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THREE STRUCTURALLY SIMILAR ODORANTS TRIGGER DISTINCT SIGNALING PATHWAYS IN A MOUSE OLFACTORY NEURON

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Abstract—In the mammalian olfactory system, one olfactory sensory neuron (OSN) expresses a single olfactory receptor (OR) gene. By calcium imaging of individual OSNs in intact mouse olfactory turbinates, we observed that a subset of OSNs (Ho-OSNs) located in the most ventral OR zone can mediate distinct signaling pathways when activated by structurally similar ligands. Calcium imaging showed that Ho-OSNs were highly sensitive to 2-heptanone (Ho), heptaldehyde (H) and cis-4-heptenal (cH). Ho-evoked intracellular calcium ($[Ca^{2+}]_i$) elevation was mediated by cAMP signaling while H triggered the diacylglycerol pathway. An increase of $[Ca^{2+}]_i$ evoked by cH was due to a combination of activation mediated by the adenylate cyclase pathway and suppression generated by phospholipase C (PLC) signaling. Pharmacological studies indicated that novel mechanisms were involved in the PLC-mediated $[Ca^{2+}]_i$ changes. Binary-mixture studies and cross-adaptation data indicate that three odorants acted on the same OR. The feature that an OR mediates multiple signaling pathways was specific for Ho-OSNs and not established in another population of OSNs characterized. Our study suggests that distinct signaling pathways triggered by ligand-induced conformational changes of an OR constitute a complex information process mechanism in olfactory transduction. This study has important implications beyond olfaction in that it provides insights of plasticity and complexity of G-protein-coupled receptor activation and signal transduction. © 2014 Published by Elsevier Ltd. on behalf of IBRO.

Key words: olfactory neuron, olfactory receptor, sensory transduction, calcium imaging, adenylate cyclase, phospholipase C.

INTRODUCTION

Olfactory receptors (ORs) are members of G-protein-coupled receptor (GPCR) superfamily that includes a list of diverse cell surface receptors. Our understanding of the canonical pathway for mammalian olfactory transduction posits that when a cognate ligand binds to an OR, the OR activates G-protein α subunit $G_{\alpha olf}$, which elevates intracellular cAMP through adenylate cyclase (AC) enzymatic reaction. Binding of cAMP to the cyclic nucleotide-gated (CNG) channel allows influx of cations, mainly calcium, into OSNs. Elevation of intracellular calcium ($[Ca^{2+}]_i$) induces the opening of the calcium-gated chloride channel that produces an efflux of chloride ions to amplify cellular depolarization. Studies have shown that gene deletion of either $G_{\alpha olf}$, CNG, or the type III of AC causes general anosmia in mice, demonstrating the importance of the cAMP pathway in olfactory transduction (Brunet et al., 1996; Belluscio et al., 1998; Wong et al., 2000; Zhao and Reed, 2001). However, recent progress shows that mice with deletion of the A2 subunit of CNG channel retain olfactory activity to certain odorants, suggesting the involvement of cAMP-independent signaling pathways for mouse olfactory transduction (Lin et al., 2004).

Biochemical and electrophysiological data indicate that cAMP-independent signaling pathways, including guanylate cyclase and phospholipase C (PLC) signaling, play roles in olfactory responses in a variety of animal species (see reviews by Schild and Restrepo, 1998; Kato and Touhara, 2009; Zufall and Munger, 2010). In the PLC system, hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by PLC yields two signaling molecules inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge, 1998; Delmas et al., 2004). Electrophysiological recordings with isolated olfactory sensory neurons (OSNs) or with the isolated olfactory cilia

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Q4 *Abbreviations:* AC, adenylate cyclase; Ace, acetophenone; 2-APB, 2-aminoethoxydiphenyl borate; Ben, benzaldehyde; $[Ca^{2+}]_i$, intracellular calcium; CC, chelerythrine chloride; cH, cis-4-heptenal (heptenal); CNG, cyclic nucleotide-gated; DAG, diacylglycerol; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol tetraacetic acid; FFA, flufenamic acid; GPCR, G-protein-coupled receptor; H, heptaldehyde; H8, H-8 dihydrochloride; HCN, hyperpolarization-activated cyclic nucleotide-gated; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Ho, 2-heptanone (heptanone); IP_3, inositol 1,4,5-trisphosphate; Kir, inwardly rectifying potassium; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; N, nanoaldehyde; O, octaldehyde; ORs, olfactory receptors; OSN, olfactory sensory neuron; PI3K, phosphoinositide-3-kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PLC, phospholipase C; ROIs, regions of interests; SAG, 1-stearoyl-2-arachidonoyl-glycerol; SOCE, store-operated Ca^{2+} entry; tH, trans-2-heptenal; tO, trans-2-octenal; TRP, transient receptor potential; TRPM5, TRP channel M5.

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fused onto an artificial lipid bilayer have shown that IP_3 induces opening of the IP_3-gated non-selective cation channel (see reviews by Schild and Restrepo, 1998). Interestingly, a study in lobster OSNs showed that cAMP- and IP_3-gated channels were located adjacently within an OSN (Hatt and Ache, 1994), suggesting that both cAMP- and IP_3-activation machineries are available for an OSN.

The concept that multiple signaling pathways are involved in mediating olfactory transduction within an OSN is challenged by the evidence that an OSN expresses one OR gene although studies on rats and zebrafish suggest existence of OSNs that can express multiple ORs (Rawson et al., 2000; Buck, 2005; Sato et al., 2007). If an OSN expresses a single OR gene, would it be plausible to activate multiple signaling pathways? Is it necessary for an OSN to invest multiple cellular machineries for differential transduction events? A recent study in rat OSNs showed that antagonistic effects of citral on octanol were alleviated by application of phosphoinositide-3-kinase (PI3K) inhibitors, suggesting the existence of distinct signaling pathways in mediating excitatory and inhibitory responses within an OSN. Questions inspired from their study include whether activation of these two signaling pathways is mediated by the same OR. If so, whether these two signaling pathways interact with each other and how so? In this report, we present evidence that an OR can trigger three distinct signaling pathways upon binding to structurally similar ligands 2-heptanone (heptanone, Ho), heptaldehyde (H) or cis-4-heptenal (heptenal, cH). Ho-evoked responses exhibit characteristics of canonical pathway for olfactory transduction that involves cAMP signaling while H-evoked receptor activation triggers the PLC pathway in which the IP_3-gated channel does not play a significant role. We further present evidence that cH-evoked $[Ca^{2+}]_i$ elevation results from a combination of activation mediated by AC signaling and suppression mediated by the PLC pathway. The feature that an OSN utilizes multiple signaling pathways to transmit the ligand-dependent information is specific for Ho-OSNs and not established in another set of OSNs, AB-OSNs, that are sensitive to acetophenone (Ace) and benzaldehyde (Ben).

We have revealed functional diversities of an OR under physiological condition. Our study exemplifies the advanced understanding of GPCR signaling in that GPCRs possess conformational plasticity and various ligands may differentially regulate distinct activities of a given GPCR by stabilizing one of the receptor conformations leading to preferential interactions with specific downstream elements (Hermans, 2003; Urban et al., 2007; Ambrosio et al., 2011). Our study has provided a new piece of evidence for functional selectivity of GPCRs under natural and physiological conditions.

EXPERIMENTAL PROCEDURES

Chemicals and solutions

All odorants used here were purchased from Sigma (St Louis, MO). Odorant stimuli were freshly prepared by directly diluting in Ringer's saline made of 145 mM NaCl, 5 mM KCl, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM Na pyruvate, and 5 mM D-glucose. Ca^{2+}-free solution was obtained by replacing $CaCl_2$ with 3 mM ethylene glycol tetraacetic acid (EGTA) in saline. High K^+ solution was made from saline by adjusting KCl to 80 mM. All solutions were adjusted to pH 7.4 and oxygen-saturated before use.

We applied various pharmacological reagents to study signaling pathways. Neomycin, $GdCl_3$, 8-Bromine-cAMP (Br-cAMP), chelerythrine chloride (CC), flufenamic acid (FFA) and MDL-12330A were purchased from Sigma. U73122, U73343, H-8 dihydrochloride (H8), 2-aminoethoxydiphenyl borate (2-APB) and 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) were bought from Cayman Chemical (Ann Arbor, MI). SQ22536 and 1-stearoyl-2-arachidonoyl-glycerol (SAG) were obtained from Calbiochem (San Diego, CA). For chemicals having low water solubility, they were first dissolved in dimethyl sulfoxide (DMSO) as stock solutions and then diluted $\geqslant$1000 times with saline just before the experiments. We tested the influence of 0.1% DMSO to $[Ca^{2+}]_i$ transients in the experiments as the background controls for pharmacological reagents. DMSO alone did not induce $[Ca^{2+}]_i$ changes. The concentrations of pharmacological reagents used in experiments were chosen on the basis of published reports and information provided by the manufacturers, Table 1 summarizes pharmacological properties of these reagents, the concentrations of the reagents we applied in the study, and a few relevant publications.

Calcium imaging to OSNs in intact turbinates and data analysis

All procedures for animal handling were carried out in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the Illinois Institute of Technology. Adult C57BL/6 mice at 2–4 months of age were used in the experiments. Samples were prepared as described previously (Zhang, 2010). Briefly, the decapitated mouse head was opened along the midline, and the endoturbinates were exposed by peeling off the septum. After further removal of the olfactory bulb and bones around the ectoturbinates, the whole turbinates, as shown in Fig. 1A, were incubated in 10 μM calcium-sensitive dye fura-2 AM (Life Technologies, Grand Island, NY) containing 0.02% nonionic dispersing agent Pluronic F-127 (Life Technologies) at 37 °C for approximately 70 min. The turbinates were then mounted in a recording chamber (a modified RC-22C, Warner Instrument, Hamden, CT) with the endoturbinates facing up as shown in Fig. 1A and were continuously perfused with oxygenated saline throughout the experiments. Ratiometric calcium imaging recording was performed with excitation of 340 nm (F340) and 380 nm (F380) in an Olympus upright microscope (BX51WI) equipped with a 20× water immersion objective (0.9 numerical aperture), a filter wheel (Sutter Instruments, Novato, CA), a 175-W xenon lamp, and a cooled CCD camera (SensiCam QE; Cooke Corporation, Romulus, MI). Images were collected every 4 s using Imaging Workbench 5.2 (Indec Biosystems, Santa Clara, CA). The ratio of F340/F380

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