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Coupling of olfactory receptor and ion channel for rapid and sensitive visualization of odorant response

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ABSTRACT

In the human smell sensing system, there are about 390 kinds of olfactory receptors (ORs) which bind to various odorants with different affinities and specificities. Characterization and odorant binding pattern analysis of the ORs are essential for understanding of human olfaction and to mimic the olfactory system in various applications. Although various cell-based odorant screening systems have been developed for this purpose, many human ORs (hORs) still remain orphan because of the time-consuming and labor-intensive experimental procedures of the available screening methods. In this study, we constructed an ion channel-coupled hOR for simple odorant detection by rapidly visualizing the odorant response to overcome the limitations of conventional screening systems. The hORs were coupled to the Kir6.2 potassium channel and the fusion proteins were expressed in HEK293 cells. In this system, when an odorant binds to the hORs coupled to the ion channel, a conformational change in the OR occurs, which consequently opens the ion channel to result in ion influx into the cell. This ion influx was then visualized using a membrane potential dye. Cells expressing ion channel-coupled hORs showed high sensitivity and selectivity to their specific odorants, and the odorant-hOR binding pattern was visualized to identify the response of individual hORs to various odorants, as well as the response of various hORs to various odorants. These results indicate that the ion channel-coupled hOR system can be effectively used not only for simple and fast high-throughput odorant screening, but also to visualize the odorant-hOR response pattern.

1. Introduction

Olfaction is an important sense in human life, not only to discriminate objects and interpret various environments, but also to enhance the quality of life. The human olfactory system has the ability to discriminate among thousands of odorant molecules. Thus, various investigations have been performed to determine the olfaction mechanism and odorant detection sensors using artificial olfactory systems, not only in industrial applications but also in daily life. The human olfactory receptor (hOR) plays an important role in odorant detection. When an odorant binds to the OR in the olfactory epithelium, a conformational change in the OR occurs, which initiates a cellular signaling pathway to allow the brain to recognize the smell [1–3]. Humans have about 390 different ORs to discriminate numerous odorants. Many researchers have developed various cell-based odorant screening systems such as a cAMP assay, calcium imaging [4–7], and a cAMP response element (CRE) reporter system [8–11] for the characterization of ORs. ORs have also been used as a sensing element for high performance odorant detection systems by combining them with the various secondary transducers such as surface plasmon resonance [12,13], quartz crystal microbalance [14,15], microelectrodes [16–20], and field effect transistors [21,22]. Characterizing ORs and analysis of the odorant binding pattern are essential for their application as a sensing element. Several researchers have conducted large-scale odorant screening for pattern analysis [4,23]; however, because of the labor-intensive and time-consuming experimental steps, the conventional cell-based odorant screening systems are limited not only for odorant screening, but also for odorant binding pattern analysis. Thus, most ORs have not been characterized, and binding pattern analysis has not been actively conducted. In a previous study, we constructed an odorant screening platform for high-throughput odorant screening and odorant binding pattern analysis by visualizing the odorant response. The odorant responses of various ORs were

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visualized by imaging the green fluorescence generated from a reporter protein on a poly ethylene glycol (PEG) microwell platform [24]. That study showed the potential advantages for visualizing the odorant response as well as for high-throughput odorant screening. However, detection using a fluorescence protein requires the protein expression time of at least a few hours. Thus, the odorant response cannot be immediately detected or visualized. To overcome this limitation, we constructed a new odorant sensing system by coupling an ion channel to the OR for fast visualization of the odorant response.

Ion channel-coupled receptors (ICCRs) were first reported by Moreau et al. as protein-based biosensors created by the covalent assembly of G-protein coupled receptors (GPCRs) with the inward rectifier Kir6.2 potassium channel [25]. The Kir6.2 channel protein is the subunit of the ATP-sensitive potassium channel, and four Kir6.2 molecules form a K^+-selective pore [26,27]. In that study, the M_2 receptor and the D_2 receptor were linked to Kir6.2 so that a conformational change in the ligand bound-receptor induced gating of the linked ion channel, resulting in a change of the ionic current. In another study, Caro et al. constructed a β_2-adrenergic ICCR by coupling the β_2AR adrenergic receptor to Kir6.2 and tested its function using patch-clamp analysis [28]. They showed that ICCRs can be used as rapid detectors in a protein-based biosensor. In this study, we coupled this ion channel to ORs to directly detect odorant binding with the OR. The odorant response was visualized by a fluorescence image scanner using fluorescence imaging plate reader (FLIPR) membrane potential dye.

2. Materials and methods

2.1. Cloning of ion channel-coupled human olfactory receptors

The human Kir6.2 potassium channel was coupled to four different hORs including hOR2AG1, hOR1A2, hOR3A1, and hOR1G1 to construct potassium channel-coupled ORs (hOR-Ks). The human olfactory receptor genes were amplified from human genomic DNA and inserted into the EcoRI/XhoI sites or EcoRV/XhoI sites of pcDNA3. The Rho-tag sequence was inserted at the 5′ end of the hOR genes for membrane expression of the hOR proteins. As Moreau et al. reported that the ion channel with the first 25 residues deleted showed optimal efficiency when coupled to the GPCRs [25], the hORs were also coupled to an ion channel with the first 25 residues deleted. The 25 residue-deleted K^+ channel (Kir6.20–25) gene was amplified from its original pCMV6-ENTRY/Kir6.2 clone (Origene, Rockville, MD, USA) using the polymerase chain reaction with complementary primers containing Sal I and Xba I sites. The Kir6.20–25 gene was inserted at the 3′ end of the hOR genes using the Xho I and Xba I sites of pcDNA3 to create the hOR-Ks. Here, a glycine linker was also introduced to one of the hOR-Ks to check for enhancement of performance. The hOR2AG1-(6G)-K was created by adding hexaglycines between the hOR2AG1 and Kir6.20–25 genes.

2.2. Heterologous expression of hOR-Ks

HEK293 cells were maintained in Dulbecoco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were treated with trypsin and transfected with pcDNA3/hOR2AG1, pcDNA3/hOR2AG1-K, pcDNA/hOR1A2-K, pcDNA3/hOR3A1-K, pcDNA3/hOR1G1-K, and pcDNA3/hOR2AG1-(6G)-K, respectively, using the Neon Transfection System (Invitrogen, Carlsbad, CA, USA) at operating conditions of 1100 V, 10 ms, and three pulses. The transfected cells were seeded in a 96 well-plate and incubated in DMEM/10% at 37 °C.

The cellular density was 8×10^5 cells/well, and the medium was added at 100 μl/well. Protein expression was confirmed after 48 h by immunocytochemistry and Western blot using anti-rho or anti-Kir6.2 antibodies. The cells were fixed for immunocytochemistry with absolute ethanol at −20 °C for 15 min, and then incubated in Tris-buffered saline (TBS) containing 0.1% bovine serum albumin (BSA) with anti-rho antibody as the primary antibody (GenScript, Piscataway, NJ, USA, 1:500 for 2 h) and anti-rabbit antibody conjugated with Alexa-594 as a secondary antibody (Invitrogen, 1:1000, 0.5% BSA for 1 h). Cells expressing the ion channel-coupled OR were trypsinized and sonicated (2 s on/off, 1 min) followed by centrifugation at 12,000*g* for 30 min to collect the membrane fraction for Western blot analysis. The hOR-K proteins, expressed in the membrane fraction, were assayed using anti-Kir6.2 antibody (Santa Cruz Biotechnology, Santa Cruz, USA, 1:2000 dilution with 1% skim milk in TBS containing 0.1% Tween20 (TBST)) as a primary antibody, and HRP-conjugated anti-goat antibody (Amersham, Piscataway, NJ, USA, 1:2000 dilution with 5% skim milk in TBST) as the secondary antibody.

2.3. Membrane potential assay

The FLIPR Membrane Potential Assay Kit was used to detect changes in the membrane potential generated by the influx of positively charged ions upon ligand stimulation (Molecular Devices, Sunnyvale, CA, USA). Two assay kits are available, containing red or blue membrane potential dyes. Both membrane potential dyes were tested, and the red dye was found to show higher and more stable change in fluorescence upon ligand stimulation. Therefore, the assay kit containing red dye was chosen for odorant response detection of hOR-K. After the pcDNA3/hOR-K-transfected cells in a 96 well-plate were incubated for 48 h, 100 μl of membrane potential dye was added to each well. The plate with mixtures was then incubated at 37 °C for 20 min. The odorants were prepared at a concentration of 1 M in DMSO and serially diluted in distilled water to concentrations of 2 nM–200 μM. The final concentration of DMSO was lower than 0.02%, which is not harmful to HEK cells [29,30]. A 50 μl aliquot of each odorant was added to the appropriate test wells and the change in membrane potential upon odorant stimulation was detected by a spectrofluorometer (Tecan, Basel, Switzerland) at an excitation wavelength of 535 nm and an emission wavelength of 590 nm. Detection was carried out at intervals of 5 s for a total of 100 s. The change in membrane potential was also detected by an image scanning method using a Typhoon 9410 (GE Healthcare, Milwaukee, WI, USA) at an excitation wavelength of 555/30 nm and an emission wavelength of 610/30 nm. Scanning was conducted at 200 μm resolution in normal sensitivity mode.

2.4. Image analysis

An image processing step was carried out with ImageJ software to visualize the odorant responses of the hOR-Ks. Fluorescence images before and after odorant stimulation were acquired using a fluorescence image scanner and background correction was carried out. The fluorescence images were normalized by subtracting the image before ligand stimulation from the image after ligand stimulation, followed by median filter processing and threshold adjustment. A pseudo color was assigned to the image through lookup tables in the plug-in menu, and the brightness/contrast range was adjusted. The fluorescence intensity of the image was calculated using ImageJ. Experiments were conducted three or four times for the error bar, which represents standard error of mean.