Ion channels incorporated in nano-lipid bilayer and cell membrane for taste sensor

QINGJUN LIU*, JINJIANG YU[a], ZHAOYIN HU, DIMING ZHANG, QIAN ZHANG, YANLI LU, PING WANG, MO YANG[a]
Biosensor National Special Laboratory, Department of Biomedical Engineering, Zhejiang University, Hangzhou, P. R. China
[a]Department of Health Technology and Informatics, The Hong Kong Polytechnic University, Hong Kong, P. R. China

Biological studies have elucidated the gustatory detection of taste receptor cells often takes place on ion channels or receptors in cell membrane. In this study, we build electronic tongues based on ion channels of channel-active peptides gramicidin and taste receptor cell membrane. Peptides gramicidin was incorporated in lipid bilayer membranes supported with highly ordered nanopores of anodic aluminum oxide (AAO). By impedance analysis, this biomimetic sensing systems can separate tastants by their functional characters to ion channels. We also fabricate a taste sensor through an electrophysiological measurement of taste cells with natural ion channels in the cell membrane by microelectrode array (MEA). Based on the above study, we indicate that the detecting platforms can separate taste with characters similar to biology detection by natural ion channels and will be useful to study special ion channels or receptors with potential detecting for taste.

(Received September 18, 2012; accepted July 11, 2013)

Keywords: Taste sensor, Ion channel, Nanopores, Lipid bilayer membranes, electrophysiological measurement

1. Introduction

Traditional taste sensors, also known as electronic tongue, with global selectivity are composed of different lipid/polymer membranes for transforming information of taste substances into electric signals [1-3]. The different output of the response electric potential patterns for chemical substances shows different taste qualities, such as saltiness, sourness, bitterness, and sweetness. These sensors, as a gustatory biomimic technique for human beings and animals, can analyze and quantify the tastants. Those lipid membranes based techniques have been successfully employed for analysis of food, drugs, and environment monitors [4-6].

However, lots of biological studies have elucidated that the gustatory detection of taste receptor cells often takes place on ion channels or receptors of cells membranes, but never on lipid membrane itself [7]. Those transmembrane proteins of ion channels, ligand-gated channels, and G-protein coupled receptors all have been well illuminated as cellular sensing component for sensory qualities in recent years [8,9]. On binding taste molecules, the receptors trigger transduction cascades and thus cause excitation of the nerve fibres. The signals are carried to the brain, where central taste processing begins, and ultimately recognized as certain kinds of taste. Therefore, ion channels and receptors are the first molecular encounters with tastants, they provide the molecular specificity of the taste response.

Ion channels and receptors can be purified and reintegrated into lipid membranes for studies of cellular biology and pharmacology. It also has spurred initial interest in the development of ion channel/receptor-based biosensors, because their immobilization conditions were closely like the natural cellular environment [10,11]. Especially, advances in the development of nanoporous substrates for electrochemical characterization of membrane protein-containing lipid bilayers have greatly improved techniques for lipid membrane self-assembly and membrane protein incorporation on these substrates [12,13]. Anodic aluminum oxide is one of the particular interested nanoporous membranes due to its excellent biocompatibility as well as the established simple fabrication process [14,15]. With precise pore diameter and length achieved, anodic aluminum oxide can be used as solid supported membrane to lipid membranes, and appear to be well suited for the development of membrane biosensors with fully functional transmembrane ion channels [16,17].

In our previous studies, we have tried to use whole cells with natural receptors and ion channels as the sensing elements for electronic nose and electronic tongue [18,19]. In this study, we try to build electronic tongue based on ion channels of gramicidin incorporated in lipid bilayer

supported by highly ordered nanopores of anodic aluminum oxide for taste sensors, and employ microelectrode array (MEA) to detect electrophysiological activities of taste epithelium with natural ion channels by an in-situ physiological monitoring.

2. Experimental

2.1 Fabrication of the nanopores

The aluminum layer was anodized to form nanoporous membrane, following the standard two-step oxidation procedures [14,15].

As shown in Fig. 1A, the first anodization process took place in 0.25 M oxalic acid, with aluminum used as anode, while copper (Cu) sheet was used as cathode. Anodization voltage was controlled by a high-precision linear DC power supply (WWL-LDX, China). Owing to the decreasing current with the increase of the thickness of the barrier layer, the formation of pores was initiated at defect positions in the aluminum surface. Then, aluminum oxide and hydroxide at the pore walls were preferentially dissolved by the acidic electrolyte which consisted of 4 wt% chromic acid and 8 wt% phosphoric acid in the etching step. The second anodization step was then repeated in 0.25 M oxalic acid for about 4 hours. Finally, the amorphous barrier layer of alumina was etched away using a solution of 8 wt% phosphoric acid and 0.1M $CuCl_2$. Fabricated nanopores were observed by scanning electron microscope (SEM, JSM-6335F, JEOL, Japan) and atomic force microscope (AFM, Digital Instruments Inc., USA).

2.2 Incorporated Ion channels in lipid bilayer

Lipid solution can be deposited on the anodic aluminum oxide surface and form bilayer via self-assemble procedure on the gold-coating surface [16,17].

In our study, gold coating of the alumina surface was achieved by sputtering 10 nm titanium as an adhesive layer followed by a 25 nm gold layer using a sputter coating. Then, the gold-coated surface was being functionalized by incubated in a 1 mM ethanolic solution of 1,2-Dipalmitoyl-sn-glycero-3-phosphothioethanol (DPPTE, Avanti Polar Lipids Inc, USA) for 12 h, which rendered the gold-coated surface hydrophobic by their -SH group linked to gold. After thoroughly rinsing with ethanol and drying by nitrogen, the porous sample was mounted in the electrochemistry detecting Teflon cell (Fig. 1B). Then, the surface surrounding was primed with 1,2-diphytanoyl-sn-glycero-3-phosphocholin (DPhPC, Avanti Polar Lipids Inc, USA) in pentane. Finally, 10 μL of DPhPC in n-decane were painted over the DPPTE-functionalized surface. The nanopores supported bilayer lipid membrane was formed with DPPTE and DPhPC.

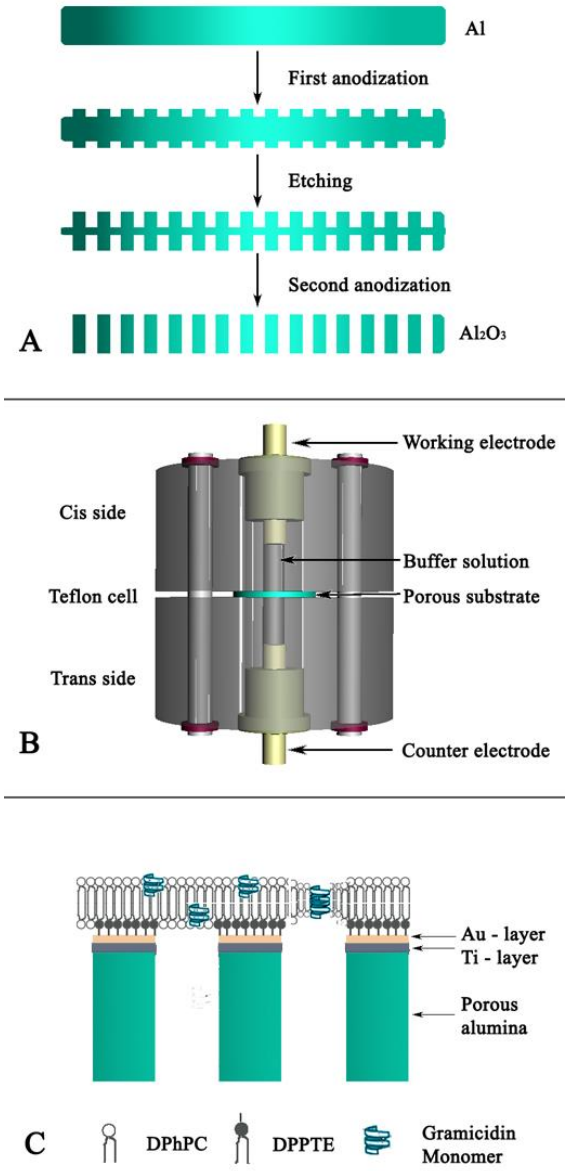

Fig. 1. Fabrication of nanoporous anodic aluminum oxide by two-step oxidation procedures (A), device of the nanoporous alumina for lipid form and impedance detection (B), and ion channels of gramicidin incorporated in lipid bilayer supported by nanopores (C).

Functionality of incorporation of channels active peptides into the supported membrane is demonstrated by inserted channel-active peptides of gramicidin. The channel-active peptides gramicidin D (in 0.1 M Na_2SO_4, Sigma-Aldrich) was inserted to the lipid bilayer. Ion channels were formed by two gramicidin monomers, each diffused in one monolayer of the lipid bilayer (Fig. 1C).

2.3 Impedance detection of tastants

The impedance spectra were performed using the impedance analyzer of HP 4192 A (Hewlett Packard, USA). Platinum and Ag/AgCl wires, respectively, immersed in the electrolyte solution on both sides, serve as working (cis compartment) and counter electrode (trans compartment) (Fig. 1B). The impedance between voltage and current was recorded within a frequency range of 1 Hz to 1 MHz. A pure sinusoidal AC voltage of 10 mV amplitude (peak-to-peak) was applied and 100 data points per frequency decade chosen to be equidistant on the logarithmic scale were recorded, which took about 7 min to complete a frequency domain scan. Then, impedance changes to tastants were measured at a fixed frequency (100 kHz) during the impedance sweep interval to obtain real time recording.

Taste is comprised of basic qualities: sourness produced by hydrogen ions of HC1, acetic acid, citric acid, etc.; saltiness produced mainly by NaCl; sweetness due to sucrose, glucose, etc.; bitterness produced by quinine, $CaCl_2$ and $MgSO_4$. So, we monitored impedance response after four basic tastants (0.3 mol/l NaCl for salty; 0.1 mol/l glumate and 0.01 mol/l HCl for sour; 0.5 mol/l sucrose for sweet; 0.01 mol/l $CaCl_2$ and 0.03 mol/l $MgSO_4$ for bitter) applied to ion channels. All of these tastansts were purchased from Sigma-Aldrich, USA.

2.4 Electrophysiological measurement

The rat was anesthetized by intraperitoneal injection of urethane. Taste epithelium (about 5 mm × 5 mm) was isolated from rat tongue and fixed on the surface of a 36-channel MEA. The electrodes were 30 μm in diameter with 200 μm center to center spacing. The USB-ME16-FAI system from Multichannel Systems (MCS, Reutlingen, Germany) was applied to record 16-channel signals. Noise of blank measurement was about 10 μV. The software of MC RACK (MCS, Reutlingen, Germany) and MATLAB were used to display and analyze the signals.

In the experiments, we used 0.01 mol/l HCl and 0.3 mol/l NaCl solutions (Sigma Aldrich) as sour and salt stimuli to taste epithelium. Before stimulation, native electrophysiological activities of the epithelium were recorded for 5 min. After one addition of solutions was injected into the MEA chamber, the recording lasted for about 5 min. Then the stimulus was washed out from the chamber by fresh standard perfusate.

3. Results and discussion

3.1 Alumina nanoporous

The two-step anodization process is capable of fabricating highly ordered nanopores array in the range of 50-120 nm depending on the anodization voltage. The first anodization time mainly affects the pore arrangement, and the second anodization time influences the pore length. Observed by SEM and AFM, alumina nanopores were successfully fabricated by the two-step anodization (Fig. 2).

Studies have found that solvent-free solid-supported lipid bilayers of aperture diameters in the sub-100 nm range, which will be more compatible with sensitive transmembrane proteins, can be formed by self-assembly compared with the membranes micro-scale apertures [12,13]. Instead of single nanopores, porous array consisting of densely packed pores will avoid leakage currents and enable working with lower protein concentrations or alternatively with proteins that have a low charge translocation rate, such as ion-transporter. Ion-channel and receptor protein functionality was measured for up to 10 h and the blocking capacity of the membrane was observed for up 6 days.

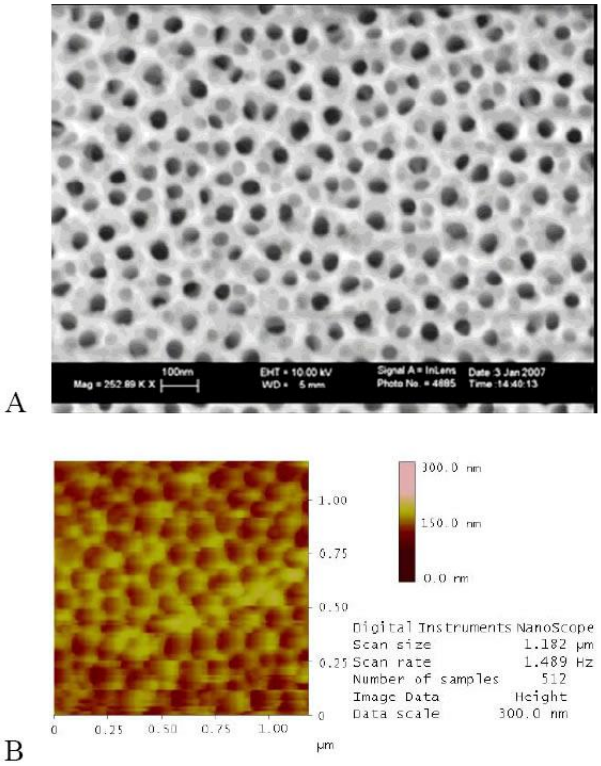

Fig. 2. Scanning electron microscope (A) and atomic force microscope (B) image of nanoporous anodic aluminum oxide.

3.2 Impedance of ion channels in lipid bilayer

The properties of alumina were demonstrated by impedance detecting. Fig. 3A shows the impedance spectrum of the nanopores with lipid layer. The impedance difference with and without lipid bilayer on the surface of nanopores was very significant, which proved the impedance change would be very sensitive to ion channels formed in it. Insertion of gramicidin took place quite

rapidly, resulting in a decrease in resistance from 10^5 to 10^4 Ωcm^2 during the measurement in 40 min. The stability of the membrane can keep intact and almost all pores covered in 70 hours.

Gramicidin is a peptide antibiotic produced by *Bacillus brevis* and toxic to gram-positive bacteria. The alternating *D*- and *L*-amino acids of the gramicidin make the formation of a single-stranded right-handed β-helix possible. The hydrophobic side chains point away from the axis and are oriented towards the lipid. The interior of the helix is an open cylindrical pore lined with polar carbonyl groups. Each gramicidin molecule can span one monolayer of a lipid membrane. By means of impedance spectroscopy as an integral method, peptide insertion and channel activity suggested this incorporation of channels active peptides into the supported membrane should demonstrate a good model of ion channel based biosensors.

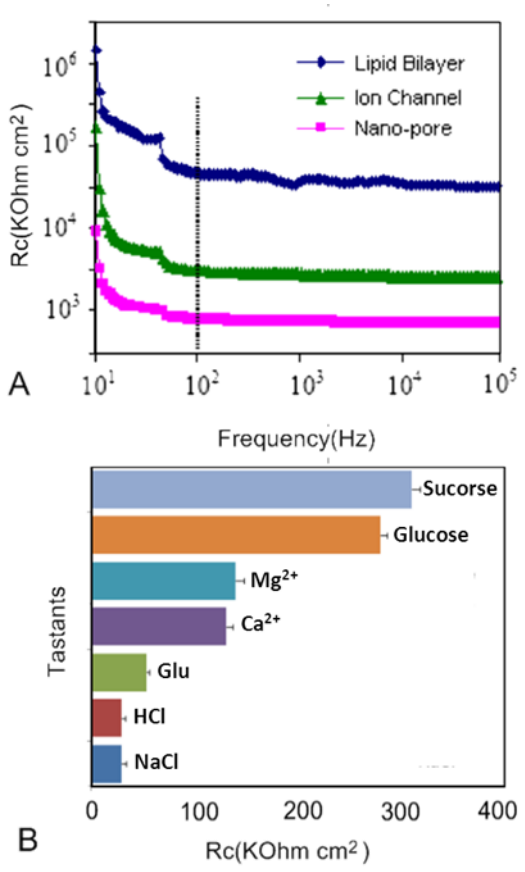

Fig. 3. Impedance detection of ion channels in lipid bilayer. (A): Impedance characterization of nanopores, lipid bilayer, and ion channels. Broken line at 100 Hz is the selected as fixed frequency for taste measurement. (B): Impedance characterization of different tastes at 100 Hz (n=6).

3.3 Tastants detection with lipid layer

Using lipid layer, existing taste sensors can detect some physics-chemical taste properties of the taste substance. However, those studies have mainly been investigated from the lipid membranes themselves, and have not paid enough attention to the protein receptors or ion channels. There are different cellular mechanisms to different taste [8,9]. Salty, sour, and some bitter tastes are elicited by ions. These stimuli function via permeation or modulation of ion channels. Transduction mechanisms for sugars, however, are believed to involve membrane receptors, G-protein and second messengers.

Our impedance results as Fig. 3B shows a significant decrease of impedance at 100 Hz for salty (NaCl) and sour (glumate and HCl). However, the impedance which decreased for bitter ($CaCl_2$ and $MgSO_4$) was only about 30% to salty or sour. However, the impedance of sweet (sucrose and glucose) changed very little. Maybe the reason is that the ion selectivity of lipid membrane containing gramicidin for transport of monovalent cation (i.e. Na^+ for salt and H^+ for sour) is greater than that for divalent cation (i.e. Ca^{2+} and Mg^{2+} for bitter), and very little for organically molecules (i.e. for sweet). Studies have shown that the ion selectivity of the bilayer lipid membranes containing gramicidin D for transport of mono-valent ions is the greatest for ammonium ion relative to sodium and lithium [20]. The ions transport through ion channels plays a significant role to above different tastants. And, if characteristic impedance to different ions illuminated, it can be used to classify tastants with a view to ion channels. At the same time, the membrane resistance of nanoporous supported lipid bilayer shown to 10^5 to 10^6 Ωcm^2 in our studies. If the high membrane resistance obtained in the GΩ regime, it should be ideally suited for low noise electrical recording of transmembrane ion currents. The current recording to single-channels also can be used to classify tastants in the next step. Taste receptors/ion channels and the transmembrane potential detection transducers can be combined as a hybrid system as a real bionic technique for tastants detection.

3.4 Tastants detection with electrophysiological measurement

Taste epithelium preserved the natural structures of basic receptor cells population with ion channels. In taste stimuli, the open of special ion channels will evoke extracellular potential changes, which can be measured with the multi channels MEA in our experiments. In fact, HCl at 0.01 mol/l and NaCl at 0.3 mol/l were applied as taste stimuli of sour and salt to active natural ion channels scattering on taste receptor cells of taste epithelium in our electrophysiological recording.

Fig. 4A showed electrophysiological signal trains detected by MEA in stimuli of PBS (phosphate buffered saline), NaCl and HCl. It indicated the potential firing of taste epithelium with natural ion channels in conditions of spontaneity, salt and sour stimulations. For each event, values of amplitudes were maintained at the range from 30

μV to 100 μV with variations between administrations of different taste stimuli, while durations were at the magnitude order of hundreds milliseconds. The amplitude characterization of potential firing was also analyzed with mathematical statistics in Fig. 4B. Indeed, amplitude of potential firing showed similar features with the impedance detection. Compared to spontaneous condition, amplitude of signals recorded in stimuli of NaCl and HCl had larger value because of the open of ion channel for taste sensing on taste receptor cells. It was believed that these electrophysiological investigations to natural ion channels on biological tissue membrane can provide a potential approach to design of artificial taste sensors.

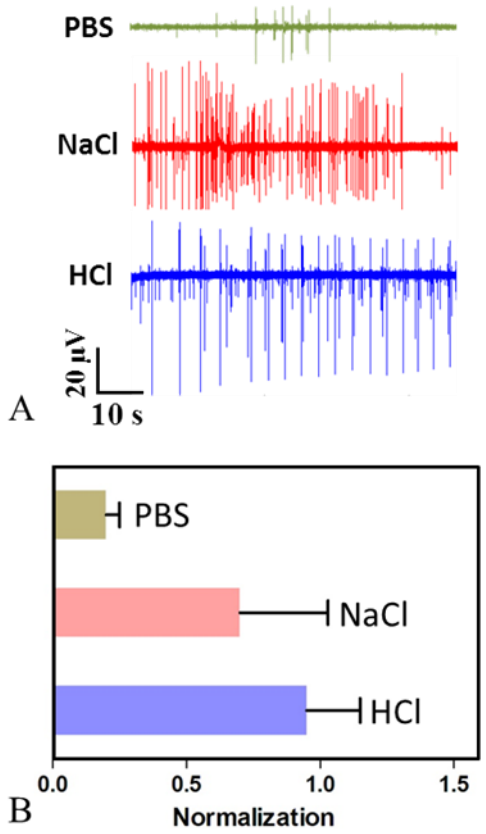

Fig. 4. Electrophysiological measurement in taste epithelium with ion channels. (A): Electrophysiological signal trains detected by MEA in stimuli of PBS, NaCl and HCl. (B): normalized amplitude characterization in stimuli of different tastes.

($n \geqslant 13$).

4. Conclusions

The nanopores of aluminum are anodized by the two-step oxidation procedures. Ion channels active peptides of gramicidin are incorporated in lipid bilayer supported by nanopores array. Electrophysiological activities of taste epithelium with natural ion channel were also detected. Results of impedance spectroscopy and electrophysiological measurement suggest this novel biomimetic taste sensing systems can separate taste by their functional characters similar to biology detection mechanism to ion channels on taste receptor cells. It displays a useful platform for special ion channels or receptors with potential detection for special kinds of taste.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant No. 81071226), the Zhejiang Provincial Natural Science Foundation of China (Grant No. LR13H180002).

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*Corresponding author: qjliu@zju.edu.cn