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Myr-Ric-8A Enhances $G_{\alpha 15}$-Mediated Ca^{2+} Response of Vertebrate Olfactory Receptors

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Abstract

The determination of ligand specificities of odorant receptors will contribute to the understanding of how odorants are discriminated by the olfactory system. To date, the ways in which some olfactory receptors (ORs) pair with their cognate ligands has been studied using a Ca^{2+} imaging technique. This approach has been used to investigate orphan G protein–coupled receptors expressed in heterologous cells; however, most attempts to functionally express ORs on the cell surface of heterologous cells have failed. Recently, receptor-transporting protein 1 and Ric-8B have been identified as proteins involved in targeting receptors to the cell membrane and amplifying receptor signals, and thus, they are able to facilitate cellular responses via ORs in a heterologous cell system. Here, we describe a technique in which we employed a myristoylation sequence–conjugated mutant of Ric-8A (Myr-Ric-8A) as a signal amplifier and show Myr-Ric-8A greatly enhances $G_{\alpha 15}$-mediated Ca^{2+} responses of ORs in HEK293 cells. Coexpression of Myr-Ric-8A enabled us to deorphanize a mouse OR and to determine its molecular receptive range. Our results suggest that Myr-Ric-8A should be helpful in functional characterization of ORs in heterologous cells using Ca^{2+} imaging.

Key words: HEK293, mouse, odorant

Introduction

Mammals employ an olfactory system to perceive a vast number of volatile chemical compounds as odors. The first step in olfactory perception is odorant binding by olfactory receptors (ORs) expressed on the cilia of olfactory sensory neurons (OSNs) in the nose. Olfactory receptors belong to the G protein–coupled receptor (GPCR) family and make up a large multigene family that in mice includes approximately 1000 members (Buck and Axel 1991; Zhang and Firestein 2002). Each OR recognizes multiple odorants and has a broad but selective ligand spectrum (Malnic et al. 1999; Kajiya et al. 2001; Katada et al. 2005). This suggests that discrimination of odor depends on different molecular receptive ranges of ORs. Therefore, identification of the cognate ligands and determination of molecular receptive ranges of individual ORs should enhance our understanding of how odorants are discriminated by the olfactory system.

To study OR function in heterologous cells such as human embryonic kidney 293 (HEK293) cells, 2 signaling pathways have been used as assay models (Katada et al. 2003; Touhara 2007). One approach relies on stimulation of the cyclic adenosine monophosphate (cAMP) cascade, which is initiated by activation of G proteins such as endogenous $G_{\alpha s}$ or exogenously transfected $G_{\alpha olf}$ that couple to ORs. An odorant-induced increase in intracellular cAMP concentration can then be monitored using an ELISA-based cAMP assay or the luciferase reporter gene assay. Another approach relies on stimulation of the Ca^{2+} cascade that can be initiated by exogenously transfected $G_{\alpha 15}$ that couples with various GPCRs, including ORs, and directs their signal to the phospholipase C (PLC) pathway. The $G_{\alpha 15}$-mediated increase in intracellular Ca^{2+} concentration can be monitored using the dye Fura-2. This Ca^{2+} imaging method is one of the most common and reliable high-throughput systems. It allows for detection of receptor activation at the single-cell level and for determination of dose dependence of OR–odorant interactions.

Multiple attempts have been made to achieve functional expression of ORs in heterologous cells by transfection of expression vectors, but most nascent cloned polypeptides are retained in the endoplasmic reticulum (McClintock et al. 1997; Lu et al. 2003). Fusing the first 20 amino acids of rhodopsin (rho-tag) to the N-terminus of OR proteins has successfully promoted their cell surface expression (Krautwurst

et al. 1998; Kajiya et al. 2001). One transmembrane protein, receptor-transporting protein 1 (RTP1), has been found to be expressed specifically in OSNs and to promote cell surface expression of ORs when expressed in HEK293T cells (Saito et al. 2004; Zhuang and Matsunami 2007). Further, Ric-8B (for resistance to inhibitors of cholinesterase), a putative guanine nucleotide exchange factor (GEF) that mediates signal transduction through G proteins, has been shown to amplify OR-mediated $G_{\alpha olf}$ signaling in HEK293T cells (Von Dannecker et al. 2006). Thus, the introduction of factors that improve proper membrane trafficking of ORs or that amplify OR-mediated signal transduction may be an effective approach for establishing functional expression of ORs on the surface of heterologous cells.

A previous study has shown that Ric-8A, another mammalian homolog of Ric-8B, functions as a GEF for $G_{\alpha i1}$, $G_{\alpha o}$, and $G_{\alpha q}$ in vitro (Tall et al. 2003). It was also demonstrated that membrane localization of Ric-8A was important for its function and thus the addition of the myristoylation signal sequence to N-terminus of Ric-8A (Myr-Ric-8A) enhances $G_{\alpha q}$-coupled receptor-mediated signal transduction in HEK293T cells due to promoting its membrane localization (Nishimura et al. 2006). In this study, we cotransfected Myr-Ric-8A into cells subjected to $G_{\alpha 15}$-mediated Ca^{2+} imaging and showed that Myr-Ric-8A enhanced signaling in heterologous HEK293 cells in response to odorants. Coexpression of Myr-Ric-8A has enabled us to determine a molecular receptive range of a previously uncharacterized mouse OR. The results suggest that use of Myr-Ric-8A is helpful in functionally expressing and deorphanizing ORs when using the Ca^{2+} imaging method.

Materials and methods

Odorants

Odorants utilized in this study were purchased from Tokyo Kasei Co. Ltd. (Tokyo, Japan), Sigma-Aldrich (Steinheim, Germany), Nacalai Tesque Inc. (Kyoto, Japan), and Wako Co. Ltd. (Tokyo, Japan). Lyral was kindly provided by T. Hasegawa Co. Ltd. (Tokyo, Japan). Odorant stock solutions were prepared immediately before each experiment at a concentration of 300 μM or 1 mM in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque Inc.) containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich) or Ringer's solutions (140 mM NaCl, 5.6 mM KCl, 2.0 mM $CaCl_2$, 2.0 mM $MgCl_2$, 2.0 mM sodium pyruvate, 9.4 mM glucose, 1.25 mM KH_2PO_4, and 5.0 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES], pH 7.4). Serial dilutions of stock solutions in DMEM containing IBMX or Ringer's solution were prepared before use.

Ca^{2+} imaging of olfactory neurons

Isolation and Ca^{2+} imaging of odorant response of olfactory neuron were performed as described previously (Touhara et al. 1999). Briefly, olfactory neurons were isolated from olfactory epithelium of 3- to 4-week-old BALB/c CrSlc mice (Japan SLC, Hamamatsu, Japan) and tissue printed on Cell-TAK (Collaborative Biomedical Products, Bedford, MA)–coated cover glass. Cells were loaded with fluorescent Ca^{2+} indicator Fura-2/AM (Molecular Probe, Eugene, OR; 5 μM final concentration) for 30 min, and Ca^{2+} responses were recorded. Odorant solutions were sequentially applied to cells using a peristaltic pump at the flow rate of 1.5 ml/min.

Cell culture

HEK293 cells were grown in DMEM supplemented with 10% fetal bovine serum (JRH Bioscience, Lenexa, KS). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were split every 2 days prior to becoming confluent; this was done because the number of responsive cells decreases after confluence. Cells were discarded after 1.5 months of passages, at which time new cultures were prepared using cells from frozen stock.

cAMP assay following transfection of expression vectors and exposure to odorants

The wells of a 24-well plate were coated with 50 μg/ml of poly-D-lysine (Sigma-Aldrich) in phosphate-buffered saline for 20 min and washed 3 times with H_2O. In all, 60–70% confluent cells in this 24-well plate were transfected with rho-tagged OR cDNAs by use of Lipofectamine 2000 (Invitrogen, Carlsbad, CA; 2.5 μl/μg DNA). The expression plasmid pME18S-Rho-OR contains genes for mouse ORs MOR23, MOR31-2, MOR204-34, and MOR139-3 (0.5 μg DNA each). Plasmids pME18S-Ric-8B, pME18S-RTP1, and pME18S-$G_{\alpha olf}$ (0.25 μg DNA each) were transfected to express the accessory proteins Ric-8B, RTP1, and $G_{\alpha olf}$, respectively. pME18S alone was cotransfected to make total amount of transfected DNA 1.0 μg.

Transfected cells were cultured for 24 h and then incubated with 1 mM IBMX for 30 min to inhibit endogenous phosphodiesterase activity. The cells were exposed to the indicated concentrations of odorants in DMEM containing 1 mM IBMX for 15 min. Intracellular cAMP levels induced by odorant stimuli were determined by use of an enzyme immunoassay kit (Applied Biosystems, Foster City, CA).

Ca^{2+} imaging following transfection and exposure to odorants

Cells were seeded onto 35-mm glass-bottomed dishes (Iwaki Inc., Chiba, Japan) coated with 100 μg/ml of poly-D-lysine. In all, 60–70% confluent cells were transfected with 2.5 μg of pME18S-Rho-OR, 1.5 μg of pME18S-$G_{\alpha 15}$, 1.0 μg of pME18S-RTP1, and/or 0.5 μg of pEGFP-N3-Myr-Ric-8A and using Lipofectamine 2000 (2.5 μl/μg DNA). pME18S was cotransfected to adjust the total amount of transfected DNA to 5.5 μg. When Ca^{2+} response of the β_2 adrenergic receptor was measured, cells were transfected with 1.5 μg

of pME18S-$G_{\alpha 15}$ and/or 1.0 μg of pEGFP-N3-Myr-Ric-8A, and the total amount of transfected DNA was adjusted with pME18S to 2.5 μg. After 24-h incubation, transfected cells were loaded with Fura-2/AM (2.5 μM final concentration) for 30 min at 37 °C. The dye-loaded cells were washed with Ringer's solution and subjected to Ca^{2+} imaging. A series of odorants in Ringer's solution were applied sequentially to the cells for 15 s at a time by use of a peristaltic pump at a flow rate of 1.5 ml/min. To exclude the effect of previous stimuli on intracellular Ca^{2+} levels, 2.5- to 3.0-min intervals in Ringer's solution were allowed between each odorant application to the subjected cells. Intracellular Ca^{2+} levels were detected as Fura-2/AM fluorescence at 510 nm by excitation at 340 or 380 nm using AQUA COSMOS (Hamamatsu Photonics, Shizuoka, Japan).

Results

Ric-8B enhances $G_{\alpha s}$-mediated cAMP response of mouse ORs

We have previously shown that ORs can couple to $G_{\alpha s}$ in HEK293 cells or to exogenously transfected $G_{\alpha olf}$, resulting in an odorant-induced intracellular cAMP increase (Kajiya et al. 2001). Ric-8B, a putative GEF expressed in OSNs, has been demonstrated to amplify OR signaling via $G_{\alpha olf}$ (Von Dannecker et al. 2006). We first tested the effect of Ric-8B coexpression on $G_{\alpha olf}$-mediated odorant responses via 3 mouse ORs, MOR23 (MOR267-13), MOR31-2, and MOR204-34 (Touhara et al. 1999; Kajiya et al. 2001; Oka et al. 2006). HEK293 cells expressing each OR alone or together with Ric-8B and/or $G_{\alpha olf}$ were analyzed to determine whether they respond to each cognate ligand (Figure 1). In all 3 ORs tested, a significant increase in intracellular cAMP was observed when an OR was coexpressed with Ric-8B when compared with that resulting from cells transfected with OR alone (Figure 1). In contrast, coexpression of $G_{\alpha olf}$ with Ric-8B did not further enhance the OR-mediated intracellular cAMP increase due to the high cAMP background observed with Ric-8B expression (Figure 1, inset). These results suggest that Ric-8B also interacts with $G_{\alpha s}$ and that the level of endogenous $G_{\alpha s}$ is sufficient for Ric-8B to function in enhancing odorant-induced cAMP response in HEK293 cells.

It has been shown that coexpression of RTP1 with an OR in HEK293T cells enhances odorant-induced increases in cAMP levels when expressed in combination with Ric-8B (Von Dannecker et al. 2006; Zhuang and Matsunami 2007). We analyzed whether coexpression of both Ric-8B and RTP1 along with ORs in HEK293 cells would improve the $G_{\alpha s}$-mediated cAMP increase. In all 3 ORs tested, coexpression of Ric-8B and RTP1 resulted in robust cAMP increases upon stimulation by odorants (Figure 2). These

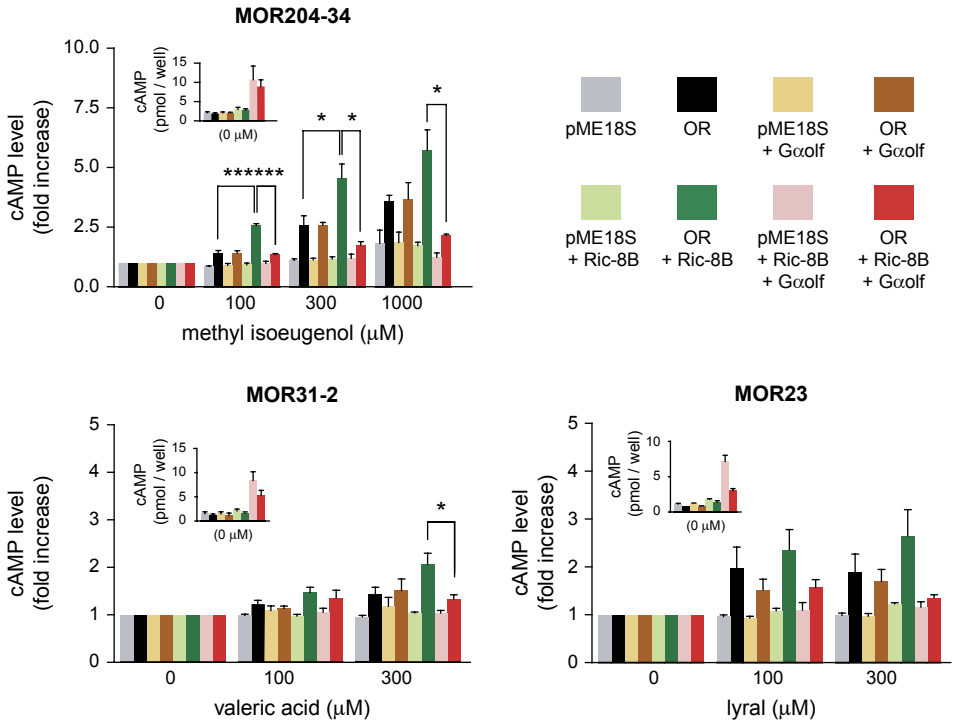

Figure 1 Ric-8B enhances $G_{\alpha s}$-mediated cAMP responses of ORs in HEK293 cells. Intracellular cAMP increases for rho-tagged ORs (MOR204-34, MOR31-2, and MOR23) cotransfected with Ric-8B and/or $G_{\alpha olf}$ in cells were measured. ORs were stimulated with their corresponding ligands (methyl isoeugenol for MOR204-34, valeric acid for MOR31-2, and lyral for MOR23) at various concentrations (100–1000 μM). *y* axis denotes cAMP level normalized by cAMP concentration without ligand stimulation in each condition (the inset figure) ±standard error (n = 3–4). For clarity, statistically significant amplification by Ric-8B and apparent inhibition by $G_{\alpha olf}$ were indicated (Student's *t*-test, $*P < 0.05$; $***P < 0.001$).

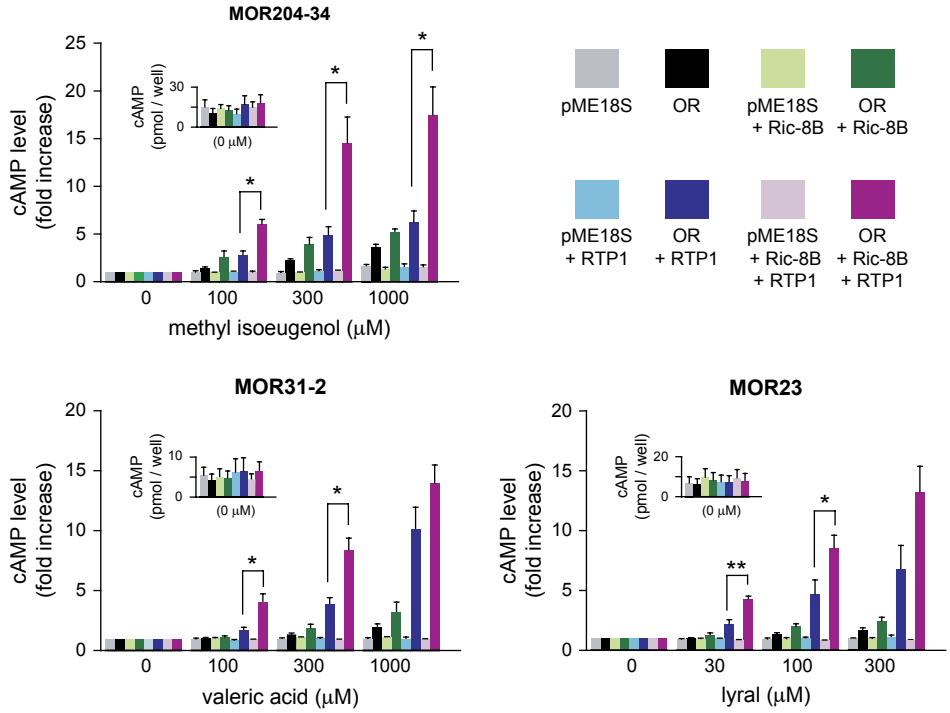

Figure 2 Coexpression of Ric-8B and RTP1 resulted in the most robust ligand-induced cAMP response of ORs. Intracellular cAMP increases for rho-tagged ORs (MOR204-34, MOR31-2, and MOR23) cotransfected with Ric-8B and/or RTP1 in HEK293 cells were measured ±standard error (n = 3–5). ORs were stimulated with their corresponding ligands (methyl isoeugenol for MOR204-34, lyral for MOR23, and valeric acid for MOR31-2) at various concentrations (100–1000 μM). Only statistically significant amplification by coexpression of Ric-8B and RTP1 compared with coexpression of RTP1 alone was indicated (Student's t-test, $*P < 0.05$; $**P < 0.01$).

results indicate that coexpression of Ric-8B and RTP1 provides the most effective conditions for functionally expressing an OR and detecting odorant-induced cAMP increases via endogenous $G_{\alpha s}$ in HEK293 cells.

Myr-Ric-8A enhances $G_{\alpha 15}$-mediated Ca^{2+} response

We next tested whether Ric-8B could enhance $G_{\alpha 15}$-mediated Ca^{2+} signaling via an OR, but introduction of Ric-8B resulted in a decrease in the Ca^{2+} response (data not shown). This is likely due to acceleration of the $G_{\alpha s}$-cAMP cascade that competes with OR coupling to $G_{\alpha 15}$, indicating that Ric-8B does not function as a GEF for $G_{\alpha 15}$. A previous study had shown that Myr-Ric-8A enhanced $G_{\alpha q}$-coupled receptor-mediated ras/extracellular signal-regulated kinase activation through the PLC pathway in HEK293T cells (Nishimura et al. 2006). This observation prompted us to test an effect of Myr-Ric-8A on $G_{\alpha 15}$-mediated signaling.

We first investigated the effects of Myr-Ric-8A on the $G_{\alpha 15}$-mediated Ca^{2+} response of the β_2-adrenergic receptor that is endogenously expressed in HEK293 cells (Krautwurst et al. 1998). HEK293 cells were transfected with $G_{\alpha 15}$ and/or Myr-Ric-8A and examined for responsiveness to isoproterenol, a ligand for β_2-adrenergic receptor, using Ca^{2+} imaging. We compared the proportion of responding cells and measured EC_{50} values for isoproterenol in the presence or absence of Myr-Ric-8A. Coexpression of Myr-Ric-8A with $G_{\alpha 15}$ greatly enhanced the isoproterenol-induced Ca^{2+} response of the β_2 adrenergic receptor (Figure 3). The proportion of cells responding to 30 nM isoproterenol was 41.0 ± 3.5% in HEK293 cells expressing $G_{\alpha 15}$ and Myr-Ric-8A, whereas the proportion of responding cells expressing $G_{\alpha 15}$ without Myr-Ric-8A was 4.0 ± 1.4% (Figure 3B). The EC_{50} value in cells coexpressing $G_{\alpha 15}$ and Myr-Ric-8A was significantly smaller than that of cells without Myr-Ric-8A (14 vs. 78 nM) (Figure 3C). These results suggest that Myr-Ric-8A enhanced $G_{\alpha 15}$-mediated signaling by acting as a GEF for $G_{\alpha 15}$ in HEK293 cells.

To examine whether Myr-Ric-8A also enhances OR-mediated Ca^{2+} response via $G_{\alpha 15}$, we expressed each OR (MOR31-2, MOR23, and MOR204-34) $G_{\alpha 15}$ along with RTP1 and/or Myr-Ric-8A in HEK293 cells and analyzed ligand responsiveness by Ca^{2+} imaging (Figure 4). We observed Ca^{2+} responses to 300 μM valeric acid in less than 2% of the cells expressing MOR31-2 and $G_{\alpha 15}$, while the proportion of responding cells increased to 5.8 ± 3.3% or 6.4 ± 2.4% when MOR31-2 and $G_{\alpha 15}$ were coexpressed with Myr-Ric-8A or RTP1, respectively. Coexpression of Myr-Ric-8A or RTP1 along with OR and $G_{\alpha 15}$ resulted in a significant decrease in the EC_{50} value (from 5790 μM to 464 or 274 μM, respectively). The improvement was most dramatic when cells coexpressed both Myr-Ric-8A and RTP1 along

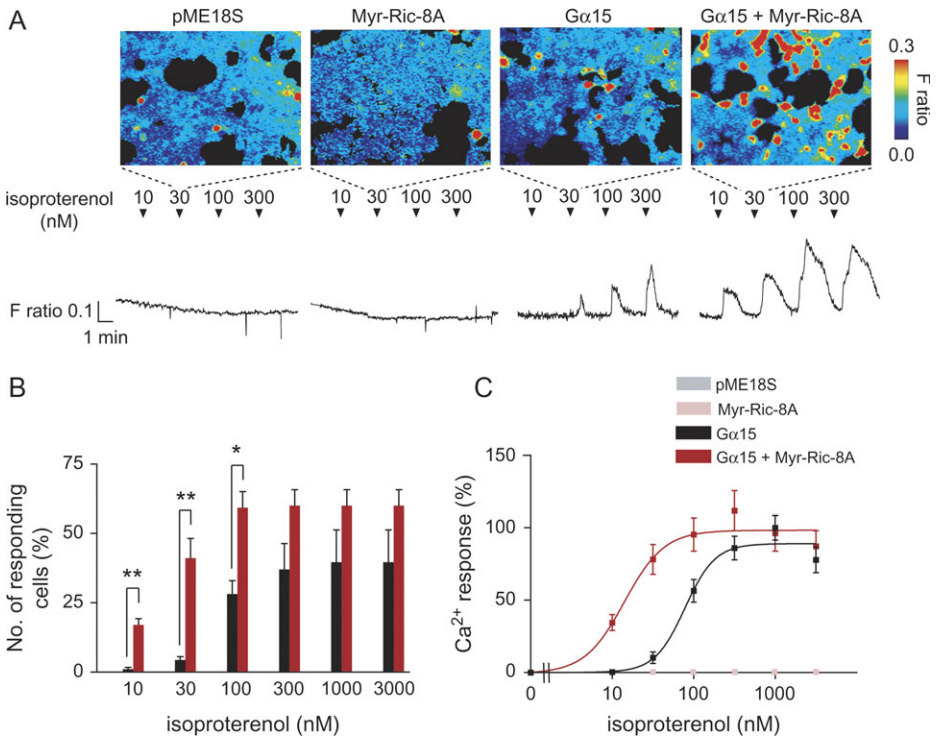

Figure 3 Myr-Ric-8A enhances $G_{\alpha 15}$-mediated Ca^{2+} responsiveness of β_2 adrenergic receptor in HEK293 cells. (**A**) Effect of Myr-Ric-8A on isoproterenol-mediated Ca^{2+} response. Top: Pseudocolored images of fura-2-loaded HEK293 cells stimulated with 30 nM isoproterenol. The pseudocolor represents the relative value of the fluorescence ratio (F-ratio). Bottom: A representative response profile of single odorant-responsive cells. Indicated concentrations of isoproterenol were applied at the times indicated by arrowheads. (**B**) Proportions of cells that responded to isoproterenol at various concentrations ±standard error ($n = 3$). Statistically significant increases by coexpression of Myr-Ric-8A compared with expression of $G_{\alpha 15}$ alone were indicated (Student's *t*-test, $*P < 0.05$; $**P < 0.01$). (**C**) Dose–response curves of isoproterenol responses of the β_2 adrenergic receptor. *y* axis denotes a percentage of the maximum response of the cells expressing $G_{\alpha 15}$ alone. Each datum point was calculated from representative ~52 cells in 3 independent experiments. The error bars represent ±standard error.

with OR and $G_{\alpha 15}$; the proportion of responding cells increased to 29.6 ± 1.0% and the EC_{50} value further decreased to 93 μM. Similar results were obtained for 2 other ORs, MOR204-34 and MOR23 (Figure 4). These results demonstrate that Myr-Ric-8A enhances odorant-induced Ca^{2+} responses of ORs via $G_{\alpha 15}$, and the condition in which Myr-Ric-8A and RTP1 were coexpressed appears to be the most efficient assay for detecting OR-mediated Ca^{2+} responses in HEK293 cells.

Deorphanizing a mouse OR

We functionally cloned MOR139-3 from an olfactory neuron that responded to *m*-cresol (1 mM) by using a single cell reverse transcriptase–polymerase chain reaction technique (Figure 5A). However, functional expression and characterization of MOR139-3 in heterologous cells were not achieved, and therefore, MOR139-3 could only be identified as a putative receptor for *m*-cresol. Here we expressed MOR139-3 together with $G_{\alpha 15}$, RTP1, and Myr-Ric-8A in HEK293 cells and tested whether MOR139-3 showed a response to the putative ligand, *m*-cresol. We observed Ca^{2+} responses upon stimulation with *m*-cresol in 3.7 ± 0.7% cells (Figure 5B). No Ca^{2+} response was observed in cells without MOR139-3, indicating that the response was dependent on MOR139-3 expression. In addition, no response was observed without Myr-Ric-8A, suggesting that coexpression of Myr-Ric-8A greatly aided the detection of OR-mediated signaling.

The *o*-cresol and the *p*-cresol, both of which are structural isomers of *m*-cresol, were inactive for MOR139-3, whereas eugenol elicited a response (5.2 ± 0.4%). Interestingly, 2-heptanone, an odorant dissimilar to cresol and eugenol, induced clear responses (2.6 ± 0.4%). In the cAMP assay, *m*-cresol, eugenol, and 2-heptanone, but not *o*-cresol or *p*-cresol, induced a cAMP increase in a dose-dependent manner when HEK293 cells were cotransfected with Ric-8B and RTP1 (Figure 5C). The ligand specificity of MOR139-3 observed in the Ca^{2+} imaging and cAMP assays was identical.

Molecular receptive range of MOR139-3

To further investigate the molecular receptive range of MOR139-3, we tested a series of odorants including various aliphatic and aromatic compounds based on the structures of 2-heptanone and eugenol using a Ca^{2+} imaging approach (Figure 6A). MOR139-3 responded to saturated aliphatic

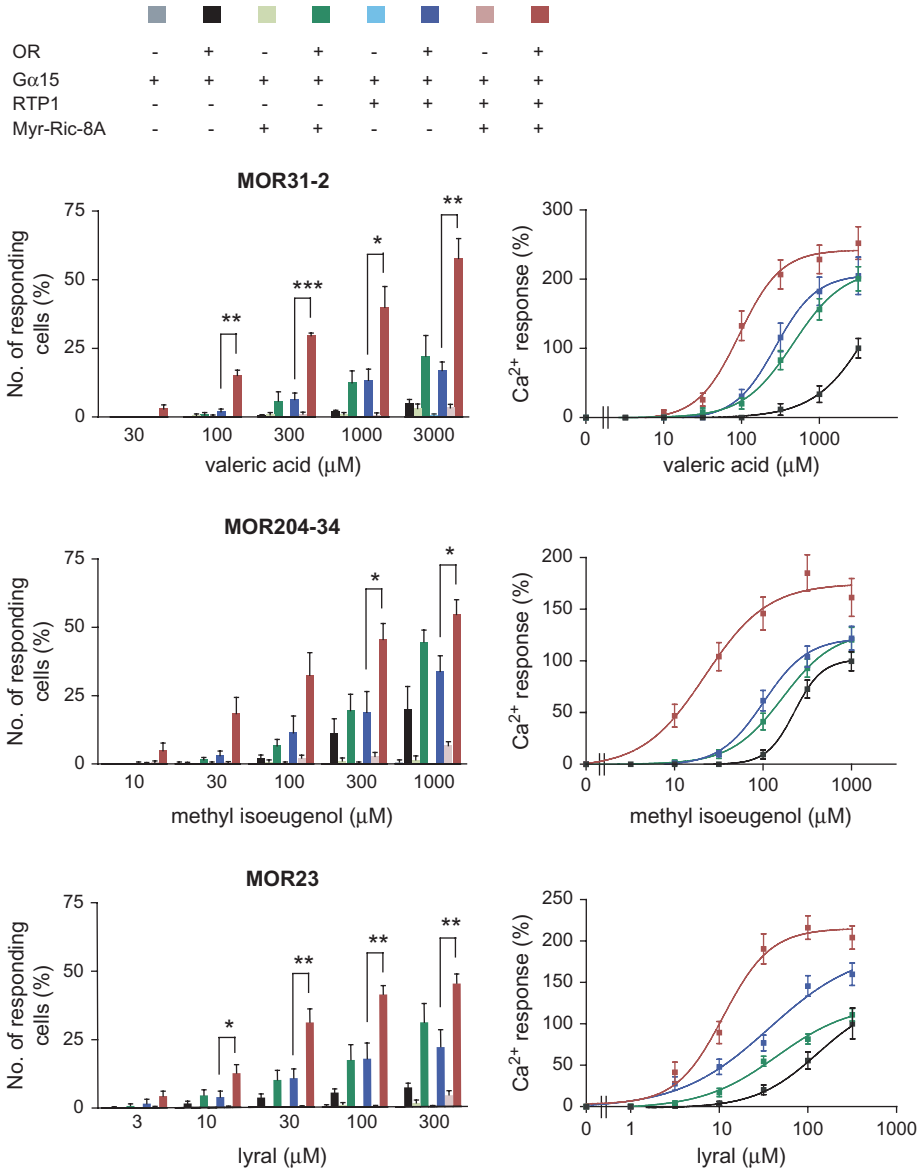

Figure 4 Coexpression of Myr-Ric-8A enhances Ca^{2+} responsiveness of ORs heterologously expressed in HEK293 cells. ORs and $G_{\alpha 15}$ were coexpressed with RTP1 and/or Myr-Ric-8A in HEK293 cells, and the Ca^{2+} responsiveness was analyzed. Left: Proportions of cells that responded to ligands at various concentrations ±standard error (n = 3–8). Only statistically significant amplification by coexpression of RTP1 and Myr-Ric-8A compared with coexpression of RTP1 alone was indicated (Student's t-test, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Right: Dose–response curves for ligands that activate MOR31-2, MOR204-34, and MOR23, respectively, in each condition. y axis denotes a percentage of the maximum response of the cells expressing ORs and $G_{\alpha 15}$. Each datum point was calculated from representative ~52 cells in 3–8 independent experiments. Error bars represent ±standard error.

aldehydes (i.e., 1-heptanal [2], 1-octanal [3], and 1-nonanal [4]) but not to 1-hexanal [1] (Figure 6B,C). MOR139-3 was responsive to the C_7–C_8 alcohols (i.e., 1-heptanol [8] and 1-octanol [9]), whereas no response was observed for C_6 or C_9 alcohols (i.e., 1-hexanol [7] and 1-nonanol [10]). The C_7 ketone, 2-heptanone [6], was a ligand, but the C_6 ketone, 2-hexanone [5], was not. These data suggest that MOR139-3 preferably recognizes the structure of C_7–C_8 carbon lengths. Consistently, stereoisomers of C_8 alcohols (i.e., 2-octanol [11] and 3-octanol [12]) also elicited responses. The C_7 acid, 1-heptanoic acid [13], however, did not elicit a response, indicating that a polar functional group was not favorable. MOR139-3 also responded to *trans*, *trans*-2, 4-nonandienal [14] that possesses double unsaturated bonds. In addition to eugenol [15], 2-methoxy-4-ethylphenol [16] also elicited a response. Consistent results were obtained using the cAMP assay (Figure 6D). These results suggest that MOR139-3 has an odorant-binding site that favors C_7–C_9 carbon lengths and accommodates both saturated and unsaturated structures.

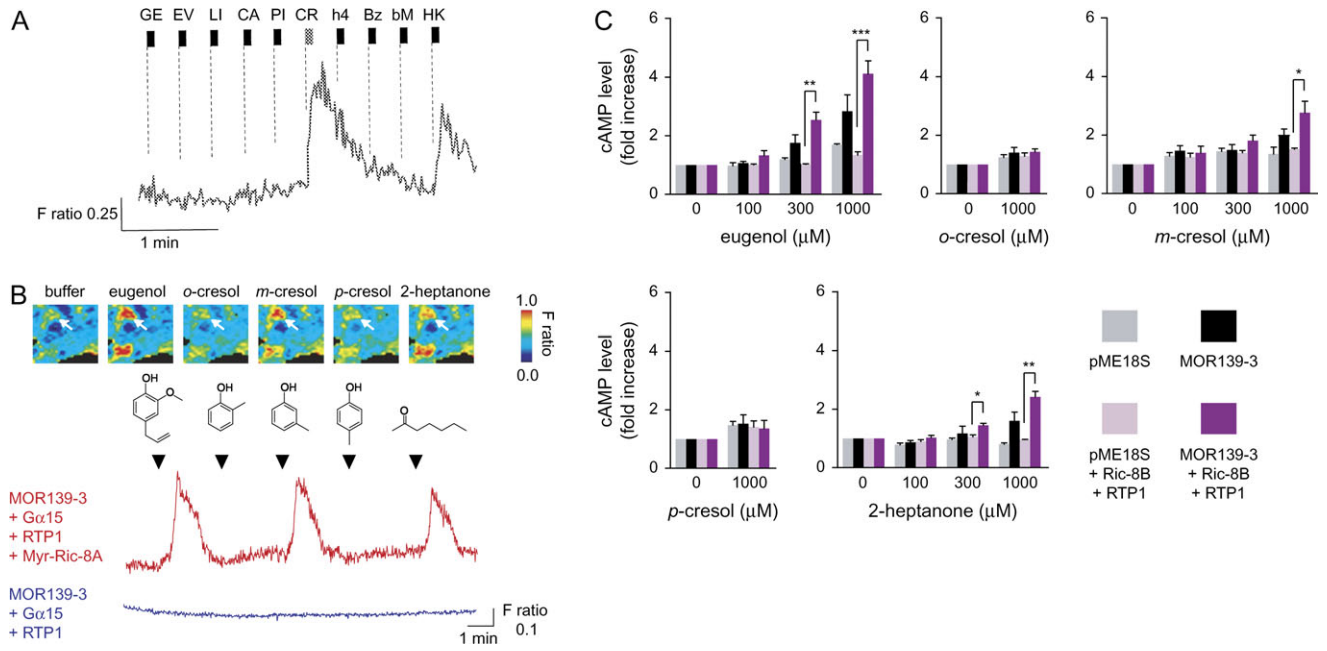

Figure 5 Deorphanization of MOR139-3 in Myr-Ric-8A–expressing HEK293 cells. (**A**) Ca^{2+} response profile of a single olfactory neuron responding to *m*-cresol. The odorants were applied to tissue-printed olfactory neurons for 4 s during times indicated by the bars. Names of odorants are abbreviated as follows: GE, geraniol; EV, ethyl vanillin; LI, lilial; CA, l-carvone; PI, pyridine; CR, *m*-cresol; h4, 1-butanol; Bz, benzene; and bM, β-mercaptoethanol (each 1 mM). HK, high KCl buffer (145.6 mM KCl, 2.0 mM $CaCl_2$, 2.0 mM $MgCl_2$, 9.4 mM glucose, and 5.0 mM HEPES, pH 7.4), was used as a positive control for cell viability. (**B**) Ca^{2+} response of HEK293 cells expressing MOR139-3, $G_{\alpha 15}$, and RTP1 in the presence or absence of Myr-Ric-8A. Top: Pseudocolored images of Fura-2-loaded HEK293 cells stimulated with each indicated odorant at a concentration of 300 μM. Pseudocolor represents relative value of fluorescence ratio (F-ratio). Bottom: Ca^{2+} response profile of the cell indicated by a white arrow in the pseudocolored images (top) and that of a cell in the absence of Myr-Ric-8A (bottom). (**C**) cAMP response of cells expressing MOR139-3 in the presence or absence of Ric-8B and RTP1. *y* axis denotes cAMP level normalized by cAMP concentration without ligand stimulation in each condition ±standard error (n = 3–4). Statistically significant increases of cAMP level in MOR139-3-expressing cells compared with control (in the absence of MOR139-3) were indicated (Student's *t*-test, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

Discussion

The major reason for the failure to characterize ORs in heterologous cells to date has been the poor translocation of ORs to the cell surface. RTP1, a molecular chaperone for ORs, turned out to be effective in enhancing cell surface expression of ORs in heterologous cells (Saito et al. 2004; Zhuang and Matsunami 2007). However, it has been shown that the time needed for the binding of an odorant to an OR is extremely short, suggesting that amplification does not occur in G protein–OR coupling (Bhandawat et al. 2005). Therefore, even in the presence of RTP1 alone, the number of ORs on the cell surface is usually insufficient to initiate Ca^{2+} signaling. Recently, Ric-8B has been shown to amplify signaling via $G_{\alpha olf}$ in heterologous cells (Von Dannecker et al. 2005, 2006). In this study, we demonstrated that Ric-8B also enhances $G_{\alpha s}$-mediated OR responses in HEK293 cells, and this effect was confirmed with 3 different ORs (Figure 7). Further, we showed that Myr-Ric-8A enhances Ca^{2+} responses of ORs in HEK293 cells by facilitating $G_{\alpha 15}$ activity (Figure 7). Coexpression of Myr-Ric-8A enabled us to deorphanize MOR139-3 using a Ca^{2+} imaging technique and to determine its molecular receptive range, of which otherwise the response was not observed.

Ric-8A has been shown to function as a GEF for a subset of G proteins of the G_{α} group, and it interacts preferentially with GDP-bound or nucleotide-free form of G_{α} but not the GTP-bound form (Tall et al. 2003). In addition, Ric-8A cannot activate a trimeric form of G proteins, which is a characteristic distinct from other GEFs such as GPCR (Tall et al. 2003). Based on these evidences, Ric-8A has been proposed as not a simple exchange factor but a signal amplifier: when an active GTP-bound form of G_{α} returns to an inactive GDP-bound form, Ric-8A binds to a GDP-bound G_{α} before $G_{\beta\gamma}$ rebinds to it, and it induces rapid release of GDP and stabilizes its nucleotide-free form, after which GTP binds to it, causing reactivation of G_{α} and amplifying signal transduction. This is a likely mechanism by which Ric-8A acts as a signal amplifier for $G_{\alpha 15}$.

Some ORs show a narrowly tuned ligand spectrum, whereas other ORs possess a broadly tuned molecular receptive range. MOR139-3, which was deorphanized using Myr-Ric-8A in this study, turned out to belong to the latter category, and thus, the binding site of MOR139-3 seems to be tolerant of various structures. Indeed, the ligands include both saturated and unsaturated aliphatic compounds and further include aromatic molecules that possess a benzene

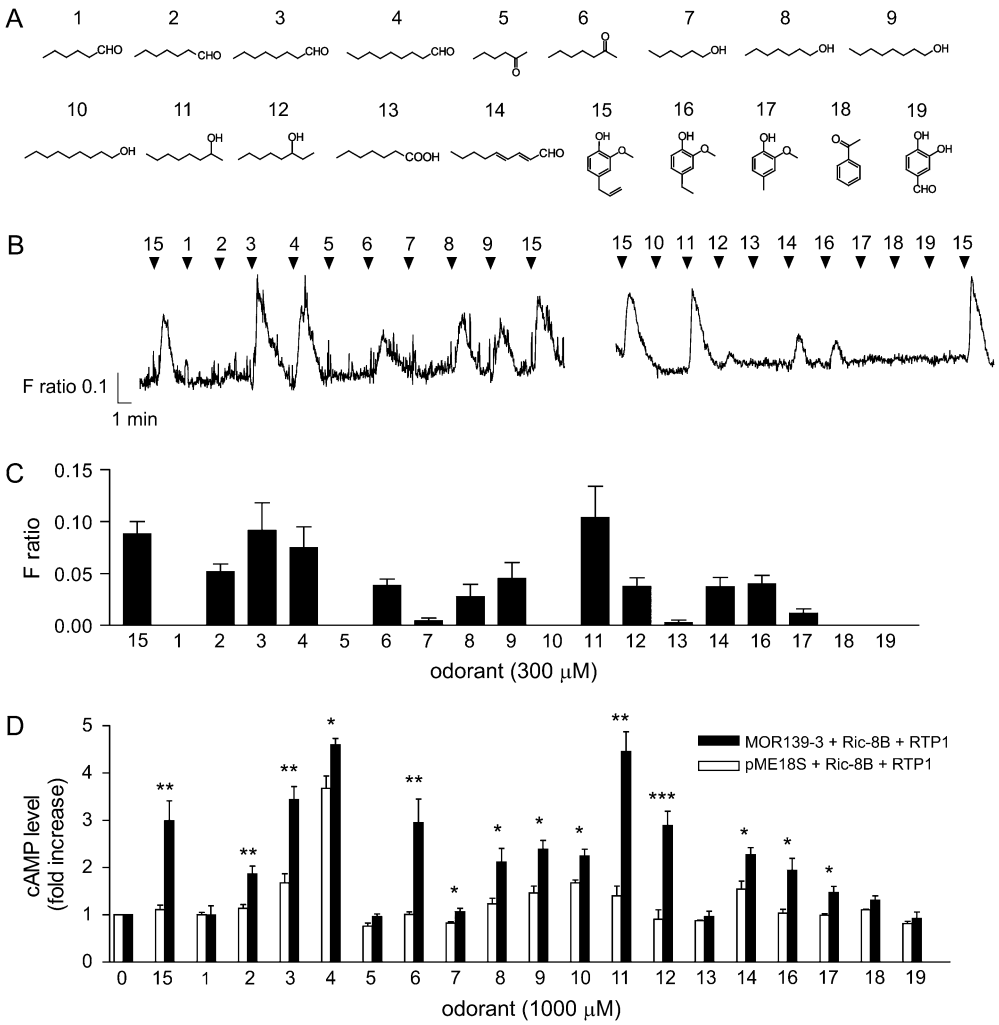

Figure 6 Molecular receptive range of MOR139-3. (**A**) Chemical structures of odorants used in these experiments. (**B**) Example of the Ca^{2+} response profile of single cells expressing MOR139-3, $G_{\alpha 15}$, RTP1, and Myr-Ric-8A. In all, 300 μM of each odorant was applied at the time indicated by arrowheads. (**C**) The average ΔF-ratios of responses to each indicated odorant ±standard error (n = 3). (**D**) cAMP responses of cells coexpressing MOR139-3, Ric-8B, and RTP1. y axis denotes cAMP level normalized by cAMP concentration without ligand stimulation in each condition ±standard error (n = 3–4). Statistically significant increases of cAMP level in MOR139-3-expressing cells compared with control (in the absence of MOR139-3) were indicated (Student's t-test, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

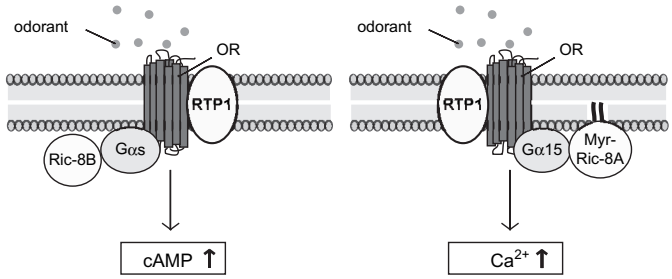

Figure 7 Schematic diagrams of the mechanisms of the 2 assay systems used in this study to measure odorant–OR interaction, the cAMP assay and Ca^{2+} imaging. Left: ORs are efficiently translocated to the cell surface upon odorant stimulation as a result of RTP1, and $G_{\alpha s}$-mediated signal transduction is amplified by Ric-8B. Right: In Ca^{2+} imaging, Myr-Ric-8A amplifies $G_{\alpha 15}$-mediated signal transduction.

ring. One explanation for this broad ligand specificity is that saturated aliphatic compounds can rotate around any of their carbon bonds, but once bound to MOR139-3, they take a structure that is similar to the orientation of a benzene ring in the aromatic compounds. Unsaturated aliphatic molecules may form a similar structure. We have previously shown that the odorant-binding site of an OR is formed by hydrophobic amino acids, and the interaction is relatively weak and flexible (Katada et al. 2005). Such a flexible binding site environment that generates a broad receptive range may account for the relatively low responsiveness of MOR139-3. It is possible that many other orphan ORs possess a similar broad ligand spectrum with relatively low affinity. This could be a reason why it has been difficult to detect odorant responses from many ORs by cloning into

a heterologous system. The assay system described here thus may provide a tool to deorphanize these remaining low affinity and broadly tuned ORs.

Although various response-based approaches have been developed to pair odorants to ORs, functional characterization of ORs in a heterologous expression system is still required to confirm the interaction (Malnic et al. 1999; Touhara et al. 1999; Oka et al. 2006). We herein introduced the use of Myr-Ric-8A as a novel cofactor that enhances OR-mediated Ca^{2+} signaling in HEK293 cells. Although this assay method is an artificial system that is different from an in vivo olfactory transduction model, the developed methodology should be helpful in successfully conducting a functional analysis of orphan ORs and large-scale ligand screening.

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