. Depending on whether FSK and OR enhance or inhibit TRPV1 activation, it seems that TRPV1 ligands can be classified into two categories: those similar to capsaicin (i.e., capsaicin-type ligands as shown in Figure 7a) and those similar to eugenol (i.e., eugenol-type ligands as shown in Figure 7b).

(Figure 7)

Discussion

Both ORs and TRPV1 are expressed in OSNs and these two types of receptors share identical ligands at least a portion of their ligands, such as eugenol and camphor. However, the mechanism of their

interaction remains largely unknown. In this study, we analyzed the effects of ORs on the ligand responsiveness of TRPV1 using HEK293T cells that co-express both receptors. In OSNs, activation of ORs increases intracellular cAMP, opening CNG channels and leading to Ca^{2+} influx (Sklar *et al*. 1986; Mombaerts *et al.* 1996; Nakamura and Geoffrey 1987). Conversely, TRPV1, being a cationic ion channel, can directly elicit Ca^{2+} influx when stimulated by TRPV1 ligands. Since HEK293T cells do not express CNG channels, they are useful for analyzing the influence of ORs on TRPV1-mediated Ca^{2+} influx.

Our results suggest that ORs can modulate the ligand responsiveness of TRPV1 by activating the cAMP production pathway. Regulatory roles of GPCRs other than ORs in TRPV1 activation have been reported, especially about nociception. Ligand-stimulated prostaglandin receptors activate the cAMP production pathway, and PKA promotes the phosphorylation of TRPV1 (Moriyama *et al.* 2005). This phosphorylation lowers the threshold of TRPV1 response to capsaicin. In addition, various other GPCRs except for ORs have been reported to regulate TRPV1 activation through its phosphorylation (Salzer *et al.* 2019). Therefore, it is suggested that either ORs induce the phosphorylation of TRPV1, which in turn, makes TRPV1 more susceptible to capsaicin, although this presumption must be confirmed by future studies.

However, the effects of ORs or FSK on Ca^{2+} influx were opposite in response to between capsaicin and eugenol. Phosphorylation would make TRPV1 insusceptible to eugenol, which is opposite to capsaicin. Generally, odors are composed of smaller molecules rather than capsaicin (molecular weight of 305.4), and eugenol (molecular weight of 164.2) is a representative odorant molecule. Our results suggest that the way TRPV1 interacts with small molecules such as odors is distinct from its interaction with capsaicin. We found that ligands of TRPV1 can be divided into two categories (i.e., capsaicin-type and eugenol-type ligands) based on the modulation of TRPV1 responses to these ligands by ORs or FSK. Future studies need to clarify which structural properties in compounds determine the two types of TRPV1 ligands.

TRPV1, a multimodal receptor, can bind various substances, leading to changes in its function and structure (Kwon *et al.* 2021; Sun *et al.* 2022; Zhang *et al.* 2021). The existence of a binding pocket in TRPV1 for capsaicin has been unveiled through the analysis of 3D structures using cryo-electron microscopy and computational modeling (Yang *et al.* 2015; Yang *et al.* 2018; Vu *et al.* 2020; Li *et al.* 2023). This pocket has been reported to be surrounded by S3-S6 in six transmembrane segments in TRPV1 (Yelshanskaya *et al.* 2022). The components of capsaicin, referred to as the head (vanillyl group), neck (amide bond), and tail (aliphatic chain) respectively, interact with different positions within the binding pocket (Yang *et al.* 2015). Capsaicin is presumed to bind the pocket in a 'tail-up and head-down' configuration (Kwon *et al.* 2021; Sun *et al.* 2022; Zhang *et al.* 2021; Yang *et al.* 2015; Yang *et al.* 2018; Vu *et al.* 2020; Li *et al.* 2023). Previous reports suggest that longer aliphatic tails enhance the affinities of capsaicin analogs through their interaction with TRPV1 via van der Waals

force (Yang *et al.* 2015). On the other hand, computational ligand docking studies suggest that eugenol attaches to a site adjacent to the TRPV1 pocket where the vanillyl group of capsaicin forms hydrogen bonds with TRPV1 amino acid residues (Harb *et al.* 2019; Yang *et al.* 2015). It is hypothesized that ORs and FSK modify TRPV1 configuration to interact with capsaicin or eugenol, possibly through phosphorylation, resulting in an increased affinity and/or channel opening capability of TRPV1 for capsaicin, while decreasing those for eugenol.

In this study, we demonstrated that the regulation of TRPV1 activation by ORs could vary depending on the specific TRPV1 ligands when both OR and TRPV1 were expressed in HEK293T cells. As future studies, it will be necessary to investigate whether such an interaction between ORs and TRPV1 exists in OSNs as well as HEK293T cells. It is also intriguing to investigate whether TRPV1 signaling influences the activation of ORs.

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Author contributions

S.M., conceptualization, methodology, validation, formal analysis, investigation, data curation, writing—original, draft preparation, writing—review & editing, funding; Y.T.; methodology, writing—review & editing; S. H., conceptualization, methodology, validation, formal analysis, investigation, data curation, writing—original, draft preparation, writing—review & editing, supervision; S.K., investigation, writing—review & editing, resources, funding, supervision.

Competing interests

The authors declare no conflict of interest.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Legends for Figures

Figure 1. Flow cytometric analyses of OR51E1 and TRPV1 protein expressions in HEK293T cells. Plasmids (a total of 800 ng plasmid DNA /well) used for transfection were as follows. Empty (■): 800 ng of empty vector plasmid; TRPV1 (■): 720 μg of TRPV1 expression plasmid and 80 ng of empty vector plasmid; OR51E1 (■): 720 μg of empty vector plasmid and 80 ng of OR51E1 expression plasmid; TRPV1 + OR51E1 (■): 720 ng of TRPV1 expression plasmid and 80 ng of OR51E1 expression plasmid. After plasmid-transfected cells were fixed and permeabilized, they were stained with the following antibody combinations. Panels (a) and (b): 1st mouse anti-Rho antibody and 2nd Alexa Flour 647-conjugated goat anti-mouse IgG antibody. Panels (c) and (d): 1st rabbit anti-Flag antibody and 2nd Alexa Flour 488-conjugated goat anti-rabbit IgG antibody. (a) and (c) display histograms, while (b) and (d) depict OR51E1 and TRPV1 protein expression levels (geometric means of FIs) determined from the histograms of (a) and (c), respectively. Geometric means were obtained from triplicate assays. OR51E1 and TRPV1 expressions were estimated from geometric mean values calculated by subtracting the geometric mean values of the 'Empty' from the geometric mean values of 'TRPV1' and 'OR51E1' transfection. Data were expressed as geometric means (n = 3) ± SD (vertical bars). Statistical analysis was performed using Tukey test. *: p <0.05.

Figure 2. cAMP production in HEK293T cells expressing OR51E1 with or without ligand stimulation. HEK293T cells were transfected with the indicated combinations of plasmids (total 300 ng/well) in a 96-well microplate. The amount of each plasmid used for transfection is shown in the table under (a) and the color code used here corresponds to lines and bars in (a) - (c). FI of GFP was utilized to normalize the RLU in each well, taking into account the transfection efficacy. Cells were treated with indicated concentrations of isovaleric acid (a) or propionic acid (b) in the presence of IBMX. After ligands were added to the wells, luminescence in each well was measured every 2 min for a total of 20 min, and the maximum value was used to calculate the RLU. Assays were performed in triplicate, and data are presented as mean values (n = 3) of normalized RLU ± SD (vertical bars). (c), a part of the panel (a), i.e., in the absence of the ligand, is enlarged and shown as a bar graph with a differential vertical scale. Statistical analysis was performed using Tukey test. *: p <0.05; **: p < 0.01.

Figure 3. Ca^{2+} influx induced in HEK293T cells transfected with TRPV1 expression plasmid or empty vector plasmid after stimulation with capsaicin or eugenol. HEK293T cells were transfected with the following plasmids (total 300 ng/well) in a 96-well microplate. In (a) and (c), filled circles (● and ●): 270 ng of TRPV1 expression plasmid and 30 ng of empty vector plasmid; blank circles (○ and ○): 300 ng of empty vector plasmid. After the addition of the indicated concentration of

capsaicin (a) or eugenol (c) dissolved in HBSS, Ca^{2+} influx in cells was measured through changes in FI every second for 3 min using a FLIPR. The assays were performed in triplicate. The data, represented by the maximal FI values in each measurement, were shown as mean values (n = 3) with standard SD (vertical bars). The specific TRPV1 responses (■ and ■) to capsaicin (b) and eugenol (d) were determined respectively by subtracting the responses of control cells to the indicated ligands from those of the TRPV1-transfected cells.

Figure 4. Modulatory effects of OR51E1 on TRPV1-mediated Ca^{2+} influx in response to capsaicin or eugenol. HEK293T cells were transfected with combinations of plasmids (total 300 ng/well) in a 96-well microplate. The amount of each plasmid used for transfection is shown in the table under (a) and the color code used here corresponds to lines in (a) - (e). Using a FLIPR, the influx of Ca^{2+} in cells was measured through changes in FI every second for 3 min. This was done after the addition of HBSS without ligands for TRPV1 (a), HBSS containing 10 μM of capsaicin (b), or HBSS containing 400 μM of eugenol (d). The assays were performed in triplicate. The data were shown as mean values (n = 3) with standard SD (vertical bars). The specific responses of TRPV1 to capsaicin (c) and eugenol (e) were determined by subtracting the responses to capsaicin or eugenol in cells without TRPV1 expression plasmid from those in cells with TRPV1 expression plasmid transfection.

Figure 5. Intensified modulations of TRPV1 responses to capsaicin and eugenol in HEK293T cells co-expressing TRPV1 and OR51E1 by ligand stimulation of OR51E1. Indicated expression plasmids were transfected into HEK293T cells cultured in a 96-well plate, totaling 300 ng/well. After 24-h culture, the cells were stimulated with either capsaicin (a) or eugenol (b), in the presence or absence of isovaleric acid. FI changes in the cells following the addition of capsaicin or eugenol were measured. Assays were performed in triplicate. Data were presented as mean values of maximal TRPV1-specific responses (n = 3) with SD (horizontal bars). Statistical analysis was performed using Tukey test. *: p<0.05; **: p<0.01 ; n.s.: not significant.

Figure 6. Effects of OR51E2 and NTCP on TRPV1-mediated Ca^{2+} influx in response to capsaicin and eugenol in HEK293T cells. Indicated expression plasmids were transfected into HEK293T cells cultured in a 96-well plate, totaling 300 ng/well. After 24-h culture, the cells were stimulated with either capsaicin (a) or eugenol (b). FI changes in the cells following the addition of capsaicin or eugenol were measured. Assays were performed in triplicate. Data were presented as mean values of maximal TRPV1-specific responses (n = 3) with SD (horizontal bars). Statistical analysis was performed using Student's t-test. *: p<0.05; **: p<0.01 ; n.s.: not significant.

Figure 7. Effects of FSK on TRPV1-mediated Ca^{2+} influx in response to various TRPV1 ligands.

568 TRPV1 expression plasmid (270 ng/well) and empty vector plasmid (30 ng/well) were transfected into
569 HEK293T cells. FSK treatment was performed 15 min before indicated TRPV1 ligands were added.
570 FI changes in the cells following the addition of ligands were measured. Assays were performed in
571 triplicate. Data were presented as mean values of maximal TRPV1-specific responses ($n = 3$) with SD
572 (horizontal bars). Statistical analysis was performed using Student's *t*-test. *: $p<0.05$; **: $p<0.01$.
573

Graphical abstract caption

The effects of OR on TRPV1 activation vary depending on whether TRPV1 is stimulated with capsaicin (a) or eugenol (b).