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SHORT COMMUNICATION

Odor-induced cAMP production in *Drosophila melanogaster* olfactory sensory neurons

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ABSTRACT

Insect odorant receptors are seven transmembrane domain proteins that form cation channels, whose functional properties such as receptor sensitivity are subject to regulation by intracellular signaling cascades. Here, we used the cAMP fluorescent indicator Epac1-camps to investigate the occurrence of odor-induced cAMP production in olfactory sensory neurons (OSNs) of *Drosophila melanogaster*. We show that stimulation of the receptor complex with an odor mixture or with the synthetic agonist VUAA1 induces a cAMP response. Moreover, we show that while the intracellular Ca^{2+} concentration influences cAMP production, the OSN-specific receptor OrX is necessary to elicit cAMP responses in Ca^{2+}-free conditions. These results provide direct evidence of a relationship between odorant receptor stimulation and cAMP production in olfactory sensory neurons in the fruit fly antenna and show that this method can be used to further investigate the role that this second messenger plays in insect olfaction.

KEY WORDS: 3′,5′-Cyclic adenosine monophosphate, Fruit fly, Odorant receptor, Orco, Or22a, Epac1-camps

INTRODUCTION

Chemical stimuli play a central role in insect ecology. Faint concentrations of odorant blends or even single compounds can inform insects, like the fruit fly *Drosophila melanogaster*, about the presence of, for example, food sources, perils or sexual partners (Hansson and Stensmyr, 2011; Mansourian and Stensmyr, 2015). The two main olfactory organs of *Drosophila* – the antennae and the maxillary palps – express three major classes of chemoreceptors: the odorant receptors (ORs), the gustatory receptors (GRs) and the ionotropic receptors (IRs), which are related to ionotropic glutamate receptors (Touhara and Vosshall, 2009; Joseph and Carlson, 2015). ORs are seven transmembrane domain proteins not related to any other receptor family and form heteromers of a neuron-specific OR protein (OrX) and a ubiquitous co-receptor (Orco) (Larsson et al., 2004).

The OR heteromers form ligand-gated cation channels (Neuhaus et al., 2005; Sato et al., 2008; Wicher et al., 2008) of undefined stoichiometry. Their functional properties, e.g. sensitivity, are regulated by multiple intracellular signaling cascades (Nakagawa and Vosshall, 2009; Wicher, 2015). The second messenger 3′,5′-cyclic adenosine monophosphate (cAMP) is an activating ligand for Orco channels (Wicher et al., 2008; Stengl, 2010; Stengl and Funk, 2013) and has been shown to enhance the activity of olfactory sensory neurons (OSNs) that express ORs (Olsson et al., 2011; Getahun et al., 2013). Insect OSNs possess the cellular machinery required to produce cAMP (Iourgenko and Levin, 2000; Boto et al., 2010) and disruption of this signaling cascade has been reported to affect the functional properties of OSNs (Martín et al., 2001; Gomez-Diaz et al., 2004; Deng et al., 2011). However, insect ORs show an inverted topology with respect to their mammalian counterparts (Benton et al., 2006), they are not related to any known G protein-coupled receptor and there is no proof of a direct interaction between insect ORs and G proteins. The current consensus model suggests that the slow metabotropic regulation of ORs by cAMP or 3′,5′-cyclic guanosine monophosphate (cGMP) is achieved indirectly by as yet undescribed membrane receptors co-stimulated by the ORs or by the influx of Ca^{2+} through the channel pore upon activation (Nakagawa and Vosshall, 2009).

Several approaches have been used to study the effect of cAMP on OR modulation, including genetic manipulations coupled with electrophysiological (Martín et al., 2001; Deng et al., 2011), behavioral (Martín et al., 2001; Gomez-Diaz et al., 2004) and optogenetic (Bellmann et al., 2010) experiments. The recent establishment of genetically encoded cAMP fluorescent indicators has broadened the number of possible approaches (Gorshkov and Zhang, 2014; Calebiro and Maiellaro, 2014). The Förster resonance energy transfer (FRET)-based sensor Epac1-camps is a notable example (Nikolaev et al., 2004; Börner et al., 2011). This single-chain indicator is constituted of the cAMP binding domain of the exchange protein directly activated by cAMP 1 (Epac1) flanked by an N-terminal cyan fluorescent protein (CFP) and a C-terminal yellow fluorescent protein (YFP), and has been used successfully to image cAMP dynamics in a variety of tissues also in *D. melanogaster* (e.g. Shafer et al., 2008). Previous work from Lissandron et al. (2007) showed the possibility of imaging cAMP dynamics in the fly mushroom body, a brain region involved in olfactory learning, using a protein kinase A-based sensor (GFP-PKA). Unfortunately, the brightness and dynamic range of these fluorescent indicators have not yet been refined like other neural activity indicators (Störtkuhl and Fiala, 2011; Broussard et al., 2014), making *in vivo* imaging of first level OSNs through the cuticle of intact antennae more complicated.

In this study we used the Epac1-camps sensor to test the hypothesis that OR stimulation leads to odor-induced cAMP production in *D. melanogaster* OSNs. We first expressed Epac1-camps in Orco-expressing OSNs and studied the change in CFP/YFP emission ratio of the sensor after stimulation of these neurons in *ex vivo* conditions, both for flies expressing a functional Orco and for those expressing a mutated form. We then used a subset of neurons, namely those expressing the OrX protein Or22a, as a model to characterize this signal, in particular its persistence in extracellular Ca^{2+}-free conditions and whether it could be silenced by pharmacological inhibition of adenylyl cyclases (ACs). Finally,

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we investigated whether Ca^{2+} influx via depolarization of OSNs lacking functional OR complexes is sufficient to induce a response. In this way, we could image the time-dependent production of cAMP in insect OSNs and study its properties.

MATERIALS AND METHODS

Insect rearing and antennal preparation

Drosophila melanogaster Meigen 1830 rearing and the antennal preparations from 4–8 day old female flies were performed as described in Mukunda et al. (2014). Parental lines were obtained from Bloomington Stock Center [UAS-Epac1-camps no. 25409, Orco-Gal4 no. 26818, UAS-Orco no. 23145, $Orco^1$ no. 23129, CKG30 (Casso et al., 2000) no. 5194], the Or22a-Gal4 line was donated by Dr L. Vosshall (Rockefeller University) and the Δhalo (Dobritsa et al., 2003) fly line by Dr J. R. Carlson (Yale University). Flies used for experiments were from stable lines with the following genotypes: $Orco^{+/+}$: *UAS-Epac1-camps, w; Orco-Gal4; +*. $Orco^{-/-}$: *UAS-Epac1-camps, w; Orco-Gal4; Orco*^{1}. Orco rescue: *UAS-Epac1-camps, w; Orco-Gal4, UAS-Orco; Orco*^{1}. $Or22a^{+/+}$: *UAS-Epac1-camps, w; +; Or22a-Gal4*. $Or22a^{-/-}$ flies were selected from an $Or22a^{-/+}$ line with the following genotype: *UAS-Epac1-camps, w; Δhalo/CKG30; Or22a-Gal4*. Δhalo homozygote larvae showing no GFP fluorescence were selected using a SteREO Discovery V20 (Zeiss, Jena, Germany) and adult genotype was confirmed individually by PCR after the experiment.

Chemicals and solutions

Solutions used in this study were as follows: regular *Drosophila* Ringer solution (5 mmol l^{-1} Hepes, 130 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 4 mmol l^{-1} $MgCl_2{\cdot}6H_2O$, 2 mmol l^{-1} $CaCl_2$, 36 mmol l^{-1} sucrose), Ca^{2+}- and Na^+-free Ringer solution (130 mmol l^{-1} *N*-methyl-D-glucamine, 10 mmol l^{-1} HCl, 5 mmol l^{-1} KCl, 4 mmol l^{-1} $MgCl_2{\cdot}6H_2O$, 5 mmol l^{-1} Hepes, 10 mmol l^{-1} EGTA, 6 mmol l^{-1} sucrose), high KCl Ringer solution (103 mmol l^{-1} NaCl, 50 mmol l^{-1} KCl, 4 mmol l^{-1} $MgCl_2{\cdot}6H_2O$, 2 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} Hepes). All solutions had an osmolarity of 323 mOsm l^{-1} and were adjusted to pH 7.3; reagents were purchased from Sigma-Aldrich (Steinheim, Germany) and Carl Roth (Karlsruhe, Germany). VUAA1 {*N*-[4-ethylphenyl]-2-[(4-ethyl-5(3-pyridinyl)-4*H*-1,2,4-triazol-3-yl)thio]acetamide, CAS no. 525582-84-7} was synthesized by the Mass Spectrometry/Proteomics group of the Max Planck Institute for Chemical Ecology (Jena, Germany). Ethyl acetate (99.8% purity), methyl acetate (99.8% purity), ethyl hexanoate (99% purity), forskolin, dideoxyforskolin, 3-isobutyl-1-methylxanthine (IBMX), SQ22536 and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Stimulus solutions were prepared by diluting the appropriate amount of stock solution (100 mmol l^{-1} for ethyl acetate, methyl acetate, ethyl hexanoate, VUAA1 and IBMX; 10 mmol l^{-1} for forskolin; 8 mmol l^{-1} for dideoxyforskolin, in DMSO and stored at −20°C) in the appropriate Ringer solution immediately before use; control solutions were prepared by dissolving 0.1% DMSO in the appropriate Ringer solution. SQ22536 was pre-solubilized in DMSO from a 100 mmol l^{-1} stock solution or, for final concentrations higher than 100 µmol l^{-1}, immediately before experiment, so that the final concentration of DMSO was 0.1%.

cAMP imaging

Imaging was performed using a BX51WI widefield fluorescence microscope (Olympus, Hamburg, Germany) equipped with a 60×/0.90 water immersion LUMPFL objective (Olympus). Epac1-camps stimulation with a 440 nm light and an exposition time of 50 ms was performed using a monochromator (Polychrome V, TILL Photonics, Gräfelfing, Germany). Emitted light was separated with a DualView imaging system (DV2 - Photometrics, Tucson, AZ, USA) with a DCXR505 dichroic mirror and emission filters for CFP (BP465/30) and YFP (HQ535/30). Images were acquired at a 0.2 Hz frequency using a cooled CCD camera (Sensicam, PCO Imaging, Kelheim, Germany) controlled by TILLVision 4.5 software (TILL Photonics). Pixels were subjected to a 2×2 binning, for a final resolution of 66.7×115.5 µm in a frame of 300×520 pixels for each channel. Odor stimulations, each consisting of 100 µl of stimulus solution, were applied via pipette in the proximity of the objective; the final volume of Ringer solution after stimulation was always 1 ml, except for the high KCl experiments, where it was 500 µl. The ratio (R) between the CFP and the YFP channels was calculated after background subtraction and the response magnitude was evaluated for each frame as the average $\Delta R/R_0$ and expressed as a percentage. Regions of interests (ROIs) were selected using the in-built tools of TILLVision 4.5 (Fig. S1) and R_0 was estimated as the mean fluorescence level calculated for each ROI from the average intensity between the 10th and the 19th frame of the recording. The mean response of each antenna was calculated by averaging the responses of all selected ROIs.

Genotype confirmation by PCR

The genomic DNA of each candidate $Or22a^{-/-}$ fly was extracted according to Gleason et al. (2004). Internal primers (Eurofins Genomics, Ebersberg, Germany) were used to amplify Or22a (forward: 5′-GTGTTTTCTGATGACGGAGG; reverse: 5′-CGAC-ACAGTGTTCTTGTAGC) and Orco (forward: 5′-CTTCATCCTG-GTCAACATGG; reverse: 5′-AATGGTAACGAGCATCCGAC).

Data analysis

Two-tailed unpaired *t*-tests between each group and the appropriate control (with Welch's correction in case of heteroscedasticity) were performed using Prism 4 software (GraphPad Software Inc., La Jolla, CA, USA). The pictures of the antennal preparation in Fig. 1A were prepared using Fiji (Schindelin et al., 2012) ImageJ v2.0.0-rc-34/1.50a software and subjected to histogram normalization for display purposes.

RESULTS AND DISCUSSION

In order to test whether the stimulation of OR complexes induces an Epac1-camps response, we first characterized the signals from OSNs bearing an intact OR complex ($Orco^{+/+}$; Fig. 1A), stimulating them with 100 µmol l^{-1} solutions of the general AC agonist forskolin, the phosphodiesterase (PDE) inhibitor IBMX, the OR agonist VUAA1, an odor mixture composed of ethyl acetate and methyl acetate (two acetate esters produced by yeasts that are highly attractive to flies; Mansourian and Stensmyr, 2015) or, as a control, a solution containing 0.1% DMSO. In all cases except the control, we could detect a strong signal with a characteristic time course (Fig. 1B–F). To confirm that the responses to VUAA1 and the odor mixture were induced by activation of ORs, we evaluated dose-dependent responses (Fig. 1G) using flies carrying intact OR complexes ($Orco^{+/+}$), a deletion of the Orco co-receptor ($Orco^{-/-}$) which also impairs the ability of the neuron-specific OrX proteins to be correctly inserted in the plasma membrane (Larsson et al., 2004), and a restored Orco function on an $Orco^{-/-}$ background (Orco rescue). We could detect a dose-dependent response to VUAA1 and the odor mixture in $Orco^{+/+}$ flies. The response was abolished in $Orco^{-/-}$ flies and recovered under the Orco rescue condition, while

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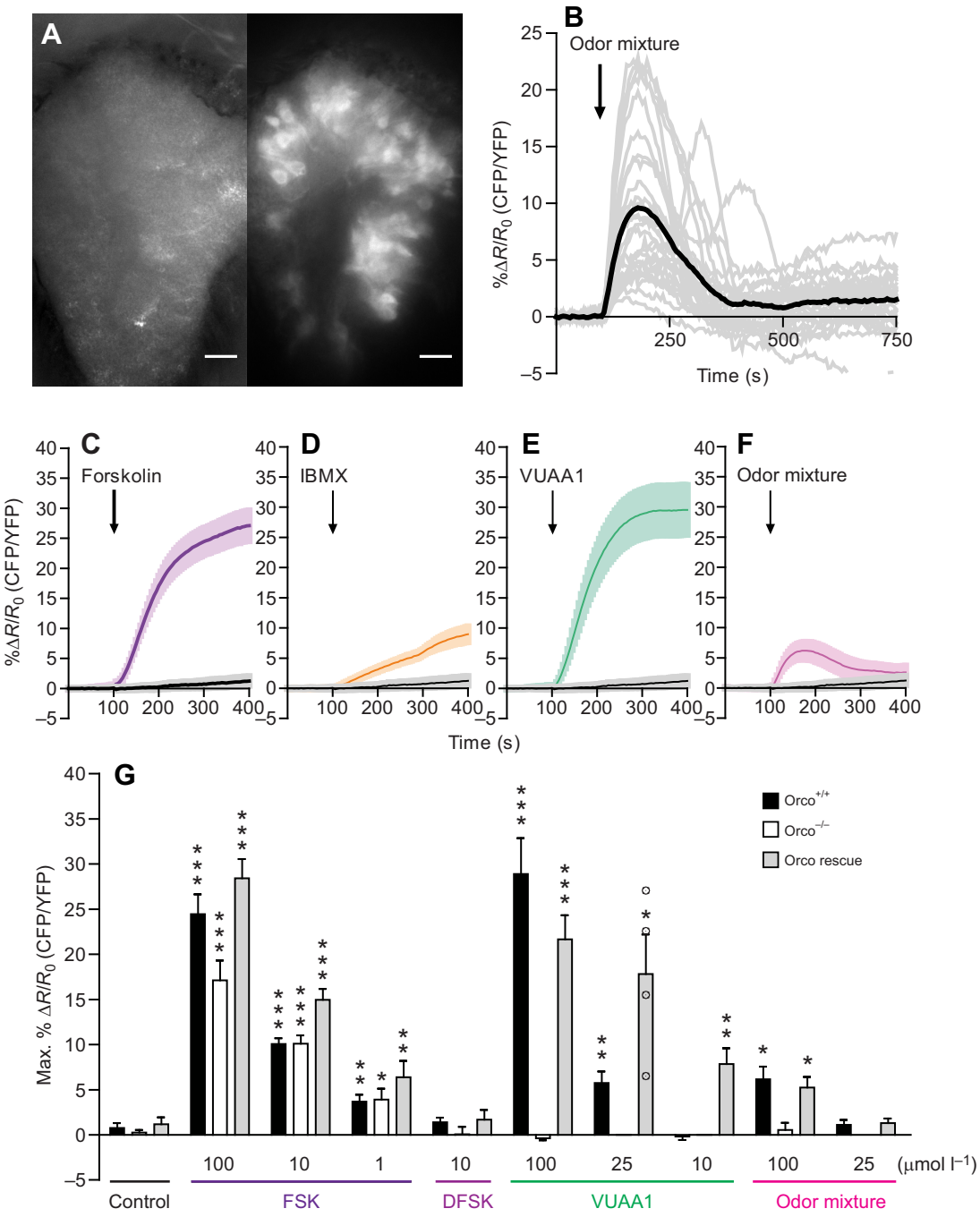

Fig. 1. Stimulation of the olfactory receptor complex induces cAMP production in *Drosophila melanogaster* olfactory sensory neurons (OSNs). (A) Left: transmission light image of an antennal preparation from an Orco$^{+/+}$ fly. Right: Epac1-camps fluorescence intensity recorded in resting conditions from the same antenna under stimulation with a 440 nm light (YFP channel). It is possible to identify multiple OSNs and to mark them as regions of interest (ROIs). Scale bars: 10 μm. (B) Plot of fluorescence intensity (% $\Delta R/R_0$) over time of the antenna in A stimulated with 100 µl of an odor mixture containing 100 µmol l^{-1} each ethyl acetate and methyl acetate (arrow). Each gray trace represents a single ROI and the black trace represents the averaged fluorescence intensity.
(C–F) Fluorescence intensity over time after stimulation at 100 s (arrows) with control solution (gray traces, N=9) or with 100 µmol l^{-1} of the adenylyl cyclase (AC) agonist forskolin (C, N=7), the phosphodiesterase (PDE) inhibitor IBMX (D, N=6), the Orco agonist VUAA1 (E, N=8) or the odor mixture (F, N=8). Plots represent means±s.e.m. (G) Maximum intensity values recorded at 300 s for control, forskolin (FSK), dideoxyforskolin (DFSK) and VUAA1 and at 175 s for the odor mixture, in Orco$^{+/+}$, Orco$^{-/-}$ and Orco rescue flies. Unpaired t-tests with Welch's correction in case of heteroscedasticity: *P<0.05, **P<0.01, ***P<0.001. Graphs represent means±s.e.m., 4≤N≤11 for N<5 single data points; no correction for multiple comparisons was used; the exact number of replicates for each treatment and the exact P-value for each test are reported in Table S1.

the dose-dependent response to forskolin was present in all three conditions, and the response to dideoxyforskolin, an inactive form of forskolin, was not significantly different from the control stimulation (Fig. 1G). This suggests that the Epac1-camps signal we detected is linked to the activation of the OR complex and is a general property of *D. melanogaster* OSNs.

Next, to characterize the nature of the Epac1-camps signal, we studied a specific receptor – Or22a – involved in the detection of

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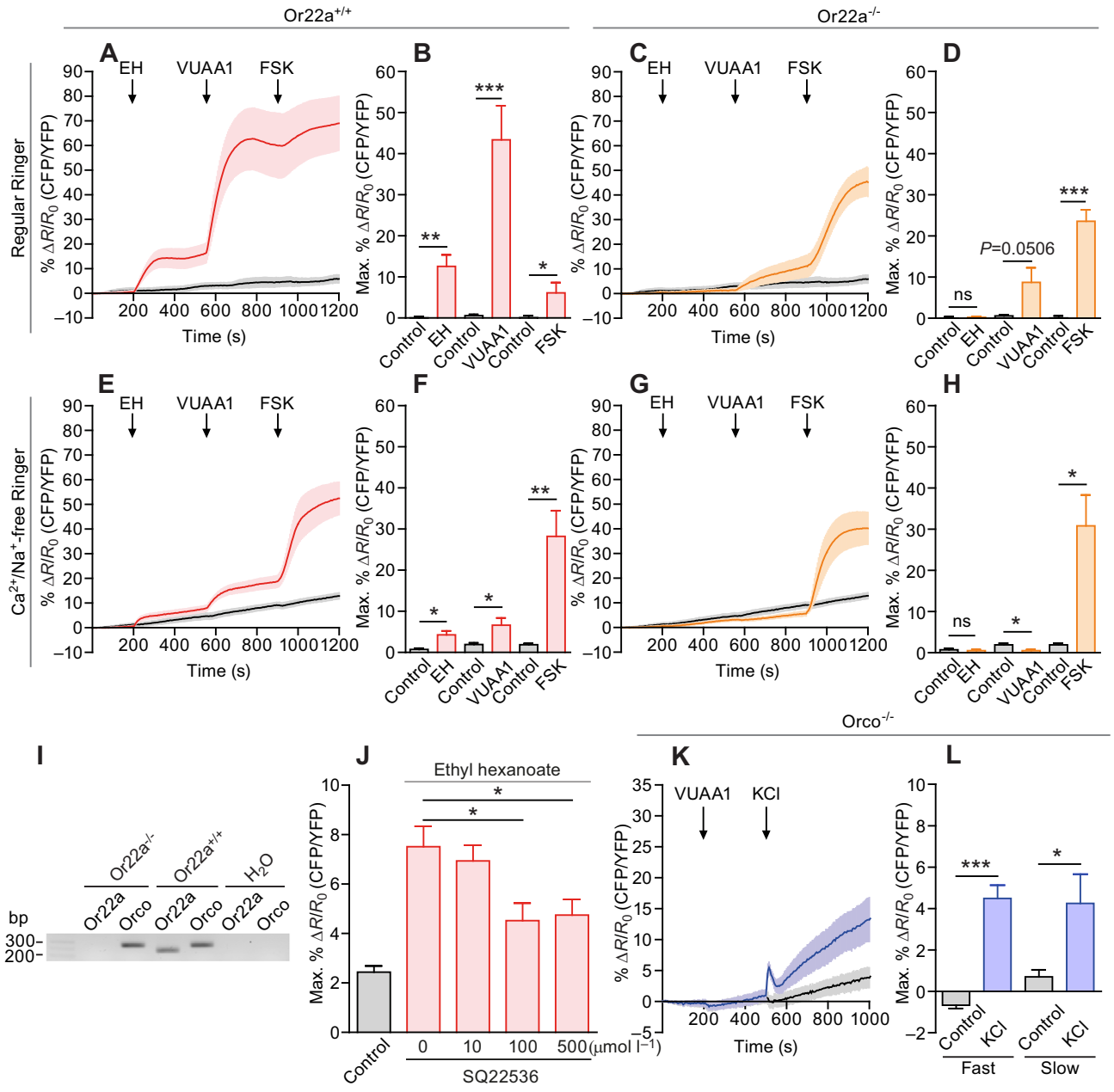

Fig. 2. Ca^{2+} influx is sufficient to induce cAMP production in OSNs, but in Ca^{2+}-free conditions, stimulation of Or22a is necessary. (A–H) Plots of fluorescence intensity over time and response intensity of Or22a OSNs expressing a wild-type receptor ($Or22a^{+/+}$) in regular Ringer solution (A,B) and in Ca^{2+}/Na^{+}-free Ringer solution (E,F), or expressing a mutated Or22a ($Or22a^{-/-}$) in the same conditions (C,D and G,H, respectively), stimulated (arrows) with 100 µl of control solution (black traces) or with solutions containing 100 µmol l^{-1} of the Or22a agonist ethyl hexanoate (EH), VUAA1 and forskolin (FSK) (red and orange traces). Response intensity plots were calculated by subtracting the baseline fluorescence from plateau levels at 100 s after stimulation with ethyl hexanoate, or 150 s after stimulation with VUAA1 and forskolin, or the respective control solution application. Graphs represent means±s.e.m., $5 \leq N \leq 9$; the exact number of replicates for each treatment and the exact *P*-value for each test are reported in Table S2. (I) Example of $Or22a^{-/-}$ fly genotype confirmation by PCR using internal primers for Or22a and Orco. Fragment length is expressed in base pairs (bp). (J) Maximum $\Delta R/R_0$ from Or22a-expressing neurons in Ca^{2+}/Na^{+}-free Ringer solution with 0.1% DMSO in control conditions ($N=8$) or stimulated with 100 µmol l^{-1} ethyl hexanoate in the presence of different concentrations of the general AC inhibitor SQ22536 (0 µmol l^{-1} $N=13$, 10 µmol l^{-1} $N=13$, 100 µmol l^{-1} $N=11$, 500 µmol l^{-1} $N=10$). Asterisks indicate significance: 0 versus 100 µmol l^{-1} SQ22536, $P=0.013$; 0 versus 500 µmol l^{-1} SQ22536, $P=0.0192$. (K) Plot of fluorescence intensity over time for $Orco^{-/-}$ OSNs stimulated with 100 µmol l^{-1} VUAA1 and 50 mmol l^{-1} KCl solutions (blue traces, $N=8$) or a control solution (black traces, $N=10$). (L) Response intensity calculated by subtracting the base level 15 s after stimulation with KCl or with control solution (fast response: control versus KCl, $P<0.0001$) and 150 s after stimulation (slow response: control versus KCl, $P=0.0446$). Unpaired *t*-tests with Welch's correction in case of heteroscedasticity: *$P<0.05$, **$P<0.01$, ***$P<0.001$. Graphs represent means±s.e.m.

food odors (Mansourian and Stensmyr, 2015). We stimulated OSNs from flies carrying wild-type Or22a ($Or22a^{+/+}$) or the Δhalo mutation ($Or22a^{-/-}$). Moreover, as the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) affects the activity of some isoforms of the eukaryotic class III ACs (Linder, 2006), stimuli consisting of 100 µmol l^{-1} ethyl hexanoate, VUAA1 or forskolin solutions were delivered in regular Ringer solution (containing both Ca^{2+} and Na^{+}) or in Ca^{2+}/Na^{+}-free Ringer solution in order to prevent extracellular

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Ca^{2+} influx and Na^{+}-induced Ca^{2+}-release from intracellular Ca^{2+} stores (Fig. S2). $Or22a^{+/+}$ OSNs responded to all three stimuli in both the presence and the absence of extracellular Ca^{2+} (Fig. 2A,B and E,F), while in $Or22a^{-/-}$ flies, VUAA1 application showed a tendency to elicit a signal in regular Ringer solution (Fig. 2C,D) and the signal intensity was slightly but significantly reduced in Ca^{2+}/Na^{+}-free Ringer solution (Fig. 2G,H). The genotype of each $Or22a^{-/-}$ fly was confirmed by PCR (Fig. 2I). To test whether the odor-induced signals were attributable to cAMP, we inhibited ACs with SQ22536 solubilized in 0.1% DMSO in Ca^{2+}/Na^{+}-free Ringer solution. We detected a significant dose-dependent reduction of the signal with increasing concentrations of SQ22536 (Fig. 2J); however, SQ22536 did not abolish the response even at high concentrations (control: 2.44±0.25, 500 µmol l^{-1} SQ22536: 4.47±0.63, mean±s.e.m., $P<0.01$, unpaired *t*-test with Welch's correction). This may reflect the inability of the drug to inhibit ACs completely. Similarly, SQ22536 did not suppress OSN activity at high stimulus concentrations (Getahun et al., 2013). As $Or22a^{-/-}$ OSNs showed a high variability in the response to VUAA1 in the presence of extracellular Ca^{2+} (Fig. S4A), we asked whether a Ca^{2+} influx alone could induce a detectable signal. For this reason, we depolarized OSNs of $Orco^{-/-}$ flies using 50 mmol l^{-1} KCl Ringer solution. We could detect a highly significant fast response, matching the time course of Ca^{2+} influx (Fig. S3), followed by a slower response (Fig. 2K,L) with variable intensity (Fig. S4B).

Taken together, these data show that stimulation of ORs with odors leads to a change in the CFP/YFP ratio of the sensor, in both the presence and the absence of extracellular Ca^{2+}, which is reduced in the case of AC inhibition (Figs 1, 2). Moreover, the presence of an OrX protein is necessary to induce a detectable signal in the absence of extracellular Ca^{2+} (Fig. 2E–H), but $[Ca^{2+}]_i$ can affect the intensity of the signals recorded because of the opening of cationic channels, like Orco (Fig. 2C,D,G,H) or voltage-gated Ca^{2+} channels (Fig. 2K,L). This suggests that the stimulation of Or22a can induce a Ca^{2+}-independent cAMP production compatible with a direct or indirect coupling to G proteins, while an eventual cAMP production induced by stimulation of Orco homomeric channels with VUAA1 is linked to the Ca^{2+} influx into the cell (Fig. 3).

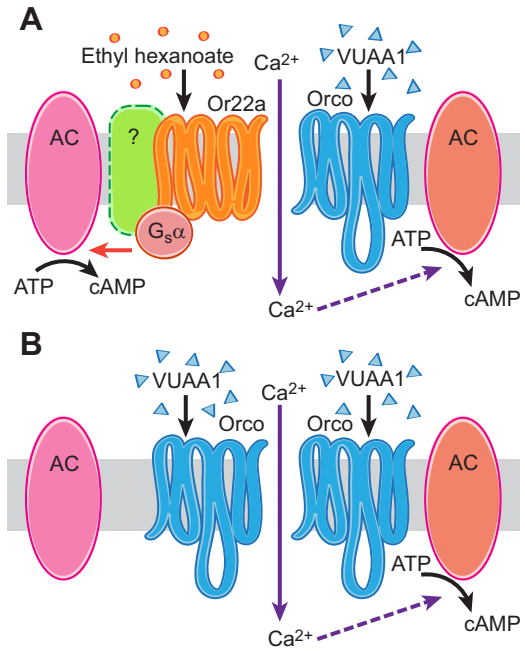

Fig. 3. Model of cAMP production induced by olfactory receptor (OR) stimulation in *D. melanogaster* Or22a-expressing OSNs. Stimulation of the heteromeric OR complex by ethyl hexanoate or VUAA1 can induce the production of cAMP via a Ca^{2+}-independent pathway, compatible with a coupling of Or22a to G_s proteins directly or indirectly by unknown proteins (red arrow). Additionally, Ca^{2+} influx through the channel pore may stimulate Ca^{2+}-activated ACs (violet arrows). (B) Stimulation of Orco homomers via VUAA1 may induce the production of cAMP only through an increase of intracellular Ca^{2+} (violet arrows), and not via Ca^{2+}-independent mechanisms.

We can thus show that functional imaging of cAMP in insect OSNs using the Epac1-camps indicator is a valid method to investigate the dynamics of this second messenger and further experiments could focus on the functional role of the cAMP we detected. The lowest concentration of the ethyl hexanoate stimulus (100 µmol l^{-1}) in the experimental chamber was of the order of a 10^{-6} dilution with respect to the pure compound, a relatively high concentration but one regularly used in physiology and behavior experiments, with the important difference that this was a chronic and not an acute stimulation. Interestingly, the signal persistence in the absence of Ca^{2+} suggests that at least part of it may originate not from Ca^{2+}/calmodulin-dependent ACs such as Rutabaga; a possible candidate is Ac3, a Ca^{2+}-inhibited AC highly expressed in fly antennae (Iourgenko and Levin, 2000), which has been shown to mediate the circadian pacemaker synchronization of M cells in adult flies (Duvall and Taghert, 2012). Additionally, as Ca^{2+} release from intracellular stores can occur following OR stimulation (Ignatious Raja et al., 2014; Mukunda et al., 2016), this may also enhance cAMP production in OSNs.

In conclusion, as we report here the effectiveness of this method in *Drosophila* OSNs in *ex vivo* conditions, future experiments targeting Epac1-camps to the olfactory cilia, or to membrane microdomains by means of fusion constructs (Calebiro and Maiellaro, 2014), could help to further investigate the role of cAMP at the first site of olfactory signal transduction, thereby allowing an understanding of the role that this second messenger plays in the modulation of insect ORs.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
F.M. performed the experiments, analyzed the data and wrote the first draft of the manuscript. All authors contributed to the design of the study and the final version of the manuscript.

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Supplementary information
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