Biomed Microdevices (2012) 14:1047–1053
DOI 10.1007/s10544-012-9702-3

An ATP sensitive light addressable biosensor for extracellular monitoring of single taste receptor cell

Chunsheng Wu · Liping Du · Ling Zou · Luhang Zhao · Ping Wang

Published online: 6 September 2012

Abstract Adenosine triphosphate (ATP) is considered as the key neurotransmitter in taste buds for taste signal transmission and processing. Measurements of ATP secreted from single taste receptor cell (TRC) with high sensitivity and specificity are essential for investigating mechanisms underlying taste cell-to-cell communications. In this study, we presented an aptamer-based biosensor for the detection of ATP locally secreted from single TRC. ATP sensitive DNA aptamer was used as recognition element and its DNA competitor was served as signal transduction element that was covalently immobilized on the surface of light addressable potentiometric sensor (LAPS). Due to the light addressable capability of LAPS, local ATP secretion from single TRC can be detected by monitoring the working potential shifts of LAPS. The results show this biosensor can detect ATP with high sensitivity and specificity. It is demonstrated this biosensor can effectively detect the local ATP secretion from single TRC responding to tastant mixture. This biosensor could provide a promising new tool for the research of taste cell-to-cell communications as well as for the detection of local ATP secretion from other types of ATP secreting individual cells.

Keywords Light addressable · Taste receptor cells (TRCs) · Adenosine triphosphate (ATP) secretion · Biosensor

C. Wu · L. Du · P. Wang (✉)
Biosensor National Special Laboratory, Key Laboratory for Biomedical Engineering of Ministry of Education, Department of Biomedical Engineering, Zhejiang University,
Hangzhou 310027, China
e-mail: cnpwang@zju.edu.cn

L. Zou · L. Zhao
Department of Biochemistry and Genetics, School of Medicine, Zhejiang University,
Hangzhou 310058, China

1 Introduction

Adenosine triphosphate (ATP) is essential to all cells since it is not only a universal energy source for all cell functions, but also a pivotal neurotransmitter for extracellular signaling in various tissue and organs (Burnstock 2008; Fields and Burnstock 2006; Abbracchio et al. 2009). In taste buds, ATP has been considered as the key afferent neurotransmitter for taste signal transmission and procession (Finger et al. 2005). It has been demonstrated that ATP is secreted from type II taste receptor cells (TRCs) through specific hemichannels in response to electrical stimulation (Romanov et al. 2007, 2008) and chemical stimulation (Hayato et al. 2007; Huang et al. 2007). However, the detailed mechanisms and functions of ATP secretion in taste buds are still far from the complete understanding. To advance the research of taste sensation, it is highly desirable to develop novel methods or tools for the measurement of ATP secretion with high sensitivity and specificity, especially for the measurement of local ATP secretion from single TRC.

The stand luciferin-luciferase assay has been employed for the measurement of bulk concentration of ATP secretion from taste buds (Finger et al. 2005). However, the bulk ATP concentration is usually different from the local ATP concentration in the vicinity of the cell surface due to the fast diffusion and degradation of ATP. To address this problem, cell-based ATP sensitive biosensors were developed using cells transfected with recombinant ATP receptors as sensitive elements and changes in intracellular Ca^{2+} concentration as readout signals (Hayashi et al. 2004). The cell-based ATP sensitive biosensors have been widely used for the measurement of local ATP secretion from single TRC (Romanov et al. 2007; Hayato et al. 2007; Huang et al. 2007; Romanov et al. 2008). However, the cell-based ATP sensitive biosensors have some intrinsic difficulties and limitations such as tedious preparation of biosensor cells, sophisticated responses of biosensor cells to ATP, and precise manipulation of biosensor cells closely to target cells

for local ATP measurement. This has greatly limited their practical application for routine measurements.

ATP measurement based on other strategies such as cell surface attached firefly luciferase (Beigi et al. 1999; Nakamura et al. 2006), myosin-functionalized cantilevers (Schneider et al. 1999), and enzyme-functionalized Pt microelectrode (Patel et al. 2011), have also been developed for local ATP measurement. However, these methods suffered from the requirements of specially designed cell lines or biomolecules as well as spatial limitation on the measurement active sites. Aptamers are nucleic acid ligands that can bind to a great variety of molecular targets with comparable affinity and specificity to those of antibodies (Tuerk and Gold 1990; Nutiu and Li 2005). ATP sensitive aptamer has become a promising candidate to serve as sensitive element in ATP detection sensors due to its decisive advantages such as high stability, high performance, and low cost (Li and Ho, 2008). On the other hand, it has been demonstrated that light addressable potentiometric sensor (LAPS) chip, which is a silicon-based surface potential detector (Hafeman et al. 1988), can achieve single cell measurement by illuminating the desired cell with a modulated laser (Xu et al. 2005; Wu et al. 2009). The combination of aptamer and LAPS chip may provide novel solutions to the problems encountered in the development of ATP sensitive biosensors for single cell extracellular monitoring.

Here, we developed an aptamer-based biosensor on the basis of LAPS chip for the detection of local ATP secretion from single TRC. In this biosensor, aptamer that can specifically bind to ATP was used as recognition element. Its DNA competitor (with full complementary sequence to ATP aptamer) was covalently immobilized on the surface of LAPS chip to capture ATP aptamer via DNA hybridization. ATP detection performance of this biosensor was investigated and sensor specificity, stability, sensitivity and reproducibility were assessed. TRCs were isolated from Sprague–Dawley rats and cultured on the surface of LAPS chip functionalized with ATP sensitive aptamer. By illuminating the desired single TRC by means of a focused laser, local ATP secretion from single TRC was detected by recording the working potentials shifts of LAPS chip.

2 Theories

ATP secretion plays important roles in cell-to-cell communications for linking taste buds to the central nervous system (Finger et al. 2005). Type II TRCs are responsible for sensing taste of sweet, bitter, and umami, which lack classical synaptic connection with taste afferent nerve (Finger et al. 2005; Romanov et al. 2007; Huang et al. 2007). The secreted ATP from Type II TRCs functions as neurotransmitter, which can stimulate P2X2 and P2X3 receptors at the afferent nerve endings transmitting the signal to the central nervous system (Romanov et al. 2007; Hayato et al. 2007). In addition, ATP can also stimulate type III TRCs to secrete a second transmitter, serotonin (5-HT) (Huang et al. 2005). Type III TRCs sense sour signals, which have synaptic contact with taste afferent nerve for taste signal transmission and use 5-HT as a neurotransmitter (Kataoka et al. 2008). Figure 1(a) shows taste signal transduction pathway of Type II TRCs in vertebrate. Type II TRCs express taste receptors, which belong to the family of G protein-coupled receptors (GPCRs). The specific binding of taste compounds to taste receptors (R) activates specific G proteins (G), which can activate PLC-β2 to generate inositol triphosphate (IP_3). IP_3 can bind to IP_3R3 resulting in the release of Ca^{2+} from intracellular Ca^{2+} stores. This activates TRPM5 and finally leads to ATP secretion.

Figure 2(a) shows the detection principle of aptamer-based ATP sensitive biosensor. In this biosensor, ATP sensitive aptamer was used as sensitive element for ATP detection. ATP sensitive aptamer was hybridized with its DNA competitor that was covalently immobilized on LAPS surface. When exposed to ATP, aptamer separates from its

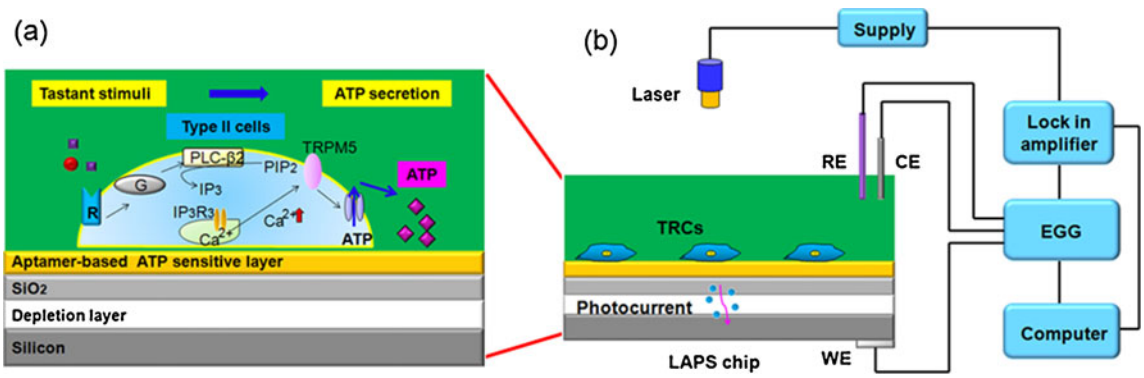

Fig. 1 Schematic diagram of (**a**) ATP secretion pathway in type II TRCs coupled to (**b**) LAPS measurement setup for the detection of local ATP secretion. *R* receptor; *G* G protein; *WE* working electrode; *RE* reference electrode; *CE* counter electrode

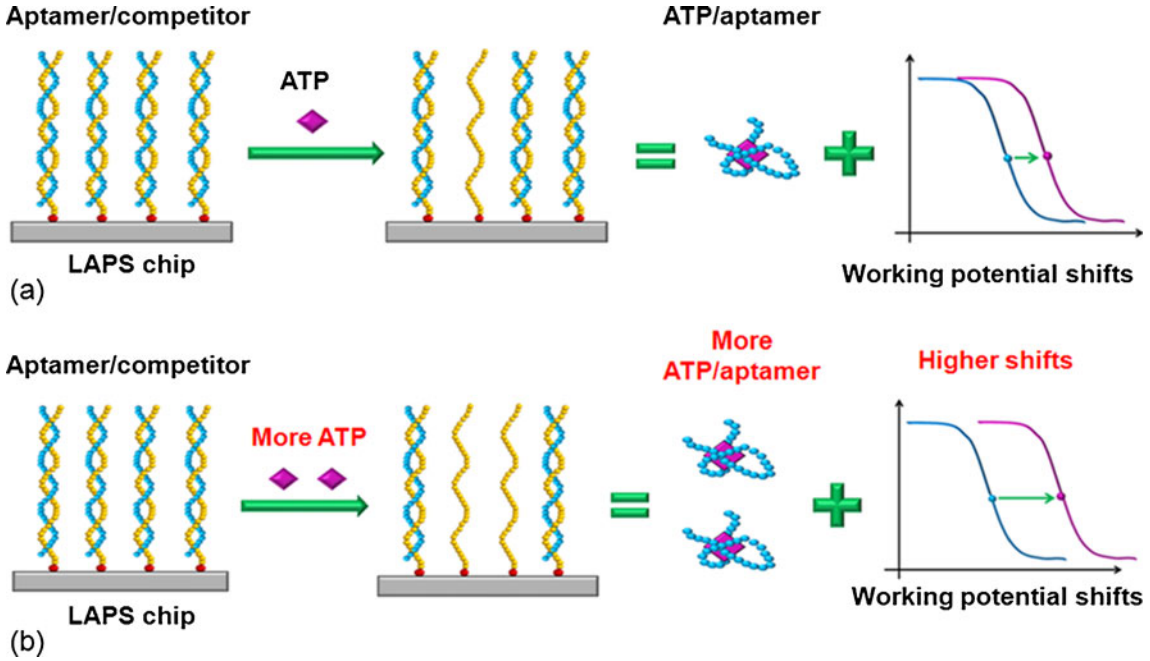

Fig. 2 Schematic diagram showing detection mechanisms of aptamer-based ATP sensitive biosensor on the basis of LAPS (**a**) at a certain ATP concentration and (**b**) at a higher ATP concentration

DNA competitor and binds to ATP. This leads to the changes in LAPS surface charges, which can be monitored by recording the working potential shifts of LAPS chip. Furthermore, as shown in Fig. 2(b), when the more ATP are presented, the more ATP sensitive aptamers will leave from the surface of LAPS chip, which resulting in the higher working potential shifts. Consequently, different concentrations of ATP could be detected by this aptamer-based ATP sensitive biosensor. LAPS chip is a silicon-based surface potential detector (Hafeman et al. 1988), which is sensitive to the local surface charge changes due to its light-addressable capability and field effect mechanisms (Poghossian et al. 2007). Figure 1(b) shows the basic detection mechanism of LAPS for local surface charge changes. When light illuminates LAPS chip, the light energy can be absorbed by the semiconductor to generate electron–hole pairs. With the bias voltage applied in depletion layer, the electron–hole pairs lead to the generation of photocurrent that can be detected by peripheral circuit. When TRCs are cultured on the surface of LAPS chip, the ATP secreted from TRCs leads to separation of aptamers from LAPS surface. This results in the surface charge changes of LAPS since aptamer is a kind of negatively charged molecules. At the illuminated local site, the surface charge changes lead to the generation of corresponding fluctuation in photocurrent, which can be detected by recording the working potential shifts, ie, shifts of I-V curve (ΔV) of LAPS chip. In addition, the more ATP presented, the more aptamers will separate from LAPS surface resulting in the larger working potential shifts. Consequently, local ATP concentration can be measured by recording the corresponding working potential shifts when the measurement site is illuminated.

3 Materials and methods

3.1 Materials

ATP sensitive aptamer (5′-ACC TGG GGG AGT ATT GCG GAG GAA GGT-3′) and DNA competitor (5′-ACC TGG GAA TAC TCC CCC AGG T −3′, 5′-end with C6-NH_2 modified) were obtained from Takara Biotechnology Co. Ltd., Dalian, China. TiO_2 sol–gel was prepared following previous reported work (Zong et al. 2009). Bovine serum albumin (BSA) was provided by Sino-American Biotechnology Co. Ltd., China. Tyrode's solution was homemade, which consisted of 126 mM NaCl, 5 mM KCl, 5 mM NaHEPES, 1.25 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$ (pH=7.4, adapted with NaOH). Tastant mixture solution consisted of 0.03 M $MgSO_4$ (bitter), 0.3 M sucrose (sweet), and 0.5 M monosodium glutamate (umami). Glutaraldehyde, serotonin, acetylcholine, noradrenaline, and glutamate were purchased from Sigma-Aldrich, USA. All other chemicals were of analytical pure grade or better quality, which were used without further purification.

3.2 LAPS chip fabrication and functionalization

The process of LAPS chip fabrication and functionalization was adopted from previous reported work (Zong et al. 2009). Briefly, LAPS chip fabrication was carried out on the basis of n-type silicon wafer with a specific resistance of 3–8 Ωcm. After extensively cleaning, the surface of silicon wafer was thermal oxidized with a layer of SiO_2 with thickness of 50 nm under 1,180 °C. A thin film of titanium dioxide (TiO_2) was deposited onto the upper side of silicon wafer by spin-coating of TiO_2 Sol–gel at the speed of 3,000 r/min for 10 s. Afterwards, the silicon wafer was dried in a furnace at 120 °C for 20 min and sintered at 500 °C for 60 min in air with the temperature raised at 10 °C/min. Then the back side of silicon wafer was treated with hydrofluoric acid (HF) to remove the SiO_2 layer and evaporated with a layer of aluminum with thickness of 1 μm to create an ohmic contact. The upper surface of silicon wafer was then irradiated with UV for 2 h to improve the following silanization level. Silanization was carried out by dipping the silicon wafer into the toluene solutions with 3-Aminopropyltriethoxysilane (APTES) concentration of 0.1 % (v/v) for 1 h and sealed under room temperature. Then the silicon was completely rinsed with toluene and ethanol, and cured at 120 °C for 1 h. A detection chamber with a 5 mm-diameter hole in the bottom center was then attached to the upper side of silicon wafer.

DNA competitor of ATP sensitive aptamer was covalently immobilized on LAPS chip surface by a crosslink method. For the first, amine residues introduced to LAPS chip surface by silanization was reacted with glutaraldehyde overnight at room temperature. Then DNA competitor was added allowing the reaction between amine residues of DNA competitor with aldehyde residues of TiO_2 film at room temperature for 12 h. After blocked by 1 % (v/v) BSA in phosphate buffered saline (PBS) to avoid any unreacted aldehyde residues and other non-specific binding sites on the TiO_2 film, ATP sensitive aptamer was added and captured by DNA competitor via hybridization for 4 h at room temperature. After rinsing with PBS to remove the excessive aptamer and the non-specific adsorption, the functionalized LAPS chips could be used for measurement or stored at 4 °C.

3.3 TRCs isolation, culture, and fluorescent staining

TRCs were isolated from Sprague–Dawley rats following previous reported method (Herness and Sun 1995; Chen et al. 2009). The intact tongue of rat was dissected after sacrifice. Tyrode's solution containing collagenase (2 mg/ml) and elastase (0.25 mg/ml) was then injected under the epithelium of the tongue. The lingual epithelium was gently peeled from the underlying connective tissue after incubation with divalent-free Tyrode's solution for 30 min. Afterwards, circumvallate taste cells were collected from lingual epithelium using a fire polished glass pipette. The collected cells were then transferred to the detection chamber allowing attachment to the surface of LAPS chip. The cells were cultured for 20 h at 37 °C under standard conditions of humidified air with 5 % CO_2 in DMEM (Dulbecco's Modified Eagle's Medium) (Gibco, UK).

For fluorescent staining experiments, cells were seeded on LAPS chip and incubated with primary antibody, mouse monoclonal anti-IP_3R3 (inositol 1,4,5-trisphosphate receptor type 3, diluted to 1:50, BD), in 0.1 M PBS overnight at 4 °C. After washing 3 times with PBS (pH 7.4), cells were incubated with secondary antibody, FITC conjugated goat anti-mouse IgG (diluted to 1:100, Jackson Lab), in 0.1 M PBS (pH=7.3) for 2 h at room temperature. After washing 3 times with PBS (pH 7.4), cells were fixed with 90 % glycerin in PBS and examined under a confocal fluorescence microscope (Leica TCS-SP, Germany).

3.4 Experimental setup and measurements

The experimental setup was similar to a previously reported one (Zong et al. 2009; Wu et al. 2009). Figure 1(b) also shows schematic diagram of experimental setup used in this study, which consisted of three electrodes. LAPS chip is used as working electrode. While Ag/AgCl electrode and Pt wire were used as reference electrode and counter electrode, respectively. The bias voltage between working electrode and reference electrode was supplied by a potentiostat (EG&G M273A Princeton Applied Research, USA). A modulated light (wavelength 543.5 nm, power 5 mW, 4 kHz) was generated by a He-Ne semiconductor laser (Coherent Co., USA) and focused to less than 10 μm, which could be moved on the surface of LAPS chip to illuminate the desired measurement site to generate local photocurrent. The photocurrent were subsequently transmitted into peripheral equipments and recorded by a lock-in amplifier (model SR830 DSP, Stanford Research Systems). A 16-bit data collection card and the software of LABVIEW were used to collect, analyze, and store the data. The shifts of I-V curve (ΔV) of LAPS chip (referred as working potential shifts) originated from local surface potential changes were recorded to reflect the local surface changes of ATP concentration.

The local ATP secretion from individual TRCs under different stimuli was detected by recording the working potential shifts of LAPS chip. The LAPS chip was fixed in a detection chamber with input and output pipes. During measurements, various stimuli were pumped alternatively into the detection chamber through input pipe by a peristaltic pump, which was controlled by a personal computer. HEK-293 cells were used as negative control. The experimental data was expressed as mean ± standard error of the mean (SEM). The significance was carried out by performing Student's *t*-test of the data. $P< 0.05$ was set as statistical significant threshold. All the data were analyzed based on different measurements from different cell culture under the same stimuli. All the measurements

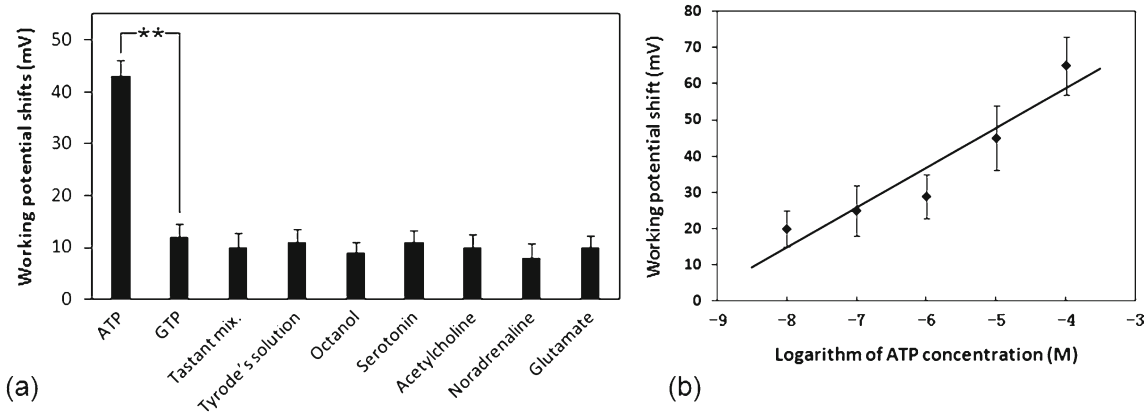

Fig. 3 Characteristics of aptamer-based ATP sensitive biosensor. (**a**) The specificity was tested using ATP and GTP at the same concentration of 5 μM. The working potential shifts of ATP (43±3.2 mV) are significantly higher than those of GTP (12±2.6 mV, $p=7.2\times10^{-8}$, $n=6$). The stability was tested by recording working potenrial shifts under various solutions including tastant mixture, tyrode's solution, 3 mM octanol, 1 mM serotonin, 0.1 mM acetylcholine, 1 mM noradrenaline, and 1 mM glutamate. (**b**) Responses of the biosensor to serial concentration of ATP. The mean and SEM of 6 experiments are shown

were carried out at room temperature about 25 °C. The whole setup was shield with a copper box to exclude ambient light and to minimize the influences of environmental factors to the measurements.

4 Results and discussion

4.1 Characterization of ATP sensitive biosensor

To access the specificity of this aptamer-based biosensor, specific target ATP and nonspecific target guanosine triphosphate (GTP) were tested. As shown in Fig. 3(a), the working potential shifts resulting from ATP introduction are significantly higher than those of GTP. The stability of this biosensor was tested using different solutions including Tyrode's solution, octanol, tastant mixture solution, serotonin, acetylcholine, noradrenaline, and glutamate. The results shown in Fig. 3(a) indicate that this biosensor shows no significant response to compounds presented in these solutions, which suggests good stability of the sensor for what concerns the tested solutions.

The reproducibility of this aptermer-based biosensor was also investigated. ATP sensitive aptamer was introduced to LAPS surface via hybridization with DNA competitor to replenish the aptamer loss after each measurement. The performance of this biosensor was stable within 5 times of regeneration (data not shown). The working potential shift in responding to the same ATP concentration decreased significantly after 5 times of regeneration. This may be due to the irreversible conformation changes of DNA competitor on LAPS surface after several measurements, which could cause dramatically loss in its affinity with aptamer. Improvements on the structure of aptamer are desirable to achieve higher reproducibility and self-renewable capability by molecular design.

In order to determine the sensitivity of this biosensor, a series of Tyrode's solutions containing different concentrations of ATP was tested. We used Tyrode's solution without any ATP addition as the negative control to obtain the baseline of working potential point by measuring the I-V

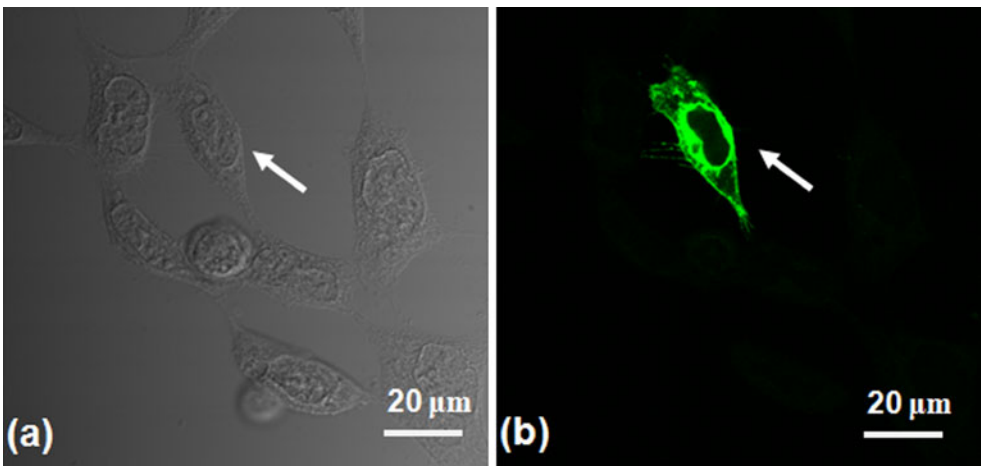

Fig. 4 Fluorescent staining images of type II TRCs taken under (**a**) optical field and (**b**) fluorescent field. *White arrow* indicates type II TRCs. Mouse monoclonal anti-IP_3R3 was used as primary antibody. FITC conjugated goat anti-mouse IgG was used as secondary antibody

curve of LAPS chip. When different concentrations of ATP were applied, I-V curve of LAPS chip were measured accordingly. The working potential shifts of each ATP concentration was calculated by comparing I-V curve shifts to that of negative control. The statistical results of the working potential shifts of each ATP concentration are shown in Fig. 3(b). The results indicate that the I-V curve moves towards more positive voltage when exposed to ATP. In the range from 10^{-8}M to 10^{-4}M, the working potential shifts increase with the ascension of ATP concentration, which indicate a linear relationship between them. We defined the sensitivity of this biosensor as follows:

$$S = \Delta V / \Delta C$$

Where S represents the sensitivity of this biosensor; ΔC represents the differences of two different ATP concentrations; ΔV represents the differences of working potential shifts resulting from the corresponding two different ATP concentrations. Following this definition, the calculation results indicate the sensitivity of this biosensor is 9 mV/μM. The limit of detection (LOD) of this biosensor is 2.6×10^{-8}M, which was calculated by the intersection point of the extrapolated linear region of the calibration curve with abscissa.

All the results demonstrate this aptamer-based biosensor can detect ATP with high specificity, stability, and sensitivity. It could be further used for the detection of local ATP secretion from individual TRCs.

4.2 Measurement of ATP secretion from single TRC

To validate that type II TRCs can functionally existed on the surface of LAPS chip, fluorescent staining experiments were carried out using mouse monoclonal anti-IP_3R3 as primary antibody, which is specific to IP_3 that was reported existed in type II TRCs but not in type III TRCs (Clapp et al. 2008). From Fig. 4, we can see the existence of type II TRCs on LAPS surface, which grows well with elongated and spindle shape.

To investigate the performance of this aptamer-based biosensor for the detection of local ATP secretion from single TRC, we used tastant mixture solution to stimulate ATP secretion from TRCs. While HEK-293 cells were cultured and stimulated by the same protocol to serve as the negative control, which is believed no ATP secretion could be induced under any stimulation mentioned above.

By illuminating the desired TRC and recording the working potential shifts of LAPS chip, the local ATP secretion from single TRC was measured. As shown in Fig. 5, the working potential shifts of TRCs under tastant mixture stimulation were significantly higher than those of HEK-293 cells. As for TRCs, the working potential shifts under tastant mixture stimulation were significantly higher than those of under ATP free tyrode's solution. The mean working potential shifts of tastant mixture stimulation was 42± 3.3 mV (n=6). Calculated by the linear relationship between working potential shifts and ATP concentration in the range from 10^{-8}M to 10^{-4}M as shown in Fig. 3(b), the local ATP concentration measured by this biosensor was around 4.87×10^{-6}M. This result is comparable with previous reported results achieved by biosensor cells (Huang et al. 2007). It has been demonstrated that ATP secretion from TRCs can be specifically inhibited by octanol, which is a compound that can specifically inhibit activity of hemichannels mediating ATP secretion (Romanov et al. 2008). In order to further confirm that our recordings of working potential shifts were originated from ATP secretion, TRCs were

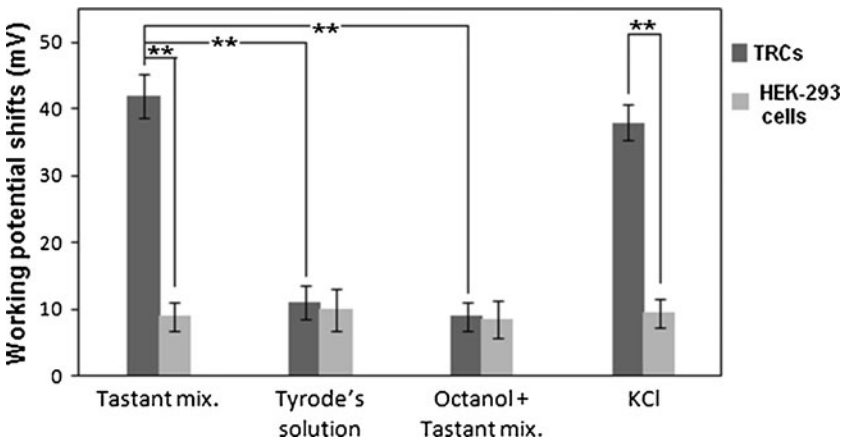

Fig. 5 Statistical working potential shifts of TRCs responding to various solutions. Working potential shifts of TRCs under tastant mixture (42±3.3 mV) are significantly higher than that of tyrode's solution (11±2.5 mV, $p=6.7\times10^{-8}$, n=6). Working potential shifts of TRCs under tastant mixture (42±3.3 mV) are significantly higher than those of 3 mM octanol pretreated TRCs under tastant mixture (9± 2.5 mV, $p=8.1\times10^{-7}$, n=6). Working potential shifts of TRCs under 100 mM KCl (38±2.6 mV) are significantly higher than those of HEK-293 cells under 100 mM KCl (9.5±2.1 mV, $p=7.6\times10^{-8}$, n=5). HEK-293 cells were used as negative control. All data are represented by the mean ± SEM. *p<0.01, Student's t-test

pretreated with octanol at concentration of 3 mM for 10 min incubation. By the comparison of recordings from the same TRCs before and after octanol treatment under tastant mixture stimulation, a significant decrease in working potential shifts can be observed, which indicates inhibitory effects of octanol on ATP secretion from TRCs. These results demonstrate that this biosensor can record the inhibitory effect of octanol to the responses of TRCs to tastant mixture. This could further prove the working potential shifts are originated from ATP secretion of TRCs.

We also monitored ATP release from TRCs upon stimulation of 100 mM KCl, which is a commonly used depolarizing solution. As shown in Fig. 5, the results indicate that ATP release from TRCs was detected when 100 mM KCl was applied, while HEK-293 cells show no response to the same stimulation of KCl. The results further demonstrate this biosensor can record the ATP release from TRCs mediated by hemichannels, which is a kind of membrane voltage-dependent release (Romanov et al. 2008).

5 Conclusion

This study aimed at developing an aptamer-based biosensor for the detection of local ATP secretion from single TRC. This biosensor showed good performances for ATP detection being able to efficiently detect local ATP secretion from individual TRCs under tastant mixture. Due to its high sensitivity, high specificity, and light addressability, this biosensor has great potential to be used as a promising tool for the research of taste cell-to-cell communications. It could also be applied in the detection of local ATP secretion from other types of individual cells. This is the first crucial step towards the development of aptamer-based ATP sensitive biosensors. In the future, we will focus on the improvement of DNA aptamer to achieve self-renewable capability after each measurement.

Acknowledgments This work was supported by the grants from the National Natural Science Foundation of China (Grant Nos. 30970765, 31000448), the China Postdoctoral Science Foundation (Grant No. 201104734) and the Key Project of Zhejiang Province (Grant No. 2010C14006).

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