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Virus-mediated Transfer of Foreign DNA into Taste Receptor Cells

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Abstract

Foreign genes can be transferred into taste cells via adenoviral vectors. The present study was undertaken to characterize the subpopulation of taste cells that are susceptible to adenovirus infection and to determine whether another viral vector, derived from herpes simplex 1 (HSV-1), infects the same subpopulation of taste cells. Using an adenovirus containing the gene for enhanced green fluorescent protein (EGFP) under the control of the human cytomegalovirus (CMV) immediate early promoter, we found that EGFP was present in blood group antigen H immunoreactive (ir) taste cells, but not in gustducin-ir or PGP 9.5-ir cells. Infection of taste buds with an HSV-1 vector containing EGFP also resulted in a subpopulation of EGFP-positive taste cells. However, both gustducin-ir and PGP 9.5-ir taste cells expressed the marker protein. In conclusion, this study shows that both adenoviral and HSV-1 vectors can be used to transfer foreign genes into the cells of isolated rat taste buds and that different viruses can be used to target specific subpopulations of taste cells.

Introduction

Taste cells are specialized receptor cells that detect a wide range of chemical stimuli, including ions and complex molecules. These stimuli interact with the taste cell via receptors or channels on the cell surface and subsequent intracellular responses vary depending on the tastant [for review see (Gilbertson and Kinnamon, 1996; Kinnamon and Margolskee, 1996; Lindemann, 1996; Stewart *et al*., 1997; Herness and Gilbertson, 1999)]. Simple species, such as protons and Na^+, interact with ion channels located on the plasma membrane, directly leading to depolarization of the cell. Other tastants, including bitter substances, sugars and amino acids, bind to protein receptors and activate intracellular second messenger systems.

Several genes encoding proteins involved in the transduction of chemical stimuli have been cloned from taste cells. These include genes for putative receptor proteins: taste-mGluR4, a metabotropic glutamate receptor (Chaudhari *et al*., 2000); two families of G-protein-coupled receptors (Hoon *et al*., 1999; Adler *et al*., 2000; Chandrashekar *et al*., 2000; Matsunami *et al*., 2000; Kitagawa *et al*., 2001; Max *et al*., 2001; Montmayeur *et al*., 2001; Sainz *et al*., 2001); and MDEG1, a proton-gated cation channel (Ugawa *et al*., 1998). In addition, PLCβ2 and the G-protein subunits α-gustducin and γ13 (McLaughlin *et al*., 1992; Huang *et al*., 1999) have been cloned. To determine the roles of specific components in taste transduction pathways, cloned genes have been characterized in heterologous systems, including CHO cells and *Xenopus* oocytes (Ugawa *et al*., 1998; Chandrashekar *et al*., 2000; Chaudhari *et al*., 2000). In addition, the G-protein α-gustducin has been studied extensively in cell-free assays (Hoon *et al*., 1995; Ming *et al*., 1998; Huang *et al*., 1999). Expression of cloned genes in their native environment, the taste cell, could define the molecular roles of specific proteins even further. For example, targeting of proteins to specific intracellular or membrane domains could be studied.

There are several methods available for transferring foreign genes into cells. These include lipofection, particle-mediated gene transfer (gene gun), electroporation and viral gene transfer. Preliminary studies in our laboratory indicate that viral transfer (Stone *et al*., 1999, 2001) can be used to transfer foreign genes into taste cells. In addition, other workers (Kishi *et al*., 2001) have recently reported successful infection of taste cells with adenoviral vectors. Following infection, a subpopulation of taste cells expresses virally encoded proteins. The present study was undertaken to examine this subpopulation of cells and to determine whether a herpes vector infects the same subpopulation of

cells. Preliminary presentations of this work have been published in abstract form (Stone *et al*., 1999, 2001).

Materials and methods

Taste bud isolation

Taste buds were isolated from 6- to 10-week-old male Sprague–Dawley rats as described in elsewhere (Ruiz *et al*., 2001). Whole taste buds were chosen for analysis rather than isolated taste cells. Taste buds consist of many different cell types based on ultrastructural and immunocytochemical characteristics and we wished to maintain both the different cell types and the normal intercellular interactions between these specialized receptor cells. To obtain taste buds for culture, tongue segments containing the designated papillae were injected with ~0.5 ml of an enzyme solution containing collagenase B, dispase II and trypsin inhibitor (see Solutions and media). Following incubation in oxygenated Tyrode's solution for 50–60 min, the lingual epithelium was gently peeled from the underlying connective tissue. The epithelial strip was pinned out on a Sylgard dish and bathed in calcium-free Tyrode's solution (plus 1 mM BAPTA) for 20 min. Taste buds were removed with gentle suction using a pipet (tip diameter ~100 μm) and placed into a drop of Tyrode's or culture medium on a lysine-coated 'Biocoat Cell Environments' culture slide (Cat. No. 40632; Becton Dickinson). Prior to plating taste buds, slides were equilibrated to room temperature for 20–30 min to enhance taste bud adhesion. After removing buds from a defined region (circumvallate, foliate or fungiform papillae), the taste buds were allowed to settle for 5–10 min before additional culture medium was added to each well. Both two- and eight-well culture slides were used, resulting in media volumes of 500–2000 μl or 200 –300 μl, respectively.

Solutions and media

Enzyme solution consisted of: 0.7 mg collagenase B (Roche Diagnostics Corp., Indianapolis, IN), 3.0 mg dispase II (Roche Diagnostics Corp.) and 1.0 mg trypsin inhibitor (Sigma, St Louis, MO) in 1.0 ml Tyrode's solution (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, 10 mM pyruvate, pH 7.4). Taste buds were cultured and infected in serum-free medium adapted from Pixley (Pixley, 1992; Ruiz *et al*., 2001). This medium consisted of: DMEM (Dulbecco's modified Eagle medium with D-glucose, L-glutamine and sodium pyruvate; Cat. No. 12800-017; Gibco, Baltimore, MD); 15 mM KCl; 18 mM HEPES; 10 ml/l MEM NEAA IX (Earle's Salts with non-essential amino acids; Gibco); 30 μm hypoxanthine; 3 μm thymidine; 100 U/ml penicillin; 0.1 mg/ml streptomycin; 25 μm 2-mercaptoethanol; ITS + Premix (20 ml/l—6.25 μg/ml transferrin, 6.25 μg/ml selenious acid, 1.25 mg/ml bovine serum albumin (BSA) and 5.35 μg/ml linoleic acid; Collaborative Biomedical Products, Bedford, MA). The medium was buffered to pH 7.4 with NaOH. Unless otherwise noted, chemicals were obtained from Sigma Chemical Corp.

Viral vectors

The ability of three viral vectors to express the marker enhanced green fluorescent protein (EGFP) in isolated taste buds was tested and compared. Two of the vectors were derived from adenovirus and differed only in the promoter used to drive expression of the marker. Ad-RSV-EGFP contained the Rous sarcoma virus (RSV) promoter and Ad-CMV-EGFP contained the human cytomegalovirus (CMV) immediate early promoter. The third vector was a recombinant herpes simplex virus type 1 (HSV-1) vector containing a fusion of the native viral protein ICP4 (infected cell peptide 4) with EGFP driven by the native ICP4 promoter. Construction of Ad-CMV-EGFP and Ad-RSV-EGFP have been described previously (Smith *et al*., 2000).

Infection of taste cells with adenoviral vectors

Adenoviral vectors were added to the medium bathing isolated taste buds within 6 h of taste bud isolation ($n = 16$ preparations). The amount of virus used to infect the buds depended on the volume of media. For wells containing 200–300 μl of media, 20 μl of virus was added (2.5×10^7 pfu/ml). For larger wells containing 500–2000 μl, 50 μl of virus was added. Due to the variability encountered with taste bud isolation and survival, it was not possible to determine the multiplicity of infection (amount of infectious virus per cell). After addition of the virus, taste buds were cultured at 35–37ºC for 18–24 h.

Infection of taste cells with the herpes vector

The herpes vector was added to the taste bud medium 0–2 h following isolation and infected cultures were incubated for 17.5–21 h at 35–37° C.

Immunocytochemistry

Adenovirus- and HSV-1-infected taste buds were fixed with 4% paraformaldehyde overnight at 4°C then washed 3 × 10 min with 0.1 M phosphate buffered saline (PBS, pH 7.2). To decrease non-specific binding, taste buds were incubated for 2–3 h in blocking solution consisting of 1% normal goat serum (Jackson Laboratories, West Grove, PA), 1% BSA (Sigma, St Louis, MO) and 0.3% Triton X-100 (United States Biochemical Corp., Cleveland, OH) in PBS. Next, anti-α-gustducin (1:500, raised against amino acids 93–113; No. sc-395; Santa Cruz, Santa Cruz, CA), anti-PGP 9.5 (1:1500; No. 7863-0504; Biogenesis, Kingston, NH), or anti-blood group antigen H antibodies (1:50; clone 92FR-A2; Dako Corp., Carpinteria, CA) were applied to the taste buds. Each primary antibody was diluted in

blocking solution and buds were incubated at 4ºC for 1–3 days, then washed 3 × 10 min in PBS. Rhodamine red-X goat anti-rabbit (1:100; No. 111-295-144; Jackson Labs) secondary antibodies were then applied to α-gustducin and PGP 9.5 labeled taste buds and rhodamine red-X goat anti-mouse (No. 115-295-100; Jackson Labs) secondary antibodies were applied to the blood group antigen H labeled taste buds. Secondary antibody incubation was carried out at room temperature for 2–3 h, after which the buds were washed 3 × 10 min in PBS and coverslipped with fluoromount G (Southern Biotechnologies Inc., Birmingham, AL). Coverslipped slides were stored at 4ºC.

Imaging

Following incubation with the viral constructs, taste buds were viewed while still in the culture dish with a Nikon Diaphot 200 inverted microscope. Fluorescent and bright-field images were captured with a Cool Snap digital camera and Image Pro software, and then processed using Adobe Photoshop 5.0 software. Confocal images of fixed, antibody-labeled taste buds were collected using an Olympus FVX-IHRT Fluoview laser scanning confocal microscope equipped with argon, green HeNe and red HeNe lasers. For immunocytochemical analysis of taste buds double-labeled with virally expressed EGFP and rhodamine-tagged secondary antibodies, images were collected sequentially. After each fluorescent image was collected, an accompanying differential interference contrast (DIC) image was taken at the same focal plane to enhance visualization of individual taste cells. Adobe Photoshop 5.0 software was used to process both epifluorescent and confocal images. Processing included adjustments for brightness and contrast as well as application of identifying labels and scale bars.

Results

Infection of taste buds with Ad-RSV-EGFP and Ad-CMV-EGFP

Rat taste buds were isolated from strips of lingual epithelium and infected with adenoviral vectors. Intact taste buds were used for these experiments instead of isolated cells, so that intercellular physiological interactions were retained during infection and to maintain the polarized structure of individual taste cells. Isolated taste cells lose their elongated shape in culture and membrane domains become less distinct. In addition, identification of isolated taste cells in culture is sometimes difficult, since both taste cells and epithelial cells are present in cultures. Two different adenoviral vectors were tested for their ability to infect and express EGFP in cells of isolated rat taste buds. The adenoviral vectors tested in this experiment differed only in the promoter used to drive the EGFP gene; one construct contained the RSV promoter and the other contained the CMV immediate early promoter. Twenty-four hours after virus application, taste buds infected with vectors containing the RSV promoter (n = 3 experiments) showed only rare, light labeling of isolated cells in taste buds, although occasional isolated cells not associated with taste buds showed bright EGFP labeling (data not shown). The isolated labeled cells were found in wells containing non-labeled taste buds. Isolated cells in our taste bud cultures appear to consist of both taste and non-taste cells based on immunocytochemical labeling (personal observations). Because bright EGFP labeling was never seen in intact taste buds, the isolated cells expressing EGFP were probably lingual epithelial cells rather than taste cells.

In contrast to the limited labeling seen with Ad-RSV-

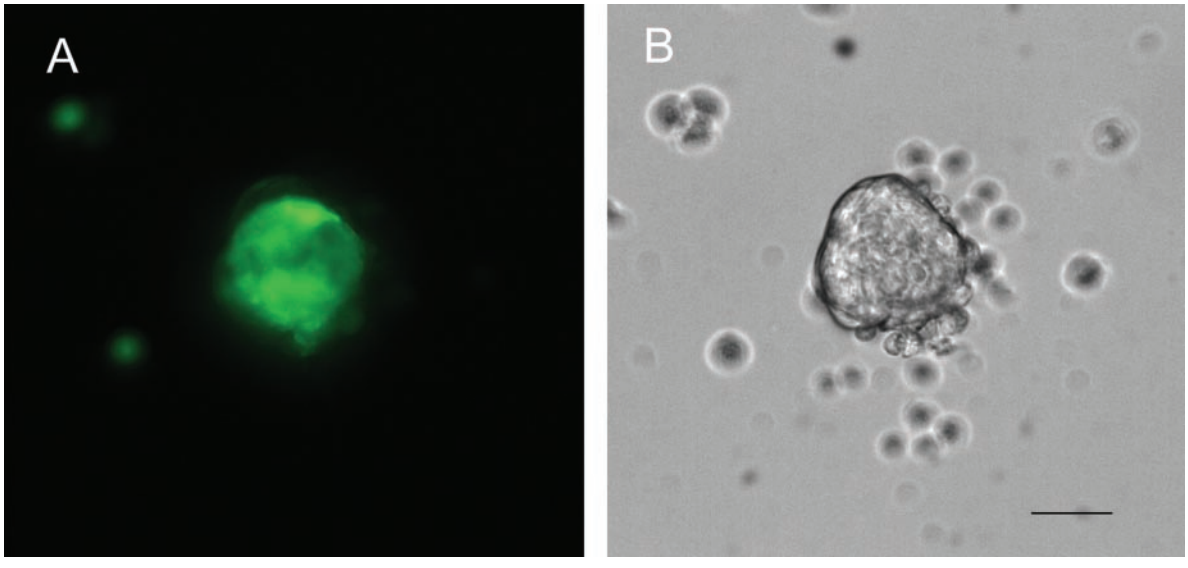

Figure 1 Isolated circumvallate taste bud infected with the adenoviral vector Ad-CMV-EGFP. **(A)** EGFP fluorescence; **(B)** bright-field image of the infected taste bud. Transmitted light images facilitated visualization of taste bud boundaries. Buds were in contact with the virus for 24 h and images were taken while buds were in fresh culture media. Scale bar = 20 μm.

EGFP, taste buds infected with the adenoviral vector containing the CMV promoter exhibited robust EGFP labeling of many taste buds. The EGFP label appeared to cover most of the bud when viewed with epifluorescence microscopy (Figure 1A). Bright-field images were taken to facilitate identification of taste bud boundaries (Figure 1B). Although many cells appeared to be labeled, the EGFP label was brighter in some cells than others and seemed to be more prevalent in the central, compact regions of the taste buds. Studies of cultured taste buds indicate that the compact region of taste buds contains the healthiest cells (Ruiz *et al*., 2001). No difference in the extent or quality of labeling was noted in taste buds isolated from fungiform, circumvallate and foliate papillae (data not shown). Following this initial study, subsequent adenovirus experiments were carried out using the vector with the CMV promoter driving expression of EGFP.

Immunocytochemical characterization of cell types expressing EGFP following adenoviral infection

Confocal imaging showed that EGFP expression following infection with Ad-CMV-EGFP was limited to a subset of cells (data not shown). To characterize the EGFP+ subpopulation of taste cells, immunocytochemical analysis was carried out. Three antibodies were tested to determine the usefulness of adenoviral vectors as investigational tools for studying certain types of taste cells. The antibodies were: (i) an antibody to PGP 9.5, an ubiquitin carboxyl-terminal hydrolase found in some type II and III taste cells (Wilkinson *et al*., 1989; Iwanaga *et al*., 1992; Kanazawa and Yoshie, 1996; Yee *et al*., 2001)—four preparations, 13 taste buds analyzed; (ii) an antibody to α-gustducin, a G-protein found in a different subset of type II cells (Boughter *et al*., 1997; Yang *et al*., 2000)—10 preparations, 50 taste buds analyzed; and (iii) an antibody to blood group antigen H, a cell surface carbohydrate that is present on a large subset of taste cells and identifies the type I or dark cells in rat taste buds (Pumplin *et al*., 1997; Smith *et al*., 1994)—two preparations, 12 taste buds analyzed. α-Gustducin appears to be involved in the transduction of some bitter, sweet and umami stimuli (Ruiz-Avila *et al*., 1995; Wong *et al*., 1996). The functions of PGP 9.5 and antigen H in taste cells are unknown. We used antibodies against these proteins as markers for cell type, rather than to imply function.

Ad-CMV-EGFP infection followed by immunocytochemical labeling showed that EGFP was not present in α-gustducin-ir (Figure 2A,B) or PGP 9.5-ir (Figure 2C,D) cells. In contrast, some blood group antigen H-ir cells expressed EGFP (Figure 2E,F). The marker protein was not present in all antigen H-ir cells, however, and EGFP was present in some cells that lacked visible blood group antigen H immunoreactivity.

Infection of taste buds with HSV-1-(ICP4)-EGFP

Because Ad-CMV-EGFP infected only a subpopulation of taste cells, we examined EGFP expression in taste buds following infection with a recombinant HSV-1 vector containing EGFP under the control of the herpes ICP4 promoter. As with adenoviruses, HSV-1 infects both dividing and differentiated cells and infects a variety of cell types. Following incubation with HSV-1-(ICP4)-EGFP, taste buds exhibited robust EGFP expression when viewed with epifluorescence microscopy (Figure 3). The nuclear EGFP signal appeared to be in many taste cells. Similar to cultures infected with the adenoviral vector, EGFP expressed from the HSV-1 vector was found in most taste buds and appeared to be present in numerous cells within each bud.

Immunocytochemical analysis of HSV-1-(ICP4)-EGFP infected buds revealed that the herpes vector was successful in transferring EGFP into α-gustducin- and PGP 9.5-ir cells (Figure 4). Whether antigen H immunoreactive cells expressed EGFP following herpes infection was unclear, due to the nuclear localization of the EGFP marker and the cell surface labeling with this antibody.

Discussion

The current study shows that both adenoviral and herpes vectors are capable of transferring foreign genes into cells of isolated rat taste buds. Interestingly, the two types of vectors infect different, although possibly overlapping, subsets of taste cells. Ad-CMV-EGFP infects a subpopulation of taste cells including some blood group antigen H-ir cells, but not α-gustducin-ir or PGP 9.5-ir cells. In addition to transferring the marker gene into blood group antigen H-ir cells, Ad-CMV-EGFP infection resulted in EGFP expression in an unidentified subpopulation of taste cells. The herpes vector HSV-1 (ICP4)-EGFP also infects a subpopulation of taste cells, including taste cells immunoreactive for α-gustducin and PGP 9.5.

The cells that make up a rat taste bud are a heterogeneous population. Based on ultrastructural characteristics, the elongated taste cells in a bud can be classified into three types: type I (dark) cells; type II (light) cells; and type III cells (Lindemann, 1996; Finger and Simon, 2000). Type I cells have electron-dense cytoplasm, their nuclei may be invaginated and many type I cells wrap around other cells. Type II cells are electron-lucent, have a round or oval nucleus and short apical microvilli. Type III cells have electron-lucent cytoplasm, nuclei that are intermediate in appearance between type I and type II cells, and have a single large microvillus. In addition to ultrastructural criteria, immunocytochemical markers can be used to classify taste cells. For example, a subpopulation of taste cells is immunoreactive for several components known to be involved in bitter taste transduction: IP_3R3, PLCβ2 and γ13 (Clapp *et al*., 2001). A portion of taste cells expressing these proteins also expresses α-gustducin. In some cases the ultrastructural and immunocytochemical subpopulations

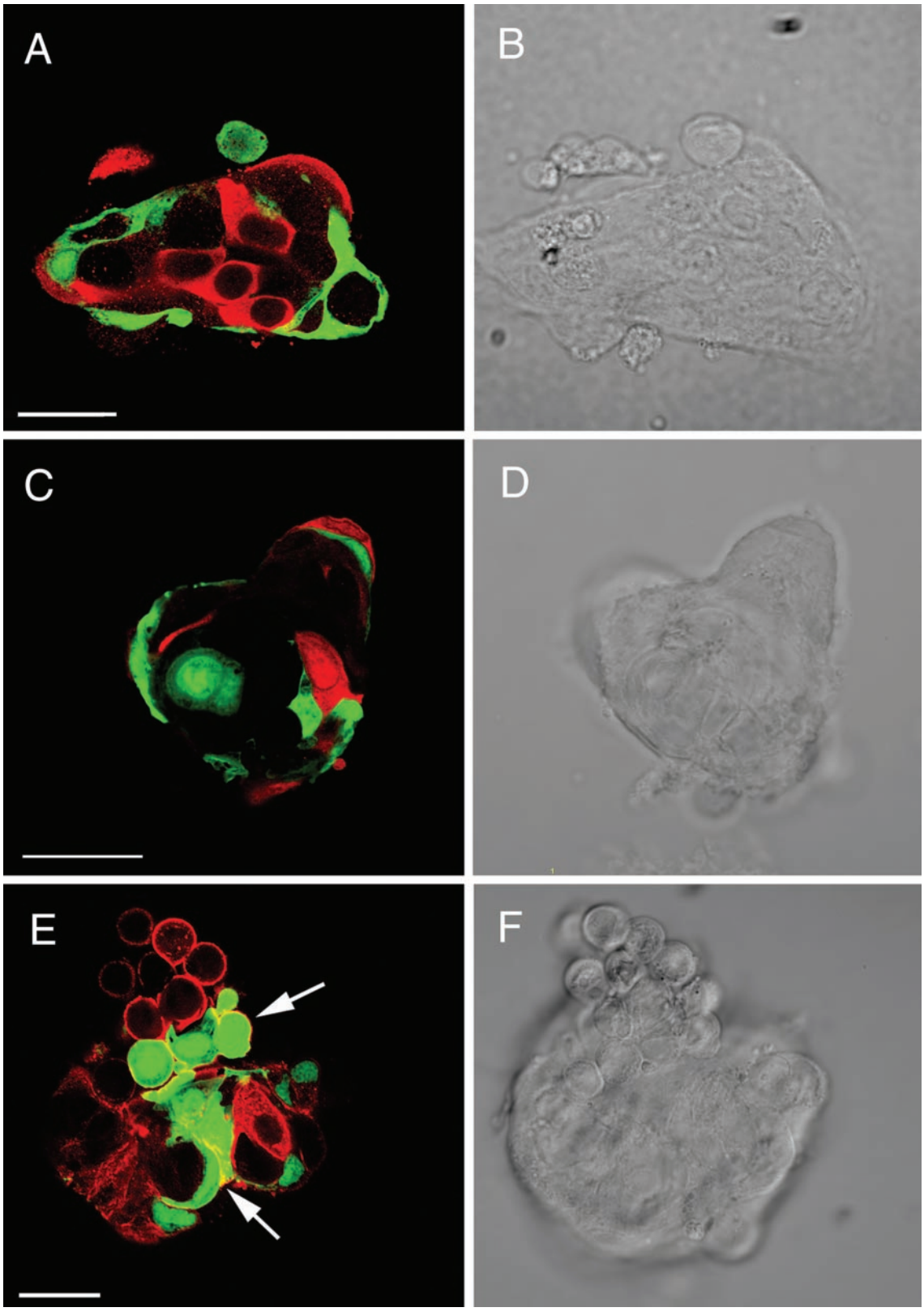

Figure 2 Confocal (left column) and differential interference contrast (DIC) images (right column) of isolated rat taste buds infected with Ad-CMV-EGFP then labeled with antibodies to α-gustducin **(A)**, PGP 9.5 **(C)** and blood group antigen H **(E)**. EGFP labeling is green and antibody labeling is in red. The EGFP label indicative of viral infection is not found in α-gustducin-ir cells (A), or in PGP 9.5-ir cells **(B)**. However, EGFP and blood group antigen H are co-expressed in some taste cells (E). Arrows indicate double-labeled cells and DIC images **(B, D, F)** are provided to clarify taste bud boundaries. Blood group antigen H is a cell surface carbohydrate, thus the yellow color indicative of double-labeled cells is restricted to the plasma membrane (E). Scale bars = 20 μm.

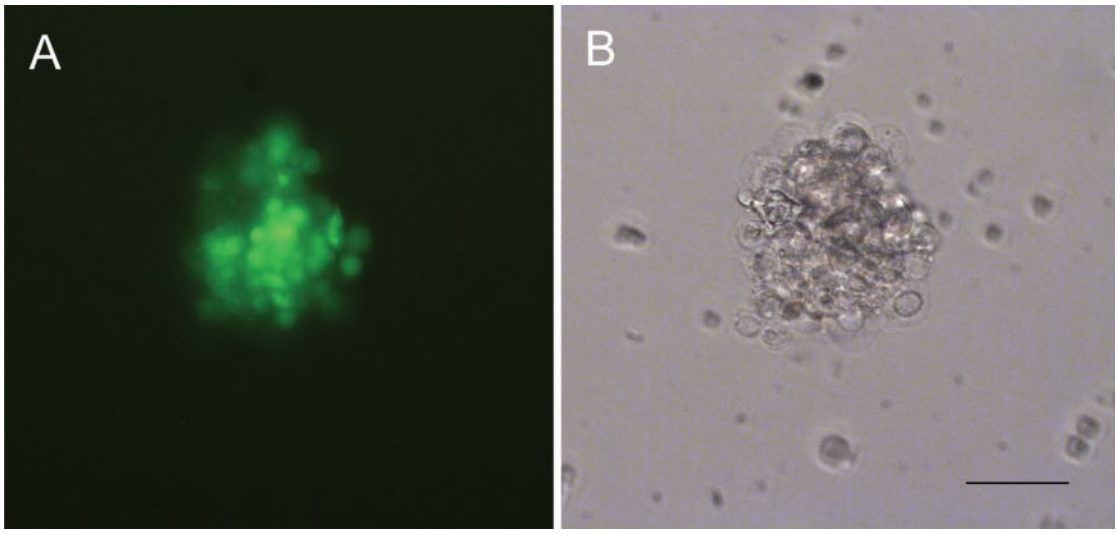

Figure 3 Isolated circumvallate taste bud infected with the herpes vector HSV-1-(ICP4)-EGFP. **(A)** EGFP fluorescence; **(B)** bright-field image of the infected taste bud. Images were taken while the taste bud was in fresh culture media. Scale bar = 20 μm.

overlap, so that antibodies can be used to roughly identify ultrastructural taste cell types. The antibodies used in this study were raised to α-gustducin, PGP 9.5 and blood group antigen H. α-Gustducin is a G-protein found primarily in some type II taste cells (Boughter *et al*., 1997), PGP 9.5 is found in some type II and type III cells (Yee *et al*., 2001) and blood group antigen H is found on the cell surface of most type I cells (Pumplin *et al*., 1997).

Our results indicate that adenoviral vectors are capable of transferring genes into type I cells, since we found cells double-labeled with virally expressed EGFP and antigen H immunoreactivity. The specific functions of type I taste cells are unclear, although both supportive and sensory roles have been suggested (Kinnamon *et al*., 1985, 1988; McPheeters *et al*., 1994). Type I cells wrap around nerve fibers and other taste cells, suggesting that, as with glial cells, type I cells may have supportive functions including control of extracellular ions and transmitters, nutrition and phagocytosis (Lindemann, 1996). The presence of the glutamate–aspartate transporter (GLAST) in rat type I taste cells supports this idea (Lawton *et al*., 2000). GLAST is found in glial cells and likely is involved in taking up glutamate from the extracellular space, thus regulating the availability of this neurotransmitter. Additional evidence for taste cells with glia-like functions comes from electrophysiological studies that identified a subpopulation of taste cells with a leakage conductance highly permeable to K^+ (Bigiani, 2001). The identity of the 'leaky' cells is unclear, although Bigiani speculates that they may be type I cells. In addition to a supportive role, ultrastructural analyses of murine taste buds and associated nerve fibers indicate that some type I cells may have synaptic contacts with nerve fibers, suggesting a chemosensory function for these cells (Kinnamon *et al*., 1985, 1988).

A recent study (Kishi *et al*., 2001) has reported successful transfer of foreign genes into isolated rat taste buds using an adenoviral vector. They reported that GFP expression was present in >90% of the cells in their cultures following infection with an adenoviral vector. In our studies, the subpopulation of EGFP-expressing cells following Ad-CMV-EGFP excludes gustducin-ir cells and PGP 9.5-ir cells, thus EGFP expression in our infected cultures is <90%. PGP 9.5-ir cells in rat constitute ~10% of the taste bud population (Kanazawa and Yoshie, 1996) and α-gustducin-ir cells also comprise at least 10% of the circumvallate or foliate taste bud population (Boughter *et al*., 1997). These two taste bud populations are non-overlapping (Yee *et al*., 2001) and therefore in our cultures no more than 80% of taste bud cells express EGFP following Ad-CMV-EGFP infection. Furthermore, qualitative analysis of our confocal images indicates that <50% of cells in our taste bud cultures express EGFP following infection. Both our vector and that used by Kishi *et al*. were constructed with Ad5 sequences, so the cause of the difference in infection efficiency is unclear. Taste bud cultures contain both taste cells and non-sensory epithelial cells, so some of the EGFP-expressing cells in both our studies and those done by Kishi *et al*. probably were not taste cells. A difference in the number of non-taste cells in the cultures may have contributed to the difference in EGFP expression.

The HSV-1 vector used in the present study was able to transfer EGFP into type II and possibly type III cells. Cells double-labeled with virally expressed EGFP and either α-gustducin-ir or PGP 9.5-ir were evident. α-Gustducin is present in some type II taste cells (Tabata *et al*., 1995; Boughter *et al*., 1997; Yang *et al*., 2000) and PGP 9.5 is present in some type II cells and some type III cells (Yee *et al*., 2001). Both type II and type III cells are likely to have

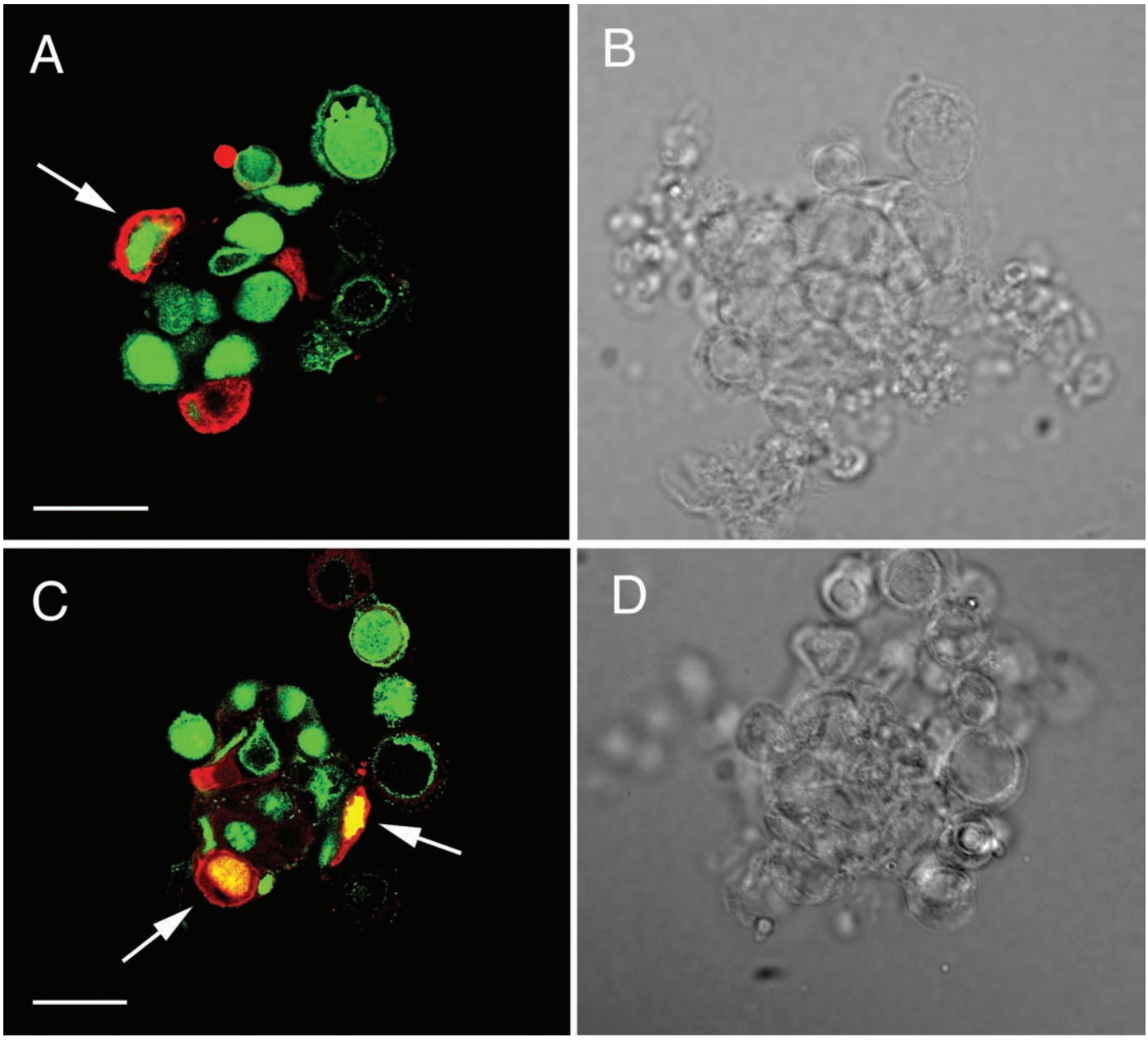

Figure 4 Confocal **(A, C)** and DIC images **(B, D)** of isolated rat taste buds infected with HSV-1-(ICP4)-EGFP then labeled with antibodies to α-gustducin (A) and PGP 9.5 (C). EGFP labeling is shown in green, antibody labeling is shown in red. Both α-gustducin-ir and PGP 9.5-ir taste cells exhibit EGFP following infection with the herpes virus. Arrows indicate double-labeled cells. Scale bars = 20 μm.

chemosensory roles. Components of the bitter transduction pathway, including α-gustducin, PLCβ2, IP_3R3 and γ13, have been identified in type II cells (Asano-Miyoshi *et al.*, 2000; Clapp *et al.*, 2001) and type III cells exhibit synapses in several species, including mouse and rabbit (Delay *et al.*, 1986; Royer and Kinnamon, 1991).

The present study indicates that adenoviral vectors can be used for transferring genes into type I taste cells and that herpes vectors can be used for transferring genes into type II and possibly type III cells. The exact functions of each of the taste cell types are unclear. All three might have sensory functions, or type I cells may have a supportive, glia-like role, with type II and type III cells responsible for taste transduction. Gene transfer via viral vectors may help define cellular roles by providing a tool to interfere with, or enhance, specific proteins in a subpopulation of cells. For example, modification of the GLAST in type I cells, or the G-protein α-gustducin in type II cells may affect taste bud physiology. Infection of taste buds with viral vectors containing apically located, tagged proteins may help determine how taste transduction machinery gets targeted to the small apical membrane of taste receptor cells. Cell

lineage studies utilizing adenoviral vectors also are possible. Although recent studies suggest that taste cells expressing serotonin constitute a separate cell population (Stone *et al*., 2002), it is possible that other markers indicate specific stages in the lifetime of a taste cell. For example, type I cells may give rise to type II cells (Delay *et al*., 1986). Infection of type I cells with an adenoviral vector expressing EGFP, followed by an incubation period of several days, may reveal whether blood group antigen H (type I) cells, mature into a cell type expressing other markers. In addition to examination of the roles of specific taste cells, molecular functions of proteins within taste cells could be studied by modification or over-expression of specific proteins followed by analysis of the effects on the expressed protein and its associated molecules. In conclusion, both adenoviral and herpes vectors will provide tools for examination of the roles of specific taste cells and taste molecules.

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