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Research Article

A Cellular Fluorescence Assessment Technique Applying to Quantitate the Sweetness Degree

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Abstract

Sweetness is a physiological taste modality activated by the receptor T1R2/T1R3 heterogenic dimer on cells of the taste bud in oral cavity. Herein, we constructed a human cell line HEK293-*T1R2/T1R3* that stably expresses human sweet taste receptors T1R2/T1R3. The cytoplasmic Ca^{2+} concentration in this cell line was detected by use of fluorescence probe Fluo-4 AM. We found that the fluorescence intensity increases in a concentration-dependent manner with sucrose addition. We further established a calculation equation to quantify the changes of cytoplasmic Ca^{2+} concentration from the variation of Fluo-4 AM fluorescence intensity, which is linearly dependent on the sweetness of sucrose in a range of 50-250mM. Moerover, we measured the relative sweetness of glucose as 0.72 by taking sucrose as standard reference sugar, and this value is similar with the reported glucose relative sweetness (0.74). Therefore, this assessment method can be used to quantify the sweetness degree consistent with variation of fluorescence intensity reflecting cytoplasmic Ca^{2+} concentration. In summary, our study established a credible method to evaluate sweetness degree in physiological environment, and this method is promising for sweet taste sensory application detection.

Keywords: Sweetness; Sucrose; Glucose; *T1R2; T1R3;* Calcium fluorescence imaging

Introduction

Taste variations distinguishable by human taste buds are categorized into five major modalities; *sour, sweet, bitter, umami and salty* [1]. Each taste modality is mediated by a unique receptor expressed on the membrane of the taste bud cells (TBCs). Earlier reports suggest that different classes of TBCs, which express one taste receptor, can exclusively respond to distinct ligand stimuli with differential signal pathway [2-4]. The taste receptors that recognize sweet and umami are evidently encoded by *T1R1*, *T1R2* and *T1R3*. Human T1R2 and T1R3 act jointly to form a heterogenic dimer, which serves as the sweet taste receptor. Human T1R1 and T1R3 also function as heterogenic dimers for the umami receptor [5].

T1Rs taste receptors originate from the C-family of G-protein-coupled receptor, which has an extracellular N-terminal, seven-helical-transmembrane domains, three extracellular loops, three intracellular loops and an intracellular carboxyl tail [6]. The seven helical transmembrane domains are closely linked to the N-terminal flytrap domain via a cysteine-rich structural domain [6]. As the primary receptors for diverse sweet ligands, T1R2/T1R3 can respond to sweet molecules and activate two different signaling pathways depending on the property of the ligands, natural sugar, or artificial sweetener. T1R2/T1R3 receptor activates the downstream Gα protein, cAMP, cNMP-gated channels, Ca^{2+} influx, and PKA orderly, important for phosphorylation of basolateral K^+ channels, thereby shutting off basolateral K^+ channels when natural sugars bound to it. This ultimately depolarizes cell membranes and the voltage-dependent influx of Ca^{2+} [7,8]. Again, binding of artificial sweetener to the T1R2/T1R3 receptor activates other signal pathways through the downstream Gα and GβGγ protein to produce IP3 (which binds to IP3 receptor on the ER) and culminates in the release of Ca^{2+} from ER into the cytoplasm. The increase of cytoplasmic Ca^{2+} concentration facilitates release of neurotransmitters, with consequent sweet perceptions [9,10].

Sweetness is usually assessed by sucrose reference solutions comparison. Thus, sucrose is used as a standard sweet substance to which all other sweeteners are compared. Several sweetness measurement methods have been developed; however, most of them lack a physiological degree value. In this study, we constructed and screened a cell line derived from HEK293 that stably expresses sweet taste receptors T1R2/T1R3 to simulate the human sweet taste receptor's perception, and to establish a credible method to evaluate the sweetness degree in physiological environment.

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Materials and Methods

Plasmids and cell lines

The cDNA sequences of human *T1R2* and *T1R3* were synthesized by the Wuhan Qingke company. Specific primers used for amplifying *T1R2* cDNA were *T1R2*-F (FW: 5'-AAGCTTGCCACCAT-

GGGGCATCATCATCATCATCATGGATCCATGGGGCCCAGGGCAAAGACC-3') and *T1R2*-R (RV: CCGCTCGAGCTAGTCCCTCCTCATGGTGTAGC). *T1R2*-F contained *Hind*III, 6×His tag coding sequence and *Bam*HI sites. *T1R2*-R contained a translation terminator and *Xho*I site. The His-*T1R2* gene sequence was ligated into the pcDNA3 vector to construct a recombinant plasmid (pcDNA3-His-*T1R2*). Specific primers used for amplifying *T1R3* cDNA were *T1R3*-F (FW: 5'-CCCAAGCTTGGTACCGACATGGACTACAAGGACGACGATGACAAGGGATCCATGCTGGGCCCTGCTGT-3') and *T1R3*-R (RV: 5'-CCGCTCGAGTCACTCATGTTTCCCCTGAT-3'). *T1R3*-F contained *Hind*III, Flag tag coding seqence and *Bam*HI sites before the initiation codon. *T1R3*-R contained a translation terminator and *Xho*I. The Flag-*T1R3* gene sequence was ligated into the pcDNA3 vector to construct a recombinant plasmid (pcDNA3-Flag-*T1R3*).

The plasmids pcDNA3-His-*T1R2* and pcDNA3-Flag-*T1R3* were co-transfected into HEK293 cells (CCTCC, Wuhan, China). The cells were cultured in RPMI-1640 complete medium containing 10 % FBS (Everyday Green, China), and screened with 600 μg/ml G418 (Thermo) for two weeks and then with 300 μg/ml G418 for three weeks. The surviving cells were dispersed and diluted into 96-well plates for monoclonal cell screening and expansion.

Western blot

Cells were split with RIPA lysate buffer and whole proteins in cells were collected for Western blotting. 8 % SDS-PAGE gel was used for electrophoresis to separate proteins. The proteins in SDS-PAGE gel were transferred to a nitrocellulose membrane. After blocking with 5% BSA, the membranes were incubated with antibodies against T1R2, T1R3 and actin (Abcam, Wuhan, China). The membranes were incubated with the diluted HRP conjugated secondary antibodies (Abcam) for 2 hours, and then exposed to autoradiographic film with ECL (Thermo) in a dark room.

Immunofluorescence detection

Sterling 10 mm coverslips were incubated with polylysine at 37°C for 30 min and washed with PBS thrice, and placed in 35 mm dishes. The expanded monoclonal cells were plated in the dishes in proportion to abundance of 50 - 70 %. After culturing for 24 hours, the cells were treated with 4 % paraformaldehyde for 15 min, and then washed with PBS buffer thrice. After blocking with 5 % BSA (Sigma) for 30 min, the cells were incubated overnight with *T1R2*/*T1R3* antibodies at 4 °C and washed with PBS buffer thrice for 5 min each. These were then incubated with diluted FITC/TRITC secondary antibodies (Abcom) for 30 min at room temperature. Finally, the cells were washed with PBS buffer thrice for 5 min and detected using a FV1000 laser confocal microscope (Olympus, Japan).

Detection of intracellular Ca^{2+} concentration with FV1000 confocal microscopy

Cells were planted in the confocal dish with an outer and inner diameter of 35 and 10 mm, respectively in abundance of 40-60 %. Following 24 hours culturing, the cells were stained with Fluo-4 AM (Beyotime) for 30-45 mins in 37 °C, and then washed once with PBS. The cells were imaged with a FV1000 confocal laser scanning microscope according to the setting: 60 × oil mirror as objective lens, helium ion laser as laser source, 488 nm excitation wavelength, 500-600 nm emission wavelength range, 8μs/Pixel scanning speed, 640 x 640-pixel scanning area, and non-invasive XYT scanning procedure applied to the cells. Continuous image scanning was recorded and at room temperature under dark conditions for 2 min. Total number of image frames was 60 and exogenous stimulus solution was added after 12 seconds. Glucose (Sigma) and sucrose (Sigma) were used to prepare exogenous stimulus solutions.

Image analysis

The images were analyzed with ImageJ software (National Institutes of Health, USA) and data were plotted and analyzed with GraphPad 7.0 Prism (GraphPad Software, San Diego, CA). Images of intracellular Ca^{2+} were collected and analyzed with ImageJ, and data were plotted as columns or scatter diagrams with GraphPad Prism.

Data analyses

Data were presented as mean ± SD of three independent replicates (triplicates). A *t*-test was used to compare the difference between groups, and values of $P < 0.05$ were considered statistically significant, unless otherwise specified.

Results

Characteristics of HEK293-T1R2/T1R3 cell line

To obtain a stable cell line which expresses human sweet taste receptor T1R2/T1R3, we first constructed plasmids, pcDNA3-His-*T1R2* and pcDNA3-Flag-*T1R3*, to express recombinant T1R2 and T1R3, respectively. Both plasmids were co-transferred into HEK293 cells by electroporation transfection technique and monoclonal cells were screened by G418 for five weeks and then expanded for following experiment. (Figure 1(a)). To identify whether sweet receptors are expressed in selected monoclonal cells and their localization in monoclonal cells, we performed western blotting with specific antibodies against T1R2 and T1R3, and identified the sweet taste receptor localization in monoclonal cells by immunofluorescence imaging. The results showed that the cells screened out could stably express the sweet taste receptor proteins T1R2 (94 kDa) and T1R3 (96 kDa) (Figure 1(b)), and the receptors are located on the cell membrane (Figure 1(c)). We named the verified monoclonal cell as T1R2/3^{H} cell.

Detection of T1R2/3^{H} cell responding to sucrose

Activated sweet taste receptors could lead to increase of Ca^{2+} concentration in the cytoplasm (Figure 2(a)). To determine whether sweet taste receptors stably expressed in T1R2/3^{H} cell can respond to sweet molecules, we chose 150mM sucrose solution as exogenous stimulus and detect the variation of intracellular calcium concentration with fluorescence probe Fluo-4 AM. Fluorescence emission from Fluo-4 AM was detected, and fluorescence images were recorded every 4s for 2 min under confocal scanning microscope with exogenous stimulation (Supplementary file 1 for T1R2/3^{H} cells and Supplementary file 2 for HEK293 cells). It was clearly observed that the fluorescence

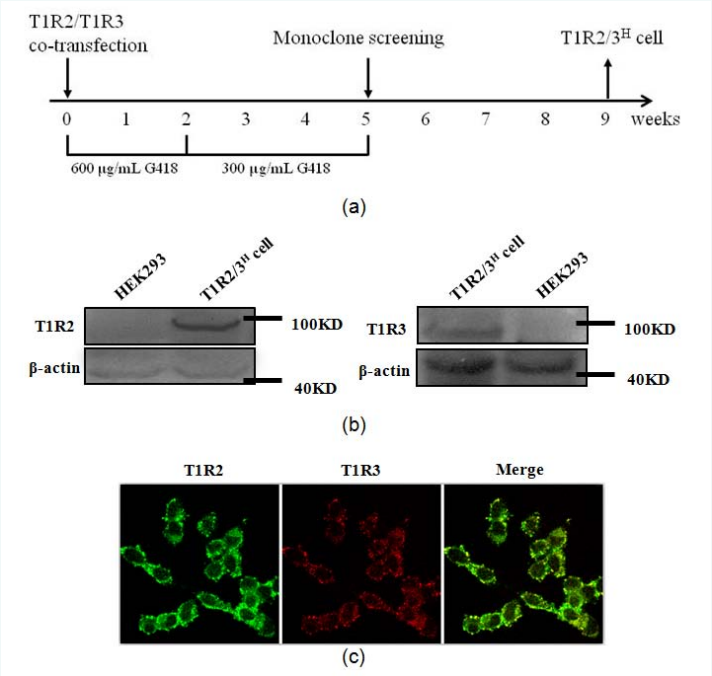

Figure 1 Construction of the cell line stable expressing human T1R2/T1R3. (a) The schematic chart of screening procedures. HEK293 cells were co-transfected with pcDNA-His-*T1R2* and pcDNA-Flag-*T1R3*, then treated with 600 μg/ml G418 in the first two weeks, and with 300 μg/ml G418 during the third to fifth weeks. T1R2/3^{H} cell indicates the monoclonal cell stable expressing T1R2 and T1R3. (b) Identification of T1R2 and T1R3 expressed in screened cell line by western blot. β-actin served as a reference. (c) Cellular localization of T1R2 and T1R3 expressed in screened monoclonal cell line. T1R2 was detected with specific T1R2-antibody and FITC secondary antibody, while T1R3 was detected with T1R3-antibody and TRITC secondary antibody..

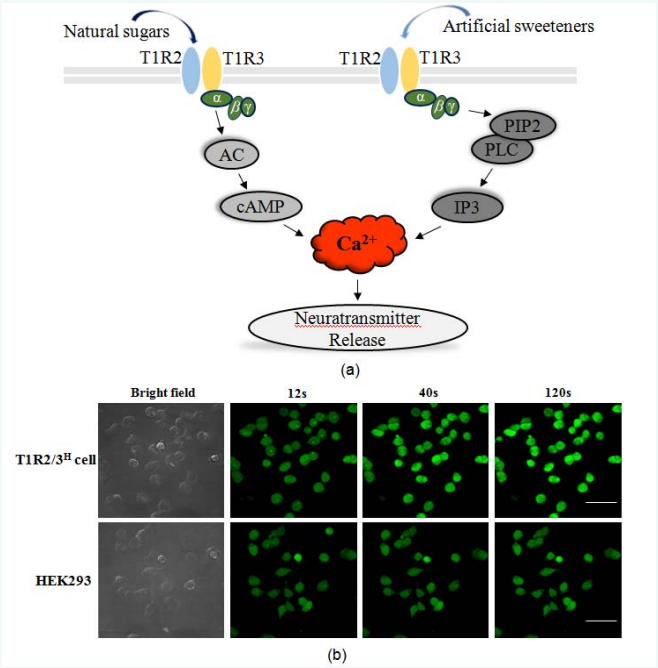

Figure 2 Sweet taste receptors expressed on cells can respond to sucrose. (a) The schematic pathways of Ca^{2+} release triggered by T1R2/T1R3 responding to natural sugars and artificial sweeteners. (b) Fluorescent and bright field image at different time spots (12s, 40s, 120s) with 150mM sucrose stimulation. The fluorescence images probing intracellular Ca^{2+} in T1R2/3^{H} cell and HEK293 were captured every 4s within 2 mins by confocal microscope, and sucrose solution was added at 12s after the scan beginning (scale bar = 50 μm).

intensity in both T1R2/3^{H} cells and HEK293 cells increased expressly responding to sucrose solution (Figure 2(b)), indicating the increase of intracellular Ca^{2+} concentration. However, the variation of Fluo-4 AM fluorescence intensity in T1R2/3^{H} cells was more significant than that in HEK293 cells. This indicates that the sweet receptors stable expressed in T1R2/3^{H} cells can respond to sucrose solution, contributing to the increasing of Ca^{2+} related fluorescence.

Establishing a method for quantifying intracellular Ca^{2+} concentration based on fluorescence intensity

To further quantify the difference between the T1R2/3^{H} cell and HEK293 response to sweet molecules, we calculated the fluorescence intensity of single cells obtained from fluorescence images. The image stacks showing representative single cells from two cell lines at separate time spots of are shown in Figure 3(a). The fluorescence intensity of each cell was calculated by ImageJ software and plotted against time (Figure 3(b)). After sucrose treatment, the cellular fluorescence intensity of T1R2/3^{H} cells increased significantly, while that of HEK293 cells had no distinct changes, reaffirming that the sweet receptors stable expressed in T1R2/3^{H} cells can respond to sucrose. We further calculated the ratio of single-cell fluorescence increasement (ΔF/Fo), defined as ΔF/Fo = (Fmax-Fo)/Fo. Fo is the average of three weakest fluorescence intensities of the cells in the first 20s, and Fmax is the maximum fluorescence intensity value in the first 120s. Six representative single cells were selected from each group for ΔF/Fo calculation. The average ΔF/Fo value of T1R2/3^{H} cell was 0.62, while that of HEK293 was as low as 0.14. The D-value of ΔF/Fo between T1R2/3^{H} cell and HEK293 is 0.48, which represents the activation of receptors by sucrose treatment (Figure 3(c)).

Fitting the standard curve of D-value of ΔF/Fo related to concentration of sucrose

To determine how sweet taste receptors in T1R2/3^{H} cells could respond to sucrose at different concentrations, the fluo-

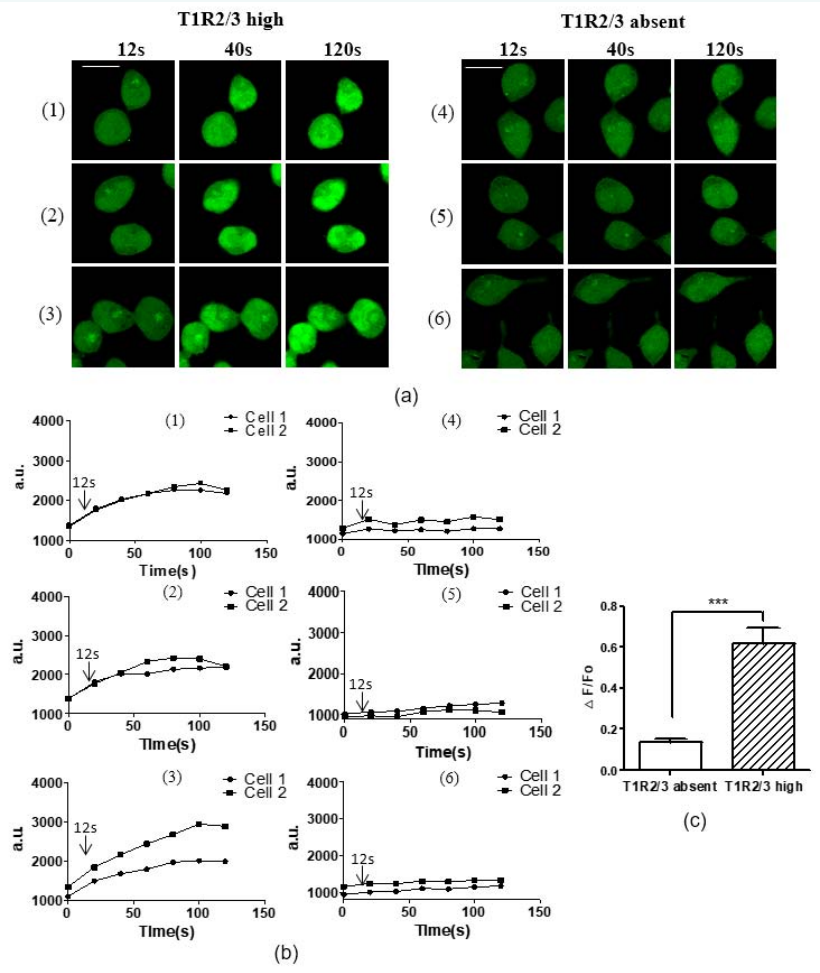

Figure 3 Quantitation of intracellular Ca^{2+} concentration based on fluorescence intensity. (a) the fluorescent images showing several representive cells in two cell lines at 12s, 40s, and 120s time points. T1R2/3 high represents T1R2/3^{H} cell, and T1R2/3 absent represents HEK293 (scale bar = 20 μm). (b) The cellular fluorescence intensity of each fluorescence image showing in (a). Curves were plotted against time within 2 mins. Sucrose solution was added into cell medium at 12s. (c) ΔF/Fo of HEK 293 (as T1R2/3 absent) and T1R2/3^{H} cell (as T1R2/3 high) with 150 mM sucrose treatment. (n=6, three independent experiments, p=0.0001, two-tailed Student's t-test.)

rescence images of two cell lines were obtained with sucrose treatment at series of concentrations. The results also showed that increased fluorescence intensity and F/Fo of T1R2/3^{H} cells were higher than that of HEK293 cells studied (Figures 4(a) and 4(b)). Furthermore, we found that the D-value of ΔF/Fo between T1R2/3^{H} cell and HEK293 is linearly dependent on the sucrose concentration within 50-250 mM, expressed as regression equation Y= 0.001945 X-0.01064 (R^2=0.9886) (Figure 4(c)). The R-squared value of 0.9886 suggests that the linear regression equation is successful when sucrose concentraion changes within 50 to 250 mM, which can be employed to quantitatively sweetness measurement.

Measuring the relative sweetness degree of glucose

In order to applying our cellular fluorescence assessment technique to measure the relative sweetness of glucose, we prepared 200 mM glucose solution as a sample for sweetness measuring and set 200mM sucrose solution as the standard sweetness solution. Fluo-4 AM fluorescence images were recorded under confocal scanning microscope with glucose solution stimulation (Figure 5). The D-value of ΔF/Fo is 0.27 for 200mM glucose. According to the regression equation we got above, equal sucrose concentration of 200mM glucose is calculated as 144mM, and the relative sweetness of glucose is 0.72 (144mM/200mM), which is similar to 0.74, the value of reported relative sweetness of glucose [11].

Discussion

Currently, sweetness detection is restricted to detecting content of sweet substances through physical or biological methods. However, these methods have limitations. For example, liquid chromatography techniques can accurately detect the content of sweet substances, but it can only detect known sweet substances with known sweetness degree and are unable to identify unknown sweet substances. Moreover, simply converting, and overlaying values of sweetness degree of each sweet substance

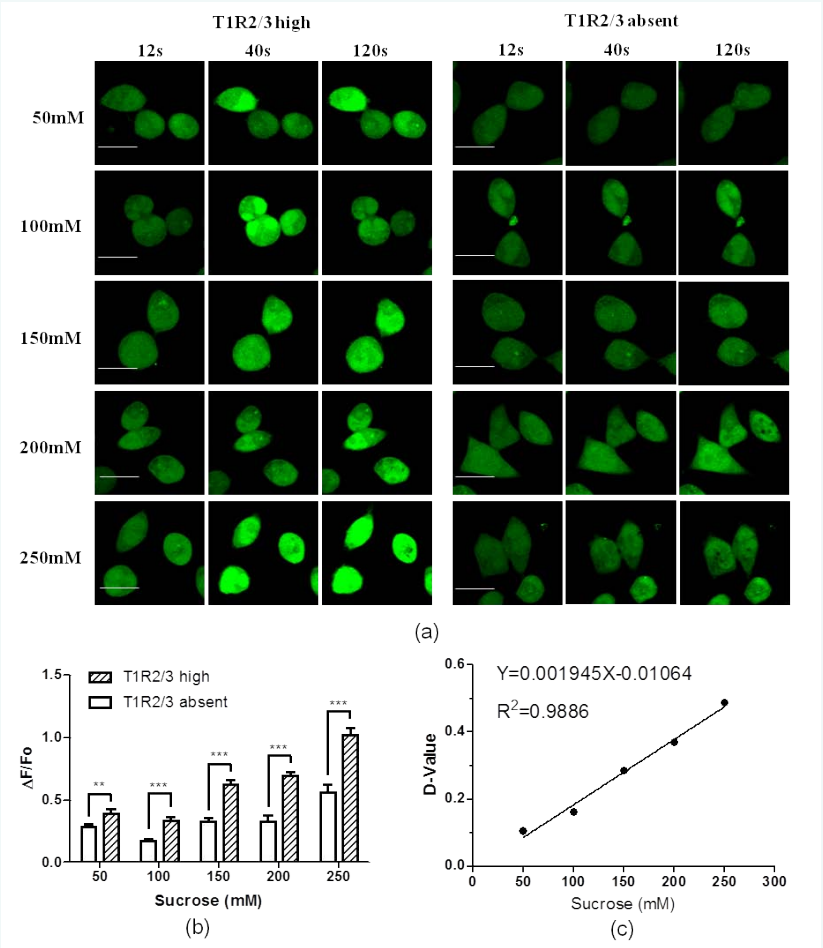

Figure 4 Fitting the standard curve of D-value of ΔF/Fo related to sucrose of different concentrations. (a) Fluorescence images probing cellular Ca^{2+} of two cell lines with sucrose treatments at different concentration. T1R2/3 high represents T1R2/3^{H} cell, and T1R2/3 absent represents HEK293 (scale bar = 20 μm). (b) ΔF/Fo of two cell lines with different sucrose treatments (n>=20, three independent experiments, p=0.0054 (50mM) and p<0.0001 (100mM to 250mM), two-tailed Student's t-test.). (c) The correlation between D-value of ΔF/Fo and sucrose concentration (from 50mM to 250mM) was fit to linear dependence (R^2=0.9886).

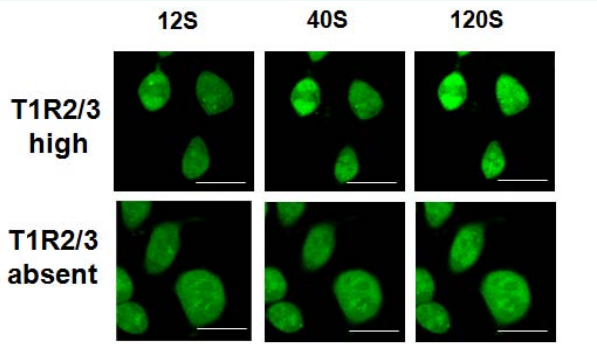

Figure 5 The fluorescent images showing several representive cells in T1R2/3^{H} cells and HEK293 cells at 12s, 40s, and 120s time points. 200mM glucose solution was added into cell medium at 12s. T1R2/3 high represents T1R2/3^{H} cell, and T1R2/3 absent represents HEK293 (scale bar = 20 μm).

may not reflect the real sweetness of a mixture. Training professionals primarily do bioassay testing to quantify sweetness by tasting sweet substances. This method can reflect the sweetness of sweet complexes at the physiological level and can also assess the sweetness of unknown sweet complexes or pure products, but the testing platform is highly unpredictable and less accurate.

In this study, we screened out a cell line derived from HEK293 which stably expresses human sweet taste receptors on the cell membrane. The cell line could respond to sweet molecules such as sucrose, which is usually regarded as a standard reference for sweetness measurement (orosensory perception measurement) [12]. In T1R2/3^{H} cell, sucrose could bind to T1R2/T1R3 receptors expressed on cellular membrane, and then activate the intracellular pathways, leading to higher Ca^{2+} concentration, which could be visualized by fluorescence probe Fluo-4 AM. D-value of ΔF/Fo calculated from the cellular fluorescence intensity could specifically reflect the activation degree of sweet taste receptors, which directly corresponds to the concentration and sweetness of ligands. D-value of ΔF/Fo between T1R2/3^{H} cells and HEK293 cells was linearly contingent on sucrose concentration (50-250mM). Our method may be employed to evaluate concentration of sucrose solution, and other sweeteners. This prototype developed is based on a physiological system to measure the sweetness degree of samples, which could be potentially useful in industrial food and drug applications.

Conclusion

In this study, we constructed the cells with stably high-expression of T1R2 and T1R3, and then established a credible method to quantify the sweetness degree using the variation of fluorescence intensity reflecting cytoplasmic Ca^{2+} concentration. This method is promising for sweet taste sensory application detection.

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