cAMP and IP3 Signaling Pathways in HEK293 Cells Transfected with Canine Olfactory Receptor Genes

NAÏMA BENBERNOU, STÉPHANIE ROBIN, SANDRINE TACHER, MAUD RIMBAULT, MICHAÈLLE RAKOTOMANGA, AND FRANCIS GALIBERT

From the Institute of Genetic and Development of Rennes, CNRS Unité de Recherche Mixte 6061, Faculté de Médecine, Rennes, F-35043 France. Sandrine Tacher is now at the Systèmes d’Elevage Nutrition Animale et Humaine, Institut National de la Recherche Agronomique, Unité de Recherche Mixte 1079, Domaine de la Prise, 35590 Saint Gilles, France.

Address correspondence to F. Galibert at the address above, or e-mail: francis.galibert@univ-rennes1.fr.

Abstract

Olfactory receptors (ORs) expressed at the cell surface of olfactory sensory neurons lining the olfactory epithelium are the first actors of events leading to odor perception and recognition. As for other mammalian ORs, few dog OR have been deorphanized, mainly because of the absence of good methodology and the difficulties encountered to express ORs at the cell surface. Within this work, our aim was 1) to deorphanize a large subset of dog OR and 2) to compare the implication of the 2 main pathways, namely the cAMP and inositol 1,4,5-triphosphate (IP3) pathways, in the transduction of the olfactory message. For this, we used 2 independent tests to assess the importance of each of these 2 pathways and analyzed the responses of 47 canine family 6 ORs to a number of aliphatic compounds. We found these ORs globally capable of inducing intracellular calcium elevation through the IP3 pathway as confirmed by the use of specific inhibitors and/or a cAMP increase in response to aldehyde exposure. We showed that the implication of the cAMP or/and IP3 pathway was dependent upon the ligand–receptor combination rather than on one or the other partner. Finally, by exposing OR-expressing cells to the 21 possible pairs of C6–C12 aliphatic aldehydes, we confirmed that some odorant pairs may have an inhibitory or additive effect. Altogether, these results reinforce the notion that odorant receptor subfamilies may constitute functional units and call for a more systematic use of 2 complementary tests interrogating the cAMP and IP3 pathways when deorphanizing ORs.

Key words: G protein, ligands, olfactory receptors, signal transduction

Olfactory receptors (ORs) constitute one of the largest group of G protein–coupled receptor (GPCR) families described to date. They play a key role in the recognition of thousands of odorant molecules by olfactory sensory neurons (OSNs) in the nasal olfactory epithelium (Buck and Axel 1991). In response to odorants, OSNs transmit signals to the brain, resulting in odor perception. Mammals have between 500 and 1500 different odorant receptors, which are highly polymorphic (Sharon et al. 2000; Young and Trask 2002; Zhang and Firestein 2002; Godfrey et al. 2004; Quignon et al. 2005; Robin et al. 2009). These ORs use a combinatorial code to detect several thousands of different odorants (Malnic et al. 1999; Gaillard et al. 2002). Mammalian ORs, like those of the frog (Xenopus laevis), can be assigned to 2 classes on the basis of phylogeny (Glusman et al. 2001; Zozulya et al. 2001; Zhang and Firestein 2002). Class I ORs resemble those found in fish and are found in the lateral diverticulum of the nose in frogs. They are thought to detect water-soluble odorants. Class II ORs are expressed in the sensory neurons of the main diverticulum and are thought to be responsible for detecting volatile compounds (Freitag et al. 1995). Five class I families and 18 class II families, representing a total of 300 subfamilies of ORs, have been identified in the dog OR repertoire made of 1094 genes and 20.3% of pseudogenes (Quignon et al. 2005).

The binding of odorants to ORs triggers the production of cAMP. OSNs express a G protein with a specific α subunit named Goαolf (Belluscio et al. 1998). This α subunit activates an olfaction-specific adenylate cyclase following the binding of an agonist to the OR. The resulting increase in cAMP concentration leads to the opening of channels, mostly permitting the entry of Ca2+, depolarizing the neuron membrane. This depolarization amplified by a Ca2+...
activated Cl\textsuperscript{−} current generates an action potential. The adenyl cyclase (AC)/cAMP pathway is essential for olfactory responses in vertebrates (Sklar et al. 1986; Brunet et al. 1996; Nakamura 2000; Wong et al. 2000). However, odorants activate more than one transduction cascade; indeed, IP\textsubscript{3} has also been shown to be an efficient second messenger (Restrepo et al. 1990; Bruch 1996; Spehr et al. 2002). More specifically, in rat cilia preparations, cAMP was shown to increase in response to citralva (3,7-dimethyl-2,6-octadiene-nitrile), a component with a citrus-like odor, but not in response to pyrazine. Pyrazine instead induced an increase in IP\textsubscript{3} (Boekhoff et al. 1990). Similar results have been obtained for many other odorants in cilia preparations and in OSNs (Breer et al. 1990; Ronnett et al. 1993; Bruch 1996). Individual OSNs express adenylate cyclase III and the phospholipase C beta-2, which are needed for the activation of the cAMP and IP\textsubscript{3} pathways, respectively (Noé and Breer 1998). Recent studies, however, have provided evidence for a novel class of sensory cells in the mouse olfactory epithelium (Elsaesser et al. 2005), which do not express adenyl cyclase III or cyclic nucleotide-gated cation channels, but do express phospholipase C beta-2 (PLC\textsubscript{B2}). Cells in which only the IP\textsubscript{3} pathway is activated make up 5% of all olfactory cells and respond to a variety of odorants, as observed by calcium imaging (Elsaesser et al. 2005).

Besides their importance in the detection of odorants and the fact that they have been discovered nearly 20 years ago, only few OR ligand pairs have been identified and most ORs remain orphan receptors. Even more, although one knows that individual OR can recognize many different compounds, often only a couple of them has been attributed to a given OR. This is due to technical difficulties and the absence of high-throughput methods.

Several in vitro tests have been developed to deorphanize ORs. Basically, they interrogate the increase of intracellular Ca\textsuperscript{2+} or cAMP resulting from the specific binding of an odorant onto an OR expressed by a transiently transfected cell. Although in vivo it was observed that different compounds could activate either the cAMP or the IP\textsubscript{3} pathways or both (Restrepo et al. 1990; Bruch 1996; Brunet et al. 1996; Nakamura 2000; Wong et al. 2000; Spehr et al. 2002), the correlation with the in vitro tests performed in heterologous cell system is not totally clear (Katada et al. 2003; Shirokova et al. 2005; Ko and Park 2006; Kato et al. 2008).

Additionally, results described in the literature are related to tests measuring either the increase in Ca\textsuperscript{2+} or the cAMP and rarely compared the results of these 2 tests with an identical set of ORs exposed to the same series of compounds (Katada et al. 2003; Shirokova et al. 2005; Ko and Park 2006; Kato et al. 2008). Additionally, most of these studies have been performed with human, rat, or mouse ORs and very few with dog ORs.

In this study, we utilized an in vitro heterologous system to explore both cAMP and IP\textsubscript{3} signal transduction pathways coupled to activated OR transiently expressed in 1E6 cells (HEK293 cells expressing the G\textalpha\textsubscript{olf} subunit). We expressed and screened 69 dog OR genes of class I and class II from different families including 47 family 6 ORs and tested the ability of 37 odorants (C6–C12 aldehydes, ketones, esters, fatty acids, and alcohols) to induce a Ca\textsuperscript{2+} or cAMP intracellular increase in 1E6 cells and compared the results with those previously obtained (Benbernou et al. 2007). Our results demonstrate first that dog family 6 OR are specific to aliphatic aldehydes and second that the implication of the cAMP and IP\textsubscript{3} pathway is dependent upon the ligand–receptor combination rather than on one partner or the other. Inversely, dog family 52 ORs were shown to specifically recognize aliphatic ketones through the IP\textsubscript{3} pathway only.

We also systematically tested the 21 possible pairs of C6–C12 aliphatic aldehydes with 47 dog family 6 ORs and confirmed that some odorants may exert antagonist effects when applied in combination with certain other odorants (Spehr et al. 2003; Oka, Nakamura, et al. 2004; Oka, Omura, et al. 2004; Sanz et al. 2005; Shirokova et al. 2005). We also showed that an odorant, which does not exert any agonist effect when applied alone, could increase the response of an agonist when applied in combination.

**Materials and Methods**

**OR Gene Cloning**

A genomic DNA sample, prepared from mongrel dog hepatocytes, was used as the starting material for cloning OR genes in frame with a leader peptide sequence derived from the influenza virus haemaglutinin (Gaillard et al. 2002) and a c-Myc epitope sequence in the pIRES plasmid vector (Clontech, Mountain View, CA). This epitope was used to assess the OR cell surface expression by immunocytochemistry with a c-Myc antibody (Benbernou et al. 2007). The liver dog sample was obtained from a dog euthanized for medical reasons by a veterinarian practitioner with the consent of the dog owner.

PCR conditions, cloning, and sequencing methods were as previously described (Benbernou et al. 2007). The sequences of all OR clones are available in GenBank (accession numbers JF33471–JF33539).

**Chemicals**

All the chemicals used in functional assays were purchased from Sigma/Aldrich (Saint Quentin Fallavier, France). Odorant stock solutions were prepared in dimethyl sulfoxide (DMSO) immediately before each experiment. Solutions were then serially diluted in phosphate-buffered saline (PBS) (final DMSO concentration was <1/500).

**Cell Culture**

Adherent HEK293 cells were cultivated as previously described (Benbernou et al. 2007). We maintained 1E6 cells, a subclone of HEK293 cells expressing the human G\textalpha\textsubscript{olf} subunit, in culture medium containing G418 sulfate (Sigma) at a concentration of 800 \mu g/ml. Cells were cultured...
at 37 °C, under an atmosphere containing 5% CO2. Cells were discarded after 7 passages and new cultures were prepared from a frozen cell stock.

HEK293 cell line was obtained from ATCC (number: CRL-1573).

The 1E6 cell clone was constructed with GMO authorization no. 12576 issued March 2003 by the genetic recombinant committee of the Ministry for Education and Research.

**OR Functional Assays**

Functional assays were carried out in poly-L-lysine–coated 96-well plates (BioCoat, BD Bioscience). We seeded each well with 7500 cells 24 h before transfection.

For the Ca\(^{2+}\) assay, we used Fugene 6 (Roche, France) to transfect cells with 100 ng of a plasmid containing the entire coding ORF of an OR gene. About 48 h after transfection, cells were loaded with Fluo-4 for 30 min and stimulated with odorant. We performed Ca\(^{2+}\) imaging as previously described (Benbernou et al. 2007). Plates were placed on an automated stage of a Leica DMIRB microscope equipped with Metamorph software. Odorant solutions were then added and images acquired every 3 s for 90 s. The Ca\(^{2+}\) signal was expressed as \(\Delta F/F = ([F - F_0]/F_0)\), corresponding to the difference in fluorescence intensity at each time point after stimulation (\(F\)) with respect to the fluorescence intensity before stimulation (\(F_0\)), in a microscopic field of 140 × 113 \(\mu\)m. As shown in Figure 1, maximum responses are observed around 30 s following the addition of odorant.

For the cAMP secreted alkaline phosphatase (SEAP) assay, cells from each well were cotransfected with 100 ng each of an OR-encoding plasmid and a pCRE-SEAP plasmid (Clontech). Cells were incubated in medium alone for 48 h at 37 °C and then with 500 \(\mu\)M 3-isobutyl-1-methylxanthine (IBMX) (Sigma Aldrich) for 30 min to inhibit endogenous phosphodiesterase activity. Cells were then exposed to odorants. After an incubation period of 18 h during which cAMP accumulates and fosters SEAP synthesis, SEAP activity was measured in an aliquot of culture supernatant from each well, following the kit manufacturer's instructions (Anaspec). A Fluoroscan plate reader was used to measure the fluorescence (LabSystems).

For the cAMP luciferase assay, cells were cotransfected with 100 ng of a plasmid encoding OR, 50 ng of pCRE-luc plasmid (Stratagene, France), and 1 ng of pRL-SV40 control plasmid (Promega, France). At 48 h post-transfection, cells were incubated with IBMX for 30 min and then exposed to various concentrations of odorants. Both firefly and Renilla luciferase were measured after 5 h incubation at 37 °C, using Dual Glo luciferase reagent following the manufacturer’s instructions (Promega, France) and a Centro XS luminometer (Berthold, France). Data were normalized to Renilla activity levels by dividing the value obtained for firefly luciferase by the Renilla luciferase value. Data were expressed as the ratio odorant/DMSO control for each OR. All experiments were carried out at least 4 times, with triplicate measurements taken for each time-point.

**Results**

**Aldehydes Induced cAMP Responses**

ORs have no identified translocation sequence at their N-termini and are difficult to express at the plasma membrane in heterologous cell systems. We thus transfected 1E6 cells (a HEK293 clone expressing G\(_a\)olf) with expression vectors encoding an individual canine OR with a peptide leader sequence added to the N-terminal end to facilitate OR expression at the cell surface (Gaillard et al. 2002; Benbernou et al. 2007). Phylogenetic and synteny comparison analyses indicated that the canine OR gene CfOR12A07 is the ortholog of the rat OR gene RnI7, which has been the subject of many studies using octanal as ligand (Krautwurst et al. 1998; Zhao et al. 1998).

CfOR12A07 belongs to canine ORs family 6, a class II family made up of 40 subfamilies and containing 88 genes and 46 pseudogenes (Quignon et al. 2005). As shown in Figure 2A, the membrane expression of these 2 ORs and of 2 additional ORs, used as controls, induced a cAMP response with the
Figure 2. Aldehyde cAMP responses of cells transfected with dog family 6 OR genes. 1E6 cells were cotransfected with expression vectors encoding OR and CRE-SEAP. SEAP activity was assayed following octanal exposure. (A) The responses obtained with CfOR12A07 and 3 other ORs used as controls are displayed. Results were expressed as the ratios of responses in the presence of odorants versus DMSO alone. (B) shows the responses obtained with 4 ORs out of 21 dog family 6 ORs responding to octanal. * indicates a statistically significant increase in the presence of odorant as compared to DMSO alone ($P < 0.001$ using the Student's t-test).
SEAP assay when they are elicited by their respective ligands, validating the use of this expression plasmid as well as the assay.

We selected 47 canine OR genes from various sub-families, representative of family 6 and assessed the increase in cAMP levels resulting from the recognition of octanal by OR-expressing cells. To this end, we transiently cotransfected 1E6 cells with an OR-encoding plasmid and a plasmid containing the cAMP CRE-SEAP reporter gene (Durocher et al. 2000; Liberles and Buck 2006). The cAMP reporter gene CRE-SEAP expresses SEAP in response to cAMP owing to its cAMP response elements (CREs). We exposed cells to octanal 48 h after transfection and then assayed for SEAP activity using the fluorescent SEAP substrate, FDP. Stimulation of CfOR12A07-transfected 1E6 cells with 500 μM octanal led to an increase in cAMP concentration, whereas no such increase was observed in nontransfected control 1E6 cells (Figure 2).

Among the 47 dog ORs tested, the strongest responses to octanal were obtained with CfOR12A07, CfOR0046, CfOR0250, CfOR0012, and CfOR5269 (Figure 2B). Intermediate responses were observed for 16 other ORs (Supplementary Figure S1). There were 26 ORs of the 47 selected that did not respond to octanal.

We then investigated responsiveness of these 47 ORs to a panel of 7 aliphatic aldehydes (C6–C12) and DMSO, as a negative control. The phylogenetic tree shown in Supplementary Figure S1 shows the 24 ORs that gave a positive response, of varying intensity, to anywhere from 1 to 7 of these C6–C12 aliphatic aldehydes in the cAMP SEAP assay.

Inhibition of SEAP Activity by Inhibitors of Adenylate Cyclase

We checked that SEAP activity was completely inhibited by ddA and SQ22536 at concentrations of 250 and 200 μM, respectively. Both of these compounds are known to inhibit the adenylate cyclase (Morita et al. 1986; Ishihara et al. 1989). Results using CfOR12A07 and CfOR0012, for example, are presented in Figure 3. They show that the observed fluorescence increase is indeed dependent on an increase in intracellular cAMP concentration and is abolished by the concomitant addition of adenylate cyclase inhibitors.

Aldehyde-Induced Calcium Responses Involve the Inositol-1,4,5-Triphosphate Pathway and Require Extracellular Ca2+

In a previous paper (Benbernou et al. 2007), we reported the binding properties toward aliphatic aldehydes of 36 of these 47 OR, not with the cAMP assay as above but with a Ca2+ assay to assess intracellular changes in Ca2+ using the fluorogenic compound, Fluo4. We have now extended this study to these 47 ORs, using the Ca2+ assay. But more importantly we also show that 3 μM xestospongin C, an IP3R antagonist (Gafni et al. 1997), blocked the octanal-stimulated intracellular Ca2+ signal (87% inhibition). See, for example, CfOR12A07 in Figure 4A. Moreover, prior incubation of the cells with SQ22536, an adenylate cyclase inhibitor, did not decrease the intensity of the Ca2+ responses (Figure 4A). Thus, utilization of these 2 inhibitors within the course of the Ca2+ test clearly demonstrated that the Ca2+ test interrogates the IP3 pathway and not the cAMP pathway.

We then incubated cells in PBS without Ca2+ for Fluo-4 loading and during odorant exposure and showed that the IP3 implication in the CfOR12A07 responses was inhibited by the absence of external Ca2+ (87.5% inhibition with 0.01 μM octanal and 75% inhibition with 1 μM octanal) (Figure 4B).

Our findings demonstrate that the increase in intracellular Ca2+ concentration, following the stimulation of family 6 ORs is dependent on the entry of extracellular Ca2+.

The cAMP and Calcium Assays Give Different Answers

Twenty-four of the 45 ORs that gave positive responses in the Ca2+ assay with at least one aldehyde (Benbernou et al. 2007), also gave positive responses in the cAMP assay (Supplementary Figure S1). Careful inspection of the data demonstrated that the patterns of responses in the 2 tests
are different. For example, CfOR12A07 displayed a positive cAMP response to heptanal, octanal, nonanal, decanal, and undecanal but a negative cAMP response to hexanal, heptanal, and undecanal in the Ca\(^{2+}\) assay despite the strong response observed with the 2 last odorants in the cAMP assay (Figure 5A). This same OR showed positive responses to octanal, nonanal, decanal, and dodecanal but a negative response for hexanal, heptanal, and undecanal in the Ca\(^{2+}\) assay despite the strong response observed with the 2 last odorants in the cAMP assay (Figure 5A). Different profiles in the cAMP SEAP and Ca\(^{2+}\) assays were also observed for other ORs. Examples are CfOR0012 (Figure 5B), CfOR0250 (Figure 5C), CfOR0046 (Figure 5D), CfOR5269 (Figure 5E), and CfOR3548 (Figure 5F).

Finally, 21 ORs, which gave a positive response for at least one compound with the Ca\(^{2+}\) assay (Benbernou et al. 2007), were negative with the cAMP SEAP assay, whereas 2 ORs gave negative results for both assays (this study). A summary of the responses observed for the ORs in the Ca\(^{2+}\) and cAMP SEAP assays are given in Table 1.

There are 2 main differences in the setting of the 2 tests: one corresponds to the odorant concentration and the second to the time elapsed between the odorant exposure and the observed response. Odorant concentration reported in the literature for the Ca\(^{2+}\) assay goes from 10\(^{-10}\) M to 1 mM, whereas for the cAMP assay, they go from 10 \(\mu\)M to 1 mM (Levasseur et al. 2003; Katada et al. 2003, 2004; Shirokova et al. 2005; Ko and Park 2006; Kato et al. 2008; Saito et al. 2009). In our studies, we used 10\(^{-11}\) M to 1 \(\mu\)M for the Ca\(^{2+}\) assay and 500 \(\mu\)M for the cAMP assay.

The timing is also very different, in the case of the Ca\(^{2+}\) test, the increase in fluorescence is recorded between 10 and 90 s postexposure, whereas in the cAMP test, the fluorescence or luminescence are recorded 6–18 h after exposure to the odorant. However, one should point to a big conceptual difference in the 2 tests. In the Ca\(^{2+}\) test, the fluorescence observed is directly correlated to the intracellular Ca\(^{2+}\) increase, whereas in the cAMP test, the recorded fluorescence or luminescence are an indirect measurement of a cAMP increase as this later is used to induce the synthesis of either SEAP or luciferase reporter genes.

Given these dramatic differences in the setting parameters of the 2 tests, it is not possible to be certain whether the response differences are real or merely reflect technical reasons. Nevertheless, it is known from in vivo analysis that OR exposed to different odorant can elicit a response via the cAMP and IP3 pathway or one of the other only; thus, the differences observed in vitro might be real. In any cases, we believe the 2 tests should be used in parallel in experiments aimed at deorphanizing OR and not only one or the other.

G\(_\text{olf}\) Is Required for the Increase in cAMP Levels but not for the Ca\(^{2+}\) Response

We compared responses to octanal in HEK293 cells and 1E6 cells expressing CfOR12A07 (Figure 6). The 2 cell lines displayed similar Ca\(^{2+}\) responses (Figure 6A). However, we observed a large increase in cAMP levels upon stimulation with odorant in the G\(_\text{olf}\)-expressing 1E6 cell line only (Figure 6B). Similar results were obtained for all family 6 ORs displaying a positive response to octanal (data not shown).

Thus, OR responses mediated by the cAMP signaling pathway required the expression of the G\(_\text{olf}\) subunit, as expressed in the 1E6 cell line, whereas OR responses resulting in increased Ca\(^{2+}\) levels might involve another G protein in HEK293, which do not express the G\(_\text{olf}\) subunit. This is in accordance with previous reports showing that the Gq and the promiscuous Gz15 and Gz16 are involved in Ca\(^{2+}\) assay (Shirokova et al. 2005; Oka et al. 2006).
Inhibitory and Cooperative Effects of Aldehydes on Family 6 ORs

We then investigated whether the aliphatic aldehydes (C6–C12) tested had inhibitory or synergistic properties by exposing each of the 47 dog OR to the 21 different pairs of odorants. We observed inhibitory effects for various OR–odorant combinations (Supplementary Table 1). In the cAMP SEAP assay, nonanal blocked the activation of CfOR0422 by undecanal and incubation with decanal inhibited the activation of CfOR0250 by heptanal (Figure 7A). These inhibitory effects were further confirmed using a luciferase-based reporter assay, which allowed normalization of the data through the Renilla values, as described in the experimental procedures (Figure 8A). These findings are consistent with previous reports showing that various odorants may act as agonists when applied alone or as antagonists when paired with another compound (Spehr et al. 2003; Oka, Nakamura, et al. 2004; Oka, Omura, et al. 2004; Sanz et al. 2005; Shirokova et al. 2005).

By contrast, the combination of heptanal with undecanal induced higher cAMP levels than heptanal alone, in CfOR0012- and CfOR0250-expressing cells, whereas undecanal alone induced no significant response in cells expressing either of these 2 ORs (Figure 7B). We also showed a higher response to octanal/nonanal than to octanal alone in CfOR12A07-transfected cells at odorant concentrations below to 500 µM (Figure 8B). A more complex response pattern was observed for the response to undecanal and heptanal in cells expressing CfOR3548

Figure 5. Dual cAMP and calcium responses of cells transfected with family 6 OR genes. For the calcium assay, dog family 6 ORs were expressed in 1E6 cells and exposed to aldehydes (1 µM). Results are presented as the ratio of odorant response (ΔF/F of odorant) to DMSO response (ΔF/F of DMSO). None of the odorants evoked a positive response in negative controls, that is, untransfected cells or cells expressing the Gαolf protein but not the OR receptor. For the cAMP assay, 1E6 cells were cotransfected with a plasmid encoding a dog OR and pCRE-SEAP and then exposed to aldehydes (500 µM). The cAMP response was expressed as a ratio of odorant value/DMSO value (triplicate results ± S.D.). *P < 0.01.
and CfOR5269. Neither of these odorants gave a positive response when applied separately to these cells at a concentration of 500 μM or 1 mM, whereas when applied together at 500 μM each, they induced an increase in cAMP concentration (Figures 7C and 8C). Thus, mixtures of 2 odorants may exert either an inhibitory or cooperative/additive effect. These findings demonstrate that the combinatorial receptor code may be highly complex, providing an efficient system for the perception of many individual odorants and a large variety of possible mixtures.

Effects of Other Functional Groups

We found that the response patterns of dog family 6 ORs to C6–C12 aliphatic chains bearing different chemical groups were dependent on the chemical functional group concerned. Dog family 6 ORs responded to aldehydes but not to C6–C12 ketones, esters, or alcohols. Figure 9A,B shows examples of cAMP responses; similar results were obtained with the Ca2+ assay. However, we observed cAMP responses in CfOR12A07-expressing cells exposed to heptanoic and octanoic acid and CfOR0426-expressing cells exposed to heptanoic acid, suggesting that a polar functional group may trigger positive responses (Figure 9C), although in general weaker. In addition, no significant response to these acids was observed with Ca2+ imaging (data not shown). These results suggested that most dog family 6 ORs have odorant-binding sites that favor recognition of aldehyde functional groups and that their ability to recognize odorants is influenced by the length of the odorant carbon chain.

Comparison with Class I and Other Class II Families

We extended our screening to several dog OR genes from other families belonging to classes I and II. None of the 7 class II ORs tested, from families 2–5, 7, 10, or 14, responded to aldehydes in Ca2+ imaging or cAMP SEAP assays (data not shown), providing additional support.
We also investigated the responses of several class I dog ORs, from families 51, 52, 55, and 57, upon exposure to various aldehydes, ketones, acids, esters, and alcohols. We found that a number of ORs, all belonging to family 52, responded to ketones (cyclohexanone, 3-octanone, 3-nonanone) but not to aliphatic aldehydes, cinnamaldehyde, or vanillin, when tested by Ca\(^{2+}\) imaging (Figure 10). We did not observe a positive response to cyclohexanone, 3-octanone, 3-nonanone nor to the other tested odorants (aldehydes, alcohols, esters), for any of these dog ORs in the cAMP SEAP assay (data not shown), suggesting that the odorant response of dog family 52 ORs does not involve the cAMP pathway, but the IP3 pathway. Consistent with our findings, the human OR52D1, which is the ortholog of the canine CfOR0130 (52 family), was previously shown to respond to cyclohexanone using a Ca\(^{2+}\) assay (Sanz et al. 2005). CfOR0130 and CfOR0276, which share a high level of sequence identity (94%), had very different ligand-binding properties. CfOR0130, but not CfOR0276, showed strong responses to cyclohexanone and both receptors were activated by 3-nonanone using a Ca\(^{2+}\) test (Figure 10), highlighting the complicated effects that some or all of the 6% of amino acids that differ between these 2 ORs have on ligand binding.

Table 1  Responses of the 47 family 6 OR to 7 aliphatic aldehydes differed according to the functional assay used

<table>
<thead>
<tr>
<th>Odorant</th>
<th>cAMP (SEAP) assay</th>
<th>Ca(^{2+}) assay</th>
<th>Both assays</th>
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</thead>
<tbody>
<tr>
<td>Hexanal</td>
<td>6</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>Heptanal</td>
<td>10</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Octanal</td>
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<tr>
<td>Nonanal</td>
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</tr>
<tr>
<td>Dodecanal</td>
<td>4</td>
<td>22</td>
<td>0</td>
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</tbody>
</table>

Calcium and SEAP assays were each performed on OR-transfected 1E6 cells as described in experimental procedures. The numbers of positive ORs are indicated for each odorant tested, using the SEAP assay only, the calcium assay only or for both assays.

Figure 6. Involvement of G\(_{olf}\) in the Ca\(^{2+}\) and cAMP responses. (A) HEK 293 cells and 1E6 cells expressing CfOR12A07 were exposed to DMSO or to octanal and Ca\(^{2+}\) levels were determined via fluorescence emission. (B) HEK293 cells and 1E6 cells were cotransfected with a plasmid encoding CfOR12A07 and pCRE-SEAP and cAMP levels measured. *P < 0.001 using the Student’s t-test.
Discussion

In vivo analysis have shown that 2 different pathways, the cAMP and the IP3, are used by OSN to transform a chemical signal, the odorant, into an electric signal that is conveyed to the brain. In parallel, 2 different in vitro tests based on the use of transiently transfected cells able to express a given OR have been developed. One is based on the measurement of the increase concentration of intracellular Ca$^{2+}$ that follows the specific binding of an odorant onto the OR expressed at the cellular membrane. The other is a measurement of the cAMP intracellular concentration.

As detailed in the Results, the 2 main setting parameters of these tests, the odorant concentration and the time elapsed between the odorant exposure and the recording of the elevated concentration of the second messenger are very different. Second these 2 tests have never been used in parallel with a large set of ORs exposed to many different odorants but only on rare occasions to a couple of ORs and a limited number of odorants (Katada et al. 2003; Shirokova et al. 2005; Ko and Park 2006; Kato et al. 2008).

Given all these differences, comparison of the results obtained by different groups are difficult if not impossible and it was one the aim of this study to compare the outcome of these 2 tests on a much larger scale.

The previously reported high selectivity of rat Rn17 OR for octanal (Krautwurst et al. 1998; Zhao et al. 1998) led us to choose its canine ortholog CfOR12A07 for our initial studies of interactions between odorants and canine ORs. We transiently expressed CfOR12A07 at the cytoplasmic...
membrane of HEK 293 cells expressing the G\textsubscript{olf} subunit. To monitor the responses induced with a large number of odorants, we used a Ca\textsuperscript{2+} assay based on the fluorescence emitted by Fluo-4 and a cAMP assay based on an SEAP reporter gene encoded by a second plasmid under the control of a CRE. The inhibition of the Ca\textsuperscript{2+} response by xestospongin C, an IP3R inhibitor, and the failure of SQ22536, an adenylate cyclase inhibitor, to prevent the increase in calcium, confirmed that the Ca\textsuperscript{2+} increase was indeed dependent on activation of the IP3 pathway. We also confirmed that the cAMP increase was dependent upon the presence of the G\textsubscript{olf} subunit, whereas the IP3 increase was dependent upon another G\textsubscript{a} subunit as already reported (Shirokova et al. 2005; Oka et al. 2006).

We also found that all ligands of the C6–C12 aliphatic series inducing a positive response in one or both of the tests contained an aldehyde group connected to an aliphatic chain of 6–12 carbons, but no other chemical group.

Figure 8. Inhibitory and cooperative effects of exposure to combinations of odorants using a normalized luciferase reporter assay. OR-expressing 1E6 cells were incubated with aldehydes applied separately or in combination. We observed an antagonistic effect (A) and a cooperative response (B and C) using the luciferase cAMP assay. Triplicate results are expressed as ratios of odorant/DMSO control. Names of odorants have been in some cases abbreviated: nona for nonanal, hepta for heptanal etc. *Indicates a significant inhibition (A) or increase (B and C) in the presence of odorant combination (P < 0.01).

We then extended our investigations to 47 dog ORs from the same family (family 6) as CfOR12A07 and 22 dog ORs from various class I and class II families (this study and Benbernou et al. 2007). Of the 47 dog family 6 ORs, 24 responded positively in both assays to at least one of the aldehydes tested, 21 responded positively in the Ca\textsuperscript{2+} assay only (Table 1). Two OR did not respond in either test with the range of odorants tested, despite displaying similar levels of cell surface expression as other ORs. As shown in Table 1, 15 ORs responded to octanal through both pathways. For example, the 15 ORs responding to octanal through both pathways can be assigned to 4 groups on the basis of their response to heptanal: 1) positive responses in both assays (n = 6), 2) negative responses in both assays...
A similar scheme can be used to classify the OR responses to octanal/undecanal. However, in no instances, the octanal/heptanal groups were identical to the octanal/undecanal groups. These results suggest that the differences observed between the 2 tests are not dose dependent and not due to experimental artifacts. Moreover, they demonstrate that the pathway activated is not dependent on the odorant alone, as first thought, but depends in a complex way on the ORs/ligand combination. As it is highly probable that the transduction pathways elicited by dog ORs are very similar or identical to those elicited by human or rodent ORs, these last results explain the discrepancies observed in earlier studies investigating the involvement of these 2 independent pathways.

One could also imagine that for ORs giving a positive response with the cAMP test, a negative response in the Ca test could correspond to a low binding affinity. Conversely, a positive Ca response in absence of cAMP response could be explained by an inhibitory effect and/or desensitization resulting from a higher odorant concentration. However, it is worth mentioning, these in vitro tests may not necessarily reflect the in vivo responses neither they are comparable one to the other because of their very different setting. Nevertheless, these results indicated that functional assays for these 2 pathways should be used in parallel when evaluating the odorant-binding properties of an OR.

Whereas dog family 6 ORs respond through either one or both of the pathways, depending upon the odorant to which they are exposed, the 10 dog ORs from class I family 52 challenged with different odorants gave positive responses with ketones only, through the IP3 pathway only.
We confirmed that the G\alphaolf subunit was required to induce an increase in cAMP levels upon ligand–ORs recognition but was dispensable for the IP3 pathway. It therefore seems likely that the presence of G\alphaq or another subunit in HEK cells results in an increase in intracellular Ca\textsuperscript{2+} concentration via the IP3 pathway and that the G\alphaolf subunit expressed by 1E6 cells leads to an increase in cAMP levels. These observations are consistent with other reports suggesting that the calcium-imaging assay is limited to GPCRs coupled to G\alphaq-type G proteins (Hansen et al. 2003; Shirokova et al. 2005; Jacquier et al. 2006; Oka et al. 2006; Luttrell 2008).

Recent studies have demonstrated the importance of the C-terminal OR sequence for interaction with the G\alpha subunit (Kato et al. 2008). Our observation that some dog ORs can activate both the IP3 and cAMP pathways, via 2 different G\alpha subunits, raises questions concerning the sequence requirements for this dual recognition and the selection of this pattern of recognition in different neurons.

Most of the critical residues involved in odorant recognition are hydrophobic and located within the binding pocket formed by transmembrane domains TM3, 5, and 6 (Singer et al. 1996; Pilpel and Lancet 1999; Floriano et al. 2000; Singer 2000; Floriano et al. 2004; Man et al. 2004; Katada et al. 2005). Analysis of amino acid sequence alignments of the ORs that differed in their response to octanal failed to identify critical residues or even to confirm the role of the 7 amino acids previously identified as critical for octanal binding by rat Rn17 (Singer 2000) or the role of the 22 amino acids identified for OR binding (Man et al. 2004). Similarly, comparison of the dog family 6 OR responses to the different C6–C12 aliphatic aldehydes, through the cAMP and/or the IP3 pathways, does not support any conclusion to be drawn regarding their subfamily membership. The phylogenetic tree (Supplementary Figure S1) showed that the ligand response profiles of dog ORs are not correlated with the dog OR subfamily.

As observed for other GPCRs, ORs may be subjected to the agonistic or antagonistic effects of their ligands, reflecting the complexity of interactions between ORs and odorants (Spehr et al. 2003; Oka, Nakamura, et al. 2004; Oka, Omura, et al. 2004; Sanz et al. 2005; Shirokova et al. 2005). Here, we confirmed, using cAMP assays (with SEAP and luciferase as reporter genes), that odorants identified as agonists may also function as antagonists depending upon the experimental conditions. However, additive effects were also observed for odorant responses, as shown in both CfOR0012- and CfOR0250-expressing cells exposed to a combination of heptanal and undecanal. Despite the fact that undecanal alone does not elicit a response in these cells, the presence of this compound in the mixture increased the intensity of the response observed with heptanal. An even more striking effect was observed for cells expressing CfOR3548 or CfOR5265 and exposed to a mixture of heptanal and undecanal. Neither of these aldehydes elicited a response when used alone, whereas a positive response was obtained when they were used together. These cooperative effects may be due to the homodimerization of receptors at the cell membrane, with each of the 2 subunits binding a different compound. Dimerization of non-ORs GPCRs has been well established (Franco et al. 2007), as is ORs heterodimerization in Drosophila (Hague et al. 2004), but there is less evidence available concerning ORs homodimerization in mammals. Thus, combinations of
odorants in complex mixtures—as is most commonly the case in nature—may lead to antagonistic or cooperative effects, thus adding to a multitude of factors determining the responses of the olfactory system in vivo.

**Supplementary Material**

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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**References**


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