Biosensors and Bioelectronics 24 (2009) 2659–2664

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Cell-based olfactory biosensor using microfabricated planar electrode

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ARTICLE INFO

Article history:
Received 7 November 2008
Received in revised form 21 January 2009
Accepted 22 January 2009
Available online 2 February 2009

Keywords:
Cell-based olfactory biosensor
Planar electrode
Olfactory receptor
Gustatory cyclic nucleotide gated (CNG) channel
Human embryonic kidney (HEK)-293

ABSTRACT

The initial event in olfactory perception is the binding of odorant molecules to specific receptor proteins in the human nose. The interaction between odorant and receptor initiates olfactory signal transduction that leads to a cation influx and change in the membrane potential of the olfactory sensory neuron. In this study, a microfabricated planar electrode was used to measure the generated membrane potential in a heterologous olfactory system. Human embryonic kidney (HEK)-293 cells expressing the olfactory receptor I7 were transfected with the gustatory cyclic nucleotide gated (CNG) channel to amplify the membrane potential. A microfabricated planar electrode was used to measure the electrical responses of odorant–receptor binding. Stimulation of the olfactory receptor with its specific odorant caused an intracellular Ca^{2+} influx, which was quantitatively measured using a planar electrode. The extracellular field potential generated by the Ca^{2+} influx through the CNGgust channel of the cells was approximately 10 mV. This cell-based olfactory biosensor, which uses a microfabricated planar electrode for detection, would be useful for screening specific ligands for binding to orphan olfactory receptors.

1. Introduction

Olfactory receptors comprise the largest multigene G protein-coupled receptor families in many organisms and play a critical role in recognizing thousands of odorant molecules. The initial step in olfactory perception is the binding of odorant molecules to specific receptor proteins on the ciliary surface of olfactory cells. Olfactory receptors coupled to G-proteins activate adenylyl cyclase leading to the generation of cyclic adenosine monophosphate (cAMP), which directly opens a cyclic nucleotide-gated (CNG) cationic channel in the ciliary membrane (Kawai and Miyachi, 2000). The opened CNG-channel leads to an influx of Ca^{2+} and Na^{+} and the subsequent opening of the Ca^{2+}-activated Cl^{-} channel, which results in the depolarization of olfactory cells (Krautwurst et al., 1998). Understanding of the function of olfactory receptors has progressed slowly due to a lack of appropriate olfactory receptor expression systems and functional assays. Attempts have been made to develop a functional assay for olfactory receptors. These methods include Ca^{2+} imaging, cAMP assay, surface plasmon resonance (SPR) and quartz crystal microbalance (QCM) (Houa et al., 2007; Joung et al., 2007; Ko and Park, 2005; Lee et al., 2006; Liu et al., 2006; Sung et al., 2006; Wetzel et al., 1999). Although these methods have their advantages, they also have other limitations. The Ca^{2+} imaging and SPR method are cost- and time-consuming, and the QCM assay only measures the interaction between the olfactory receptor and the odorant, without detecting changes in the intracellular molecules that result from signal transduction (Lee et al., 2006). However, cell-based biosensors, which use living cells as the sensing elements, have many advantages over these traditional techniques and can be used to detect functional information of biologically active analytes. These cell-based biosensor techniques, characterized with high sensitivity, excellent selectivity and rapid response, have been used in many fields ranging from biomedicine to environmental detection (Liu et al., 2006).

In this work, a microfabricated planar electrode was used to measure the extracellular potential generated by odorant stimulation in a cell-based system. This type of measurement is essentially electrophysiological detection. Electrophysiological characteristics of cells have been traditionally measured using the patch-clamp technique. Although electrophysiological assays including the patch-clamp technique are typically very sensitive and have a high resolution, these methods require the user to have a high level technical expertise (Haruyama et al., 2003). A planar electrode has many advantages over the conventional patch clamp technique. The planar electrode can be easily microfabricated as array-type electrodes to permit multi-cell or multi-site measurements. In addition, non-invasive extracellular measurements of the electrical activity occurring inside in cells would provide a potentially more robust method than the conventional patch clamp technique. Furthermore, amplifier electronics can be readily integrated into the electrode substrate to improve the noise performance (Haruyama et al., 2003; Klemic et al., 2002). The aim of this study was to develop a

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doi:10.1016/j.bios.2009.01.035

cell-based olfactory biosensor. To achieve this, an olfactory receptor protein was expressed in a heterologous system and the electrophysiological signal generated from this system was measured using a planar electrode in a non-invasive manner. To amplify the electrophysiological signal, CNGgust channels were introduced into the heterologous olfactory cell by transfecting the cells with the CNGgust gene. The performance of this cell-based olfactory biosensor was evaluated and discussed.

2. Materials and methods

2.1. Fabrication of planar electrode

The planar electrode was fabricated using a semiconductor process as described previously (Jun et al., 2007). Briefly, thin titanium adhesion (30 nm) and gold (300 nm) layers were deposited on substrates (Pyrex #7740). An additional titanium layer (10 nm) was deposited on the gold layer to allow for adhesion to the insulation layer and to prevent the breakdown of the insulation layer during repetitive culture cycles. The metal layers were patterned by wet etching with HF (1%) for titanium and HCl, HNO_3 (3:1) solutions for gold. A triple stack insulating layer ($SiO_2/Si_3N_4/SiO_2$) was deposited by plasma enhanced chemical vapor with stress compensated thicknesses of each layer. A photoresist (Clariant Corporation, USA) mask was used for reactive ion etching of the electrode and contact sites. The upper titanium layer was etched in the same cycle. Finally, a teflon ring was attached to the planar electrode with polydimethylsiloxane (PDMS; Dow Corning, USA) to create a culture chamber. The planar electrode was designed to have 4 electrodes (2×2 arrays). The exposed area of each electrode site was 1 mm^2. Before beginning new cultures, the planar electrode was sonicated in acetone, ethyl alcohol, and deionized water (15 min for each step).

2.2. Reagents

The Ca^{2+} standard buffer used while recording the extracellular potential consisted of the following: 140 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 5 mM glucose and 2 mM $CaCl_2$. The Ca^{2+}-free buffer contained 2 mM EGTA instead of 2 mM $CaCl_2$, and other components were the same as the Ca^{2+} standard buffer. The phosphate-buffered saline (PBS, pH 7.4) consisted of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4. These chemicals and octanal, dimethyl sulfoxide (DMSO), 99% ethanol, poly-D-lysine, Fura PE3-AM were purchased from Sigma–Aldrich (USA). *Taq* polymerase, restriction enzymes and DNase were purchased from Takara (Japan). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were all purchased from GIBCO BRL (USA). The lipofectamine reagent was purchased from Invitrogen (Netherlands).

2.3. Cloning of I7 and CNGgust channel genes

The rat olfactory receptor I7 gene was amplified by PCR of the pVL-I7 gene using the following primers: I7-N (EcoRI); 5′-GAATTCATGGAGCGAAGGAAC-3′; I7-C (XhoI); 5′-TCTCTCGAGGACCTAACCAATT-3′. PCR was performed using a mixture of *Taq* polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 mM of each primer, and 100 ng of plasmid template DNA with the following temperature cycle; 35 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min. The Rho-tag import sequence was fused at the N-terminus of the receptor protein to target the olfactory receptor protein to the cell membrane surface. The rho-tag import sequence was obtained by digesting the pBK-CMV vector, which contains the *rho-tag* gene, with BamHI–EcoRI. The resulting 60-bp fragment was subcloned into the pcDNA3 vector (Invitrogen, Netherlands) that was digested with EcoRI–XhoI. The resulting sequence was inserted into this vector previously digested with the same restriction enzymes. The *rho-tag* and *I7* fusion genes were cloned into the pcDNA3 mammalian expression vector. This pcDNA3-rho-I7 construct was directly sequenced. The cDNA encoding the CNGgust channel in the pUC18 vector was kindly provided by Dr. Keiko Abe, who originally cloned the gene from the tongue epithelia of the rat (Misaka et al., 1997). The CNGgust channel gene was amplified by PCR using the primers: CNGgust-N (SalI); 5′-ATGTCGACATGCATCAGATGGAAACA-3′, CNGgust-C (KpnI); 5′-TAGGTACCTCAGCTTTGAAGCA-3′. The temperature cycle used for PCR was as follows; 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The coding region of the CNGgust channel gene was subcloned into the pIRES2-EGFP vector (Clonetech, Japan), and *E. coli* DH5α was transformed with this vector. This vector was used for the transfection of HEK-293 cells. The CNGgust gene was confirmed by DNA sequencing.

2.4. Culture and transfection of HEK-293 cells

The stable cell line expressing I7 gene was established first, and then this cell line was transiently transfected with the above mentioned plasmid containing CNGgust channel gene. HEK-293 cells were grown in DMEM, supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml), in 5% CO_2 at 37 °C. The cells were transfected with pcDNA3-rho-I7 using Lipofectamine, according to the manufacturer's instructions. Geneticin (G418) was used at a final concentration 500 μg/ml to select stable clones. 24 h after transfection, the growth medium was replaced with the selection medium, and cells were further cultured for 2–3 weeks to obtain stable clones. Cell surface expression of the olfactory receptor I7 was identified by immunofluorescence and RT-PCR. The stable cell line expressing I7 gene was transiently transfected with the plasmid containing CNGgust channel gene.

2.5. RT-PCR

HEK-293 cells were seeded on two 6-well plates and cultivated. The cells on one plate were transfected with the I7 clone and cultivated in DMEM supplement with 10% FBS for 1 day. The transfected cells were then harvested and the cells on the other plate were harvested without transfection. Total RNA was isolated using PURE-script (GENTRA Systems, USA), treated with DNase at 37 °C for 1 h, and heat-treated at 80 °C for 10 min. The cDNA of the I7 was synthesized from the total RNA, and then amplified by PCR. The forward and reverse primers were 5′-GAATTCATGGAGCGAAGGAAC-3′, 5′-TCTCTCGAGGACCTAACCAATT-3′, respectively. The temperature cycle used for PCR was as follows; 35 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min. The PCR product was analyzed by gel electrophoresis.

HEK-293 cells expressing I7 were transfected with pIRES2-EGFP-CNGgust channel using the lipofectamine reagent. The cells co-expressing I7 and CNGgust were harvested, and RT-PCR for I7 and CNGgust was carried out using the same procedure described above. The forward and reverse primers for the CNGgust were 5′-ATGTCGACATGCATCAGATGGAAACA-3′, 5′-TAGGTACCTCAGCTTTGAAGCA-3′, respectively. The temperature cycle for PCR was as follows: 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min.

2.6. Measurement of the extracellular field potential using a planar electrode

The cells were cultured on the surface of the planar type electrode coated with poly-D-lysine and grown in DMEM supplemented with 10% FBS in 5% CO_2. The transfected cells were used in the