Odorant receptor-mediated sperm activation in disease vector mosquitoes

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Insects, such as the malaria vector mosquito, Anopheles gambiae, depend upon chemoreceptors to respond to volatiles emitted from a range of environmental sources, most notably blood meal hosts and oviposition sites. A subset of peripheral signaling pathways involved in these insect chemosensory-dependent behaviors requires the activity of heteromeric odorant receptor (OR) ion channel complexes and ligands for numerous A. gambiae ORs (AgOrs) have been identified. Although AgOrs are expressed in nonhead appendages, studies characterizing potential AgOr function in nonolfactory tissues have not been conducted. In the present study, we explore the possibility that AgOrs mediate responses of spermatozoa to endogenous signaling molecules in A. gambiae. In addition to finding AgOr transcript expression in testes, we show that the OR coreceptor, AgOrco, is localized to the flagella of A. gambiae spermatozoa where Orco-specific agonists, antagonists, and other odorant ligands robustly activate flagella beating in an Orco-dependent process. We also demonstrate Orco expression and Orco-mediated activation of spermatozoa in the yellow fever mosquito, Aedes aegypti. Moreover, we find Orco localization in testes across distinct insect taxa and posit that OR-mediated responses in spermatozoa may represent a general characteristic of insect reproduction and an example of convergent evolution.

To date, studies of odorant receptor (OR) expression and function in mosquitoes and other insects have been limited to adult and larval appendages where the fundamental properties of insect chemosensation continue to be elucidated (1–6). Unlike their mammalian counterparts, which function strictly as G protein-coupled receptors (GPCRs), insect ORs generally act as heteromeric ion channels of at least two subunits: a highly conserved coreceptor (Orco) and a ligand-recognizing receptor (ORx) (7–11), although evidence for second messenger signaling has also been observed (12, 13), especially in sex pheromone signaling (14). Although their exact stoichiometry remains unresolved, OR channels serve as nonspecific channels of monovalent and divalent cations, including calcium, whose relative permeabilities depend upon ORx (9). Within this paradigm, ligands for numerous members of the Anopheles gambiae OR family (AgOrs) have been identified (4, 15–17). Although AgOrs are expressed in tissues beyond adult head appendages, studies regarding AgOr function in nonolfactory tissues have not, until now, been conducted. One intriguing possibility is that AgOrs act to mediate spermatozoa responses to endogenous signaling molecules. Indeed, several studies have suggested the existence of signaling pathways in insect sperm, including proteomics analyses in Aedes aegypti (18) and Drosophila melanogaster (19), although ORs were not identified in those studies. Importantly, OR expression in male germ cells has been reported for numerous mammalian species (20–22) and evidence for functional expression of ORs in human and mouse sperm have been described (23–27), although the requirement for human ORs in ligand recognition and fertilization has been seriously challenged (28). In a potentially striking example of convergent evolution, we describe the expression of a subset of ORs in male germ cells of A. gambiae where they act to modulate activation and perhaps orientation of spermatozoa, which are critical to male reproductive fitness.

Results and Discussion

Nonolfactory Expression of A. gambiae OR Transcripts. A previous RNA sequencing (RNAseq) study in A. gambiae adults revealed that a subset of AgOrs is enhanced in whole male bodies (5). One interpretation of those data is that AgOrs are functional in nonhead tissues in males where they are used in noncanonical chemosensory roles. Given the previous characterizations of functional OR expression in mammalian sperm (23, 26, 27), we speculated that AgOrs may also contribute functionally to male reproductive tissues in A. gambiae. To address this hypothesis, RNAseq was used to examine relative transcript abundances in A. gambiae testes (Table 1) where more than 30 AgOrs were detected, nine of which had reads per kilobase per million (RPKM) values greater than 1 (Table 1) and their percentile ranks ranged between 20 and 45. Interestingly, seven of the 10 most abundant transcripts, AgOrs 3, 4, 5, 6, 8, 34, and 37, are predominantly expressed in tissues other than antennae (Table 1) including the maxillary palps, probosces, and larval antennae (3, 4, 29). Highly correlated results were obtained from age-matched, mated versus unmated testes samples (Fig. S1, Dataset S1), suggesting that mating itself does not alter AgOr abundance in male testes (Fig. S1B). In these studies, AgOrco was present at a very low level in one sample, but absent in the other (Dataset S1). The expression of the most abundant AgOr in testes was confirmed by reverse-transcription PCR, whereas attempts to amplify AgOrco were marginally successful in

Significance

Mosquitoes use neuronal-expressed odorant receptors in their antennae to locate blood meal sources via chemical cues emitted by hosts. Although their expression in nonsensory tissues is known, the potential for odorant receptors to also mediate endogenous signaling events in insects has remained unexplored. In this study, we have identified a subset of odorant receptors showing transcript expression in the testes of the malaria mosquito, Anopheles gambiae. In addition, we provide functional evidence that the broadly conserved insect coreceptor, Orco, mediates flagellar activation in mosquito spermatozoa. These results are reminiscent of odorant receptor function in human sperm and may represent an intriguing example of convergent evolution.


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two of five biological replicates (Fig. S2). Independently, expression of AgOrs in testes, including AgOrco and three other AgOrs identified in our RNAseq studies (Table 1), was found in a previous microarray study by Baker et al., where AgOr37 was specifically recognized as being enhanced in male testes (30). These multiple lines of evidence support the hypothesis that AgOr transcripts are expressed in testes, and in some cases, their expression is similar in antennal-expressed AgOr transcripts where RPKMs were often observed to be less than 10 (5). Additionally, several testes/sperm-specific transcripts were found in high abundance in our RNAseq samples, indicating both tissue/RNA integrity and the comparatively low abundances of AgOrs (Dataset S1).

Expression of AgOrco Protein in Male Reproductive Tissues. Detection of AgOr transcripts in testes raised the possibility that some Ors are expressed as functional proteins in spermatozoa. However, the lack of apparent AgOrco transcript might also indicate that AgOrs in testes function in a unique manner, one that does not rely on AgOrco. Alternatively, AgOrco protein may be present, despite the near absence of detectable transcript, and is stable throughout spermatogenesis. To examine this possibility, we first used Multidimensional Protein Identification Technology (MudPIT) to investigate the A. gambiae testis proteome and were unable to identify any AgOr proteins. By comparison, only two AgOrs (AgOr47 and AgOr39) were detected in a parallel MudPIT analysis of the proteome of A. gambiae antennae, where AgOrs were expected to be highly enriched. These results strongly suggest that more sensitive techniques are needed to identify AgOr protein expression in A. gambiae testes. Therefore, a previously characterized Orco antibody (11) that specifically labels the Orco protein in adult antennae of A. gambiae and D. melanogaster (Fig. S3) was used in immunohistochemical (IHC) examinations of AgOrco protein expression in testes. In these studies, AgOrco was detected in testes sections, including multiple developmental zones where the most robust fluorescence was detected in immature regions (Fig. 1). AgOrco expression appeared as puncta along the flagella of mature spermatozoa, coincident with α-tubulin that did not extend into the midpiece or head region (Fig. 1B, Inset and Fig. 2A). Antibody labeling was effectively blocked by an Orco antigen-specific peptide (Fig. 2B, Fig. S3), but not by a nonspecific peptide (Fig. 2C, Fig. S3). These results indicate that the AgOrco protein is expressed in male sperm where it may colocalize with testes-expressed tuning AgOrs that were identified in our RNAseq analyses to form functional ligand-gated ion channels.

One potential explanation for the apparent absence of AgOrco transcripts in testes is that its expression occurs at an earlier life stage, perhaps during larval gonad development, and that the translated protein is highly stable, such that it is active throughout the adult male life. The apparent persistence of AgOrco across multiple stages of spermatogenesis in the adult testis is an indication of this stability (Fig. 1). Altogether, our data raise the possibility that AgOrs perform previously unknown functions in A. gambiae spermatozoa where they may mediate responses to chemical signals.

Activation of Spermatozoa. To explore the possible biological function of AgOrs in A. gambiae testes, and in light of the known responses of vertebrate spermatozoa to exogenous chemical stimuli (23, 26, 27), we have developed a simple video-based bioassay (Fig. S4) to examine the amplitudes of flagellar movements of bulk spermatozoa nascent to ruptured testes in response to a range of natural and synthetic compounds. Responses were scored by post hoc examination of video clips in a double-blinded fashion (Movie S1). The stimuli used in these assays were comprised of a range of unitary odorants as well as highly specific Orco modulators that have been recently characterized (8, 31, 32). Flagellar beating responses were significantly elevated in the presence of two Orco agonists, VUAA1 and VUAA4, but not in the presence of a nonpotent structural analog, VUAA0 (32) (Fig. 3A). Moreover, the Orco antagonist, VU0183254 (hereafter VUANT) (31), did not activate spermatozoa flagella on its own, whereas VUAA1 and VUAA4 responses were significantly reduced when VUANT was coapplied (Fig. 3B). The presence of other AgOr transcripts in testes suggests the presence of heteromeric Or complexes in spermatozoa. We therefore speculated that a subset of the known AgOr ligands (15, 16) would also activate flagella, mimicking the effect of Orco agonists. To examine this, we used a panel of odorant ligands in the spermatozoa flagella bioassay, revealing that fenchone, which can activate several AgOrs, including the testes-expressed AgOr11 (15, 16), induced significant spermatozoa movements in a concentration-dependent manner (Fig. 3C). The fenchone responses increased from 10−6 to a peak activity at 10−4 molar and then decreased at 10−3 and 10−2 molar, becoming insignificant compared with buffer alone (Fig. 3C). A newly identified AgOr6 ligand, indole-3-carboxyaldehyde, also activated A. gambiae spermatozoa in a concentration-dependent manner with highest activity at 10−2 molar (Fig. 3B). Importantly, both the fenchone and indole-3-carboxyaldehyde responses were inhibited by the coapplication of the Orco antagonist VUANT (Fig. 3B and C), indicating that

![AgOrco protein expression in tests and spermatozoa. (A) Differential interference contrast (DIC) image of A. gambiae testis showing zones of sperm development. (B) Immunolabeling of AgOrco (green) in whole testis counterstained with the nuclear acid dye, propidium iodide (magenta). Germ cell/spermatogonia regions demarcated with dotted line. (Inset) Higher magnification of single spermatozoa; h, head; m, midpiece; f, flagellum. (C) AgOrco (green) in germ cell/spermatogonia region of A. gambiae testis; a, anterior; p, posterior.](image-url)
flagellar responses to both compounds require a functional Orco subunit. These results support the hypothesis that flagellar beating responses of sperm can be modulated by heteromeric AgOr complexes and constitutes evidence for their function outside of sensory neurons in *A. gambiae*.

Interestingly, a membrane-permeable form of cyclic adenosine monophosphate (8-Br-cAMP) also induced a significant increase in flagellar beating at several concentrations (Fig. 3D). Both cAMP and cyclic guanosine monophosphate (cGMP) are important second messengers that regulate flagellar beating in response to activators and chemoattractants of mammalian and marine invertebrate sperm (33, 34). The cAMP activation of *A. gambiae* sperm was unaffected by VUANT, suggesting the presence of a second messenger-mediated activation pathway that is either independent of Orco or performs downstream of Orco in *A. gambiae* (Fig. 3D). Furthermore, the lack of VUANT antagonism of the cAMP activation response also demonstrates that the VUANT receptor is not inherently toxic to *A. gambiae* spermatozoa and that the reductions in VUAA1, VUAA4, fenochrome, and indole-3-carboxylic acid–evoked flagellar beating responses in the presence of VUANT are specific to their Orco and tuning Or targets, respectively. Numerous other AgOr-activating compounds were tested in our bioassay but failed to elicit flagellar responses. These compounds included geranyl acetone, 1-oxetan-3-ol, 2-acetoephene, butylamine, and 4-methyl cyclohexanol. The lack of responses to these compounds could indicate technical impediments to their delivery to receptors in our assay or real differences in their contextual recognition in this tissue. Further studies will be needed to clarify these issues.

Although the concentrations of compounds that elicited spermatozoa activation are arguably high and likely to be outside the range of physiological relevance, we propose that several factors may be responsible for these high response thresholds.

**Fig. 2.** AgOrco protein expression in spermatozoa. IHC labeling of spermatozoa with an Orco antibody. (A) Left, anti-Orco (green); Center, anti–α-tubulin (blue); Right, overlay of green and blue signals (cyan) plus propidium iodide (magenta). (B) Left, anti-Orco preincubated with AgOr18 peptide (green); Center, anti–α-tubulin (blue); Right, overlay of green and blue signals (cyan) plus propidium iodide (magenta). Scale bar in C applies to all images.

First, the effective dose that is experienced by receptors on individual spermatozoa may not correlate exactly with the compound dilutions because of the potential effects of the numerous other testes-derived cells and the compounds that they may release into the preparation. Second, other factors may be impacting the threshold concentrations required to elicit flagellum activation in these assays. For example, unknown factors released by male accessory glands during mating might prime the sperm for subsequent activation in the female reproductive tract. Such factors could conceivably lower the threshold responses of AgOr complexes in vivo by impacting their localization or activity, but were not explored in our bioassay. Third, the concentrations of VUAA1 and VUAA4 that activate spermatozoa in our bioassays are actually comparable to concentrations that have been shown to elicit activity in AgOrco channels expressed in heterologous systems and in endogenous olfactory neurons (8). The bioassay data suggest that heteromeric complexes of AgOrs represent one of potentially several signaling pathways that participate in the activation of spermatozoa in *A. gambiae*.

In light of the *A. gambiae* bioassay results, we used a recently described orco−/− mutant strain of *A. aegypti* (35) to examine the specificity of spermatozoa flagellar responses to the Orco agonist, VUAA4 (Fig. 4A). Strikingly, spermatozoa from a wild-type strain of *A. aegypti* responded robustly to the application of VUAA4, whereas spermatozoa from the orco−/− strain were unresponsive (Fig. 4A). Similar to *A. gambiae*, *A. aegypti* sperm flagellar beating was also stimulated by the application of 8-Br-cAMP in both the wild-type and orco−/− mutant strains (Fig. 4A).

These results further implicate Orco in the VUAA spermatozoa response and strongly support the hypothesis that the cAMP response is independent of Orco. Importantly, IHC labeling also confirmed the presence of Orco protein in wild-type *A. aegypti* testes as well as the absence of Orco in orco−/− mutant testes (Fig. 4B and C). IHC labeling was evident throughout *A. aegypti* testes developmental zones, but strongest in the immature zones (Fig. 4B), mirroring the observations of Orco expression in *A. gambiae* testes (Fig. 1). We also attempted to perform bioassays on spermatozoa in wild-type and orco−/− mutant *D. melanogaster* (36). However, *D. melanogaster* sperm exhibited a very high background flagellar beating in our experimental conditions, thus precluding discrimination of activating responses to compounds. Nonetheless, antibody labeling in *D. melanogaster* demonstrated Orco antibody labeling in wild-type but not in orco−/− mutant testes (Fig. 5A and B).

Additional IHC studies revealed the potential presence of highly conserved Orco protein orthologs within spermatozoa of other holometabolous insects, including the parasitic wasp, *Nasonia vitripennis* (Fig. 5C and D) and the mosquito *Aedes albopictus* (Fig. 5E and F). These results raise the possibility that Orco expression in testes/spermatozoa is broadly conserved across insect lineages. If so, the functioning of OR complexes in sperm activation that are suggested by our bioassay data may be a general feature of insect reproduction.

Although we recognize that overt viability and fecundity defects have not been reported for laboratory-reared orco mutants in *D. melanogaster* (35) and *A. aegypti* (36), such conditions do not preclude the presence and biological importance of a subtle yet significant OR-based reproductive fitness advantage being active in natural insect populations. Furthermore, it is also possible and indeed likely that other ion channel and chemosensory receptor gene families may also facilitate parallel signaling functions in spermatozoa. Our RNAseq transcriptome profiling studies revealed that transcripts for multiple members of *A. gambiae* variant ionotropic receptor (*Agr*), gustatory receptor (*AgGr*), and odorant-binding protein (*AgObp*) gene families are present in the testes of *A. gambiae* males (Dataset S1). In total, we found 14 AgGrs, 17 Agrs, and six AgObps with RPKMs greater than 1, among which two AgGrs, four Agrs, and five AgObps had transcript abundances above the

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median of the entire testis transcriptome (Dataset S1). These highly expressed chemosensory genes include AgGr22, which encodes a carbon dioxide receptor, and several conserved AgOrs with significant antennal expression (5).

*Fig. 4.* Orco function and expression in *A. aegypti* sperm/testes. (A) Activation indices (y axis; + SEM) at given log_{10}[M] (x axis) for wild-type (white bars) or orco−/− mutant spermatozoa (striped bars) in response to buffer (Left), VUAA4 (Center), or 8-Br-CAMP (Right). n = 6 for all compound treatments and n = 7 for buffer control. (B and C) IHC labeling of *A. aegypti* testes. (B) Wild-type and (C) orco−/−. Left, anti-Orco (green); Right, overlay of anti-Orco (green), anti-α-tubulin (blue), and propidium iodide (magenta) signals.

and it is unlikely that the volatile AgOr ligands used here comprise the endogenous signals involved in *A. gambiae* spermatozoa activation. Indeed, endogenous ligands for human sperm ORs have recently been characterized, which are distinct from their previously identified volatile ligands (39). Examples of directed movement of sperm have been extensively characterized in marine invertebrates and mammals (reviewed in ref. 40) as well as several insect species. For example, in the beetle, *Drosophila melanogaster*, sperm swim backward upon entering the female reproductive tract, and genetic ablation of the spermatic gland in the boll weevil, *Anthonomus grandis*, is required for sperm activation, storage, clearance, and fertility (42, 43). In *D. melanogaster*, sperm swim backward upon entering the female reproductive tract, and genetic ablation of the spermatic gland in the boll weevil, *Anthonomus grandis*, is required for sperm activation, storage, clearance, and fertility (42, 43). In *D. melanogaster*, sperm swim backward upon entering the female reproductive tract, and genetic ablation of the spermatic gland in the boll weevil, *Anthonomus grandis*, is required for sperm activation, storage, clearance, and fertility (42, 43).

Reproductive fitness is an important component in establishing and maintaining insect populations, and accordingly, the vectorial capacity of malaria vectors. Despite ongoing efforts to characterize the functions of accessory gland proteins and sperm in the formation of the *A. gambiae* mating plug and fertilization (49–55), the potential signals that induce sperm activation, spermatozoa localization, retention, or fertilization, within the female reproductive tract remain unknown. An intriguing possibility is that females produce and release chemicals that activate male sperm before fertilization that also act as chemotactic cues to orient or otherwise direct sperm motility. Importantly, the overall reproductive success of *A. gambiae* males correlates positively with the presence of motile spermatozoa in mated female spermatozoa and negatively with sperm length (56, 57). In this context, an enhanced understanding of *A. gambiae* sperm activation/motility and the molecular processes that impinge upon them will be significant in terms of both basic biology and as a potential means to develop new vector and, more broadly, insect control methods.
The activation of insect sperm via ionotropic ORs is reminiscent of capacitance of mammalian sperm, which has been linked to signaling pathways mediated by metabotropic ORs (23, 26, 27) as well as the activity of several ionotropic channels (58, 59). These include calcium channels, most notably the sperm cation channels, CatSpers (60–63), potassium channels such as Kcn1 (64, 65) as well as sodium, proton, bicarbonate, and chloride channels that are localized along the sperm flagella and speculated to act downstream of receptors for diverse extracellular ligands (33, 59). The modulation of insect and mammalian sperm via proteins of distinct evolutionary origins yet that encompass conserved modes of signal transduction represents a potent example of convergent evolution impacting upon a singularly essential biological process.

Materials and Methods

RNAseq. Testes were dissected from sexually mature, unmated or mated males at 4–6 days posteclosion into TRIzol reagent for subsequent total RNA isolation. Messenger RNA was isolated, and sample libraries were prepared for RNAseq on the Illumina HiSeq platform by the Hudson Alpha Institute for Biotechnology. Approximately 20 million, 50 bp paired-end reads were generated for each sample. Quality filtered reads were mapped to the A. gambiae genome using the TopHat2 short read mapper (66) and quantified using generalized fold change (GFOLD) differential expression analysis program (67). Transcript abundance values were calculated for unmated and mated samples separately.

Immunolocalization of AgOrco. Cryosections of paraformaldehyde-fixed A. gambiae testes were collected on gelatin-coated glass slides and dried. Slides were processed according to a previously published protocol (2) and used as substrates for immunohistochemistry with an Orco-specific antibody (11).

Spermatozoa Bioassay. We developed a bioassay to examine sperm flagellum activation in response to a range of chemical cues. We took advantage of previous AgOr deorphanization studies that uncovered ligands and modulators for both a sexually mature, 4–6-day-old A. gambiae male and placed in 2 μL assay buffer [145 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1.3 mM CaCl₂, 5 mM 8-deoxy-cAMP, 0.1% BSA, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.4] containing 10% (vol/vol) DMSO and test chemicals on a clean glass microscope slide (24 × 50 mm GOLD SEAL, LOT# 121311–9) using a pair of blunt-end forceps to prevent tissue damage. A coverslip (22 × 22 mm VWR, 040912–9) was placed on the top of preparation and gently pressed for four times to squeeze open the testis wall and release spermatozoa into the assay buffer (Fig. S4). The slide was placed under an inverted microscope equipped with a digital video camera (Kagem DigitalZeiss Axiovert 35 at 200x magnification). Videos were recorded for ~2 min using Ethovision software (Noldus), while the microscope slide was slowly manipulated in the XY and focal planes every 10 to scan around the entire testis area (Fig. 54). Each compound and vehicle treatment was repeated 5–21 times with spermatozoa isolated from different individuals. 8-Bromo-cAMP was obtained from Sigma-Aldrich, Inc. (Cat# B5386). VUAA-class compounds were prepared as previously described (8, 31, 32). All other compounds were obtained from Sigma-Aldrich at the highest purity available. Video-recorded bioassays were arranged in randomized orders and processed using premier pro software (Adobe Inc.) to remove unnecessary focal adjustment as well as stage moving so that a minimum of four fields of view were obtained for subsequent scoring. Each video clip was viewed by four independent observers who were blinded to the treatment conditions and trained to provide a general assessment on the activation level of the spermatozoa by assigning an “activation index” (AI) (Fig. 54). The qualitative AI scale ranges from 0, no flagella moving, to 3, nearly all flagella moving. All spermatozoa within the field of view were considered. This assay has proven to be very robust and allowed us to rapidly assess sperm responses to chemical treatments. The JMP10 statistical software package (SAS Institute, Inc.) was used to identify statistically significant differences between mean AIs of test compounds and vehicle, via the nonparametric Mann–Whitney U test (P < 0.01).

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