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Odorant-stimulated phosphoinositide signaling in mammalian olfactory receptor neurons

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Abstract

Recent evidence has revived interest in the idea that phosphoinositides (PIs) may play a role in signal transduction in mammalian olfactory receptor neurons (ORNs). To provide direct evidence that odorants indeed activate PI signaling in ORNs, we used adenoviral vectors carrying two different fluorescently tagged probes, the pleckstrin homology (PH) domains of phospholipase Cδ1 (PLCδ1) and the general receptor of phosphoinositides (GRP1), to monitor PI activity in the dendritic knobs of ORNs *in vivo*. Odorants mobilized $PI(4,5)P_2/IP_3$ and $PI(3,4,5)P_3$, the substrates and products of PLC and PI3K. We then measured odorant activation of PLC and PI3K in olfactory ciliary-enriched membranes *in vitro* using a phospholipid overlay assay and ELISAs. Odorants activated both PLC and PI3K in the olfactory cilia within 2 sec of odorant stimulation. Odorant-dependent activation of PLC and PI3K in the olfactory epithelium could be blocked by enzyme-specific inhibitors. Odorants activated PLC and PI3K with partially overlapping specificity. These results provide direct evidence that odorants indeed activate PI signaling in mammalian ORNs in a manner that is consistent with the idea that PI signaling plays a role in olfactory transduction.

1. Introduction

It is increasingly clear that the mammalian sense of smell is organized into subsystems, including functional subsets of olfactory receptor neurons (ORNs) within the main olfactory epithelium (OE) itself [1–3]. This organizational complexity presumably extends to individual ORNs since it is long known that odorants can inhibit as well as excite canonical ORNs in the OE, as they can in most animals [4]. It is generally agreed that in canonical ORNs the binding of odorant molecules to their cognate odorant receptors (ORs) triggers a cyclic nucleotide signaling cascade that targets a cyclic nucleotide-gated (CNG) ion channel [5]. Activation of the CNG channel leads to Ca^{2+}-influx into the ORN. The elevated Ca^{2+}-concentration secondarily targets a Ca^{2+}-activated chloride channel, amplifying the output of the cell [6]. Negative feedback from the elevated Ca^{2+}-concentration causes a Ca^{2+}-calmodulin-dependent decrease in the sensitivity of the CNG channel to cAMP [7, 8]. How

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odorants inhibit these ORNs, however, is unclear, but potentially could involve phosphoinositide (PI) signaling [9, 10].

The idea that PI signaling is involved in signal transduction in mammalian ORNs has been controversial [11–15]. This controversy is perhaps understandable in light of the complexity of PI signaling that includes not only the canonical phospholipase C (PLC)-dependent pathway but also phosphoinositide-3-kinase (PI3K)-dependent signaling. PLC can cleave $PI(4,5)P_2$ into soluble inositol trisphosphate (IP_3) and the membrane-bound diacyglycerol (DAG). The production of IP_3 triggers the release of Ca^{2+} from intracellular stores (e.g. ER), while the production of DAG leads to the activation of protein kinase C [16]. In conjunction, activation of PI3K leads to 3′-phosphorylation of inositol lipids, especially phosphatidylinositol-(4,5)-bisphosphate [$PI(4,5)P_2$] to generate phosphatidylinositol (3,4,5)-trisphosphate [$PI(3,4,5)P_3$] *in vivo* [17, 18].

Early studies of PI signaling in olfaction focused on the canonical PLC-dependent pathway, where it could be shown, for example, that odorants can elevate levels of IP_3 in insect [19] and rodent [14, 20–22] ORNs. Furthering the idea that PLC-mediated signaling may play a role in olfactory signal transduction, more recently it was found that some ORNs express a transient receptor potential channel (TRPM5, [23]), which in many systems, including chemosensory cells, are the downstream targets of PLC signaling [24, 25]. It was also recently shown that exogenous $PI(3,4,5)P_3$ negatively regulates the olfactory CNG channel [26] through a complex interaction with Ca^{2+}/calmodulin at the N-terminus of the channel [27], and that inhibition of PI3K can increase the odorant-evoked Ca^{2+}-signal in rodent ORNs [9], suggesting that PI3K-mediated signaling, too, may play a role in olfactory signal transduction.

If indeed PI signaling is involved in olfactory transduction in mammalian ORNs, it should be possible to show that odorants are capable of activating PLC and/or PI3K in these cells and that one or both enzymes can be activated sufficiently fast to play a role in olfactory transduction. Here we extend the earlier evidence for the involvement of PI signaling in mammalian ORNs by showing that odorants stimulate both PLC and PI3K in the dendritic knob of rodent ORNs *in vivo*, as well as rapidly and transiently in olfactory cilia *in vitro*. We also initially characterize the specificity with which odorants activate PI3K and PLC, indicating that odorants activate the two enzymes with partially overlapping specificity. We interpret these results as providing direct evidence that odorants indeed activate PLC and PI3K signaling in mammalian ORNs in a manner that is consistent with PI signaling playing a role in olfactory transduction.

2. Methods

2.1 Chemicals

Unless stated otherwise, chemicals and odorants were purchased from Sigma Aldrich (St. Louis, MO). All drugs were prepared as stock solutions in DMSO according to the manufacturers' recommendations and diluted to the final concentrations in artificial cerebrospinal fluid (ACSF) for application. The final concentration of DMSO in the carrier did not exceed 0.1%. Two complex odorant mixtures were used, Henkel 100 (H100; [28]) and Symrise 100 (S100). S100 consists of: irolene, Citrowanil® B, citronitrile, Mandaril, tridecen-2-nitrile, irisnitrile, Parmanyl®, Cantryl®, mimonil, marenil, hydrocitronile, ambrinol S, freesiol, terpinenol-4, Synambran®, rose oxide high cis super, rose oxide I, Symroxane®, palisandin, ambroxide crystals, mevanyl, Vertacetal Coeur®, magnolan, Jacinthaflor®, Ysamber® K, palisandal, Indoflor®, leguminal, vetirox, frutalan, phenyl acetaldehyde dimethyl acetal, Timberol®, cyclohexyl magnol, mugetanol, Madranol®, sandel 80, fleursandol, ethyl phenyl carbinol, floranol, cedramol, Vetival®, Aldron®,

boisanol, passifloran, thiogalbanat, 3-thio hexanol, Isodamascon®, Globanone® 100%, aureliole, isocivet, coumarone H&R, chrysantheme, projasmon B, Vetikon®, tabanon coeur, nerolione H&R, Claritone®, citronone, frutanone, Vertral®, Farenal®, florazon, profarnesal, boronal, Rosaphen®, p-tolyl acetaldehyde 50%, limonelal, vertocitral, Vetikolacetat®, cyclopentadecanolide supra, peacholide, isoananat, datilat, frutinat, pyroprunat, lactojasmone, lactoscatone, rholiate, Mahagonat®, Globalide® 100%, mintonat, benzyl cinnamate, prenyl salicylate, prenyl benzoate, methyl cinnamate, allyl cyclohexyl propionate, allyl cinnamate, muguetalcohol, Majantol®, Sandranol®, cinnafix, farbesol P, Buccoxime®, corps racine, vertosine, Brahmanol®, menthol, and eucalyptol. Only Brahmanol®, menthol and eucalyptol are in common with H100.

2.2 Preparation of the intact olfactory epithelium

All experiments were performed on adult Sprague-Dawley rats or CD1 mice at least 6 weeks old. All procedures were carried out in accordance with protocols approved by the IACUC of the University of Florida and the European Guidelines for Laboratory Animal Care. Animals were euthanized by inhalation of carbon dioxide and decapitated. The head was opened to keep the septum and the underlying olfactory turbinates intact. The septal olfactory epithelium (OE) was dissected free of the head and maintained in a Petri dish filled with ice-cold modified ACSF [10].

2.3 Adenoviral vectors

Plasmids encoding the adenoviral backbone genes and the shuttle vector were provided by B. Vogelstein (Howard Hughes Medical institute, Baltimore, USA). The plasmid encoding the fusion construct of the PH domain of PLCδ1 and GFP was cloned into a pShuttleCMV vector (Stratagene, La Jolla, CA) according to the description of Stauffer *et al.* [29]. The PH domain of GRP1 was amplified using primers described in Venkateswarlu *et al.* [30] and fused to RFP in the pShuttleCMV vector. In order to construct the adenoviral vectors, the plasmids pShuttleCMV-PH_{PLC}GFP and pShuttleCMV-PH_{GRP1}RFP were transformed into pAdEasier bacteria (BJ5183), which already contained the pAdEasy-1 vector (Stratagene, La Jolla, CA) as the backbone plasmid of the virus. After homologous recombination, positive clones were selected for kanamycin resistance and tested with *Bam*H1 and *Pac*1 digestions. Adenoviruses lacking the E1 gene are replication-incompetent and can only grow in HEK293 cells, which provide the E1 gene in trans. HEK293 cells were transiently transfected with 6 μg pAdeno-PH_{PLCd}GFP or pAdeno-PH_{GRP1}RFP DNA with Lipofectamine (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Virus was purified after 7 to 10 days [31].

2.4 Infection of mice

Adult mice were anesthetized with a ketamine/ rompun mixture. 75 μl of the viral solution was delivered to the OE by letting the mice sniff the solution into their right nostrils. We used a viral stock solution of 6.82×10^{12} viral particles/ ml for AdVPH_{PLC}GFP and of 5.14×10^{12} viral particles/ ml for AdVPH_{GRP1}RFP. Mice were used for the experiments at 7 days post infection.

2.5 Confocal laser scanning microscopy

Real-time confocal microscopy was performed with the 488 nm line of an argon laser and LSM510 Meta laser scanning microscope equipped with a 40× objective lens (Zeiss, Jena, Germany). Experiments were performed in assay buffer containing (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 HEPES, pH 7.4. H100 was diluted in the assay buffer and delivered to the cells using a custom made application system that allows solenoid valve controlled superfusion of the cells in the visual field [9].

2.6 Analysis of probe translocation

The translocation of PH_{PLC}GFP is indicated by an increase in cytosolic and a decrease in membrane-associated fluorescence and the translocation of PH_{GRP1}RFP is indicated by a translocation vice versa (LI-Index) [32]: $LI = (((IM1 + IM2) / 2) - IZ) / ((IM1-IM2) / 2)$.

2.7 Odorant stimulation

Odorants were delivered as solutions. H100 and S100 were dissolved 1:1 in anhydrous DMSO as stocks. Final solutions were prepared fresh daily by diluting the stocks in ACSF with extensive vortexing and sonication. Normal Ringer's solution supplemented with 0.1% DMSO as a carrier for odorants served as the control.

2.8 Overlay dot blot assay for PIP_3

Membrane fractions enriched in rat olfactory cilia were prepared as described in Washburn *et al.* [33]. The membrane fractions or dissociated OE cells, prepared as described in Spehr *et al.* [9], were stimulated with odorants. The reactions were stopped by immediate freezing in liquid N_2 followed by the addition of ice cold 0.5 M trichloroacetic acid (TCA). Unstimulated and mock-treated membrane fractions were used as controls. The lipids were extracted and detected using a protein-lipid overlay assay, PIP_3 Mass Strip Kit, following the manufacturer's protocols (Echelon Biosciences, Salt Lake City, UT). The blots were incubated with ECL detection reagent (Millipore, Billerica, MA), and the signal was captured with a CCD camera (Fluor-S, Bio-Rad Laboratories, Hercules, CA). Grayscale PIP_3 signal strength was quantified with ImageJ software (National Institutes of Health, Bethesda, MD).

2.9 ELISA for PI3K and PLC activity

For the ELISAs, membrane fractions enriched in rat olfactory cilia or dissociated OE cells were incubated with odorants. In some cases, the preparations were pre-incubated with 10 μM LY294002 (Calbiochem, San Diego, CA), 100 nM wortmannin, 50 μM U73122 (Calbiochem, San Diego, CA) or 10 μM edelfosine for 10 min before adding H100. The lipids were extracted and detected using an ELISA assay, either a PIP_3 Mass ELISA Kit (Echelon Biosciences, Salt Lake City, UT) or an IP-One-ELISA (Cisbio Bioassays, Bedford, MA), following the manufacturer's protocol. The plates were read on a multiwell plate reader (Bio-Rad Laboratories, Hercules, CA) at 450 nm. The data were quantified with standards, 0.625 to 20 pmol $PI(3,4,5)P_3$ or 17 to 5000 nM IP_3, per well, and analyzed with Microplate Manager 4.0 software (Bio-Rad Laboratories, Hercules, CA).

3. Results

3.1 Odorants activate both PLC and PI3K in the dendritic knobs of mouse ORNs in vivo

IP_3 and $PI(4,5)P_2$ were localized within the dendritic knobs of mouse ORNs using the PH domain of PLCδ1 fused to green fluorescent protein (PH_{PLC}GFP) [34, 35]. $PI(3,4,5)P_3$ was localized using the PH domain of the general receptor of phosphoinositides (GRP1) fused to red fluorescent protein (PH_{GRP1}RFP) [36]. Adenoviral vectors carrying the genes for these markers were used to infect ORNs. The olfactory septum and the turbinates were infected 7 days prior to imaging, on average yielding 52 infected cells in a visual field at 20× magnification (Fig. 1a and b). The PH_{PLC}GFP probe was targeted to the plasma membrane in unstimulated cells, where it could be seen as a distinct green rim on the knobs of unstimulated cells (Fig. 1c). The complex odorant mixture H100 caused PH_{PLC}GFP to translocate from the cell membrane to the cytoplasm (Fig. 1c) in 17.4% (4/23) of the infected knobs surveyed. It is likely that not all of the infected cells were able to respond to H100 due to the presence of cells with receptors for odorants not present in the mixture and

this indicates that the odorants do not directly activate the signaling pathways. H100-induced translocation of PH_{PLC}GFP took 10 ± 8 sec (n = 4), while relocation, indicating regeneration of $PI(4,5)P_2$ in the cell membrane, took 360 ± 47 sec (n = 4). In contrast, PH_{GRP1}RFP was localized throughout the cytoplasm in resting cells (Fig. 1d). H100 caused PH_{GRP1}RFP to translocate from the cytoplasm to the cell membrane (Fig. 1d) in 9.6% (5/52) of the infected knobs surveyed. The odorant-induced translocation of PH_{GRP1}GFP took 380 ± 53 sec (n = 5), while recovery, indicating a dephosphorylation of $PI(3,4,5)P_3$, in the cell membrane, took 1020 ± 217 sec (n = 5).

3.2 Odorants rapidly activate both PLC and PI3K in the olfactory cilia in vitro

To confirm activation of both PLC and PI3K in the actual transduction compartment, the olfactory cilia, which extend from the dendritic knobs but are too narrow in diameter to monitor translocation of fluorescent probes, we tested the ability of odorants to activate the enzymes in an olfactory cilia-enriched membrane preparation. Up to 2–5% of the total protein in the cilia-enriched membrane fraction of the OE can be from cell types other than ORNs [37], but components of the PI3K and PLC signaling pathways, such as PI3Kβ and γ [10], can be localized to the olfactory cilia from which the membrane preparation was derived. Due to the tissue-intensive nature of these assays, these experiments were performed with rat OE. PLC activation was measured with an IP-One ELISA, which uses IP_1, a stable downstream metabolite of IP_3, as an indirect measure of IP_3 levels. PI3K activation was measured with a PIP_3-Mass Strip overlay assay, which specifically detects $PI(3,4,5)P_3$. Measurable activation of PLC occurred within 2 sec of stimulation with H100 and could continue to be detected for as long as 1 hr (Fig. 2a). The IP-One ELISA measures the total accumulation of IP_1 and blocks degradation of the response, thereby preventing assessment of any transience in PLC activity. Activation of PI3K occurred within 2 to 5 sec of stimulation with H100 and was transient, returning to baseline within 30 sec of stimulation (Fig. 2b). Both IP_1 and $PI(3,4,5)P_3$ were essentially undetectable in the absence of any treatment (Fig. 2b), in mock-treated samples, and in response to stimulation with forskolin (data not shown).

3.3 Odorants activate both PLC and PI3K in the OE in vitro

In order to develop a higher throughput assay we tested the ability of the ELISAs to measure PLC and PI3K activation in dissociated rat OE tissue. H100 elicited a dose dependent increase in both PLC and PI3K activation in OE cells (Fig. 3). For PLC, the response to a 1:5000 dilution was 1.31 fold greater than that to a 1:10000 dilution (Fig. 3a). For PI3K, the response to a 1:5000 dilution was 4.56 fold greater than that to a 1:10000 dilution (Fig. 3b). Similar results were obtained in mouse OE (data not shown). The 1:10000 dilution of H100, in which each odorant is present at approximately 6.6 μM based on an average molecular weight, was used for further testing. The IP-One ELISA was specific for the activity of PLC. The response to H100 was reduced by two known PLC inhibitors U73122 and edelfosine (Fig. 4). U73122 (50 μM) reduced PLC activation to 51.53% and edelfosine (10 μM) to 17.41% of pretreatment levels. Similarly, the PIP_3-Mass-ELISA was specific for the activity of PI3K. The response to H100 was reduced by two pan-specific PI3K inhibitors, LY294002 and wortmannin (Fig. 4). LY294002 (10 μM) reduced activation of PI3K in response to a 1:10000 dilution of H100 to 17.94% and wortmannin (100 nM) to 5.6% of pre-treatment levels. Although the activation of PI3K by H100 was not affected by the PLC-specific inhibitors U73122 and edelfosine, the PI3K–specific inhibitors LY294002 and wortmannin reduced the activation of PLC by H100 to 17.61% and 20.63% of pre-treatment levels, respectively (Fig. 4).

3.4 Odorants activate PLC and PI3K with partially overlapping specificity

To determine the specificity, if any, of the odorants activating PLC and PI3K we compared the stimulatory capacity of H100 with that of Symrise 100 (S100), another equally complex odorant mixture but with only three components in common (see Methods). S100 at the same overall concentration activated PLC, but much less so than H100, and did not activate PI3K (Fig. 5). S100 stimulated PLC 28.74% of the response to H100, while S100 did not stimulated PI3K compared to the response of H100 (1.12%), suggesting that all odorants are not equally potent at stimulating the two enzymes across the receptor population.

To further evaluate this apparent specificity, we screened a panel of 25 single odorants that included 17 odorants chosen at random and 8 previously shown to activate PLC [19, 21]. Each of the single odorants was tested at a 1:10000 dilution, producing a final average concentration of 65 μM, which was approximately 100 times the individual odorant concentration in H100. Of the 17 odorants chosen at random, 11 activated PLC to levels greater than the mock-treated control (Fig. 6a). Included in this group were cineol, octanol, citral, acetophenone, benzaldehyde, eugenol, menthone, citronellal, isoamyl acetate, octyl aldehyde, and methyl-iso-eugenol. All 8 odorants previously shown to activate PLC, including pyrazine, pyrollidine, Lyral®, Lilial®, triethylamine, isovaleric acid, phenylethylamine and ethylvanillin, did so in our survey as well (Fig. 6b). Six of the 25 single odorants, including octanol, citral, citronellal, isoamyl acetate, octyl aldehyde and methyl-iso-eugenol, activated PI3K to levels greater than the mock-treated control (Fig. 7a). None of the 8 odorants previously shown to activate PLC activated PI3K (Fig. 7b). All 6 odorants that activated PI3K as determined by the PIP_3-Mass-ELISA, also did so as determined by the protein-lipid overlay assay specific for $PI(3,4,5)P_3$ (Fig. 7c), showing that these odorants indeed activated class I PI3K(s) and raised the level of $PI(3,4,5)P_3$. Two of the three odorants that failed to give a detectable signal with the ELISA also did not raise the level of $PI(3,4,5)P_3$ above that of the mock-treated sample.

4. Discussion

The adenovirus-based, PI-specific probes allowed direct monitoring of odorant-dependent mobilization of $PI(4,5)P_2$/ IP_3 and $PI(3,4,5)P_3$ in the dendritic knobs of mouse ORNs. This technique is based on the idea that the GFP signal linked to the PH domain of PLCδ1 follows $PI(4,5)P_2$ during its metabolism, including hydrolysis by PLC, while the PH domain of GRP1 follows production of $PI(3,4,5)P_3$ by PI3K [30, 34, 38]. Because the PH domain of PLCδ1 also has a strong affinity for IP_3, a downstream product of $PI(4,5)P_2$ hydrolysis, it has been proposed that rather than solely tracking changes in $PI(4,5)P_2$, the probe also binds to the newly produced IP_3 [30, 34, 38, 39]. However, it is generally agreed that translocation of PH_{PLC}GFP cannot be completely explained by increases in IP_3, and that activation of other pathways, such as PI3K, also influence movement of the probe [38, 40]. H100 evoked translocation of the PH_{PLC}GFP probe in almost twice as many cells compared to the PH_{GRP1}RFP probe. Since PH_{PLC}GFP potentially detects both $PI(4,5)P_2$ and IP_3, the difference between the percentage of cells that respond to each probe may suggest that the pathways are co-activated. However, we have yet to establish whether one odorant is capable of activating both pathways in the same cell so perhaps more odorants in the H100 mixture activate the PLC pathway. This remains to be determined. Nonetheless, the odorant-evoked translocation of both PI-specific probes establishes that both PI3K and PLC are activated in the dendritic knobs *in vivo*.

Olfactory signal transduction is initiated and terminated within tens to hundreds of milliseconds, yet translocation of the PI-specific probes required an average of 10 sec for PH_{PLC}GFP and 6 minutes for PH_{GRP1}RFP. The slow response of the PH_{GRP1}RFP probe at least is thought to result from the limited ability of the probes to diffuse within the cells [30].

Similar time courses of translocation occur, for example, in response to GPCR activation of PI3Kγ transfected into HEK cells [41]. Also, since the fluorescence was measured at the maximum level of probe translocation, initial accumulation of the target lipids likely began much earlier and thus based on these data alone we cannot establish whether the changes in the lipids are sufficiently fast to play a role in transduction. In addition, while at least some elements of the transduction cascade are expressed in the knob, it is not clear that olfactory signal transduction actually occurs there. Our ability to show that odorants activate both enzymes in the olfactory cilia *in vitro*, therefore, extends our findings in the dendritic knob to the transduction compartment per se. Our ability to confirm previous evidence that increases in $PI(3,4,5)P_3$ [10] and IP_3 [19] can be detected in the olfactory cilia within as little as 2 sec following odorant treatment shows that activation of both enzymes by odorants is sufficiently fast in the transduction compartment for them to play a role in olfactory transduction. Given possible delays in the solution exchange due to the manual application of the odorants and termination of the reactions, it is likely that the actual production of IP_3 and $PI(3,4,5)P_3$ occurred even faster than 2 sec, which would place it even closer to the time course of olfactory signal transduction.

Finding that odorant stimulated IP_3 and $PI(3,4,5)P_3$ were decreased by PLC (U73122 and edelfosine)- and PI3K (LY294002 and wortmannin)-specific inhibitors, respectively, validates the biochemical assays. Yet, we noted that while the PLC inhibitors did not alter the PI3K signal, the PI3K inhibitors decreased the accumulation of IP_3. This suggests that activation of PI3K may influence the level of PLC activity in the signal transduction cascade downstream of odorant activation. Given the evidence that PI3K mediates inhibitory input [10], these results suggest that PLC may also be involved in odorant-evoked inhibition pathway in ORNs, which is consistent with the previously described amplification of the cAMP response in olfactory cilia by the PLC inhibitor U73122 [42]. If PLC were instead mediating or contributing to excitatory input, it would be expected that blocking PI3K, and thus the inhibitory pathway, would increase PLC signaling. The PI3K–dependent activation of PLC in ORNs has been described in other systems, including keratinocytes, B cells, macrophages and platelets [43–46], where PI3K–dependent $PI(3,4,5)P_3$ production has been implicated in the recruitment of PLC to the cell membrane to be activated by G proteins [47]. The interesting possibility that the inhibitory pathway mediated by PI3K may also involve signaling through PLC in ORNs requires further investigation.

PI3K and PLC were activated by single odorants with only partially overlapping specificity. Not all of the single odorants tested activated both PI signaling pathways and some did not activate either. Of the twenty-five different single odorants tested for the ability to activate the PLC and PI3K, only six odorants activated both. The remaining thirteen single odorants that activated PLC did not activate PI3K. As there is inevitably a ‘threshold’ effect associated with such biochemical assays that would prevent detection of low-level activation, we cannot eliminate the possibility that both enzymes were activated by the same odorants but to different, and in some cases undetectable, extents. Even so, the relative response spectra of the two enzymes would not be the same. Partially overlapping specificity is also consistent with the response to stimulation with the complex mixtures. H100 strongly activated both PLC and PI3K signaling, while S100, which has only three components in common with H100, was considerably less effective activating PLC and PI3K, indicating lack of broad overlap in specificity between the two pathways. The functional significance of this partial overlap is unclear, although given that PI signaling appears to mediate inhibitory input to the olfactory signal transduction cascade [9, 10], perhaps the two enzymes mediate different types (e.g., rates) of inhibitory input.

Cursory analysis of the nature of the single odorants that activated the enzymes based on their perceptual (e.g., floral, fruity, putrid, etc.) and chemical (e.g., alcohol, aldehyde,

ketone, etc.) properties did not reveal any obvious pattern that would allow predicting which odorants would activate PI3K and/or PLC. For example, although a majority of the odorants that activated both pathways can be classified as fruity, pentyl acetate, which has a banana/apple-like odor, did not activate either pathway, and methyl-iso-eugenol, which has a spicy clove-like odor, activated both. Further, of the aldehydes tested: octyl aldehyde activated both pathways, benzaldehyde, cinnamaldehyde, Lyral® and Lilial® activated only PLC, and bourgeonal and helional did not detectably activate either. Thus, even the limited sample of single odorants tested indicates there is nothing perceptually or chemically unique about the odorants per se that active PI signaling. This point is also supported by the fact that nine of the single odorants we tested were identified earlier [48] to activate adenylyl cyclase. A caveat here is that we are assaying odorant specificity across all cells; our results would not necessarily translate to a specific subpopulation of ORNs that was not widely represented in the overall receptor cell population. We tentatively conclude that there is no obvious difference in the perceptual or chemical properties of the odorants activating PLC and PI3K, although the validity of this generalization needs to be substantiated by testing many more odorants.

5. Conclusion

Collectively, our results considerably strengthen the contention that PI signaling plays a role in mammalian olfactory transduction. We provide direct evidence that odorants indeed activate both PLC and PI3K signaling in rodent ORNs *in vivo*, that they do so in the transduction compartment of the cells, and that they do so sufficiently fast to account for a role in olfactory transduction. We also show that odorants activate the two enzymes with partially overlapping specificity, with no obvious chemical or perceptual characteristics of the odorants per se. An important next step in this line of research will be to further explore the functional significance of PI signaling in mammalian olfaction by identifying the odorants that activate PLC and PI3K in individual ORNs, the type(s) of receptor through which they act, and their relationship to odorants that activate cyclic nucleotide signaling in these cells.

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Literature Cited

1. Breer H, Fleischer J, Strotmann J. Cell Mol Life Sci. 2006; 63(13):1465–1475. [PubMed: 16732429]
2. Ma M. Crit Rev Biochem Mol Biol. 2007; 42(6):463–480. [PubMed: 18066954]
3. Munger SD, Leinders-Zufall T, Zufall F. Annu Rev Physiol. 2009; 71:115–140. [PubMed: 18808328]
4. Ache BW, Young JM. Neuron. 2005; 48(3):417–430. [PubMed: 16269360]
5. Kaupp UB, Seifert R. Physiol Rev. 2002; 82(3):769–824. [PubMed: 12087135]
6. Kleene SJ. Neuron. 1993; 11(1):123–132. [PubMed: 8393322]
7. Bradley J, Bonigk W, Yau KW, Frings S. Nat Neurosci. 2004; 7(7):705–710. [PubMed: 15195096]
8. Bradley J, Reisert J, Frings S. Curr Opin Neurobiol. 2005; 15(3):343–349. [PubMed: 15922582]
9. Spehr M, Wetzel CH, Hatt H, Ache BW. Neuron. 2002; 33(5):731–739. [PubMed: 11879650]
10. Ukhanov. 2009
11. Gold GH. Annu Rev Physiol. 1999; 61:857–871. [PubMed: 10099713]
12. Noe J, Breer H. J Neurochem. 1998; 71(6):2286–2293. [PubMed: 9832126]

13. Restrepo D, Teeter JH, Schild D. J Neurobiol. 1996; 30(1):37–48. [PubMed: 8727981]
14. Schandar M, Laugwitz KL, Boekhoff I, Kroner C, Gudermann T, Schultz G, Breer H. J Biol Chem. 1998; 273(27):16669–16677. [PubMed: 9642220]
15. Wong ST, Trinh K, Hacker B, Chan GC, Lowe G, Gaggar A, Xia Z, Gold GH, Storm DR. Neuron. 2000; 27(3):487–497. [PubMed: 11055432]
16. Suh PG, Park JI, Manzoli L, Cocco L, Peak JC, Katan M, Fukami K, Kataoka T, Yun S, Ryu SH. BMB Rep. 2008; 41(6):415–434. [PubMed: 18593525]
17. Fruman DA, Meyers RE, Cantley LC. Annu Rev Biochem. 1998; 67:481–507. [PubMed: 9759495]
18. Vanhaesebroeck B, Waterfield MD. Exp Cell Res. 1999; 253(1):239–254. [PubMed: 10579926]
19. Boekhoff I, Tareilus E, Strotmann J, Breer H. Embo J. 1990; 9(8):2453–2458. [PubMed: 2164471]
20. Boekhoff I, Breer H. Proc Natl Acad Sci U S A. 1992; 89(2):471–474. [PubMed: 1370581]
21. Breer H, Boekhoff I, Tareilus E. Nature. 1990; 345(6270):65–68. [PubMed: 2158631]
22. Ronnett GV, Cho H, Hester LD, Wood SF, Snyder SH. J Neurosci. 1993; 13(4):1751–1758. [PubMed: 8385207]
23. Lin W, Ogura T, Margolskee RF, Finger TE, Restrepo D. J Neurophysiol. 2008; 99(3):1451–1460. [PubMed: 18160424]
24. Liu D, Liman ER. Proc Natl Acad Sci U S A. 2003; 100(25):15160–15165. [PubMed: 14657398]
25. Nilius B. Bull Mem Acad R Med Belg. 2007; 162(3–4):244–253. [PubMed: 18075054]
26. Zhainazarov AB, Spehr M, Wetzel CH, Hatt H, Ache BW. J Membr Biol. 2004; 201(1):51–57. [PubMed: 15635812]
27. Brady JD, Rich ED, Martens JR, Karpen JW, Varnum MD, Brown RL. Proc Natl Acad Sci U S A. 2006; 103(42):15635–15640. [PubMed: 17032767]
28. Wetzel CH, Oles M, Wellerdieck C, Kuczkowiak M, Gisselmann G, Hatt H. J Neurosci. 1999; 19(17):7426–7433. [PubMed: 10460249]
29. Stauffer TP, Ahn S, Meyer T. Curr Biol. 1998; 8(6):343–346. [PubMed: 9512420]
30. Venkateswarlu K, Gunn-Moore F, Oatey PB, Tavare JM, Cullen PJ. Biochem J. 1998; 335(Pt 1): 139–146. [PubMed: 9742223]
31. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. Proc Natl Acad Sci U S A. 1998; 95(5):2509–2514. [PubMed: 9482916]
32. Karpova TS, Baumann CT, He L, Wu X, Grammer A, Lipsky P, Hager GL, McNally JG. J Microsc. 2003; 209(Pt 1):56–70. [PubMed: 12535185]
33. Washburn KB, Turner TJ, Talamo BR. Chem Senses. 2002; 27(7):635–642. [PubMed: 12200344]
34. Balla T, Varnai P. Curr Protoc Cell Biol. 2009 Chapter 24:Unit 24 24.
35. Varnai P, Balla T. J Cell Biol. 1998; 143(2):501–510. [PubMed: 9786958]
36. Vanhaesebroeck B, Leevers SJ, Ahmadi K, Timms J, Katso R, Driscoll PC, Woscholski R, Parker PJ, Waterfield MD. Annu Rev Biochem. 2001; 70:535–602. [PubMed: 11395417]
37. Mayer U, Ungerer N, Klimmeck D, Warnken U, Schnolzer M, Frings S, Mohrlen F. Chem Senses. 2008; 33(2):145–162. [PubMed: 18032372]
38. Halet G. Biol Cell. 2005; 97(7):501–518. [PubMed: 15966865]
39. Nash MS, Young KW, Willars GB, Challiss RA, Nahorski SR. Biochem J. 2001; 356(Pt 1):137–142. [PubMed: 11336645]
40. Matsu-ura T, Michikawa T, Inoue T, Miyawaki A, Yoshida M, Mikoshiba K. J Cell Biol. 2006; 173(5):755–765. [PubMed: 16754959]
41. Brock C, Schaefer M, Reusch HP, Czupalla C, Michalke M, Spicher K, Schultz G, Nurnberg B. J Cell Biol. 2003; 160(1):89–99. [PubMed: 12507995]
42. Vogl A, Noe J, Breer H, Boekhoff I. Eur J Biochem. 2000; 267(14):4529–4535. [PubMed: 10880977]
43. Bierne H, Dramsi S, Gratacap MP, Randriamampita C, Carpenter G, Payrastre B, Cossart P. Cell Microbiol. 2000; 2(6):465–476. [PubMed: 11207601]
44. Razzini G, Ingrosso A, Brancaccio A, Sciacchitano S, Esposito DL, Falasca M. Mol Endocrinol. 2000; 14(6):823–836. [PubMed: 10847585]
45. Rhee SG. Annu Rev Biochem. 2001; 70:281–312. [PubMed: 11395409]

46. Xie Z, Singleton PA, Bourguignon LY, Bikle DD. Mol Biol Cell. 2005; 16(7):3236–3246. [PubMed: 15872086]
47. Maffucci T, Falasca M. Biochem Soc Trans. 2007; 35(Pt 2):229–230. [PubMed: 17371245]
48. Sklar PB, Anholt RR, Snyder SH. J Biol Chem. 1986; 261(33):15538–15543. [PubMed: 3536906]

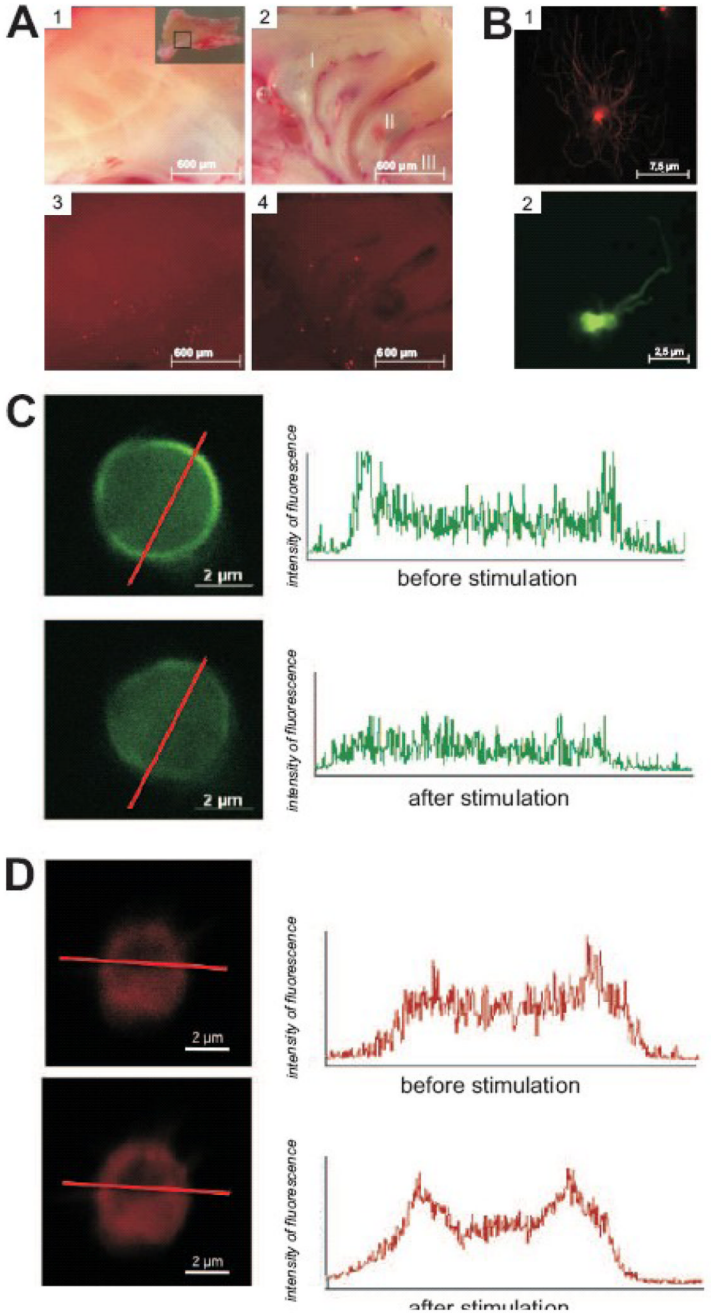

Figure 1.

Odorant-evoked translocation of the PI-specific probes PH_{GRP1}RFP and PH_{PLC}GFP in the dendritic knobs of mouse ORNs. (A) Brightfield images of mouse olfactory (1) septum and (2) turbinates at 7 days post infection with AdVPH_{GRP1}RFP. Corresponding fluorescent images of RFP expression in the (3) septum and (4) turbinates. Images were collected at 20× magnification. Approximately 52 fluorescent knobs could be detected in one visual field. (B) Fluorescent images of representative knobs infected with (1) AdVPH_{GRP1}RFP and (2) AdVPH_{PLC}GFP. Infected knobs were treated with H100 for 3 min for PH_{PLC}GFP and 6 min for PH_{GRP1}RFP. Images were collected for 10 min at 2 min intervals for PH_{PLC}GFP and for 30 min at 3 min intervals for PH_{GRP1}RFP. Representative fluorescent images of knobs

infected with (C) AdVPH_{PLC}GFP and (D) AdVPH_{GRP1}RFP captured before (top images and graphs) and 3 min after (bottom images and graphs) stimulation with Henkel 100 (1:10000). Results are representative of 4/23 knobs for AdVPH_{PLC}GFP and 5/52 knobs for AdVPH_{GRP1}RFP. Right: plots of the level of fluorescence before and after stimulation with H100 measured along one section represented by the red line through the corresponding image.

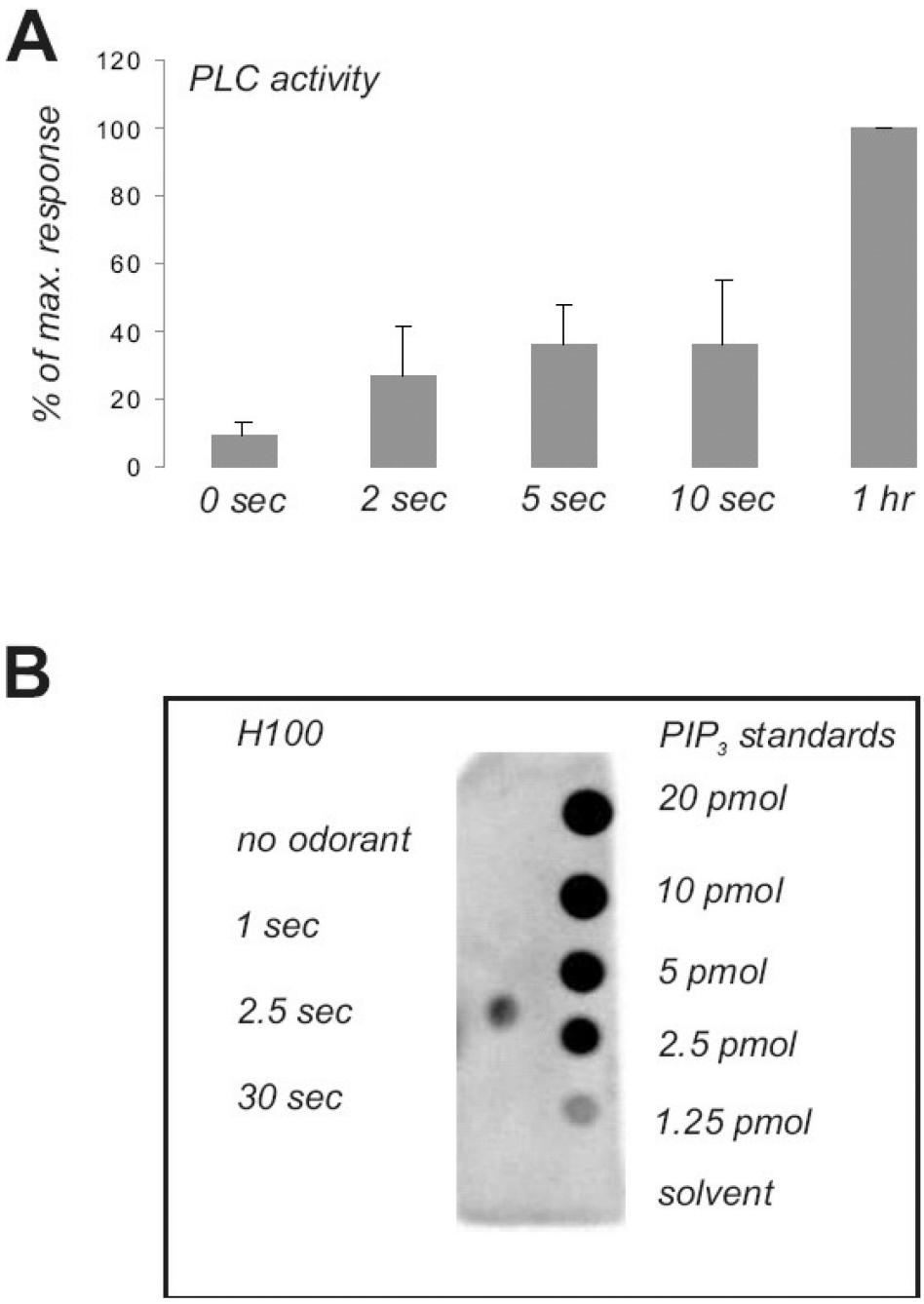

Figure 2.

Odorant-evoked activation of PLC and PI3K in rat ORNs. (A) Bar graphs of PLC activity in ORN cilia-enriched membrane preparations determined by ELISA at various time points after treatment with a 1:10000 dilution of H100. Bars denote the mean ± standard deviation of at least three independent experiments. Activity presented as the percentage of the maximum response. (B) A protein lipid overlay-assay specific for PI(3,4,5)P_3 shows a transient increase in class I PI3K activation in response to H100 (left column) in ORN cilia-enriched membrane preparations. PI(3,4,5)P_3 standards (right column) allowed estimation of the actual concentration.

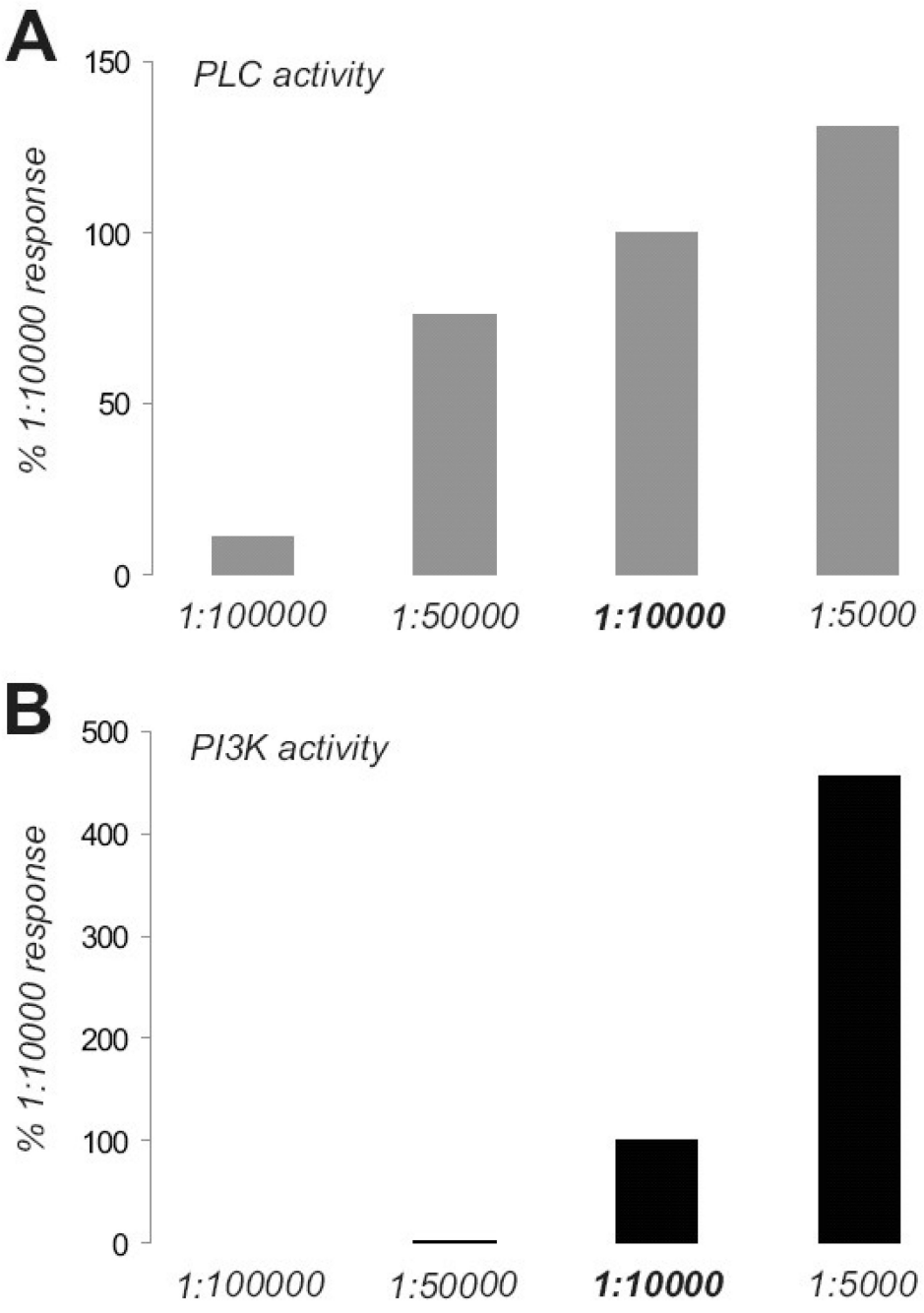

Figure 3.

Concentration-dependence of odorant-evoked PLC and PI3K activity in rat OE cells. Bar graphs of PLC (A) and PI3K (B) activity determined by ELISA as a function of H100 concentration indicated by the dilution ratio under each bar. Bars denote the mean ± standard deviation of at least three independent repetitions and are presented as the percentage of the response to the 1:10000 dilution.

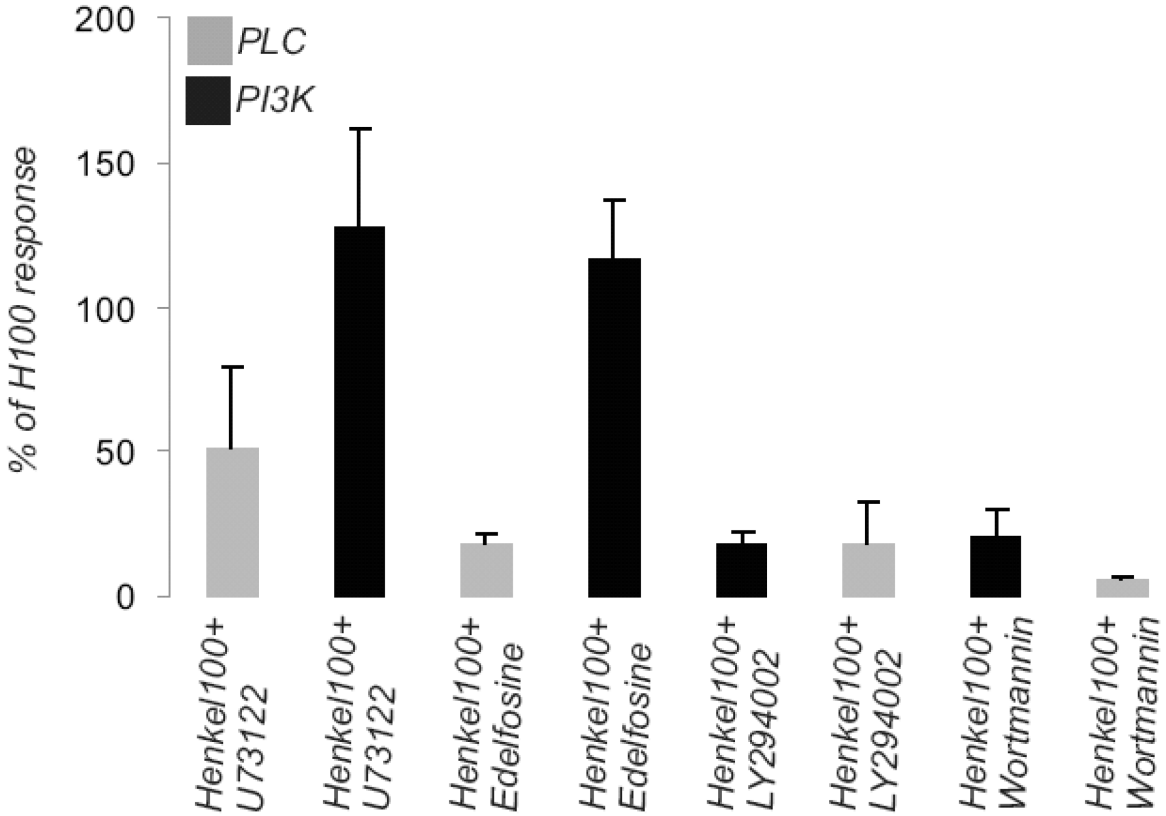

Figure 4.

Inhibition of PLC and PI3K decreases odorant-evoked enzyme activity in rat OE cells. Cells were pretreated with the PLC inhibitors U73122 and edelfosine or the PI3K inhibitors LY294002 and wortmannin for 2 minutes prior to treatment with a 1:10000 dilution of H100 for 5 sec time. Bar graph of PLC (light bars) and PI3K (dark bars) activity measured by ELISA. Bars denote the mean ± standard deviation of at least three independent repetitions and are presented as the percentage of the response of uninhibited OE cells to H100.

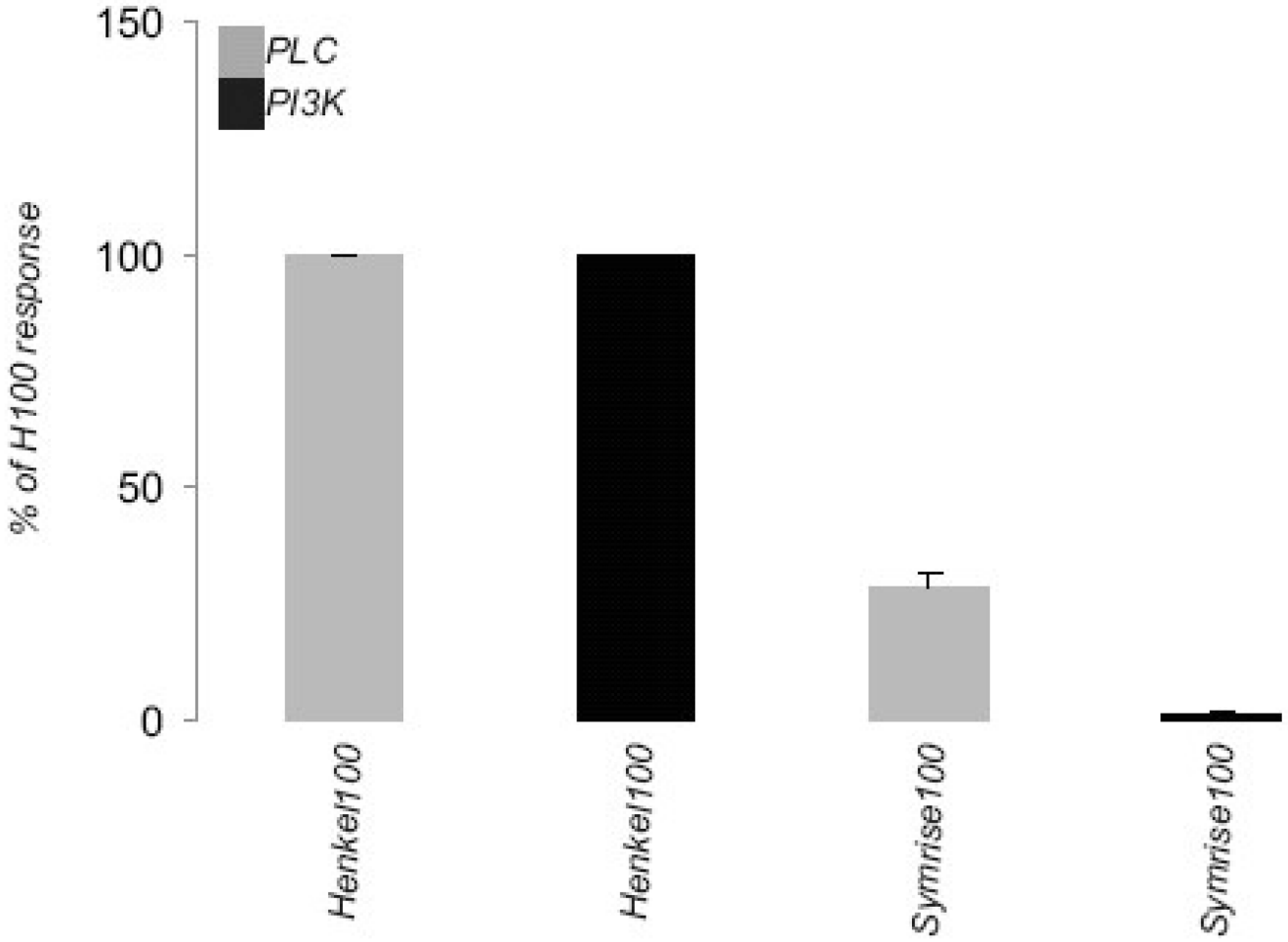

Figure 5.

Specificity of odorant-evoked PLC and PI3K activity in rat OE cells. Bar graph of PLC (light bars) and PI3K (dark bars) activation by two different complex odorant mixtures, H100 and S100, which differ in all but 3 components. Dissociated cells were treated with a 1:10000 dilution of either H100 or S100 for 5 sec time prior to measuring PLC and PI3K activity with ELISAs. Bars denote the mean ± standard deviation of at least three independent repetitions and are presented as the percentage of the response to H100.

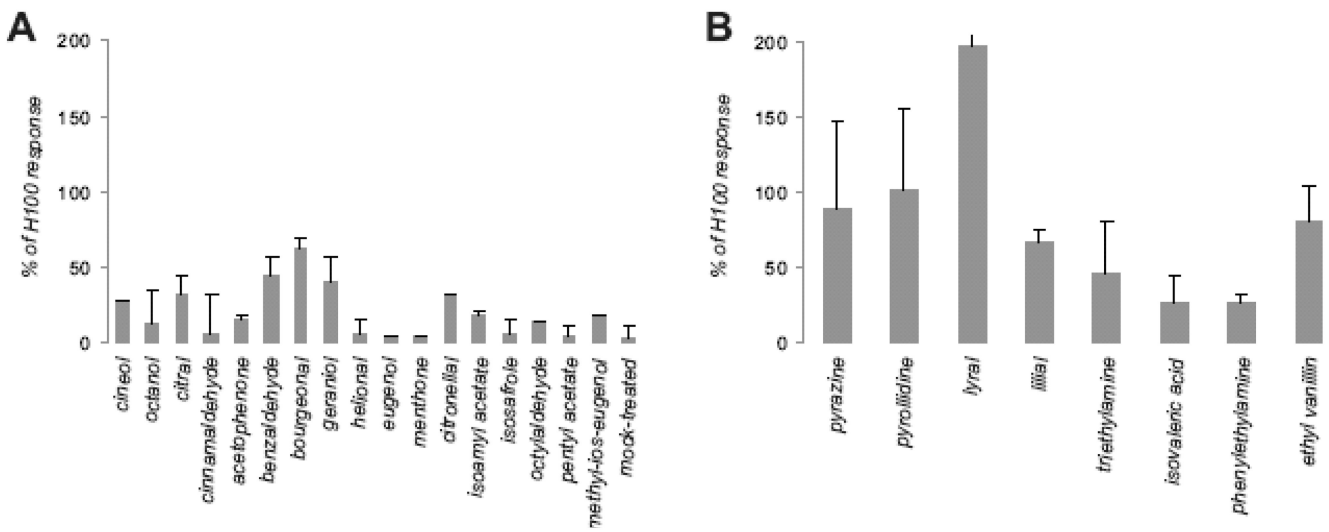

Figure 6.

Specificity of odorant-evoked PLC and PI3K activity in rat OE cells. Bar graphs of PLC activity in rat OE cells evoked by a panel of 25 single odorants. Dissociated rat OE cells were treated with (A) 17 single odorants chosen at random and (B) 8 odorants previously known to activate PLC. Cells were treated for 30 min prior to measuring the level of PLC activity by ELISA. Bars denote the mean ± standard deviation of at least three independent repetitions and are presented as the percentage of the response to H100.

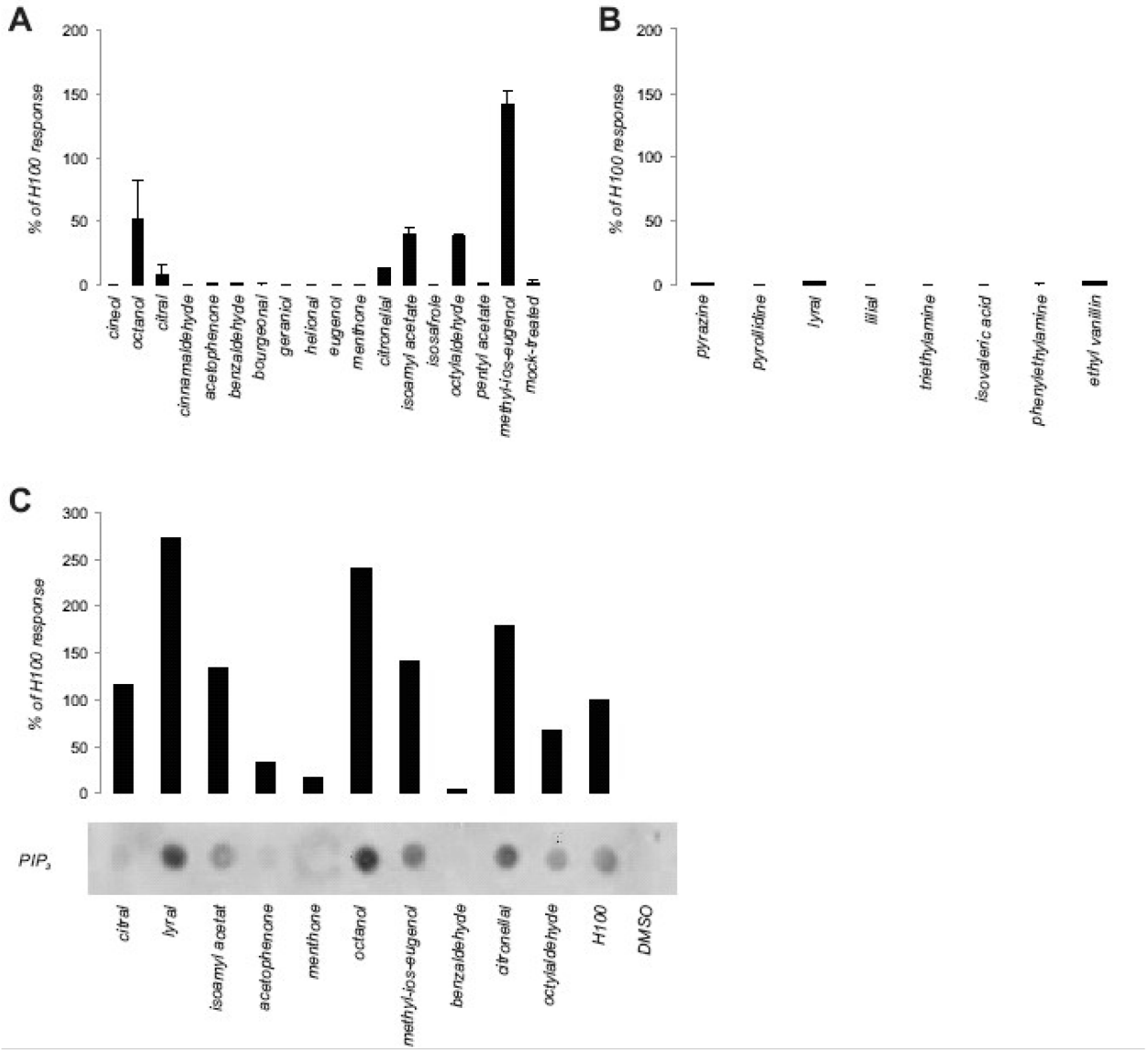

Figure 7.

Specificity of odorant-evoked PLC and PI3K activity in rat OE cells. (A, B) Bar graphs of total PI3K activity in rat OE cells evoked by the same panel of single odorants tested in Fig. 6. Dissociated rat OE cells were treated with (A) 17 single odorants chosen at random and (B) 8 odorants previously known to activate PLC. Cells were treated for 5 sec prior to measuring the level of PI3K activity by ELISA. Bars denote the mean ± standard deviation of at least three independent repetitions and are presented as the percentage of the response to H100. (C) Bar graph of PI3K activity determined by a protein lipid overlay-assay specific for PI(3,4,5)P_3 evoked by 10 representative single odorants from the panel of 25. Dot blots below each bar show the actual signal, which was captured with a CCD camera and the intensity of grayscale quantified.