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Functional analysis of a *Drosophila melanogaster* olfactory receptor expressed in Sf9 cells

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Abstract

Olfactory receptors (ORs) are seven transmembrane proteins that are responsible for the transduction of volatiles into neuronal signals. Their low sequence homology means that the prediction of ligands for ORs based on extrapolation from empirical data of other ORs is difficult, so an experimental approach must be used. Here, we report a functional assay for insect ORs using calcium-imaging in Sf9 cells. We find that the interaction of the odorant, ethyl butyrate, with the *Drosophila melanogaster* olfactory receptor Or22a is both dose-dependent and highly sensitive, with Or22a responding to ethyl butyrate with an EC_{50} of $(1.58 \pm 0.82) \times 10^{-11}$ M. This degree of sensitivity does not require the addition of odorant binding proteins or downstream signal transduction elements. Furthermore, we demonstrate that Or22a expressed in Sf9 cells has a similar response profile to a range of odorants previously tested *in vivo*. This functional assay system will provide a useful tool for the de-orphaning of ORs from a wide range of insect species that are yet to have ligands assigned, and will help provide insight into OR specificity and mechanism of activation.
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1. Introduction

The sense of olfaction, or smell, is mediated by the interaction of volatile ligands with a set of specialized seven transmembrane receptors known as olfactory receptors (ORs). ORs are the largest multigene family in animals, comprising 347 OR genes in humans, 913 in mice and 60 in the model insect, *Drosophila melanogaster* (Godfrey et al., 2004; Robertson et al., 2003; Zozulya et al., 2001). ORs are highly divergent both within and between species, for example the *D. melanogaster* ORs share as little as 20% amino acid identity (Vosshall, 2001). Such levels of divergence leave little avenue for the predictive assignment of ligands for these receptors based on extrapolation from empirical data of other ORs. The de-orphaning, or identification of ligand specificity of these receptors, can therefore only be achieved through experimental means.

ORs, unlike other seven transmembrane receptors, are broadly tuned, able to recognize a wide range of different compounds with varying degrees of specificity (Duchamp-Viret et al., 1999; Malnic et al., 1999). Typically, a volatile compound is able to interact with more than one OR, and can even be an agonist for one OR and an antagonist for another (Oka et al., 2004b). Thus, an organism's perception of its olfactory environment is based on the combination of signals from its full complement of ORs (Malnic et al., 1999). This explains why an organism with a complement of ORs numbered in the tens or hundreds can recognize and discriminate between thousands of different compounds or blends. These properties of the olfactory system complicate the experimental determination of ligands for ORs.

Several approaches to de-orphaning ORs have previously been applied to insect ORs. The *in vivo* response pattern of odorant receptor neurons (ORNs) expressing known ORs has been measured electrophysiologically (Dobritsa et al., 2003; Stortkuhl and Kettler, 2001). This approach has also been used where particular ORs have been expressed in an 'empty' olfactory sensory neuron in *D. melanogaster* (Hallem et al., 2004; Hallem and Carlson, 2006). In general, one OR appears to give

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each ORN its odorant specificity (Vosshall et al., 2000). In addition to the OR that confers odorant specificity, the non-canonical potential chaperone OR, Or83b, is also expressed in every ORN (Benton et al., 2006).

A further strategy to de-orphan ORs involves the use of heterologous cell systems. Patch-clamp electrophysiological assays of *Xenopus* oocytes expressing mouse and *D. melanogaster* ORs have been used to monitor the opening of ion channels as an indicator of OR activation (Katada et al., 2003; Wetzel et al., 2001). Fluorometric assays based on the detection of changes in intracellular calcium levels have also been utilized to determine odorant responses of both human and mouse ORs in a range of cell types, including human embryonic kidney 293 (HEK 293) cells (Sanz et al., 2005), *Cercopithecus aethiops* kidney (COS-7) cells (Levasseur et al., 2003) and *Spodoptera frugiperda* 9 (Sf9) cells (Matarazzo et al., 2005). Such an approach has also been used to monitor the activation of the insect ORs *D. melanogaster* Or43a and Or22a in HEK 293 cells to show the effect of their co-expression with Or83b (Neuhaus et al., 2005).

These heterologous systems have often required exogenous factors. Gα proteins, for example, can amplify signal strength by enhancing the coupling of the OR to the cell lines downstream signaling system (Sanz et al., 2005). The co-expression of insect ORs with Or83b increases the sensitivity of a calcium assay in HEK 293 cells by 1000-fold (Neuhaus et al., 2005). No insect cell system, however, has yet been tested for assaying insect OR function. Here, we report the development of a heterologous cell assay system for the determination of insect OR response profiles using insect cells. This response assay does not require exogenous factors, gives response profiles similar to *in vivo* electrophysiological profiles, and demonstrates that insect ORs are highly sensitive.

2. Materials and methods

2.1. Materials and reagents

All odorants were purchased from Sigma–Aldrich (St. Louis, MO) at the highest purity possible. Odorant solutions were made up at a stock concentration of 10^{-3} M in sterile water and stored at −20 °C. For each assay, odorant solutions were made fresh from the stock solution to the desired concentration in sterile water. Fluo-4 AM ester (excitation at 494 nm, emission at 516 nm) was obtained from Invitrogen (Carlsbad, CA) as a lyophilized powder and made to 1 mM in dimethylsulfoxide for storage at −20 °C. Assay buffer had the following constituents: 21 mM KCl, 12 mM NaCl, 18 mM $MgCl_2$, 3 mM $CaCl_2$, 170 mM D-glucose, 1 mM probenecid (Sigma–Aldrich) and 10 mM PIPES. The buffer's pH was modified to 7.2, then filter-sterilised (0.22 μm) prior to use.

2.2. Expression vector

The plasmid pIB-Or22a was constructed by inserting the cDNA for *D. melanogaster* Or22a into the multiple cloning site of the pIB/V5-His vector (Invitrogen) using the restriction enzymes *Kpn*I and *Sac*II.

2.3. Cell culture and transfection of Sf9 cells

S. frugiperda Sf9 cells (Invitrogen) were maintained as an adherent culture in Sf-900 II Serum Free Media (SFM; Invitrogen) at 28 °C in T-25 tissue culture flasks (Corning Inc., NY). Sf9 cells were grown to approximately 90% confluent as judged under a light microscope. The cells were dislodged from the growing surface by washing with the media contained in the flask. A small aliquot was then taken and the cell density measured using a hemocytometer. 1×10^6 Sf9 cells were added to 2 mL of Sf-900 II SFM in each well of a Nunclone six-well tissue culture plate (Nunc, Copenhagen, Denmark).

Transfection of Sf9 cells with plasmid DNA was performed using Escort IV (Sigma–Aldrich) according to the manufacturer's instructions. Briefly, the media covering the cells in the six-well plates was removed, the cells washed twice with fresh media and then replaced with 800 μL of fresh Sf-900 II SFM. One microgram of plasmid DNA of each construct and 6 μL of Escort IV transfection reagent was then added to 200 μL of Sf-900 II SFM per well, gently mixed and incubated at room temperature for 30 min. The DNA/Escort IV mix was then added to the well, and the plate incubated as above for 7 h. The media containing plasmid DNA and Escort IV was then removed, the cells washed twice with fresh Sf-900 II SFM, covered in 2 mL of fresh SFM, and incubated for 48 h prior to calcium imaging.

2.4. Calcium imaging

The six-well plate was tilted and the media removed with a pipette before washing with 1 mL of sterile assay buffer. Five hundred microliters of assay buffer containing 2 μM Fluo-4 acetoxymethyl ester and 0.01% pluronic acid (Sigma–Aldrich) in DMSO was added to each well and the plates incubated in the dark for 20 min. The Fluo-4 solution was removed and the cells were washed twice with sterile assay buffer to remove excess dye and replaced with 900 μL of fresh buffer. Each plate was then incubated in the dark for a further 20 min prior to calcium imaging.

Calcium imaging was performed using the 10× objective lens on a Leitz Fluovert FS inverted fluorescence microscope fitted with an I3 filter set (excitation filter 450–490 nm, dichroic mirror 510 nm, long pass emission filter 520 nm; Carl Zeiss, Jena, Germany) and a MicroMax CCD camera system (model RTE/CCD-1300-Y/HS; Princeton Instruments, Trenton, NJ). Images of the Fluo-4 loaded cells were recorded at 10 s intervals. One hundred microliters of an odorant solution was then added and further images acquired at 10 s intervals. In order to help identify cells responding to low concentrations of ethyl butyrate in the dose–response study, an additional step was incorporated in which the concentration of ethyl butyrate was raised to a final concentration of 5×10^{-4} M. Finally, to determine maximal fluorescence, ionomycin was added to the well to a final concentration of 2 μM and images collected for a further minute at 10 s intervals. Photobleaching was minimized as much as possible by switching off the UV light source between image acquisitions.

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