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Activation of SRE and AP1 by olfactory receptors via the MAPK and Rho dependent pathways

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ABSTRACT

Whereas the activation of MAPKs (mitogen activated kinases) and Rho dependant pathways by GPCR (G protein coupled receptors) has been the subject of many studies, its implication in the signalling of olfactory receptors, which constitute the largest GPCR family, has been far less analysed.

Using an in vitro heterologous system, we showed that odorant activated ORs activate SRE containing promoters via the ERK pathway. We also demonstrated that RhoA and Rock kinases but not Rac were involved in ORs-induced SRE/SRF activation and that AP1 was activated, via JNK and p38 MAPKinase. Using real time PCR we found that mOR23, RnI7 and CfOR12A07 induced elevated levels of transcription factors ELK-4, srf, c-fos and c-jun mRNAs whereas mOREG induced an elevated transcription levels of c-fos and c-jun mRNA only. We showed also that odorant activated ORs stimulate the downstream MAPKs and Rho pathways in primary cultures of rat olfactory sensory neurons (OSNs). Similar results were also obtained with OE (olfactory epithelium) extracts prepared from rats exposed to odorants in vivo. Finally, we showed the important role of the AKT and MAPK signalling pathways in OSNs survival.

Taken together, these data provide direct evidence that the binding of odorants onto their ORs activates the MAPK and Rho signalling pathways that are involved in OSNs survival events. This suggests that these pathways could be implicated in the regulation of OSNs homeostasis.

1. Introduction

Olfactory receptors (ORs) constitute the largest G-protein-coupled receptor (GPCR) family described to date. ORs play a critical role in the recognition of many odorant molecules by olfactory sensory neurons (OSN) present in the nasal olfactory epithelium [1]. In response to odorants, OSNs transmit electric signals to the brain, resulting in odour perception. Mammals have between 500 and 1500 highly polymorphic odorant receptors [2–7]. It was shown by several studies that the binding of odorants to ORs triggers the production of cAMP and IP3, as second messengers via activation of adenylate cyclase and PLC respectively [8–14]. The effects of OR activation on other downstream pathways and cellular functions have been the subject of very few studies [15,16].

It is known that GPCRs, other than ORs activate mitogen activated protein kinases (MAPKs) [17–21]. MAPKs are essential components of many signalling pathways, linking extracellular signals to changes in transcription factor activity and expression of genes that are important for cell homeostasis [22,23]. Thus, the ternary complex factor (TCF) and the serum response factor (SRF), each of them controlled by a distinct mechanism [24,25], bind to the serum response element (SRE) included in the c-fos promoter.

Multiple signals induce the transcriptional activity of TCF via the activation of MAPKs, such as extracellular regulated kinases (ERK), Jun N-terminal kinases (JNK), and p38 [26,27], which phosphorylate the TCF trans-activation domain. In contrast, SRF is activated by RhoA, independently from the activity of MAPKs. The RhoA pathway is linked to the activation of SRF through the Rock kinase, which in turn phosphorylates the LIM kinase [28].

Among the MAPK pathways, the Jun N-terminal kinase (JNK) and p38 MAPK pathways, are activated by different types of stresses and have key roles in tissue homeostasis, as they control cell proliferation, differentiation, survival and cell migration. Depending on the type of stimuli, the cellular response can range from apoptosis to survival [29]. JNKs phosphorylate transcription factors, including c-Jun and ATF2 [30]. AP-1, which is formed by a dimer of Jun and Fos family members, regulates the expression of genes involved in cell proliferation and differentiation [31].

We thus wondered whether ORs would interfere with other downstream pathways and more specifically the MAPK and the RhoA pathways upon the binding of their cognate odorants. To this end we selected the rat OR gene RnI7, which has been the subject of

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many studies using octanal as ligand [8,32] as well as CfOR12A07, its canine orthologue [5,12,14]. We also included in this study two other ORs, which have been the subject of numerous reports in the literature, the mouse olfactory receptors mOR23 and mOREG, which bind lyral and eugenol respectively [4,33,34]. Using several luciferase reporter gene constructs and an in vitro heterologous system, we showed that ligand stimulated ORs are able to activate SRE transcriptional activity not only via MAPK but also via Rho dependent pathways. Also, we showed that ORs stimulation induced AP1 activation. Through in vivo analysis and OSN primary cultures, we confirmed that odorant stimulation does activate various MAPK pathways known to be implicated in cell survival and/or proliferation.

Altogether our results point to the additional role that ORs would have in inducing signalling pathways that may be involved in maintaining the homeostasis of the olfactory system, and more specifically the number of mature OSNs and the choice of OR expressed at their membrane surface.

2. Materials and methods

2.1. ORs cloning and plasmids

Olfactory receptors RnI7, CfOR12A07, mOR23 and mOREG were cloned in frame with a leader peptide sequence derived from Influenza haemagglutinin and a c-Myc epitope sequence in the pIRES plasmid vector (Clontech, Mountain View, CA) as previously described [12].

The plamids pSerum response element (SRE)-luciferase, pSerum response factor (SRF) binding site-luciferase, and pCRE-luciferase were from Stratagene (Agilent, France), pAP1 was from Clontech (France) and pRL-SV40 was from Promega (France).

2.2. Reagents

JNK, phospho JNK, phospho ERK1/2, phospho p38 and p38 antibodies were purchased from Cell Signalling Technology Inc (Ozyme, France). Erk2 antibody was purchased from Santa Cruz Biotechnology, Inc. (Tebu, France).

The following pharmacologic inhibitors and controls Y294002, UO126, UO124, SP600125, SB203580, Y-27632, CCG-1423, NSC23766 and SQ22536 were obtained from Merck–Calbiochem (VWR, France) and were used as indicated in the legend figures. The Rho inhibitor cell permeable exoenzyme C3 transferase was purchased from Cytoskeleton (Tebu, France). The AKT inhibitor IV was purchased from Santa Cruz Biotechnologies (Tebu).

All the odorants used were purchased from Sigma/Aldrich (Saint Quentin Fallavier, France). Stock solutions were prepared in DMSO immediately before each experiment. Solutions were then serially diluted in PBS (final DMSO concentration was <1/500). The composition of the cocktail of odorants for in vivo experiments was as described in the Fig. 7 legend.

2.3. 1E6 cell cultures

All culture reagents were from Invitrogen (France). We used a subclone (1E6) of a human embryonic kidney HEK293 expressing the Gα-olf as described previously [12]. Briefly, cells were maintained in complete medium (DMEM plus 10% FBS, nonessential amino acids, antibiotics) containing 800 μg/ml of G418. Cells were cultured at 37 °C, under an atmosphere of 5% CO_2. Cells were discarded after seven passages and new cultures were prepared from a frozen cell stock. The 1E6 cell clone was constructed with GMO authorization n° 12576 issued March 2003 by the genetic recombinant committee of the Ministry for Education and Research (France).

2.4. Dual-luciferase reporter assay

CRE, SRE or SRF-driven promoter activity was assayed using the PathDetect Signal Transduction Pathway cis-reporting Systems (Stratagene, France). AP1 activity was assayed using the Pathway profiling system of Clontech (France). Cells were co-transfected with 100 ng of plasmids encoding OR, 50 ng of pCRE-luc, pSRE-luc, p-SRF-luc or pAP1-Luc plasmid and 1 ng of pRL-SV40 control plasmid (Promega, France). At 24 h post-transfection, 1E6 cells were starved for an additional 15 h in DMEM-F12 without FBS and containing 0.1% bovine serum albumin (BSA). For the cAMP assay, cells were incubated with IBMX for 30 min and then exposed to various concentrations of odorants. Both firefly and *Renilla* luciferase were measured after 4–5 h incubation at 37 °C, using Dual Glo® luciferase reagent following the manufacturer's instructions (Promega, France) and a Fluostar Omega plate reader equipped with luminescence detectors (BMGLabtek). Data were normalised to *Renilla* activity levels by dividing the values obtained for firefly luciferase by the *Renilla* luciferase values. For some experiments, data were expressed as the ratio odorant/DMSO control. All experiments were carried out at least four times, with duplicate or triplicate technical measurements taken for each time-point.

2.5. Western blot analysis

For Western blot analysis, proteins from activated and non-activated 1E6 cells were separated on 12% SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were then incubated for 2 h with primary antibodies. Anti-rabbit IgG conjugated with HRP (Jackson Laboratories, Interchim), was used as a secondary antibody. Immunoreactivities were detected by ECL Reagents (Pierce, Thermofisher Scientific).

2.6. Real time PCR

OR-transfected 1E6 cells were incubated with the corresponding ligand for the indicated time periods. Total RNA was extracted and purified with RNAII kit following the manufacturer's instructions (Macherey Nagel, France). Total RNA (1 μg) was used as a template for cDNA synthesis. cDNA was prepared using High capacity cDNA RT reagent kit (Applied Biosystems, France). Real-time PCR was performed using the following primer sets:

- Primers for the detection of human transcription factors in 1E6 cells were as follows:
  c-jun forward: CCAAAGGATAGTGCGATGTTT;
  c-jun reverse: CTGTCCCTCTCCACTGCAAC.
  c-fos forward: CTACCACTCACCCGCAGACT;
  c-fos reverse: AGGTCCGTGCAGAAGTCCT.
  srf forward: AGCACAGACCTCACGCAGA;
  srf reverse: GTTGTGGGCACGGATGAC.
  ELK-1 forward: GCTTCCTACGCATACATTGACC;
  ELK-1 reverse: GGTGCTCCAGAAGTGAATGC.
  ELK-4 forward: CTCGAGTTTCCAGCGTGAG;
  ELK-4 reverse: CAGGGTGATAGCACTGTCCAT.
  18S forward: TAGTTGGTGGAGCGATTTGTCTG;
  18S reverse: CTAAGCGGCATAGTCCCTCTAAG.
- Primers for the detection of rat transcription factors in OSNs or in vivo were as follows:
  c-jun forward: GCTGAACTGCATAGCCAGAA;
  c-jun reverse: GCCCCACTGACAGGTTGT.
  c-fos forward: CAGCCTTTCCTACTACCATTCC;
  c-fos reverse: ACAGATCTGCGCAAAAGTCC.
  srf forward: GCACAGACCTCACGCAGA;
  srf reverse: ATGTGGCCACCCACAGTT.
  ELK-1 forward: GCTCCCCACACATACCTTGA;