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Maintenance of Rat Taste Buds in Primary Culture

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Abstract

The differentiated taste bud is a complex end organ consisting of multiple cell types with various morphological, immunocytochemical and electrophysiological characteristics. Individual taste cells have a limited lifespan and are regularly replaced by a proliferative basal cell population. The specific factors contributing to the maintenance of a differentiated taste bud are largely unknown. Supporting isolated taste buds in culture would allow controlled investigation of factors relevant to taste bud survival. Here we describe the culture and maintenance of isolated rat taste buds at room temperature and at 37°C. Differentiated taste buds can be sustained for up to 14 days at room temperature and for 3–4 days at 37°C. Over these periods individual cells within the cultured buds maintain an elongated morphology. Further, the taste cells remain electrically excitable and retain various proteins indicative of a differentiated phenotype. Despite the apparent health of differentiated taste cells, cell division occurs for only a short period following plating, suggesting that proliferating cells in the taste bud are quickly affected by isolation and culture.

Introduction

The differentiated taste bud is a complex, dynamic organ. Multiple cell types exist in taste buds and there is continual turnover of cells with accompanying re-modeling of taste cell–neuron synapses (Beidler and Smallman, 1965; Conger and Wells, 1969; Farbman, 1980). With regard to heterogeneity, several different types of taste cells have been described. Based on ultrastructural features such as cytoplasmic density and the presence or absence of dense cored granules, taste buds contain basal, type I (dark), type II (light) and type III cells (Farbman, 1965; Kinnamon *et al.*, 1985; Delay *et al.*, 1986). The presence or absence of specific proteins can also be used to differentiate subsets of taste cells. For example, gustducin (McLaughlin *et al.*, 1992, 1994), PGP 9.5 (Huang and Lu, 1996; Kanazawa and Yoshie, 1996; Wakisaka *et al.*, 1996), serotonin (Reutter, 1971; Toyoshima and Shimamura, 1987; Kuramoto, 1988; Delay *et al.*, 1993) and neural cell adhesion molecule (NCAM) (Nolte and Martini, 1992; Nelson and Finger, 1993; Smith *et al.*, 1993) each exist in subsets of taste cells. In some cases protein markers are expressed in the same cells, but others are mutually exclusive (Böttger *et al.*, 1997). Physiologically, taste cells exhibit voltage-gated Na^+, K^+ and Ca^{2+} currents and the types and magnitudes of the currents vary between different taste cells (Akabas *et al.*, 1990). Another distinguishing feature is the cellular response to chemical stimuli. For example, nearly 70% of fungiform taste cells express the amiloride-sensitive Na^+ channel, the primary basis of Na^+ salt taste in rats (Lin *et al.*, 1999), whereas only 15% of taste cells respond to sweet compounds (Bernhardt *et al.*, 1996; Cummings *et al.*, 1996). The limited lifespans of taste cells also contribute to the complexity of a taste bud, as cells are lost and replaced with accompanying re-modeling of synapses.

The mechanisms underlying the regulation of proliferation, cell replacement and phenotypic maturation in taste buds are largely unknown. A controlled *in vitro* environment would facilitate study of these mechanisms. Culture systems utilizing lingual explants have been developed to begin investigating questions about formation of taste buds and papillae during development (Farbman, 1972; Mbiene *et al.*, 1997; Morris-Wiman *et al.*, 2000). Farbman cultured embryonic rat circumvallate papillae with and without sensory ganglia and found that only cultures containing ganglia developed structures resembling taste buds (Farbman, 1972). More recently the development of fungiform papillae has been examined using fetal tongue explants placed in organ culture. Although fungiform papillae begin to develop normally in the absence of innervation, taste

buds do not form (Farbman and Mbiene, 1991; Mbiene *et al.*, 1997). This may be due to lack of innervation in the explants. However, increased survival times of the cultured explants may be necessary to fully examine the inductive role of nerves in lingual cultures (Barlow, 1999). Using a different approach, Morris-Wiman *et al.* used branchial arch explants cultured in roller tubes. In these studies papillae morphogenesis seemed to correlate with innervation, although taste bud development was not addressed in these studies (Morris-Wiman *et al.*, 2000).

Adult taste tissue has been cultured as lingual explants in the anterior chamber of the eye. As with embryonic tissue, taste buds formed in adult tongue tissue when it was co-cultured with ganglia but failed to form when explanted alone (Zalewski, 1972). These studies indicate that the ability of an adult mammalian taste bud to maintain its differentiated phenotype may be dependent on factors associated with innervation. This idea is consistent with denervation studies *in vivo*. Denervation of the circumvallate papilla results in complete degeneration of vallate taste buds in 1–2 weeks (Guth, 1957; Kennedy, 1972; Miyawaki *et al.*, 1998; Seta *et al.*, 1999). Fungiform taste buds are affected less by denervation and persist, at least in some mammals, in a relatively dedifferentiated state (Whitehead *et al.*, 1987; Oliver and Whitehead, 1992). Restoration of gustatory innervation *in vivo* results in regeneration of taste buds occurring in 1.5–2 months (Iwayama and Nada, 1969; Hosley *et al.*, 1987; Miyawaki *et al.*, 1998; Seta *et al.*, 1999).

To date, culture systems have been used primarily to investigate differentiation and development of embryonic lingual tissue. A culture system for isolated adult taste buds would be useful to investigate the conditions necessary for the maintenance of differentiated taste buds. Thus, the goals of the present study were to develop a procedure for maintaining differentiated isolated rat taste buds for a short time in a defined culture medium, describe the changes that occur during culture and provide a general time course for these changes. Also, we wished to determine if taste buds in culture retain histochemical and physiological properties. A defined culture medium was used in these studies and the taste buds were maintained in the absence of nerve fibers. This study was meant to investigate the length of time taste buds retain critical features in culture and to develop a starting point for investigating the specific factors that contribute to a normal taste bud phenotype. Preliminary accounts of this work have been published previously (Ruiz *et al.*, 1993, 1995a,b).

Materials and methods

Taste bud isolation

All use and handling of the animals was performed with the approval of the Colorado State University and University of Colorado Health Sciences Center Institutional Animal Care and Use Committees. Fungiform, foliate and circumvallate taste buds were isolated from adult Sprague–Dawley rats by a procedure adapted from Béhé *et al.* (Béhé *et al.*, 1990). Briefly, the rat was killed with CO_2 and the tongue removed proximal to the circumvallate papillae. Approximately 500–1000 μl dispase/collagenase enzyme solution was injected uniformly under the epithelium of the dissected tongue. After incubating for 40–50 min in Ca^{2+}-free oxygenated Tyrodes solution the lingual epithelium was gently separated from the underlying connective tissue. The posterior part of the epithelium containing the circumvallate and foliate taste buds often required somewhat longer incubations, approaching 70–90 min. The epithelium was pinned out serosal side up in a Sylgard-coated dish containing Ca^{2+}-free Tyrodes solution and incubated for 20 min at room temperature. Individual taste buds were viewed at 50× with a dissecting microscope and buds were removed with a fire-polished pipette (tip diameter 75–150 μm) using gentle suction.

Plating of isolated taste buds

Experimental cultures were incubated at either room temperature (18–22°C) or 37°C. For room temperature experiments Sylgard ring culture wells or lysine-coated Cell Environments culture slides (Becton Dickinson, Bedford, MA) were used. Sylgard culture wells were constructed with Sylgard rings and coverslips. The Sylgard rings were fabricated from sheets of cured Sylgard (1–2 mm deep; Dow Corning, Midland, MI) using two different sized cork borers (i.d. 5 and 14 mm). The larger diameter cork borer was used to cut disks from the Sylgard, while the smaller diameter borer was used to remove the center of each disk, thus creating a ring with an internal diameter of 5 mm. The area of the coverslip contained within the Sylgard ring was then coated with several layers of Cell-Tak (Collaborative Research, Bedford, MA), allowed to dry, rinsed with 70% EtOH, dried again and rinsed with deionized H_2O. Sylgard rings were attached to coverslips (no. 1; Dow Corning) with vacuum grease (Dow Corning) to ensure a watertight seal and sterilized for 20 min with UV radiation. Culture wells were filled with 50 μl of culture medium and, following addition of isolated taste buds, the wells were placed into covered 35 mm sterile plastic culture dishes and wrapped with Parafilm (American National Can, Chicago, IL) to prevent evaporation of the medium. The medium was changed every 2 days for most experiments. Exchange of medium often resulted in a loss of taste buds, so some of the long-term cultures were maintained for 4–14 days with fewer changes of medium. For 37°C experiments, and some room temperature experiments, taste buds were placed in individual wells of poly-D-lysine-coated Biocoat Cell Environments culture slides (Becton Dickinson). Depending on well size, 200 (8-well slides) or 1000–1500 μl (2-well slides) of medium was used during culture.

Solutions

Tyrode's solution contained 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $CaCl_2$, 10 mM glucose, 1 mM $MgCl_2$ and 10 mM Na pyruvate, adjusted to pH 7.4 with NaOH. For Ca^{2+}-free Tyrodes the Ca^{2+} and Mg^{2+} were replaced with 2 mM BAPTA (Molecular Probes, Eugene, OR).

The enzyme mix for taste bud isolation consisted of 2.5–4.0 mg dispase (grade II; Boehringer Mannheim, Indianapolis, IN), 1.0 mg collagenase A (Boehringer Mannheim) and 1.0 mg trypsin inhibitor (Type I-S; Sigma Chemical Corp., St Louis, MO), dissolved in 1 ml of Tyrode's solution.

The culture medium used for both room temperature and 37°C experiments was a serum-free medium modified from Pixley (Pixley, 1992). The medium consisted of DMEM (Dulbecco's modified Eagle's medium containing D-glucose, l-glutamine and sodium pyruvate; Gibco BRL, Grand Island, NY), 15 mM KCl, 18 mM HEPES, 10 ml/l MEM NEAA IX (Earle's Salts with non-essential amino acids; Irvine Scientific, Santa Ana, CA), 30 μM hypoxanthine, 3 μM thymidine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 25 μM 2-mercaptoethanol and 2 ml/100 ml ITS + Premix (6.25 μg/ml transferrin, 6.25 μg/ml selenous acid, 1.25 mg/ml bovine serum albumin and 5.35 μg/ml linoleic acid; Collaborative Biomedical Products, Bedford, MA). The medium was adjusted to pH 7.4 with NaOH. For 37°C experiments 49.3 ml/l sodium bicarbonate (7.5% w/v; Gibco BRL) was added to the medium before adjusting the pH. All chemicals were obtained from Sigma Chemical Corp. unless otherwise noted

Patch-clamp recording

For electrophysiological studies the medium was removed from the culture dishes and replaced with Tyrode's solution. In some cases two drops of Trypan blue (0.4% in 0.1% NaCl; Sigma) was added to the culture wells to identify viable taste cells for recording. After 1–2 min the taste buds were rinsed with Tyrode's solution and maintained in this solution for recording. Viable cells were identified by their exclusion of the Trypan blue stain. Patch pipettes were made from micro hematocrit capillary tubes (Scientific Products, McGaw Park, IL) on a two-stage vertical puller (model PB-7; Narishige, Tokyo, Japan). Pipette resistances were 3–7 MΩ when filled with intracellular saline, con- taining 140 mM KCl, 10 mM HEPES, 11 mM EGTA, 1 mM $CaCl_2$ and 2 mM $MgCl_2$, adjusted to pH 7.4 with KOH.

Voltage-dependent currents were monitored in the whole cell configuration (Hamill *et al.*, 1981). Membrane currents were filtered at 2 kHz and recorded with an Axopatch patch-clamp amplifier (model 1D; Axon Instruments, Foster City, CA). An Indec laboratory computer system (Sunnyvale, CA) or pCLAMP8 software (Axon Instruments, Foster City, CA) was used to apply voltage steps and to analyze voltage-dependent currents. Membrane potential was measured as the 0 current potential and input resistance was measured by applying a 25 mV hyperpolarizing pulse to the pipette.

BrdU labeling

Incorporation of 5-bromo-2-deoxyuridine (BrdU) was studied in taste buds maintained at room temperature and 37°C. To label dividing cells cultures were incubated with BrdU (15 μg/ml) immediately following isolation or after 48 h in culture. The BrdU remained in contact with the cultured taste buds for 24–72 h before removal. Taste buds were kept in culture for an additional 2–7 days and then fixed with 100% cold methanol for 30–60 min. Following evaporation of the methanol samples were either processed for immunocytochemistry immediately or stored at –20°C for up to 2 months. Sylgard rings were removed before further processing and fixed taste buds were rinsed in 0.1 M phosphate-buffered saline (PBS) (pH 7.2). To reveal the BrdU, fixed taste buds were treated with 1 N HCl for 10 min, then rinsed in 0.1 M PBS (pH 8.5) and 0.1 M PBS (pH 7.2) to restore the pH. Non-specific binding was reduced by incubation of the cultures with 4% bovine serum albumin and 1% normal horse serum for 30 min. Primary antibody (mouse anti-BrdU, 1:20; Amersham Pharmacia Biotech, Piscataway, NJ) was then applied and cultures were incubated for 1 h at room temperature. After being washed for 3 × 10 min in PBS taste buds were incubated in rhodamine anti-mouse secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h and then washed again in PBS for 3 × 10 min. A drop of Fluoromount G (Fisher Scientific, Burr Ridge, IL) was placed on a slide and the coverslip containing the labeled taste buds was mounted upside-down on the slide. Taste buds were viewed with a Zeiss standard microscope equipped for epifluorescence or with an Olympus Fluoview Laser Scanning Confocal microscope.

Immunocytochemistry

Cultures were fixed in methanol or 4% paraformaldehyde, then washed in 0.1 M PBS for 3 × 10 min. After blocking non-specific binding sites for 30–60 min (in 1–4% bovine serum albumin, 1% normal horse serum and 0.3% Triton X-100 in PBS) the cultured buds were incubated in anti-gustducin, anti-PGP 9.5 or anti-blood group antigen A antibodies. The antibodies and dilutions were as follows: rabbit anti-gustducin (sc-395, directed against amino acids 93–113 of α-gustducin; Santa Cruz Biotechnology), 1:500–1:1000; rabbit anti-PGP 9.5 (7863-0504; Biogenesis, Kingston, NH), 1:1500; mouse anti-blood group antigen A (A0581; Dako Corporation, Carpinteria, CA), 1:30. Taste buds were incubated in primary antibodies for 1.5–2 h at room temperature or overnight at 4°C. Following primary antibody incubation, cultures were washed for 3 × 10 min in PBS and then incubated with appropriate secondary antibodies for 1.5–2 h. Secondary antibodies were purchased from Jackson and included rhodamine anti-rabbit

antibodies for gustducin and PGP 9.5 studies and rhodamine anti-mouse antibodies for blood group antigen A studies. Following incubation in secondary antibodies taste buds were washed in PBS for 3 × 10 min and coverslipped with flouromount G (Southern Biotechnology Associates, Birmingham, AL or Fisher, Burr Ridge, IL).

Results

Gross morphology of taste buds

Room temperature cultures

Isolated taste buds from fungiform, foliate and vallate papillae were maintained in room temperature cultures for up to 2 weeks. When first plated the taste buds consisted of loosely aggregated clusters of cells; outlines of individual cells were clearly visible within each cluster. After a few hours in culture the central cells of the taste buds became more compact in appearance. At this time individual cells became difficult to distinguish in the compact areas and there was a smooth appearance to the outer layer of the central ball of cells. Surrounding this compact region there was often a collection of loosely aggregated peripheral cells. The apparent health of taste buds following isolation varied between experiments and was assessed visually a few hours after plating. For our purposes taste buds were considered healthy if they attached to the substrate and formed the compact centers described above (Figure 1, days 0 and 3). To determine the effect of extended culture on taste bud phenotype isolated buds were examined every day for 2 weeks. The cultures remained on the microscope stage during this time so that the same taste buds could be examined and photographed daily. The general appearance of the cultures over time is illustrated in Figure 1. Immediately following isolation taste buds possessed a distinct conical form, with apical and baso-lateral regions clearly distinguishable (not shown). Within a few hours after isolation the taste buds lost this initial form and became spherical, with clearly defined, compact centers (see Figure 1, days 0 and 3). Despite the rounding up of taste bud organs, individual cells within the buds retained their elongated shapes as described below. During the first 4 days in culture the taste buds appeared to shift and settle, with taste buds occasionally merging to form larger aggregates, as seen in Figure 1. Two foliate taste buds in the lower right hand corner of day 0 are partially merged on day 3 and are completely merged by day 7. During the first 4 days of culture ~25% of the taste buds were lost, due to their propensity to detach from the substrate. The approximate size of the remaining taste buds appeared to stay constant during the first 10 days following isolation, but taste bud diameter began to decrease at later time points and dead cells began to slough off the periphery of the taste bud (Figure 1). After 14 days in culture most of the taste buds had lost clear definition and appeared as loose clusters of cells. However, a small number of the taste buds remained with clearly defined borders and little loss of size. No obvious differences were noted among cultures of fungiform, foliate or vallate taste buds.

37°C cultures

Taste buds isolated from circumvallate and foliate papillae were maintained in culture at 37°C for up to 2 weeks (Figure 2). The required use of the incubator for these experiments and the increased propensity of taste buds to detach from the substrate at higher temperatures resulted in an inability to photograph the same taste buds each day. However, the same region of the culture slide was viewed at each time. For the first 2 days taste bud cultures at 37°C resembled room temperature cultures. Taste buds formed compact centers and attached to the substrate; few cells sloughed off the periphery of the bud. However, beginning with day 3 taste bud diameter began to decrease and there was an increase in the number of dead cells surrounding the cultured buds. In addition, taste buds began to lose their compact appearance and fewer taste buds were firmly attached to the substrate. In some cultures non-taste cells, possibly fibroblasts, increased in number with longer times in culture. These cells were often closely associated with taste buds, growing around or under the taste bud aggregates, and could be identified by their flatter appearance. By day 13 many taste buds had become detached from the substrate and the remaining taste buds became loose and disaggregated.

Assessment of cell viability

Some room temperature taste bud cultures were stained with Trypan blue to assess taste cell viability (Figure 3). The compact aggregates of cells always excluded Trypan blue, regardless of culture duration up to 14 days, indicating that these central cells were alive. However, isolated cells at the periphery of the taste buds often stained blue, suggesting that these outer cells were not viable. The number of blue staining cells associated with individual buds tended to increase with time in culture (data not shown).

Electrophysiological properties

Rat taste cells possess voltage-gated K^+ conductances and ~75% possess voltage-gated Na^+ conductances, which confer electrical excitability to the taste cells (Béhé *et al.*, 1990). We used Giga-seal whole cell recording to examine the membrane properties of cells in cultured taste buds to compare them with the properties of cells in acutely isolated taste buds. Seals of several gigaohms were readily obtained from cultured cells from all times tested. Interestingly, whole cell recordings were obtained much more readily from the cultured cells than from freshly isolated cells, where balloons of membrane are often sucked into the pipette during attempts at seal formation. Membrane currents were elicited by stepwise depolarizing pulses from a holding potential of –80 mV. Voltage-dependent K^+ currents were recorded from

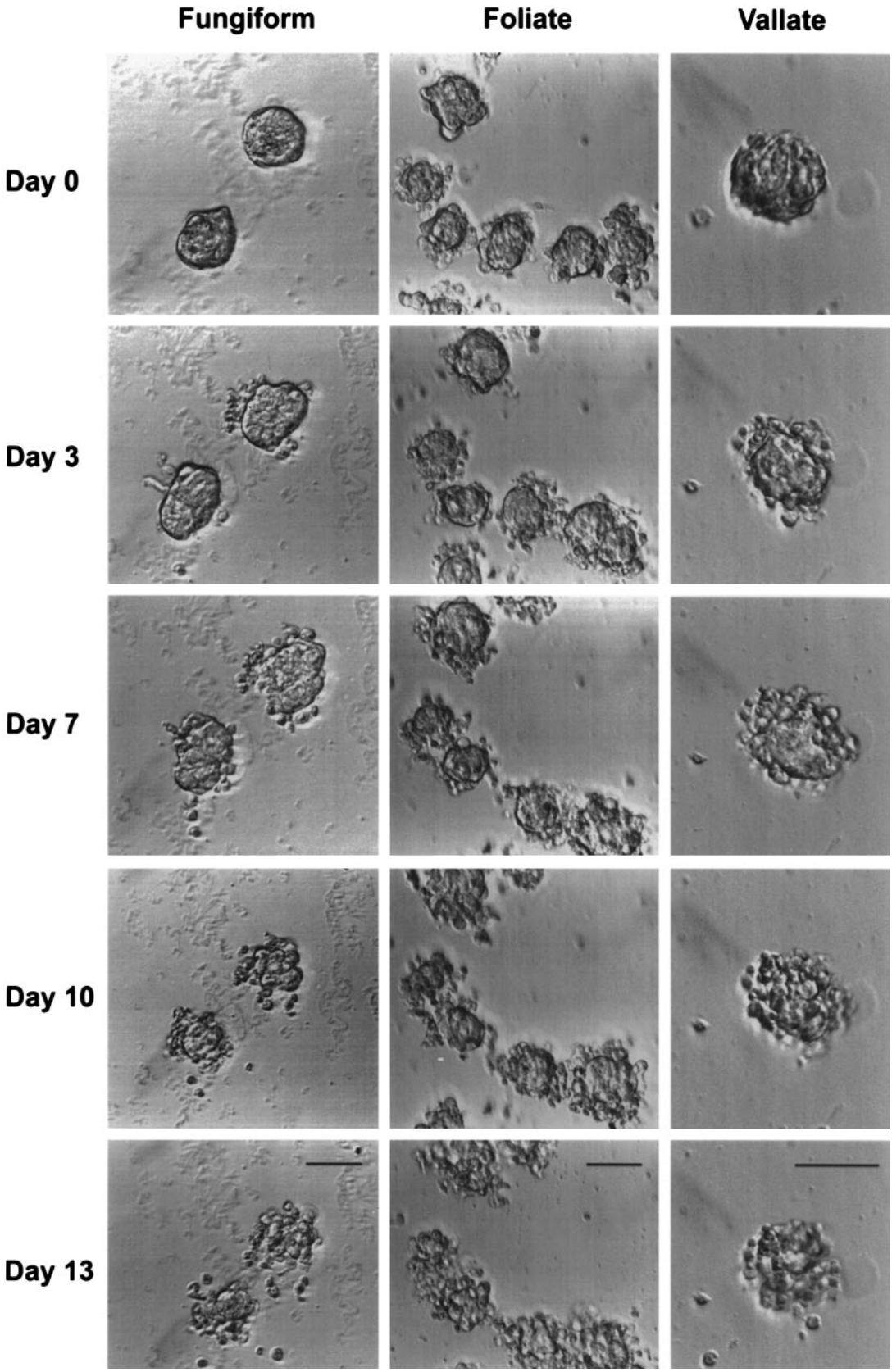

Figure 1 Micrographs of isolated fungiform, foliate and circumvallate taste buds maintained in culture at room temperature for 13 days. For each type of taste bud the culture dishes were affixed to the microscope stage and the same taste buds were photographed at each time.

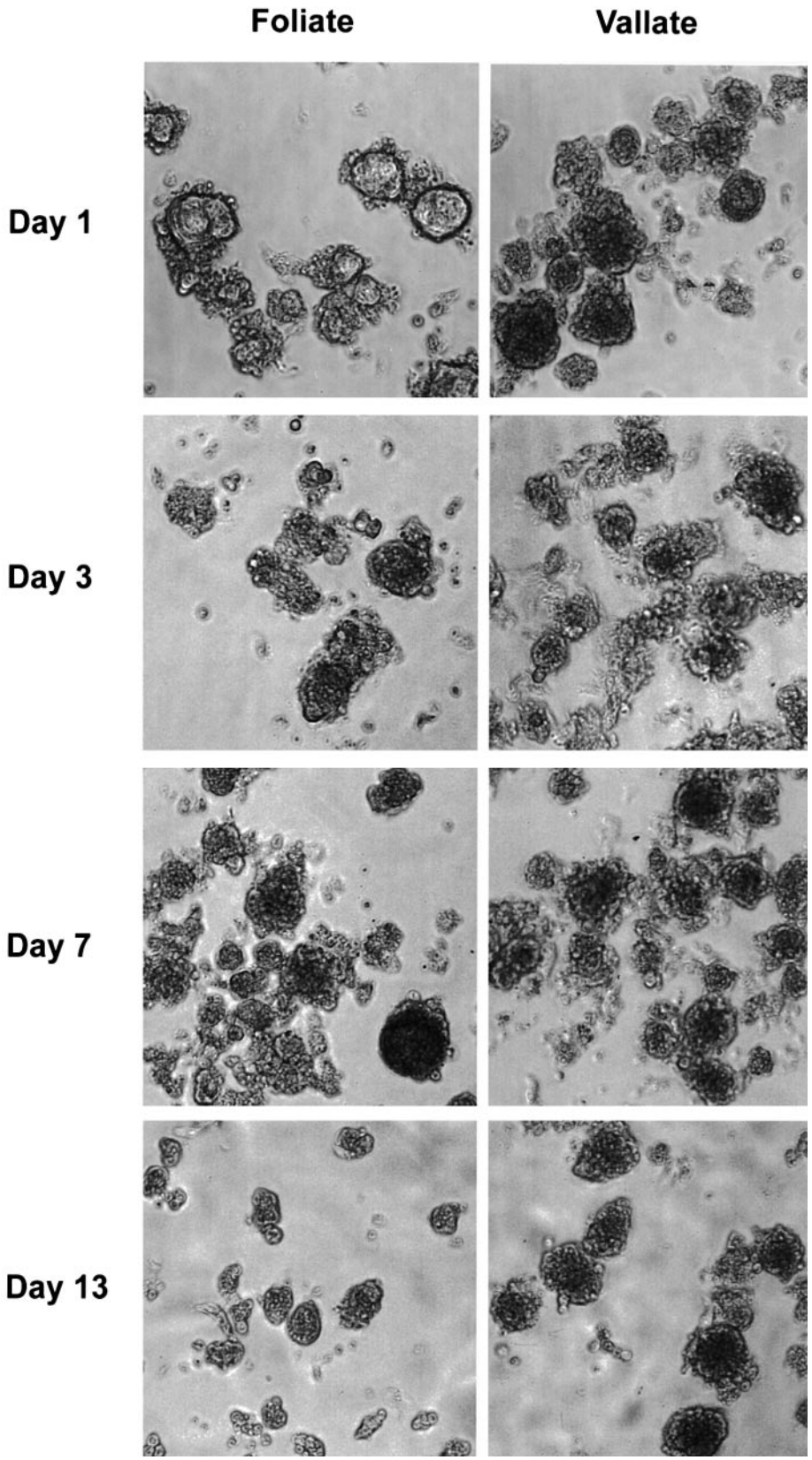

Figure 2 Micrographs of isolated foliate and vallate taste buds maintained in culture at 37°C for 13 days. Since taste buds were cultured in the incubator, cultures had to be removed for photography, thus preventing following the same taste buds over time.

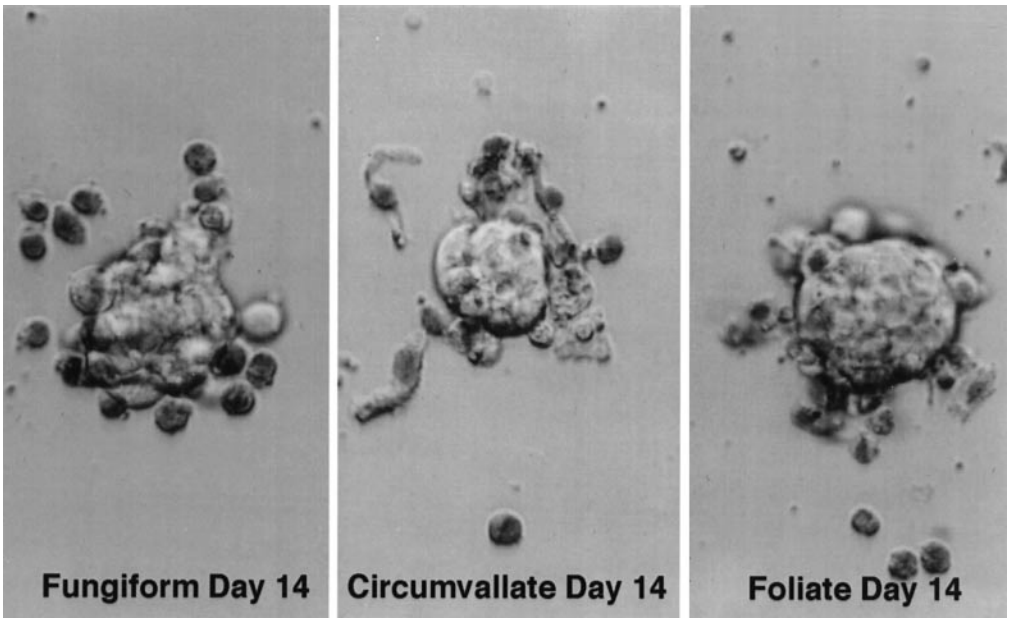

Figure 3 Light micrograph of Trypan blue staining in taste buds cultured at room temperature for 14 days. Trypan blue-labeled cells are indicated by dark gray. Absence of Trypan blue staining in the compact, central regions of the taste buds identifies viable taste cells; Trypan blue is excluded from healthy tissue.

Table 1 Membrane properties of cultured taste buds maintained at room temperature and at 37°C

Parameter	Room temperature		37°C	
	Day 2/3	Day 5/7	Day 1	Day 2
$\%I_K$	100% (9)	100% (17)	100% (6)	100% (4)
$\%I_{Na}$	55% (9)	41% (17)	66% (6)	75% (4)
Mean I_K (pA)	666 ± 68 (9)	458 ± 89 (17)	500 ± 157 (6)	200 ± 0 (4)
Mean I_{Na} (pA)	–220 ± 49 (5)	–428 ± 83 (7)	–600 ± 40 (4)	–216 ± 61 (3)
Resting V_m (mV)	–38 ± 3 (8)	–38 ± 3 (15)	–30 ± 3 (4)	–32 ± 4 (2)

$\%I_K$ and $\%I_{Na}$ refer to the percentage of taste cells that displayed voltage-gated K^+ and Na^+ currents, respectively. Mean I_K refers to the K^+ current recorded at +60 mV, while mean I_{Na} refers to the peak Na^+ current; values are expressed as means ± SEM. Only cells expressing Na^+ currents were used to calculate mean I_{Na}. The values in parentheses represent the number of taste cells sampled for each category.

all cultured taste cells tested (up to 7 days; Table 1), but the magnitude of the currents was somewhat lower than K^+ currents of freshly isolated taste buds (Béhé *et al.*, 1990). Voltage-gated Na^+ currents ranged from 55% of the taste cells tested in 2–3 day cultures to ~41% of the cells tested in 5–7 day cultures (Table 1). When Na^+ currents were present their magnitudes were similar to those reported previously for freshly isolated taste buds (Behe *et al.*, 1990; Herness and Sun 1995). After 7 days the cultures were quite variable, with some cultures still showing taste cells with voltage-gated currents (see for example Figure 4) and other cultures showing few if any taste cells with voltage-gated currents. Resting potentials were consistent in the cultures (Table 1), but the values were somewhat lower than what is typically

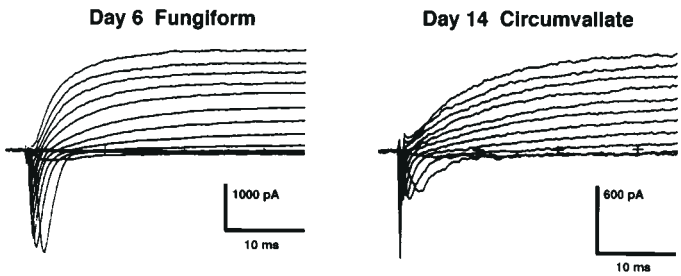

Figure 4 Electrophysiological properties of taste buds cultured at room temperature for 6 and 14 days. Giga-seal whole cell recording was used to examine the membrane properties of cultured taste cells. Currents were recorded in response to depolarizing voltage steps from –60 to +60 mV from a holding potential of –80 mV.

found in freshly isolated taste buds (Behe *et al.,* 1990). We observed that with time in culture an increasing number of taste cells would fail to seal well and thus would exhibit large leak conductances. In some cells membrane capacitance increased as well, suggesting that cells may be electrically coupled in older cultures. Taste cells that had leak currents >10 pA at –80 mV were excluded from analysis, since it was not possible to separate a cell with a 'leaky' membrane from a poor seal. We obtained recordings from cultured circumvallate, foliate and fungiform taste cells (Figure 4), however, we analyzed in detail only currents from circumvallate taste buds (Table 1). Voltage-gated currents were also observed in taste cells maintained at 37°C (Table 1). No apparent differences in membrane properties were observed in taste cells cultured at room temperature and 37°C for the first 2 days in culture. However, membrane properties of taste cells appeared to degrade in cultures that were maintained for longer periods at 37°C. It was difficult to obtain good seals and non-leaky whole cell recordings.

Expression of taste cell-specific markers

Normally taste cells express a variety of histochemical markers intracellularly, including the G protein α-gustducin and protein gene product 9.5 (PGP 9.5), and carbohydrate blood group antigens, such as antigen A, on the cell surface (McLaughlin *et al*., 1992; Smith *et al.*, 1993; Huang and Lu, 1996; Kanazawa and Yoshie, 1996; Wakisaka *et al.*, 1996). However, these proteins are detectable only in differentiated taste cells and disappear within 7–14 days following denervation of taste buds in the intact animal (McLaughlin *et al*., 1992; Smith *et al*., 1994a). Isolated taste buds in culture are completely separated from their nerve supply. Thus, an interesting question is whether cultured taste buds retain cellular and surface proteins that are affected by denervation *in vivo*. To address this question cultures containing circumvallate and foliate taste buds were exposed to anti-α-gustducin, anti-PGP 9.5 and anti-blood group antigen A antibodies and appropriate secondary antibodies. Cells in cultured taste buds exhibited immunoreactivity for each of the markers tested and the approximate number of labeled cells per bud was similar to that seen following fixation of an intact animal (Smith *et al.*, 1994a; Boughter *et al*., 1997). Gustducin immunoreactivity was observed in taste buds cultured for up to 14 days at room temperature, with no apparent differences in taste buds isolated from the different papillae (data not shown). Gustducin immunoreactivity was similar in cultures maintained at room temperature and at 37°C (Figure 5). Immunoreactivities to PGP 9.5 (Figure 6) and to blood group antigen A (not shown) were also present in taste buds cultured at room temperature and at 37°C. For each antibody the longest time period tested for 37°C was 4 days. Immunocytochemical analysis became problematical after longer periods because the taste buds detached from the substrate during processing. In both the room temperature and 37°C cultures some immunoreactive taste cells displayed elongated morphologies with distinct apical processes (see, for example, Figure 5), suggesting that apical/basolateral polarities of some taste cells are maintained in culture.

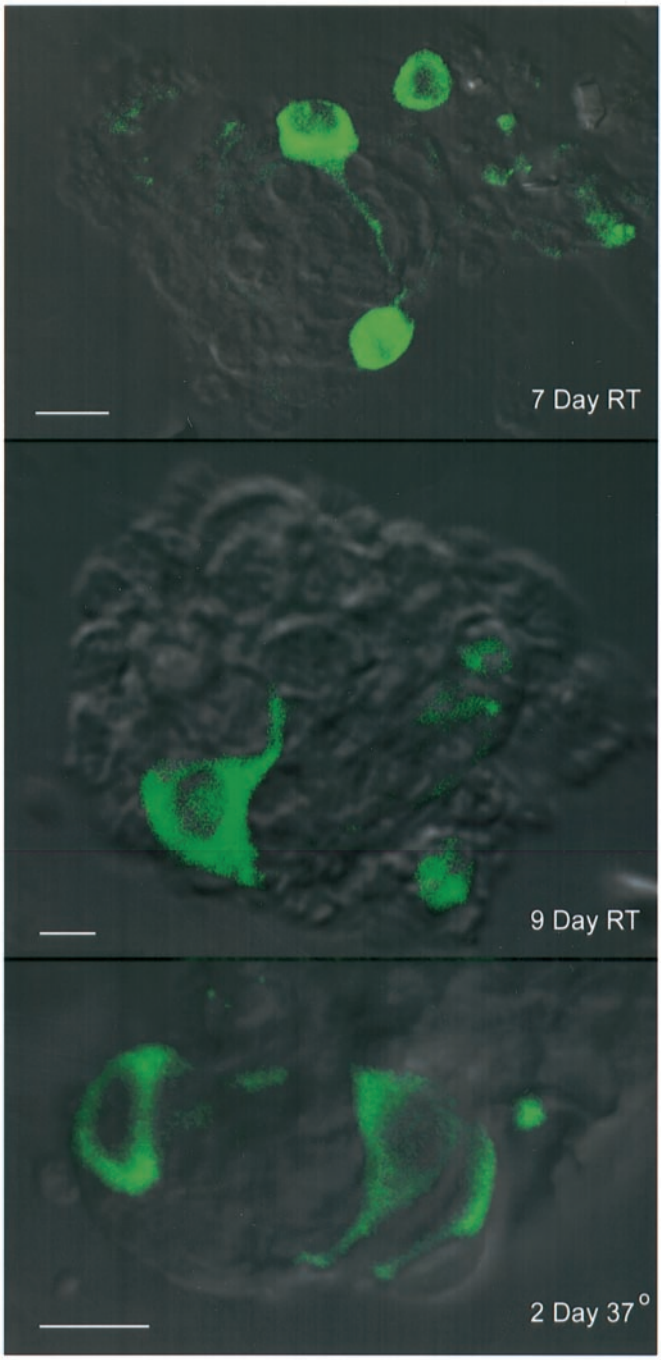

Figure 5 Gustducin immunoreactivity of taste buds cultured for 7 and 9 days at room temperature and for 2 days at 37°C. Taste bud cultures were labeled with an antibody to α-gustducin to determine if G protein expression is maintained in culture. Gustducin immunoreactive cells are green.

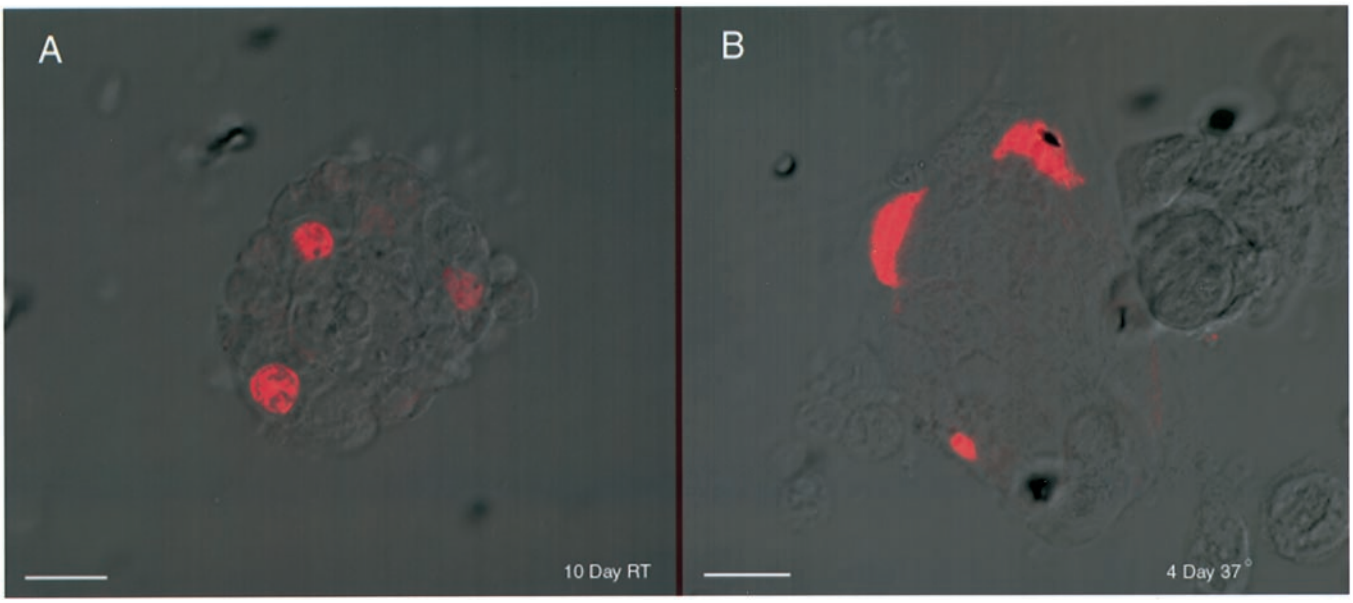

Figure 6 PGP 9.5 immunoreactivity in taste buds cultured for 10 days at room temperature and for 4 days at 37°C. PGP 9.5 immunoreactive cells are red. Scale bars represent 10 μm.

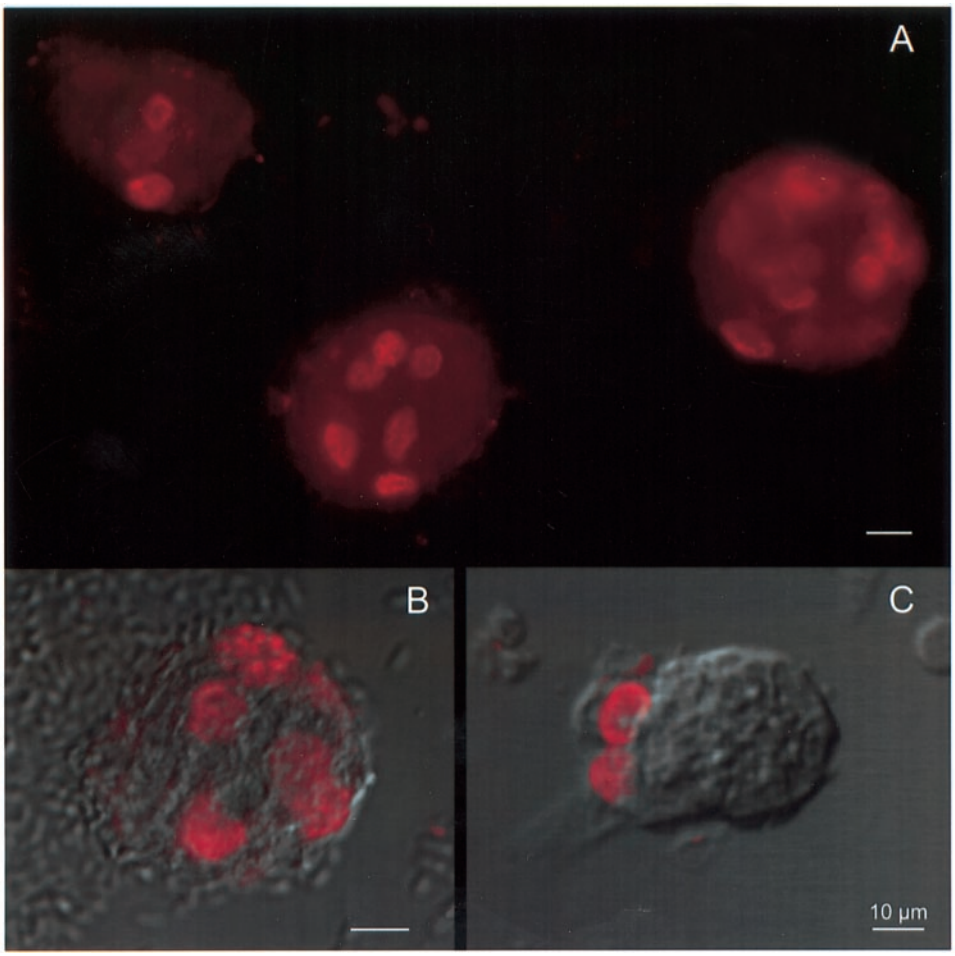

Figure 7 BrDU labeling in cultured taste buds. To label dividing cells, cultures were incubated with BrdU at 15 μg/ml for 48 h beginning on day 0. The cultures were fixed 2 days later and processed for BrdU immunoreactivity. **(A)** and **(B)** illustrate labeled buds cultured at room temperature and **(C)** shows labeling following culture at 37°C.

Cell division in culture

To test whether cultured taste buds contained proliferative cells BrdU was added to the cultures immediately following isolation and 24 h after isolation. The BrdU remained in contact with the cultures for up to 72 h to increase the probability of labeling slowly dividing cells. When BrdU was added to the culture medium at the time of plating and buds were cultured at room temperature for 2 days in the presence of BrdU some taste buds had as many as 5–8 labeled nuclei following immunocytochemical processing (Figure 7A,B). However, in some cultures incorporation of BrdU was substantially less (maximaum 2–3 labeled cells/bud; data not shown). Addition of BrdU to taste buds that already had been in culture for 48 h resulted in essentially no BrdU label. Thus, cell division continues in culture immediately following isolation, but then either stops or is reduced to a rate undetectable by the short-term techniques employed in our studies.

To determine if cell division occurred in taste buds cultured at 37°C BrdU was added to cultures immediately following isolation and taste buds were incubated for 2 days at 37°C. Following fixation and immunocytochemical processing taste buds were analyzed for BrdU incorporation. Only a small number of taste buds exhibited BrdU labeling and only 1–2 cells were labeled per bud (Figure 7C). In some cases it was not clear whether the labeled cells were part of the taste bud or associated non-taste cells.

Discussion

The current study demonstrates that adult rat taste buds can be maintained in a differentiated state in culture for up to 14 days at room temperature and for at least 3–4 days at 37°C. These cultured taste buds retain electrophysiological and immunocytochemical properties and individual cells within the buds maintain elongated morphologies. In addition, some cells in isolated taste buds are able to divide when initially placed in culture.

In our studies taste buds were separated from their normal microenvironment. Major components that were lost during this process included surrounding epithelial cells, associated nerve fibers and saliva. In place of these elements a defined medium was used. We did not expect this medium to replicate *in vivo* conditions. The goals of these experiments were to establish a defined culture system for maintaining differentiated taste buds for short periods of time, to describe the characteristics and changes exhibited by taste buds in culture and to provide a general time course for these changes. Ultimately, the global purposes of these studies were to create a short-term culture system for expressing foreign proteins and to provide a starting point for investigating specific components (e.g. growth factors) that could produce a normally functioning taste bud *in vitro*.

Taste buds cultured at room temperature and 37°C exhibited a series of changes that ended with degeneration. Under both temperature conditions the taste buds demonstrated the same sequence of events, although the timing of these events differed dramatically. Room temperature culture likely slows down most cellular processes, including degeneration. This idea is consistent with studies in catfish indicating that decreasing temperatures increase taste bud survival times following denervation (Torrey, 1934) and that the average lifespan of a taste cell is temperature dependent (Raderman-Little, 1979).

When cultured at room temperature isolated taste buds maintained most of their phenotypic properties for 10–14 days. Some taste buds were lost prior to day 10, but this was likely due to detachment from the substrate or damage during isolation and plating. With the exception of these damaged taste buds, the most obvious change prior to day 10 was rounding up of the taste buds, which occurred soon after plating. Despite this change in overall shape of the cultured end organ, individual taste cells retain an elongated morphology. Taste bud cultures kept at 37°C also exhibited initial taste bud loss and rounding up of the remaining healthy taste buds soon after plating. The two temperatures produced no obvious differences in timing with respect to this overall change in taste bud shape.

The next observed change was degeneration and loss of peripheral cells from taste bud aggregates. This resulted in smaller taste buds and occurred after ~10 days at room temperature and ~2 days at 37°C. Sloughing of dead cells from the compact taste bud in culture may mimic the extrusion of dead or dying taste cells from the bud *in vivo* (Guth, 1957). In contrast to the demise of peripheral cells, the central compact regions of the taste buds remained healthy for up to 14 days at room temperature and for 3–4 days at 37°C. These central cells excluded Trypan blue, exhibited immunoreactivity for specific proteins and had voltage-gated currents. Elongated taste cells were apparent in the taste bud clusters throughout these time periods. In contrast to the apparent limited effect of culture on differentiated taste cells, proliferative cells associated with taste buds were quickly and dramatically affected by isolation and culture. Cell division was detectable only initially in room temperature cultures and was quickly abolished at 37°C. The labeling of cells in taste buds at the initial time points indicates that proliferative cells were included in the cultures and that isolation and culture resulted in an inability of these cells to divide at later times.

Specific environmental features appear to be important for taste bud survival in the intact animal and loss of these associations in culture likely contributed to the decline of taste bud health in cultures over time. Denervation studies *in vivo* indicate that loss of innervation results in dramatic decreases in taste cell number within 4–5 days following nerve injury and complete loss of normal taste cells 7 days after surgery (Guth, 1957; Farbman, 1969). In addition, Smith *et al.* reported a reduction in taste cells and NCAM expression 4 days after nerve crush and an absence of

NCAM immunoreactivity in taste buds 8 days after nerve injury (Smith *et al*., 1994b). Thus, taste bud loss following denervation is a gradual process and resembles changes seen in cultured taste buds. Although most recognizable taste buds are gone following 14 days in room temperature cultures, a few taste buds remain. Taste buds cultured at 37°C decline much more rapidly than those cultured at room temperature, with a time course similar to that seen following denervation in the intact animal.

The degree and timing of taste cell loss *in vivo* following denevation is dependent on several factors, including the papilla examined and the length of nerve left attached to the taste bud. In the intact animal fungiform buds are affected less by denervation than other lingual taste buds (Whitehead *et al*., 1987). We see no obvious differences in survival *in vitro* of the taste buds from different papillae. However, fungiform taste buds are difficult to isolate, resulting in fewer healthy taste buds at the start of culture relative to circumvallate and foliate cultures. This makes direct comparisons of culture viability between the different types of taste buds difficult.

Denervation studies in intact animals indicate that nerve fragments associated with taste buds influence taste bud survival (Torrey, 1934; State and Dessouky, 1977). During denervation experiments the nerve is typically sectioned or crushed proximal to the lingual epithelium. This results in the retention of nerve fragments associated with taste buds distal to the nerve injury. State and Dessouky compared the length of the remaining nerve fragment with taste bud survival times and found that longer nerve fragments resulted in slower degeneration of taste buds from the injury (State and Dessouky, 1977). In contrast to denervation studies in the intact animal, in culture there is a complete and uniform removal of innervating fibers except for the extreme distal processes ramifying between the taste cells. Following the principle that shorter nerve stumps make for more rapid loss of taste buds, taste buds in 37°C culture degenerate somewhat more quickly than denervated taste buds *in vivo*. However, this is a qualitative observation complicated by extensive variability in the cultures. If this observation is accurate, the quicker demise may be due to complete loss of innervation or to the combined loss of several elements in culture.

Cultured taste buds not only lack innervation, but are missing other factors and tissue relationships as well. For example, cultured taste buds must survive in the absence of saliva. Removal of salivary glands adversely affects taste buds in the intact animal and these changes have been attributed largely to the loss of EGF, which occurs in high concentrations in the saliva (Cano and Rodriguez-Echandia, 1980; Nanda and Catalanotto, 1981, Morris-Wiman *et al*., 2000). Epithelium, connective tissue and other lingual cells may also contribute to the health of a normally functioning taste bud. For example, Suzuki *et al*. reported that OX62 (an antibody that recognizes macrophages and dendritic cells) immunoreactive cells invade the circumvallate epithelium following degeneration and suggest that these cells may produce keratinocyte growth factor and influence the proliferation of cells within the lingual epithelium (Suzuki *et al*., 1997). Cells from surrounding tissue, most likely fibroblasts, reportedly phagocytose dying taste buds following denervation (Suzuki *et al*., 1996), although taste bud cells have also been shown to perform this function (Takeda *et al*., 1996). Despite our attempts to produce pure taste bud cultures, some non-taste cells are present. In 37°C cultures there was a proliferation of non-taste cells surrounding some taste buds. No attempt was made to specifically identify these cells, but they might have been fibroblasts. Their proliferation at this temperature may be a factor in the shorter survival of the cultured taste buds.

In summary, we have developed a culture system for maintaining differentiated adult taste buds *in vitro*. Current studies are focused on using these cultured taste buds to investigate specific components that contribute to a normally functioning taste bud and to express foreign gene products in taste buds (Stone *et al*., 1999).

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