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Ligand-selective activation of heterologously-expressed mammalian olfactory receptor

K. Ukhanov¹, Y. Bobkov¹, E.A. Corey¹, and B.W. Ache^{1,2}

¹Whitney Laboratory, Center for Smell and Taste, and McKnight Brain Institute, University of Florida, Gainesville, FL 32610

²Depts. of Biology and Neuroscience, University of Florida, Gainesville, FL 32610

Abstract

Mammalian olfactory receptors (ORs) appear to have the capacity to couple to multiple G protein-coupled signaling pathways in a ligand-dependent selective manner. To better understand the mechanisms and molecular range of such ligand selectivity, we expressed the mouse eugenol OR (mOR-EG) in HEK293T cells together with Gα15 to monitor activation of the phospholipase-C (PLC) signaling pathway and/or Gαolf to monitor activation of the adenylate cyclase (AC) signaling pathway, resulting in intracellular Ca²⁺ release and/or Ca²⁺ influx through a cyclic nucleotide-gated channel, respectively. PLC-dependent responses differed dynamically from AC-dependent responses, allowing them to be distinguished when Gα15 and Gαolf were co-expressed. The dynamic difference in readout was independent of the receptor, the heterologous expression system, and the ligand concentration. Of 17 reported mOR-EG ligands tested, including eugenol, its analogs, and structurally dissimilar compounds (mousse cristal, nootkatone, orivone), some equally activated both signaling pathways, some differentially activated both signaling pathways, and some had no noticeable effect even at 1-5 mM. Our findings argue that mOR-EG, when heterologously expressed, can couple to two different signaling pathways in a ligand selective manner. The challenge now is to determine the potential of mOR-EG, and perhaps other ORs, to activate multiple signaling pathways in a ligand selective manner in native ORNs.

Introduction

G-protein coupled receptors (GPCRs) can show a hierarchy of downstream signaling imposed primarily by the conformational stage of the ligand-GPCR complex, such that different ligands reproducibly shift the balance of the cell's signaling network towards different transduction pathways. This concept, originally introduced in the 1990s [1-3] and most commonly referred to as ligand induced selective signaling or biased agonism, has since become a well-recognized phenomenon of significance to both basic and clinical

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Corresponding author: Kirill Ukhanov, Telephone +1-352-3924217, Fax: +1-352-2945365, UKHANOV@MBI.UFL.EDU.

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science [4-6]. Mechanisms mediating ligand selective signaling can be complex, including processes such as activation of multiple G-protein isoforms [7], β -arrestins [8], the heteromerization of GPCRs [6], and direct interaction of GPCR and G-protein subunits with a variety of ion channels, including Ca^{2+} channels [9, 10] and G-protein-gated inwardly rectifying potassium channels, GIRKs [11], among others.

Despite the fact that olfactory receptors (ORs) represent the largest family of mammalian GPCRs [12], their potential for ligand-selective signaling has received little attention, notwithstanding indirect evidence that activation of native ORNs can involve adenylyl cyclase (AC)-as well as phosphoinositide (PI)-dependent signaling [13] and that activation of the two signaling pathways in native ORNs can be ligand selective [14, 15]. A major constraint to implicating ligand-induced selective signaling in ORNs is the identification of odorants that target each signaling pathway for a given OR, given that deorphanizing ORs for even a single ligand is not trivial. Most attempts to deorphanize mammalian ORs involve heterologous expression of ORs with a specific isoform of G-protein. Co-expression of the OR with $\text{G}\alpha_{\text{olf}}$ allows coupling the OR to AC signaling, which can be monitored by an increase in intracellular Ca^{2+} through a co-expressed cyclic nucleotide-gated channel [16] or by a variety of biochemical assays [17, 18], although $\text{G}\alpha_{\text{s}}$ alone can serve the same function as $\text{G}\alpha_{\text{olf}}$ [18, 19]. Co-expression of the OR with the promiscuous $\text{G}\alpha_{15/16}$ allows coupling the OR to PI (phospholipase-C, PLC) - dependent signaling, yielding release of Ca^{2+} from intracellular stores [20]. Interestingly, monitoring PLC-dependent signaling appears to bias the ligand ranking from that obtained by monitoring AC-dependent signaling [16], potentially reflecting ligand specific bias in the activation of the two different G proteins in the heterologous system.

In order to better understand the potential for ligand selective signaling by mammalian ORs, we used a Ca^{2+} imaging based approach that allows us to monitor activation of both pathways when activated by the mouse eugenol receptor mOR-EG, an OR with numerous known ligands. We expressed mOR-EG in HEK293T cells together with $\text{G}\alpha_{15}$ to target PLC signaling (mOR-EG/PLC) and/or $\text{G}\alpha_{\text{s(olf)}}$ to target AC signaling (mOR-EG/AC) and differentiated the two Ca^{2+} signals in real time based on their different kinetic parameters. We tested 17 of the 38 reported mOR-EG ligands [21, 22], including eugenol and some of its analogs, as well as structurally dissimilar compounds, including muscic acid, muscatone, and muscivone. Six of the ligands failed to give a measurable response to either output. The remaining eleven ligands activated both outputs, seven of them equally and four preferentially favoring the PLC-dependent output. Interestingly, small alterations in molecular structure could significantly bias the output towards the PLC pathway. Our findings argue that mOR-EG when heterologously expressed can couple to two different signaling pathways in a ligand-induced selective manner. The challenge now is to determine the potential of mOR-EG, and perhaps other ORs, to indeed use ligand-selective activation of multiple signaling pathways in native ORNs.

Materials and Methods

Cell Culture and Transfection

HEK293T cells were grown in a high glucose Dulbecco's minimum essential medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin and streptomycin, and 2 mM L-glutamine and maintained at 37 °C (5% CO₂). MMDD1 macula densa cells (provided by Dr. Jurgen Schnermann, NIH, Bethesda, Maryland) were grown under similar conditions after an initial two weeks of treatment with plasmocin (Invivogen). New cells were thawed every 2 to 3 months. A rhodopsin-tagged mouse eugenol olfactory receptor (mOR-EG) encoded by the Olf73 gene (provided by Dr. H.Matsunami, Duke University) was co-expressed with RTP1s, a short version of the Receptor Transporting Protein 1 ensuring proper surface trafficking of the receptor (provided by Dr. K.Touhara, University of Tokyo), mouse Gα15 (Thermo Scientific Open Biosystems), human Gαolf (obtained from the Missouri S&T cDNA Resource Center) and/or a mutated A2 subunit of the rat olfactory cyclic nucleotide gated channel (CNGC-camp). Two mutations C460W and E583M ensured high sensitivity and selectivity of the channel to cAMP (provided by Dr.T.Rich, University of Alabama). Transfections were performed using Calfectin (SigmaGen) or standard calcium phosphate DNA precipitation. Cells at 70-80% confluence were transfected with 1.5 µg of the rho-mOR-EG plasmid, 0.9 µg of RTP1s, 0.9 µg of Gα15, 0.9 µg of Gαolf and/or 1.0 µg of the CNGC-camp per 35 mm cell culture dish (BioLite, Fisher Scientific). Transfected cells were incubated overnight and then split onto new 35-mm dishes or poly-lysine coated glass cover slips in fresh media for analysis between 36 and 72 hours after transfection. Cells were transfected with 1 µg of human M3-AChR (obtained from the Missouri S&T cDNA Resource Center) as described and used at 24 hours post-transfection. Mosquito OR-expressing HEK293T cells (provided by Dr. L. Zwiebel, Vanderbilt University) were incubated with 0.3 µg/mL tetracycline for 16 hours before the assay to induce OR expression.

Reagents and solution application

Single odorants were of highest purity obtained from Sigma-Aldrich, Acros Chemicals, Alpha Aesar. Some mOR-EG ligands were kindly provided by Dr. Boris Schilling (Givaudan, Switzerland). Several structural analogs of muscristal were a generous gift of Dr. Michael Doyle (University of Maryland). Pilocarpine (MP Biochemicals) was used as a PLC biased muscarinic ligand [23] for the M3 AChR receptor. Stocks were prepared as 0.5 M solution in anhydrous DMSO and were kept frozen at -20°C. The final aqueous solutions of odorants were prepared on the day of experiment in a magnesium-free Ringer's solution containing (in mM): 140 NaCl, 5 KCl, 1.8 CaCl₂, 0 MgCl₂, 10 HEPES, 1.25 sodium pyruvate, 10 glucose, pH 7.6, and diluted immediately before experiments to yield the indicated concentrations. Extracellular Mg²⁺ ions were removed to reduce inhibition of the cyclic nucleotide gated channels by the divalent cation [24]. No significant difference due to reduced extracellular Mg²⁺ was observed in the response evoked due to the intracellular Ca²⁺ release in HEK293T cells expressing either M3 AChR or mOR-EG/Gα15 (data not shown).

All odorants used in the study are listed in Fig.1 in order to better illustrate their structural relationship to each other. Odorants were applied to cells using a computer-controlled fast perfusion system (RSC-200, BioLogic) ensuring precise delivery of the stimulus with a minimal time lag. The perfusion system was controlled by Clampex 9.2 or 10.2 software (Molecular Devices).

Single Cell Ca^{2+} Imaging

Cells were incubated (30 min/37°C) in a DMEM containing 6-8 μM Fluo-4/AM (Invitrogen) or Fluo-2/AM (TefLabs) containing 0.04% Pluronic F127. After removal of extracellular dye, cells were washed with Ringer's solution for at least 15 min. Odorant solutions were applied sequentially to the cells for 5 s with a 3 min interval between each application to allow recovery from desensitization resulting from previous application of odorants. 35 mm dishes with attached cells were transferred to the stage of an inverted microscope (Axiovert 200, Zeiss) equipped with a 10 $\times$ /0.5NA Fluar objective. Additional measurements were performed using an inverted microscope Olympus IX-71 equipped with 20 $\times$ /0.45NA U-Plan objective lens. Both microscopes were equipped with 12-bit cooled CCD cameras (ORCA R2, Hamamatsu, Japan). A standard FITC filter set (excitation at 510 nm, emission at 530 nm, dichroic mirror 516 nm) was used for a single-wavelength measurement. The illumination system (Lambda DG-4 or Lambda L10-BS with a Smart Shutter controller, Sutter Instruments) and image acquisition were controlled by Imaging Workbench 6 software (INDEC BioSystems) under master control of Clampex software (Molecular Devices) to ensure syncing of image acquisition and odorant application.

Each cell was assigned a region of interest (ROI) and changes in fluorescence intensity within each ROI were analyzed and expressed as the fractional change in fluorescent light intensity F/F_0 where F_0 is the baseline fluorescence before stimulus application. Cells characterized by a stimulus evoked change in fluorescence intensity exceeding two standard deviations above the median noise level were included in the analysis. For quantitative comparison, the peak amplitudes of the responses of different cells were normalized to the responses elicited by application of a saturating concentration (1-5 mM) of eugenol.

Data analysis

All data are expressed as mean $\pm$ SEM. Each type of experiment was done at least in triplicate or as indicated in the text including also a number of cells used for analysis. All analyses were performed with pClampfit 10.2 (Molecular Devices), Microsoft Excel and SigmaPlot 11 (Systat Software, USA) software.

RESULTS

As a dual read-out assay to test for differential activation of the mOR-EG/PLC and mOR-EG/AC responses, we used a previously established HEK293T heterologous expression system in conjunction with Ca^{2+} imaging. We co-expressed mOR-EG and Gaolf with a mutated cyclic nucleotide-gated (CNG) channel (CNGC-camp) as a cAMP sensor and monitored Ca^{2+} entry from the extracellular medium [24, 25]. Gas is expressed endogenously in HEK293T cells and likely contributed to activation of the AC signaling

pathway, but the relative contribution of exogenous G α olf vs endogenous G α s to the assay was not explored. In a separate set of cells, we co-expressed mOR-EG with G α 15 to target the endogenous PLC and monitored Ca²⁺ release from intracellular stores [20]. Ligands were applied for a 5-s period using a computer-controlled fast perfusion system and changes in intracellular Ca²⁺ were analyzed in individual single cells to resolve the details of the response kinetics. The overall number of cells sensitive to eugenol varied and presumably was dependent on the signal transduction elements expressed, i.e., the percentage of cells transfected with mOR-EG/G α 15 and mOR-EG/G α olf/CNGC-camp. The relatively brief application of the agonists approximated the odor stimulation typically used in studies of native ORNs and avoided unnecessary saturation of the response, especially at higher odorant concentrations.

We initially established three response parameters (rise time, *Trise*; time of peak, *Tpeak* and decay time, *Tdecay*) to distinguish between the pathways using the response to eugenol at 100 μ M (Fig.2). We found that the mOR-EG/AC responses were slow in comparison to the mOR-EG/PLC responses (Fig.2). For the mOR-EG/AC responses (Fig.2), *Tpeak* (relative to stimulus application time) ranged from 61.7 to 116.5s (mean=76 $\pm$ 6); *Trise* from 19.7 to 46.1s (mean=32 $\pm$ 3); and *Tdecay* from 74 to 125.4s (mean=102 $\pm$ 11s, n=24). For the mOR-EG/PLC responses (Fig.2), *Tpeak* ranged from 26.1 to 40.1s (mean=31.5 $\pm$ 0.7); *Trise* from 5.4 to 12.9s (mean=8.4 $\pm$ 0.3); and *Tdecay* from 32.9 to 72.4s (mean=49.4 $\pm$ 1.8, n=30).

We next used pharmacological probes to provide evidence that the kinetically distinct responses resulted from activation of PLC- and AC-dependent pathway as predicted. As would be expected, the mOR-EG/PLC-dependent Ca²⁺ response persisted upon removal extracellular Ca²⁺ (Fig.3A) and was sensitive to the PLC inhibitor U73122 (1 μ M, Fig.3B). In contrast, the mOR-EG/AC-dependent response was abolished in the absence of extracellular Ca²⁺ (Fig.3C) and could be induced by treatment with the AC activator forskolin (10-50 μ M) in combination with the phosphodiesterase inhibitor IBMX (100 μ M) or with forskolin (10-50 μ M) by itself. Forskolin induced Ca²⁺ influx that was kinetically similar to that resulting from application of eugenol (Fig.3D): *Tpeak* (relative to stimulus application time) ranged from 49.8 to 212.6s (mean=127.5 $\pm$ 4.5s, n=74); *Trise* from 17.3 to 148.3s (mean=67.4 $\pm$ 3.1s, n=74); and *Tdecay* from 78.8 to 284.6s (mean=173.6 $\pm$ 14.5s, n=18). HEK293T cells expressing mOR-EG and G α olf without CNGC-camp did not generate noticeable Ca²⁺ responses to either eugenol (0.5-2mM, n>500) or forskolin (20 μ M, n>500). Neither untransfected HEK293T cells nor cells expressing mOR-EG alone were sensitive to eugenol (0.5-5mM, n>1000). Saturating stimulation of the endogenous purinergic G α q-dependent pathway with 100 μ M ATP did not induce appreciable activation of CNGC-camp, indicating that the two readouts are largely independent of one another (data not shown).

To assure that the use of the CNGC-camp ion channel did not impose any dynamic constraints on measuring the level of cAMP, we performed a control experiment by applying 10 μ M forskolin in the absence of extracellular divalent cations. Removal of extracellular Ca²⁺ completely abolished the CNGC-camp dependent readout and re-addition of 2 mM Ca²⁺ to the media was accompanied by a relatively quick rebound response due to Ca²⁺ influx through already open CNG channels (Fig.3F). In order to maximize the

projected cAMP build-up and opening of the CNG-camp channel, extracellular Ca^{2+} was added 60 s after the stimulation. Under these conditions the rate of Ca^{2+} influx exceeded the rate of response to forskolin more than 3-fold, Trise 24.3 ± 3.1 s ($n=18$, 2 experiments) in comparison to 76.7 ± 5.7 s ($n=16$, 2 experiments, compare Fig.3E and 3F). We conclude that CNGC-camp faithfully reports the cAMP gradients generated as a result of AC-dependent activity in our system.

We also tested the effects of eugenol on the mOR-EG/PLC or mOR-EG/AC pathways expressed in a different cell type to determine whether the Ca^{2+} signal kinetics for the two pathways were inherent characteristics of the pathways or the cell type. For these experiments, we used MMDD1 macula densa cells which reportedly endogenously express ORs as well as components of the olfactory signal transduction pathway [26]. For the mOR-EG/PLC responses: Tpeak ranged from 6.7 to 24s (mean= 13 ± 0.8 s, $n=26$), Trise 10% to 90% - 0.8 to 12.6s (mean= 3.6 ± 0.5 s, $n=26$); and Tdecay 90% to 10% - 5.9 to 32s (mean= 12.5 ± 1.1 s, $n=26$). For the mOR-EG/AC responses: Tpeak ranged from 46.7 to 207.9s (mean= 131.1 ± 16.5 s, $n=9$); Trise from 10% to 90% - 17.3 to 148.4s (mean= 80.6 ± 12.9 s, $n=9$); and Tdecay from 90% to 10% - 131.1s. These results argue that the kinetics of the two pathways are determined by the signaling pathway components.

After establishing that the Ca^{2+} kinetics would allow us to distinguish between the mOR-EG/PLC and mOR-EG/AC responses, we compared the responses at several ligand concentrations to generate and compare EC_{50} values for eugenol. For mOR-EG/PLC, Tpeak decreased from 18.6 ± 0.8 s for 25 μM to 15.4 ± 0.4 s for 1 mM, Trise decreased from 10.8 ± 0.9 s to 5.6 ± 0.3 s (25 μM -5mM), and Tdecay increased from 35.0 ± 0.8 s to 57.9 ± 3.8 s (25 μM -5mM, Fig.4A, middle panel). The cumulative concentration-dependence of the mOR-EG/PLC responses to eugenol yielded an EC_{50} of 53 ± 17 μM with a Hill coefficient of 1.2 (Fig.4A, right panel, $28 < n < 161$, 3 experiments). The responses to eugenol at close to saturating concentrations (0.5-1mM) exhibited minimal run-down/desensitization and were used for normalization in further experiments. The parameters of the mOR-EG/AC response kinetics also changed in a concentration-dependent manner (100 μM vs 5mM): Tpeak increased from 75.9 ± 6.1 s to 90.5 ± 2.9 s, Trise increased from 32.2 ± 3.0 s to 38.5 ± 1.5 s, and Tdecay increased from 101.9 ± 10.9 s to 125.1 ± 4.9 s (Fig.4B, middle panel). The cumulative concentration-dependence of the mOR-EG/AC response to eugenol yielded an EC_{50} of 55 ± 11 μM with a Hill coefficient of 0.97 (Fig.4A, right panel, $n=28$, 2 experiments). Given the likely differences in the expression levels of the exogenous signaling elements among individual cells, it is not surprising that the response potency varied substantially at the single-cell level, creating the variability seen within the dose-response curves (Fig.4A,B, right panels).

Having established functional read-outs for the comparison of the mOR-EG/PLC and mOR-EG/AC responses using eugenol, we then screened a panel of 16 additional compounds previously published as mOR-EG ligands. The saturating response parameters for all 17 compounds are shown in Table 1. Four of the compounds previously reported to be agonists (nootkatone, raspberry ketone, 4-isopropylphenol, and 4-tert-butylphenol), as well as two previously reported antagonists [27] methyl isoeugenol (MIEG, Fig.6) and isosafrole failed to activate any response in our assay (Fig.1, Table 1) and were not investigated further. The

remaining 11 compounds activated both pathways. Although there was a wide range in overall stimulus strength, seven compounds (eugenol, methyl eugenol, acetyl eugenol, vanillin, ethyl vanillin, orivone and 4-propylguaiacol) activated both pathways with similar EC₅₀ values. Four of the 11 compounds (isoeugenol, mousse cristal, 4-ethylguaiacol and 4-vinylguaiacol) showed biased activation of the PLC pathway (Fig.7B-D).

Isoeugenol is structurally similar to eugenol, differing only by a double bond in the R4 residue of the phenol ring (Fig.1). However, isoeugenol evoked a substantially smaller mOR-EG/AC response in comparison to eugenol, yielding only 0.14±0.11 of the normalized peak magnitude (Fig.5A). The concentration-dependence of the mOR-EG/AC response to isoeugenol yielded an EC₅₀ of 286±40 µM (n=88-345). Similarly, isoeugenol activated the mOR-EG/PLC response much less robustly than eugenol, with a normalized to eugenol peak magnitude of 0.38±0.03 (Fig.5A) and a longer time to reach the peak magnitude with a Trise of 14.8±1.6 s and Tpeak of 37.1±3.5 s (Table 1). However, the concentration-dependence yielded an EC₅₀ of 35.4±4.8 µM (n=90-374; Fig.5A, right panel, Table 1), indicating that it has ~8-fold greater potency in activating mOR-EG/PLC signaling in comparison to the mOR-EG/AC signaling (Fig.5A).

Three other compounds were tested for structural variation (4-propylguaiacol, 4-ethylguaiacol and 4-vinylguaiacol) also have small variations at the position R4 of the phenol ring in comparison to eugenol (Fig.1). Like isoeugenol, 4-propylguaiacol differs from eugenol by just a double bond in the R4 residue and demonstrates a dramatically smaller activation of the mOR-EG/AC response, yielding an EC₅₀ of 205±15 µM. However, in contrast to isoeugenol, it also demonstrated reduced activation of the mOR-EG/PLC response (EC₅₀ of 174±88 µM). It should be noted that a similar pattern of activation was found for vanillin, which also differs from eugenol at just the R4 residue but more substantially with an aldehyde group (–CHO) in place of the allyl group, yielding EC₅₀ of 162±68 and 218±27 µM for the mOR-EG/AC and mOR-EG/PLC response, respectively. The other two compounds, 4-ethylguaiacol and 4-vinylguaiacol were more functionally similar to isoeugenol with a bias towards activation of the mOR-EG/PLC response. Despite the structural similarities between 4-ethylguaiacol and 4-vinylguaiacol, which differ by only a double bond, 4-ethylguaiacol demonstrated ~5-fold lower potency in activating the mOR-EG/AC response, further highlighting the influence of the R4 residue in signaling by the receptor.

The structures of the R1 and R3 residues of eugenol-related compounds also appear to be important for activation and interaction with mOR-EG. Most notably, methylation at the R1 position transforms isoeugenol from an agonist biased towards activation of the mOR-EG/PLC response into the inactive ligand methyl isoeugenol which does not activate either pathway in our system (Fig.6). Even prolonged application of methyl isoeugenol did not evoke any measurable response in our assay (data not shown). However, methyl isoeugenol acts as a potent antagonist that inhibits eugenol activation of both the mOR-EG/PLC and mOR-EG/AC responses (Fig.6B; [27, 28]), indicating that although it does not act as an agonist it may interact with the receptor. Similarly, alteration of the R3 residue can also dramatically reduce the ability of eugenol-related compounds to activate mOR-EG as demonstrated by the comparison of vanillin and ethyl vanillin. While vanillin robustly

activated both the mOR-EG/PLC and mOR-EG/AC responses with EC₅₀ of 218±27 and 162±68 μM, respectively, ethyl vanillin was much less potent, with EC₅₀ of 1487±716 and 2165±131 μM, respectively (Fig.7A, Table 1).

In contrast to isoeugenol and other related compounds, mousse cristal is a derivative of tartaric acid that is structurally distinct from eugenol (Fig.1). Activation of the mOR-EG/AC response by mousse cristal was even stronger than by eugenol, maximal peak response 1.12±0.13 (n=128, 7 experiments) and with a 30% faster Trise (Fig.5B, Table 1). It also robustly and fully activated the mOR-EG/PLC response (Fig.5B, Table 1) but with slower kinetics (T_{peak} of 30.7±1.4 s and Trise of 14.1±0.8 s (n=45, Table 1) than did eugenol. Despite what would be expected based on the slower response dynamics, the EC₅₀ value for mousse cristal was more than 2-fold higher for the mOR-EG/AC response (253±81 μM) than that for the mOR-EG/PLC response (551±22 μM). Several other structural analogs of mousse cristal [29] were also screened, but were ineffective at activating either pathway up to millimolar concentration (data not shown).

To estimate functional bias between all tested mOR-EG agonists we did a simple pair-wise comparison of EC₅₀ values calculated for each signaling pathway. Mostly the agonists positioned along the diagonal suggesting little if any functional selectivity within this group (Fig. 7D). However, four different ligands preferentially activating PLC-dependent readout, positioned below the diagonal forming a distinct group (Fig.7D grey dots).

Previously it was found that when both readouts are expressed together in HEK293T cells, the mOR-EG/AC response can be completely inhibited by co-transfection of Gα15/16 [30]. However, the responses in that study were analyzed with an endpoint cAMP assay at the population level rather than in individual cells. Thus, we asked if it is possible to monitor simultaneous activation of the mOR-EG/PLC and mOR-EG/AC responses within a single cell. HEK293T cells were co-transfected with mOR-EG, Gα15, Gαolf and CNNG-camp. The majority of the odorant sensitive cells produced either the mOR-EG/PLC or the mOR-EG/AC response (Fig.8A,B), likely reflecting a low incidence of cells that are transfected with all of the necessary components at the optimal ratio to create the dual response. However, in each experiment cells were detected that were capable of generating a dual response with the initial fast rising phase that we found to be associated with mOR-EG/PLC signaling followed by a slower mOR-EG/AC Ca²⁺ signal (Fig.8C). The overall incidence of cells capable of generating a dual response from mOR-EG was rather low at around 1%. Nevertheless these cells not only generated a dual response in a concentration-dependent manner but did so according to the previously characterized functional bias. Isoeugenol even at the highest concentration of 1 mM was not able to evoke a slow Gαs(olf)-dependent response (Fig.8C left panel) which was obvious in the signal evoked by eugenol (Fig.8C right panel). The non-olfactory GPCR M3 AChR, one of several non-olfactory GPCRs known for their pluripotency [23, 31], was also capable of evoking such a dual response mediated by coupling to endogenous Gαq and Gαs proteins (data not shown). We conclude that at least in heterologous system a mammalian OR may couple to at least two different signaling pathways.

DISCUSSION

As noted in the Introduction, our study builds on previous studies of heterologously expressed ORs. In addition to activating the Gas(olf)/AC pathway, heterologously expressed ORs are known to also couple to the promiscuous Gα15/16, an isoform of Gαq, to activate the PLC signaling pathway [20, 32]. With few exceptions [16, 33], these previous studies typically used fixed end point assays that rank ligands by their overall ability to activate a particular pathway [34, 35] and did not consider other response characteristics that may reflect ligand-GPCR and GPCR-G-protein interactions such as the degree of receptor activation, cellular amplification of the signal by other pathway components, and the transitory nature of the interactions that may affect the overall potency of the ligand. To circumvent some of these limitations, we adapted insight from previous studies of non-OR GPCRs where the kinetics of their Ca²⁺ responses was used to explore various parameters of ligand-receptor interactions [36]. A similar approach has been used to show that the parathyroid hormone receptor couples to both Gαq and Gas based on the PLC-dependent response preceding the AC-dependent response [37-39], suggesting its potential general utility.

We assume the faster activation of the mOR-EG/PLC response reflects the fast, highly cooperative opening of IP₃-gated Ca²⁺ channels mediating Ca²⁺ release from intracellular stores [40]. In contrast, we assume the significantly slower activation of the mOR-EG/AC response (Fig. 2, Table 1) reflects the on-rate of the response, which as shown for other Gas-dependent GPCRs is determined by the rate of activation of AC and in general is a much slower, more linear process [41]. Support for this assumption comes from our finding that the differential kinetics are consistent for the same receptor expressed in two different cell lines, HEK293T and MMDD1, despite the differences in cellular morphology and endogenous signaling elements, suggesting that the dynamic differences in the Ca²⁺ signal patterns are inherent characteristics of these pathways. Together with our finding that the differences in the Ca²⁺ signal dynamics persists across different ligands and concentrations, these results suggest that this is a reliable system for studying ligand-receptor interactions associated with selective downstream signaling.

It is possible that the observed differences in response kinetics could also arise from different levels of cell surface expression of GPCRs, preventing the direct comparison of signaling by different receptors. This is particularly relevant for ORs which in general show highly variable levels of surface expression. However, we found that applying eugenol to mOR-EG/Gα15-expressing cells generates a coherent, fast Ca²⁺ response with a Trise and Tpeak of 7.6±0.2s and 15.4±0.4s (n=161), respectively, that closely resembles that evoked by pilocarpine stimulation of M3 AChR-expressing cells (Trise and Tpeak of 6.8±0.6s and 13.9±1.0s (n=87), respectively. The consistency in the Ca²⁺ responses observed for these two different GPCRs, which likely have different levels of surface expression in HEK293T cells, indicates that receptor density is probably not the prime determinate of the response kinetics.

Although “ligand-induced selective signaling” could imply that a given ligand will cause a receptor to adopt a single conformation and activate one particular downstream signaling

pathway, it is more commonly held that most ligands probably do not activate one pathway to the exclusion of all others but, instead, shift the balance towards one outcome, thus “biasing” the signaling [42]. Four of the ligands tested (isoeugenol, mouse crystal, 4-ethylguaiacol and 4-vinylguaiacol) fit this model in activating both pathways, but with bias towards one (the PLC-dependent pathway). This preferential activation of the PLC-dependent pathway persisted when both readouts were measured in the same cell, suggesting the bias was not imposed by testing the two readouts independently. Interestingly, the remaining seven active ligands, including eugenol, vanillin, and several other known strong agonists [19, 21, 43] also activated both the mOR-EG/PLC and mOR-EG/AC responses, but did so without apparent bias. It is unclear if these ligands also acted with bias but saturated the readout so the difference in signal amplitude was not measurable, or they had effectively equal ‘bias’. Further experimentation is required to address this distinction. We conclude that at least some reported mOR-EG ligands can activate $G\alpha_{15}$ - and $G\alpha_{s(olf)}$ -coupled signaling in a ligand-induced selective manner when mOR-EG is heterologously expressed.

Question arises as to why some previously reported active mOR-EG ligands were without effect, or had different effects from those in our study. Methyl isoeugenol, for example, originally was reported to be an inactive ligand that potently blocks the effect of eugenol [27]. We observed the same agonistic and antagonistic properties of methyl isoeugenol when applied to the heterologously expressed mOR-EG for both the mOR-EG/PLC and mOR-EG/AC responses. However, a recent study using a cAMP-dependent Cre reporter assay identified methyl isoeugenol as a strong agonist [22]. One major difference between the two assay formats is the duration of ligand application. In our case methyl isoeugenol was applied for a relatively short period of time, 5-10 sec, considerably shorter than the response duration and therefore may represent non-equilibrium binding of the ligand to the receptor. The Cre reporter assay requires incubation times of at least 15 min and presumably reflects equilibrium ligand binding, which could account for the different outcome when measured at the level of reporter gene expression [34]. However, in our $G\alpha_{s(olf)}$ /AC assay we were not able to detect any significant activation under a similar prolonged stimulation with methyl isoeugenol. Noteworthy is that four other mOR-EG ligands identified as agonists in the same study [22] failed to evoke any activity in our assay (Table 1, green). Such differences emphasize the limitations of using heterologous systems to study receptor function and the importance of at least comparing ligands of interest in the same system using a common type of readout, as done here.

Although the mechanism of ligand-induced selective signaling by mOR-EG is not explored here, it was previously demonstrated that the C-terminus of the receptor is involved in regulating $G\alpha_{15}$ vs $G\alpha_{s(olf)}$ interaction and specificity [44, 45]. Additionally, site-directed mutagenesis identified residues within intracellular loop 3 (IC3) that are critical for $G\alpha_{15}$ and $G\alpha_{s(olf)}$ activation by eugenol. While some mutations in IC3 had similar effects on the ability of mOR-EG to activate both $G\alpha_{15}$ and $G\alpha_{s(olf)}$, others at residues I222, L227, S231 and G234 had differential effects on the two pathways [45], indicating that coupling of mOR-EG to $G\alpha_{s(olf)}$ and other G-proteins is mechanistically different and could be influenced by conformational differences of the receptor caused by binding to structurally distinct ligands. Such a ‘multi-state’ model with distinct ligand-specific receptor

conformations resulting in activation of different downstream signaling pathways has been proposed for several non-OR GPCRs [46, 47].

In summary, while we show that mOR-EG can activate Gα15 and Gαs(olf)-coupled signaling pathways in a ligand-induced selective manner when heterologously expressed, this does not necessarily reflect the behavior of the OR in native ORNs. The challenge remains to directly demonstrate that mOR-EG can selectively activate a signaling pathway other than the conventional Gαolf induced activation of AC in native conditions. However, since many ORs have the capacity to activate PI3K signaling in native ORNs [48], we suggest that ligand-induced selective signaling may be an inherent property of mammalian ORs worthy of further exploration.

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Highlights

- Mammalian olfactory receptor
- Dual signaling calcium readout
- Ligand-induced selective signaling

Figure 1.

Chemical structures of mOR-EG ligands used in the study, including eugenol, some eugenol derivatives, and the structurally dissimilar compounds mousse cristal, nootkatone, and orivone. Some of the ligands failed to give a measurable response (green). Some ligands activated both PLC- and AC-mediated outputs, seven of them equally (black) and four preferentially favoring the PLC-dependent output (blue). Methyl isoeugenol and isosafrole are known antagonists (red).

Figure 2.

Activation of the mouse olfactory receptor mOR-EG transiently co-expressed either with $G_{\alpha 15}$ or $G_{\alpha olf}$ proteins. Note the cognate agonist eugenol ($100\mu\text{M}$) activates both the $G_{\alpha 15}$ -dependent (red trace) and $G_{\alpha s(olf)}$ -dependent (blue) metabotropic signaling pathways. For comparison, similar expression of the heteromeric mosquito *A. gambiae* OR (AgOR65+AgOrco) resulted in a much faster response to the cognate agonist eugenol ($1\mu\text{M}$, light grey trace) reflecting direct Ca^{2+} influx through the ionotropic OR. Traces represent normalized averaged response to a 5-s stimulation recorded in a single experiment from 20-30 different cells. Stimulus was always triggered at 14.7 s time point. Three parameters of the response were measured: time of peak (T_{peak} , a time of peak since stimulus onset), rise time (10% to 90%, T_{rise}), and decay time (90% to 10%, T_{decay}).

Figure 3.

Pharmacological properties of the heterologous expression system. (A) Removal of extracellular Ca^{2+} (step profile under the traces) decreased resting intracellular Ca^{2+} otherwise not affecting the Ca^{2+} response to a 5-s pulse (vertical bar) of 1mM eugenol in HEK293T cells expressing mOR-EG/G α 15. (B) Blocking PLC with U73122 (10 μM) suppressed the response in mOR-EG/G α 15 cells. (C) Removing extracellular Ca^{2+} (step profile under the traces) completely abolished the response to 1mM eugenol in mOR-EG/G α s(olf) cells. (D) The adenylate cyclase activator forskolin (10 μM , 5 sec pulse, vertical

bar) increased the Ca^{2+} concentration in mOR-EG/G α s(olf) cells. (E, F) Comparison of the kinetics of Ca^{2+} influx evoked by application of forskolin (E, vertical bar) and by replacement of extracellular Ca^{2+} from virtual 0 to 2mM indicated by a step profile (F). Forskolin was applied to 0- Ca^{2+} solution prior to application of 2mM Ca^{2+} (F). The difference in the kinetics suggests that the CNGC-camp ion channel does not impose any dynamic constraints on measuring the level of cAMP.

Figure 4.

Concentration-dependence of the response to eugenol measured in HEK293T cells expressing either mOR-EG/Gα15 (A, red symbols) or mOR-EG/Gαs(olf) (B, blue symbols). Left panels - examples of Ca^{2+} signals recorded from HEK293T cells in a single experiment. Each data point is the mean $\Delta F/F \pm \text{SD}$ (grey bars) of fluorescence intensity of individual cells. Middle panels - concentration dependence of the kinetic parameters of the response. Light grey symbols and scales correspond to T_{peak} , dark grey symbols and scales – T_{rise} , and black – T_{decay} (see Fig.2. for parameters definition). Values represent mean $\pm$ SEM. Right panels – concentration dependence of the response peak amplitude. Grey circles and lines show concentration dependence for individual cells. Large color symbols represent average values. All values were normalized to the maximum response activated by 1mM eugenol in individual cells. Solid smooth lines show best fit to the Hill equation with $\text{EC}_{50} = 55 \pm 38$ and 53 ± 11 μM for HEK293T cells expressing mOR-EG/Gα15 (red) and mOR-EG/Gαs(olf) (blue), respectively.

Figure 5.

Isoeugenol and mousse cristal as examples of biased mOR-EG ligands. (A) Isoeugenol represents a group of compounds that evoke substantially reduced $G_{\alpha s(olf)}$ -dependent responses as compared to $G_{\alpha 15}$ -dependent responses. (B) Mousse cristal appears to be a full agonist for mOR-EG, activating both $G_{\alpha 15}$ - and $G_{\alpha s(olf)}$ -dependent response even stronger than eugenol yet with a different apparent affinity. The response to mousse cristal is also characterized by a different kinetics. Apparently, the $G_{\alpha 15}$ -dependent response to mousse cristal was considerably slower than that to eugenol. Left panels (A,B) show averaged Ca^{2+} signals recorded in a single experiment. Each data point is the mean $F/F \pm SD$ (grey bars)

of fluorescence intensity of individual mOR-EG/Gα15 cells (red) and mOR-EG/Gαs(olf) cells (blue). Middle panels (A,B) show concentration dependence of parameters of the response kinetics for the respective ligand. Light grey symbols and scales correspond to Tpeak, dark grey symbols and scales – Trise, and black – Tdecay (see Fig.2 for parameters definition). Values are mean ± SEM. Right panels (A, B) show concentration dependence of the response amplitude for the respective ligand. Dose response of individual cells is shown in grey. Color symbols represent averaged values. All values were normalized to the response maximum activated by 1mM eugenol in individual cells. Solid smooth lines are the best fit to the Hill equation (see Table 1 for the respective parameters).

Figure 6.

Methyl isoeugenol acts as an antagonist on mOR-EG independent of the heterologous system. (A) Even at the highest concentration of 1 mM methyl isoeugenol did not evoke any appreciable Ca^{2+} signal in $\text{G}\alpha 15$ (red) or $\text{G}\alpha s(\text{olf})$ -expressing cells (blue). (B) Adding 1 mM methyl isoeugenol (MIEG) completely eliminated Ca^{2+} response evoked by 100 μM eugenol. Control response to 1 mM eugenol is shown for reference (shaded box). At least three independent experiments of this kind were performed. Each data point is the mean $F/F \pm \text{SD}$ (grey bars) of fluorescence intensity of individual mOR-EG/ $\text{G}\alpha 15$ cells (red) and mOR-EG/ $\text{G}\alpha s(\text{olf})$ cells (blue).

Figure 7.

Concentration response profiles of the mOR-EG ligands supporting the ligand-induced selective signaling of the OR. Data of the mOR-EG/ $\text{G}\alpha 15$ and mOR-EG/ $\text{G}\alpha s(\text{olf})$ assay is shown in red and blue, respectively. Ligands are grouped according to their ability to differentially signal through the mOR-EG/ $\text{G}\alpha 15$ and mOR-EG/ $\text{G}\alpha s(\text{olf})$ pathways. (A) Ligands in this group activate both pathways with a similar affinity albeit their different efficacy. (B) Three ligands in this group show substantial selectivity towards activation of the mOR-EG/ $\text{G}\alpha 15$ pathway, and (C) mousse cristal is a unique ligand strongly activating

both pathways yet with a different apparent affinity. Ligand concentration dependences were generated after normalization of the data to the maximal response evoked by 1mM Eugenol. Each data point is a mean $F/F_0 \pm \text{SEM}$ measured in 28-554 cells in 2-9 independent experiments. Solid lines show the best fit to the Hill equation (with 1.0 set as the maximum function constraint in a few cases e.g. vanillin, where response saturation could not be reached due to the solubility limit). (D) Pair-wise comparison plot of the EC50 values calculated for each ligand in mOR-EG/G α 15 (PLC based) and mOR-EG/G α s(olf) cells (AC based). Proximity of ligands close to diagonal suggests their little if any functional selectivity. A number of ligands however appear to preferentially activate the G α 15-dependent pathway resulting in a shift off the diagonal (grey symbols).

Figure 8.

Ligand binding to the mOREG activates both signaling pathways simultaneously in the same cell in a ligand-induced selective manner. HEK293T cells were co-transfected with mOR-EG, Ga15, Gas(olf) and CNGC-camp. Representative recordings of each type of the response measured in nine different cells are shown in each panel. Application of isoeugenol (left, open panel) and eugenol (right, shaded panel) evoked in the majority of cells either (A) Ga15- or (B) Gas(olf)-dependent response. (C) However, a small but significant fraction of cells generated a biphasic response with the initial fast rising phase associated with the

Gα15-dependent pathway followed by a slower Gαs(olf)-dependent Ca²⁺ signal. The cell response patterns are consistent throughout the range of concentrations. Notably even saturating concentration of a putative selective mOR-EG ligand isoeugenol failed to evoke noticeable Gαs(olf)-dependent Ca²⁺ signal. The response of each cell was normalized to the respective maximum F/F.

ligand	solubility (pH7, mmol/l)/logP	AC-/PLC- mediated Ca^{2+} response, EC_{50} , μM	time of peak, s, AC-/PLC- dependent Ca^{2+} response	time to rise 10% to 90%, s, AC-/PLC- dependent Ca^{2+} response	decay time 90% to 10%, s, AC-/PLC- dependent Ca^{2+} response
eugenol	11/2.4	55±38/53.3±11	90.5±4.4/15.4±0.4	39.4±2.2/7.6±0.2	128.2±4.9/52.6±1.1
methyl eugenol	4.3/2.7	177±11/165.3±7.2	92±3/19.8±1.2	41.6±2.2/5.4±0.6	101.2±6/39.8±2.3
vanillin	28/1.2	162±68/218.1±27.1	85.3±2.9/18.6±0.9	31.4±1.3/8.3±0.5	101.7±2.6/48±2.9
ethyl vanillin	11/1.7	2165±131/1487±716	91.6±5.8/22.6±1.5	36.9±3.3/9.5±0.8	115.7±10.8/52.3±4.8
acetyl eugenol	1.4/2.7	373±49/348±25	90.8±3.4/19.6±0.9	27.6±1.7/7.7±0.4	125.5±5.1/44±1
orivone	2.4/3.1	4600±535/4370±471	94.9±5.3/29.8±2.3	36.4±2.6/8±1	101±5/47.6±4.1
methyl isoeugenol	3.6/3.05	non agonist, antagonist			
isosaifrole	0.6/3.9	non agonist, antagonist (*)			
isoeugenol	7.3/3.08	286±40.3/55.4±4.8	95.3±4.8/37.1±3.5	36.7±2.7/14.8±1.6	104±4.8/54.5±2.6
4-propylguaiaicol	6/2.9	205±15/174±88	95.8±3.9/23±1	46±3/5.7±0.4	128.2±8.5/36.3±2.8
4-ethylguaiaicol	14/2.4	1057±861/66±44	79.3±2.3/22.1±0.8	31.5±1.7/6.1±0.3	101.6±6.7/71.3±4.9
4-vinylguaiaicol	15/2.6	211±226/56.2±4.9	85.1±4.3/21.5±0.8	34.2±2.1/5.6±0.4	113±102/49.7±4.2
mousse cristal	10/2.8	551±22/253±81	107.3±2.4/30.7±1.4	32.6±1.6/14±1	117.6±5.3/74±4
nookatone	0.24/3.8	no effects			
raspberryketone	31/1.3	no effects			
4-isopropylphenol	15/3	no effects			
4-tert-butylphenol	6.3/3.4	no effects			