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A novel label-free bioengineered cell-based biosensor for salicin detection

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ABSTRACT

Salicin is a bitter compound with important pharmaceutical values. The detection of salicin in pharmaceutical industry usually employs standard methods of analytical chemistry such as high performance liquid chromatography, which is not suitable for primary detection due to the complicated operation process and expensive systems. In this study, we explore the feasibility of using a label-free bioengineered cell-based biosensor to detect salicin. A human bitter taste receptor, T2R16, which could specifically respond to salicin, was expressed in HEK-293 cells as sensing elements of the biosensor. The structural and functional expression of T2R16 was validated by immunofluorescent staining and calcium imaging. The cell-impedance sensor was utilized as secondary transducer to detected cellular responses to salicin. The cell-impedance response of the bioengineered cells to salicin was investigated by analyzing the response intensity under various concentrations. The cell-impedance results revealed that this bioengineered cells could respond to salicin in a dose-dependent relationship. Finally, the performance of this bioengineered cell-based biosensor was evaluated. In cell-impedance measurement, non-transfected HEK-293 cells were not responsive to salicin, which proved the specificity of the biosensor. The detection of salicin at two unmeasured concentrations in standard response curve also proved this biosensor was sensitive in salicin detection. With the advantage of label-free measurement, this bioengineered may provide a promising and valuable approach for primary and quick detection of salicin.

1. Introduction

Bitter detection is an important issue in pharmaceutical industry as most drugs taste bitter. Salicin, a bitter tasting substance, is also an anti-inflammatory agent with high pharmaceutical values. It is commonly found in the bark of willow and Populus species. The chemical oxidation of salicin resulted in a salicylic acid, whose acetylated derivative is the one of the most successful drug in history, Aspirin. The quantitative detection of salicin is a common issue in pharmaceutical studies of plant medicine. Traditional detection of salicin usually employs standard methods of analytical chemistry such as thin layer chromatography [1], high performance liquid chromatography [2–5], which are superior in precise quantification but are not applicable in primary detection due to complicated and time-consuming operation process and expensive detection system. In recent years, label-free cell-based biosensor for drug detection has attracted great attention in drug discovery and screen due to numerous advantages of the assay. First of all, the cell-based biosensor presents specific cellular responses to various chemicals, which provides cell-level evaluation on drug pharmacology such as toxicity and function. Besides, no labeling of the ligand or G-protein-coupled receptor (GPCR), the largest family of the drug targets, simplifies the assay design and minimizes artifacts and liabilities created by the labeling process. Moreover, the detection process is usually quick and operation is simple and convenient.

Bitter taste is mediated by a subfamily of G-protein-coupled receptors (GPCRs), T2Rs (taste receptor, type II), which are ~40 highly specialized membrane receptors for bitter signal transduction [6,7]. As a bitter tasting compound, salicin is known to activate a member of T2Rs, bitter receptor T2R16. The activation of the bitter

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receptors induces a series of specific intracellular and extracellular responses including molecular and electrophysiological changes [8,9], which could be detected by cell-based sensing assay. Thus it is assumedly feasible to detect salicin by a bitter receptor cell-based biosensor.

In the development of cell-based biosensor for bitter detection, most studies use the primary cells or tumor-derived taste cell lines as sensing elements with natural elaborate taste receptors, which induce relatively specific cellular responses when being activated by bitter compounds. However, they are still insufficient for sensitive and selective detection of bitter compounds. The most important reason is that a bunch of bitter receptors were expressed in a single taste cell, which responds to various bitter compounds with similar cellular responses, namely lack of selectivity. Besides, the acquisition of taste receptor cells is relatively difficult due to the small cell quantity and sophisticated isolation operation. Thus, bioengineered cells specifically expressing certain bitter receptors provide a more feasible and valuable cell source as sensing elements for cell-based bitter biosensor.

Bioengineered taste receptor cells, usually used in functional characterization of receptors, are effective and sensitive in response to taste stimuli. It is reported that T2R16 can be activated by the β-glucopyranosides (salicin and related compounds), a family of bitter phytonutrients with specific chemical structures [10]. In the present study, T2R16, specifically respond to salicin, was expressed in heterologous HEK-293 cells as sensing unit. Studies have reported that the addition of the agonist for GPCRs will induce cell morphological changes in a very specific manner [11,12], which can be monitored by cell-impedance sensor [13–15]. Thus cell-impedance sensor was employed as secondary transducer in order to detect the cellular responses, mainly the morphological changes, of T2R16 activation. Non-transfected HEK-293 cells were used as control. To couple the T2R16 with bitter signaling pathway as well as to enhance the cellular responses, G protein chimera Gα16gust44 was also introduced into this heterologous expressing system. Prior to the cell-impedance measurement, immunofluorescent staining and calcium imaging were used to determine the structural and functional expression of bitter receptor. To testify the effectiveness of cell-impedance measurement in monitoring cellular responses induced by GPCR activation in our study, HEK293 cells' impedance responses to isoproterenol (ISO) were determined. HEK293 cells endogenously express G protein coupled receptors (β2 adrenergic receptor), which can be specifically activated by ISO. Then the biosensor responses to salicin was monitored and analyzed. Finally, the performance of this bioengineered cell-based biosensor was assessed to determine the selectivity and specificity of this biosensor. All the details are discussed in the following sections.

2. Material and methods

2.1. Reagents and animals

T2R16 agonist, salicin, and ISO were purchased from Sigma–Aldrich (St. Louis, MO). T2R16 plasmid and Gα16gust44 as virus assay were gifts from Dr. Liquan Huang. Experiments with animals were performed according to the protocols approved by the Institutional Animal Care and Use Committee of the Monell Chemical Senses Center.

2.2. Bioengineered bitter receptor cell establishment

Human bitter receptor, hT2R16, was expressed on the plasma membrane of HEK-293 cells. The transfection protocol was shown in Fig. 1b. Briefly, the expression plasmid hT2R16 was pCEP4/rho-hT2R16. After confirmed by DNA sequencing, the correct expression plasmids were used transfect HEK-293 cells using Lipofectamine™ 2000 (Invitrogen, USA) as DNA carrier. After 24 h, the chimeric G-protein Gα16gust44 was introduced into T2R16 transfected HEK-293 cells (HEK-293/T2R16/Gα16) and parental HEK-293 cells (HEK-293/Gα16) by virus assay [16]. HEK-293/Gα16 cells were used as positive control. These HEK-293 cells were ready for further measurements another 24 h later. Cells were 90% confluent or more or more upon the time of calcium or impedance measurement.

The expression of T2R16 was validated by immunofluorescent staining using anti-His6-tag rabbit IgG as the primary antibody and FITC conjugated anti-rabbit IgG as secondary antibody. The nucleus of HEK-293 cells was stained by 4, 6-diamidino-2-phenylindole

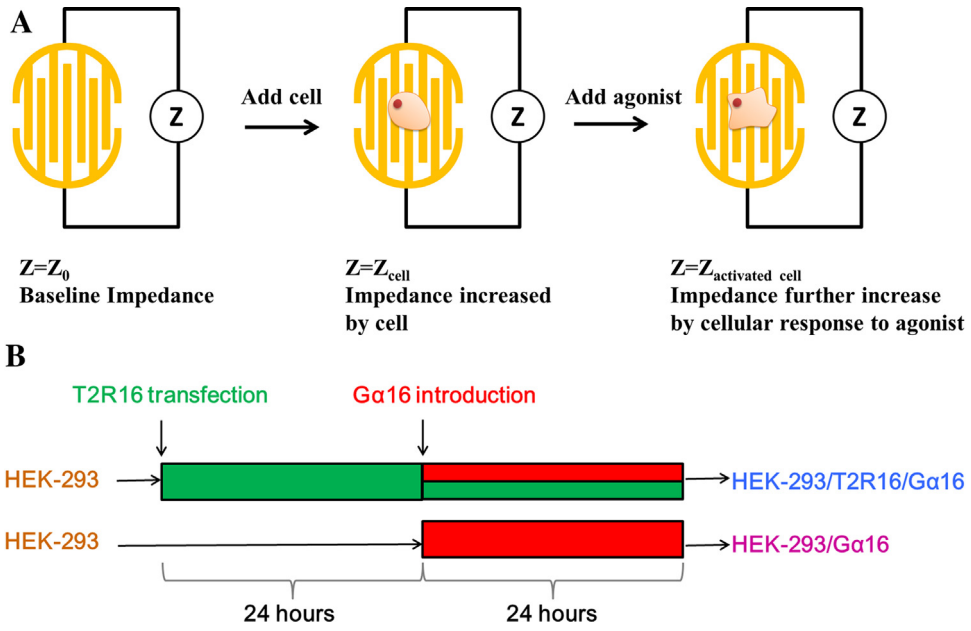

Fig. 1. A. Schematic principle of cell-impedance measurement in detecting cellular responses induced by GPCR activation. B. The protocol of T2R16 plasmid transfection and G protein chimera Gα16 introduction into HEK-293 cells. The upper is for HEK-293/T2R16/Gα16 cells and the lower is for HEK-293/Gα16 cells.

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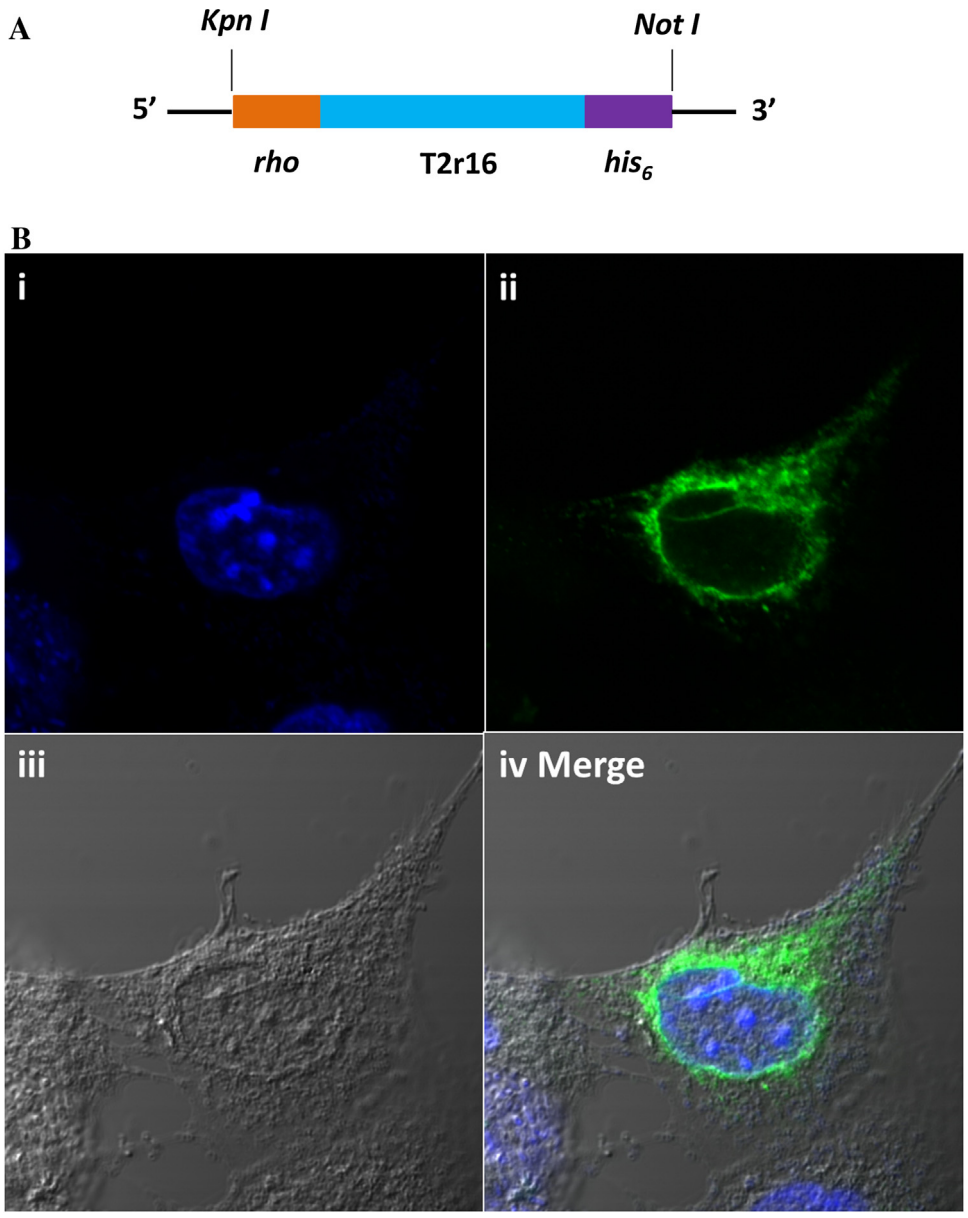

Fig. 2. A. The expression vector *pCEP4/rho-T2R16-His*$_6$. B. The fluorescent images show the expression of a human bitter receptor, T2R16, on the membrane of HEK-293 cells. i: The fluorescent image of nucleus of HEK-293 cell stained blue by DAPI. ii: The fluorescent image of bitter receptor T2R16 labeled green by FITC. iii: The image of HEK-293 cell in bright field. iv: The merge of the former three images. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

(DAPI) (Sigma–Aldrich, USA), which can pass through an intact cell membrane and bind strongly to A-T rich regions in DNA. Images were taken using a fluorescent microscope.

2.3. Calcium imaging of bioengineered taste receptor cells

To further validate the functional expression of T2R16 in HEK-293 cells, calcium imaging techniques were applied in our study. When the T2R16 transfected cells were 90% confluent or more in 96-well-plates, cytoplasmic calcium concentration was determined. Cells were loaded with a Ca^{2+} sensitive, membrane-permeable fluorescent dye Fluo-4/AM using a calcium assay kit (Molecular Probes, USA) for 30 min at 37 °C. The calcium signal was recorded by Flex Station III (Molecular Device, USA). Cells were excited at 494 nm, and signals at 516 nm were captured. For each well, the scanning duration is 40 s. All the procedures were out of light to avoid bleaching effects.

2.4. Cell-impedance measurement

The electrodes in the E-plates were made of thin gold film and coated with poly-lysine and laminin prior to experiments. The cell electrode impedance was mainly determined by cell quantity, adhesion and some dynamic changes such as morphology and migration. To quantify the cell-electrode impedance, a parameter termed cell index (CI) was derived as

$$\mathrm{CI} = \frac{Z_{\mathrm{cell}}(f)}{Z_{\mathrm{b}}(f)} - 1$$

where $Z_{\mathrm{b}}(f)$ and $Z_{\mathrm{cell}}(f)$ are the frequency-dependent electrode impedance without or with cells in the presence, respectively. For the cell-impedance measurement, the iCelligence system (ACEA Biosciences Inc., China) was applied. A sinusoidal voltage with amplitude of 20 mV and frequency of 10 kHz is applied on the interdigitated electrodes (IDEs) (Fig. 1a). Impedance values are usually

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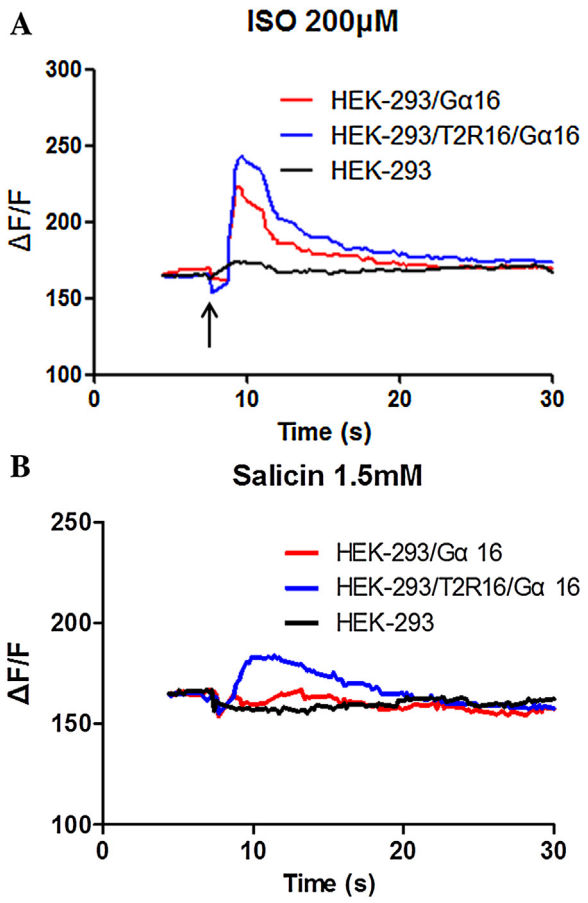

Fig. 3. A. Calcium response of three types of cells to ISO. ISO could elicit calcium release in the cell types with Gα16. However, in parental HEK-293 cells, ISO did not evoke calcium response. B. Calcium response of three types of cells to salicin. Only T2R16 expressing cells respond to salicin.

normalized by cell index (CI) values for comparison. The principle of cell-impedance measurement was shown in Fig. 1a. When the cells are located on the IDEs surface, the ion current is impeded, so the impedance of IDEs increases. The activation of GPCR by agonist leads to transient changes in cell morphology that also affect impedance values. Thus CI is a quantitative measure of the cell status in real-time.

2.5. Chemical application for cell-impedance measurement

Salicin and ISO solutions at 10-fold concentrations were freshly made as stimulating solutions with distilled H_2O from stock solutions. Transfected cells were cultured in the E-plates. All the solutions were kept at 37 °C in water bath upon application. Prior to chemical stimulation, the original medium were replaced with 180 μl fresh medium. After 30 min incubation, 20 μl of salicin or ISO was added to each well. The impedance responses to taste stimulation were recorded for 90 min.

2.6. Statistics

To quantify the CI response under various concentrations, the baselines of CI curves was normalized as 1 and ΔCI_t was derived as $\Delta CI_t = CI_t - CI_s + 1$where CI_t is the CI value at each time point andCI_s is the CI value at certain time point before bitter stimulation. Response intensity was defined as Maximal CI. At certain time when ΔCI_t reached peak or stable (when peak was not obvious), the Maximal CI at each concentration, namely $MaximalCI_c$, was calculated as

$MaximalCI_c = \Delta CI_c - \Delta CI_{buffer}$where ΔCI_c is the peak value at each concentration and ΔCI_{buffer} is the CI value of buffer added group at same time as ΔCI_c.

3. Results

3.1. Expression of bitter receptor T2R16 in HEK-293 cells

Bitter receptors can respond to bitter compounds with high sensitivity and specificity, which could be promising candidates to be sued as sensitive elements in biosensors. In this study, bioengineered cells specifically expressing human bitter receptor hT2R16 were used as sensitive elements for bitter detection. Bitter taste receptors are G-proteins-coupled receptors containing 7 transmembrane segments. The localized expression in the plasma membrane is crucial for its function. Hence, it is necessary to validate the structural and functional expression of this bitter receptor.

The structural expression of T2R16 was validated by immunofluorescent staining. As shown in Fig. 2b, the nucleus of HEK-293 cell was labeled blue using DAPI and the membrane receptor T2R16 was labeled green. Furthermore, as indicated by the nucleus staining of HEK-293 cells, the expressed T2R16 was mainly distributed on the plasma membrane of HEK-293 cells, verifying the structural expression of the bitter receptor T2R16. The results demonstrate the successful expression of T2R16 in HEK-293 cells.

The functional expression of T2R16 in HEK-293 cells was confirmed by calcium imaging. HEK-293 cells endogenously express β_2 adrenergic receptor (β_2AR), which is also a GPCR. Gα16 chimeras can translate β_2AR activation into Ca^{2+} mobilization, which can

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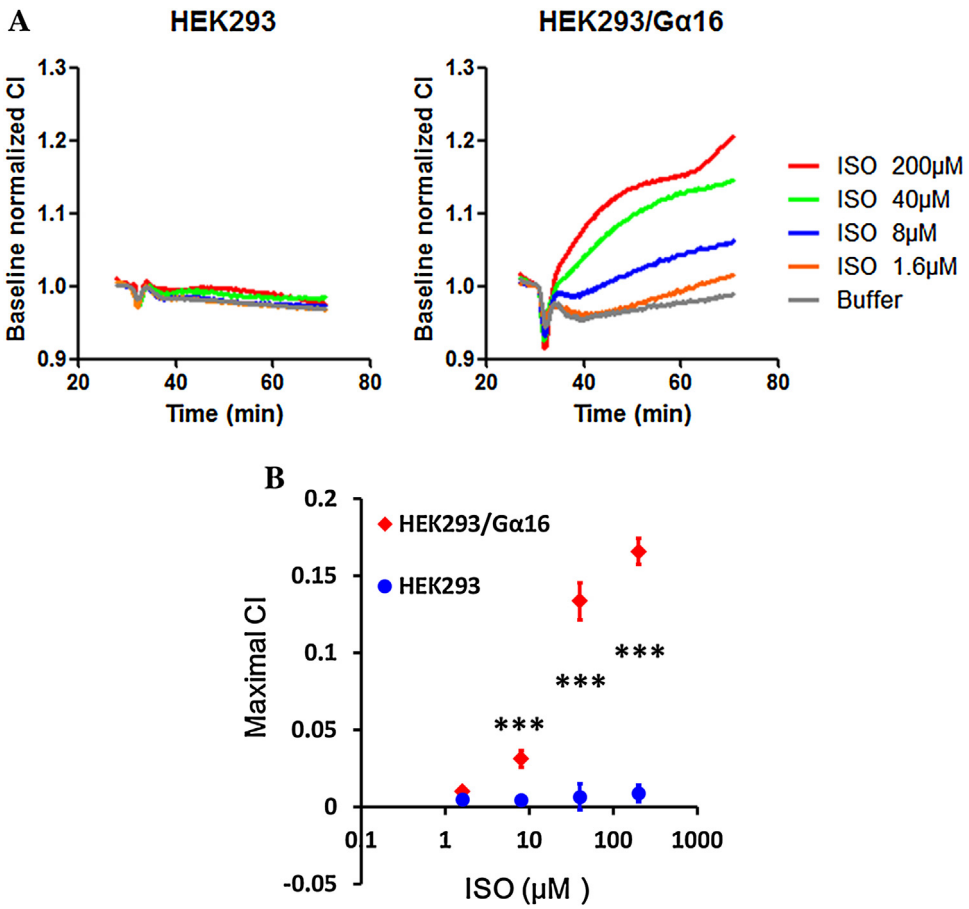

Fig. 4. Cell-impedance responses of HEK-293 cells to ISO. A. CI curves of parental HEK-293 cells (left panel) versus HEK-293/Gα16 (right panel) in responses to ISO. B. Quantitative analyses of response intensity (represented as Maximal CI) of HEK-293 and HEK-293/Gα16 cells at each ISO concentration. There was significant difference between the response intensity of HEK-293 and that of HEK-293/Gα16 cells to ISO (***, $p < 0.001$, two tailed *t*-test analysis). Data were represented as mean ± SD from at least three independent experiments.

be monitored by fluorescence imaging. Thus the expression of Gα16 was determined using the prototypical β_2AR-selective agonist isoproterenol (ISO). In the present study, the calcium imaging results in Fig. 3a showed that ISO application increased intracellular Ca^{2+} in HEK-293/T2R16/Gα16 and HEK-293/Gα16 cells, but did not affect the parental HEK-293 cells, proving the effective transfection of Gα16. Subsequently, the expression of T2R16 was validated by salicin, the T2R16 agonist. The calcium response in Fig. 3b revealed that salicin evoked the intracellular Ca^{2+} mobilization only in HEK-293/T2R16/Gα16 cells but not in HEK-293/Gα16 and parental HEK-293 cells. This result confirmed that the T2R16 was functionally expressed in HEK-293 cells. As a whole, the expression of bitter receptor T2R16 in HEK-293 cells was confirmed in both structural and functional levels.

3.2. Cell impedance measurement for GPCR activation

Cell impedance measurement has been illustrated to detect the GPCR activation in many studies [14,15]. In the present study, cell impedance was utilized to detect the T2R16 activation in heterologous HEK-293 cells. The intracellular calcium response of HEK-293/Gα16 to ISO has been confirmed by fluorescence, in which β_2-adrenergic receptor was activated by ISO. To ensure the efficiency of cell-impedance sensing in detecting cellular status changes induced by GPCR activation, the impedance response of HEK-293/Gα16 to ISO was measured at first. The parental HEK-293 was used as control. The typical cell impedance response curves of HEK-293/Gα16 and HEK-293 cells were shown in Fig. 4a. In HEK-293/Gα16 cells ISO induced a dose-dependent impedance response while in parental HEK-293 cells there was no obvious dose-dependent relation (Fig. 4b). The result confirmed both the successful introduction of Gα16 into HEK293 cells and the significant responses recorded by the impedance system. More importantly, the results demonstrated effectiveness of cell-impedance measurement in detecting cellular responses induced by GPCR activation.

3.3. Bitter receptor activation detected by cell impedance sensor

Cell-impedance sensor was proved to be effective in detecting cellular responses induced by GPCR activation. Thus the cell-impedance sensing technique could be applied in bitter detection by measuring changes of the cellular status induced by bitter receptor activation. The structural and functional expression of T2R16 has been proved in Section 3.1, which provide biological evidence and support for cell-impedance measurement. In order to detect salicin, the bioengineered HEK-293/T2R16/Gα16 cells were used as sensing elements to establish a hybrid cell-based biosensor combined with cell-impedance sensor. The typical cell-impedance responses to salicin were shown in Fig. 5a. The addition of salicin in HEK-293/T2R16/Gα16 cells, the impedance increased rapidly following an obvious decrease. The impedance increased until reached peak at around 20 min after salicin addition. Then the impedance gradually decreased to baseline of control state. When the salicin concentrations were lower, the peak of impedance response appeared lower. The impedance response intensity was

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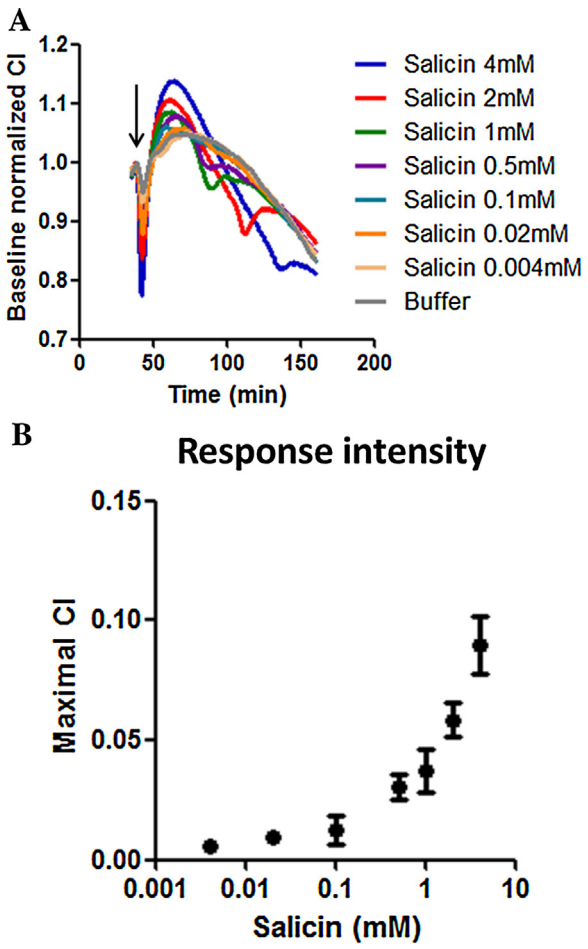

Fig. 5. Bioengineered cell-based biosensor in salicin detection. A. Typical cell-impedance responses to salicin at various concentrations. The black arrow indicates the time for salicin application. B. The cell-impedance response presented a dose-dependent relation. Data were represented as mean ± SD from at least three independent experiments.

presented dose-dependent relation (Fig. 4), which is consistent with calcium response examinations in other report [10]. The calibration line of response intensity was fitted by an exponential equation and the limit of detection for this biosensor was calculated as about 0.055 mM. These results indicate that the cell-impedance sensor can detect the T2R16 activation induced cellular responses in a dose-dependent manner. Furthermore, it demonstrates that this bioengineered bitter-receptor-cell based biosensor is promising in detecting bitter compound salicin.

3.4. Performance evaluation of the bioengineered cell-based biosensor for salicin detection

To evaluate the performance of this bioengineered cell-based biosensor for salicin detection in terms of sensitivity and specificity, we have applied this biosensor in quantitative assessment of bitter intensity in salicin solutions at 3 mM and 0.6 mM, which were not measured in the standard biosensor response (Fig. 5b). The HEK-293/Gα16 cells were taken as control. The results in Fig. 6a showed that the application of salicin at 3 mM and 0.6 mM also led similar impedance response curves as in Fig. 4a. The impedance reached to peak at around 20 min post-stimulation. However, salicin application did not elicit obvious impedance response in HEK-293/Gα16 cells, which confirmed the specific contribution of T2R16 in biosensor response to salicin. As human bitter receptor T2R16 can be activated by β-glucopyranosides, a family of bitter phytonutrients with specific chemical structure, this biosensor assumedly also responds to those bitter phytonutrients. The T2R16 activation links the recognition of a specific chemical structure to perception of bitter taste, which renders this biosensor specificity in bitter detection among various bitter compounds with diverse chemical structures.

The response intensities at these two stimulations also generally fitted in the dose-dependent relation in standard response curve, which proved the sensitivity of the biosensor (Fig. 6b). Overall, there results imply this bioengineered taste-receptor-cell-based biosensor is promising in sensitive and specific detection of salicin.

4. Conclusion

In the present study, a bioengineered cell-based biosensor was established to detect salicin, a bitter tasting compound with high pharmaceutical values. T2R16, a bitter receptor specifically respond to salicin was expressed in heterologous HEK-293 cells as sensing element of the biosensor and cell-impedance sensor was used as secondary transducer which could detected the cellular status changes induced by receptor activation. The structural and functional expression of T2R16 in HEK-293 cells was confirmed by immunofluorescent staining and calcium imaging respectively. The dose-dependent cellular responses of T2R16 expressing cells to salicin were detected and quantified by the cell-impedance measurement. Moreover, the sensitivity and specificity of this bioengineered biosensor was validated by quantitative assessment of the salicin response intensity at unmeasured concentrations. With the advantages of label-free measurement, this bioengineered cell-based biosensor can be applied in monitoring cellular responses to chemicals in a long term, which is valuable for drug detection

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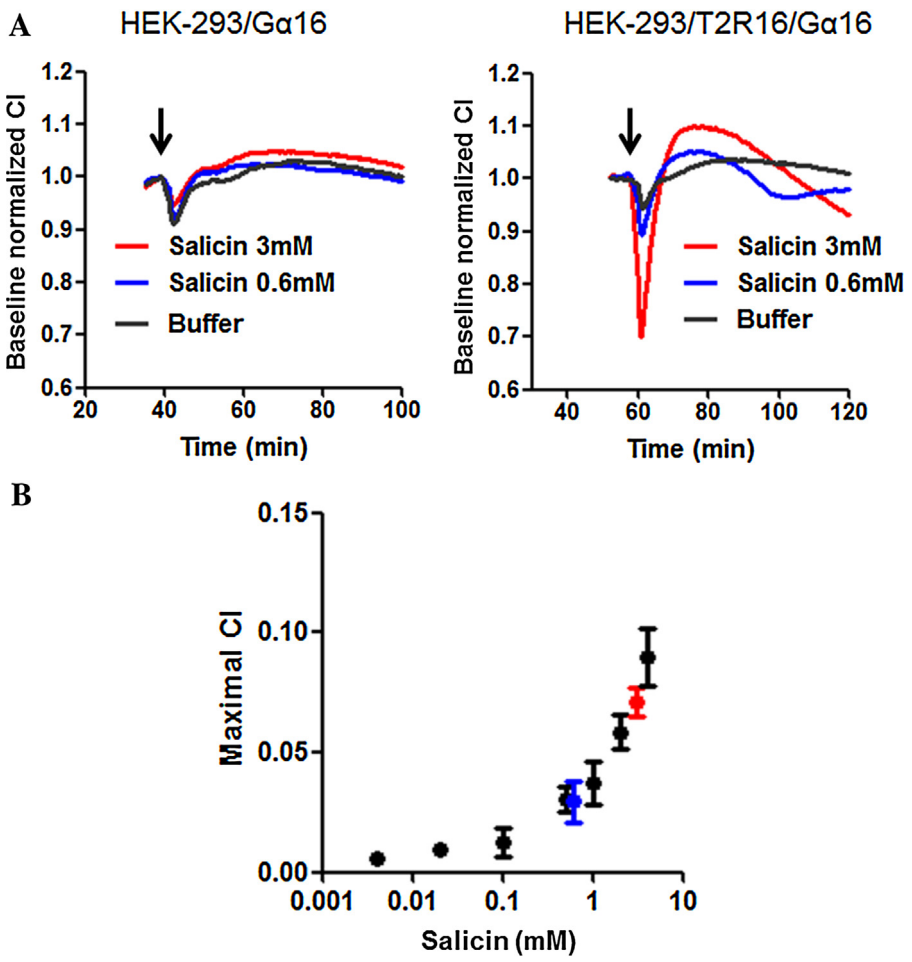

Fig. 6. Performance assessment of this bioengineered cell-based biosensor. A. Cell-impedance responses of HEK-293/Gα16 (left panel) and HEK-293/T2R16/Gα16 cells (right panel) to salicin at two unmeasured concentrations (blue and red). The black arrows indicate the time for salicin application. Salicin led similar cell-impedance response only in T2R16 expressing cells whereas salicin had no obvious effects on HEK-293/Gα16 cells. B. The corresponding dose-response relations of HEK-293/T2R16/Gα16 cells with two unmeasured concentrations. Data were represented as mean ± SD from at least three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

and evaluation. Overall, the results indicated this bioengineered cell-based biosensor provides a novel and promising cell-based approach for detection of salicin.

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Biographies

Liang Hu received her B.E. and Ph.D degree of biomedical engineering in Zhejiang University, P.R. China in 2010 and 2015. Now she is a postdoctor in the Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, China. Her research includes cell-based biosensor, bitter taste transduction.

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