Mutant cycle analysis identifies a ligand interaction site in an odorant receptor of the malaria vector *Anopheles gambiae*

Received for publication, August 3, 2017, and in revised form, September 27, 2017. Published, Papers in Press, September 29, 2017, DOI 10.1074/jbc.M117.810374

Suhaila Rahman1 and Charles W. Luetje2

From the Department of Molecular and Cellular Pharmacology, University of Miami Miller School of Medicine, Miami, Florida 33101

Edited by Joseph Jez

Lack of information about the structure of insect odorant receptors (ORs) hinders the development of more effective repellents to control disease-transmitting insects. Mutagenesis and functional analyses using agonists to map the odorant-binding sites of these receptors have been limited because mutations distant from an agonist-binding site can alter agonist sensitivity. Here we use mutant cycle analysis, an approach for exploring the energetics of protein–protein or protein–ligand interactions, with inhibitors, to identify a component of the odorant-binding site of an OR from the malaria vector, *Anopheles gambiae*. The closely related odorant-specificity subunits Agam/Or15 and Agam/Or13 were each co-expressed with Agam/Orco (odorant receptor co-receptor subunit) in *Xenopus* oocytes and assayed by two-electrode voltage clamp electrophysiology. We identified (−)-fenchone as a competitive inhibitor with different potencies at the two receptors and used this difference to screen a panel of 37 Agam/Or15 mutants, surveying all positions that differ between Agam/Or15 and Agam/Or13 in the transmembrane and extracellular regions, identifying position 195 as a determinant of (−)-fenchone sensitivity. Inhibition by (−)-fenchone and six structurally related inhibitors of Agam/Or15 receptors containing each of four different hydrophobic residues at position 195 served as input data for mutant cycle analysis. Several mutant cycles, calculated from the inhibition of two receptors by each of two ligands, yielded coupling energies of ≥1 kcal/mol, indicating a close, physical interaction between the ligand and residue 195 of Agam/Or15. This approach should be useful in further expanding our knowledge of odorant-binding site structures in ORs of disease vector insects.

Many debilitating and deadly diseases are transmitted by hematophagous insects, such as mosquitoes (malaria, yellow fever, dengue, Zika virus, West Nile virus), triatomine bugs (Chagas disease), tsetse flies (sleeping sickness), and sand flies (leishmaniasis) (1). Mosquitoes use a variety of olfactory cues, as well as heat and visual cues, to locate and feed on humans (2, 3). Olfactory cues are detected using receptors from several families, including odorant receptors (ORs), glutamate receptor-like “ionotropic receptors,” and some members of the gustatory receptor family (4–9). The multimodal approach that mosquitoes use to locate humans means that the development of new, more effective control strategies can’t be achieved by attacking any single component. For example, genetic ablation of ORs, or of a gustatory receptor that mediates CO2 detection, impairs but does not eliminate the attraction of *Aedes aegypti* to humans (8, 10). Effective control of disease vector insects will require a multimodal attack (8), targeting multiple receptor families. Including the ORs in this attack is critical, because ORs help mediate a preference for humans and final targeting to specific skin regions (4, 6, 8, 10, 11).

Insect ORs are a novel class of ligand (odorant)-gated ion channel located on the dendrites of olfactory sensory neurons (12–14). ORs are heteromeric complexes composed of an invariant subunit (the odorant receptor co-receptor subunit, known as Orco (15)) that is highly conserved across species (16–22) and one of a large number of highly variable subunits that confer odorant specificity. The specificity subunits are thought to be the primary binding site for odorants (4, 9, 23–25), whereas both Orco and the specificity subunits are thought to contribute to the structure of the ion pore (13, 26, 27). The number of subunits needed to form an OR and the stoichiometry of these subunits are currently unknown. These receptors may also initiate, or be modified by, second messenger cascades (14, 28, 29).

Although we currently lack an atomic structure for any insect OR, a variety of approaches have recently been used to gain some insight into the structural basis for insect OR function (12). Of particular interest is identification of residues that form the binding site for odorant ligands. Several studies have used mutagenesis and functional analysis to identify residues in transmembrane domain (TMD) 2, the extracellular ends of TMDs 3 and 4, as well as extracellular loop 2, as being determinants of sensitivity to various odorants (24, 25, 30–32). However, limitations of these various approaches precluded defini-

---

1 Present address: Dept. of Molecular Physiology & Biophysics, Vanderbilt University, Nashville, TN 37232.

2 To whom correspondence should be addressed: Dept. of Molecular and Cellular Pharmacology, R-189, University of Miami Miller School of Medicine, P.O. Box 016189, Miami, FL 33136. Tel.: 305-243-4458; E-mail: cluetje@med.miami.edu.

3 The abbreviations used are: OR, odorant receptor; TMD, transmembrane domain; ACE, acetophenone; FEN, (−)-fenchone; ADM, 2-adamantanone; DEB, 7,7-diethylbicyclo[2.2.1]heptan-2-one; NOR, norcamphor; CAM, d-camphor; CIN, 1,4-cineole; EUC, eucalyptol.
tive assignment of the identified residues to the odorant-binding site. For example, the odorant ligands used in most studies are agonists, and mutations almost anywhere in a receptor structure can alter agonist sensitivity (33). Here, we sought to identify one or more residues that physically interact with a ligand molecule occupying the odorant-binding site in an OR from the malaria vector *A. gambiae*. To do this, we used thermodynamic mutant cycle analysis, an approach developed for exploring the energetics of protein–interactions (34–38) that has been adapted to the study of ligand–receptor interactions (39–43).

**Results**

**Identification of inhibitors of Agam/Or15 and Agam/Or13**

To use mutant cycle analysis to identify a site of ligand interaction within an odorant-binding site, two things are required: a series of structurally related competitive inhibitors of odorant activation of an OR and a site within the odorant-binding subunit of this OR at which mutagenesis alters sensitivity to the competitive inhibitors. We chose to use Agam/Or15 and Agam/Or13 to develop the latter requirement because these two odorant specificity subunits are closely related (82% amino acid identity) and because a panel of Agam/Or15 mutants, encompassing all residue differences between Agam/Or15 and Agam/Or13 within the extracellular and transmembrane domains of these subunits, was available (30). Although a large number of activating odorant ligands (agonists) have been identified for ORs formed by these subunits (4, 9, 30), little is known about inhibitors. Thus, we first set about identifying inhibitors of receptors formed by these subunits.

Interestingly, when Agam/Ors were functionally characterized using the *Drosophila melanogaster* “empty neuron” approach (4), some compounds decreased the basal spike rate of OSNs expressing these receptors. Although the mechanism of this action is unknown, the basal spike rate was OR-dependent, suggesting that such compounds might be inhibitors of these ORs (4, 44). (→)-Fenchone, (+)-fenchone, and 3-ocotane decreased basal spike rates of OSNs expressing Agam/Or15 or Agam/Or13, whereas geranyl acetate, 2-ethyl-1-hexanol, and linalool oxide decreased basal spike rates of OSNs expressing Agam/Or15. In Fig. 1 we test whether these and related compounds can function as inhibitors. We screened against Agam/Or13 and Agam/Or15 expressed in *Xenopus* oocytes (each in combination with Agam/Orco, which will not be subsequently mentioned). Acetophenone (ACE), a known agonist of both Agam/Or13 and Agam/Or15 (4, 9), was used at an approximate EC<sub>75</sub> concentration for each receptor (30), to activate these receptors. Current responses were recorded under two-electrode voltage clamp (see “Experimental procedures”), and ACE responses in the presence of inhibitor candidate (1 mM) were compared with the preceding response to ACE alone. To avoid problems caused by potential decreases in response amplitude caused by repeated application of ACE, each inhibition value was normalized to the value obtained when the protocol was performed in the absence of the inhibitor candidate (sham).

With the exception of linalool oxide, each of the compounds tested in Fig. 1 was able to achieve some inhibition of the ACE responses of both the Agam/Or13 and Agam/Or15 receptors. Most of the cyclic compounds showed a differential effect, inhibiting Agam/Or13 to a greater extent than Agam/Or15. We decided to further investigate (→)-fenchone (FEN) because it showed the largest difference in ability to inhibit these two receptors. Concentration–inhibition analysis showed FEN to be a more potent inhibitor of the Agam/Or13 receptor than of the Agam/Or15 receptor (Fig. 1C). The concentration–inhibition data were obtained with approximately equipotent ACE concentrations (approximate EC<sub>75</sub>) of 10 and 30 μM for the Agam/Or13 and Agam/Or15 receptors, respectively (30). Because of our intention to use a panel of Agam/Or15 mutants later in this study, we examined the mechanism of FEN inhibition of the Agam/Or15 receptor. Concentration-response analysis for ACE activation of Agam/Or15 receptors in the absence and presence of 3 mM FEN showed a significant increase in EC<sub>50</sub> in the presence of FEN, whereas the maximal response remained unchanged (Fig. 1D). Concentration–inhibition analysis for FEN inhibition of Agam/Or15 receptors activated by 1 μM ACE showed a significant decrease in IC<sub>50</sub> when compared with the IC<sub>50</sub> value obtained when 30 μM ACE was used to activate the receptors (Fig. 1E). These results indicate that FEN is a competitive inhibitor of ACE activation of the Agam/Or15 receptor. Together with the differing effect of FEN on Agam/Or15 and Agam/Or13, these results identify FEN as a useful tool for screening a series of receptor mutants.

**Identification of a determinant of inhibitor sensitivity**

To identify one or more determinants of inhibitor sensitivity, we screened a series of Agam/Or15 mutants with 300 μM FEN, a concentration that has little effect on the WT Agam/Or15 receptor but reduces the ACE response of the Agam/Or13 receptor by approximately half. The previously constructed mutation series (30) covers the 37 positions that differ between Agam/Or15 and Agam/Or13, within the predicted TMDs and extracellular domains of these subunits (Fig. 2A). Each residue was changed from what occurs in Agam/Or15 to what occurs at the same position in Agam/Or13. Two mutants did not form functional receptors (L81W and T206I), so we ultimately screened 35 mutant receptors, activating each with 30 μM ACE. Application of 300 μM FEN had little effect on 34 of the 35 mutant receptors, similar to what was observed for the WT Agam/Or15 receptor. Only the Agam/Or15-A195I mutant receptor displayed sensitivity to FEN that differed from that of the WT Agam/Or15 receptor and was similar to what was observed for the WT Agam/Or13 receptor (Fig. 2B). When we tested the reverse mutation at this position in Agam/Or13 (I195A), the effect of 300 μM FEN was eliminated, making the Agam/Or13-I195A mutant receptor similar to the WT Agam/Or15 receptor. We examined the FEN sensitivity of the Agam/Or15-A195I and Agam/Or13-I195A receptors in more detail by generating concentration–inhibition curves. The IC<sub>50</sub> value for FEN inhibition of Agam/Or13-I195A (1816 ± 303 μM) was similar to the value for inhibition of WT Agam/Or15, whereas the IC<sub>50</sub> value for FEN inhibition of Agam/
**Insect odorant receptor ligand–binding site**

**Figure 1. Identification of inhibitors of Agam/Or13 and Agam/Or15.** A, *left trace*, current recording from an oocyte expressing Agam/Or15 + Agam/Orco. A 30-s application of 30 μM ACE was followed by a 10-min wash period. A 90-s application of 1 mM FEN was then immediately followed by a 30-s co-application of 1 mM FEN and 30 μM ACE. B, *left trace*, current recording from an oocyte expressing Agam/Or15 + Agam/Orco. A 30-s application of 30 μM ACE was followed by a 10-min wash period. A 90-s application of 0.1% DMSO (Sham) was then immediately followed by a 30-s co-application of 0.1% DMSO and 30 μM ACE. A–C, concentration–inhibition analysis shows that FEN inhibition of 10 μM ACE activation of Agam/Or13 + Agam/Orco (IC_{50} = 240 ± 29 μM) and 30 μM ACE activation of Agam/Or15 + Agam/Orco (IC_{50} = 240 ± 29 μM) differ (p < 0.0001, F-test, n = 4–8). C, concentration–response analysis of ACE activation of Agam/Or15 + Agam/Orco in the absence (filled circles) and presence (open circles) of 3 mM FEN. Responses were normalized to the response of the same oocyte to 10 μM ACE (means ± S.E., n = 10). The apparent EC_{50} values for ACE activation in the absence and presence of FEN (21 ± 4 and 77 ± 15 μM, respectively) were different (p < 0.0001, F-test, n = 10). The maximal responses did not differ (p = 0.51, F-test). E, concentration–inhibition analysis shows that FEN inhibition of 1 μM ACE activation of Agam/Or15 + Agam/Orco (IC_{50} = 54 ± 6 μM) and 30 μM ACE activation of Agam/Or15 + Agam/Orco (IC_{50} = 1200 ± 53 μM) differ (p < 0.0001, F-test, n = 4–9).

Or15-A195I (275 ± 32 μM) was similar to the value for inhibition of WT Agam/Or13. These results identify position 195, located at the interface of extracellular loop 2 and transmembrane domain 4, as a determinant of sensitivity to inhibition by FEN in the Agam/Or15 and Agam/Or13 receptors. This position was previously identified as a determinant of sensitivity to several agonists, including ACE, in these receptors (30).

Next, we determined the effect of mutation at position 195 on sensitivity to a series of cyclic compounds that are structurally related to FEN (Fig. 2C). Each compound was screened at a concentration of 300 μM against WT Agam/Or15 and Agam/Or15-A195I receptors. Most of the compounds displayed greater inhibition at the Agam/Or15-A195I receptor, as compared with the WT Agam/Or15 receptor. The exceptions were 2-adamantanone (ADM), which had little or no effect on the receptors at 300 μM, and 7,7-diethylbicyclo[2.2.1]heptan-2-one (DEB), which inhibited both receptors by more than half. The ability of the A195I mutation to alter sensitivity to multiple inhibitors and the differential effect of the mutation across the inhibitor panel provides further support for position 195 as a determinant of inhibitor sensitivity.

**Mutant cycle analysis reveals an interaction between residue 195 and inhibitor molecules**

To further explore the role of position 195 in determining inhibitor sensitivity, we brought additional mutations at this site into our analysis. Because both alanine (native in Agam/Or15) and isoleucine (native in Agam/Or13) have hydrophobic side chains, we decided to also test the hydrophobic residues valine and leucine. We then performed concentration–inhibition analysis for seven cyclic inhibitors: FEN, ADM, DEB, norcamphor (NOR), d-camphor (CAM), 1,4-cineole (CIN), and eucalyptol (EUC). These compounds were tested against WT Agam/Or15 and the three mutant Agam/Or15 receptors (A195I, A195V, A195L), each activated by ACE (Fig. 3, A–C, and supplemental Fig. S1). The IC_{50} values obtained from these analyses were corrected for the slight differences in ACE responsiveness among these receptors (see “Experimental procedures”). This array of corrected IC_{50} values for inhibition of
Insect odorant receptor ligand–binding site

Figure 2. Mutation at position 195 alters inhibitor sensitivity of the Agam/Or15 receptor. A, positions of mutated residues within a predicted secondary structure of Agam/Or15. Residue locations that differ between Agam/Or15 and Agam/Or13 in predicted extracellular regions and transmembrane domains are colored: blue indicates mutations that did not significantly alter sensitivity to FEN; red indicates a mutation (A195I) that significantly altered sensitivity to FEN; and black indicates mutant receptors that were nonfunctional (L81W and T206I). The image was constructed as previously described (30), based on TMD locations estimated using TMPrs2D (48). B, WT Agam/Or15, Agam/Or15 mutants, WT Agam/Or13, and Agam/Or13-A195I, each co-expressed with Agam/Orco, were screened for sensitivity to antagonism by 300 μM FEN, WT Agam/Or15 and Agam/Or15 mutants were activated with 30 μM ACE, WT Agam/Or13, and Agam/Or13-A195A were activated with 10 μM ACE. ACE responses in the presence of 300 μM FEN are presented as percentages of the preceding response to ACE alone and normalized to the effect of sham applications run in parallel (mean ± S.D., n = 4–8; see “Experimental procedures”). Comparison of the values for Agam/Or15 mutants to the values for WT Agam/Or15 was done by one-way analysis of variance and Dunnett’s post-test. Only Agam/Or15-A195I differed from Agam/Or15 (p < 0.001). The values for Agam/Or13-I195A differed from those of WT Agam/Or13 (unpaired t test, p < 0.001). C, WT Agam/Or15 (white symbols) and Agam/Or15-A195I (black symbols), each co-expressed with Agam/Orco were screened for sensitivity to antagonism by a series of cyclic compounds (300 μM), WT Agam/Or15 and Agam/Or15-A195I receptors were activated with 30 μM ACE. ACE responses in the presence of 300 μM of each compound are presented as percentages of the preceding response to ACE alone and normalized to the effect of sham applications run in parallel (mean ± S.D. are shown, n = 3–7; see “Experimental procedures”). Comparison of the effect of each compound on the ACE responses of the WT Agam/Or15 and Agam/Or15-A195I receptors was done using an unpaired t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001; NS, not significant. The data for FEN is from B.
Discussion

The current lack of knowledge about the tertiary and quaternary structure of insect ORs is a hindrance to the development of more effective means of insect control. Understanding the structure of the odorant-binding site of behaviorally relevant ORs of disease vector insects could aid in the development of better repellants, deterrents, or baits. In the absence of receptor structure, several studies have examined the effect of mutations in moth and mosquito ORs using functional assays (30–32). Residues in TMD3, TMD4, and the EC2 loop between them were identified as determinants of odorant sensitivity. A limitation of these studies comes from the fact that nearly all known OR ligands are agonists because of the nature of the various screening assays used to identify these ligands. The use of agonist sensitivity to analyze the effects of mutations makes it difficult to definitively assign identified residues to the odorant-binding site, because mutations almost anywhere in a receptor structure can alter sensitivity to agonists (33). Inhibitors offer a

Table 1

IC₅₀ values (mM) for inhibition of receptors formed by WT Agam/Or15 and three mutant Agam/Or15 mutant receptors

| Compounds | Residue at position 195 of Agam/Or15 | Ala | Ile | Leu | Val |
|-----------|-------------------------------------|-----|-----|-----|-----|
| FEN       | 1.20 ± 0.05                         | 0.28 ± 0.03 | 0.81 ± 0.11 | 0.22 ± 0.04 |
| NOR       | 20.20 ± 1.56                        | 1.83 ± 0.14 | 17.26 ± 1.23 | 7.34 ± 0.81 |
| CAM       | 1.43 ± 0.21                         | 0.36 ± 0.04 | 1.11 ± 0.15 | 1.24 ± 0.12 |
| EUC       | 1.07 ± 0.10                         | 0.52 ± 0.04 | 2.70 ± 0.28 | 0.54 ± 0.08 |
| ADM       | 1.85 ± 0.11                         | 0.65 ± 0.08 | 2.31 ± 0.23 | 1.55 ± 0.19 |
| DEB       | 3.74 ± 0.50                         | 0.95 ± 0.08 | 2.52 ± 0.19 | 1.87 ± 0.15 |

D

Figure 3. Example mutant cycles. A–C, concentration–inhibition analysis of the inhibition of WT Agam/Or15 (Ala, black), Agam/Or15-A195I (Ile, red), Agam/Or15-A195L (Leu, blue), and Agam/Or15-A195V (Val, green) receptors by FEN (A), NOR (B), and DEB (C). IC₅₀ values may be found in Table 1. The data are presented as means ± S.E. (n = 3–8). D, schematic representation of the 18 mutant cycles that can be derived from the IC₅₀ values obtained in A. Each cycle is represented by a rectangle with the contributing ligand–residue pairs at the vertices. IC₅₀ values for each cycle are displayed in the same color as the cycle.
cycles with high $\Omega$ values allow us to conclude that residue 195 is a component of the odorant-binding site of the Agam/Or15 odorant specificity subunit.

The $\Omega$ values we observed were sufficiently high so as to assign residue 195 to the ligand-binding site of Agam/Or15, but much larger $\Omega$ values have been observed in other mutant cycle studies (37, 38). However, these exceptionally high $\Omega$ values were derived from altering the interactions between charged residues of a $K^+$ channel and a peptide inhibitor. The hydrophobic ligand–receptor interaction that we have identified would not be expected to yield such high $\Omega$ values. Residue 195, which we identify here as a component of the ligand-binding site of Agam/Or15, was also identified in our previous study as a determinant of agonist specificity (30). Although assignment of this residue to the binding site was not possible in the previous study, the combination of that work with our present results provides strong evidence that this residue is a component of the binding site and that this residue is interacting with odorant ligands, both agonists and inhibitors. Interestingly, 10 other positions identified as determinants of agonist specificity in the previous study do not affect inhibitor specificity in the present study. This result further emphasizes the concern about the use of agonists in structural studies (33).

Insect ORs are unrelated to other classes of receptors and Channels (16). This has precluded the use of homology modeling to generate estimates of OR structure. However, the vast numbers of OR subunit sequences that have been revealed through genomic sequencing of a variety of insect species has allowed the use of amino acid evolutionary co-variation to generate ternary structure models of odorant specificity and Orco subunits (45). In such a model, position 195 and the various other residues identified in previous studies (24, 25, 31, 32) are located to the same surface of the model, suggesting a binding site structure. Further exploration of the OR odorant-binding site using the mutant cycle approach we demonstrate here, as well as other methodologies such as bi-functional cross-linking from position 195, should allow further elucidation of the structure of the odorant-binding site of insect ORs.

**Experimental procedures**

**Materials**

*Xenopus laevis* frogs, for use as an oocyte source, were purchased from Nasco. The care and use of frogs in this study were approved by the Animal Research Advisory Committee of the Office of Animal Care and Use at the National Institutes of Health. Odorants and other chemicals were purchased from Sigma–Aldrich. Structures and CID (PubChem Compound Identifier) numbers for all odorants used in this study are provided in [supplemental Table 1](#). Agam/Or13 and Agam/Or15 in the pSP64T-Oligo vector, and Agam/Orco in pT7TS vector (9) were generously provided by L. Zwiebel. The Agam/Or15 mutant series and the Agam/Or13-I195A mutant were previously constructed (30).
Insect odorant receptor ligand–binding site

Expression of ORs in X. laevis oocytes

Oocytes were surgically obtained from mature X. laevis frogs. Follicle cells were removed using collagenase B (Roche) for 2 h at room temperature. Capped cRNA encoding each OR subunit was generated using SP6 (Agam/Or13, Agam/Or15, and all Agam/Or mutants) or T7 (Agam/Orco) mMessage mMachine kits (Thermo Fisher Scientific). The oocytes were injected with 25 ng of cRNA encoding each subunit and incubated at 18 °C in Barth’s saline (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.3 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 15 mM HEPES, pH 7.6, 0.05 g/liter tetracycline hydrochloride, 0.1 g/liter amikacin, and 0.05 g/liter ciprofloxacin) for 3–5 days prior to electrophysiological recording.

Electrophysiology and data capture

Odorant-induced currents were recorded under two-electrode voltage clamp using an automated electrophysiology system (OpusXpress 6000A; Molecular Devices). The oocytes were perfused with ND96 (96 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.5). Odorant stock solutions (usually 1 nM) were prepared in DMSO. Odorants were diluted from stock into ND96 on the day of experimentation. Micropettes 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 the OpusXpress 1.1 software (Molecular Devices).

Experimental protocols and data analysis

To measure inhibitor activity, oocytes expressing the various ORs were exposed to 30-s applications of agonist (acetophenone) followed by a 10-min wash period. The oocytes were then exposed to a 90-s application of inhibitor candidate, immediately followed by a 30-s co-application of inhibitor candidate and agonist. The current response in the presence of inhibitor candidate was compared with the preceding response in the absence of inhibitor candidate to calculate the percentage of response remaining. Repeated application of odorants to ORs expressed in oocytes can result in a decline in response amplitude (24, 26, 30). To avoid having potential declines in response confound our results, each inhibition value was normalized to the value obtained when the protocol was performed in the absence of the inhibitor candidate (a sham application of the DMSO vehicle). The computer-controlled, robotic electrophysiology system used for these experiments (OpusXpress 6000A) allowed reproducible duration and timing for experimental and sham applications. Example current recordings showing application of an inhibitor compound and the sham application are shown in Fig. 1A.

Initial analysis of electrophysiological data were done using Clampfit 9.1 software (Molecular Devices). Curve fitting and statistical analyses were done using Prism 7 (GraphPad). Concentration inhibition data were fit to the equation: \[ I = I_{\text{max}} / (1 + (X/IC_{50})^n) \], where \( I \) represents the current response at a given concentration of inhibitor, \( X \); \( I_{\text{max}} \) is the maximal response in the absence of inhibitor; \( IC_{50} \) is the concentration of inhibitor yielding 50% of the maximal response; and \( n \) is the apparent Hill coefficient. \( IC_{50} \) values were corrected for differences among

the concentration-response curves for acetophenone activation of each receptor (30) using the method of Leff and Dougal (46). Statistical significance was assessed using a two-tailed unpaired \( t \) test, an \( F \) test, or a one-way analysis of variance followed by the Dunnett’s post-test, as appropriate.

Mutant cycle analysis

We used the corrected \( IC_{50} \) values for inhibition of receptors formed by Agam/Or15 subunits with four different residues at position 195 (alanine, valine, isoleucine, and leucine) by seven different inhibitor ligands (FEN, norcamphor, d-camphor, 1,4-cineole, eucalyptol, 2-adamantanone, and 7,7-diethylbicyclo [2.2.1]heptan-2-one) as the input data for assessing the degree of interaction between the ligands and residue 195 of Agam/Or15. A mutant cycle consists of the \( IC_{50} \) values from the four receptor–inhibitor pairs that can be generated from two receptors and two inhibitors. A coupling coefficient (Ω) can then be calculated using the equation: \[ \Omega = (K_{R1,L1}/K_{R1,L2})/(K_{R2,L1}/K_{R2,L2}) \], where R1 and R2 represent two different receptors, and L1 and L2 represent for the two different inhibitors (43). The standard errors for Ω values were estimated by propagation of errors (47). Lack of interaction between the ligand and receptor residue would yield \( Ω = 1 \), whereas \( Ω ≠ 1 \) would indicate an interaction of some sort, although not necessarily a close physical interaction. However, \( Ω \) can be converted to a coupling energy by \( RT \ln (Ω) \), where \( R \) is the gas constant, and \( T \) is temperature (37). Coupling energies of ≥1 kcal/mol indicate a physically close interaction between ligand and residue (34, 39). This corresponds to an Ω value of 5.4. Whether \( Ω > 1 \) or \( Ω < 1 \) is irrelevant, so the analysis was simplified for all Ω < 1 by using the reciprocal value.

Author contributions—S. R. and C. W. L. designed the study. S. R. performed the experiments. S. R. and C. W. L. analyzed the data. S. R. and C. W. L. wrote the paper.

Acknowledgments—We thank A. Castro and B. Sherman for Xenopus care and oocyte preparation.

References

1. Guidobaldi, F., May-Concha, I. J., and Guerenstein, P. G. (2014) Morphology and physiology of the olfactory system of blood-feeding insects. J. Physiol. Paris 108, 96–111
2. Gibson, G., and Torr, S. J. (1999) Visual and olfactory responses of haematophagous Diptera to host stimuli. Med. Vet. Entomol. 13, 2–23
3. Zwiebel, L. J., and Takken, W. (2004) Olfactory regulation of mosquito-host interactions. Insect Biochem. Mol. Biol. 34, 645–652
4. Carey, A. F., Wang, G., Su, C. Y., Zwiebel, L. J., and Carlson, J. R. (2010) Odorant reception in the malaria mosquito Anopheles gambiae. Nature 464, 66–71
5. Erdelyn, C. N., Mahood, T. H., Bader, T. S., and Whyard, S. (2012) Functional validation of the carbon dioxide receptor genes in Aedes aegypti mosquitoes using RNA interference. Insect Mol. Biol. 21, 119–127
6. Hallem, E. A., Nicole Fox, A., Zwiebel, L. J., and Carlson, J. R. (2004) Olfaction: mosquito receptor for human-sweat odorant. Nature 427, 212–213
7. Lu, T., Qiu, Y. T., Wang, G., Kwon, J. Y., Rutzler, M., Kwon, H. W., Pitts, R. J., van Loon, J. J., Takken, W., Carlson, J. R., and Zwiebel, L. J. (2007) Odor coding in the maxillary palp of the malaria vector mosquito Anopheles gambiae. Curr. Biol. 17, 1533–1544
