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Biophysical and functional characterization of the human olfactory receptor OR1A1 expressed in a mammalian inducible cell line

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ABSTRACT

Olfactory receptors (ORs) play a crucial role in detecting the odorant molecules present in the surrounding environment. These receptors, which belong to class A G-protein-coupled receptors, constitute the largest transmembrane protein family in the human genome. Functional studies showed that the OR family includes members that are able to respond to a large set of odorants and members that are activated by a relatively small number of related odorants. To understand the molecular mechanisms that govern the receptor–ligand interactions, we overexpressed the human OR hOR1A1 in a stable tetracycline-inducible HEK293S cell line. This receptor was engineered by inserting a C-terminal rho1D4 epitope tag and an N-terminal FLAG epitope tag to allow its purification and detection. The functional activity of the FLAG-rho1D4-tagged hOR1A1 in heterologous HEK293S cells was analysed using a real-time cAMP assay. A two-step purification using monoclonal anti-FLAG immunoaffinity purification and gel filtration was then employed to purify the detergent-solubilized receptor. A size exclusion chromatography-multi-angle light scattering analysis showed the presence of monomeric and dimeric forms of FLAG-rho1D4-tagged hOR1A1. The amounts of the monomeric and dimeric forms purified from sixty T175 flasks were approximately 1.6 and 1.1 mg, respectively. The circular dichroism analysis showed that the purified receptor was properly folded. Ligand binding was quantified using an intrinsic tryptophan fluorescence assay and revealed that the detergent-solubilized FLAG-rho1D4-tagged hOR1A1 bound its cognate odorant, dihydrojasmone, with an affinity in the micromolar range. These results pave the way for future crystallographic and NMR studies.

1. Introduction

In vertebrates, the olfactory sensory neurons (OSNs) located in the olfactory epithelium of the nasal cavity are responsible for detecting the odorant molecules present in the surrounding environment. Odorant molecules bind to olfactory receptors (ORs) that are embedded in the cilia of OSNs. This interaction triggers an increase in the intraciliary concentration of cAMP through the activation of the olfactory G protein G_{olf} and type III adenylyl cyclase. The increased cAMP level opens a cyclic nucleotide-gated cation channel, allowing an influx of Na^+ and Ca^+ ions that ultimately contributes to OSN depolarization. This transduction cascade results in action potentials that are conducted along the axons towards the olfactory bulb [1–3]. The peripheral coding of olfactory signals by the ORs has been explored by analysing the responses of dissociated mouse OSNs or ORs expressed in heterologous cell systems to various odorant molecules [4–8]. These studies showed that one OR is able to respond to multiple odorants and that one odorant may be recognized by several ORs. These observations support a combinatorial coding scheme that allows OSNs to discriminate thousands of olfactory stimuli.

The ORs belong to class A G-protein-coupled receptors (GPCRs) and constitute the largest gene family in the human genome [9,10]. From a structural and functional viewpoint, class A is the largest and best studied GPCR family. Over the last three decades, numerous cellular and biochemical studies have provided a general appreciation of GPCR activation patterns [for reviews, see i.e. 11, 12, 13]. It has been demonstrated that several receptors can bind to different ligands that elicit various types of responses, depending on their potency and their efficiency. In some cases, ligands with a

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Abbreviations

CD	circular dichroism
CMC	critical micelle concentration
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
FC14	Fos-choline-14
GPCR	G-protein-coupled receptor
HEK	human embryonic kidney
MALS	multi-angle light scattering
NaBu	sodium butyrate
OR	olfactory receptor
OSN	olfactory sensory neuron
PBS	phosphate-buffered saline
SEC	size exclusion chromatography
TRITC	tetramethyl-rhodamine isothiocyanate

very low efficacy have been found to prevent the response to full or partial agonists (neutral antagonists). More recently, important technological advances in GPCR crystallization allowed researchers to determine the three-dimensional atomic structures of several class A GPCRs, such as the rhodopsin, adrenergic and muscarinic receptors [14]. These studies provided a significant contribution to our understanding of the molecular mechanisms of GPCR signalling. However, the crystal structures of the ORs are not yet available. The mechanisms underlying OR activation have been explored using molecular modelling approaches that are often combined with site-directed mutagenesis experiments. These studies led to the identification of specific amino acid residues that are involved in ligand binding or in the conformational changes of the ORs during the signal transduction (inactive/active states) [15–19]. Biophysical and structural characterization studies are required to obtain more insights into the activation processes of ORs, but access to large quantities of proteins is a prerequisite for such investigations.

With the exception of rhodopsin, most GPCRs are expressed in small amounts *in vivo*, making their purification from native tissues very difficult or even impossible. An alternative that has been extensively developed consists of overexpressing recombinant, functional GPCRs in a variety of hosts, such as bacteria (*Escherichia coli*), yeast, insect cells, mammalian cells or cell-free expression systems [14,20]. Mammalian cell expression systems have an advantage over prokaryotic systems in that the recombinant proteins undergo post-translational modifications, thus mimicking endogenous human protein production [21,22]. In addition, these cells promote near-native protein folding and disulphide bond formation. Several cell lines derived from human embryonic kidney (HEK) 293 cells (HEK293) have recently been developed for the overexpression of various mammalian GPCRs, including rhodopsin, β2-adrenergic receptor, neurotensin receptor 1, parathyroid hormone-1 receptor and formyl peptide receptor 3 [23–27].

Significant progress has recently been made in producing a recombinant OR (hOR17-4) using a specific HEK293 cell line that contains the tetracycline inducible system, HEK293S GnTI$^-$ cells [28,29]. The tetracycline inducible system allows the cells to grow to a high density, and then the expression of the receptor is induced by the addition of tetracycline. This inducible expression is particularly useful for over-expressing exogenous proteins that may inhibit the subsequent growth of the cells. The HEK293S GnTI$^-$ cells lack N-acetylglucosaminyltransferase I (GnTI$^-$) and, consequently, are unable to synthesize complex N-glycans, which can disrupt the purification step and biophysical techniques, such as spectroscopy and crystallography. The conditions for the over-expression, solubilization and purification of the OR produced by these cells have been optimized. The fos-choline detergent FC14 was found to be optimal detergent for solubilizing and stabilizing ORs [28–30]. Moreover, it has been demonstrated that sodium butyrate (NaBu), a histone deacetylase inhibitor, enhances the expression of the OR protein in this cell line [29]. These conditions allow to obtain large quantities of the recombinant OR protein that are suitable for structural and functional investigations.

We took advantage of these recent advances to overexpress and purify the detergent-solubilized human OR hOR1A1 from stable tetracycline-inducible HEK293S GnTI$^-$ cells. We selected this OR because several of its ligands have been identified in cellular function tests. This OR was particularly activated by aliphatic and cyclic ketones (notably dihydrojasmone) and aldehydes [5,15]. Combinatorial approaches involving homology modelling, site-directed mutagenesis studies and computational analyses have been performed to identify the key residues in the binding pocket of this receptor that are involved in ligand binding [15,31]. However, additional studies are needed to obtain more information on the structure of this receptor and to determine the binding constants that govern the receptor–ligand interactions.

As a step towards this objective, two-step purification using monoclonal anti-FLAG immunoaffinity purification and gel filtration was employed to purify the receptor. The purified, detergent-solubilized receptor was structurally and functionally characterized using several methods, including size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS), circular dichroism and intrinsic tryptophan fluorescence.

2. Materials and methods

2.1. Chemicals

The odorant molecules (dihydrojasmone, CAS # 1128-08-1; (±)-citronellal, CAS # 106-23-0, citral, CAS # 5392-40-5; (−)-carvone, CAS # 6485-40-1, 3-octanone, CAS # 106-68-3), EZview™ Red ANTI-FLAG® M2 Affinity Gel, monoclonal ANTI-FLAG® M2 antibody and FLAG® peptide were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). The odorant stock solutions (100 mM) were prepared in dimethylsulfoxide and stored at −20 °C. The detergent was purchased from Anatrace (Affymetrix, High Wycombe, United Kingdom). Dulbecco's modified Eagle's medium (DMEM) and all tissue culture media components were purchased from Life Technologies (St Aubin, France), unless indicated otherwise. The horseradish peroxidase-conjugated goat anti-mouse secondary antibody was obtained from Bio-Rad (Life Science, Marnes-la-Coquette, France). The anti-rho1D4 antibody was obtained from Merck-Millipore (Molsheim, France).

2.2. hOR1A1 gene design and construction

The 309 amino acid sequence of human OR1A1 (hOR1A1) was obtained from the online NCBI Protein database (NCBI accession # NP_055380.2). To facilitate expression of the protein and its purification from mammalian cells, the following modifications were made: 1) codon optimization using the GeneOptimizer™ software (GeneArt, Life Technologies); 2) the addition of a Kozak sequence (GCCACCATGG) immediately before the start codon; 3) the addition of the FLAG epitope tag (DYKDDDDK) to the N-terminus of the hOR1A1 gene after the start codon; 4) the addition of the bovine rhodopsin C9 (rho1D4) epitope tag (TETSQVAPA) to the C-terminus of the protein; and 5) the addition of the *Eco*RI and NotI restriction sites at the 5′ and 3′ ends, respectively to enable subcloning into the