

Functional Characterization of Two Human Olfactory Receptors Expressed in the Baculovirus Sf9 Insect Cell System

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Abstract

Olfactory receptors (ORs) are the largest member of the G-protein-coupled receptors which mediate early olfactory perception in discriminating among thousands of odorant molecules. Assigning odorous ligands to ORs is a prerequisite to gaining an understanding of the mechanisms of odorant recognition. The functional expression of ORs represents a critical step in addressing this issue. Due to limitations in heterologous expression, very few mammal ORs have been characterized, and so far only one is from human origin. Consequently, OR function still remains poorly understood, especially in humans, whose genome encodes a restricted chemosensory repertoire compared with most mammal species. In this study, we have designed cassette baculovirus vectors to coexpress human OR 17-209 or OR 17-210 with either G_{α_{olf}} or G_{α₁₆} proteins in Sf9 cells. Each OR was found to be expressed at the cell surface and colocalized with both G_α proteins. Using Ca²⁺ imaging, we showed that OR 17-209 and OR 17-210 proteins are activated by esters and ketones respectively. Odorant-induced calcium response was increased when ORs were coexpressed with G_{α₁₆} protein, whereas coexpression with G_{α_{olf}} abolished calcium signaling. This strategy has been found to overcome most of the limitations encountered when expressing an OR protein and has permitted odorant screening of functional ORs. Our approach could thus be of interest for further expression and ligand assignment of other orphan receptor proteins.

Key words: baculovirus, calcium imaging, confocal microscopy, GPCR, insect cell system, olfactory receptor

Introduction

Early olfactory discrimination of higher vertebrates is accomplished via the recognition of odorous ligands by olfactory receptors (ORs) which belong to the G-protein-coupled receptor (GPCR) superfamily (for review, see Breer, 1994). A single OR could bind different odorants sharing the same odotope while one odorant molecule could be recognized by multiple ORs, suggesting the existence of a combinatorial receptor scheme to encode odorous entities (Malnic *et al.*, 1999). Odorant discrimination has been shown not only to proceed through the recognition of numerous molecules of widely different structures but also to markedly vary upon odorant concentration (Kajiya *et al.*, 2001).

Over the last decade, thousand of sequences of putative ORs have been made available in the Olfactory Receptor Data Bank (<http://senselab.med.yale.edu/senselab/ORDB/default.asp>), which currently contains the largest family of

heptahelical receptors, covering up to 3% of the genome superfamily (for review, see Mombaerts, 2001). The human OR genome includes numerous dysfunctional sequences, or pseudogenes (Rouquier *et al.*, 1998; Menashe *et al.*, 2003), since mutations disrupting OR coding region have been accumulated fourfold faster than any other species sampled (Gilad *et al.*, 2003). About 350 genes in our olfactory repertoire are potential candidates for full-length OR sequences (Zozulya *et al.*, 2001; Malnic *et al.*, 2004), while almost 50% are pseudogenes (Malnic *et al.*, 2004). Such a limited repertoire is of particular biological relevance since the human ortholog corresponding to an identified mammal OR could be nonfunctional (Sharon *et al.*, 1999; Gaillard *et al.*, 2002). Furthermore, not all ORs play a role in perception since human OR genes are also expressed in non-olfactory tissues, like testicular tissue, where they might be

involved in the fertilization process (Vanti *et al.*, 2003; Volz *et al.*, 2003). In this respect, we have recently shown that the expression of two human OR genes, OR 17-209 and OR 17-210, was restricted to the olfactory neuroepithelium (Matarazzo *et al.*, 2002). Both ORs are located in chromosome 17 OR gene cluster, a region of special interest as it originates from a copy of an ancestral OR repertoire (Ben-Arie *et al.*, 1994). With their rather restricted chemosensory repertoire, humans represent a good model for understanding olfaction because of the intensive information available on olfactory faculties in *Homo sapiens*, gathered from earlier work (Beets, 1971) to the more recent modelling of human olfactory stimulus–response function (Chastrette *et al.*, 1998), including data on phylogenetic evolution of human ORs (see <http://biportal.weizmann.ac.il/HORDE/>).

Starting from gene sequences provided by genome analysis, identifying OR proteins and assigning their ligands represent a crucial step in the elucidation of the combinatorial olfactory code. However, functional properties of ORs still remain poorly understood since no more than a few dozen mammal OR proteins have been successfully obtained so far, including only one receptor of human origin. This receptor, OR 17-40, has been expressed in mammalian cells and its ligand assigned by screening of odorant libraries (Hatt *et al.*, 1999; Wetzel *et al.*, 1999). It was found to discriminate an aldehydic molecule, helional, between structurally related odorants.

Limitation in heterologous expression is due to impaired OR protein translocation to the cell surface (Ivic *et al.*, 2002; Lu *et al.*, 2003) and largely accounts for the limited functional data on OR obtained so far. Among the heterologous systems available for gene expression, the Sf9 cell system has been established as a useful intact cell setting to reconstitute GPCRs for ligand binding studies (Butkerait *et al.*, 1995; Barr *et al.*, 1997). The choice of such a system is of particular interest for human protein expression since Sf9 cells carry out a variety of co- and post-translational processing events (Butkerait *et al.*, 1995; Poul *et al.*, 1995). These processing events support viable targeting of receptors and G-protein subunits to the cell membrane. In distinction of mammalian cells, the Sf9 cell model is free of potential interference with endogenous GPCRs and the low level of endogenous G proteins permits to test the interaction of mammalian subunits with the coexpressed receptor. Also, the insect cells can be used for the functional expression of mammalian receptors linked to changes in Ca²⁺ homeostasis (Hu *et al.*, 1994). To provide tools for OR deorphanization, we have thus developed a strategy based on the expression of human OR protein in Sf9 cells together with G_α protein in order to transduce a measurable biological signal in response to odorant stimulation. We designed recombinant baculoviruses to coexpress OR 17-209 or OR 17-210 with either G_{αolf} protein, a subunit involved in olfactory transduction through cAMP production (Jones and Reed, 1989), or G_{α16} protein, an ubiquitous subunit of the

phosphoinositide pathway. Quantitative pharmacological odorant screening of both ORs has been then performed using a Ca²⁺ imaging method to monitor change in intracellular Ca²⁺ concentration in Sf9 cells.

Material and methods

Chimera constructs

The cDNA encoding rat G_{αolf} protein sequence was cloned by RT-PCR using a whole-brain total RNA preparation, kindly provided by Dr. Gilles Toumaniantz (IPMC, Nice, France). RNAs (4 µg) were primed (5 min at 70°C and 5 min at 4°C) with 1 µg of oligo dT primer (Invitrogen, Netherlands) in a final volume of 15 µl. A 10 µl aliquot of RNA was used as a template for RT (42°C for 70 min) to cDNA using the cDNA cycle kit from Invitrogen. The reaction was then inactivated at 94°C for 5 min. First-strand cDNA synthesis was used for further PCR amplification. The HA sequence was also introduced by PCR in frame with the methionine of the G_{αolf} gene. The following oligonucleotide sense (S) and antisense (AS) primers were used: G_{αolf}/Bg/II HA S: 5'-TTACGATATCAGATCTGCCACCATGTACCCCTACG-ACGTCCCTGACTCGCCATGGGGTGTITG-GGCAAC-3'; G_{αolf}/HindIII AS: 5'-CTATAAGCTTTCACAAGAGT-TCGTACTGCTTGAG-3'.

The cDNA encoding human G_{α16} gene was obtained from the Human Leukemia, promyelocytic HL60 cDNA library (Clontech, France). The HA sequence was introduced by PCR in frame with the methionine at the 5' end of cDNA. The following oligonucleotide sense (S) and antisense (AS) primers were used (Eurogentec, Belgium): G_{α16}/Bg/II HA S: 5'-TTACGATATCAGATCTGCCACCAT-GTACCCCTACGACGTCCCT-GACTACGCCA-TGGCCCGCTCGCTGACC-3'; G_{α16}/HindIII AS: 5'-CTATA-AGCTTTCACAGCA-GGTTGATCTCGTCCAG-3' (restriction sites are underlined and HA sequence is double underlined).

The intronless genes encoding OR 17-209 and OR 17-210 were amplified by PCR using the cosmid no. ICR F105cF06137 (GenBank HSU53583) as described previously (Matarazzo *et al.*, 2002). The following oligonucleotide S and AS primers (Eurogentec) were used: OR 17-209/*Nco*I S: 5'-TAAGAAGCTTGCCACCATGGAGGGGAAAA-ATCTG-3'; OR-209/*Kpn*I AS: 5'-TAACGGTACCGCGGCCGCTAAGGGGAATGAATTTTCCG-3'; OR 17-210/*Nco*I S: 5'-CAATAAGCTTCCATGGCTATGTAT-TTGTGTCTCAGCAAC-3'; OR-210/*Kpn*I AS: 5'-TAACGGTACCG-CGGCCGCTTAAGCCACTGATT-TAGAGTG-3'. In all PCR experiments, 10 ng of cDNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.001% gelatin, 0.2 mM dNTP, 1.5 U of high-fidelity Expand™ mix of polymerases (Roche Molecular Biochemicals) and 100 pmol of each primer were mixed according to the following schedule: 94°C (60 s, 1 cycle), 94°C (45 s), 58°C (45 s), 72°C (90 s, 35 cycles) and 72°C (120 s, 1 cycle). PCR products were

then digested and subcloned into the transfer vectors p119 and pGmAc 217T respectively.

Transfer vectors

The unique *Bgl*II and *Hind*III cloning sites of the p119 transfer vector (Marchal *et al.*, 2001), located downstream of the baculovirus p10 promoter, were used to insert either the HA- $G_{\alpha\text{olf}}$ cDNA or HA- $G_{\alpha16}$ cDNA. Two plasmids containing the p10 promoter, namely p119-HA- $G_{\alpha\text{olf}}$ and p119-HA- $G_{\alpha16}$, were thus obtained. An 108 pb oligonucleotide, corresponding to the EGT (ecdysteroid UDP-S-glycosyltransferase) signal peptide (Riegel *et al.*, 1994) from AcMNPV followed by the FLAG epitope tagging sequence and two unique restriction sites (*Nco*I and *Kpn*I), was inserted into the *Bgl*II/*Sac*I sites of the pGmAc217T transfer vector (Gaymard *et al.*, 1996). The OR 17-209 or OR 17-210 sequences were then inserted into the *Nco*I and *Kpn*I sites. Two plasmids containing the polyhedrin promoter were thus obtained: pGmAc217T-FLAG-OR 17-209 and pGmAc-FLAG-OR 17-210.

Recombinant baculoviruses

Sf9 insect cells (ATCC CRL 1711), maintained at 28°C in medium containing 5% serum, were used for virus propagation, transfection, plaque assays and limiting dilutions. Baculoviruses DNA from AcMNPV wild type and AcSLP10 (Blanc *et al.*, 1993) were prepared from budding particles. Transfections were performed as previously described (Poul *et al.*, 1995) with minor modifications. Sf9 cells were cotransfected with AcMNPV baculovirus and then with pGmAc217T (see Figure 1B). This recombination procedure permitted to obtain two single baculoviruses expressing either the FLAG-OR 17-209 or the FLAG-OR 17-210 under control of the polyhedrin promoter, which were plaque-purified as an occlusion-bodies negative (ob^-) virus phenotype and designated as AcMNPV209 and AcMNPV210 respectively. A three-step recombination procedure (see Figure 1C) was carried out to construct four double-recombinant baculoviruses (FLAG-OR 17-209 with either HA- $G_{\alpha\text{olf}}$ or HA- $G_{\alpha16}$ and FLAG-OR 17-210 with either HA- $G_{\alpha\text{olf}}$ or HA- $G_{\alpha16}$). The first step consisted of cotransfecting Sf9 cells with AcSLP10 baculovirus and p119 transfer vector. Recombinant baculoviruses expressing either HA- $G_{\alpha\text{olf}}$ or HA- $G_{\alpha16}$, under the control of the p10 promoter, were plaque-purified as ob^- virus phenotype and designated as AcSLP10 $G_{\alpha\text{olf}}$ and AcSLP10 $G_{\alpha16}$. In a second step, AcSLP10 $G_{\alpha\text{olf}}$ or AcSLP10 $G_{\alpha16}$ DNA were cotransfected with the pGmAc150 transfer vector (including the polyhedrin sequence and its promoter, as illustrated in Figure 1A) into Sf9 cells. Recombinant baculoviruses expressing either G_{α} protein and the polyhedrin were plaque-purified ob^+ virus phenotype and designated as AcSLP10 $G_{\alpha\text{olf}}$ Ph and AcSLP10 $G_{\alpha16}$ Ph. Insertion of polyhedrin gene in the recombinated viral genome

was assessed by *Eco*RI digestion and Southern blot analysis. Finally, in order to obtain double-recombinant baculoviruses, AcSLP10 $G_{\alpha\text{olf}}$ Ph or AcSLP10 $G_{\alpha16}$ Ph DNA were cotransfected with pGmAc 217T-transfer vector into Sf9 cells and selected as ob^- virus phenotype. The final four double-recombinant baculoviruses were designated as Ac $G_{\alpha\text{olf}}$ -209, Ac $G_{\alpha16}$ -209, Ac $G_{\alpha\text{olf}}$ -210 and Ac $G_{\alpha16}$ -210.

Western blot analysis

Lysates (TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) of 36–48 h post-infected cells were electrophoresed on 12.5% SDS-polyacrylamide gel and transferred to nitrocellulose filter papers. After a blocking step (30 min in TS buffer, 5% dry milk, 0.05% Tween 20), blots were incubated overnight at 4°C with an 1:50 dilution of anti-HA monoclonal antibody 3F10 (Roche) or 1:1000 dilution of anti-FLAG M2 monoclonal antibody (Sigma) and revealed with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Promega) at 1:500 dilution. Peroxidase-conjugated antibody (Sigma) was used to probe the in-house anti-polyhedrin antibody.

Immunofluorescence and confocal microscopy

All fixation steps were made on ice to prevent receptor internalization. Post-infected cells (36 h) were fixed in 3% of PFA buffer for 20 min and washed with a 0.05 M NH_4Cl solution for 10 min, then twice with PBS for 5 min. After a blocking step (30 min with 10% inactivated goat serum buffer), cells were incubated for 1 h with anti-FLAG (1:500 dilution) then with FITC-coupled monoclonal anti-mouse (Sigma) at 1:128 dilution. For HA-tagged G-proteins, a 1:20 dilution of the HA-rho conjugated rhodamine (Roche) was used after a permeabilization step with 0.1% of Triton X-100. For the double-staining procedure, after anti-FLAG labelling, cells were fixed and blocked again before permeabilization. Coverslips were mounted on slides with Mowiol (Calbiochem), air-dried for 24 h prior to of fluorescent analysis with a Leica TCS 4D confocal microscope with an oil-X63 lens.

OR cell surface expression

An indirect ELISA protocol was applied to quantify the expression of FLAG epitope-tagged OR constructs. Briefly, after a 36, 48 or 72 h transfection period, Sf9 cells were fixed, blocked and incubated with anti-FLAG antibody (1:800 dilution). Plates were washed and incubated with a 1:500 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Promega) for 1 h at room temperature. After three washings, the *p*-nitrophenyl phosphate substrate (2 mg/ml) diluted in ethanolamine (1 M, pH 9.8) was added and further incubated in the dark for up to 1 h. Absorbance was measured at 405 nm. Calibration was made using commercial preparation of bovine alkaline phosphatase-FLAG (Sigma).

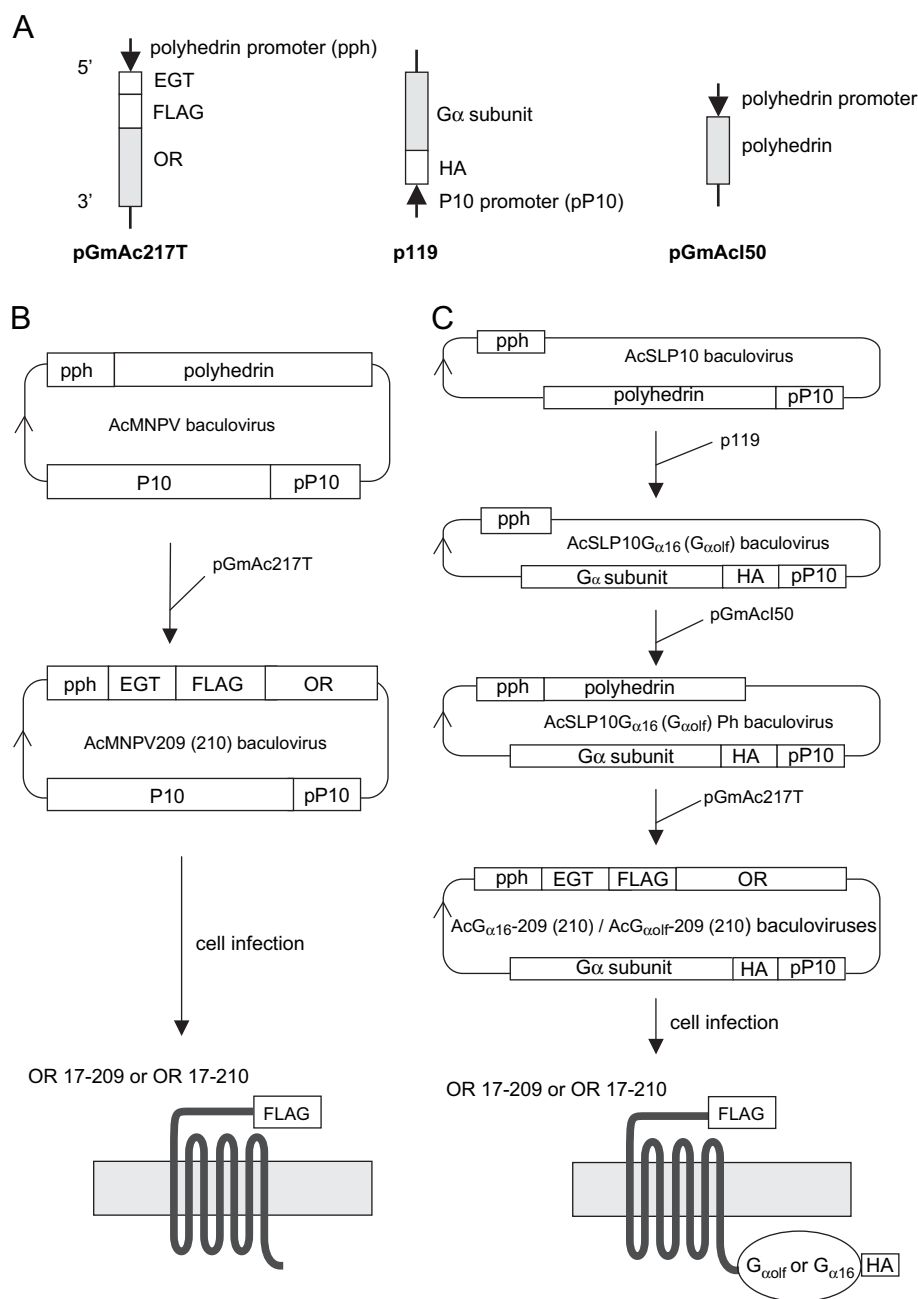


Figure 1 Schematic representation of obtention of recombinant baculoviruses. **(A)** Transfer vectors used for recombination with baculovirus, obtention of **(B)** monorecombinant baculovirus OR 17-209 or OR 17-210 and **(C)** double-recombinant baculovirus OR 17-209 or OR 17-210 with either $G_{\alpha 16}$ or $G_{\alpha olf}$ protein subtype.

Measurement of intracellular calcium concentration

Fura-2 fluorescence Ca^{2+} imaging was used as previously described (Marcet *et al.*, 2003) to measure $[Ca^{2+}]_i$ in non-infected or 24 h post-infected cells. Briefly, Sf9 cells, cultured on glass coverslips and loaded (1 h at room temperature) with 4 μ M fura-2-AM (Molecular Probes), were placed into a microperfusion chamber (200 μ l bath volume) and gravity superfused with test solutions (1 ml/min). A new glass coverslip was used for each odorant mixture. Each test solution

was applied in the syringe linked to the perfusion chamber with a manifold. Using a TILLVISION system (Germany), $[Ca^{2+}]_i$ was calculated from the formula proposed by Grynkiewicz *et al.* (1985). Minimal and maximal experimental ratio values (R_{min} and R_{max}) were calculated for Sf9 cells. R_{max} and R_{min} are the values of R under saturating (5 μ M A23187, a Ca^{2+} -ionophore) and Ca^{2+} -free conditions (4 mM EGTA combined with 5 μ M A23187), respectively. The $[Ca^{2+}]_i$ of individual cells was thus calculated every 5 s

and values were plotted versus time. Resting $[Ca^{2+}]_i$ values of individual Sf9 cells oscillated between 80 and 100 nM. For comparison purposes, we determined the ΔCa^{2+} for each cell, defined as the value of the $[Ca^{2+}]_i$ increase after ligand application minus the value of $[Ca^{2+}]_i$ at the resting state. For each experiment, the most responsive cells (5–10 cells out of 20–30 in the camera field of view) were selected. Data were expressed as the mean \pm SEM. Student's *t*-test was used to determine statistical significance. Differences were considered significant if $P < 0.05$.

Odorant screening

All odorants were tested at a concentration of 1% (w/w). Stock solutions were stored at 4°C and diluted (10^{-6} M) in MBS medium (Mes-buffered saline) before use. Odorant screening was performed using successive sets of odorants. First a mixture of 100 odorant or Henkel 100 mix, as detailed by Wetzel *et al.* (1999), and a set of six odorants (camphor, limonene, isoamyl acetate, acetophenone, 2-heptanone and anisole), named the minimal set, were used. Next, the following sets of chemically defined groups were prepared: lactones (pentadecanolide, whisky lactone), ketones (acetophenone, camphor, β -ionone), esters (benzylacetate, 8-cineole, isobornylacetate, hexyl acetate, benzyl benzoate, isoamyle acetate, ethyl butyrate, 2-phenyl ethyl acetate), phenols (thymol, eugenol, guaiacol), alcohols (menthol, citronellol, geraniol, linalool, octanol, benzyl alcohol, cinnamyl alcohol, phenylethanol, 1-octanol) and aldehydes (citral, amyl cinnamaldehyde, cinnamaldehyde, piperonal, C7/C8/C11/C12/C13 aldehydes, octanal aldehyde, vanillin, para-anisaldehyde). When a set was positively identified, individual odorant solutions of isoamyle acetate (from the ester set) and acetophenone (from the ketone set) were further prepared. In all experiments, octopamine (Sigma) was used to stimulate an endogenous GPCR (Hu *et al.*, 1994) to control the intactness of infected Sf9 cells. Thapsigargin (Sigma) was also used to assess genuine intracellular calcium release response upon odorant application.

Results

Human olfactory receptor expression

Baculovirus transfer vector pGmAc217T has been designed for cloning PCR-amplified OR 17-209 or OR 17-210 cDNAs. Each full-length OR coding sequence was included in a plasmid cassette containing the FLAG-tagged OR cloned downstream from an EGT signal sequence under the polyhedrin promoter (Figure 1A, left). Both $G_{\alpha olf}$ and $G_{\alpha 16}$ sequences were tagged with an HA sequence and cloned separately in transfer vector p119 under the P10 promoter (Figure 1A, middle). Transfer vector pGmAcI50 (Figure 1A, right) was used for double recombinating steps as described below. Recombination of transfer vector pGmAc217T with AcMNPV baculovirus enabled monorecombinant OR baculoviruses

designated as AcMNPV209 or AcMNPV210 (Figure 1B). To construct double-recombinant viruses, AcSLP10 baculovirus was recombined with transfer vectors p119, pGmAcI50 and pGmAc217T sequentially (Figure 1C). Four double-recombinant baculoviruses, Ac $G_{\alpha olf}$ -209, and Ac $G_{\alpha olf}$ -210, Ac $G_{\alpha 16}$ -209 and Ac $G_{\alpha 16}$ -210, were thereby designed.

PCR product analysis of OR and G_{α} protein DNAs is represented in Figure 2A. Southern blot analysis of the modified AcSLP10 baculovirus DNA revealed that the polyhedrin gene has been re-integrated in its proper locus, as compared with the wild type baculovirus (Figure 2B), allowing proper selection of recombinant viruses and subsequent OR cloning. Western blot analysis of cell lysates showed that polyhedrin, G_{α} subunits and OR recombinant proteins were found at the expected size for each recombinated baculovirus (Figure 2C).

Expression and localization of ORs and G_{α} proteins

Cellular localization of OR and G_{α} proteins were assessed by immunofluorescence using anti-FLAG and anti-HA mAbs as presented in Figure 3. Confocal microscopy of Sf9 cells infected with monorecombinant viruses AcMNPV209 or AcMNPV210 showed a remarkably typical punctuated localization at the cell surface in absence of permeabilization (Figure 3A, left panels), while cells expressing only $G_{\alpha olf}$ or $G_{\alpha 16}$ subunits exhibited an intense cytoplasmic labelling after a required permeabilization step (Figure 3A, right panels). When the two labelling procedures were carried out sequentially on infected cells with any of the four double-recombinant viruses, a significant portion of the G_{α} protein labelling was found to colocalized with OR staining (Figure 3B, right panel). No significant differences in OR staining were noticed whether OR proteins were expressed alone (Figure 3A, left panels) or together with a G_{α} subunit (Figure 3B, left panel). Using an in-house immunoassay with commercial anti-FLAG antibodies and a FLAG-protein calibrator, up to of 250 ng of recombinant tagged protein per 10^6 cells were estimated for each OR over a 96 h post-infection period. Both the intensity of cell surface labelling and the estimation of OR production indicated a satisfactory level of OR expression in the baculovirus/Sf9 cell system.

Functional characterization of OR 17-209 and OR 17-210

To assess the functional expression of OR 17-209 and OR 17-210, we used fura-2 fluorescence Ca^{2+} imaging. Ratiometric fluorescence (340/380) was used to calculate $[Ca^{2+}]_i$. For each experiment, the resting $[Ca^{2+}]_i$ in unstimulated cells was determined before odorant application (90 ± 10 nM, $n = 100$). Pharmacological screening was based on the measurement of an increase of $[Ca^{2+}]_i$ in OR expressing cells upon application of various sets of odorants. For each cell response to a given odorant, the change in intracellular Ca^{2+} concentration ($\Delta[Ca^{2+}]_i$) was calculated by subtracting the resting value of $[Ca^{2+}]_i$ from the maximal value of $[Ca^{2+}]_i$ induced by

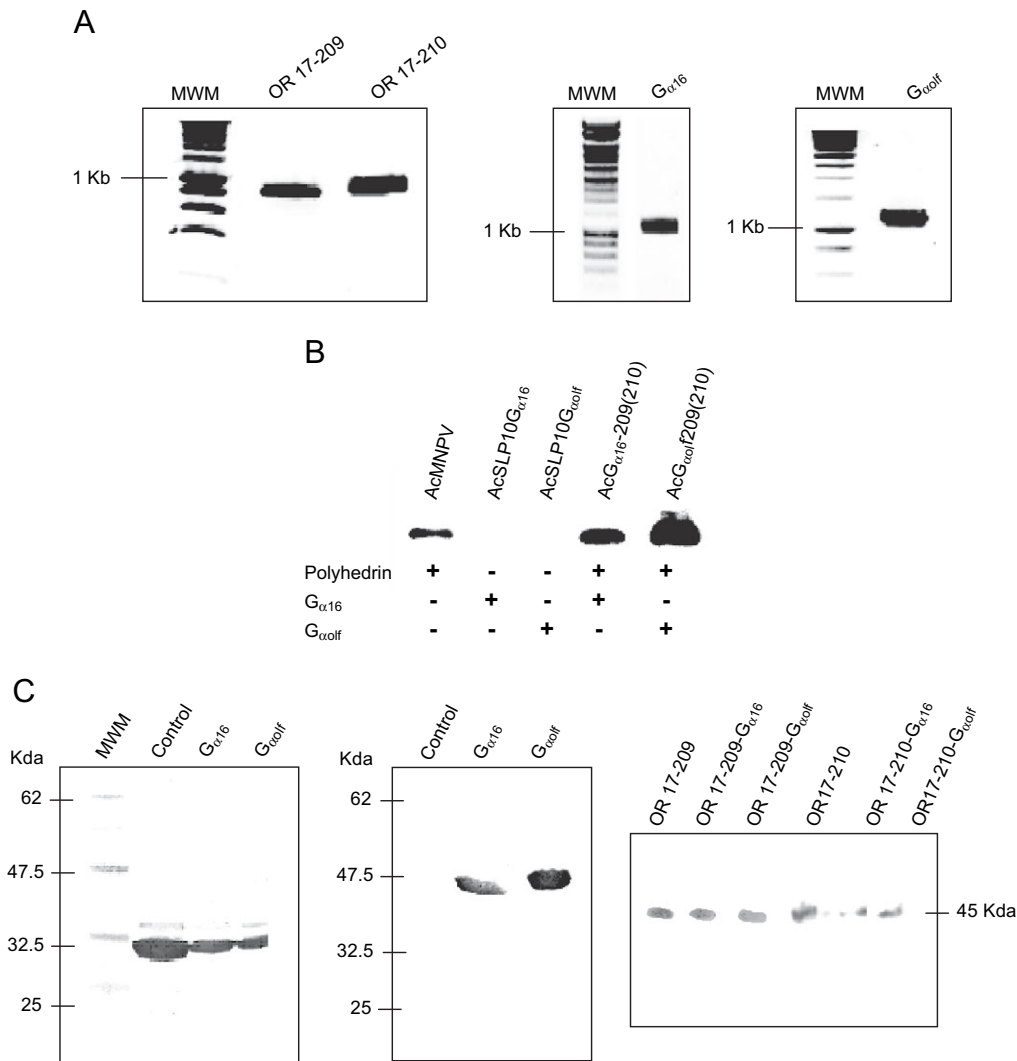


Figure 2 Analysis of recombinant materials. **(A)** PCR products of human OR 17-210 and OR-17-209 obtained from cosmid DNA (left). RT-PCR product analysis of $G_{\alpha 16}$ (middle) and $G_{\alpha olf}$ (right) obtained respectively from human HL60 cDNA bank and a total cDNA brain preparation. **(B)** *EcoRI* enzymatic restriction and Southern blot mapping of the incorporated polyhedrin gene in each recombinated baculovirus and in the wild type baculovirus ACMNPV. **(C)** Western blot analysis of polyhedrin protein expression (left), G-protein expression (middle) and OR expression (right). The control lane corresponds to Sf9 cells infected by wild type baculovirus. Polyhedrin, $G_{\alpha 16}$ and $G_{\alpha olf}$ proteins were detected similarly in Sf9 cells infected with any of the four double-recombinant baculovirus (Ac $G_{\alpha 16}$ -209, Ac $G_{\alpha 16}$ -210, Ac $G_{\alpha olf}$ -209, Ac $G_{\alpha olf}$ -210). For simplification purpose only two lanes, $G_{\alpha 16}$ or $G_{\alpha olf}$, are represented in the left and middle Western blot experiments.

the odorant application. In all experiments, stimulation of an endogenous GPCR by octopamine has permitted to control the intactness of infected Sf9 cells (Figures 4–6). In a first step, Henkel 100 mixture (50 μ M) was used to assess large-scale screening of OR 17-209 or OR 17-210 expressed in Sf9 cells. We observed that Henkel 100 elicited an increase in $[Ca^{2+}]_i$ for both ORs (data not shown). Screening was then carried out by using selected chemical members of qualitative groups, consisting in a minimal set of six odorants (camphor, limonene, isoamyl acetate, acetophenone, 2-heptanone, anisole). Application of the minimal set evoked a transient $[Ca^{2+}]_i$ rise in cells expressing either OR 17-209 ($\Delta[Ca^{2+}]_i = 40 \pm 5$ nM, $n = 4$) or OR 17-210 ($\Delta[Ca^{2+}]_i =$

50 ± 5 nM, $n = 4$), as illustrated in Figure 4A. To assess whether odorant application induced a genuine calcium release signal, experiments were performed with thapsigargin-pretreated Sf9 cells. Preincubation with thapsigargin (5 μ M, 20 min) prior to odorant stimulation inhibited the calcium response of Sf9 cells (Figure 4B). Also, when cells were incubated in free Ca^{2+} MBS medium, responses were still observed upon minimal set application (Figure 4B), supporting the hypothesis that the measured $[Ca^{2+}]_i$ increase originated from intracellular stores.

The identification of the odotope family was then pursued by constructing from the composition of the Henkel 100 and the minimal set six distinct chemical families of purified

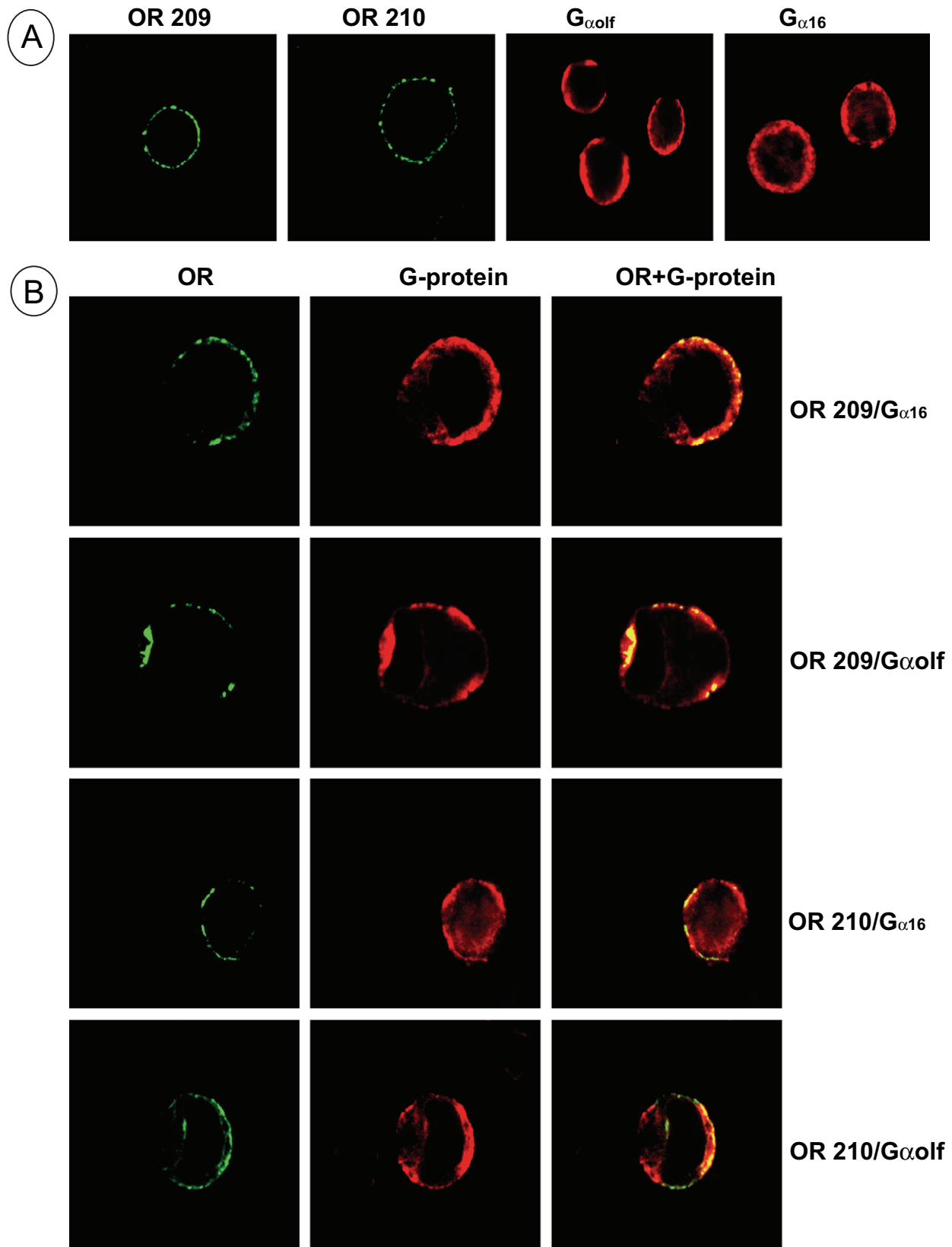


Figure 3 Confocal microscopy images of Sf9 cells infected by recombinant baculoviruses. Cells were infected with either **(A)** monorecombinant or **(B)** double-recombinant baculoviruses as indicated, prior to immunostaining procedure. Cells expressing OR 17-209 or OR 17-210 are visualized using a anti-FLAG mAb (green fluorescence) while expressed $G_{\alpha16}$ or $G_{\alpha\text{olf}}$ proteins are visualized with the anti-HA-rhodamine-conjugated mAb (red fluorescence). For both ORs,

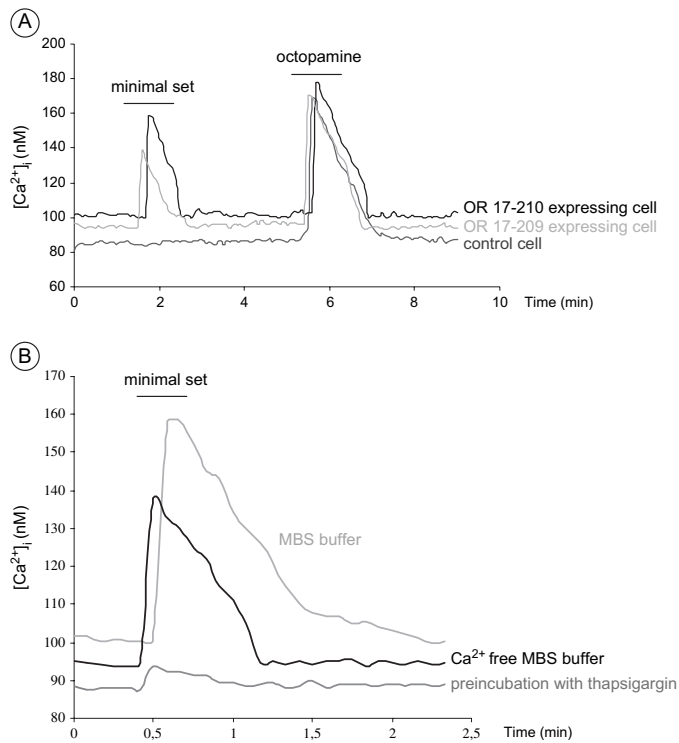


Figure 4 Calcium response of Sf9 cells after minimal set stimulation. **(A)** A typical recording of the Ca^{2+} response of a single control cell (uninfected or wild type baculovirus infected) after minimal set application ($50 \mu\text{M}$ to 1mM) is represented by the red line. For comparison purposes, the representative response of a cell infected with OR 17-209 recombinant baculovirus (green line) and one infected with OR 17-210 recombinant baculovirus (blue line) after minimal set application ($50 \mu\text{M}$) are superimposed. In all experiments, cell responsiveness was assessed by a second stimulation with octopamine ($50 \mu\text{M}$). **(B)** Comparison of OR expressing cell responses to minimal set application ($50 \mu\text{M}$). Cells were incubated in different conditions: standard buffer (MBS medium, green line), in the absence of extracellular Ca^{2+} (free Ca^{2+} MBS medium, blue line) and after depletion of intracellular calcium stores prior to odorant stimulation (20 min pre-treatment with $5 \mu\text{M}$ thapsigargin in MBS medium, red line).

odorants sharing the same functional group (ketone, ester, lactone, alcohol, aldehyde and phenyl). Non-infected cells did not respond to any application of each of the six mixtures (Figure 5A). Tested sequentially, we found that esters induced an elevation of $[\text{Ca}^{2+}]_i$ only in OR 17-209 expressing cells ($\Delta[\text{Ca}^{2+}]_i = 45 \pm 5 \text{ nM}$, $n = 10$) whereas ketones provoked a Ca^{2+} response ($\Delta[\text{Ca}^{2+}]_i = 55 \pm 5 \text{ nM}$, $n = 10$) only in OR 17-210 expressing cells (Figure 5B,C). Finally, single odorants from the sets positively identified were tested. Isoamyl acetate (from the ester set) and acetophenone (from the ketone set) induced a calcium response in OR 17-209 or OR 17-210 expressing cells respectively (Figure 5B,C, right).

Application of $50 \mu\text{M}$ of each odorant induced an elevation of $[\text{Ca}^{2+}]_i$ ($\Delta[\text{Ca}^{2+}]_i = 40 \pm 5 \text{ nM}$, $n = 4$, for isoamyl acetate; $\Delta[\text{Ca}^{2+}]_i = 50 \pm 5 \text{ nM}$, $n = 4$, for acetophenone). Application of $10 \mu\text{M}$ of odorant also induced a response in OR 17-209 or OR 17-210 expressing cells, but with a slight calcium increase for both isoamyl acetate ($\Delta[\text{Ca}^{2+}]_i = 7 \pm 3 \text{ nM}$, $n = 4$) and acetophenone ($\Delta[\text{Ca}^{2+}]_i = 12 \pm 5 \text{ nM}$, $n = 4$).

Importantly, no response was observed when any of the odorant (single or in mixture) was applied at concentration ranging from micromolar to millimolar to control cells, i.e. non-infected cells or cells infected with wild type baculovirus (as illustrated in Figures 4A and 5A). Also, no odorant stimulation was observed when cells were infected with p119 baculovirus containing $G_{\alpha\text{olf}}$ or $G_{\alpha 16}$ protein (data not shown). These results indicate that both ORs are functionally expressed and could be efficiently activated by application of a restricted combination of odorants or individual odorous molecule. The calcium responses are odorant specific since only esters (single or set) activated OR 17-209 expressing cells while only ketones activated OR 17-210 expressing cells. It is thus concluded that each OR specifically binds to chemically distinct odorants. Ongoing study with structurally related molecules will further help to determine the fine specificity of each OR protein for odorants.

As shown in Figure 6, esters (A) and ketones (B) induced a significant increased Ca^{2+} responses (t -test; $P < 0.05$) in cells coexpressing the $G_{\alpha 16}$ subunit with OR 17-209 ($\Delta[\text{Ca}^{2+}]_i = 56 \pm 3 \text{ nM}$, $n = 8$ for esters) or OR 17-210 ($\Delta[\text{Ca}^{2+}]_i = 72 \pm 5 \text{ nM}$, $n = 8$ for ketones) respectively, compared with those observed in cells expressing OR alone ($\Delta[\text{Ca}^{2+}]_i = 46 \pm 3 \text{ nM}$, $n = 10$ for esters; $\Delta[\text{Ca}^{2+}]_i = 55 \pm 5 \text{ nM}$, $n = 10$ for ketones). The coexpression of the $G_{\alpha 16}$ subunit with OR 17-209 or OR 17-210 improved the Ca^{2+} response induced by esters or ketones by 22 ± 1.5 and $31 \pm 3\%$ respectively. Similarly, $G_{\alpha 16}$ coexpression induced also an improved calcium response of $20 \pm 5\%$ upon application of isoamyl acetate (Figure 6A) and $30 \pm 2\%$ upon application of acetophenone (Figure 6B). Calcium response to the minimal set (Figure 6A,B) was also improved by $\sim 20\%$ in cells coexpressing $G_{\alpha 16}$ with either OR. These findings therefore indicate that the coexpression and coupling of the $G_{\alpha 16}$ subunit with OR increase the odorant calcium response in Sf9 cells. Alternatively, in Sf9 cells coexpressing OR 17-209 or OR 17-210 together with the $G_{\alpha\text{olf}}$ subunit, no change in $[\text{Ca}^{2+}]_i$ was observed when any ligand ($50 \mu\text{M}$ to 1mM) was applied to both ORs, while the octopamine ($50 \mu\text{M}$)-induced Ca^{2+} response was unaffected (Figure 6B,C, left). This observation suggests that both ORs may have been preferentially coupled to the recombinant human $G_{\alpha\text{olf}}$ protein than to

immunostaining is visualized at the plasma membrane and shows typical punctuated localization at the cell surface when OR is expressed alone (A, left panels) or coexpressed with either $G_{\alpha 16}$ or $G_{\alpha\text{olf}}$ protein (B, left panels). Similarly, either G_{α} subtype staining shows intense cytoplasm labelling (A, right panels and B, middle panels). Superposition of immunostaining (yellow fluorescence) of cells infected with double-recombinant baculovirus reveals colocalization of OR and G_{α} protein at the plasma membrane (B, right panels).

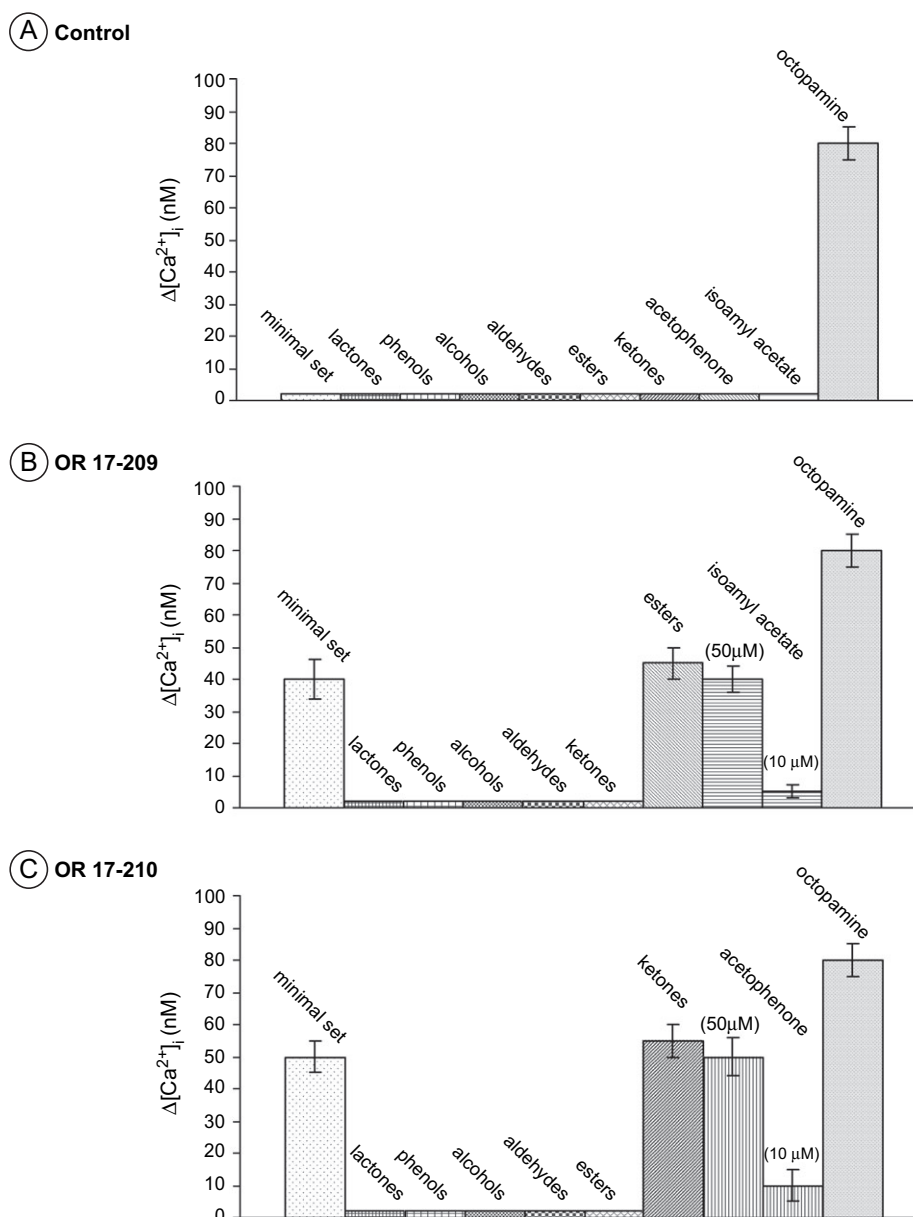


Figure 5 Odorant-induced calcium responses of Sf9 cells expressing OR 17-209 or OR 17-210. **(A)** Control cells (uninfected or wild type baculovirus infected) or **(B)** cells expressing OR 17-209 or **(C)** cells expressing OR 17-210 were stimulated by different odorant sets (50 μM) or individual molecules (50 μM or 10 μM) as indicated. For each Sf9 cell, the value of $\Delta[\text{Ca}^{2+}]_i$ was calculated. It represents the value of the maximal $[\text{Ca}^{2+}]_i$ increase obtained upon odorant or octopamine application (50 μM) minus the value of $[\text{Ca}^{2+}]_i$ at the resting state. Data are means \pm SEM for n independent experiments ($n = 4-10$; total number of cells = 30-120).

endogenous G_α subunits of Sf9 cells, inducing a shift of the $\text{Ca}^{2+}/\text{IP}_3$ signaling pathway to the cAMP pathway. Consequently no odorant-induced calcium response could be detected in these conditions.

Taken together, the present results demonstrate that the baculovirus/insect cell system coupled to Ca^{2+} imaging represents a valuable method to express functional human ORs and investigate their odorant recognition properties. Ligand screening was successfully performed and has permitted to identify the respective odorant families of the human OR

17-209 and OR 17-210. Coexpression and coupling of OR with $G_{\alpha 16}$ subunit of the same human origin may also significantly improved the odorant-induced Ca^{2+} response in the Sf9 cell system.

Discussion

Difficulties in expressing OR in heterologous systems are caused by impaired intracellular traffic to the surface of the recombinant protein (McClintock *et al.*, 1997; Lu *et al.*,

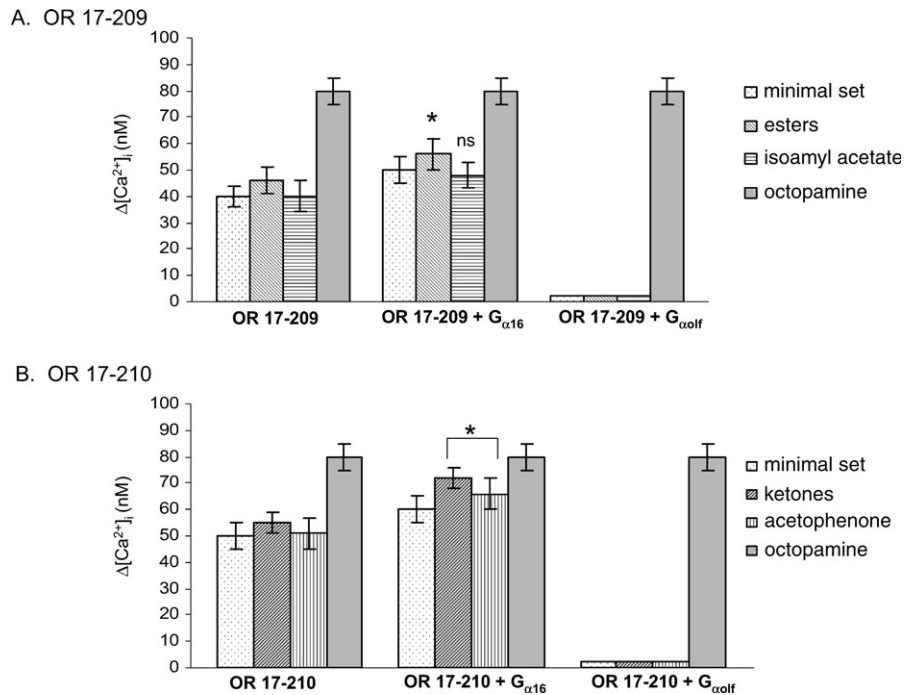


Figure 6 Odorant-induced calcium responses of Sf9 cells coexpressing OR together with G_{αolf} or G_{α16} subunits. For OR 17-209 (**A**) or OR 17-210 (**B**), cells expressing OR alone (left) and cells coexpressing OR with G_{α16} subunit (middle) or G_{αolf} (right) are represented. The value of $\Delta[\text{Ca}^{2+}]_i$ of each cell was determined upon odorant (50 μM) or octopamine application (50 μM). Data are means \pm SEM for n independent experiments ($n = 8\text{--}10$; total number of cells = 60–100). For ester and ketone molecules (single or set), t -tests between OR and OR-G_α protein responses were calculated (ns, not significant; * $P < 0.05$).

2003). These GPCRs have specific requirements for maturation and targeting to the plasma membrane, as hypothesized by McClintock *et al.* (1997). Indeed, poor surface expression of OR in heterologous cells is caused by a combination of ER retention due to inefficient folding and ER export, aggregation and degradation (Lu *et al.*, 2003). As a result, functional properties of ORs have remained poorly understood, especially in the human, whose genome encodes a restricted chemosensory repertoire compared with other mammal species. While the mechanisms responsible for inefficient translocation and surface expression of OR in heterologous cells are now in part elucidated, it still remains a technological bottleneck in the field of OR production and deorphanization. Alternatively, performing odorant screening on OR expressed in olfactory receptor neurons may suffer from several other disadvantages. The presence of non-neuronal surrogate cells, high level of background activity and lack of response reproducibility may prevent odorant identification (Reed *et al.*, 1998; Wetzel *et al.*, 1999).

The insect cell system has been established as a useful intact cell setting for GPCR expression. Co- and post-translational processing events permit appropriate targeting of receptors and G subunits to the cell membrane. The variety of protein processing events performed by the Sf9 cell has provided valuable examples of reconstituted GPCRs, in particular for receptors of human origin (Parker *et al.*, 1991; Butkerait *et al.*, 1995; Barr *et al.*, 1997). The fact that folding of helical secondary structure is better promoted at lower tempera-

tures (Sf9 cells are cultivated at 27°C) is also of relevant importance when dealing with seven transmembrane segment receptors. Nevertheless, since the successful expression of rodent OR5 (Raming *et al.*, 1993), no other OR has been produced in this system so far. Mammalian cell lines have rather been used as expression system with variable success, as reported from our previous work (Matarazzo *et al.*, 2002) and in the literature. Based on these considerations, we aimed at developing an alternate expression strategy and used the baculovirus/Sf9 cell system to express our human ORs. As described in this study, we have designed cassette baculovirus vectors containing tagged OR gene fused to an insect signal sequence. This specific leader sequence was selected to facilitate the addressing of the protein to the secretory pathway while the inherent properties of Sf9 cell allow appropriate folding of the recombinant product. Confocal microscopy associated to immunodetection experiments showed that human ORs are well expressed at the membrane of Sf9 cell together with a high level of receptor protein. Our genetically engineered baculovirus cassette is not restricted to human protein expression since OR sequences from any origin could also be expressed with the proper inserts.

Ligand identification of engineered ORs requires the detection of an efficient coupling second messenger system that produces a measurable response upon application of odorant molecules. Some inherent disadvantages in using calcium imaging for odorant response measurement have been

reported, e.g. lack of reversibility, saturation and low transfection rate preventing calcium quantification (Wetzel *et al.*, 1999). However, we did not encounter such limitations in our Sf9 system. Specific calcium responses were measured with cells expressing either OR, indicating that the necessary use of small epitope tag (FLAG) at the N-terminus of the expressed protein did not affect ligand binding or downstream signaling, as also demonstrated in the case of other mammalian ORs (Ivic *et al.*, 2002). Indeed, ester or ketone application generated a rapid and quantitative $[Ca^{2+}]_i$ increase, with fully reversible signals in OR 17-209 or OR 17-210 expressing cells, respectively. Earlier work on human OR 17-40 indicated that odorant recognition was not affected by the choice of a given OR expression system (Wetzel *et al.*, 1999). Recently, however, it has been reported that odotope affinity may differ according to the system used (Levasseur *et al.*, 2003). Such contradictory findings may be explained by a low level of OR expression, thus reinforcing the need for an efficient expression system for screening and identification of the odotope.

Since hundreds of different odorant molecules exist, we have developed a strategy based on chemical dichotomy in order to functionally characterize each OR. First, Henkel 100, a mixture of 100 diverse odorants from commercial mixtures (Wetzel *et al.* 1999), was used to assess large-scale screening of OR 17-209 and OR 17-210 expressed in Sf9 cells. Screening was then carried out by using selected chemical members of qualitative groups. These odorants, defined as the minimal set, were originally determined on a neuro-olfactory basis (Duchamp-Viret *et al.*, 1999) and were used previously for odorant stimulation of a mammal OR (Gaillard *et al.*, 2002). From there, identifying the odotope family was pursued by constructing six distinct chemical families of purified odorants sharing the same functional group (ketone, ester, lactone, alcohol, aldehyde and phenyl). Induced calcium responses obtained with single members of a positive odorant set (isomyl acetate or acetophenone from the ester and ketone sets respectively) represent the first step in odotope identification of both ORs. Current investigation is now aimed at refining ligand identification with the use of other structurally related odorous molecules and, if possible, defining the odorant specificity parameters for each expressed OR.

Odorant response obtained with cells expressing OR alone or together with a G_α subtype confirmed that mammalian GPCRs can couple to different G_α proteins, including endogenous G_α proteins of Sf9 cells (Leopoldt *et al.*, 1997). Coexpression with the $G_{\alpha 16}$ subunit improves the odorant-induced calcium response (+20 to +30%), suggesting an efficient OR coupling. Such enhanced response obtained when coexpressing a human OR together with a human $G_{\alpha 16}$ subunit might help to discriminate among related odorants. Also, our observation underlines the putative role of the $G_{\alpha 16}$ subtype protein in promoting intracellular calcium response to odorants (Krautwurst *et al.*, 1998). Alternatively, this preferential coupling may also result from a shut-off of

cellular genes as a result of baculovirus gene expression which concomitantly reduced endogenous G-protein number and favoured OR association with recombinant $G_{\alpha 16}$ protein.

No $[Ca^{2+}]_i$ increase has been observed upon ligand application in Sf9 cells coexpressing either OR together with the $G_{\alpha olf}$ protein. This observation might indicate that OR could interact with the $G_{\alpha olf}$ subunit (as suggested by confocal microscopy experiments) and thus activate the cAMP pathway but not the IP3/ Ca^{2+} pathway. Unfortunately, no increase in cAMP level can be recorded because its endogenous level is highly elevated in infected Sf9 cells and does not change upon odorant application (Raming *et al.*, 1993). It is therefore technically difficult to explore whether or not a shift from the IP3 to the cAMP pathway occurred as a result of a putative coupling of OR to $G_{\alpha olf}$ protein. Of interest, such a potential coupling would be relevant to the physiological situation because odorant binding to ORs in olfactory neurons induced $G_{\alpha olf}$ protein activation (Jones and Reed, 1989). Further work is needed to determine whether ORs can efficiently couple to both transduction pathways and maintain a functional cross-talk in our heterologous system, as previously reported for the mouse OR-EG expressed in HEK cells (Katada *et al.*, 2003). However, we cannot rule out that the over-expression of $G_{\alpha olf}$ subunit may alternatively play a role of 'scavenger' for endogenous $G_{\beta/\gamma}$ subunits. In this case, the lack of available $G_{\beta/\gamma}$ subunits may prevent OR signaling via an endogenous Ca^{2+} release-mediating G_α subunit.

In conclusion, our strategy for OR expression in the Sf9 model has been validated. Two ORs from distinct genetic human subfamilies have been functionally expressed and their respective odorant family identified by screening of odorous sets based on calcium imaging. It is hoped that the enhanced responses to odorants observed through efficient homologous coupling of $G_{\alpha 16}$ subunit to OR will help further to discriminate quantitatively among competitive odorants. This in turn will permit the refining of odotopes identified by deconvolution of structural odorant libraries. As our cassette strategy is adaptable to different gene constructs, this system is convenient for expressing other ORs and assigning their respective odorants. More generally, our approach may not be limited to orphan OR and could be also useful for expression of various GPCRs.

Acknowledgements

We thank J.M. Botto and J.C. Guillemot for technical assistance. This work was supported by a grant (ANS 'odorant perception') from the Institut National de la Recherche Agronomique and part of the work has been patented jointly by CNRS and INRA under the accession number 0209377.

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Accepted December 22, 2004