Characterization and Long-Term Maintenance of Rat Taste Cells in Culture

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Abstract

Taste cells have a limited life span and are replaced from a basal cell population, although the specific factors involved in this process are not well known. Short- and long-term cultures of other sensory cells have facilitated efforts to understand the signals involved in proliferation, differentiation, and senescence, yet few studies have reported successful primary culture protocols for taste cells. Furthermore, no studies have demonstrated both proliferation and differentiation *in vitro*. In this study, we have developed an *in vitro* culture system to maintain and utilize rat primary taste cells for more than 2 months without losing key molecular and biochemical features. Gustducin, phospholipase C-β_2 (PLC-β_2), T1R3, and T2R5 mRNA were detected in the cultured cells by reverse transcriptase–polymerase chain reaction. Western blot analysis demonstrated gustducin and PLC-β_2 expression in the same samples, which was confirmed by immunocytochemistry. Labeling with bromo-2-deoxyuridine (BrdU) demonstrated proliferation, and a subset of BrdU-labeled cells were also immunoreactive for either gustducin or PLC-β_2, indicating differentiation of newly generated cells *in vitro*. Cultured cells also exhibited increases in intracellular calcium in response to several taste stimuli. These results indicate that taste cells from adult rats can be generated and maintained under the described conditions for at least 2 months. This system will enable further studies of the processes involved in proliferation, differentiation, and function of mammalian taste receptor cells in an *in vitro* preparation.

Key words: culture, gustducin, imaging, proliferation, taste receptor

Introduction

Mammalian taste cells are heterogeneous in terms of phenotypical structure and immunochemical features. There are four subtypes of taste cells identified based on structural characteristics: basal, type I, type II, and type III (Farbman, 1965; Kinnamon *et al.*, 1985; Delay *et al.*, 1986; Yee *et al.*, 2001; Stone *et al.*, 2002). The significance of these different morphological cell classifications in terms of function, structure, and lineage are not known because taste cells are continuously renewed from basal cells and may vary with age (Beidler and Smallman, 1965; Farbman, 1980). Furthermore, cytochemical signatures vary among species (Takeda *et al.*, 1992; Yang *et al.*, 2000), and it is not known how these signatures relate to maturational stage, functional status, or lineage (Yee *et al.*, 2001).

It has been hypothesized that formation of taste buds is induced by cranial nerve fibers during embryonic development (Fujimoto and Murray, 1970; Hosley and Oakley, 1987; Oakley *et al.*, 1993; Whitehead and Kachele, 1994).

Studies have also shown that denervation of gustatory nerves leads to the disappearance of taste buds (Morris-Wiman *et al.*, 1999; Huang and Lu, 2001; Sollars *et al.*, 2002; Uchida *et al.*, 2003). Contrary to these neuronal induction theories, other studies have shown that taste cells can differentiate fully without requiring any nerve innervation. The induction of taste cell development may be triggered by signals from other tissues or may arise independently through intrinsic signaling mechanisms that are associated with the epithelium during late embryonic development (Barlow and Northcutt, 1997; Barlow, 1999). Individual taste buds arise from multiple progenitors. However, it is not known if these progenitors give rise to basal cells that generate different taste cell types or to multipotent epithelial stem cells that generate lineage-restricted basal cells (Stone *et al.*, 2002).

Cell culture techniques can be a valuable approach to advance our knowledge and understanding of the molecular structure and cellular physiology of taste cells and the

processes of proliferation, differentiation, and regeneration. One study reported that taste cells were maintained for up to 2 weeks at room temperature but with a loss of almost 25% of cells occurring within the first 4 days and cell death around day 10 (Ruiz et al., 2001). This study also reported that cells maintained at 37°C disappeared after 3–4 days. Another study isolated a particular type of cell from mouse taste epithelium, sorted based on their expression of integrin β1 marker, and these cells expressed neural cell adhesion molecule (NCAM). However, this integrin β1–positive mouse cell culture did not generate cells similar to those responsible for the primary detection of taste stimuli (Ookura et al., 2002).

Currently, there are no longer term in vitro taste cell models to study function and development because of the limitations of primary cultures and the difficulties in maintaining sensory cells in vitro. These limitations may be caused by taste cell isolation procedures, mechanical stresses, and harsh enzyme treatments, which may degrade structures and reduce cellular viability (Spielman et al., 1989; Kishi et al., 2001; Ookura et al., 2002; Landin et al., 2005). Previous studies indicated that maintaining rat primary cells beyond 3–5 days was not possible based on the specified isolation procedures and culture techniques (Spielman et al., 1989; Kishi et al., 2001; Ruiz et al., 2001). We aimed to revisit the problem in view of the established need for such a system.

We report for the first time the establishment of an in vitro culture system for the long-term maintenance of isolated rat taste cells by developing isolation methods and selecting culture media and supplements that are essential for cell attachment, growth, and differentiation. Isolated rat taste cells from circumvallate and foliate papillae were maintained for more than 2 months in culture with minimal loss of viability and which maintained the expression of characteristic molecular and physiological features. 5-Bromo-2-deoxyuridine (BrdU) immunoreactivity, expression of mRNA for specific taste biomarkers [i.e., gustducin, phospholipase C-β_2 (PLC-β_2), T1R3, and T2R5] by polymerase chain reaction (PCR), and expression of gustducin and PLC-β_2 by western blot demonstrated cellular proliferation and differentiation in our long-term taste cultures. Calcium imaging demonstrated that cultured taste cells also responded to different taste stimuli, indicating functional maturation. The establishment of this long-term taste cell culture protocol provides an important in vitro model for studying growth and differentiation of taste cells during aging, the impact of trophic or toxic agents on taste cell growth and function, and the assessment of chemical stimuli that may elicit taste responses and/or modulate responses to other stimuli.

Materials and methods

Primary cell culture

The use and handling of animals were performed with the approval of the Monell Chemical Senses Center Institutional Animal Care and Use Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Rats ranging from 1 to 2 months old were euthanized by CO_2 inhalation followed by cervical dislocation. The tongue was dissected proximal to circumvallate papillae and immediately placed into an isolation solution [26 mM $NaHCO_3$, 2.5 mM NaH_2PO_4, 20 mM glucose, 65 mM NaCl, 20 mM KCl, and 1 mM ethylenediaminetetraacetic acid (EDTA)] for 5–10 min on ice. The preparation was then removed from ice and approximately 1 ml of the isolation buffer mixed with 1.5 mg/ml pronase E (Sigma, St Louis, MO) and 1 mg/ml elastase (Sigma) was uniformly injected with a 25-gauge Norm-Ject syringe under and around the lingual epithelium of cicumvallate and foliate papillae of dissected tongue. After 15–20 min of incubation in isolation buffer at room temperature, the epithelium was gently peeled from the underlying muscle layer under a dissecting microscope (Stereomaster, Fisher Scientific, Pittsburgh, PA) and placed in isolation solution. The isolated epithelium was then transferred to Iscove's modified Dulbecco's medium (IMDM, Gibco BRL, New York, NY) containing 10% fetal bovine serum (FBS, Biomedical Technologies, Inc [BTI], Stoughton, MA), 1:5 ratio of MCDB 153 (Sigma), 10 ng/ml insulin, and a triple cocktail of antibiotics (100 U/ml/100 μg/ml, penicillin/streptomycin, 2.5 μg/ml gentamycin, and 0.5 μg/ml fungizone) and cut into small pieces with a razor blade. The pieces were seeded onto 18-mm round glass coverslips (Fisher) coated with rat tail collagen type 1 (3.96 mg/ml diluted 1:4 in distilled nuclease-free water, BD Sciences, San Diego, CA) and incubated at 37°C in a humidified environment containing 5% CO_2. Culture medium was replaced after 48 h and then every 5–7 days. Prior to use, coverslips were treated with 2 M NaOH for 1 h and left overnight in 70% nitric acid (HNO_3). After 1 h in an HCl acid wash, the coverslips were autoclaved in water, rinsed with 70% and 100% ethanol, and then air dried.

In preliminary studies, we also examined different coating materials and medium supplements to determine the optimal culture conditions. Attachment, viability, growing conditions, and composition of culture medium were compared between cells seeded on coverslips coated with mouse fibroblasts (ATCC, Manassas, VA), matrix gel (2 ml/l, ATCC), or poly-D-lysine (BD Biosciences, San Jose, CA) or on the uncoated polystyrene plate (Corning, Corning, NY). We also examined different compositions of tissue culture medium for maintaining taste cells [i.e., Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) either with 10% FBS (BTI) or MCDB 153 medium (Sigma)]. We also examined IMDM (Gibco BRL) containing 10% FBS (BTI). Assessment of cultured taste cell viability was done by staining with 0.04% trypan blue (Sigma). Based on cell attachment and viability over 7–10 days in culture observed in these preliminary experiments, we selected the protocol described above for subsequent studies.

Western blot

Western blots were conducted using standard immunoblotting techniques as previously described (Sambrook *et al.*, 1989). Cultured primary rat taste cells were lysed, and tissue samples from rat cicumvallate and foliate papillae were homogenized in radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 10 mM Tris (pH 7.2), 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% deoxycholate, and 5 mM EDTA] containing protease inhibitors (104 mM 4-(2-Aminoethyl)benzenesulphonyl fluoride [AEBSF], 80 μM aprotinin, 2 mM leupeptin, 4 mM bestatin, and 1.5 mM pepstatin A). Protein concentration was estimated for each sample using Bio-Rad Dc Protein estimation kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol. Protein samples were mixed with SDS loading buffer containing β-mercaptoethanol, boiled for 5 min, and then placed on ice for 5 min. The cellular homogenates were separated by a sodium dodecyl sulfate–polyacrylamide (5–15%) gradient gel (Bio-Rad Laboratories) electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories) that was incubated at 4°C overnight with 1% nonfat dry milk. Polyclonal rabbit anti-gustducin (G$_{\alpha gust}$, I-20; sc-395, dilution 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) and polyclonal rabbit anti–PLC-β_2 (Q-15; sc-206, dilution 1:1000, Santa Cruz Biotechnology) were used to identify taste cell proteins. After 1.5-h incubation with primary antibodies at room temperature, the membrane was rinsed in three 15-min washes with 0.1 M phosphate buffer solution with 0.05% Tween 20 (PBS/T), reacted with horseradish peroxidase–conjugated secondary anti-rabbit antibody (NA934, dilution 1:5000, Amersham Biosciences, Piscataway, NJ) for 1 h at room temperature, followed by three 15-min washes with PBS/T. Signal was detected with the enhanced chemiluminescence immunoblot detection system (Amersham Biosciences) following the manufacturer's instructions. X-ray films were later scanned for documentation and analysis.

Immunoprecipitation

Epithelial tissues were isolated as described above and homogenized into 1 ml of cold RIPA buffer supplemented with protease inhibitor cocktail. Homogenate was clarified at 3500 rpm for 4 min. Supernatant was incubated at 4°C for 30 min with 5 μl of rabbit IgG (Vector Laboratories, Burlingame, CA). To this was added 25 μl of a 50% suspension of protein A–Sepharose beads (Invitrogen), and the mixture was incubated with constant rotation at 4°C for 1 h. The beads were centrifuged, and precleared homogenate was incubated with 10 μl of rabbit anti-gustducin antibody (Santa Cruz Biotechnology, sc-395) at 4°C for 30 min with the subsequent addition of 25 μl of a 50% protein A–Sepharose suspension. The mixture was then incubated at 4°C with rotation overnight. The beads were washed three times with 0.5 ml of RIPA buffer, after being

centrifuged at 3500 rpm for 3 min. Washed beads were resuspended in 55 μl of SDS gel loading buffer [50 mM Tris–HCl (pH 6.8), 5% 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol], heated at 100°C for 3 min, and centrifuged, and the supernatants were separated by SDS–polyacrylamide gel electrophoresis as described above.

Reverse transcription–polymerase chain reaction

Total RNA from cultured cells and rat tongue (foliate, vallate, and nontaste epithelium) was extracted with TRIzol reagent (Invitrogen Corp., Carlsbad, CA) following the manufacturer's instructions. RNA (4 μg) was reverse transcribed for 90 min at 42°C using the Superscript First Strand Synthesis System for reverse transcription–polymerase chain reaction (RT–PCR) (Invitrogen Corp.). As a control to check genomic DNA contamination and nonspecific amplification, samples of RNA were treated in parallel in the presence and absence of reverse transcriptase and used for PCR by amplifying with primers designed for detection of gustducin, PLC-β_2, β-actin, T2R5, and T1R3 (see Table 1). Primers were chosen to span one or more introns to exclude confusion with amplified fragments from genomic DNA. PCR amplification of cDNA for each RT reaction was performed in a final volume of 50 μl containing 2 μl of RT reaction, 1× AmpliTaq Gold PCR buffer, 2.5 mM MgCl$_2$, 1 mM deoxynucleoside triphosphates, 0.4 μM of each primer, and 0.25 U/μl of AmpliTaq Gold polymerases (Applied Biosystems, Foster City, CA). PCR amplification consisted of initial denaturation at 95°C for 5 min followed by cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 30 s. After 40 cycles of amplification, the final extension was at 72°C for 10 min. PCR products were separated on 2% agarose gels and stained with 0.2 μg/ml of ethidium bromide to verify their expected size.

BrdU labeling and immunocytochemistry

BrdU incorporation was used to monitor cell proliferation. Isolated taste cells were seeded onto collagen-coated coverslips, maintained for the indicated time, and treated with 50 μM BrdU (Sigma) dissolved in dimethyl sulfoxide (Sigma) for 24–48 h, when BrdU was replaced with fresh medium. The BrdU-treated cells were maintained for 3 additional days in culture and were then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 10 min at room temperature. After washing in PBS, the cells were treated with H$_2$O$_2$ solution (4 ml PBS + 0.5 ml 100% methanol + 0.5 ml 30% H$_2$O$_2$) for 20 min to block endogenous peroxidase and denatured with 2 N HCl at 37°C for 30 min. SuperBlock blocking buffer in PBS (Pierce Chemical Company, Rockford, IL) was used as the blocking buffer to reduce nonspecific binding for 1 h at room temperature. Cells were then incubated with mouse anti-BrdU (dilution 1:100, Sigma B-2531) diluted in 10% SuperBlock with 0.05% Tween 20 overnight at 4°C. Alexa Fluor 488–conjugated

Table 1 Primers used for detecting taste-specific molecules

Gene	Sequence	Expected size	Reference
Gustducin	Forward: 5′-gat gct agc caa tcc gag aag tag aga gg-3′	450	Hofer *et al.* (1996)
	Reverse: 5′-cgg aga tct gct gtt gaa gag gtg aag ac-3′		
PLC-β_2	Forward: 5′-ctg gag gct gaa gta aag gag-3′	454	Rossler *et al.* (2000)
	Reverse: 5′-gcc cct gca tgt atg tta gg-3′		
β-Actin	Forward: 5′-tca tgt ttg aga cct tca a-3′	493	Rossler *et al.* (2000)
	Reverse: 5′-gtc tt gcg gat gtc cac g-3′		
T2R5	Forward: 5′-tgg caa atc cac atg aag aa-3′	330	Accession no. NM_023996
	Reverse: 5′-gca ggg ata gag gaa tgc aa-3′		
T1R3	Forward: 5′-gat cag tgg tcc cca gaa aa-3′	682	Accession no. NM_130818
	Reverse: 5′-taa gct agc atg gcg aag gt-3′		

anti-mouse IgG (dilution 1:500, Molecular Probes Inc., Eugene, OR) diluted in 10% SuperBlock with 0.05% Tween 20 was used for immunofluorescence detection. Coverslips were thoroughly washed with PBS and water and then mounted onto slides with Vectashield (Vector Laboratories). For immunofluorescence double labeling, the coverslips were reblocked with 0.3% Triton X-100, 4% normal goat serum, and 3% bovine serum albumin in 0.1 M PBS and then incubated with either polyclonal rabbit anti-gustducin (dilution 1:500–1:1000, Santa Cruz Biotechnology) or polyclonal rabbit anti–PLC-β_2 (dilution 1:500–1:1000, Santa Cruz Biotechnology) overnight at 4°C. After washing with PBS, cells were then incubated with Alexa Fluor 633 anti–rabbit-IgG (dilution 1:500, Molecular Probes Inc.) diluted in blocking buffer for 1 h. After washing in PBS and water, coverslips were mounted with either Vectashield or Vectashield with 4′,6-Diamidino-2-phenylindole (DAPI) (Vector Laboratories). To determine the specificity of gustducin staining, gustducin antibody preincubated with fivefold (by weight) of its specific peptide (Santa Cruz Biotechnology, sc-395 P) for 2.5 h at room temperature was applied to cultured taste cells as described above. Controls for immunofluorescence consisted of omitting the primary antibody or substituting the primary antibody with the host IgG from which the antibody was generated. In addition, for double-labeling experiments, controls were done in which either the first or second primary antibody was omitted with all other steps in the protocol maintained to check for nonspecific interactions. In all cases, these controls revealed no artifactual labeling. Immunoreactive cells were counted in at least three sampling fields.

Confocal imaging

Fluorescent images were captured with the Leica TCS SP2 Spectral Confocal Microscope (Leica Microsystems Inc., Mannheim, Germany) using UV, argon, and HeNe lasers, and the coverslips were viewed under a HC PL APO CS 20.0× (0.070 NA) objective. Excitation wavelengths used were at 405 nm for DAPI, 488 nm for Alexa Fluor 488, and 633 nm for Alexa Fluor 633 with emissions detected at appropriate wavelengths. The pinhole diameter was set at the first minimum diameter of the Airy disc for the objective used, giving acceptable resolution of the *z*-axis for the fluorescent focal plane. The power for the laser beam and gain of the photomultiplier were adjusted to optimize the signal/noise ratio and held constant for comparison of antibody labeled and control slides. Sequential acquisition of each wavelength was used for some double-labeling experiments to prevent cross talk or bleed through between fluorophores. Leica Scanware software was used to acquire confocal images scanning unidirectionally at a 1024 × 1024 pixel format with two lines plus three frames averaging. Computer-controlled digital zoom was used to increase magnification to a maximum of 2.5× under 20× objectives. Digital images were arranged and adjusted for contrast and brightness using LCS software (Leica Microsystems Inc.) and Photoshop v8 (Adobe Systems Inc., San Jose, CA).

Calcium imaging

Cultured taste cells grown for 1 or 4 weeks on collagen-coated coverslips were loaded for 15–30 min with the calcium sensitive dye fura-2 by incubating the cells in Mammalian Ringer's solution (80 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na-pyruvate, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-Na, pH 7.2 with osmolarity adjusted to 300–310 by 5 M NaCl) supplemented with 1 mM fura-2 pentakis(acetoxymethyl) ester (FURA-2 AM) (Molecular Probes Inc.) and 10 mg/ml Pluronic F127 (Molecular Probes Inc.). Coverslips were then placed in a recording chamber and continuously bathed with MHNK solution superfusion. Stimuli were dissolved in MHNK and then pH and osmolarity were readjusted if needed. Cells were exposed to various chemical stimuli: 0.5 and 2 mM denatonium, 25 μM cycloheximide, 80 mM potassium, and 1 mM sucralose (all purchased from Sigma).

The chemical stimuli were applied to the coverslip by switching the superfusion to the stimulus solution, which allowed for a complete change of bath solutions in the chamber within 10 s. Calcium-imaging recordings were performed using standard imaging techniques (Rawson *et al.*, 1997). Illumination was via an LSR SpectraMASTER monochromator coupled to the microscope. Cells were illuminated with light emitted by a 75-W Xenon lamp alternately filtered with narrow band-pass filters at 340 nm, then 380 nm. Emitted light from the fura-2 in the cells under 200× microscopic magnification was filtered at 510 nm and passed through an image intensifier coupled with a cooled charge-coupled device (CCD) camera (Olympix, Perkin Elmer Life Sciences, Bethesda, MD). Exposure times were minimized and light shuttered between acquisitions to minimize photobleaching. Images were digitized using a Merlin Imaging Workstation (Perkin Elmer Life Sciences), which controlled the illuminator and camera, and acquisition, and performed the image ratioing and display of pseudocolor images. Cells remained viable in the recording setup for over 2 h without visible effects of dye bleaching. Stimuli were diluted in MHNK buffer and applied via a gravity-flow superfusion apparatus for 10–60 s, depending on the stimulus.

Data analysis

To facilitate data analysis and visualize trends in intracellular calcium over time, the ''average $[Ca^{2+}]_i$'' was determined by computing the mean $[Ca^{2+}]_i$ over three data points (approximately 20 s) at sequential points along the data trace. Responses to taste stimuli were determined by calculating the average $[Ca^{2+}]_i$ for the three points immediately prior to the application of a stimulus solution (prestimulus baseline), at the highest point during or within 10 s after stimulus delivery (''peak'' $[Ca^{2+}]_i$), and at the low point after at least 30–60 s of washout (poststimulus baseline). A response was defined as follows: a distinct and sustained change in $[Ca^{2+}]_i$ for which the average ratio at the highest point poststimulation was at least 0.03 greater than the average baseline ratio following stimulation by the tastant. Only records in which calcium levels returned to within 0.01 ratio points of the original baseline within 100 s of taste stimulus removal (at the portion of the trace where $[Ca^{2+}]_i$ attained a steady state) were included.

Results

Cell culture conditions and cell viability

The method for taste cell isolation was evaluated with respect to enzymatic treatment for cell isolation, coating of the cell culture plates and coverslips, and composition of tissue culture medium. The isolation procedure was rendered more efficient by using a single isolation solution with a shorter incubation time than described in previous studies and utilizing two protein digestion enzymes, which required less

treatment time. Of the five different coatings tested, rat tail collagen type 1 provided a consistent attachment rate of approximately 15–20% on the collagen-coated coverslips and tissue culture plates (Figure 1A). Culturing rat taste cells with UV-irradiated mouse fibroblast cells as cofeeder had technical and experimental limitations since mouse fibroblast cells became detached from tissue culture plates (Figure 1B). The number of cells attached to matrix gel–coated coverslips was less than 5%, and the cells did not grow well (Figure 1C). No cell attachment was observed on the surface of uncoated tissue culture plates (polyprene) and glass coverslips coated with (Figure 1D) or without poly-D-lysine. Different tissue culture media and isolation conditions were also tested. Growth was minimal in the presence of DMEM, IMDM, or MCDB 153 alone. We found that IMDM supplemented with 20% MCDB 153 medium and 10% FBS provided good cell adhesion on collagen-coated tissue culture plates and coverslips over long periods of time. Viability and growth were maintained by changing the medium every 7–10 days.

The morphology and viability of cultured taste cells changed over time (Figures 2 and 3). Although individual cells and bud-type cells were visible 24–48 h after plating (Figure 2A–D), cells grew for up to 7–8 days under attached cell clusters, which seemed to give rise to daughter cells (Figure 2E–H). After 5–7 days, the cell clusters started to detach (Figure 2I–J). Most cells maintained their original compact appearance of round cell bodies with or without one or more processes up to 15–20 days but after 20 days; most of the long-term cultured cells had a flatter appearance
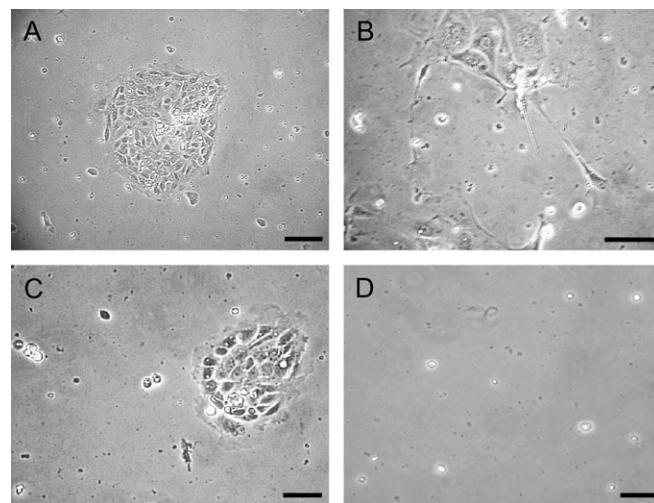

Figure 1 Attachment of cultured cells varies on different adhesion materials. Primary rat taste cell cultures were grown under the same culture medium for 10 days on coverslips coated with different adhesion materials: rat tail collagen-1 **(A)**, mouse fibroblast **(B)**, matrix gel **(C)**, and poly-D-lysine **(D)**. Rat tail collagen-1 provided a good initial attachment rate of about 20% and was used for subsequent experiments. Scale bars = 50 μm (B) and 100 μm (A, C–D).
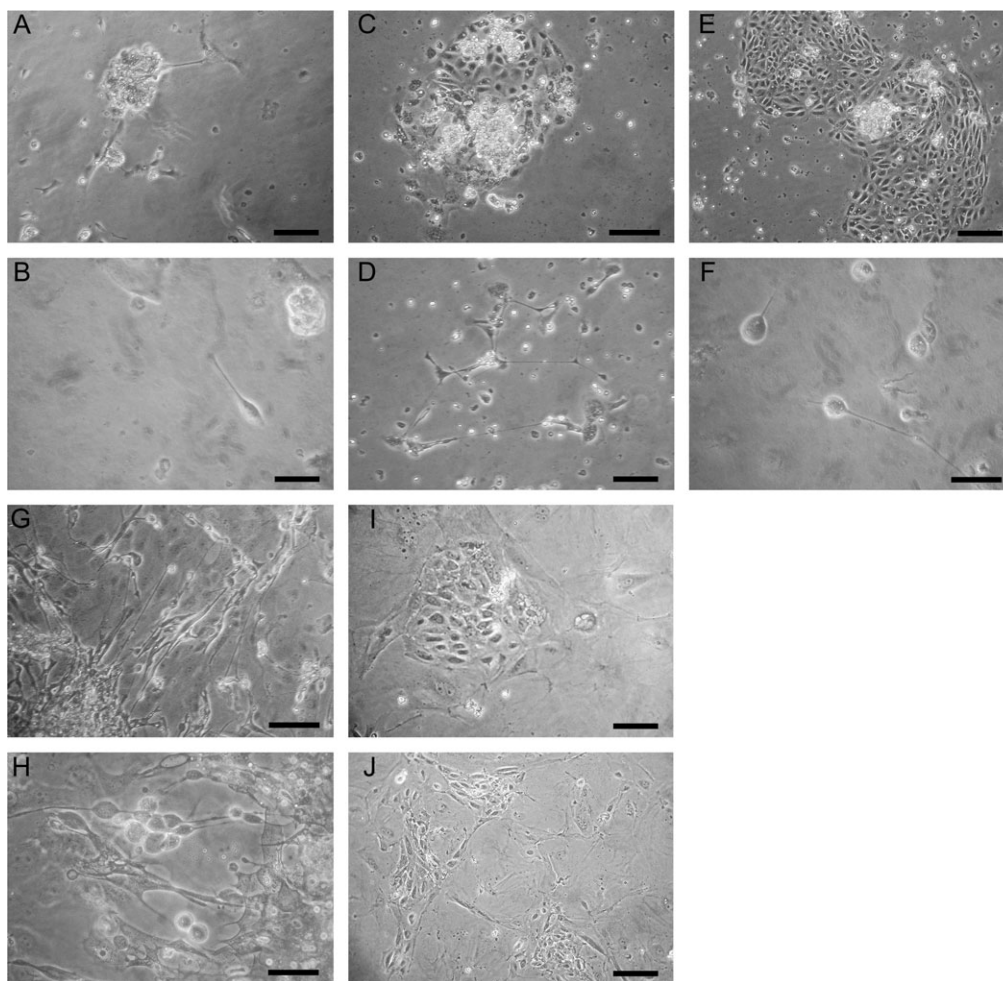
Figure 2 Morphology of short-term cultured cells. Live taste cell cultures grown on collagen-1–coated plates were imaged after 1 day **(A, B)**, 2 days **(C, D)**, 3 days **(E, F)**, 4 days **(G, H)**, and 13 days **(I, J)**. Individual and bud-type cells were observed 24–48 h after culturing, and cell clusters were observed after 1 week. Each culture represents independent experiments from different starting cultures. Scale bars = 50 μm (C, F–H) and 100 μm (A, B, D, E, I, J).

(Figure 3A–D). There were an increasing number of dead cells by 3 weeks, however in some cultures, a significant number of cells remained viable for more than 2 months (Figure 3C–F). Staining with trypan blue showed that over 95% of the cells were alive during the first week of culturing with only 5–10% dead (data not shown). Under our culturing conditions, taste cells maintained 98–99% viability for the first 3–4 weeks and maintained 90–95% viability after 2 months. We did not observe any bacterial or fungal contamination problems throughout the study, indicating the elimination of contamination in the cultures.

Expression of specific taste cell marker proteins and mRNA

It has been reported that the expression of specific bio-markers such as gustducin, PLC-β_2, blood group antigens, and protein gene product 9.5 disappeared in differentiated taste cells within 7–10 days after denervation (Smith *et al.*, 1994). This suggests that the absence of a neuronal population may represent a limitation for long-term culturing of

taste cells. We utilized cultured rat foliate and circumvallate cells along with control samples obtained from freshly isolated rat tongue foliate and circumvallate tissues to determine whether cells expressed gustducin and PLC-β_2 after 1 month in culture. Gustducin and PLC-β_2 proteins were detected in all the samples albeit at apparently different levels of expression (although these were not quantified). Freshly isolated samples (Figure 4A) exhibited an apparently higher concentration of PLC-β_2 compared to cultured cells (Figure 4B), but there were no differences evident in gustducin levels between the two samples. This may indicate differences in gene regulation in cultured versus intact taste cells and independent control of gustducin versus PLC-β_2 protein levels, but additional studies are needed to address this. Two distinct bands were observed for the anti–PLC-β_2 antibody which corresponded with those previously reported (Zhang and Neer, 2001). The homogenates from taste and nontaste tissues were subjected to immnunoprecipitation with polyclonal antisera.
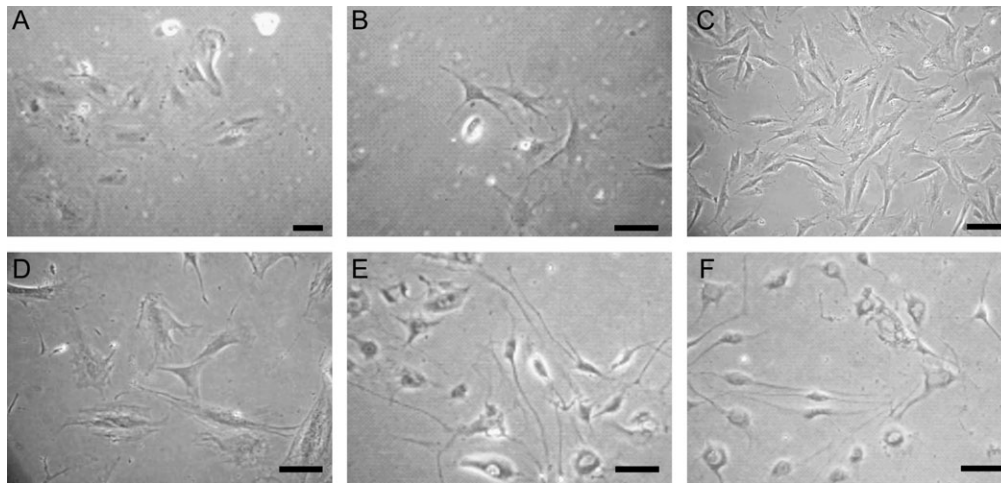
Figure 3 Morphology of long-term cultured cells. Live taste cells grown on collagen-1–coated plates were imaged at 1 month **(A, B)**, 2.5 months **(C, D)**, and 3 months **(E, F)**. Unlike the morphology of short-term cultures, cells cultured longer than 20 days had a flattened appearance. Each culture represents independent experiments from different starting cultures. Scale bar = 50 μm (A–F).
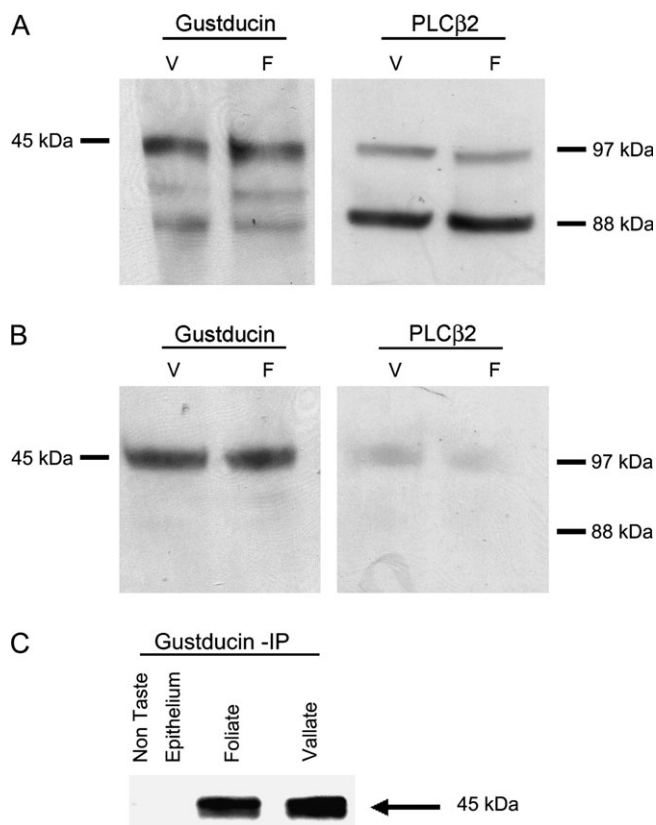

Figure 4 Gustducin and PLC-β_2 proteins were present in cultured cells. Western blot analysis showed both proteins were expressed in freshly isolated rat tongue foliate and circumvallate tissue **(A)**. Similar results were observed in 1-month cultured rat foliate and circumvallate cells **(B)**. Immunoprecipitation of freshly isolated tissues obtained from nontaste lingual epithelium, foliate, and vallate with gustducin antibody showed specificity of detection of gustducin expression **(C)**. Protein concentrations were consistent for each sample (see Methods). V = vallate and F = foliate.

Homogenates from taste tissue (foliate and vallate) showed specific immunoprecipitation of 45- to 46-kDa proteins which were absent in homogenate of nontaste lingual tissue (Figure 4C).

We also examined mRNA expression of gustducin, PLC-β_2, T2R5, and T1R3 with β-actin as the housekeeping marker in the freshly isolated taste cell (foliate and vallate) and nontaste lingual epithelium and in short- and long-term cultured cells from the vallate and foliate papillae. RT–PCR results demonstrated that all four specific taste cell biomarkers were expressed in freshly isolated cells (Figure 5A) and in cDNA from taste cells cultured for 7–10 days (Figure 5B) and 2.5 months (Figure 5C), with amplification products of the expected size. T1R3 and T2R5 amplification products were also detected in all samples. As a control, PCR reactions carried out with RNA samples amplified in the absence of reverse transcriptase enzyme yielded no products using all sets of primers, indicating the lack of genomic DNA contamination. Additionally, gustducin, T1R3, and T2R5 expression were not observed in nontaste lingual epithelium, although β-actin was detected (Figure 5D).

Immunocytochemical detection of taste cell markers

Immunocytochemical experiments were conducted to determine proliferation, differentiation, and the presence of specific taste cell biomarkers in cultures.

Approximately 30–40% of taste cells were labeled with BrdU after 3–5 days in culture, indicating cellular proliferation (Figure 6A–C and G–P). A subset of 7- to 10-day-old taste cells treated with BrdU (approximately 10–20%) were immunoreactive for both BrdU and either gustducin (Figure 6G–L) or PLC-β_2 (Figure 6P–R), indicating that
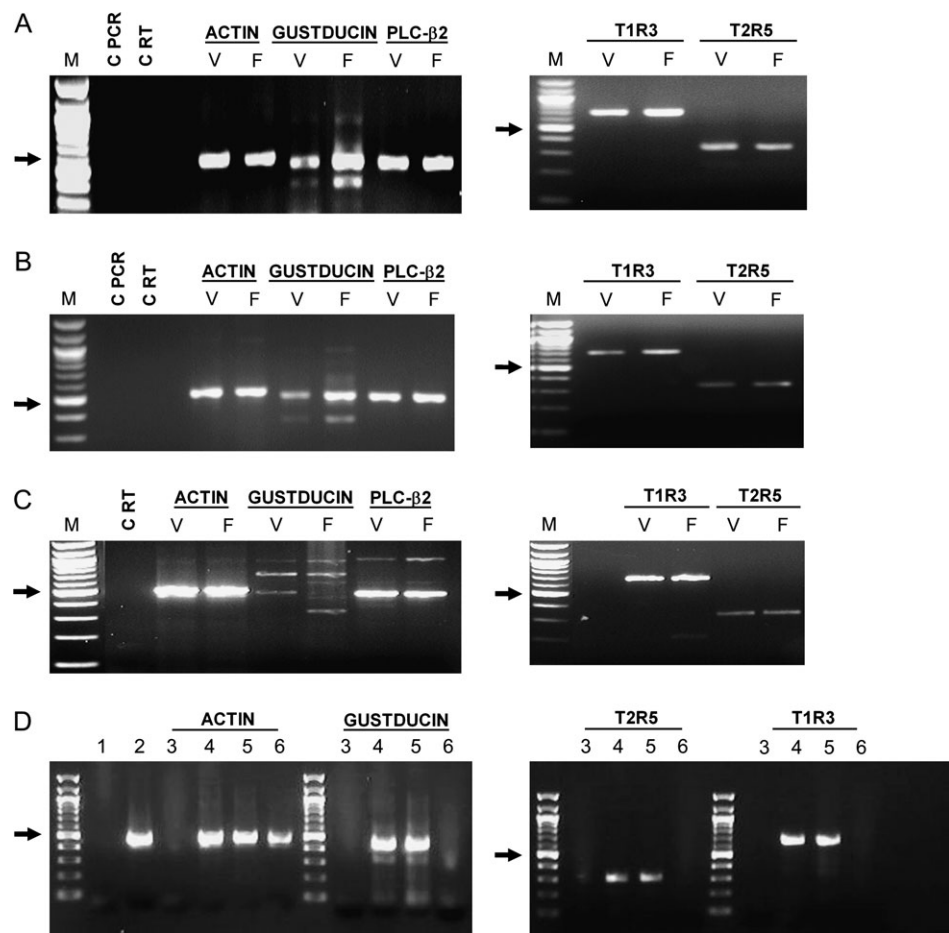
Figure 5   The expression of gustducin, PLC-β_2, T1R3, and T2R5 mRNA in tongue tissue and cultured taste cells. RT–PCR amplifications were conducted in rat tongue taste tissue **(A)**, 9-day-old primary cultured taste cells **(B)**, and 2.5-month-old primary cultured taste cells **(C)**. Each of the taste cell biomarkers was expressed in all samples. In control experiments, gustducin, T1R3, and T2R5 mRNA were not detected in nontaste epithelium **(D)**. Numbers indicate samples which were PCR mixture without RNA (1), RT-positive control (2), sample RNA without RT (3), foliate (4), vallate (5), and nontaste lingual epithelium (6). Arrows indicate 300, 500, and 700 bp. C RT = reverse transcriptase control, M = marker (100-bp division), V = vallate and F = foliate.

cells which had divided *in vitro* had subsequently differentiated. These tended to be within clusters containing many cells immunoreactive for the biomarker. Some cultured cells were immunoreactive to only a single biomarker and many cells, particularly those not associated with clusters, were not immunolabeled with any of these antibodies. After 1 month, the presence of BrdU-immunoreactive cells decreased to 5–10% of total cells, suggesting a decline in proliferation with time (data not shown).

The specificity of immunostaining was evaluated in a variety of control experiments. No cross-reactivity with the gustducin or PLC-β_2 secondary antibodies was detected when the BrdU-reacted cells were further processed in the absence of the second primary antibody (Figure 6B, gustducin; Figure 6N, PLC-β_2). Cells not exposed to BrdU showed no BrdU immunoreactivity, demonstrating the specificity of BrdU antibody (Figure 6D). Immunostaining with antibody-specific immunoglobulin demonstrated the absence of nonspecific immunoreactivity (data not shown). Finally, peptide inhibi-

tion of gustducin antibody showed complete removal of gustducin immunostaining, indicating antibody specificity (data not shown).

Taken together, these results demonstrate that a variety of cells at different stages persist in the cultures for at least 1 month and that some cells dividing *in vitro* progress to express proteins found in mature taste cells *in vivo*.

Chemical stimuli induce intracellular Ca^{2+} responses

The responses of 1- to 4-week-old cultured taste cells to chemical stimuli were analyzed using the Ca^{2+}-sensitive dye fura-2. A total of 937 cells representing 18 cultures maintained for 4–10 days (young) and seven cultures maintained for 2–4 weeks (older) were tested with bitter and sweet stimuli. Approximately 15.5% of the cells exhibited increased [Ca^{2+}]$_i$ levels in response to one or more of the stimuli (Table 2). Overall response frequency was similar for young and older cultures. The highest frequency of responses was
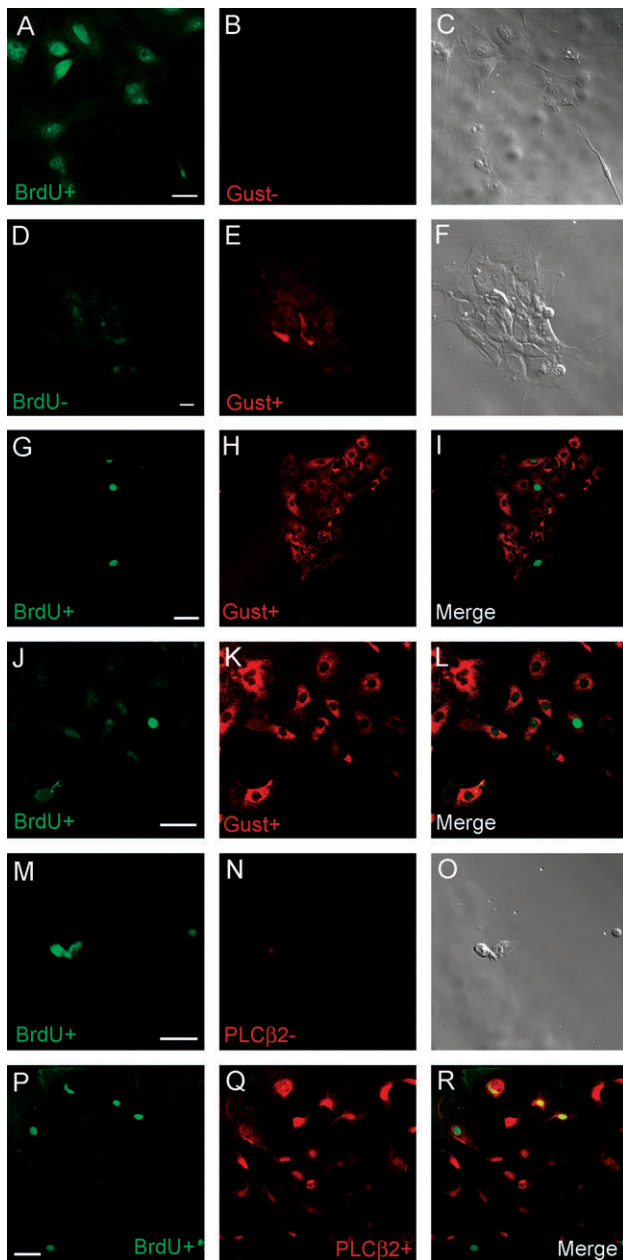
Table 2 Response frequency of 1- to 4-week-old cultured taste cells to different chemical stimuli

Stimuli	Response frequency (%)	Responsive cells/ total cells tested
0.5 mM denatonium	12.7	14/110
2 mM denatonium	34.2	36/105
25 µM cycloheximide + 2 mM denatonium	20.7	46/222
1 mM sucralose	10	50/500

elicited by 2 mM denatonium (34.2%) and a mixture of 2 mM denatonium and 25 µM cycloheximide (20.7%). Figure 7 (top trace) shows a representative tracing of the transient increase in $[Ca^{2+}]_i$ to different concentrations of denatonium. The increase in $[Ca^{2+}]_i$ was greater after exposure to 2 mM denatonium than to 0.5 mM denatonium. Note the return of $[Ca^{2+}]_i$ to baseline levels over time after washing with buffer. The increase of $[Ca^{2+}]_i$ to the mixture of 2 mM denatonium and 25 µM cycloheximide elicited responses of similar magnitude when repeated after several minutes (Figure 7, middle trace). The artificial sweetener sucralose (1 mM) elicited an increase in $[Ca^{2+}]_i$ in about 10% of cells tested (Table 2) and the response was repeatable but it exhibited some adaptation (Figure 7, lower trace). We did not observe any taste cells responding to both sweet and bitter stimuli, consistent with previous work showing that bitter and sweet responses in rat taste cells were independently distributed (Caicedo and Roper, 2001). We also tested whether potassium (K^+) could elicit $[Ca^{2+}]_i$ responses by depolarizing the cells with a buffer solution containing 80 mM K^+ (equimolar substitution of NaCl with KCl). Only two of 220 cells tested responded to KCl depolarization and neither cell responded to bitter or sweet stimuli (data not shown). This result is consistent with other reports, indicating that not all taste cells express voltage-gated K^+ channels and/or voltage-gated Ca^{2+} channels (Herness and Gilbertson, 1999; Medler *et al.*, 2003).

Figure 6 Cultured taste cells undergo proliferation. Double immunofluorescence labeling was conducted on 10-day-old cultured cells with BrdU (green) and either gustducin (red) or PLC-β_2 (red) antibodies. BrdU immunocytochemistry detected proliferating cells in the culture **(A, M)**, and there was no cross-reactivity when further processed in the absence of the second primary antibody **(B, N)**. Furthermore, cells not exposed to BrdU showed no immunoreactivity demonstrating the specificity of BrdU antibody **(D)**. In the same double-labeling experiment, no cross-reactivity was detected between BrdU and gustducin antibodies **(E)**. Immunoreactivity for BrdU **(G, J)** and gustducin **(H, K)** overlapped in some cells, indicating that gustducin-labeled cells divided in culture **(I, L)**. Immunoreactivity for BrdU **(P)** and PLC-β_2 **(Q)** also overlapped **(R)** in some cells. Transmission images of corresponding cells are shown **(C, F, O)**. Scale bars = 20 µm (D–F), 40 µm (A–C and M–O), and 80 µm (G–L and P–R).

Discussion

The results of this study demonstrate a protocol for an *in vitro* culture method in which cells obtained from foliate and vallate regions of rat tongue epithelium remained viable for up to 3 months at 37°C. Although cultured taste cells changed morphology after 1 month, immunocytochemical and physiological properties were retained. In addition, we have demonstrated that this new culture method can generate new taste cells from the primary cells that mature *in vitro* to express appropriate markers of mature taste receptor cells.

It has been hypothesized that taste cells originated from the epithelial cell lineage with an average life span of 10 days
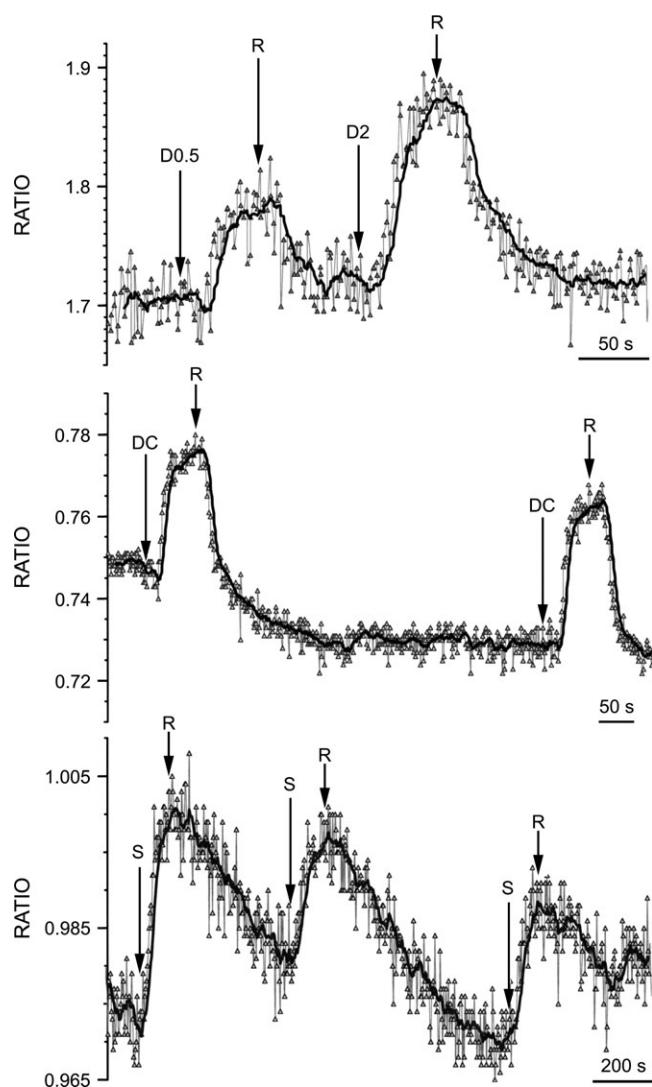
Figure 7 Taste cells cultured for 1–4 weeks responded to sweet and bitter stimuli. Graphs illustrate representative responses of $[Ca^{2+}]_i$ levels in individual cells during exposure to 0.5 and 2 mM denatonium (upper trace), a mixture of 2 mM denatonium and 25 µM cycloheximide (middle trace), and 1 mM artificial sweetener sucralose (lower trace). Arrows indicate the time that the superfusion solution was added to cultures — actual time of arrival at the cells was within 20 s after switching. D0.5 = 0.5 mM denatonium, D2 = 2 mM denatonium, DC = 2 mM denatonium and 25 µM cycloheximide mixture, S = 1 mM sucralose, R = rinse with MHNK (see Methods). Time scale = 50 s (upper and middle traces) and 200 s (lower trace).

and were replaced by basal cells. In our culture system, cell clusters which supported taste cell growth dissociated after 7–10 days, suggesting the need for additional neurotrophic factors for the production of newly generated cells. Previous studies indicated the importance of specific microenvironmental features controlling the survival and regeneration of mammalian taste cells (Farbman, 1969; Farbman and Mbiene, 1991; Kishi *et al.*, 2001; Ruiz *et al.*, 2001; Stone *et al.*, 2002). The loss of these features may alter the gener-

ation of new taste cells from their precursors, hence limiting the use of taste cell cultures over a long period of time. In a culture environment, taste cells lose the innate components such as nerve fibers, growth factors, and neurotrophins surrounding epithelial cells and saliva. The results from our study demonstrated that the medium described and surface coating with rat collagen type 1 was able to substitute for at least some of these environmental factors and maintain differentiated taste cells for longer periods of time than previously reported (Spielman *et al.*, 1989; Kishi *et al.*, 2001). Further studies to identify factors influencing proliferation and differentiation of cultured taste cells will inform future investigations using *in vivo* systems to develop therapeutic approaches.

An important criterion for a model system is the presence of relevant functional properties. Calcium responses have been studied in a variety of taste cell preparations, including both dissociated cells (Kishi *et al.*, 2002) and slices (Caicedo *et al.*, 2002). In these studies, sweet, bitter, and umami stimuli elicit an increase in intracellular calcium that may correspond to a depolarization (Hayashi *et al.*, 1996, 1997). Although the details of the transduction pathways are not definitively established for all taste stimuli, the increase in intracellular calcium elicited by bitter, sweet, and umami stimuli is thought to be dependent on activation of PLC, release of calcium from intracellular stores, and activation of the calcium-dependent TRPM5 channel which also allows calcium influx. However, activation of adenylyl cyclase and cyclic adenosine monophosphate production has also been observed in response to sweet stimuli and may play a role in transduction as well (Gilbertson *et al.*, 2000; Lindemann, 2001; Margolskee, 2002; Scott, 2004). The pathway responsible for calcium responses in these cultured cells remains to be established, but our data support the use of this model system for further studies of these pathways.

In conclusion, this protocol represents a substantial improvement over earlier methods in its ability to maintain differentiated cells for up to 3 months and to generate new taste cells *in vitro* that proceed to differentiate to express taste cell markers. The development of this long-term culture will provide a model system for studies of proliferation and differentiation, stimulus specificity, cross talk, and adaptation properties of mammalian taste receptor cells. In addition, the effects of conditions that impair taste, such as infection, medications, radiation, toxic, or chemical exposures, can be examined at molecular and physiological levels (Nelson, 1998).

Acknowledgements

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