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Cell-based olfactory biosensor using microfabricated planar electrode

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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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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

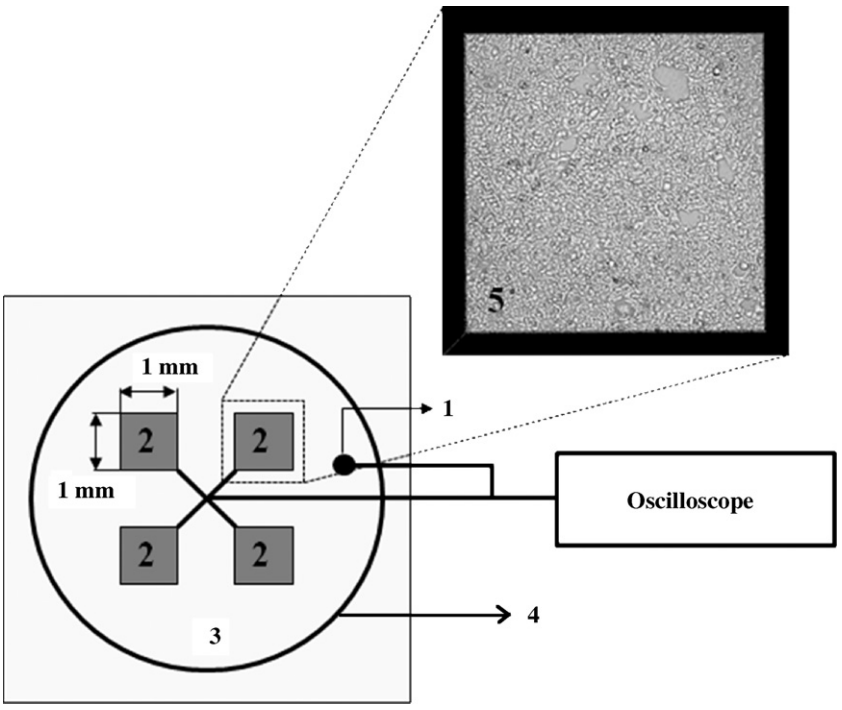

Fig. 1. Schematic diagram of the planar electrode culture system (top view). (1) Reference electrode (cell-noncontacted electrode), (2) recording electrode (cell-contacted electrode), (3) insulation layer, (4) cell culture chamber, (5) micrograph of cultured cell on the planar electrode. Micrograph image of cultured cells on the electrode surface was obtained using upright metallurgical microscope (Nikon, Eclipse LV150, Japan).

extracellular potential recording experiments 48 h after transfection. The extracellular potential of the cell was monitored with an oscilloscope (Tektronix TDS2024, USA, gain: 1, sample rate: 10 Hz). The experimental system is shown in Fig. 1. The planar electrode was mounted into a custom-made connector. Odorants were freshly prepared before experiments by diluting 1 M stock solutions (DMSO) with the Ca^{2+} standard solution to the appropriate concentrations for each experiment. Odorants were supplied to the cells for 30 s with a syringe pump at a flow rate of 400 μl/min. All measurements were performed at 25 ± 1 °C.

2.7. Spectrofluorimetric intracellular calcium assay

Cells in a 96-well plate were incubated with 5 μM fura PE3-AM in the Ca^{2+} standard solution or Ca^{2+}-free solution containing 0.1% Pluronic F-127 for 30 min at 37 °C. The cells were then washed with each solution and incubated in each solution for an additional 1 h at 37 °C to allow the acetoxymethyl (AM) ester group to be cleaved by intracellular esterase. Nonspecific esterases, which were present in most cells, hydrolyzed the AM esters to a Ca^{2+}-sensitive form. After washing three times in each solution, the Ca^{2+} dependent fluorescence signal was measured at 510 nm by dual excitation at 340 nm and 380 nm using a spectrofluorophotometer (Tecan, Switzerland). Fluorescence signals were acquired at 2 s invervals. By using the ratio of fluorescence intensities produced by excitation at these two wavelengths, factors such as uneven dye distribution and photobleaching are minimized because both measurements are affected to the same extent (Tran et al., 2007). Using this ratio, the level of intracellular Ca^{2+} was estimated.

3. Results and discussion

3.1. Expression of olfactory receptor and CNGgust in HEK-293 cells

The rat olfactory receptor I7, was used as a model olfactory receptor in this study. To express the I7 receptor protein on the cell surface, a rho-tag import sequence was fused to the N-terminus of the receptor protein. The *rho-tag* gene was inserted into the BamHI–EcoRI sites of the pcDNA3 vector. To measure the odorant-induced extracellular potential, we established stable and functional expression of rhodopsin-tagged I7 olfactory receptor in HEK-293 cells for which octanal has been identified as a cognate odorant. The expression of I7 was confirmed by RT-PCR (Fig. 2(a)). After gel electrophoresis of the RT-PCR products, a major band, which corresponded to the I7 gene, was detected near 1 kb in the transfected cells. In addition, this band was not observed in the control cells. This indicates that the I7 gene was expressed, at least at the mRNA level, in the transfected HEK-293 cells. The presence of the I7 protein on the cell surface was confirmed by an immunocytochemical method (results not shown). This result was consistent with our previous study (Ko and Park, 2005). HEK-293 cells stably expressing I7 were transiently transfected with the rat CNGgust channel gene to amplify the membrane potential, which is generated by an ion influx into cells through ion channels. The expression of the CNGgust channel was confirmed in HEK-293 cells expressing I7 by RT-PCR (Fig. 2(b)).

3.2. Measurement of response to odorant using planar electrode

The extracellular potential generated by the membrane potential in cells was recorded using a planar electrode. The planar electrode was coated with 0.1 mg/ml poly-D-lysine for 1 h, and then washed with deionized water for three times. Cells were then directly cultured on the surface of the planar electrode. The planar electrode was connected to an oscilloscope in order to record and analyze the field potential output. Using this set-up, the field potential was measured between a cell-contacted electrode (recording electrode) and a cell-noncontacted electrode (reference electrode). The recording electrode surface was 4 mm^2 (four electrodes, 1 mm × 1 mm for each) and the number of cultured cells within this area was approximately 4×10^4 cells. The field potential response was the sum of the responses generated from the cells

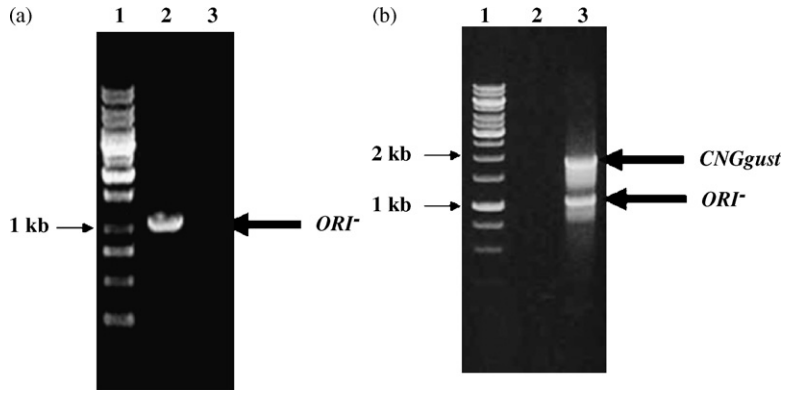

Fig. 2. (a) RT-PCR of I7. Lane 1, size marker; lane 2, HEK-293 cells expressing I7; lane 3, wild type HEK-293 cells. (b) RT-PCR of I7 and CNGgust. Lane 1, size marker; lane 2, wild type HEK-293 cells; lane 3, HEK-293 cells expressing I7 and CNGgust channel. The 1080 bp band and 1800 bp bands correspond to the mRNA of I7 and CNGgust channel, respectively.

on each electrode. After injection of the odorant, the generated field potential of the cells was monitored by the oscilloscope. Odorant solutions were applied to the cells with a syringe pump for 30 s.

Without administration of the odorant, the recorded potential was stable (Supplementary Fig. S1(a)). Injection of the odorant solution (dissolved in the Ca^{2+} standard solution) onto the planar electrode did not result in any shifts in the field potential when wild type HEK-293 cells were used (Supplementary Fig. S1(b)). However, injection of octanal (final concentration of 10 mM) induced a change in the field potential when HEK-293 cells expressing I7 were used (Fig. 3(a)). The maximum change in the field potential for HEK-293 cells expressing I7 was approximately 4 mV. After odorant injection, the signal intensity reached a maximum value and then slowly decreased. In addition, the field potential did not fully return to the baseline 200 s after injection. The generation of the field potential after injection of the odorant was believed to have occurred because of an increase in the flux of ions. This result suggests that the HEK-293 cells may be expressing an endogenous ion channel that responds to the cAMP generated by the I7. To verify that this response was not due to the presence of DMSO in the odorant solution, a DMSO solution without the odorant was used as a control solution (0 mM). When this was done no changes in the field potential were observed (Fig. 3(a)).

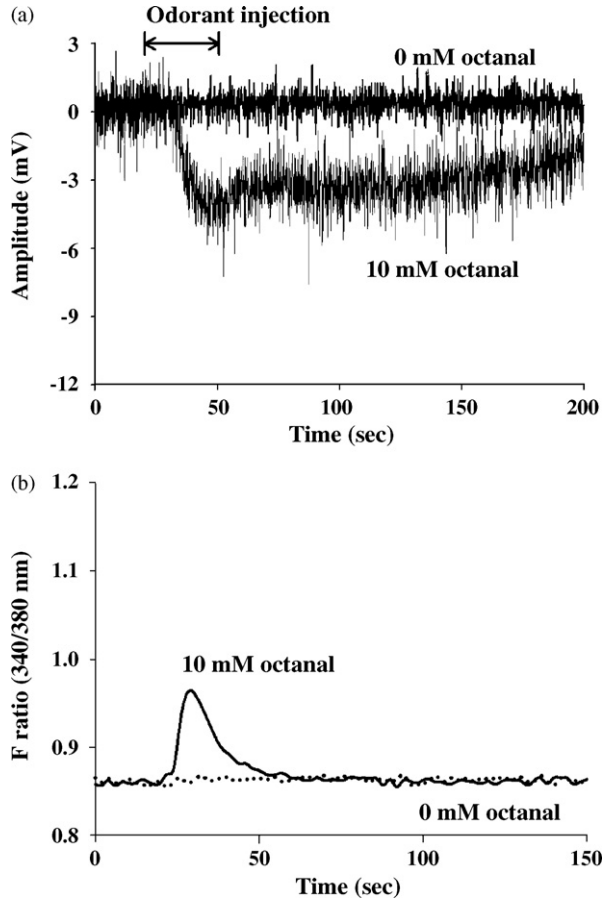

Fig. 3. Field potential and intracellular Ca^{2+} profiles of HEK-293 cells expressing I7. (a) Field potential profile of HEK-293 cells expressing I7 in the Ca^{2+} standard solution. (b) Intracellular Ca^{2+} concentration when cells were in the Ca^{2+} standard solution. Data were obtained from 3 independent experiments. The profile shown here is representative. The peak height is 0.08 ± 0.03 (mean $\pm$ S.D.). Intracellular Ca^{2+} ions were measured using 5 μM Fura PE3-AM. Cells were exposed to 10 mM octanal in the Ca^{2+} standard. The *Y*-axis in (b) represents the ratio of fluorescence intensity produced by the change in intracellular Ca^{2+} ions in HEK-293 cells expressing olfactory receptor I7. Solid line represents the response to 10 mM octanal, and dotted line represents the response to 0 mM octanal.

Fig. 3(b) shows the induction profile of intracellular Ca^{2+}. This intracellular Ca^{2+} response is faster than the field potential response shown in Fig. 3(a). As mentioned above, the change in the field potential was believed to have resulted from the inflow of Ca^{2+} ions from outside of the cell into the cytoplasm. The olfactory receptor I7 expressed on the surface of HEK-293 cells is known to be activated through the cAMP pathway. It has been shown that the increase in cAMP concentration after odorant–olfactory receptor binding induces an influx of Ca^{2+} ions (Ko and Park, 2006). The Ca^{2+} influx may subsequently activate Cl^- channels, and then Cl^- efflux across the cell membrane ensues. This makes the inside of the cell much more positive (Reisert et al., 2003). The different temporal dynamics between the membrane potential shift and intracellular Ca^{2+} concentration measurement was observed (Fig. 3(a) and (b)). This difference is considered to be due to the subsequent Cl^- efflux. Consequently, the subsequent change of Cl^- efflux in response to the increase in Ca^{2+} influx sustains the membrane potential shift for over 100 s, while the Ca^{2+} influx by odorant stimulation was transiently increased and returned to the basal level within 30 s as shown in Fig. 3(b). When the Ca^{2+}-free solution was used, a change in the field potential was not observed (Supplementary Fig. S1(c)). This result supports the hypothesis that the change in the field potential, shown in Fig. 3(a), was initially caused by an influx of Ca^{2+}.

3.3. *Effect of CNGgust channels on the field potential*

A large amount of ion influx into cells would be desirable to obtain highly sensitive measurements of the change in the field

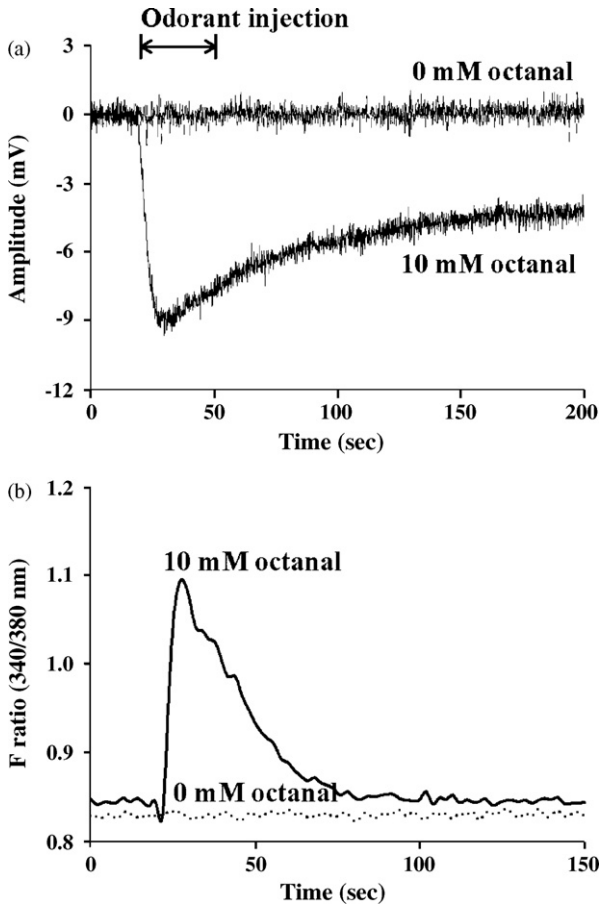

Fig. 4. Field potential and Ca^{2+} profiles of HEK-293 cells co-expressing the I7 and CNGgust channel. (a) Field potential profile of HEK-293 cells expressing co-expressing the I7 and CNGgust channel when cells were in the Ca^{2+} standard solution. (b) Intracellular Ca^{2+} concentration when cells were in the Ca^{2+} standard solution. Data were obtained from 3 independent experiments. The profile shown here is representative. The peak height is 0.23 ± 0.03 (mean ± S.D.).

potential. A plausible method for achieving this would be to transfect cells with a gene that encodes for an ion channel that when expressed generates a high influx of ions, such as the CNGgust channel. Therefore, to amplify the change in the field potential in this study, HEK-293 cells stably expressing I7 were transiently transfected with the rat CNGgust channel gene. The expression of the CNGgust channel in HEK-293 cells expressing I7 was confirmed by RT-PCR (Fig. 2(b)). HEK-293 cells co-expressing the I7 and CNGgust channel were stimulated with 10 mM octanal in the Ca^{2+} standard solution (Fig. 4(a)). The change in the field potential in HEK-293 cells expressing the I7 and CNGgust channel was approximately 10 mV, while it was about 4 mV in cells expressing only I7 (Fig. 3(a)).

Fig. 4(b) shows that the intracellular concentration of Ca^{2+} ions increased after odorant stimulation. Compared with the intracellular Ca^{2+} concentration shown in Fig. 3(b), the signal intensity was much higher in cells containing the CNGgust channel. The peak heights were 0.08 ± 0.03 and 0.23 ± 0.03 without and with CNGgust channel, respectively. These results indicate that the expressed ion channel protein was effective in increasing the amount of Ca^{2+} influx. There was no shift in the membrane potential after odorant injection when the Ca^{2+}-free solution was used (Supplementary Fig. S2(a)). These results indicate that the higher membrane potential was generated by the influx of Ca^{2+} ions through the CNGgust channel. It is also worth noting that this ion channel did not generate a spontaneous response, the response was only observed after the injection of an odorant.

In the presence of 10 mM $MgCl_2$, which blocks the CNG channel, a field potential was not generated in HEK-293 cells co-expressing the I7 and CNGgust channel (Supplementary Fig. S2(b)). This result indicates that the CNG channel blocker completely blocked the CNGgust channel and other endogenous ion channel, preventing the influx of Ca^{2+} ions. This result supports the notion that the change in the field potential was caused initially by an inflow of Ca^{2+}. These combined results indicate that the odorant-induced membrane potential was generated through the CNGgust channel together with other intrinsic transmembrane ion channels in HEK-293 cells co-expressing the olfactory receptor and CNGgust channel.

3.4. Dose-dependent extracellular potential response to odorant

In order to confirm the dose-dependent relationship between odorant concentration and field potential in the presence of the CNGgust channel, cells co-expressing the I7 and CNGgust channel were stimulated with various concentrations of octanal. From these experiments, the membrane potential was shown to increase with odorant concentration (Fig. 5). The most important factor contributing to the successful recording of a change in the field potential in this study was a high expression of the olfactory receptor and CNGgust channel on the cell surface. However, the sensitivity of the olfactory receptor I7 to octanal in this planar electrode turns out to be relatively low in comparison with other methods. The dose–response profiles have been reported in the range of 10^{-6} to 10^{-1} M for cell-based electroolfactogram (Zhao et al., 1998), 10^{-3} M for cell-based extracellular potential recording which is non-olfactory technique (Haruyama et al., 2003), 10^{-6} to 10^{-5} M for olfactory cell-based light-addressable potentiometric sensor (Liu et al., 2006), 10^{-9} to 10^{-5} M for nanosomes containing olfactory receptor-based SPR (Vidic et al., 2006), and 10^{-11} to 10^{-2} M for olfactory receptor-based QCM (Ko and Park, 2005). The low sensitivity of this system is most likely due to a slow ionic flow into the cell, the geometrical characteristics of the cell-substrate contact, and the coupling capacity of the olfactory receptor-CNGgust channel.

In this work, the sensitivity of the microelectrode-based olfactory biosensor was improved by introducing a high influx ion channel into the cell membrane. The sensitivity might be further improved if we activated the cGMP signaling pathway rather than the cAMP signaling pathway. The CNGgust channel in the gustatory neuron has been reported to be activated by both cAMP- and cGMP-signaling pathways (Meyer et al., 2000; Misaka et al., 1997). The sensitivity of the CNGgust channel to cGMP is about 100 times higher than of cAMP. Therefore, if the heterologous cell system

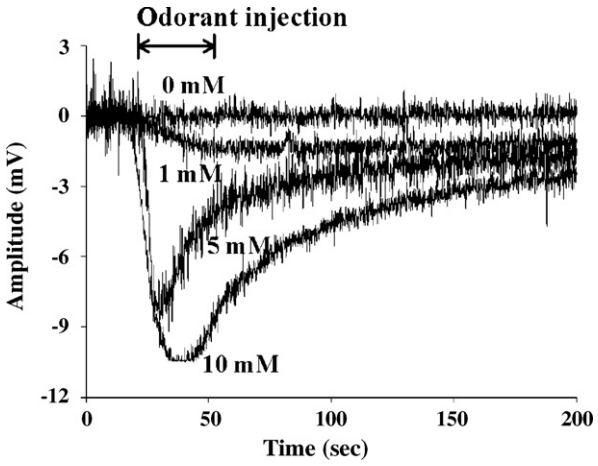

Fig. 5. Dose-dependent response of HEK-293 cells co-expressing the I7 and CNGgust channel.

can be designed to use the cGMP pathway rather than the cAMP pathway, the sensitivity would be further enhanced.

4. Conclusion

A microfabricated planar electrode was used to measure odorant-induced cellular responses. To increase the sensitivity of this measurement, high influx ion channels were introduced into the cell membrane. For this purpose, CNGgust channels were expressed in the cell membrane by transfecting the cells with the CNGgust channel gene. The presence of CNGgust channels in the cell membrane increased the Ca^{2+} influx into the cell, which resulted in a higher field potential. The field potential increased 2.5 times by introducing the CNGgust channels. This cell-based olfactory biosensor, which used a microfabricated planar electrode for detection, effectively converted the odorant concentration information into an electrical signal. This would provide a simple and convenient way to quantitatively measure the presence of odorant molecules.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.01.035.

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