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Review

Deorphanizing vertebrate olfactory receptors: Recent advances in odorant-response assays

Kazushige Touhara *

Department of Integrated Biosciences, University of Tokyo, Chiba 277-8562, Japan

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Abstract

Olfactory receptors (ORs) comprise the largest multigene G protein-coupled receptor families in organisms from fish to primates, and play a critical role in recognizing thousands of odorant molecules. Recent achievement of functional OR expression in heterologous cells led to identification of ligands for some ORs, revealing a combinatorial receptor coding scheme in the olfactory sensory system. Using the functional assay, the odorant-binding site in ORs has been elucidated, showing that a binding pocket constructed by transmembrane helices provides the molecular basis for agonist and antagonist specificity. To retrospectively identify ORs that recognize a particular odorant of interest, two functional cloning strategies have been developed: one is a strategy wherein OR genes are amplified from single olfactory neurons that show odorant responsiveness in Ca^{2+} imaging, and another is an approach based on glomerular activity by combining *in vivo* bulbar Ca^{2+} imaging and retrograde dye labeling of innervating olfactory neurons. The conventional ligand-screening approach and the functional cloning strategies in an odorant-directed manner have allowed us to match ORs to the cognate odorants both *in vitro* and *in vivo*.

Keywords: Odorant; Olfactory receptor; Olfaction

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* Tel.: +81 471 36 3624; fax: +81 471 36 3626.
E-mail address: touhara@k.u-tokyo.ac.jp.

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1. Olfactory receptors

1.1. The multigene superfamily

Olfactory receptors (ORs) belong to the G protein-coupled receptor family and play a critical role in recognizing thousands of odorant molecules in the olfactory sensory system. It has been estimated that the OR family includes at least several hundred members in the rat (Buck and Axel, 1991). In the last decade or so, the genome sequence projects have enabled the comprehensive analysis of the OR gene family and have revealed the genomic structure and distribution of the OR genes from various organisms (Mombaerts, 2004). In mammals, the OR repertoire comprises 800–1500 members, whereas in fish, the OR family has only approximately 100 members (Niimura and Nei, 2005). Thus, expansion of the OR gene family occurred when animals shifted their living space from an aquatic to a terrestrial environment.

A significant portion of the OR gene family has been pseudogenized in vertebrates. Hominoids possess a high pseudogene content (∼50%), whereas only ∼20% of OR genes are pseudogenes in mouse and dog and 25–35% are pseudogenes in primates (Gilad et al., 2004; Niimura and Nei, 2005; Quignon et al., 2005). The OR gene dynamics could also be pressured by the state of the environment and the type of sense that is utilized for social and sexual behavior. The fraction of pseudogenes in the OR family has increased during evolution in the order of rodents, monkeys, and humans, suggesting that the reduced sense of smell correlates with the loss of functional OR genes. Indeed, in whale and dolphin, in which the auditory system is dominant, 70–80% of OR genes appear to be pseudogenes (Y. Go, personal communication).

1.2. Olfactory receptor proteins

Based on phylogenetic analysis, the mammalian ORs can be classified into two different groups: classes I and II (Glusman et al., 2001; Zozulya et al., 2001; Zhang and Firestein, 2002) (Fig. 2). This classification is based on the finding that frog (*Xenopus laevis*) has two different classes of OR genes: one (class I) that is similar to fish OR genes and a second (class II) that is similar to mammalian OR genes (Freitag et al., 1995). For example, the numbers of intact class I OR genes in human and mouse are 57 and 115 out of 388 and 1037 total intact OR genes, respectively (Niimura and Nei, 2005). Later studies revealed, however, that mammalian class I genes formed a distinct clade from fish OR genes that can be further divided into nine subgroups (Niimura and Nei, 2005). The amphibian OR repertoire turns out to be similar to both fish and mammal ORs, suggesting that there are at least three evolutional lineages (i.e., fish, amphibian, and mammal) in the vertebrate OR gene family (Niimura and Nei, 2005).

OR proteins possess seven hydrophobic transmembrane domains, a disulfide bond between the conserved cysteines in the extracellular loops, a conserved glycosylation site in the N-terminal region (Katada et al., 2004), and several amino acid sequences that are conserved in the OR family (Zozulya et al., 2001; Zhang and Firestein, 2002). The OR consensus sequences reside within the cytoplasmic side of each transmembrane regions and include PMYFFL (transmembrane domain [TM] 2), MAYDRYVAIC (TM3), KAFSTC (TM6), and PMLNPXXY (TM7). Although these consensus sequences vary somewhat between species, they have been widely used to retrieve OR genes from genome. In addition, extensive motif analysis has revealed more than 80 specific short motifs, some of which constitute signature sequences for the OR subfamily of a certain species or have implications for the function and evolution the ORs (Liu et al., 2003). These conserved motifs likely contribute to the proper folding of ORs in the plasma membrane so that ORs can bind odorants and couple to appropriate G proteins. The transmembrane regions, on the other hand, help form the odorant-binding pocket. The sequences in the binding pocket are relatively variable, allowing ORs to bind a wide spectrum of odorant molecules (Singer et al., 1996; Pilpel and Lancet, 1999; Floriano et al., 2000, 2004; Singer, 2000; Man et al., 2004; Katada et al., 2005).

2. Screening of ligands for olfactory receptors

2.1. General consideration

Understanding of OR function has progressed slowly due to a lack of appropriate heterologous systems for expressing and assaying odorant responses (McClintock and Sammeta, 2003). Several attempts have been made to achieve functional expression of ORs on the cell surface in heterologous systems. In some cases, adding an N-terminal leader sequence from another GPCR resulted in a limited expression of functional ORs in the plasma membrane and in a successful odorant-response in a heterologous system such as HEK293 cells (Krautwurst et al., 1998; Wetzel et al., 1999; Kajiya et al., 2001). Addition of the N-terminal epitope tag appears to facilitate expression and membrane trafficking of ORs (Katada et al., 2004). Glycosylation of the N-terminus of ORs is required for proper translocation to the plasma membrane (Katada et al., 2004). The one-transmembrane protein RTP1, which has been referred to as an OR chaperone, appears to enhance cell surface expression of ORs, and many ORs have been deorphanized by co-expressing them with RTP1 (Saito et al., 2004). Ric8B, a putative guanine nucleotide exchange factor for Gαolf, promotes efficient signal transduction of ORs (Von Dannecker et al., 2006). Thus, introduction of the factors required for OR expression present in olfactory neurons is expected to solve the problems of heterologous expression. Finally, a method for maintaining cell surface localization is needed because the ORs undergo continuous internalization and recycling in heterologous cells (Jacquier et al., 2006). Thus, the level of surface expression appears to be critical for OR assays.

2.2. cAMP assay

The odorant signals of ORs are converted to electrical signals in olfactory sensory neurons. The first step in signal transduction is the activation of an olfactory Gαs-type G-

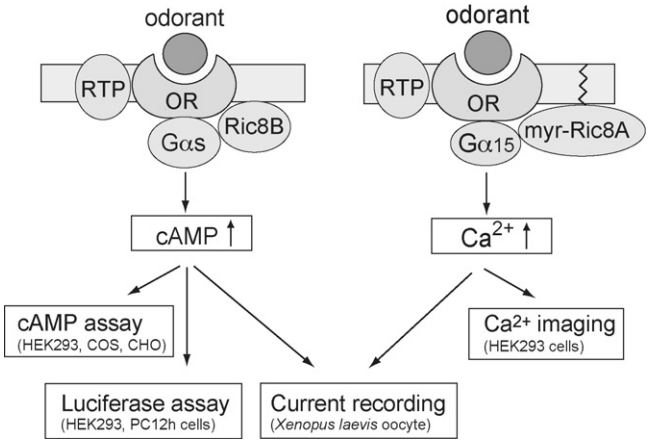

Fig. 1. Schematic diagrams of various assay systems for measuring odorant responses in heterologous cells. ORs activate Gαs in heterologous cells, resulting in cAMP increases that could be directly measured, or monitored by the reporter gene expression. ORs also activate Gα15 in HEK293 cells, resulting in intracellular Ca^{2+} increases that could be directly measured. Odorant-induced activation of Gαs and Gα15 via ORs in *Xenopus larvis* oocytes was monitored electrophysiologically by changes in membrane currents due to activation of cAMP-dependent and Ca^{2+}-dependent Cl^- channels, respectively. Expression of ORs in cell membrane is facilitated by co-expressing an OR chaperone, RTP1. Efficiency of G protein activity is greatly enhanced by co-expressing Ric8B for cAMP assay and myristylated Ric8A for Gα15-mediated Ca^{2+} assay.

protein, Gαolf, by the activated odorant-bound OR. Thus, ORs expressed in various mammalian cell lines (e.g., HEK293, COS-7, and CHO-K1 cells) activate endogenous Gαs upon ligand stimulation (Fig. 1) (Kajiya et al., 2001; Katada et al., 2003). Co-expression of Ric8B greatly facilitates signal transduction of ORs by enhancing GDP-GTP exchange activity of Gαs (Von Dannecker et al., 2006). Odorant-induced cAMP increases via Gαs have been measured using an enzyme-linked immunoassay (Touhara et al., 2006). Alternatively, a luciferase-reporter assay system using the zif268 promoter allows luminescent detection of cAMP increases upon stimulation with an odorant (Katada et al., 2003; Saito et al., 2004). For example, in PC12h cells cotransfected with an OR and the luciferase-reporter construct, odorant stimulation produces dose-dependent increases in luciferase activity (Katada et al., 2003). The *X. laevis* oocyte is another good heterologous expression system for ORs. Gαs-mediated cAMP increases can be electrophysiologically detected by coexpressing a cAMP-dependent Cl^- channel, cystic fibrosis transmembrane conductance regulator (CFTR) (Katada et al., 2003; Abaffy et al., 2006; Touhara et al., 2006).

2.3. Calcium imaging

Ca^{2+} imaging has been one of the most common and reliable methods for detecting activation of orphan GPCRs in high-throughput ligand screening. The Ca^{2+}-imaging assay, however, is limited to GPCRs that couple to Gαq-type G-proteins. For GPCRs that couple to unknown G-proteins, the G-protein α subunit, Gα15/16, is often used to force the signaling to an inositol phosphate-mediated cascade because of its promiscuity for GPCRs. This strategy has been successfully applied to ORs that couple to the Gαolf. Thus, in HEK293 cells co-expressing Gα15 and ORs, Ca^{2+} responses are observed when the cells are stimulated with their ligands (Fig. 1) (Krautwurst et al., 1998; Kajiya et al., 2001; Touhara et al., 2006). In addition, we recently found that co-expression of myristylated Ric8A greatly enhanced Gα15-mediated odorant signaling (Yoshikawa, Touhara, unpublished results). In *X. laevis* oocytes, odorant-induced Ca^{2+} increases via an endogenous Ca^{2+}-dependent Cl^- channel can be electrophysiologically detected by coexpressing Gα15 (Wetzel et al., 1999; Katada et al., 2003; Touhara et al., 2006).

2.4. Problems

Despite recent development of efficient odorant-response assays in heterologous systems, many vertebrate ORs have remained to be orphan. It is because the expression system requires additional components that allow for expression of enough OR proteins on the cell membrane or because cognate ligands do not exist in the currently available odorant repertoire that is widely utilized. It is also a question whether the ligand specificity we determine in heterologous cells really reflect *in vivo* responsiveness of the target OR. Indeed, we sometimes observe a difference in ligand-responsiveness as measured by Gα15-mediated Ca^{2+} imaging and Gαs-mediated cAMP elevation, probably due to the difference in receptor coupling to Gα15 and Gαs (Shirokova et al., 2005; Oka et al., 2006). In addition, as I will describe later, *in vivo* odorant responsiveness of an OR at the level of the olfactory bulb, wherein odorants are

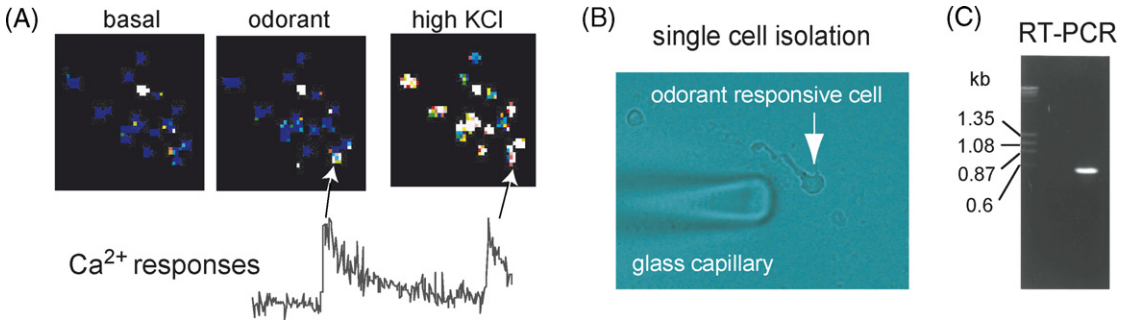

Fig. 2. Functional cloning of an OR expressed by a single odorant-responsive neuron. (A) Identification of odorant-responsive olfactory sensory neurons from mice in Ca^{2+}-imaging experiments. (B) Isolation of single odorant-responsive cells by a glass micro-capillary tube. (C) Amplification of an OR gene by single cell RT-PCR.

delivered through the air, does not necessarily exactly reflect the pharmacological properties of the OR in a heterologous expression system or in isolated olfactory neurons, wherein odorants are delivered through an aqueous solution without mucosa (Oka et al., 2006). Therefore, we have to be careful when we evaluate structure–activity relationship of ORs in *in vitro* expression systems.

3. Functional cloning of olfactory receptors

3.1. From peripheral sensory neurons

Each olfactory sensory neuron expresses just one of the 1000 ORs (Serizawa et al., 2004; Shykind, 2005). The one neuron–one receptor rule has been confirmed by a variety of techniques, namely, in situ hybridization (Ngai et al., 1993; Ressler et al., 1993; Vassar et al., 1993), single cell RT-PCR analysis (Malnic et al., 1999; Touhara et al., 1999), and transgenic experiments (Qasba and Reed, 1998; Serizawa et al., 2000, 2003; Vassalli et al., 2002). Further, the selected OR appears to be transcribed from only one of two alleles, either the maternal or paternal allele (Chess et al., 1994; Ishii et al., 2001). This mutually exclusive expression pattern is preserved even between transgenes and endogenous copies, supporting the stochastic model (Qasba and Reed, 1998; Serizawa et al., 2000, 2003; Vassalli et al., 2002). Indeed, the single OR choice is observed from a minigene that contains a few kilobases upstream of the transcription start site, implicating the involvement of *cis*-regulatory elements (Qasba and Reed, 1998; Vassalli et al., 2002; Oka et al., 2006).

Based on one neuron–one receptor rule, we reasoned that the combination of an odorant-response assay with single cell RT-PCR analysis would lead to the isolation of odorant receptors expressed by single olfactory neurons (Touhara, 2001). The physiological responses of single olfactory neurons should reflect the ligand specificity of ORs expressed by the corresponding neurons. To monitor responses of olfactory neurons to various odorants, we adopted a fura-2 based Ca^{2+}-imaging technique to measure increases in intracellular Ca^{2+} levels in response to odorant stimuli (Fig. 2A). The Ca^{2+}-imaging method was suitable for simultaneous recording of odorant responses in several cells, which greatly facilitated the screening of odorant-responsive cells, compared to the single cell-based electrophysiological approach. Sequential applications of various odorants by injection into a continuous stream of wash buffer flowing over the cells, allowed for the identification of odorant-responsive cells from which the expressed odorant receptor cDNA was amplified by single cell RT-PCR using primers designed from conserved amino acid sequences in the olfactory receptor family (Fig. 2B and C) (Malnic et al., 1999; Touhara et al., 1999; Kajiya et al., 2001).

The combination of Ca^{2+}-imaging and single cell RT-PCR techniques enabled us to isolate specific receptor genes that encode a receptor for a particular odorant. In order to ascertain the reliability of this functional cloning approach and to be able to proceed with further biochemical characterization of the cloned receptors, functional expression and reconstitution of the receptors were required. Initially, to overcome the difficulties in expressing odorant receptors in typical expression systems, including mammalian cell lines, we decided to target the olfactory neuron itself as an expression system for the reconstitution of odorant receptors using recombinant adenoviruses as gene transfer mediators (Touhara et al., 1999). A receptor isolated from a cell that responded to lyral, MOR23, was expressed via recombinant adenovirus in olfactory neurons, and the response to lyral was successfully demonstrated for reconstituted MOR23 (Touhara et al., 1999). Although the adenovirus-mediated expression system was also successfully utilized for ligand screening of the rat orphan receptor I7 (Zhao et al., 1998), we observed that not all of the functionally cloned receptors were successfully reconstituted by this adenovirus approach. Recently, as I mentioned earlier, we mainly utilize heterologous expression systems to reconstitute the ORs isolated from single odorant-responsive neurons. In any case, it is definitely required to express the ORs and recapitulate the odorant responses because the single cell approach often gives false positive due to contamination.

3.2. From the olfactory bulb in vivo

The olfactory bulb (OB) is the first part of the brain to relay the neural signals of olfactory sensory neurons to secondary neurons, called mitral/tufted cells, which in turn project their axons into the central olfactory system (Mori et al., 1999; Shepherd et al., 2004). Approximately 10,000 olfactory sensory neurons expressing the same OR send their axons to a few topologically fixed glomeruli, resulting in a pattern of activated glomeruli that reflects the original pattern of activated ORs in the olfactory epithelium (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996). In other words, individual odorants activate distinct subsets of ORs, resulting in a glomerular activation pattern that is unique for each odorant in a stereotyped region of the OB called the ‘‘odor map’’ (Xu et al., 2000; Leon and Johnson, 2003; Mori et al., 2006).

The odor maps in the OB can be assessed using several imaging methods such as Ca^{2+} imaging (Friedrich and Korsching, 1997; Wachowiak and Cohen, 2001; Oka et al., 2006), intrinsic imaging (Rubin and Katz, 1999; Uchida et al., 2000), and functional MRI (Xu et al., 2003), as well as by methods employing various neuronal activity markers such as 2-deoxyglucose and immediate early gene expression (see Shepherd et al., 2004). Spatial patterns of odorant-activated glomeruli can also be measured by using transgenic mice that expressed synapto-pHluorin, a pH-sensitive protein that reports synaptic vesicle fusion, in olfactory sensory neurons (Bozza et al., 2004). Different odorants elicit different glomerular activity patterns, but structurally related odorants activate similar sets of glomeruli, reflecting the fact that similar odorants are recognized by similar sets of ORs in the olfactory epithelium. Even for the same odorant, different concentrations elicit different patterns of activation in that more glomeruli are recruited at higher concentrations of odorants (Oka et al., 2006).

To determine if the odor map in the OB truly corresponds to the receptor code in the olfactory epithelium, Ca^{2+}-imaging

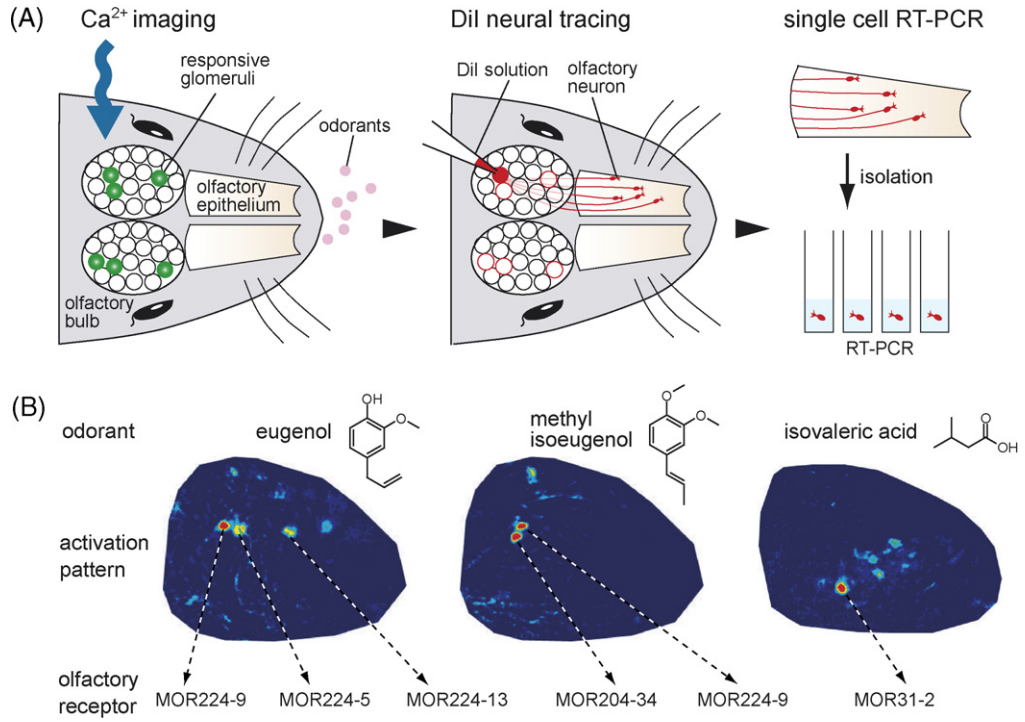

Fig. 3. Identification of OR genes expressed in olfactory sensory neurons innervating odorant-activated glomeruli. (A) A scheme for the functional cloning strategy. (B) Odorant-induced glomerular activity pattern of the mouse olfactory bulb by Ca^{2+} imaging and the type of OR genes expressed in activated glomeruli.

techniques were applied to transgenic mice where defined OR-expressing neurons were tagged with fluorescent probes. The glomerulus innervated by mOR-EG (MOR174-9)-expressing neurons showed responses to eugenol, a cognate ligand for mOR-EG (Oka et al., 2006). Similarly, I7, M71, and MOR23 glomeruli exhibited responses to their corresponding ligands, octanal, acetophenone, and lyral, respectively (Belluscio et al., 2002; Bozza et al., 2002; Grosmaitre et al., 2006). These studies provided evidence that the responsiveness of a glomerulus was a reflection of that of the OR expressed by the innervating sensory neurons. In other words, if we can identify the ORs that are expressed in these activated glomeruli, we can construct odorant–ORs combinatorial matrices *in vivo*.

A functional OR identification methodology was established based on glomeruluar activity by combining Ca^{2+} imaging, retrograde dye labeling, and single cell RT-PCR (Fig. 3A). This approach has been successfully performed to isolate the four most sensitive ORs for eugenol and methyl isoeugenol, and one sensitive OR for isovaleric acid in the dorsal olfactory bulb area (Fig. 3B) (Oka et al., 2006). The expression of the identified OR in the defined glomerulus was confirmed by single-glomerulus RT-PCR. Moreover, the identified ORs expressed in HEK293 cells recognized the odorants that were shown to elicit responses in the olfactory bulb. Importantly, we were able to identify ORs from all target glomeruli tested in the study (5/5 glomeruli), demonstrating the high efficiency and reliability of our methodology. Unlike Ca^{2+} imaging, it was difficult for dye injection after intrinsic imaging because the depth of glomerulus from the surface of the olfactory bulb was obscure without any landmark. In the future, expansion of imaging areas in the olfactory bulb would allow us to determine a complete set of ORs for a given odorant, which corresponds to an *in vivo* receptor code representing the odor quality of the odorant.

4. Olfactory receptors pharmacology

Both the ligand-screening approach and the functional cloning strategy that I described above have allowed for pairing more and more ORs with their cognate ligands. Among them, mouse mOR-EG (MOR174-9), which was originally isolated from a single eugenol (EG)-responsive neuron by Ca^{2+}-imaging and single cell RT-PCR techniques (Kajiya et al., 2001), is the best-characterized OR so far. Mouse mOR-EG recognizes at least 22 odorants that share certain molecular determinants with EC_{50} values ranging from a few micromolar to several hundred micromolar (Fig. 4A) (Katada et al., 2005). Structure–activity relationship studies with mOR-EG have suggested that ORs have a broad but selective molecular receptive range, and that the selectivity is determined by the shape, size, functional group, and length of the ligand. This pharmacological concept is a common feature of many ORs, as has been shown by studies on rat I7 (Araneda et al., 2000) and other deorphanized ORs (Wetzel et al., 1999; Bozza et al., 2002; Gaillard et al., 2002; Levasseur et al., 2003; Spehr et al., 2003; Matarazzo et al., 2005; Shirokova et al., 2005). Thus, odorant information about a specific odorant results from the combined activation of the array of ORs activated by the odorant (Malnic et al., 1999; Kajiya et al., 2001; Touhara, 2002).

As is the case of other GPCRs, odorants can function both as agonists and antagonists of ORs, suggesting that the interactions between ORs and odorants is quite complicated. For

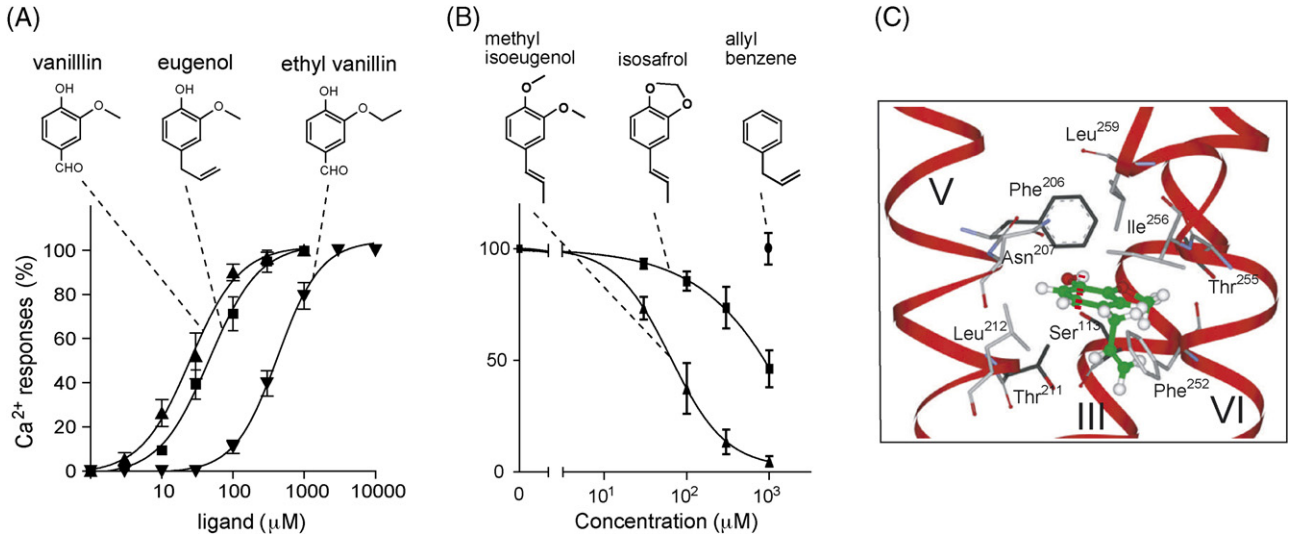

Fig. 4. Pharmacology of mOR-EG and a model for the binding site structure. (A) Dose–response curves obtained from Ca^{2+} imaging of HEK293 cells expressing mOR-EG to each odorant ligand. (B) Dose-dependent inhibition curves of mOR-EG by two antagonists, methyl isoeugenol and isosafrol, and a non-ligand, allyl benzene, as a percentage of the response to 100 μM eugenol. (C) A model for binding of eugenol (depicted by green) to mOR-EG and the orientation of amino acids in the binding site formed by three transmembrane helices (TMIII, V, and VI) shown by red ribbons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

example, the eugenol response of mOR-EG was potently blocked by some structurally related odorants, such as methyl isoeugenol and isosafrol (Fig. 4B) (Oka et al., 2004a,b). This antagonism was observed in olfactory neurons expressing mOR-EG in an intact olfactory epithelium slice preparation (Omura et al., 2003; Oka et al., 2004b). Further, quantitative recording using the Ca^{2+}-imaging technique demonstrated that the antagonist response was partially inhibited in the OR-defined glomerulus (Oka, Touhara, unpublished observation). Thus, in complex mixtures of odorants, competition between the odorants for antagonism of the OR is likely to be another factor affecting the encoding of receptor information in the olfactory system.

Multiple alignment analysis of the OR superfamily has revealed the existence of highly conserved and variable regions that are likely to be involved in structural organization and ligand recognition, respectively (Buck and Axel, 1991; Zhang and Firestein, 2002). A systematic experimental approach to decipher the odorant-binding site was undertaken for mOR-EG (Katada et al., 2005). Functional analysis of site-directed mutants and ligand docking simulation studies were employed to define the odorant-binding environment. Most of the critical residues involved in odorant recognition were found to be hydrophobic and located within the binding pocket formed by transmembrane domains TM3, 5, and 6 (Fig. 4C) (Katada et al., 2005). The spatial location of the binding pocket is similar to that of other biogenic GPCRs; however, in contrast to typical GPCRs where multiple electrostatic interactions with ligands are the rule, hydrophobic amino acids appear to be the rule for odorant recognition by ORs. Further, the accuracy of the binding model was validated by the fact that single amino acid changes caused predictable changes in agonist and antagonist specificity (Katada et al., 2005). Thus, ligand information appears to be transduced from the three-dimensional configuration that the binding pocket adopts when bound to a specific odorant ligand.

OR sensitivity to odorants in heterologous expression systems turns out to be relatively high in comparison to the odorant sensitivity of the olfactory system; the threshold concentrations for the best ligand for each OR range from a few μM to several hundred μM (Mombaerts, 2004). This is not simply a problem with the heterologous expression system as the sensitivity range of olfactory sensory neurons expressing the same OR is similar (Oka et al., 2004b, 2006). However, the odorant sensitivity of glomeruli innervated by olfactory neurons expressing a defined OR appears to be about 1000-fold higher than that of peripheral neurons (Oka et al., 2006). In addition, the specificity of *in vivo* odorant response in an OR-defined glomerulus was different from that suggested by *in vitro* OR pharmacology in a heterologous system (Oka et al., 2006). The apparently higher odorant sensitivity and specificity *in vivo* seems to be a consequence of the olfactory mucus which provides a place for efficiently concentrating and carrying odorants to the receptor site.

5. Conclusion

Thanks to the recent development of efficient functional OR assays, increasing numbers of ORs have been deorphanized, leading to construction of odorant–OR matrices *in vitro*. Co-expression of the OR chaperon and G protein regulators appears to be a critical consideration to improve functional OR expression in heterologous cells. Using the various odorant assays, the molecular environment of the odorant-binding site has been revealed, shedding light on how the OR family has evolved the ability to recognize such a variety of chemical structures. Further, functional OR identification techniques in an odorant-directed manner have allowed us to characterize OR

function *in vivo*. An important caveat that needs to be considered based on the study of odorant sensitivity and specificity of OR *in vitro* versus glomerulus *in vivo*, is the possibility that ligand specificity of ORs obtained in heterologous OR expression systems may not always reflect the specificities observed in the olfactory bulb under physiological conditions *in vivo*. Nonetheless, recent advances in odorant-response assays in heterologous systems would provide a tool to characterize ORs especially in human because *in vivo* experiments could not be easily done for the human olfactory system. How broadly the human OR repertoire cover odorant molecules would be an interesting question to be solved in the near future.

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