Mutational Analysis of Cysteine Residues of the Insect Odorant Co-receptor (Orco) from Drosophila melanogaster Reveals Differential Effects on Agonist- and Odorant-tuning Receptor-dependent Activation

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Background: Orco is a highly conserved co-receptor required for insects to respond to odorants.

Results: Mutation of two cysteine residues in the third intracellular loop of Orco increases direct agonist, but reduces odorant-tuning receptor-dependent channel activation.

Conclusion: Intracellular loop 3 is important for activation of the Orco channel.

Significance: The research identifies a region of Orco that may regulate odorant sensing by insects.

Insect odorant receptors are heteromeric odorant-gated cation channels comprising a conventional odorant-sensitive tuning receptor (ORx) and a highly conserved co-receptor known as Orco. Orco is found only in insects, and very little is known about its structure and the mechanism leading to channel activation. In the absence of an ORx, Orco forms homomeric channels that can be activated by a synthetic agonist, VUAA1. Drosophila melanogaster Orco (DmelOrco) contains eight cysteine amino acid residues, six of which are highly conserved. In this study, we replaced individual cysteine residues with serine or alanine and expressed Orco mutants in Flp-In 293 T-Rex cells. Changes in intracellular Ca$^{2+}$ levels were used to determine responses to VUAA1. Replacement of two cysteines (Cys-429 and Cys-449) in a predicted intracellular loop (ICL3), individually or together, gave variants that all showed similar increases in the rate of response and sensitivity to VUAA1 compared with wild-type DmelOrco. Kinetic modeling indicated that the response of the Orco mutants to VUAA1 was faster than wild-type Orco. The enhanced sensitivity and faster response of the Cys mutants was confirmed by whole-cell voltage clamp electrophysiology. In contrast to the results from direct agonist activation of Orco, the two cysteine replacement mutants when co-expressed with a tuning receptor (DmelOR22a) showed an ∼10-fold decrease in potency for activation by 2-methyl hexanoate. Our work has shown that intracellular loop 3 is important for Orco channel activation. Importantly, this study also suggests differences in the structural requirements for the activation of homomeric and heteromeric Orco channel complexes.

Many of the behaviors important for insects depend on olfaction. Odorant receptors are one of the main chemosensory receptor families in insects and are responsible for detecting a wide variety of volatile molecules in the environment (1). These receptors are located on the dendrites of odorant receptor neurons, where they interact with ligands, resulting in depolarization and signals that reach the corresponding glomerulus in the antennal lobe.

Insect ORs are novel seven-transmembrane domain proteins with an inverted topology compared with G protein-coupled receptors (2–4). They have been shown to function as odorant-gated, non-selective cation channels (5, 6). These channels may also be regulated by metabotropic pathways (1, 5, 7–9). Insect ORs comprise heteromeric complexes containing both a conventional, odorant-sensing, or tuning receptor (ORx), and a co-receptor, now known as Orco (2, 10). The stoichiometry of OR complexes is unknown. The conventional ORs are highly divergent and provide selectivity to a broad range of odorant compounds (11). Their expression is restricted to specific odorant receptor neurons (11–13). In contrast, Orco is broadly expressed in odorant receptor neurons and is essential for the response of ORs to odorants (14). Orco is highly conserved across insects, and in vivo, it is required for conventional OR trafficking and the localization of ORs to ciliated dendrites of odorant receptor neurons (2, 15). Because Orco is required for every insect OR, chemicals that affect Orco function may disrupt insect behavior and provide a means of pest control. When heterologously expressed, Orco is capable of forming functional channels in the absence of a conventional receptor (5, 16). Screening of cell lines expressing these homomeric channels led to the discovery of VUA1 as a novel allosteric agonist of insect ORs (16). Further exploration of the structure-
activation relationships of VUAA1 identified several more potent Orco allosteric agonists (17–19). A number of compounds structurally related to VUAA1 that are inactive as agonists act as competitive inhibitors of VUAA1 (17, 18, 20). Both heteromeric and homomeric Orco complexes can be activated by VUAA1 (16). Heteromeric and homomeric OR complexes are also blocked by the general cation channel blocker ruthenium red (21, 22) and by amiloride derivatives (23, 24).

Currently, there is little knowledge about the structure of the Orco channel, how Orco interacts with an ORx, and the mechanisms involved in channel activation and permeability. The predicted topology of Orco consisting of seven transmembrane domains with the N terminus facing the cytoplasm and C terminus on the extracellular surface is supported by a range of experimental techniques (2, 4). Bioluminescence and Förster/fluorescence resonance energy transfer experiments have been used to show interactions between Orco and an ORx (25, 26). The predicted intracellular loop 3 (ICL3) regions of both DmelOR43a and DmelOrco were shown to interact in a yeast two-hybrid assay, consistent with the observation that the C-terminal region of OR43a is sufficient for Orco dependent transport to olfactory cilia (2). Although Orco can form channels on its own (5, 16) and Orco does not contribute to the odorant binding site (21, 27), studies indicate the presence of odorant-specific ORs may influence ion permeability and inhibitor sensitivity of heteromeric OR complexes (21, 22, 28).

Taken together, these studies support the idea that both the odorant-specific tuning OR and Orco contribute to the channel pore. Alternatively, it is possible that the interaction of specific ORs with Orco may indirectly affect the properties of the channel pore. There is some information on the contribution of individual amino acid positions of Orco to OR function. Modification of a TVVGYLG sequence in TM6 of DmelOrco reduced K⁺ permeability (5) and a Y464A mutation in TM7 of the Bombyx mori Orco in combination with B. mori OR-1 results in a small increase in K⁺ selectivity (28). Furthermore, we recently showed that a conserved aspartic acid residue in TM7 is important for the activation of both homomeric channels by VUAA1 and heteromeric channels by odorants (29).

DmelOrco contains a number of cysteine residues in predicted ICLs (see Fig. 1). We hypothesized that these may contribute to structural features important for function. Here, we have carried out a mutagenesis study and find that replacement of two cysteines in ICL3 has differential effects on agonist- and odorant-tuning receptor-dependent activation.

**EXPERIMENTAL PROCEDURES**

*Expression Plasmids for DmelOrco and DmelOR22a—*The modification of DmelOrco to include an N-terminal Myc epitope and its cloning into the pcDNA5/FRT/TO vector has been described previously (29). The *Drosophila melanogaster* OR22a cDNA was obtained from Dr. Coral Warr (Monash University, Melbourne, Australia). This was cloned into pBluescript II using KpnI and SacII sites and subsequently transferred into pcDNA3.1+ (Invitrogen). Lastly, a FLAG epitope (DYKDDDK) was inserted after the initiator methionine, and the sequence around the initiation code was altered to a mammalian Kozak consensus sequence by PCR.

*Site-directed Mutagenesis and Preparation of Flp-In 293 T-Rex Cell Lines—*The pcDNA5/FRT/TO-DmelOrco template was mutated to encode the C87S, C216A, C221S, C228S, C409S, C429S, C446S, and C449S Orco variants by GenScript USA, Inc. The C429S/C449S double mutant was prepared using C429S as a template and a method adapted from the QuikChange site-directed mutagenesis kit (Stratagene) and Ref. 30. Two complementary oligonucleotides (29–33 bp) encoding the C449S mutation were obtained from Integrated DNA Technologies. The cycling reaction used *Pfu* Turbo DNA Polymerase (Agilent Technologies) and the presence of 1X PCR Enhancer solution (Invitrogen). The PCR products were treated with DpnI (Invitrogen) to remove the template DNA and used to transform competent *Escherichia coli* DH5α cells prepared as described in Ref. 30. The presence of the desired mutation and absence of introduced mutations were confirmed by sequencing the N-terminal Myc DmelOrco insert. KpnI and NotI sites were used to transfer the insert into fresh vector. Plasmids encoding the cysteine replacement mutants were transfected into Flp-In 293 T-Rex cells that were grown and selected for hygromycin resistance as described previously (29).

*Ca²⁺ Imaging—*Flp-In 293 T-Rex cells encoding WT DmelOrco and the Cys replacement mutants were plated (50,000 cells/well) in 96-well clear bottom, black-walled plates (BD Biocat, catalog no. 356640). After 1 day, cells were treated with 0.1 μg/ml doxycycline for 24 h to induce Orco expression. The medium was then removed, and the cells were loaded (30 min at 37 °C, followed by 1 h at room temperature) with Fluo-4 NW (Invitrogen) prepared as suggested by the manufacturer in Hank’s buffer containing Ca²⁺ and Mg²⁺. To investigate odorant activation, WT DmelOrco and Orco Cys replacement mutants were plated in six-well plates (400,000 cells/well), left for 24 h, and transfected with DmelOR22a (2 μg/well) using FuGENE 6 (Promega, 6 μl/well) for 12 h. Cells (80,000 cells/well) were transferred to 96 well assay plates prior to being induced with 0.3 μg/ml of doxycycline for 12–13 h prior to the assay. The cells were loaded with Fluo-4 AM (Molecular Probes) and washed prior to the assay as described previously (29). Ca²⁺ fluorescence was measured in an Envision multilabel plate reader (PerkinElmer Life Science). The following settings were used: excitation filter, FITC 485 nm; emission filter, 520 nm; bottom-fitted dichroic mirror, FITC 505; bottom excitation, bottom sensor; measurement distance: 6.5 mm. Fluorescence readings were taken every 0.4 s, except for the analysis of the time course kinetics when readings were taken every 0.1 s. The Orco allosteric agonist, VUAA1 (N-(4-ethylphenyl)-2-((4-ethyl-5-(3-pyridinyl)-4H-1,2,4-triazol-3-yl)(thio)acetamide), purchased from Interbioscreen, Ltd. (ID STOCS-70586); or odorant methyl hexanoate (Sigma, CAS 106-70-7) were freshly diluted from dimethyl sulfoxide stocks into Hank’s buffer to six times the desired test concentration and injected (20 μl into 100 μl) automatically after 8 s. The final concentration of dimethyl sulfoxide did not exceed 0.25%, and all experiments included controls with buffer and dimethyl sulfoxide alone.

*Cell Surface Biotinylation and Western Blotting—*Flp-In 293 T-Rex cell lines expressing DmelOrco and its variants were plated (700,000 cells/well in six-well plates, grown for 24 h, and induced with 0.1 μg/ml doxycycline for 24 h. Proteins at the cell
surface were labeled with a membrane impermeable biotinylation reagent. The procedure for biotinylation was based on that used previously (29). Briefly, the cells were washed with PBS and labeled with EZ-Link-Sulfo-NHS-SS-Biotin (ThermoScientific, 1.5 mg/ml; 0.6 ml/well) for 20 min on ice. The labeling was repeated once with fresh reagent. The reagent was removed, and the cells were washed and lysed in 350 μl of 20 mM Tris, 137 mM NaCl, 1 mM EDTA, pH 7.6 buffer, containing 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and protease inhibitors (Complete™ Mini protease inhibitor mixture, Roche Molecular Biochemicals). The lysate was obtained by centrifugation, and the protein concentration was determined (Bio-Rad DC Protein Assay). Lysates were adjusted if necessary by dilution with lysis buffer. Biotinylated proteins were purified from lysate samples (typically 286 μl, 1.4 mg/ml protein) by the addition (50 μl of a 50% suspension) of Neutravidin beads (ThermoScientific). Following incubation overnight at 4 °C, the beads were recovered by centrifugation and washed to remove non-specifically bound proteins. The beads were resuspended with an equal volume of a high-SDS reducing buffer (125 mM Tris, 8% SDS, 20% glycerol, and 10% β-mercaptoethanol) and incubated for 1 h at room temperature. Aliquots (17.5 μl) of the lysate and biotinylated samples were run on 10% SDS-polyacrylamide gels and subjected to Western blotting (31). The blot was probed with mouse anti-Myc antibodies (Santa Cruz Biotechnology, sc-40) followed by goat anti-mouse horseradish peroxidase conjugate (Bio-Rad, 170-6516). Chemiluminescence was detected using a Fuji LAS-1000 digital imaging system. A WesternBright ECL detection kit (Advansta) was used for samples from total cell lysates, whereas the more sensitive WesternBright Sirius kit (Advansta) was used for biotinylated samples. Exposure times were between 20–60 s for both detection systems. Bands were quantified using MultiGauge software (version 2.2, Fujifilm). Band intensity for each lane was calculated from the number of black pixels in a box placed around the band minus the black pixels in a box of the same size placed around a background region from the same blot. The expression of Orco Cys-replacement mutants is expressed relative to samples of WT Orco run in every experiment.

Whole-cell Patch Clamp Electrophysiology—Stably transfected cells were induced using 0.1 μg/ml doxycycline for 12 to 24 h before recording. Whole-cell recording was carried out as described previously (16). All cells were voltage clamped at −60 mV and Clampfit 10 (pClamp 10, Axon Instruments) was employed to calculate the rise time, which was defined as the time during which the magnitude of the inward current remained within 10 and 90% of the maximal inward current. Therefore, the changes in Ca2⁺ fluorescence in response to Orco activation were defined as the fluorescence minus the background fluorescence, all divided by the background fluorescence (determined from the average of the first 20 readings prior to the addition of agonist). Log values of the relative activity of Cys-replacement Orco mutants compared with WT Orco determined in the same experiment were used for statistical analysis.
variations in cell surface expression. To address this, Western blot experiments were carried out to compare the expression of Orco mutants with WT Orco in both total cell lysates and in a purified “biotinylated” fraction obtained after labeling of intact cells with sulfo-NHS-SS-biotin. Here, Orco was detected with antibodies against the Myc epitope, and data from individual representative experiments are shown in Fig. 3, A and B. The C409S mutant appeared to be present at higher levels than WT Orco in both total and cell surface fractions. A quantitative comparison of the relative expression of mutants confirmed that C409S Orco was expressed at significantly higher levels (3-fold) than WT Orco (Fig. 3C). A small but significant increase in total and surface expression was also observed for C446S Orco. Otherwise, the expression levels of the Cys replacement mutants were similar to WT Orco.

The properties of mutants (C409S, C429S, C449S, and the C429S/C449S double mutant) showing increased levels of VUAA1-stimulated activity in the Ca²⁺ flux assay (Fig. 2B) were studied in more detail. Fig. 4A shows representative concentration response curves for the Cys replacement variants, and WT Orco and indicates that the curves of variants containing the C429S and C449S mutations individually and in combination are “left-shifted” compared with WT Orco. The EC₅₀ values determined from multiple experiments were ~2-fold lower than WT Orco (Fig. 4B), indicating the enhanced sensitivity of these variants to activation by VUAA1. The EC₅₀ value of C409S was similar to WT Orco but gave a higher E₅₀ (max) value in accordance with the increased expression level this mutant.

Although the concentration response curves indicate that the C429S, C449S, and the C429S/C449S mutants have enhanced sensitivity to activation by VUAA1, the experiments in Fig. 2 suggested that they may also respond faster than WT Orco. However, the time course for the change in intracellular Ca²⁺ in response to VUAA1 is complex (Fig. 2A). There are multiple components, most obviously an increase followed by a decrease in intracellular Ca²⁺ (most clearly seen for the mutants showing the highest Ca²⁺ response). To further clarify this point, we more closely analyzed the response of WT Orco during the phase where intracellular Ca²⁺ is increasing, revealing that the initial Orco response exhibits a sigmoid time course (Fig. 5). We further asked whether a simple two-step reaction mechanism A → B → C → D could explain the observed kinetics. As can be seen, a mathematical simulation based on this equation generates a curve that provides an excellent fit to the
observed kinetics (Fig. 5). Data collected from the response of WT Orco and the Cys replacement mutants at different VUAA1 concentrations were used to calculate the second order rate constant ($k_1$) for the first reaction (A $\rightarrow$ B) and the rate constant ($k_2$) of the second step (C $\rightarrow$ D). This modeling suggests that each of the Cys replacement mutants facilitate the activation response in two respects: a concentration-dependent interaction of Orco with the agonist VUAA1 and also a non-VUAA1 dependent step required for entry of Ca$^{2+}$ into the cell.

The rate constants also suggest that the overall response of the Cys replacement mutants to VUAA1 is faster than for WT Orco.

Measurements of changes in intracellular [Ca$^{2+}$] are fast and sensitive and have been used extensively to characterize the dose-response characteristics of allosteric Orco agonists and antagonists (16, 18, 19) as well as Orco mutations that affect the activation of both homomeric channels by VUAA1 and heteromeric channels by odorants (29). Although these assays do not directly measure voltage changes, the response to VUAA1 does however depend on fluxing of extracellular Ca$^{2+}$ (data not shown), so increases in Ca$^{2+}$ fluorescence do reflect cation channel activity. Nevertheless, we did elect to provide additional data of these new Orco mutants through recording of ionic currents in patch clamped single cells (Fig. 6). As expected, VUAA1 elicited concentration-dependent increases in the inward current of WT and mutant Orco cell lines (Fig. 6A). The concentration response curves indicated that the two
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Cys replacement mutants were more sensitive to VUAA1 than WT Orco (Fig. 6B), although only the EC_{50} values for C449S and the C429S/C449S double mutant were significantly different. To compare the relative rates of response, we defined “rise time” as the time during which the magnitude of the inward current remained within 10 and 90% of the maximal inward current. A comparative analysis of the rise times suggested that the Cys replacement mutants responded faster than WT Orco, although the values were only significantly different from WT Orco for the C429S/C449S mutant (Fig. 6).

We wanted to determine whether the increased activation responses of C429S, C449S, and C429S/C449S Orco to VUAA1 (Figs. 4–6) extended to odorant activation of Orco when co-expressed with a conventional ORx. Cells stably expressing WT Orco or the Cys replacement mutants were transiently transfected with DmelOR22a and the change in intracellular Ca^{2+} determined in response to the addition of the OR22a agonist, methyl hexanoate (Fig. 7). The concentration response curves for the Cys replacement mutants were all “shifted to the right” compared with Orco. This change reflects an ~10-fold decrease in the potency of the Cys replacement mutants to methyl hexanoate compared with WT Orco. Thus, it appears that the replacement of cysteine residues at these positions impairs the activation of Orco by odorants in the presence of an ORx.

DISCUSSION

Although the molecular mechanisms by which direct agonists and odorants through interactions with specific odorant receptors activate the Orco channel remain largely unknown, the studies described here show the involvement of the ICL3 region in the activation of Orco. Interestingly, our functional analyses of cysteine replacement mutants suggest perturbation of ICL3 differentially affects direct Orco agonist- versus odorant-tuning receptor-dependent activation of the channel.

The conservation of six of the eight cysteines in DmelOrco in equivalent positions in Orco orthologues across a wide range of insect taxa provided a strong rationale to investigate their functional importance. We chose initially to characterize cysteine replacement mutants in the absence of a tuning receptor. This approach avoids complications from the possible effects of mutations on the interactions of Orco with the tuning receptor or from the presence of mixtures of both homomeric and heteromeric Orco complexes. Cys replacement Orco mutants were stably expressed in Flp-In 293 T-Rex cells lines to avoid potential difficulties from differences in transfection efficiency. These tetracycline-inducible lines are suitable for studies of Orco function by both microplate assays of Ca^{2+} influx and direct current recording of patch clamped cells (16, 29). Ideally, the surface expression of all mutants would be compared with WT Orco through direct binding assays with specific agonists or antagonists. In the absence of specific reagents for Orco, we used an N-terminal Myc epitope to compare the expression of mutants relative to WT Orco by cell-surface biotinylation and Western blotting. C409S Orco was the only mutant found to be expressed at substantially higher levels than WT Orco (Fig. 3). Western blotting. C409S Orco was the only mutant found to be expressed at substantially higher levels than WT Orco (Fig. 3).
with WT Orco (Figs. 2 and 4). The increased sensitivity of the cysteine replacement mutants to VUAA1, shown by assays of Ca\(^{2+}\)/H\(_{11001}\) influx, was supported by direct current analysis of WT and mutant Orco cell lines (Fig. 6B), although the effects in these whole cell patch clamp studies were not as dramatic. The differences between the EC\(_{50}\) values of the WT and Cys replacement mutants were greater in the Ca\(^{2+}\)/H\(_{11001}\) flux assays than for the patch clamp experiments. There are several inherent differences between whole cell patch clamp studies and Ca\(^{2+}\)/H\(_{11001}\) flux assays that likely contribute to this partial disconnect. The most obvious difference is that data from the Ca\(^{2+}\)/H\(_{11001}\) assays is obtained from the collective response of large numbers of cells to a single concentration of VUAA1, whereas in the whole cell patch clamp experiments, direct voltage recordings are taken from individual cells exposed to increasing doses of VUAA1 separated by washing. In this light, we cannot rule out that this paradigm does not result in some degree of Orco channel desensitization. It is also apparent that in some cases the inward current has not reached a steady state that may also likely affect the EC\(_{50}\) values. That said, it is especially salient that the increased sensitivity to VUUA1, most notably for the C449S and the C429S/C449S double mutant, is also seen in the whole cell current recording experiments.

There are many possible reasons for the increases in sensitivity to VUAA1 shown by the mutants. Replacement of a cysteine with a serine is commonly used in mutagenesis experiments, but nevertheless, differences between the properties of the two amino acid side chains may affect the structure of the ICL3 region. Such an alteration may improve the direct interactions with VUAA1 resulting in increased sensitivity to activation by this agonist. Some possibilities include improved access for VUAA1 to its binding site or by making it easier for VUAA1 binding to initiate the conformational changes required for activation of the channel.

We wanted to probe the mechanism for the observed increase in the efficacy of VUAA1 to activate the C229S, C449S, and the C229S/C449S variants in more detail. First, we investigated whether a simple two-step reaction (A → B → C → D) could be used to model the kinetics for the increase in intracellular Ca\(^{2+}\) following VUUA1 activation. This is very similar to the del Castillo-Katz mechanism for the transduction mechanism for the endplate nicotinic acetylcholine receptor, as
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FIGURE 7. The sensitivity of WT and the Cys replacement mutants of Orco from D. melanogaster to methyl hexanoate in the presence of the D. melanogaster OR22a (DmelOR22a). WT Orco, C429S, C449S, and C429S/C449S Orco Flip-In 293 T-flox cells were transfected with DmelOR22a, and Orco expression was induced with doxycycline. A, concentration response analysis to methyl hexanoate. The data for the concentration response curves were obtained from a single experiment (mean ± S.D. of four to six replicates). B, log EC_{50} values for the response of WT Orco and C429S, C449S, and C429S/C449S Orco to methyl hexanoate. The values represent the mean ± S.E. from three to five experiments. The values for C429S, C449S, and C429S/C449S Orco were significantly different from WT Orco. ***: p < 0.001; n.s., not significant. The mean EC_{50} values for WT, C429S, C449S, and C429S/C449S Orco are 1.17, 11.93, 9.64, and 10.94 μM methyl hexanoate, respectively. n.s., not significant.

reviewed in Ref. 32. In this mechanism, the agonist binds to a receptor; the receptor in the “bound” state is still inactive but can undergo a conformational change to the active (open) state. Mathematical modeling of this mechanism was found to fit the experimental data very well (best fits, typically displayed r^2 values better than 0.998). The equation enabled the calculation of the two rate constants: k_1 (A + B → C); and k_2 (C → D) for a range of VUA1 concentrations for both WT Orco and the Cys replacement mutants (Fig. 5). Separate graphical analysis identified k_1 as a second order rate constant (dependent on the concentration of VUA1), whereas k_2 was not concentration-dependent, consistent with it being associated with a step subsequent to VUA1 binding. Interestingly, the k_1 values for the Cys replacement mutants were higher than WT Orco, which would be consistent with VUA1 binding occurring more rapidly. The k_2 rate constant indicates that the C → D step is also faster for the Cys replacement mutants. The faster response kinetics for the cysteine replacement mutants was supported by the direct measurement of inward currents in the mutant cell lines (Fig. 6C). The rise time for the C429S/C449S double mutant was significantly shorter than for WT Orco, confirming that the mutant does indeed respond faster. We have only considered the “forward” reaction leading to opening of the channel gate and appreciate that k_1 and k_2 will be affected by both the forward and reverse reactions, e.g. improved efficacy to VUA1 could result from mutations that facilitate binding or access of VUA1, or alternatively, decrease the “off” reaction for VUA1 binding. Similarly, our studies have not considered alternatives such as deactivation of the Orco channel.

ICL3 is predicted to act as a cytoplasmic link between TM6 and TM7, regions known to contain amino acid residues important for channel activity (5, 28, 29). Thus, this region is well positioned to play a role in the activation of insect odorant receptors. Previously, we have shown that substitution of Asp-466 in TM7 of DmelOrco with Glu (D466E) increased the sensitivity of DmelOrco activation to both VUA1A1 and also to odorants in the presence of a specific odorant receptors (29). An intriguing feature of the current work is that the increased sensitivity of the C229S and C449S mutants in homomeric Orco complexes for VUA1 was not matched by increased sensitivity of an Orco/odorant receptor complex to an odorant. In fact, reduced sensitivity to the odorant was observed. We suggest that the Orco mutations in the ICL3 region impact the ability of OR22a, in the presence of methyl hexanoate, to induce the conformational changes required for activation of the channel. An interaction between the ICL3 region of Orco and the ICL3 region of an ORx has been demonstrated in yeast two-hybrid studies, a finding also consistent with regions required for Orco-dependent trafficking of OR complexes to olfactory cilia (2). We consider the similar F_{max} values determined for the Cys replacement mutants and Orco make it unlikely that the 10-fold increase in the EC_{50} values of the C229S, C449S, and C229S/C449S Orco variants for methyl hexanoate can be explained by these mutations affecting the interaction of Orco with DmelOR22a or the trafficking of heteromeric complexes to the cell membrane. Unfortunately, we were unable to confirm that similar amounts of OR22a were expressed at the surface of cells expressing WT and mutant Orco. Despite the presence of an N-terminal FLAG epitope, we could not detect OR22a in whole cell lysates or biotinylated fractions of transiently transfected cells by Western blotting.

The D466E mutation was suggested to induce a conformational state that favors opening of the channel common to both VUA1 and odorant/ORx agonism (29). In separate experiments, we combined the C429S/C449S mutation with the previous D466E gain-of-activation mutant. The concentration response curve of the triple mutant transfected with OR22a to methyl hexanoate (data not shown) indicated that the additional mutation partially (~50%) reversed the decrease in sensitivity resulting from the C229S and C449S mutations. This suggests that the ICL3 and TM7 mutations target different stages of the activation response as the addition of D466E improves but does not restore the loss of sensitivity to activation of Orco by OR22a.

In summary, we have provided evidence that ICL3 of Orco plays an important role in the activation of insect odorant channels by both direct agonists and odorants through tuning ORs. We suggest that an alteration to the structure of the ICL3 region is responsible for the decreased responsiveness seen to odorants. It would seem quite plausible for a structural change that improves activation by VUA1, a non-physiological allosteric agonist, to interfere with activation by a tuning receptor in the presence of an odorant.

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