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Activation of Pheromone-Sensitive Neurons Is Mediated by Conformational Activation of Pheromone-Binding Protein

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SUMMARY

Detection of volatile odorants by olfactory neurons is thought to result from direct activation of seven-transmembrane odorant receptors by odor molecules. Here, we show that detection of the *Drosophila* pheromone, 11-*cis* vaccenyl acetate (cVA), is instead mediated by pheromone-induced conformational shifts in the extracellular pheromone-binding protein, LUSH. We show that LUSH undergoes a pheromone-specific conformational change that triggers the firing of pheromone-sensitive neurons. Amino acid substitutions in LUSH that are predicted to reduce or enhance the conformational shift alter sensitivity to cVA as predicted in vivo. One substitution, LUSH^{D118A}, produces a dominant-active LUSH protein that stimulates T1 neurons through the neuronal receptor components Or67d and SNMP in the complete absence of pheromone. Structural analysis of LUSH^{D118A} reveals that it closely resembles cVA-bound LUSH. Therefore, the pheromone-binding protein is an inactive, extracellular ligand converted by pheromone molecules into an activator of pheromone-sensitive neurons and reveals a distinct paradigm for detection of odorants.

INTRODUCTION

The antenna is the principal anatomical substrate for olfactory signaling in *Drosophila*. On the antenna, there are three morphologically distinct types of sensilla that contain odor and pheromone-sensitive receptor neurons: basiconic and coeloconic sensilla house neurons that detect general odorants, while trichoid sensilla are thought to be specialized for pheromone reception (reviewed in Benton, 2007; Smith, 2007; Ebbs and Amrein, 2007; Ejima et al., 2007; Hallem et al., 2006; Vosshall and Stocker, 2007). A subset of trichoid sensilla, the T1 sensilla, contains neurons that are activated specifically by the male-produced pheromone, 11-*cis* vaccenyl acetate (cVA) (Brieger and Butterworth, 1970; Butterworth, 1969; Clyne et al., 1997; Kurtovic et al., 2007; Xu et al., 2005). T1 sensilla are present in both males and females and show similar responses to cVA (Xu et al., 2005). cVA perception mediates a variety of behaviors, including aggregation, mate recognition, and sexual behavior (Ejima et al., 2007; Kurtovic et al., 2007; Vander Meer et al., 1986; Xu et al., 2005).

Like vertebrates, insects express a large family of odorant receptors in the olfactory structures. Each receptor is expressed in a small subset of olfactory neurons that innervate the same glomerulus, the first synaptic relay in the olfactory perception pathway (Buck and Axel, 1991; Clyne et al., 1999; Vosshall et al., 2000). Members of the *Drosophila* odorant receptor family are necessary and sufficient to confer sensitivity to many food odors, as misexpression of individual Or genes in fly olfactory neurons lacking endogenous receptors often confers the odor specificity of the misexpressed receptor (Hallem and Carlson, 2004b, 2006). However, cVA pheromone perception is more complex, requiring a specific odorant receptor, Or67d, and at least two additional gene products, SNMP and LUSH. Or67d is expressed exclusively in T1 neurons and mediates cVA responses (Ha and Smith, 2006; Kurtovic et al., 2007). The CD36 homolog SNMP is expressed in most or all trichoid neurons and is required for sensitivity to cVA (Benton et al., 2007; Jin et al., 2008). SNMP colocalizes with the odorant receptor complex in T1 neuron dendrites (Benton et al., 2007), and antiserum to SNMP infused into the lymph of T1 sensilla phenocopies SNMP loss-of-function mutants, suggesting that SNMP directly mediates pheromone sensitivity (Jin et al., 2008).

Unlike the neuronal products Or67d and SNMP, LUSH is secreted into the sensillum lymph of all trichoid sensilla by non-neuronal support cells where it bathes the dendrites of these neurons. An analysis of mutants lacking LUSH-binding protein revealed that it is required for cVA-induced behavior and normal cVA sensitivity of the T1 neurons (Xu et al., 2005). Indeed, when Or67d is misexpressed in other trichoid neurons that are normally unresponsive to cVA, this receptor confers cVA sensitivity, but only in the presence of LUSH (Ha and Smith, 2006). How

odorant-binding proteins influence pheromone reception is poorly understood, but it is an important question because their essential role in pheromone perception makes them potential targets to manipulate insect behaviors triggered by pheromones (reviewed in Benton, 2007; Smith, 2007). The current model for pheromone-binding protein function is that they act as carriers that transport and release the pheromone molecules at the dendritic surface, and released pheromone binds and activates pheromone receptors on the dendrites (Benton et al., 2007; Horst et al., 2001; Leal, 2005; Lee et al., 2002; Sandler et al., 2000; Wojtasek and Leal, 1999). However, we noted an inconsistency between the phenotype of the *lush*^{1} mutants and this model. In addition to their loss of cVA sensitivity, *lush*^{1} mutants also have a 400-fold reduction in spontaneous neuronal activity that is restricted to the pheromone-sensitive neurons. This reduced basal activity is reversed by introducing a wild-type *lush* transgene or by directly infusing recombinant LUSH protein into the sensillum lymph through the recording pipette (Xu et al., 2005). Loss of an extracellular carrier protein would not be expected to affect the spontaneous activity of these neurons. To account for this activity, we postulated that perhaps the binding protein is actually a component of the ligand that normally activates these neurons. For example, pheromone-bound LUSH might be a specific trigger for T1 neurons, and LUSH itself is a weak agonist. Using a combination of structural analysis together with in vivo pheromone sensitivity assays with engineered binding proteins, we now show that conformational changes in the binding protein are sufficient for activation of the pheromone-sensitive neurons.

RESULTS

LUSH Tunes Pheromone-Sensitive Neurons to Physiologically Relevant Levels of 11-*cis* Vaccenyl Acetate

Insect pheromone detection systems are among the most sensitive chemosensors known, approaching single pheromone molecule sensitivity (Kaissling and Priesner, 1970). Recent work has shown that cVA pheromone can activate Or67d-expressing neurons in the absence of LUSH, but high concentrations are required that are orders of magnitude over the threshold levels that activate T1 neurons (Benton et al., 2007; van der Goes van Naters and Carlson, 2007; and see Figure S5 available online). Therefore, we sought to estimate the contribution of LUSH toward sensitizing the T1 neurons to cVA pheromone. Figure 1 shows the responses of T1 neurons from wild-type, *lush*^{1} mutants, and *lush*^{1} mutants reconstituted with recombinant LUSH protein infused through the recording pipette (Xu et al., 2005) to various levels of cVA pheromone. Wild-type T1 neurons respond to all cVA concentrations over 0.03% (see Experimental Procedures) with a burst of action potentials, and the spike rates correlate well with the cVA concentration (Figure 1A). *lush*^{1} mutants, completely lacking LUSH protein (Kim et al., 1998), fail to detect cVA except for very weak responses to the 100% cVA pulse (Figure 1B, squares). This represents a loss of sensitivity of over 500-fold when LUSH is absent. The loss of cVA sensitivity in *lush*^{1} mutants is clearly due to the absence of LUSH protein, because the sensitivity deficit is completely reversed when recombinant LUSH protein is added to *lush*^{1} mutant T1 sensilla through the recording pipette (Figure 1B, diamonds). Remarkably, LUSH does not sensitize T1 neurons to structurally similar volatiles, including 11-*cis* vaccenyl alcohol or 11-*cis* vaccenic acid (Figures 1A and 1B). Therefore, LUSH protein selectively increases the sensitivity of T1 neurons to cVA.

We attempted to estimate the cVA levels that are encountered between single flies by measuring the action potential rates elicited in virgin female T1 neurons by a single male fly. cVA is secreted onto the cuticle surface of males where it is thought to mediate sexual recognition behavior. Figure 1C shows that action potentials are elicited in T1 neurons from wild-type virgin females (with no previous exposure to cVA pheromone) as a male abdomen is moved into proximity. Significant increases in spikes (above 5 spikes/s) are first observed when the male fly is within 1 cm, suggesting that cVA perception between single animals normally operates over a limited distance. We correlated these spike rates with the distance from the source. The action potential rates increased inversely with distance up to 50 spikes/s at 0.1 mm. Based on these data, action potentials in the 5–50 spikes/s range probably represent levels of cVA stimulation that are physiologically relevant during single fly interactions. These spike rates correlate to cVA stimulation of 0.03% to 10% cVA delivered by our apparatus (see Experimental Procedures). Consistent with our current and previous findings, *lush*^{1} mutant flies are completely insensitive to cVA at these levels (Figures 1B and 1C and Xu et al., 2005). T1 responses were not elicited by female abdomens, confirming that cVA is the active volatile component (Figure 1D).

Structure of the LUSH-cVA Complex

LUSH specifically sensitizes T1 neurons to cVA, but what is the mechanism of this sensitization? Because the loss of an extracellular carrier protein would not be predicted to affect spontaneous activity of neurons, we suspected that a unique LUSH-cVA surface or perhaps a pheromone-induced conformational change in LUSH might mediate T1 neuron activation. Therefore, we solved the X-ray crystal structure of LUSH bound to cVA pheromone and compared it to the previously solved structure of the apoprotein (Thode et al., 2008) and alcohol-bound forms (Kruse et al., 2003). Figure 2A shows the crystal structure of the LUSH-cVA complex solved at a resolution of 1.4 Å. The overall structure is similar to the previously solved structures and has six α helices (α1–α6) surrounding a central ligand-binding cavity (Figures 2A–2C). In the crystal, there are two protein molecules in the asymmetric unit (arbitrarily labeled A and B), and one of these monomers (monomer A) exists in two distinct conformations (described below). The average pairwise backbone root mean squared deviation (RMSD) between these two monomers is 0.55 Å. The average RMSD compared to the LUSH-butanol structure (PDB ID 1OOH) is 0.64 Å and is 0.63 Å compared to the apo-LUSH structure (PDB ID 1T14). In contrast, the RMSD between the crystal structures of the apo-protein and the LUSH-alcohol structures is 0.14 Å, showing that they are essentially identical.

Each monomer of the LUSH-cVA complex contains a single molecule of cVA (Figure 2D) that completely fills the central ligand-binding pocket. The cVA molecule itself is almost