

Subunit Contributions to Insect Olfactory Receptor Function: Channel Block and Odorant Recognition

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Abstract

Insect olfactory receptors are heteromeric ligand-gated ion channels composed of at least one common subunit (Orco) and at least one subunit that confers odorant specificity. Little is known about how individual subunits contribute to the structure and function of the olfactory receptor complex. We expressed insect olfactory receptors in *Xenopus* oocytes to investigate 2 functional features, ion channel block and odorant recognition. The sensitivity of *Drosophila* olfactory receptors to inhibition by ruthenium red, a cation channel blocker, varied widely when different specificity subunits were present, suggesting that the specificity subunits contribute to the structure of the ion pore. Olfactory receptors formed by DmelOr35a and Orco subunits from several different species displayed highly similar odorant response profiles, suggesting that the Orco subunit does not contribute to the structure of the odorant-binding site. We further explored odorant recognition by conducting a detailed examination of the odorant specificity DmelOr67a + DmelOrco, a receptor that responds to aromatic structures. This screen identified agonists, partial agonists, and an antagonist of DmelOr67a + DmelOrco. Our findings favor specific subunit arrangements within the olfactory receptor complex and provide a preliminary odorphore for an olfactory receptor, offering a useful foundation for future exploration of insect olfactory receptor structure.

Key words: electrophysiology, insect olfactory receptors, *Xenopus* oocytes

Introduction

The insect olfactory system detects the presence of volatile molecules in the surrounding environment. Accurate identification of these molecules is essential for survival. Although a constant survey of the molecular constituents surrounding an animal provides general information on dynamic conditions, specific identification within complex mixtures is also required. Therefore, the olfactory system must be simultaneously capable of both general and specific molecule detection. The first stage in this process is the recognition of odors by a large array of receptors. Using a combinatorial coding approach, where multiple odors are detected by a single receptor and multiple receptors detect a single odorant, a high level of discriminatory power is obtained (Firestein 2004; Hallem and Carlson 2004). In addition, insects employ receptors with high selectivity, such as pheromone receptors, providing a mechanism for specificity (Nakagawa et al. 2005; Wanner et al. 2007, 2010). In *Drosophila*, the known odorant-detecting chemosensory receptors are

expressed in olfactory sensory neurons (OSNs) of the antennae and maxillary palp and are represented by 2 gene families, the ionotropic receptors (IRs) and the odorant receptors (ORs). While the IRs are closely related to ionotropic glutamate receptors (Benton et al. 2009), the ORs do not share any obvious features with other protein families (Vosshall et al. 1999). Thus, there is considerable interest in understanding the structure and function of the OR class of insect ORs.

ORs were recently revealed to be ligand-gated ion channels that respond to odorant binding by opening a nonselective cation pore (Sato et al. 2008; Wicher et al. 2008). Topology algorithms predict and biochemical analyses demonstrate that insect OR subunits contain 7 transmembrane domains, an intracellular N-terminus and an extracellular C-terminus (Benton et al. 2006; Lundin et al. 2007; Smart et al. 2008). This topology is opposite to that of mammalian ORs, a large family of G protein-coupled receptors (GPCRs). In fact, insect ORs

bear no resemblance to any known receptor or channel type, GPCR or otherwise (Benton et al. 2006). G proteins have been implicated in insect OSN activation and signal transduction (reviewed in Nakagawa and Vosshall 2009). However, although G-protein signaling has been shown to modulate insect OSN activity (Gomez-Diaz et al. 2004, 2006; Kain et al. 2008, 2009; Wicher et al. 2008), it is presently unknown whether direct association of G proteins with ORs is the mechanism. Regardless of the specifics of insect OSN activation, it is clear that insect ORs constitute a novel receptor class.

Functional ORs are multimeric complexes of unknown stoichiometry. These receptors contain at least one copy of a widely expressed common subunit, now known as Orco (Vosshall and Hansson 2011), and at least one copy of a non-Orco subunit (Vosshall et al. 2000; Dobritsa et al. 2003; Elmore et al. 2003; Larsson et al. 2004; Benton et al. 2006; Wanner et al. 2007, 2010; Sato et al. 2008; Wicher et al. 2008). The Orco subunit has, until recently, gone by a variety of names in different species, including Or83b in *Drosophila melanogaster* (Larsson et al. 2004), OR2 in the European honey bee *Apis mellifera* (Wanner et al. 2007) and the European corn borer *Ostrinia nubilalis* (Wanner et al. 2010), and OR7 in various mosquito species (Bohbot et al. 2011). The non-Orco subunits, or “specificity” subunits, are thought to be involved in odorant recognition because changing this subunit can alter the odorant preference of the receptor (Hallem et al. 2004; Hallem and Carlson 2006; Carey et al. 2010; Wang et al. 2010) and mutagenesis within a specificity subunit can alter odorant sensitivity (Nichols and Luetje 2010). The specificity subunits in *Drosophila* exhibit a high degree of sequence variability (Vosshall et al. 1999), and this variability is thought to enable a wide range of odorant structures to be recognized. The presence of Orco in ORs with widely divergent odorant specificities suggests that Orco may not be involved in odorant binding (Nakagawa and Vosshall 2009), but the potential contributions of Orco toward odorant specificity have not been investigated in detail. Orco is conserved among insect species, pointing to an essential and common role for this subunit (Jones et al. 2005). It is thought to provide a trafficking function, targeting the OR complex to the plasma membrane of the OSN dendrite (Benton et al. 2006). Orco does not respond to odorants when expressed alone but is required for odorant-activated ion flux (Elmore et al. 2003; Hallem and Carlson 2006; Wanner et al. 2007; Sato et al. 2008; Wicher et al. 2008). Deletion of 2 residues within transmembrane segment 6 of Dmel\Orco alters the current–voltage relationship of a homomeric complex (Wicher et al. 2008), suggesting that Orco contributes to the structure of the ion pore. Further support for the involvement of Orco in forming the ion pore comes from the recent discovery of an activator of ion channels formed by Orco when expressed alone or in combination with an odorant specificity subunit (Jones et al. 2011). Whether specificity subunits also contribute to ion pore structure is unknown.

Here, we use the *Xenopus* oocyte heterologous expression system and electrophysiology to investigate the roles of

Drosophila OR specificity subunits and the Orco subunit in mediating the odorant binding and ion pore functions of the receptor. We also examine the odorant recognition function of these receptors in more detail, by conducting a rational expansion of the molecular receptive range (MRR) of receptors formed by Dmel\Or67a, a *Drosophila* OR that is responsive to aromatic odorants.

Experimental procedures

Materials and methods

Xenopus laevis frogs were purchased from Nasco. The care and use of *X. laevis* frogs in this study were approved by the University of Miami Animal Research Committee and meet the guidelines of the National Institutes of Health. Odorants and other chemicals were from Sigma-Aldrich. CAS numbers for all odorants used in this study are provided in Supplementary Table 1.

Receptor cloning

To clone Dmel\Or67a, wild-type (Canton S) *D. melanogaster* flies were frozen in liquid nitrogen and shaken through a 3-stage sieve. Antennae were collected and homogenized in a tapered glass tube. Total RNA was extracted with Trizol (Invitrogen) and used as template for first-strand cDNA synthesis with gene-specific primers. Products were amplified by polymerase chain reaction, subcloned into pGEMHE (Liman et al. 1992), and verified by sequencing. Dmel\Or85a and Dmel\Or85b were previously cloned (Nichols and Luetje 2010), as were Amel\Or2 (Wanner et al. 2007) and Onub\Or2 (Wanner et al. 2010). Dmel\Or35a and Dmel\Orco were generously provided by J. Carlson and L. Vosshall, respectively.

Expression of ORs in *Xenopus* oocytes

Oocytes were surgically removed from mature *X. laevis* frogs. Follicle cells were removed by treatment with Collagenase B (Boehringer Mannheim) for 2 h at room temperature. Capped cRNA encoding each OR subunit was generated using mMessage mMachine kits (Ambion). Twenty-five nanograms of cRNA encoding each OR subunit were injected into Stage V–VI *Xenopus* oocytes. Oocytes were incubated at 18 °C in Barth’s saline (in mM: 88 NaCl, 1 KCl, 2.4 NaHCO₃, 0.3 CaNO₃, 0.41 CaCl₂, 0.82 MgSO₄, 15 N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.6, and 100 µg/mL amikacin) for 2–7 days prior to electrophysiological recording. The wide range of incubation times was needed to achieve suitable expression levels because expression levels can vary from oocyte to oocyte within one batch, as well as from batch to batch (frog to frog, as well as surgery to surgery with the same frog). There is also a seasonal variation, with expression tending to be poorer in the summer. In addition, there is variation in expression levels among the various receptors,

which may be due variation in RNA stability, protein stability, receptor complex assembly efficiency, or other factors. For all these various reasons, direct comparison of current amplitudes from one oocyte to another, whether with the same receptor or different receptors, is not appropriate. Comparisons must always be made relative to normalization standards that are applied to each oocyte in a given experiment.

Electrophysiology and data capture

Odorant-induced currents were recorded under 2-electrode voltage clamp from *X. laevis* oocytes expressing ORs, using an automated parallel electrophysiology system (OpusExpress 6000A; Molecular Devices). Oocytes were perfused with ND96 (in mM: 96 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.5). Odorant stock solutions (usually 1 M) of each odorant were prepared in dimethyl sulfoxide. Odorants were diluted in ND96 and, unless otherwise noted, applied for 20 s at a flow rate of 1.65 mL/min, with extensive washing in ND96 (10 min at 4.6 mL/min) between applications. Micropipettes were filled with 3 M KCl and had resistances of 0.2–2.0 MΩ. The holding potential was –70 mV. Current responses, filtered (4-pole, Bessel, low pass) at 20 Hz (–3 dB) and sampled at 100 Hz, were captured and stored using OpusXpress 1.1 software (Molecular Devices).

Experimental protocols and data analysis

The ruthenium red (RR) susceptibility screens presented in Figures 1 and 2 were performed as follows. Application of odorant for 210 s resulting in a steady-state activation was followed by application of RR plus odorant for 30 s (or 60 s for DmelOr67a). Finally, all reagents were washed out in ND96. The extent of inhibition by RR was measured as the current amplitude after 30 s of RR application (240 s after initial application of odorant) divided by the current amplitude immediately before RR application (210 s after initial application of odorant). Investigation of DmelOr67a/DmelOrco inhibition by heliotropyl acetone (Figure 6B) was performed similarly except heliotropyl acetone was applied for 60 s. The extent of inhibition by heliotropyl acetone was measured as the current amplitude immediately before washout (270 s after initial application of the activator methyl benzoate) divided by the current amplitude immediately before heliotropyl acetone application (210 s after initial application of methyl benzoate).

Initial analysis of electrophysiological data was done using Clampfit 9.1 software (Molecular Devices). Statistical analyses, curve fitting, and EC₅₀ calculations were done using Prism 5 (Graphpad). Concentration–response data were fit to the equation: $I = I_{\max}/(1 + (EC_{50}/X)^n)$ where *I* represented the current response at a given concentration of odorant, *X*; *I*_{max} was the maximal response; EC₅₀ was the concentration of odorant yielding a half maximal response; *n* was the apparent Hill coefficient. Statistical significance was assessed using a one-way analysis of variance followed by the Bonferroni's posttest.

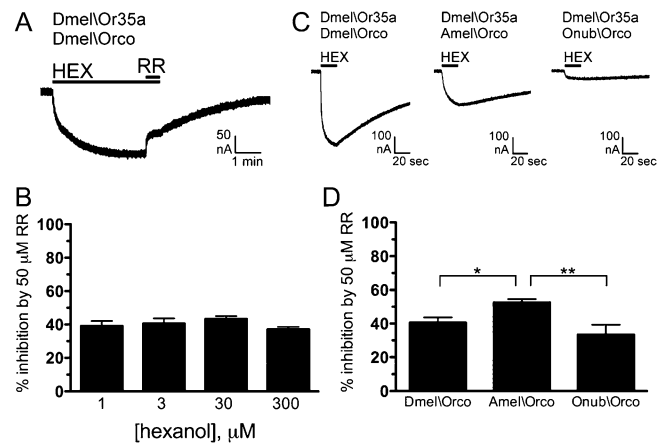


Figure 1 Inhibition of DmelOr35a-containing receptors by RR. **(A)** An oocyte expressing DmelOr35a + DmelOrco was challenged with 3 μM hexanol (HEX) for 210 s, followed by coapplication of 3 μM HEX and 50 μM RR for 30 s. **(B)** Block of DmelOr35a + DmelOrco currents by 50 μM RR at various hexanol concentrations. Blockade was assessed as described in Experimental Procedures. Values are presented as mean ± standard error of the mean (SEM) (1 μM: *n* = 4; 3 μM: *n* = 11; 30 μM: *n* = 9; 300 μM: *n* = 6). The mean values did not differ (one-way analysis of variance, *P* = 0.47). **(C)** Oocytes expressing DmelOr35a + DmelOrco (left), AmelOrco (center), or OnubOrco (right) were challenged with a 20 s application of hexanol (HEX). **(D)** Block by 50 μM RR of receptors formed with DmelOr35a and various Orco orthologs. Values are presented as the mean ± SEM (DmelOrco: *n* = 11; AmelOrco: *n* = 9; OnubOrco: *n* = 6). Statistical significance was assessed by one-way ANOVA (*P* = 0.004), followed by Bonferroni's multiple comparison test (**P* < 0.05; ***P* < 0.01).

Results

Specificity subunits influence sensitivity to channel blockade

A critical functional component of insect OR structure is the ion pore (Sato et al. 2008; Wicher et al. 2008). Although the Orco subunit has been implicated in forming this structure (Wicher et al. 2008), a potential role for specificity subunits in contributing to channel structure has not been investigated. RR, a cation channel blocker, has previously been shown to differentially inhibit insect ORs of various species, with 50 μM RR fully inhibiting the *Bombyx mori* BmorOr1 + BmorOrco receptor but failing to inhibit the *Anopheles gambiae* AgamOr2 + AgamOrco receptor (Nakagawa et al. 2005; Sato et al. 2008). These observations suggested to us that RR blockade might serve as a probe to assay the contribution of different OR subunits to the structure of the ion pore.

We first asked whether RR could block function of DmelOr35a + DmelOrco. Application of hexanol to oocytes expressing this subunit combination yielded an inward current response (Figure 1A). No inward currents in response to hexanol application were observed in the absence of DmelOrco (Wanner et al. 2007), in the absence of DmelOr35a (Supplementary Figure 1A), or in sham (water)-injected oocytes (Supplementary Figure 1B). Application of 50 μM RR in the presence of hexanol resulted in

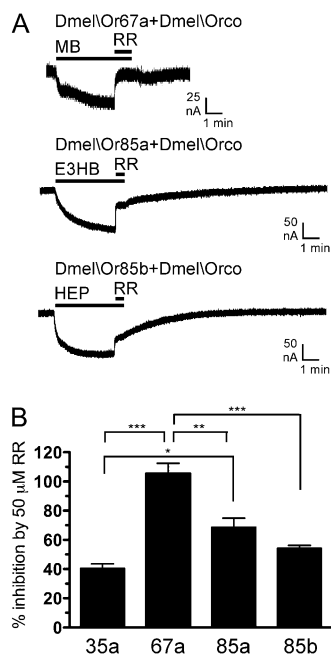


Figure 2 Odorant specificity subunits confer variable sensitivity to blockade by RR. **(A)** Current responses of OR-expressing oocytes challenged with odorant for 210 s, followed by coapplication of odorant plus 50 μ M RR for 30 s (DmelOr85a + DmelOrco and DmelOr85b + DmelOrco) or 60 s (DmelOr67a + DmelOrco). Odorant abbreviations: MB (300 μ M methyl benzoate), E3HB (300 μ M ethyl 3-hydroxybutyrate), and HEP (300 μ M 2-heptanone). **(B)** Quantification of blockade by 50 μ M RR for several ORs. Values are presented as the mean \pm standard error of the mean (DmelOr35a + DmelOrco, $n = 11$; DmelOr67a + DmelOrco, $n = 11$; DmelOr85a + DmelOrco, $n = 5$; DmelOr85b + DmelOrco, $n = 5$). Statistical significance was assessed by one-way analysis of variance ($P < 0.0001$), followed by Bonferroni's multiple comparison test ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

partial inhibition ($41 \pm 3\%$ inhibition, $n = 11$) of the DmelOr35a + DmelOrco response to 3 μ M hexanol (Figure 1A,B). RR application had no effect on sham (water)-injected oocytes (Supplementary Figure 1C). RR did not cause receptor desensitization when applied in the absence of hexanol (Supplementary Figure 2A) and extended application of hexanol in the absence of RR did not cause receptor desensitization (Supplementary Figure 2B,C). The tested hexanol concentration (3 μ M) was the EC_{83} for hexanol activation of the DmelOr35a + DmelOrco receptor in our expression system (Wanner et al. 2007). Decreasing the hexanol concentration to 1 μ M or increasing the hexanol concentration to 30 or 300 μ M did not alter the extent of blockade by 50 μ M RR (Figure 1B). The failure of a 300-fold change in agonist concentration to alter the extent of blockade by RR indicated that RR is noncompetitive, as would be expected for a channel blocker. Application of 50 μ M RR caused a similar extent of blockade when the DmelOr35a + DmelOrco receptor was activated by 3 μ M heptanol ($36 \pm 4\%$ inhibition, $n = 5$).

In each insect species, a single Orco subunit is a constant component of each functional OR (Touhara and Vosshall 2009; Kaupp 2010) and the Orco subunits of different species have been shown to be, at least to some extent, functionally

interchangeable (Nakagawa et al. 2005). AmelOrco and OnubOrco, the respective Orco orthologs of the European honey bee *A. mellifera* (Wanner et al. 2007) and the European corn borer *O. nubilalis* (Wanner et al. 2010), were each coexpressed in oocytes with DmelOr35a and screened for function. Similar to the DmelOr35a + DmelOrco receptor, both interspecies receptor complexes (DmelOr35a + AmelOrco, DmelOr35a + OnubOrco) responded to the odorant hexanol (Figure 1C). The presence of functional receptors indicated that AmelOrco and OnubOrco were incorporating into the receptor complexes because we have previously demonstrated that DmelOr35a does not form functional receptors when expressed alone (Wanner et al. 2007). Small (but significant) differences in RR inhibition were observed among ORs formed by DmelOr35a and either DmelOrco, AmelOrco, or OnubOrco (Figure 1D, Supplementary Figure 3). RR (50 μ M) blocked $53 \pm 2\%$ of the response of DmelOr35a + AmelOrco to 3 μ M hexanol ($n = 9$) but only $34 \pm 6\%$ of the hexanol response of DmelOr35a + OnubOrco ($n = 6$). Thus, differences in Orco ortholog sequence can influence susceptibility to blockade by RR.

The differences in RR sensitivity that we observed when substituting various Orco orthologs into a receptor were small compared with the large disparity in RR sensitivity reported for a *B. mori* OR (complete block) and an *A. gambiae* OR (no block) (Nakagawa et al. 2005; Sato et al. 2008), suggesting that the Orco subunit may not be the primary determinant of sensitivity to RR inhibition. We next asked whether changing the specificity subunit, while keeping the Orco subunit constant, would impart greater variability in RR sensitivity. Our demonstration of the noncompetitive nature of RR blockade of DmelOr35a + DmelOrco (Figure 1B) suggested that RR blockade could be accurately compared across a wide range of agonist concentrations. However, to ensure an appropriate comparison, each OR was activated with approximately equipotent concentrations of their cognate odorants. Odorant concentrations were chosen by examining the concentration–response relationship for each odorant–receptor pair (Supplementary Table 2): for DmelOr35a + DmelOrco, 3 μ M hexanol = EC_{83} ; for DmelOr67a + DmelOrco, 300 μ M methyl benzoate = EC_{89} ; for DmelOr85a + DmelOrco, 300 μ M ethyl-3-hydroxybutyrate = EC_{71} ; for DmelOr85b + DmelOrco, 300 μ M 2-heptanone = EC_{70} . ORs formed by DmelOr67a, DmelOr85a, and DmelOr85b, each in combination with DmelOrco, were tested for susceptibility to blockade by 50 μ M RR (Figure 2). RR block of DmelOr35a + DmelOrco was also included for comparison. Although RR was able to inhibit function of each OR tested, the extent of block was highly variable (percent inhibition: DmelOr35a + DmelOrco = $41 \pm 3\%$, $n = 11$; DmelOr67a + DmelOrco = $105 \pm 7\%$, $n = 11$; DmelOr85a + DmelOrco = $69 \pm 6\%$, $n = 5$; DmelOr85b + DmelOrco = $54 \pm 2\%$, $n = 5$). Similar to what we observed for DmelOr35a + DmelOrco, when an alternate odorant (300 μ M butyl

acetate) was used to activate Dmel\Or85b + Dmel\Orco, a similar extent of blockade by 50 μ M RR was achieved ($52 \pm 3\%$ inhibition, $n=12$). Our results identified the odorant specificity subunit of an OR as a major determinant of sensitivity to blockade by the cation channel blocker RR and strongly suggested an involvement of specificity subunits in forming at least part of the ion pore structure.

Substitution of Orco subunits has little effect on odorant specificity

The presence of Orco in OR complexes with a variety of odorant specificities suggests that Orco does not participate in odorant binding, but this idea is difficult to test using the OR subunits of one species. However, the ability of Orco subunits of different species to functionally substitute for one another (Nakagawa et al. 2005; Sato et al. 2008) (Figure 1C,D) allowed us to perform a detailed comparison of the odorant response profiles of OR complexes formed by a constant specificity subunit (Dmel\Or35a) coexpressed with each of several Orco orthologs. Dmel\Or35a was coexpressed in *Xenopus* oocytes with Dmel\Orco, Amel\Orco, or Onub\Orco and then challenged with a panel of primary alcohols to compare relative alcohol sensitivities. Each receptor was differentially activated by the alcohols in a biphasic pattern as carbon chain length was varied (Figure 3, Supplementary Table 3). The pattern of responsiveness to the alcohol panel was remarkably similar for each of the 3 receptors, despite Dmel\Orco sharing only 63% and 64% amino acid identity with Amel\Orco and Onub\Orco, respectively.

We next sought to compare odorant responsiveness to an expanded set of odorants, selected by rational expansion of the chemical space around hexanol. These odorants included aliphatic hydrocarbons ranging from 2 to 12 carbons in

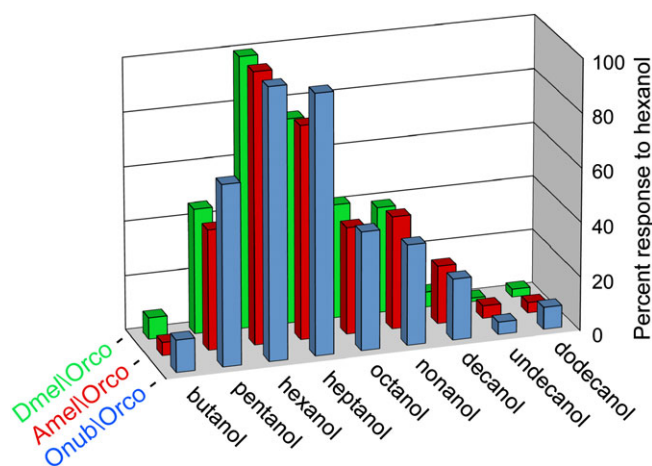


Figure 3 Highly similar alcohol specificity profiles of receptors formed by Dmel\Or35a and various Orco orthologs. Receptors formed by Dmel\Or35a and Dmel\Orco, Amel\Orco, or Onub\Orco were screened with a panel of primary alcohols (30 μ M). Responses were normalized to the response of each oocyte to hexanol (mean values are displayed; error and n values may be found in Supplementary Table 3).

length and containing various functional groups such as aldehydes, monocarboxylic acids, dicarboxylic acids, bromocarboxylic acids, ketones. We also tested the ester and amyl acetate. The relatively low current amplitudes obtained for Dmel\Or35a + Onub\Orco (Figure 1C) precluded use of this receptor complex in the screen. Comparison of the Dmel\Or35a + Amel\Orco and Dmel\Or35a + Dmel\Orco response profiles again revealed remarkably similar patterns of odorant responsiveness (Figure 4, Supplementary Table 3). We conclude that the Orco subunit exerts little or no influence on the odorant responsiveness of the receptor, suggesting that the Orco subunit does not make an important contribution to the structure of the odorant-binding site.

Exploration of the MRR of an OR that detects aromatic compounds

To further explore the role of a specificity subunit in insect OR function, we examined the odorant specificity of the receptor

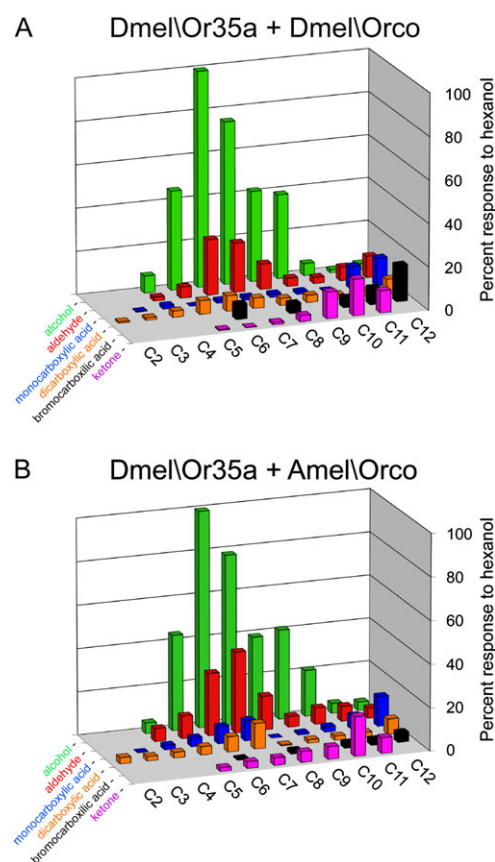


Figure 4 Highly similar aliphatic odorant specificity profiles of receptors formed by Dmel\Or35a and various Orco orthologs. Receptors formed by Dmel\Or35a and Dmel\Orco (panel A) or Amel\Orco (panel B) were screened with a panel of aliphatic odorants (30 μ M). Odorants are organized by functional group (z axis) and carbon length (x axis). Responses were normalized to the response of each oocyte to hexanol (mean values are displayed; error and n values may be found in Supplementary Table 3). Data for activation by alcohols are from Figure 3 and are shown for comparison. Data for activation by amyl acetate may be found in Supplementary Table 3.

formed by Dmel\Or67a, which is known to recognize and respond to aromatic compounds (Hallem and Carlson 2006). Information about the recognition of aromatic compounds, with their relatively rigid structure and potential for elaboration with multiple functional moieties, will be particularly useful in future studies of the odorant-binding site of insect ORs.

We have previously shown that several DmORs expressed in *Xenopus* oocytes display odorant specificities similar to what is observed in an in vivo neuronal context (Wanner et al. 2007; Nichols and Luetje 2010). Here, we show that the odorant specificity of Dmel\Or67a + Dmel\Orco was accurately reproduced when this receptor was expressed in *Xenopus* oocytes. Dmel\Or67a + Dmel\Orco has been screened with a broad panel of odorants in the in vivo “empty neuron” system (Hallem and Carlson 2006), and we selected 9 odorants from this panel to test the specificity of Dmel\Or67a in oocytes. We chose 6 odorants shown to activate the receptor (methyl benzoate, ethyl benzoate, amyl acetate, acetophenone, pentanoic acid, and 2-heptanone) and 3 odorants that did not activate the receptor (hexanal, heptanoic acid, and octanoic acid). The structures of these compounds are shown in Figure 5A. When each odorant was tested at the relatively high concentration of 1 mM, Dmel\Or67a + Dmel\Orco responded to each of the known agonists but was not activated by hexanal, heptanoic acid, or octanoic acid (Figure 5C). Thus, the odorant responsiveness displayed by Dmel\Or67a + Dmel\Orco when expressed in *Xenopus* oocytes is similar to the odorant specificity of this OR when assayed in vivo.

Based on the structures of the active and inactive compounds, we tested a panel of 17 additional structurally related compounds (Figure 5B,C). We explored the positioning of the ester group with respect to the phenyl ring in methyl benzoate and ethyl benzoate by testing methyl phenyl acetate. This compound, with the ester group moved away from the phenyl ring by one carbon, was also highly active at Dmel\Or67a. 2-coumaranone (essentially a cyclized methyl phenyl acetate) also activated Dmel\Or67a, albeit to a lesser extent. The activity of pentanoic acid suggested that a carboxylic acid moiety could substitute for the ester and, indeed, phenyl acetic acid strongly activated the receptor. The activity of phenyl acetic acid allowed us to ask several questions about the structural requirements for agonists of Dmel\Or67a. An aromatic ring was favorable as cyclohexylacetic acid only weakly activated the receptor. The single carbon between the carboxylic acid and the phenyl ring in phenyl acetic was optimal because benzoic acid, hydrocinnamic acid, and transcinnamic acid were all distinctly less active. Hydrocinnamaldehyde was also only modestly active. Interestingly, adding a second acetic acid moiety yielded a compound (1,2 phenylene diacetic acid) that retained moderate activity. However, 1,3 phenylene diacetic acid and 1,4 phenylene diacetic acid were distinctly less active. We also tested the aromatic ethers anisole and 2-methylanisole, as well as guaiacol. While anisole and guaiacol showed little or no activity, 2-methylanisole was moderately active at this receptor (Figure 5C). Agonist activity of an

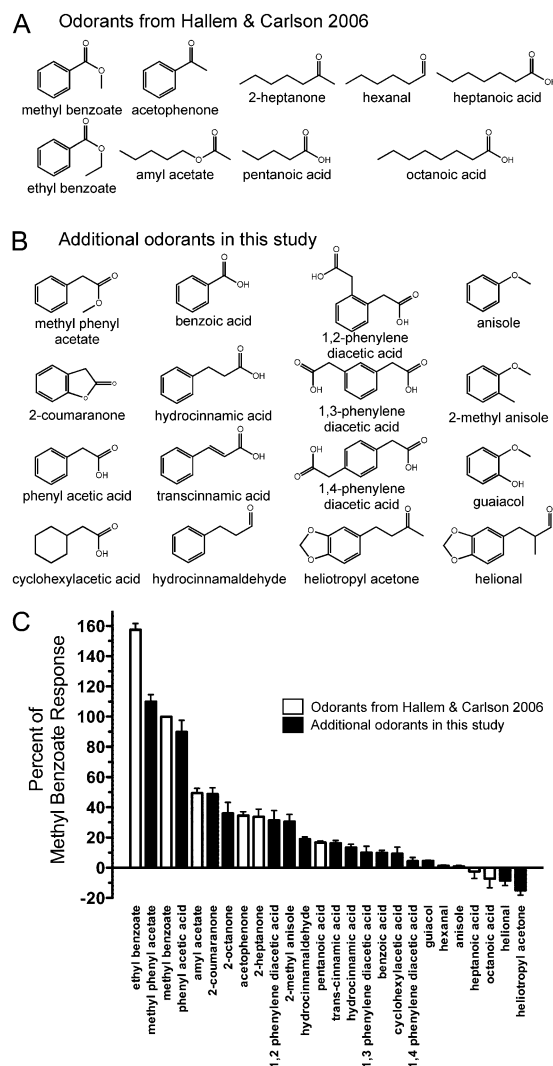


Figure 5 Exploring the odorant specificity of Dmel\Or67a + Dmel\Orco. **(A)** Structures of odorants previously tested in vivo (Hallem and Carlson 2006). **(B)** Structures of additional compounds tested in this study. **(C)** Dmel\Or67a + Dmel\Orco responsiveness to a panel of structurally related odorants (screened at 1 mM). Responses to each odorant are presented as a percentage of the response elicited by 1 mM methyl benzoate (mean \pm standard error of the mean, $n \geq 3$). White bars represent responses to odorants that were previously tested in vivo (Hallem and Carlson 2006). Black bars represent responses to additional compounds tested in this study.

ether suggested that the role of oxygen atoms in the active compounds was to provide a region of high electron density, rather than hydrogen bonding potential.

Next, we investigated the relative potency and efficacy (maximal response) of 5 of the most active Dmel\Or67a agonists by performing concentration–response analysis (Figure 6A). Methyl benzoate was used as a reference for normalization. These compounds exhibited a range of potency and efficacy values (Table 1). For example, while methyl phenyl acetate displayed potency and efficacy values ($EC_{50} = 21 \pm 3 \mu\text{M}$; efficacy = $84 \pm 4\%$) similar to that of

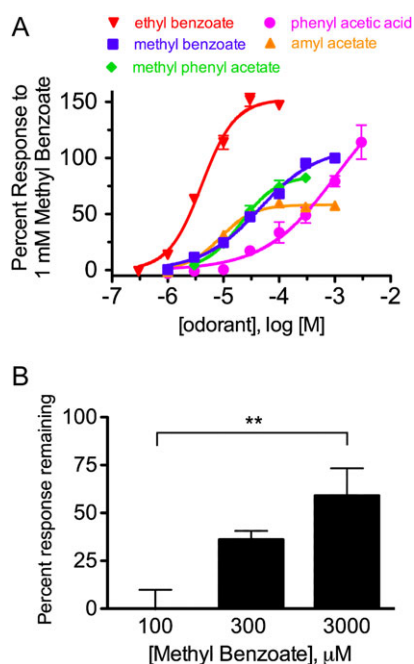


Figure 6 Agonists, partial agonists, and an antagonist of DmelOr67a + DmelOrco. **(A)** Concentration–response relationships for the activation of DmelOr67a + DmelOrco by various odorants. Responses were normalized to the response of each oocyte to 1 mM methyl benzoate and are displayed as mean \pm standard error of the mean (SEM) ($n \geq 3$). Parameters from the fit curves may be found in Table 1. **(B)** Heliotropyl acetone is a competitive antagonist of DmelOr67a + DmelOrco. Oocytes expressing DmelOr67a + DmelOrco were challenged with methyl benzoate (100, 300, or 3000 μ M) for 210 s, followed by coapplication of methyl benzoate and heliotropyl acetone (1000 μ M) for 60 s. Quantification of the block achieved by heliotropyl acetone was calculated by comparing the response to methyl benzoate alone to the response in the presence of methyl benzoate and heliotropyl acetone (bars are mean \pm SEM, 100 μ M: $n = 5$; 300 μ M: $n = 6$; 3000 μ M: $n = 6$). Statistical significance was assessed by one-way analysis of variance ($P = 0.0075$) followed by Bonferroni's multiple comparison test (** $P < 0.01$).

methyl benzoate ($EC_{50} = 44 \pm 7 \mu$ M; efficacy = 100%), amyl acetate was more potent ($EC_{50} = 9 \pm 1 \mu$ M) but less efficacious odorant ($57 \pm 3\%$). Phenyl acetic acid was the least potent of the tested compounds ($EC_{50} = 1070 \pm 183 \mu$ M), whereas ethyl benzoate was the most efficacious and most potent odorant tested ($EC_{50} = 4 \pm 0.4 \mu$ M; efficacy = $147 \pm 3\%$). Therefore, although DmelOr67a + DmelOrco was activated by many odorants at high concentrations, concentration–response analysis revealed a range of effectiveness in both potency and efficacy.

1,3 Phenylene diacetic acid and 1,4 phenylene diacetic acid, with acetic acid moieties on opposite sides of the aromatic ring, displayed only weak activity. This suggested that heliotropyl acetone and helional, with a methylenedioxy moiety opposite the ketone group, should not activate the receptor. Indeed, not only do these compounds not activate the receptor, they seem to inhibit the baseline current (Figure 5C). We decided to examine heliotropyl acetone in detail. To determine whether heliotropyl acetone was having a nonreceptor

Table 1 Quantification of DmelOr67a + DmelOrco agonist potency and efficacy

Odorant	EC_{50} (μ M)	Maximal response (as a percent of the response to methyl benzoate)
Ethyl benzoate	4 ± 0.4	147 ± 3
Phenyl acetic acid	1070 ± 183	114 ± 15 (at 3 mM)
Methyl phenyl acetate	21 ± 3	84 ± 4
Methyl benzoate	44 ± 7	100
Amyl acetate	9 ± 1	57 ± 3

EC_{50} values were derived from fitting the data in Figure 6A as described in Experimental Procedures. Efficacy (maximal response) values are presented as a percentage of the response elicited by 1 mM methyl benzoate. Values are mean \pm standard error of the mean.

mediated effect on the oocytes, we applied 1 mM heliotropyl acetone to sham (water)-injected oocytes. No effect was observed (Supplementary Figure 1D). It was also possible that what we were observing was an inhibition of a basal activity of the receptor (as has been suggested in Hallem et al. (2004)). To test this possibility, we applied 50 μ M RR to oocytes that were expressing DmelOr67a + DmelOrco but that had not yet been exposed to an agonist. No change in baseline current was observed (Supplementary Figure 1E). Thus, the most likely explanation was that what we were observing was an inhibition of a residual current that was present due to previous odorant applications. This suggested that heliotropyl acetone was an antagonist of DmelOr67a + DmelOrco. To test this idea, we coapplied 1 mM heliotropyl acetone with 100 μ M methyl benzoate. The methyl benzoate response was completely inhibited (Figure 6B). Importantly, the inhibition by heliotropyl acetone did not extend beyond baseline, providing further support for a lack of observable basal activity in our experimental preparation. When heliotropyl acetone was coapplied with higher concentrations of methyl benzoate, the extent of blockade was significantly reduced. We conclude that heliotropyl acetone is a competitive antagonist of methyl benzoate activation of DmelOr67a + DmelOrco.

Discussion

Some insect species exert a negative effect on human health by acting as disease vectors or by causing agricultural damage. Because a properly functioning olfactory system is essential for these insect behaviors, insect ORs are attractive targets for the development of olfactory-based interventions and manipulations. However, a lack similarity to any known class of receptor or channel (Benton et al. 2006) means that little is known about the structure of these receptors. Insect ORs are heteromeric complexes capable of gating a nonselective cation current upon odorant binding (Sato et al. 2008; Wicher et al. 2008). A functional OR can be reconstituted in heterologous

systems by coexpression of 2 OR subunits: a subunit common to all ORs (Orco) and one of many non-Orco subunits, but the total number of subunits in and subunit stoichiometry of the OR complex is currently unknown. Also, the relative contributions of each subunit type to basic functional features of ORs (odorant-binding, ion pore structure, gating mechanisms) are poorly understood. Here, we explore the contributions of insect OR subunits to various receptor functions.

The high conservation of the Orco subunit across insect species suggests a common role for this subunit in OR function (Jones et al. 2005). Although insect ORs can differ greatly in odorant specificity (Hallem et al. 2006; Carey et al. 2010), all are thought to contain a cation pore, suggesting that this highly conserved subunit may contribute to the structure of the pore. Heterologously expressed Orco homomers have been reported to mediate a cation current, although this current was reported to be gated by cyclic nucleotides, not odorants (Wicher et al. 2008). Interestingly, deletion of 2 residues within transmembrane segment 6 of Orco altered the current–voltage relationship of this presumed homomeric complex (Wicher et al. 2008). More recently, an Orco activating compound has been reported (Jones et al. 2011). This compound activated channels formed by Orco orthologs from various insect species when expressed alone or when Orco was expressed in combination with an odorant specificity subunit (Jones et al. 2011). Thus, the Orco subunit is likely to make structural contributions to the ion pore of insect ORs. To determine whether the odorant specificity conferring non-Orco subunits might also contribute to the structure of the ion pore, we used RR, which inhibits a wide variety of cation channels by lodging within the ion pore (Cibulsky and Sather 1999). It had been previously reported that the cation channel blocker RR (50 μ M) could fully inhibit the *B. mori* Bmor\Or1 + Bmor\Orco receptor, whereas having no effect on the *A. gambiae* Agam\Or2 + Agam\Orco receptor (Nakagawa et al. 2005; Sato et al. 2008). This differential sensitivity suggested RR as a probe for channel pore subunit contributions. Replacement of Dmel\Orco with Orco orthologs from other species (each receptor containing the same odorant specificity subunit, Dmel\Or35a) caused significant variation in RR sensitivity (Figure 1), further supporting the involvement of the Orco subunit in forming the ion pore. In addition, we found that formation of receptors with a series of non-Orco, odorant specificity subunits (each receptor containing the same Orco subunit, Dmel\Orco) resulted in highly variable sensitivity to RR inhibition (Figure 2). This result supports a role for the non-Orco subunits in forming the ion pore. If both Orco and a non-Orco subunit contribute to the structure of the ion pore, then the simplest model for OR structure would be a heterodimer with the pore formed at the interface between the subunits. However, the observation that Orco can participate in both heteromeric and homomeric interactions (Benton et al. 2006) suggests that more than one Orco may be present in the receptor. If so,

then the structure of an insect ORs may be a multimeric rosette of subunits surrounding a central ion pore. However, until more information about the number and stoichiometry of subunits in insect ORs is obtained, a variety of multisubunit models can be entertained.

It has been proposed that, in addition to a direct activation of the channel by odorant binding, odorant specificity subunits can also initiate a second messenger cascade that then activates the channel (Wicher et al. 2008). In support of this idea, an involvement of the $G\alpha_S$ G protein in olfactory signal transduction in *Drosophila* has recently been demonstrated (Deng et al. 2011). In contrast, a recent study demonstrated that ion channels formed by Agam\Orco, alone or in combination with an odorant specificity subunit, are not sensitive to cyclic nucleotides (Jones et al. 2011). Our finding of differential RR sensitivity supports a model of insect ORs as multisubunit complexes containing both an odorant-binding site (or sites) and an ion pore. However, our experiments do not directly address the potential involvement of a second messenger cascade.

The non-Orco specificity subunits exhibit high sequence variation, suggesting a role in the recognition of diverse odorant structures (Hallem and Carlson 2006; Carey et al. 2010). Changing specificity subunits in vivo is sufficient to confer odorant specificity (Hallem and Carlson 2004, 2006; Carey et al. 2010; Wang et al. 2010), and alteration of residues within a specificity subunit can change odorant preference (Nichols and Luetje 2010). Use of Quantitative Structure Activity Relationship analysis and comparative sequence analysis has identified a group of “specificity-determining residues” within the extracellular halves of the transmembrane domains of specificity subunits (Guo and Kim 2009). These lines of evidence strongly implicate specificity subunits in contributing to the structure of the odorant-binding sites in insect ORs. Our finding that the Orco subunit exerts little or no influence on odorant specificity (Figures 3 and 4) provides further support for a model in which the odorant-binding site is formed entirely by the non-Orco odorant specificity subunit. Although some portions of the Orco protein show significant variation among the various orthologs in different species, there are some regions in this protein that display extraordinarily high identity across species. For this reason, it is possible that lack of effect on odorant specificity that we observed in Figures 3 and 4 was due to the different Orco subunits contributing an identical component to the structure of the odorant-binding site. However, it is difficult to imagine how such an Orco contribution could play a role in the structure of the odorant-binding sites in ORs with widely varying odorant specificities. Thus, we continue to favor a model in which the odorant-binding site is formed entirely by the non-Orco odorant specificity subunit.

A pharmacophore is the collection of essential molecular features responsible for the activity of a drug at a receptor (Ehrlich 1909). Similarly, a collection of molecular features responsible for the ability of an odorant to interact with an

OR can be referred to as an “odorophore.” Although the general specificity of insect ORs has been investigated (Hallem and Carlson 2006; Carey et al. 2010; Wang et al. 2010), detailed odorophores for insect ORs have not been constructed. We have examined a series of structurally related compounds for activity at Dmel\Or67a + Dmel\Orco (Figures 5 and 6, Table 1) and used this information to construct a preliminary odorophore for this receptor. We propose a preliminary odorophore structure for Dmel\Or67a + Dmel\Orco agonists containing an aromatic ring, a region of high electron density and a region of hydrophobicity. Odorants containing all 3 of these features in an appropriate configuration (e.g., methyl benzoate, ethyl benzoate, and methyl phenyl acetate) display high potency and high efficacy agonist activity. Odorants containing 2 of these features can activate the receptor but appear to do so with lower potency (phenyl acetic acid) or lower efficacy (amyl acetate). Odorants lacking at least 2 of these features, or with inappropriately placed features (e.g., hydrocinnamic acid and benzoic acid), display little or no agonist activity. Placement of additional groups on the opposite side of the ring is particularly detrimental to agonist activity and one such compound (heliotropyl acetone) was found to be a competitive antagonist of the receptor. This preliminary odorophore can serve as the basis for more detailed exploration of the structural requirements for activation and inhibition of the Dmel\Or67a + Dmel\Orco receptor. A more detailed odorophore can assist in developing structural models of the odorant-binding sites of insect ORs. For example, identification of residues responsible for interaction with different odorant moieties within the binding pocket can provide spatial constraints used to render more accurate receptor models and ligand docking simulations. Such an approach has been highly successful in examining the structural features of odorant-binding sites of mammalian ORs (Araneda et al. 2000; Katada et al. 2005; Abaffy et al. 2007; Schmiedeberg et al. 2007; Peterlin et al. 2008)

Understanding the structure of insect ORs is of great interest due to the novel nature of these odorant-gated ion channels, which may serve as targets for future olfactory-based insect control strategies. The results we present here, as well as results previously published by us and others, suggest that these receptors are multimeric rosette structures, with each subunit contributing to the structure of a central ion pore and that the odorant-binding site is located among the transmembrane domains of the non-Orco odorant specificity subunit. However, many basic features remain to be elucidated, such as the total number of subunits and the subunit stoichiometry of these receptors. At a finer level of detail, it is not yet possible to model the structure of the odorant-binding site of an insect OR. Generation of odorophores as we have done here, combined with more extensive mutational and functional analysis of the sort that we recently reported (Nichols and Luetje 2010), will provide valuable structural constraints that will aid in future insect OR modeling attempts.

Supplementary material

Supplementary material can be found at <http://www.chemse.oxfordjournals.org/>.

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