Inhibition of Anopheles gambiae Odorant Receptor Function by Mosquito Repellents*

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Background: The mode of action of insect repellents on odorant receptor (OR) function remains unclear.

Results: Anopheles gambiae OR function in vitro is inhibited by specific repellents.

Conclusion: The identified inhibitory effects are due to functional blocking of Orco, the common subunit of OR heteromers.

Significance: The specific mechanism of action is distinct from the proposed modes of DEET function.

The identification of molecular targets of insect repellents has been a challenging task, with their effects on odorant receptors (ORs) remaining a debatable issue. Here, we describe a study on the effects of selected mosquito repellents, including the widely used repellent N,N-diethyl-meta-toluamide (DEET), on the function of specific ORs of the African malaria vector Anopheles gambiae. This study, which has been based on quantitative measurements of a Ca2+-activated photoprotein biosensor of recombinant OR function in an insect cell-based expression platform and a sequential compound addition protocol, revealed that heteromeric OR (ORx/Orco) function was susceptible to strong inhibition by all tested mosquito repellents except DEET. Moreover, our results demonstrated that the observed inhibition was due to efficient blocking of Orco (olfactory receptor coreceptor) function. This mechanism of repellent action, which is reported for the first time, is distinct from the mode of action of other characterized insect repellents including DEET.

Insect odorant receptors (ORs), which constitute a novel family of heteromeric ligand-gated cation channels (1–3), have been traditionally regarded as the main if not sole molecular targets for insect repellents. The great progress made recently in our understanding of the molecular mechanism(s) of insect olfactory function (4–6) has thus created hope for rational development of improved repellents and/or attractants that could effect a significant reduction in the rate of transmission of malaria and other infectious diseases transmitted by different insect and other arthropod vectors (4, 7).

Prominent among the insect repellents is DEET. This was one of the first to be tested for effects on various insect ORs, with the relevant studies yielding rather contradictory results. Specifically, different mechanisms of DEET action on ORs have been suggested (summarized in Refs. 8 and 9), which include activation of specific ORs, inhibition of specific ORs responding to attractants, and/or modulation of multiple ORs causing olfactory “confusion.” In addition, the effects of a small number of other repellents, such as IR3535, picaridin, and others whose action on insect ORs has been characterized in a more limited fashion, have also been reported (10, 11).

More recently, the modulation of ORs by a number of other compounds, such as amiloride derivatives (12, 13), trace amines (14), and synthetic Orco agonists and antagonists (15–18), has suggested that, because Orco ligands and modulators affect OR function and Orco is highly conserved across insect species, Orco might be a potential target for broadly active insect repellents. Despite their importance for the pharmacological characterization of the receptors, however, most synthetic compounds are expected to have limited usefulness in field application tests due to their low solubility and lack of volatility (15–18). Consequently, more studies are needed to understand the modulation of insect olfactory function by physiologically active compounds, especially repellling compounds, and develop new classes of repellents that may work effectively in the field.

The functional characterization and deorphanization of insect ORs, including most of the Anopheles gambiae repertoire, has been largely achieved through the use of two major test systems, functional receptor expression in Xenopus laevis oocytes (19) or an “empty neuron” of transgenic Drosophila melanogaster (20), in conjunction with electrophysiological methods. Besides these assay systems, a number of insect odorant receptors, including pheromone receptors, have also been expressed in mammalian or insect cells (17, 21–28), which employed as main reporter probes fluorescent calcium indicators and were coupled to imaging of individual cells or measurements of fluorescence changes in multiple well formats. Although rather complex preparation, instrumentation, and handling requirements are needed in the case of the Xenopus and Drosophila systems, the assays involving tissue culture cells with fluorescent probes impose other types of limitations including susceptibility to photobleaching, narrow dynamic

* This work was supported by a grant from the European Union (FP7-222927, ENAROMaTIC – European Network for Advanced Research on Olfaction for Malaria Transmitting Insect Control).

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2 The abbreviations used are: OR, odorant receptor; DEET, N,N-diethyl-meta-toluamide; DPDPE, [d-Pen2,d-Pen5]enkephalin; DMSO, dimethyl sulfoxide; Orco, olfactory receptor coreceptor; OX3a, Orco antagonist 3a; OrcoRAM2, Orco receptor activator molecule 2

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range, and potential for interference by some compounds that either quench the fluorescent signals or autofluoresce, thus causing low signal-to-noise ratios. Consequently, the development and use of alternative cell-based systems employing reporting tools that may provide more robust and quantitative readouts of insect odorant receptor activity while being amenable to miniaturization are highly desirable.

Here we are reporting on an alternative, lepidopteran insect cell-based assay system for functional expression of mosquito ORs that we have used for characterizing the effects of specific mosquito repellents on receptor function. By reconstituting A. gambiae odorant receptors in the specific heterologous expression system together with a Ca\(^{2+}\)-activated photoprotein biosensor allowing quantitative assessments of receptor function, we were able to characterize the effects of specific mosquito repellents including DEET on the function of specific ORx/Orco heteromer combinations. We show that the specific repellents we tested but not DEET block the function of multiple ORs by inhibiting the function of the common co-receptor subunit Orco.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Odorants, Orco agonists, repellents, and OR inhibitors used in the current study are summarized in Table 1. Specifically, benzoaldehyde, 2-, 3-, and 4-methylphenol, ethyl butyrate, 2-ethylphenol, cyclohexanone, DEET, and ethyl trans-cinnamate were from Sigma-Aldrich, indole and cuminic alcohol were from Acros Organics, isopropylnitromethane was from Alfa Aesar, and carvacrol from Beaucorvilles Flavors SAS (Peynier, France). VUAA1 (17) was generously provided by Dr. Richard Newcomb (New Zealand Institute for Plant & Food Research, Auckland, New Zealand), while OrcoRAM2 (11) was obtained from Hit2lead (Chembridge Corp., San Diego, CA) and Vitas-M Laboratory, Ltd. (Apeldoorn, the Netherlands). OX3a (16) was purchased from Vitas-M Laboratory. Coelenterazine was from Promega, BIOMOL GmbH (Hamburg, Germany) and Biosynth (Staad, Switzerland), while [\(C^3\)]-Pen2,\(D^3\)-Pen2]enkephalin (DPPDE) was obtained from Tocris Bioscience (Bristol, UK). Ruthenium red was a gift of Dr. Dimitrios Kontogiannatos (Agricultural University of Athens, Greece). Initial stock solutions and dilutions for OrcoRAM2 and OX3a (50 mM), as well as for all repellents, were prepared in DMSO, with subsequent working dilutions prepared freshly in Ringer's solution at room temperature in the dark for at least 2 h. Luminescence was measured in an Infinite M200 microplate reader (Tecan Group Ltd). The addition of chemicals was either by using the autoinjector, allowing rapid injection and simultaneous reading, or manually outside the plate reader. In the latter case, baseline luminescence was usually recorded for 3–7 s for a further period of up to 120 s.

To test the suitability of the expression system for use as screening platform for olfactory receptor agonists and antagonists in a single compound screen, as was previously reported for other ion channels (38), olfactory receptors (28), and nuclear receptors (39), cells co-expressing selected A. gambiae ORs with Orco and Photina were transferred to 96-well plates. Following coelenterazine loading, the cells were subjected to two cycles of compound additions (see Fig. 1B). In the first cycle, compounds screened for effects on olfactory receptors were added to the cells, while in a second application, the cognate ligand for each receptor (odorant for ORx-Orco and Orco agonist for Orco alone) was added to all wells. Between the two applications, cells were allowed to return to baseline luminescence levels (15–20 min). The same design was used for testing the effects of the selected repellents.

**Data Analysis and Curve Fitting**—Initial data acquisition and analyses were performed using i-Control 1.3 (Tecan), while curve fitting and EC\(_{50}\)/IC\(_{50}\) calculations were done using...
| Chemical [CAS number] | Abbreviation | Purity (%) | Solvent-highest [C] used* | Structural formula |
|-----------------------|--------------|------------|--------------------------|--------------------|
| 2-Methylphenol (o-Cresol) [95-48-7] | 2MP | 99+ | Methanol-1mM | ![Structural formula](image1) |
| 3-Methylphenol (m-Cresol) [108-39-4] | 3MP | 99 | Methanol-1mM | ![Structural formula](image2) |
| 4-Methylphenol (p-Cresol) [106-44-5] | 4MP | 99 | Methanol-1mM | ![Structural formula](image3) |
| 2-Ethylphenol [90-00-6] | 2EP | 98.5 | Methanol-1mM | ![Structural formula](image4) |
| Benzaldehyde [100-52-7] | BA | >99 | Ethanol-1mM | ![Structural formula](image5) |
| Indole [120-72-9] | IN | 99+ | Methanol-300µM | ![Structural formula](image6) |
| Ethyl butyrate [105-54-4] | EB | 99 | Ethanol-100µM | ![Structural formula](image7) |
| Cyclohexanone [108-94-1] | CH | ≥99.9 | Methanol-100µM | ![Structural formula](image8) |
| Ethyl trans-cinnamate [103-36-6] | EC | 99 | DMSO-2mM | ![Structural formula](image9) |
| Carvacrol [499-75-2] | CRV | >98 | DMSO-100µM | ![Structural formula](image10) |
| Cuminic alcohol[536-60-7] | CMA | 97 | DMSO-100µM | ![Structural formula](image11) |
| N,N-Diethyl-m-toluamide[134-62-3] | DEET | 98 | DMSO-100µM | ![Structural formula](image12) |
| Isopropyl cinnamate [7780-06-5] | IPC | 98 | DMSO-1mM | ![Structural formula](image13) |
| Ruthenium red [11103-72-3] | RR | ND | Water-100µM | ![Structural formula](image14) |
| N-(4-ethylphenyl)-2-((4-ethyl-5-(3-pyridinyl)-4H-1,2,4-triazol-3-yl)thio)acetamide [525582-84-7] | VUAA1 | ND | Methanol-100µM | ![Structural formula](image15) |
| N-(4-ethylphenyl)-2-((4-ethyl-5-(4-pyridinyl)-4H-1,2,4-triazol-3-yl)thio)acetamide [618427-06-8] | OrcoRAM2 (OA) | >90 | DMSO-500µM | ![Structural formula](image16) |
| N-(4-methylbenzyl)-2-thiophenecarboxamide | OX3a | >90 | DMSO-100 µM | ![Structural formula](image17) |
GraphPad Prism 4.0 for Windows. Specifically, concentration-response data were fitted to the equation for non-linear regression, sigmoidal dose response: 

\[ Y = \frac{\text{Bottom} + (\text{Top} - \text{Bottom})}{1 + 10^{\left(\frac{\text{LogEC}_{50} - X}{\text{MCS}}\right)}} \]

where \( Y \) is percentage of response at a given concentration for a given odorant; \( X \) is logarithm of concentration, with Top and Bottom values being the maximal and minimal percentage of responses for the given odorant, as normalized to 100%, set for the maximal response for a specific OR against all tested odorants, and the \( \text{EC}_{50} \) is the odorant concentration yielding a half-maximal response.

Luminescence value comparisons between independent experiments were made relative to normalization standards with cognate ligands (i.e. 100 µM 4-methylphenol and indole for OR1 and OR2, respectively) applied in every given experiment and considered to provide 100% of maximal response for the specific set of experiments. Unless otherwise stated, results represent the means of 2–3 independent experiments.

RESULTS

Mosquito ORs Produce Orco-dependent and Odorant-specific Ionotropic Responses in Lepidopteran Cells—We have previously reported on the expression of \( A. \text{gambiae} \) ORs in a lepidopteran insect cell-based system that directs efficient synthesis and correct localization of recombinant receptors in the expressing cells (34). To establish the functionality of the expressed receptors in this system and develop a platform suitable for quantitative assessments of receptor activity, we introduced into the cells a reporter construct for a \( \text{Ca}^{2+} \)-activated photoprotein, Photina® (36) (Fig. 1A), which functions similarly to aequorin, but has enhanced quantum yield (36, 40). Upon activation of OR ligand-gated cation channels, which cause \( \text{Ca}^{2+} \) influx into the cells, Photina® undergoes a conformational change leading to oxidation of coelenterazine and emission of blue light proportional to the \( \text{Ca}^{2+} \) influx (Fig. 1A).
Following co-expression of OR1 and OR2 with Orco and Photina®, cellular luminescence responses were monitored after the addition of specific ligands known to activate each OR (19, 20, 41, 42). As shown in Fig. 2A, specific responses could be detected in cells expressing OR1/Orco upon administration of 4-methylphenol, whereas cells expressing OR2/Orco responded, as expected, to indole. On the other hand, cells expressing OR9/Orco responded, as expected, to the addition of 2-ethylphenol (data not shown). The presence of Orco was obligatory for functional responses to occur as no responses were obtained in its absence (Fig. 2A). Co-expression of Gα16 was also not able to substitute for a functional Orco in stimulating agonist-dependent activity of OR2 and OR1 to any appreciable degree (Fig. 2, B and C).

To further deduce OR response specificity and potency differences, luminescence responses were monitored after the addition of selected compounds known to activate each OR (19, 20, 41, 42). As shown by the examples presented in Fig. 2D and further quantified in Fig. 2E, specific responses could be detected in cells expressing OR1/Orco upon administration of 4-methylphenol (and to a lesser extent 3-methylphenol), whereas cells expressing OR2/Orco responded, as expected, to indole and, to a lesser extent, benzaldehyde. In contrast, no responses could be detected in cells expressing either OR1/Orco or OR2/Orco following administration of ethyl butyrate (Fig. 2D). With a higher concentration of 4-methylphenol, a small response could also be detected in OR2/Orco-expressing cells (data not shown and Fig. 3A). Besides the spe-
cific odorants discussed here, other chemicals have also been tested with comparable results relative to previously reported studies (19, 20, 41, 42).

The dose-response curves and derived EC_{50} values from the agonist-induced luminescence for OR1/Orco and OR2/Orco heteromers distinguish agonists of varying efficacies and potencies (Fig. 2E). Thus, EC_{50} values of 2.8 and 3.4 μM were determined for OR1/Orco against 4-methyl phenol and for OR2/Orco against indole, respectively (Fig. 2E and Table 2). In general, when expressed in lepidopteran cells, all tested mosquito receptors were found to be functional and display the same basic specificities as those determined using the Xenopus oocyte and Drosophila empty neuron systems (19, 20, 41). Specifically, OR1 was found to respond to 4- and 3-methylphenol, and OR2 was found to respond to indole, benzaldehyde, and 2-methylphenol, with the overall response patterns and dose-response profiles for the examined ligands and receptors (4MP > 3MP for OR1, and IN > BA > 2MP for OR2 (see Table 1 for definitions and structures)) being in good general agreement with those obtained for the same receptors from frog oocytes with two-electrode voltage clamp (19).

**A Convenient Assay for Initial Assessment of Specific Chemical Compound Effects on the Functionality of Olfactory Receptors**—To investigate the effects of specific chemical compounds on the functionality of olfactory receptors, we employed an assay that permitted the easy classification of examined compounds into agonists, partial agonists, antagonists, or inert, i.e. not displaying any activity against the tested receptors, in one round of compound testing. The assay, which is shown diagrammatically in Fig. 1B, relies on two sequential additions of compounds to cells expressing specific receptor heteromers and the reporter Photina\textsuperscript{®} construct, and recordings of the differential luminescence responses obtained after each addition, which depend on the nature of the chemical of the first addition. Specifically, the compound of unknown function under examination is added first at a relatively high concentration (100 μM), and this is followed 10–20 min later by the addition of the specific agonist at the same concentration of 100 μM, which usually corresponds to an EC_{50} or greater, in the

FIGURE 3. Analysis of compound effects on Anopheles OR function. **A**, responses of cells expressing OR2/Orco to two applications of compounds, as described for Fig. 1A. In the first application (blue bars), cells in different wells were challenged with solvent, indole (IN), benzaldehyde (BA), 2-methylphenol (2MP), 4-methylphenol (4MP), cyclohexanone (CH), and ethyl butyrate (EB), each at a concentration of 100 μM. The second addition (red bars; specific agonist indole at 100 μM added to same cells) was performed after the luminescence had returned to baseline levels (n = 3). **B**, cells expressing OR2, Orco, JOR, and Gα_{16} were initially challenged with indole (100 μM), and subsequently, in the second cycle, with either 100 μM indole or 1 μM of the δ-opioid receptor agonist DPDPE. The secondary response was only abolished in the former but not the latter case (n = 3). **C**, partial inhibition of responses to indole of OR2/Orco-expressing cells by 100 μM of ruthenium red (RR) or OX3a could be demonstrated with this design (n = 3 and 5, respectively). % max response, percentage of maximum response. Error bars indicate mean ± S.D.

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same microtiter well. This assay, performed in 96-well format with no wash steps in between, allows the distinction of compounds under investigation into receptor agonists (+/− for primary/secondary receptor responses, respectively; Fig. 1B, #1), non-active (−/+ responses; Fig. 1B, #2), or antagonists (−/− responses; Fig. 1B, #3). It should be noted that following a maximal primary response triggered by a receptor-specific agonist in cells expressing the corresponding receptor heteromer, the cells do not respond to a second addition of the same or a different agonist of similar specificity and potency (Fig. 1B, #1 and Fig. 3A), presumably due to temporary receptor inactivation that is gradually reversed over time following removal of the agonist (data not shown). On the other hand, partial agonists producing a lower than maximal primary response, progressively reduced receptor inactivation.

Typical system validation examples employing the OR2/Orco heteromer are presented in Fig. 3A. Indole, the specific OR2 agonist, triggers a primary response but no secondary functional response upon a new addition of indole to the cells. This effect appears to be specific for the cognate pair of OR heteromer/ligand used rather than being caused by exhaustion of the photoprotein as cells co-transfected with OR2+Orco and δ-opioid receptor respond positively to the addition of DPDPE, the specific δ-opioid receptor ligand, following the primary indole addition (Fig. 3B). On the other hand, benzaldehyde, 2-methylphenol, and 4-methylphenol, partial OR2 agonists (Fig. 2E and data not shown), trigger partial agonist primary and secondary responses (Fig. 3A), whereas ethyl butyrate and cyclohexanone, which do not represent ligands for the specific receptor, do not trigger a primary functional response but allow the opening of the olfactory channel upon a secondary addition of indole (Fig. 3A). Controls were included both at the beginning and at the end of each set of experiments to ascertain the stability of OR2 responses to indole during the course of the experiments (Fig. 3A).

Given the absence of known antagonists for any ORx receptors, the case of inhibition in the context of a receptor heteromer was tested through the use of one of the recently reported synthetic phenyl-thiophene-carboxamide Orco antagonists, OX3a (16), and ruthenium red (43). OX3a has been shown to inhibit non-competitively odorant activation of a heteromeric OR of *Culex quinquefasciatus* and was assumed to block general odorant-dependent OR activation as the originally characterized chemical of this class of Orco antagonists (16). On the other hand, ruthenium red, a nonspecific cation channel blocker, inhibits the function of many insect odorant receptors (1, 44, 45). In the case of these inhibitors, their addition to the cells did not induce any functional responses, but did reduce receptor activation upon a secondary addition of indole (Fig. 3C). Similar results were obtained from other tested ORx/Orco heteromers involving ORx subunits of known ligand specificities (data not shown).

**Olfactory Receptor Inhibition by Specific Mosquito Repellents—**

A number of repellents previously reported to be active against different *Anopheles* and *Aedes* species were subsequently examined for their possible effects on various olfactory receptors of *A. gambiae*. Prominent among the initially examined repellents were DEET (46, 47), ethyl cinnamate (EC [47, 48]), isopropyl cinnamate (IPC [47–49]), cuminic alcohol (CMA [47, 50]), and carvacrol (CRV [47, 51]) (Table 1). Except for DEET, which has been studied extensively, to the best of our knowledge, no information concerning the molecular mechanism of action has been available for these repellents.

The chosen repellents were tested initially at a concentration of 100 μM on cells expressing OR1/Orco and OR2/Orco heteromers. As is shown in Fig. 4A, none of the tested repellents displayed agonist activity upon addition to the cells. Judging from the results of the secondary addition of 4-methylphenol or indole to the cells expressing OR1+Orco or OR2+Orco, respectively, EC, CRV, and IPC caused essentially complete inhibition of OR1/Orco and OR2/Orco receptor function at this concentration (Fig. 4A). The effect of CMA on both receptor heteromers was considerably less pronounced with the inhibition amounting to 30–40%, whereas no noticeable inhibitory action was exerted by DEET on the tested receptors. Similar inhibitory effects were also recorded with other mosquito receptor heteromers examined (data not shown).

To ensure that the observed inhibition in Ca2+ influx into the cells was due to specific inhibition of olfactory receptor function, rather than general off-target effects, e.g. at the level of the cellular membranes or the calcium photoprotein, the same compounds were examined for their effects on cells transfected

| TABLE 2 | Mosquito Repellents Inhibiting Orco Function |
| --- | --- |
| **I. Activation** |  |
| Receptor | Chemical | EC50 (pEC50±Std.error) | R² |
| OR1+Orco | 4-Methylphenol | 2.8μM (5.55±0.198) | 0.9416 |
| OR2+Orco | Indole | 3.4μM (5.47±0.119) | 0.9829 |
| Orco | OrcoRAM2 | 58.5μM (4.23±0.034) | 0.9655 |
| **II. Inhibition/blockade** |  |
| Receptor | Chemical | IC50 (pIC50±Std.error) | R² |
| OR1+Orco | Ethyl cinnamate | 25.9μM (4.59±0.263) | 0.859 |
| | Isopropyl cinnamate | 22.2μM (4.66±0.32) | 0.8309 |
| OR2+Orco | Ethyl cinnamate | 28.9μM (4.54±0.079) | 0.9814 |
| | Isopropyl cinnamate | 34μM (4.47±0.253) | 0.9162 |
| Orco | Ethyl cinnamate | 64.5μM (4.19±0.054) | 0.9903 |
| | Isopropyl cinnamate | 41.7μM (4.38±0.061) | 0.9883 |
with the murine δ-opioid receptor (52, 53) along with the human Gα16 (54) (presented in Figs. 2C and 3B). As shown in Fig. 4B, administration of the δ-opioid agonist DPDPE to cells expressing the specific receptor in the presence of a high concentration of the tested repellents did not affect plasma membrane-anchored opioid receptor signaling and the ensuing Ca\(^{2+}\) release from intracellular endoplasmic reticulum membrane stores (Fig. 4B).

Dose-response curves for the inhibition exerted by two of the repellents displaying highly inhibitory actions, ethyl cinnamate and isopropyl cinnamate, against OR1/Orco and OR2/Orco heteromers were constructed using 100 μM concentrations (>EC\(_{90}\)) of the cognate ligands 4-methylphenol and indole, respectively, as agonists for the secondary additions. For ethyl cinnamate, the rates of inhibition (IC\(_{50}\) values) of the two heteromers were found to be very similar, 25.9 and 28.9 μM, respectively (Fig. 4C). Inhibition by isopropyl cinnamate was also found to be in the same order of magnitude, with IC\(_{50}\) values of 22.2 and 34 μM, respectively, for OR1/Orco and OR2/Orco (Fig. 4C).

**FIGURE 4.** Screening for effects of selected mosquito repellents on *A. gambiae* ORs. A, OR1/Orco and OR2/Orco heteromer-expressing cells were tested for primary responses to 100 μM of each repellent (ethyl cinnamate (EC), carvacrol (CRV), cumin alcohol (CMA), DEET, and isopropyl cinnamate (IPC) (blue bars), and inhibition of secondary responses to the cognate agonists 4-methylphenol (4MP) and indole (IN), respectively, added in the same wells at 100 μM (red bars). Responses are presented as the percentages of the response of each receptor heteromer to the cognate ligand in the absence of any candidate antagonist (n = 2–8). % max response, percentage of maximum response. B, the effects of the same repellents at 100 μM on the functionality of the δ-opioid receptor and its downstream phospholipase C-coupled signaling responses to 10 μM DPDPE (n = 2). C, dose-response inhibition curves for ethyl cinnamate and isopropyl cinnamate against OR1/Orco- and OR2/Orco-expressing cells following stimulation with 100 μM of the respective cognate agonists, 4-methylphenol and indole. (n = 2 and 3, respectively). Error bars indicate mean ± S.D.
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DISCUSSION

In this study, we present the use of a lepidopteran insect cell-based assay for quantitative assessments of the functional properties of *A. gambiae* olfactory receptors. Besides high levels of receptor expression achieved in the insect cells (30, 34) through the use of plasmid-based expression vectors employing genetic control elements of the silkworm and its baculovirus (31, 32), the major functional component of the assay system is a construct directing robust expression of Photina®, a Ca²⁺-activated photoprotein (36). The activation of this photoprotein provides a luminescence-based readout reporting quantitatively on increases of intracellular Ca²⁺ ions; hence, in this study, ion channel activity upon administration of cognate olfactory receptor agonists could be studied.

Our system may be considered as an effective alternative to the two major systems used for studying insect olfactory receptor function, the *Drosophila* empty neuron (20) and *Xenopus* oocytes (19), as well as other heterologous cell-based expression systems employing cultured insect (21, 24–26) or mammalian cells (22, 23, 27, 28) in conjunction with fluorescent calcium indicator dyes. In contrast to these systems, which have some important drawbacks (see the Introduction), the new system combines the simplicity of handling with a reporter photoprotein that only luminesces when the levels of intracellular Ca²⁺ increase as a result of olfactory channel activation. Because it allows automated luminescence measurements of total cell populations seeded in microtiter plates in a mix-and-measure fashion and with high signal-to-noise ratios in the absence of wash steps, this system is very suitable for functional screens for ligand discovery at a medium-to-high throughput scale.

As already mentioned, a version of Photina® targeted to the mitochondria via a specific leader sequence (36) was employed in our optical cell-based assay. The reasons for choosing the mitochondrially targeted version of Photina® over the cytoplasmic were, first, the ~10-fold higher responses reported for the mitochondrial photoprotein over the cytoplasmic one, at least for the case of studied G-protein-coupled receptors and, secondly, the slower, and thus more accurate, detection of channel activation reaction kinetics due to the longer time assumed to be needed for the Ca²⁺ wave to reach the mitochondria-localized Photina®. Moreover, we have not noticed any artifactual responses related to metabolic stress in our system as functional responses from cells expressing specific receptors were obtained only after administration of cognate ligands at functionally relevant concentrations.

The specificities of OR responses determined in the lepidopteran insect cell system have been in good agreement with those determined using the *Drosophila* empty neuron and *Xenopus* oocytes expression systems (19, 20). As far as relevant potencies and efficacies are concerned, our results can be compared directly with those determined using the frog oocyte system (19), which can be considered as more accurate and quantitative descriptors of receptor pharmacology, as in the *Drosophila* system only one very high, and thus not physiologically relevant, concentration was applied. Briefly, the tested ORs were found to recognize the same ligands, from the panel

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**FIGURE 5. Effects of selected mosquito repellents on *A. gambiae* Orco.** A, responses of Orco homomer-expressing cells to 100 μM of the tested repellents, ethyl cinnamate (EC), carvacrol (CRV), cumin alcohol (CMA), DEET, and isopropyl cinnamate (IPC) (blue bars), and 100 μM OrcoRAM2 (OA) as secondary addition in the same wells (red bars). Responses are presented as the percentages of the response to the Orco agonist in the absence of any candidate antagonist (*n* = 2). % max response, percentage of maximum response. B, dose-dependent inhibition curves for Orco-expressing cells, in the presence of increasing concentrations of ethyl cinnamate and isopropyl cinnamate, to 100 μM of the Orco agonist OrcoRAM2 (*n* = 3 and 4, respectively). Error bars indicate mean ± S.D.

Orco Antagonism Is a Cause of Interference with Mosquito Olfactory Receptor Function—Given the similarity of the inhibitory effects of the tested repellents on different olfactory receptor heteromers, we examined the possibility that the inhibition may be exerted at the level of Orco, the common subunit of olfactory receptor heteromers. Although we found Orco to be similarly responsive to both Orco agonists tested, VUAAl (17) and OrcoRAM2 (11) (data not shown), the latter was used for detailed investigations (shown also in Fig. 2E). Indeed, as shown in Fig. 5A, when 100 μM of each repellent was added to cells expressing Orco, all examined repellents, except DEET, exerted some level of inhibitory effect on Orco function as the responses to the secondary addition of 100 μM of the Orco agonist OrcoRAM2 to the cells were considerably reduced (Fig. 5A).

The dose-response curves for the inhibition exerted on Orco homomers by the tested repellents (Fig. 5B) revealed IC₅₀ values of 64.5 and 41.7 μM for ethyl cinnamate and isopropyl cinnamate, respectively, somewhat higher than those exerted on the function of OR1/Orco and OR2/Orco heteromers (Fig. 4C). This finding suggests that the repellent action observed in the context of the heteromer (Fig. 4) likely originates from interference with Orco function. Interestingly, DEET did not conform to the behavior of the rest of the repellents at the tested concentration.
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of chemicals used for confirmation, with similar dose-dependent responses, 4MP > 3MP for OR1 and IN > BA > 2MP for OR2, respectively (Fig. 2E) (see Table 1 for definitions and structures). The calculated EC_{50} values (Table 2) were somewhat higher than the ones reported with the electrophysiologically methods, which were in the high nanomolar range (19). The Orco homomer was also found to respond to Orco agonists, as expected.

The sequential addition protocol employed in this study, initially validated using known odorants, ruthenium red and an Orco antagonist, made possible the classification of compounds added in the first instance to cells expressing specific receptors into partial or full receptor agonists, antagonists, or inert in terms of ligand activities (Figs. 1B and 3A). Using this assay, it has been possible to deduce that all but one of several examined compounds known to represent powerful mosquito repellents act as olfactory receptor inhibitors reducing receptor activation upon subsequent agonist addition (Fig. 4A). Interestingly, the exception has been the most widely used repellent DEET, which was not found to act as an agonist or antagonist for any of the examined A. gambiae olfactory receptors in the tested concentration of 100 μM (Fig. 4A).

The fact that the tested repellents were found to inhibit the function of all receptors examined in this study led to the examination of the possibility and demonstration that Orco, the common receptor subunit of the functional receptor complex, was a target of inhibition (Fig. 5, A and B). Although further studies are warranted, this finding and, most importantly, the dose-response curves for the inhibition, suggest that the inhibitory action of the tested repellents on the receptor heteromers under examination is likely due to Orco inhibition, with the ORx constituents contributing little, if any, to it. Whether the somewhat lower IC_{50} values of the repellents for the receptor heteromers (Table 2) indeed reflect enhanced affinities for Orco because of structural changes of the latter in the context of the heteromers should be investigated further.

It should be noted further that although the synthesis of several compounds acting as Orco antagonists has been reported recently (15, 16, 18), to our knowledge, this is the first study demonstrating antagonistic action on an anopheline Orco associated with powerful repellency on anopheline and other mosquitoes. Likely important factors that contribute to the repelling capacity of the Orco antagonists reported here are their physical properties, such as volatility and efficient recognition by several mosquito odorant-binding proteins (55), at least in vitro, which has been demonstrated for some of these compounds in the A. gambiae system. The fact that a highly conserved protein such as Orco is a major target of the tested repellent compounds may also explain why the specific repellents are effective against mosquito species other than A. gambiae, as well as other insects (47, 48, 50, 51). The demonstrated Orco involvement also leads us to postulate that, at least in the case of the two repellents studied in more detail here, their mosquito “repellence” action should probably be interpreted as a passive disorientation effect, which reflects an essential anosmia caused by the blocking of a large complement of olfactory receptors rather than an active one causing repulsion to the approaching mosquitoes.

Finally, the finding that DEET behaves differently than the remaining repellents tested in this study was not unexpected, as a number of studies have suggested different modes of function for this repellent. These include: activation of specific ORs (the excitoto-repellent hypothesis (56–58)); inhibition of specific ORs responding to attractants (59, 60); and/or modulation of many ORs causing an olfactory confusion similar in effect but not in molecular terms to the one postulated above for the repellents tested in this study (10, 61). In addition, the involvement of ionotropic (62) and gustatory receptors (63) in Drosophila was also demonstrated. Therefore it seems that, in contrast to the case of the Orco function-inhibiting repellents presented in this study, the molecular targets for DEET are fundamentally different. In fact, no inhibition of Aedes aegypti Orco could be observed even at 10 mM DEET (11). Nevertheless, the inhibitory effects of DEET on a Drosophila OR heteromer, OR47a/OR83b, with a calculated IC_{50} value of 929 μM in the Xenopus oocyte system have been reported (13). In the same assay system, some inhibitory effects of DEET were also documented for a number of insect olfactory receptors including A. gambiae OR1/Orco and OR2/Orco (59). In these latter cases, the inhibition ranged from a low of 30% to a maximum of ~55% at a DEET concentration of 1 mM (59).

In view of the fact that the effects of repellents with secondary (allosteric) recognition sites may be observed at high repellent concentrations (9), we consider questionable whether modulations of odorant receptor activity by high concentrations of DEET are physiologically relevant. Such reservations notwithstanding, however, it should also be of interest to investigate whether the repellents studied here have additional sensory targets, in analogy to DEET or citronella, which has been reported to target both TRPA1 and Orco-dependent pathways in Drosophila (64).

Acknowledgments—We thank Dr. Sabrina Corazza (AXXAM SpA, Milan, Italy) for provision of Photina® plasmid, Prof. Richard D. Newcomb (The New Zealand Institute for Plant and Food Research Limited, Auckland, New Zealand) for the generous gift of a sample of VfAA1, used in initial studies, Dr. Dimitrios Kontogiannatos (Agricultural University of Athens) for the gift of ruthenium red, and Dr. Maria Konstantopoulou (Chemical Ecology and Natural Products Laboratory, National Centre for Scientific Research (NCSR) “Demokritos”) for provision of carvacrol. We also thank Alexandra Amaral-Psarris for expert technical assistance, our laboratory colleagues Drs. Luc Swevers and Vassiliki Labropoulou for their constructive comments, Dr. Zafiroula Georgoussi (Signal Transduction and Molecular Pharmacology Group, IB-A, NCSR “Demokritos”) for the long and helpful discussions and suggestions, and Dr. Spyros Zografos (National Hellenic Research Foundation) for useful suggestions.

REFERENCES

1. Nakagawa, T., Sakurai, T., Nishioka, T., and Touhara, K. (2005) Insect sex-pheromone signals mediated by specific combinations of olfactory receptors. Science 307, 1638–1642
2. Sato, K., Pellegrino, M., Nakagawa, T., Nakagawa, T., Vosshall, L. B., and

---

3. K. Iatrou, K. Koussis, M. Konstantopoulou, S. E. Zografos, and G. Kythereoti, unpublished data.
Mosquito Repellents Inhibiting Orco Function

Touhara, K. (2008) Insect olfactory receptors are heteromeric ligand-gated ion channels. Nature 452, 1002–1006

Wicher, D., Schäfer, R., Bauerfeind, R., Stensmyr, M. C., Heller, R., Heinemann, S. H., and Hansson, B. S. (2008) Drosophila odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. Nature 452, 1007–1011

Carey, A. F., and Carlson, J. R. (2011) Insect olfaction from model systems to disease control. Proc. Natl. Acad. Sci. U.S.A. 108, 12987–12995

Leal, W. S. (2010) Robust odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. Annu. Rev. Entomol. 58, 373–391

Suh, E., Bobbot, J. D., and Zwiebel, L. J. (2014) Peripheral olfactory signaling in insects. Curr. Opin. Insect Sci. 6, 86–92

Leal, W. S. (2010) Behavioural neurobiology: the treacherous scent of a human. Nature 464, 37–38

DeGennaro, M., McBride, C. S., Seeholzer, L., Nakagawa, T., Dennis, E. J., Goldman, C., Jasinskievi, N., James, A. A., and Vosshall, L. B. (2013) orco mutant mosquitoes lose strong preference for humans and are not repelled by volatile DEET. Nature 498, 487–491

Dicks, J. C., and Bobbot, J. D. (2013) Mini review: Mode of action of mosquito repellents. Pestic. Biochem. Physiol. 106, 149–155

Bobbot, J. D., and Dickens, J. C. (2010) Insect repellents: modulators of mosquito odorant receptor activity. PLoS One 5, e12138

Bobbot, J. D., and Dickens, J. C. (2012) Odorant receptor modulation: ternary paradigm for mode of action of insect repellents. Neuropharmacology 62, 2086–2095

Pask, G. M., Bobkov, Y. V., Corey, E. A., Ache, B. W., and Zwiebel, L. J. (2013) Blockade of insect odorant receptor currents by amiloride derivatives. Chem. Senses 38, 221–229

Röllecke, K., Werner, M., Zemba, P. M., Neuhaus, E. M., Hatt, H., and Gisselmann, G. (2013) Amloride derivatives are effective blockers of insect odorant receptors. Chem. Senses 38, 231–236

Chen, S., and Luetje, C. W. (2014) Trace amines inhibit insect odorant receptor function through antagonism of the co-receptor subunit. F1000Res 3, 84

Chen, S., and Luetje, C. W. (2012) Identification of new agonists and antagonists of the insect odorant receptor co-receptor subunit. PLoS One 7, e36784

Chen, S., and Luetje, C. W. (2013) Phenylthiophenecarboxamide antagonists of the olfactory receptor co-receptor subunit from a mosquito. PLoS One 8, e84575

Jones, P. L., Pask, G. M., Rinker, D. C., and Zwiebel, L. J. (2011) Odorant agonism of insect odorant receptor ion channels. Proc. Natl. Acad. Sci. U.S.A. 108, 8821–8825

Jones, P. L., Pask, G. M., Romaine, I. M., Taylor, R. W., Reid, P. R., Waterston, A. G., Sulikowski, G. A., and Zwiebel, L. J. (2012) Allosteric antagonism of insect odorant receptor ion channels. PLoS One 7, e30304

Wang, G., Carey, A. F., Carlson, J. R., and Zwiebel, L. J. (2010) Molecular basis of odor coding in the malaria vector mosquito Anopheles gambiae. Proc. Natl. Acad. Sci. U.S.A. 107, 4418–4423

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

Anderson, A. R., Wanner, K. W., Trowell, S. C., Warr, C. G., Nagiec, J. Q., Vargas, E. C., Carraher, C., A, V., and Zwiebel, L. J. (2009) Molecular basis of female-specific odorant responses in the light brown apple moth (Epiphyas postvittana). Int. J. Biol. Sci. 5, 745–757

Grosse-Wilde, E., Svatos, A., and Krieger, J. (2006) A pheromone-binding protein from Drosophila melanogaster elicits sex-biased expression of odorant receptors in antennae and palps of the African malaria vector Anopheles gambiae. Insect Biochem. Mol. Biol. 36, 268–274

Bovolenta, S., Sari, M., Lohmer, S., and Corazza, S. (2007) Development of a Ca<sup>2+</sup>-activated photoprotein, Photina, and its application to high-throughput screening. J. Biomol. Screen. 12, 694–704

Wickham, T. J., Davis, T., Granados, R. R., Shuler, M. L., and Wood, H. A. (1992) Screening of insect cell lines for the production of recombinant proteins and infectious virus in the baculovirus expression system. Biotechnol. Prog. 8, 391–396

Thompson, A. J., Verheij, M. H., Leurs, R., De Esch, I. J., and Lummis, S. C. (2010) An efficient and information-rich biochemical method design for fragment library screening on ion channels. BioTechniques 49, 822–829

Swevers, L., Mortu, E., Balatou, N., Iatrou, K., and Georgoussi, Z. (2005) Functional expression of mammalian opioid receptors in insect cells and high-throughput screening platforms for receptor ligand mimetics. Cell. Mol. Life Sci. 62, 919–930

Tsitoura, P., Andronopoulou, E., Tsikou, D., Agalou, A., Papakonstantinou, M. P., Kotzia, G. A., Labropoulou, V., Swevers, L., Georgoussi, Z., and Iatrou, K. (2010) Expression and membrane topology of Anopheles gambiae odorant receptors in lepidopteran insect cells. PLoS One 5, e15428

Iatrou, K., and Biessmann, H. (2008) Sex-biased expression of odorant receptors in antennae and palps of the African malaria vector Anopheles gambiae. Insect Biochem. Mol. Biol. 38, 268–274

Nichols, A. S., Chen, S., and Luetje, C. W. (2011) Subunit contributions to insect olfactory receptor function: channel block and odorant recognition.
Mosquito Repellents Inhibiting Orco Function

Chem. Senses 36, 781–790
45. Pask, G. M., Jones, P. L., Rützler, M., Rinker, D. C., and Zwiebel, L. J. (2011) Heteromeric Anopheline odorant receptors exhibit distinct channel properties. PLoS One 6, e28774
46. Kröber, T., Kessler, S., Frei, J., Bourquin, M., and Guerin, P. M. (2010) An in vitro assay for testing mosquito repellents employing a warm body and carbon dioxide as a behavioral activator. J. Am. Mosq. Control Assoc. 26, 381–386
47. King, W. V. (1954) Chemicals Evaluated as Insecticides and Repellents at Orlando, Fla, Entomology Research Branch, Agricultural Research Service, U.S. Department of Agriculture, Entomology Research Branch, Washington, D. C.
48. Hall, S. A., Travis, B. V., and Jones, H. A. (December 4, 1945) Insect-repellent composition. U. S. Patent 2,390,249 A
49. Christophers, S. R. (1947) Mosquito repellents; being a report of the work of the mosquito repellent inquiry, Cambridge, 1943–5. J. Hyg. (Lond.) 45, 176–231
50. Kwon, H. W., Kim, S. I., Chang, K. S., Clark, J. M., and Ahn, Y. I. (2011) Enhanced repellency of binary mixtures of Zanthoxylum armatum seed oil, vanillin, and their aerosols to mosquitoes under laboratory and field conditions. J. Med. Entomol. 48, 61–66
51. Tabanca, N., Bernier, U. R., Ali, A., Wang, M., Demirci, B., Blythe, E. K., Khan, S. I., Basar, K. H., and Khan, I. A. (2013) Bioassay-guided investigation of two Monarda essential oils as repellents of yellow fever mosquito Aedes aegypti. J. Agric. Food Chem. 61, 8573–8580
52. Kieffer, B. L., Befort, K., Gaveriaux-Ruff, C., and Hirth, C. G. (1992) The δ-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. Proc. Natl. Acad. Sci. U.S.A. 89, 12048–12052
53. Yasuda, K., Raynor, K., Kong, H., Breder, C. D., Takeda, J., Reisine, T., and Bell, G. I. (1993) Cloning and functional comparison of κ- and δ-opioid receptors from mouse brain. Proc. Natl. Acad. Sci. U.S.A. 90, 6736–6740
54. Amatruda, T. T., 3rd, Steele, D. A., Slepak, V. Z., and Simon, M. I. (1991) Gβ16, a G protein α subunit specifically expressed in hematopoietic cells. Proc. Natl. Acad. Sci. U.S.A. 88, 5587–5591
55. Biessmann, H., Andronopoulou, E., Biessmann, M. R., Dimitratos, S. D., Eliopoulos, E., Guerin, P. M., Iatrou, K., Justice, R. W., Kröber, T., Marinotti, O., Tsitoura, P., Woods, D. F., and Walter, M. F. (2010) The Anopheles gambiae odorant binding protein 1 (AgamOBP1) mediates in-dole recognition in the antennae of female mosquitoes. PLoS One 5, e9471
56. Syed, Z., and Leal, W. S. (2008) Mosquitoes smell and avoid the insect repellent DEET. Proc. Natl. Acad. Sci. U.S.A. 105, 13598–13603
57. Xu, P., Choo, Y. M., De La Rosa, A., and Leal, W. S. (2014) Mosquito odorant receptor for DEET and methyl jasmonate. Proc. Natl. Acad. Sci. U.S.A. 111, 16592–16597
58. Liu, C., Pitts, R. J., Bohbot, J. D., Jones, P. L., Wang, G., and Zwiebel, L. J. (2010) Distinct olfactory signaling mechanisms in the malaria vector mosquito Anopheles gambiae. PLoS Biol 8, e1000467
59. Ditzen, M., Pellegrino, M., and Vosshall, L. B. (2008) Insect odorant receptors are molecular targets of the insect repellent DEET. Science 319, 1838–1842
60. Dogan, E. B., Ayres, J. W., and Rossignol, P. A. (1999) Behavioural mode of action of deet: inhibition of lactic acid attraction. Med. Vet. Entomol. 13, 97–100
61. Pellegrino, M., Steinbach, N., Stensmyr, M. C., Hansson, B. S., and Vosshall, L. B. (2011) A natural polymorphism alters odour and DEET sensitivity in an insect odorant receptor. Nature 478, 511–514
62. Kain, P., Boyle, S. M., Tharadra, S. K., Guda, T., Pham, C., Dahanukar, A., and Ray, A. (2013) Odour receptors and neurons for DEET and new insect repellents. Nature 502, 507–512
63. Lee, Y., Kim, S. H., and Montell, C. (2010) Avoiding DEET through insect gustatory receptors. Neuron 67, 555–561
64. Kwon, Y., Kim, S. H., Ronderos, D. S., Lee, Y., Akitake, B., Woodward, O. M., Guggino, W. B., Smith, D. P., and Montell, C. (2010) Drosophila TRPA1 channel is required to avoid the naturally occurring insect repellent citronellal. Curr. Biol. 20, 1672–1678