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Interaction between cAMP and intracellular Ca^{2+}-signaling pathways during odor-perception and adaptation in *Drosophila*

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ABSTRACT

Binding of an odorant to olfactory receptors triggers cascades of second messenger systems in olfactory receptor neurons (ORNs). Biochemical studies indicate that the transduction mechanism at ORNs is mediated by cyclic adenosine monophosphate (cAMP) and/or inositol,1,4,5-triphosphate (InsP3)-signaling pathways in an odorant-dependent manner. However, the interaction between these two second messenger systems during olfactory perception or adaptation processes is much less understood. Here, we used interfering-RNAi to disrupt the level of cAMP alone or in combination with the InsP3-signaling pathway cellular targets, InsP3 receptor ($InsP_3R$) or ryanodine receptor (RyR) in ORNs, and quantify at ORN axon terminals in the antennal lobe, the odor-induced Ca^{2+}-response. In-vivo functional bioluminescence Ca^{2+}-imaging indicates that a single 5 s application of an odor increased Ca^{2+}-transients at ORN axon terminals. However, compared to wild-type controls, the magnitude and duration of ORN Ca^{2+}-response was significantly diminished in cAMP-defective flies. In a behavioral assay, perception of odorants was defective in flies with a disrupted cAMP level suggesting that the ability of flies to correctly detect an odor depends on cAMP. Simultaneous disruption of cAMP level and $InsP_3R$ or RyR further diminished the magnitude and duration of ORN response to odorants and affected the flies' ability to detect an odor. In conclusion, this study provides functional evidence that cAMP and InsP3-signaling pathways act in synergy to mediate odor processing within the ORN axon terminals, which is encoded in the magnitude and duration of ORN response.

1. Introduction

Olfactory transduction in olfactory sensory neurons (ORNs) is initiated by the binding of an odorant to an olfactory receptor (OR), which triggers cascades of second messenger systems [1,2]. Odor binding to ORNs can increase the concentration of cyclic adenosine monophosphate (cAMP) through the activation of the G-protein coupled receptor (GPCR) and adenyl cyclase that in turn, activates the cyclic nucleotide-gated channels (CNGs) and increases Ca^{2+}-concentration within the ORNs [2–10]. Odorants also induce the formation of inositol, 1,4,5 triphosphate (InsP3), leading to an increase in Ca^{2+} concentration within the ORNs [1,7,11–13]. Odorant-induced increases in Ca^{2+} can activate a second conductance (e.g. Cl^-, cation or K^+) leading to the generation of receptor potential [14–16], which finally results in the generation of action potentials conveying the chemosensory information to the primary olfactory relay center (e.g. antennal lobe in insects/olfactory bulb in mammals) in the brain.

In contrast to the well-established transduction mechanism at vertebrate ORNs [2,17], the transduction mechanism at insect ORNs still remains partly elusive. Formerly, insects ORs were considered as a member of GPCRs [18–20]. However, more recently, new evidence suggests that unlike the mammalian ORs, insect ORs are not GPCRs and exhibit a different membrane topology [21–23]. Insect ORs serves both as odorant receptors and as ion channels [24,25] and thus, in insects, both metabotropic and ionotropic signaling mediate odor-transduction at ORNs [24,26]. Nevertheless, despite these fundamental differences in transduction machineries, the odor coding strategy at ORNs remains similar between insects and mammals [27–33].

Transduction mechanism at ORNs is mediated by cAMP and/or InsP3-signaling pathways in an odorant-dependent manner [34]. Some odorants cause an intracellular increase of the second messenger cAMP [3,4,9,35,36], while others induce an increase of InsP3 [11–13,37,38]. However, it is not completely understood how these two second messenger systems interact during odor processing in the ORNs. In addition, the roles of cAMP or InsP3-signaling pathways during odor-adaptation at ORNs have been reported previously [39,40]. Odor-adaptation is reported to occur at the level of sensory neurons by

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modulation of transduction machinery, in particular, the cAMP-gated channel [3]. Previously, we showed in *Drosophila* that odor-adaptation at ORN axon terminals is modulated by Ca^{2+}-dependent mechanisms, presumably through the $InsP_3R$ and RyR [41] but it remains unclear how simultaneous perturbation of cAMP and $InsP_3R$ or RyR affect odor-adaptation mechanisms at ORN axon terminals at the antennal lobe.

In the present study, we combine the *Drosophila* transgenic approach, using a targeted expression of RNAi to knock-down a specific gene, with in-vivo functional bioluminescence Ca^{2+}-imaging approach and behavior to study first, how cAMP modulates odor-processing at ORN axon terminals and second, how cAMP and intracellular Ca^{2+}-signaling pathways ($InsP_3R$ and RyR) interact during two phenomena: i) odor-perception/acuity (i.e. responses that occur after first odor-exposure) and ii) odor-adaptation (i.e. responses to repetitive odor-exposure).

2. Materials and methods

2.1. Animals

Drosophila melanogaster Meigen were maintained on standard medium at room temperature (24 °C). We generated double transgenic lines where two second messenger systems, for example, cAMP and InsP3 were perturbed. We used P[UAS–GFP-aequorin] (GA) transgenic flies developed previously as described in Martin et al. [42] and used specific RNAi: P[UAS-*itpr*-RNAi] (1063-R1), P[UAS-*Ryr*-RNAi] (10844-R3), P[UAS-*dnc*-RNAi] (10792-R1), and P[UAS-*rut*-RNAi] (9533-R2), courtesy of R. Ueda, NIG, Japan, to genetically knock-down the adenyl cyclase gene [*rutabaga* (*rut*)], or the phosphodiesterase gene [*dunce* (*dnc*)], and InsP3-signaling pathway cellular targets, the InsP3 receptor gene (*itpr*) or the ryanodine receptor gene (*Ryr*) at ORN axon terminals located at the antennal lobe. In this study, odor neural responses and behavior were investigated in the following genotypes: i) OR83b,GA5/CS, ii) OR83b,GA5/*rut*-RNAis, iii) OR83b,GA5/*dnc*-RNAis, iv) OR83b,GA5/*rut*,*itpr*-RNAis, v) OR83b,GA5/*dnc*,*itpr*-RNAis, vi) OR83b,GA5/*rut*,*Ryr*-RNAis, and vii) OR83b,GA5/*dnc*,*Ryr*-RNAis.

2.2. In-vivo calcium imaging of odor-neural activities at ORNs

Female flies, 4-days old were used for in-vivo brain imaging. The flies were briefly cold (ice) anesthetized, inserted in a truncated 1 ml commercial pipette tip in such a way that the head protruded through the tip of a truncated pipette and fixed in that position using a dental glue (Protemp III, ESPE, Seefeld, Germany). The assembly was then placed in an acrylic block and secured with Parafilm™. A drop of Ringer's solution [42] was deposited on the head, and a tiny window in the head capsule was cut out to expose the ORNs. Care was taken to not damage the antennae. Exposed brains were then incubated in fly Ringer's solution containing 5 μmol^{l-1} native coelenterazine (Uptima, Interchim, Montluçon, France) for 2 h prior to experiments.

To record odor neural responses, we stimulated the flies' ORs located at the antenna with three different odors: spearmint, citronella, and octanol (3-octanol) and monitored changes in calcium dynamics across ORN axon terminals at the antennal lobe. Odor-evoked bioluminescence signals in the ORNs were monitored with an electron multiplier CCD camera (EM-CCD, Andor, iXon, Belfast, Ireland; cooled to −80 °C) fitted onto a microscope (Nikon, Eclipse-E800). The setup was placed inside a tight dark box (Science Wares, Inc., Falmouth, MA, USA). Using a 20X air objective lens (N.A. 0.75; Plan Apochromat, Nikon France S.A., Champigny-sur-Marne, France), the field of view was 400 × 400 μm (512 × 512 pixels). To improve the signal-to-noise ratio, data were acquired with a 2 s integration time, and 2 × 2 binning was used (1 pixel = 1.56 μm × 1.56 μm). To acquire and store data, each detected photon was assigned x and y-coordinates and a time point.

2.3. Odor stimulus delivery

Odorants were delivered using a custom-made olfactometer that consisted of an air pump, moistening bottle and four identical channels, one of which was devoted to control air (without odor) and the rest to the odorants. Each channel was connected to a 50 ml bottle with a solenoid activated pinch valve (Sirai S-104). All connecting tubes were made of silicone. An air stream was delivered to the fly's antennae through a small glass tube that was placed a few millimeters away. During the experiment, air flowed continuously (500 ml min^{-1}) through the control channel except when a logic command issued by the imaging software switched the flow to one of the odorant-containing channels. Each odorant reservoir contained 5 μl of undiluted pure odor (all from Sigma-Aldrich, Saint-Quentin Fallavier, France), deposited on a piece of filter paper.

2.4. Olfactory behavioral assay

Olfactory behavioral responses were determined using an olfactory T-maze test, slightly modified from that used by Störtkuhl et al. [43] and Murmu et al. [41,44]. The apparatus consisted of two side arms (chambers) and a central sliding chamber. Odorant containing airstreams were drawn, from a 50 ml bottle containing 5 μl of undiluted odorant deposited on a filter paper, through one side arm while the other side arm was supplied with the control air. Air was continuously drawn through the system by a pump at a rate of 1 l min^{-1}. To measure odor perception/acuity, a batch of 10 flies, starved beforehand for 6 h, was placed at the center of the T-maze and was given 15 s to choose either the control or odor-containing arms. For olfactory-adaptation experiments, the flies were pre-exposed to a given odorant at the top chamber for 5 min after which they were moved down via the central chamber and given 15 s to choose between the control (air) and odor-containing airstreams. In both cases, the total number of flies trapped in control and odor-containing arms was counted. The performance of flies in the T-maze assay was measured by generating a response index (RI) with values ranging from 1.5 to −1.5, which was obtained by subtracting the number of flies in an odorant-containing arm from the number of flies in the control arm and dividing it by the total number of flies. In this experiment, negative RI meant that the flies were repelled by a given odorant; positive RI meant that they were attracted to a given odorant and 0 meant that they were indifferent to odorants.

2.5. Data and statistical analysis

Imaging data were analyzed using the Photon Viewer (1.0) software (Science Wares) written in LabView 7.1 (National Instruments, Nanterre, France). In the current study, we investigated two different phenomena: 1) odor-perception/acuity, and 2) odor-adaptation. Odor-perception/acuity was measured by calculating changes in three parameters that occurred immediately after the first odor-exposure: i) average amplitude of the ORN response, ii) average sum of photons (i.e. area below the curve) and iii) average of total duration (i.e. total amounts of time) that the ORNs respond to each odorant. Odor-adaptation was measured by calculating changes in the same three parameters generated by odorants during five successive odor-applications at 5-min intervals. For statistical comparison of imaging data in experiment 1, one-way ANOVA followed by a Tukey's multiple comparisons test was used to measure significant differences between control and experimental groups. For statistical comparison of imaging data in experiment 2, one-way ANOVA followed by a Tukey's multiple comparisons test was used to measure decreases in amplitude, sum or duration of odor-generated photons during five successive odor-applications. Adaptation was measured by comparing Ca^{2+}-responses evoked by odorants during first odor-exposure to those generated during second, third, fourth and fifth odor-stimulations. Animals are considered to have adapted only if there