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Espin cytoskeletal proteins in the sensory cells of rodent taste buds

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Abstract

Espins are multifunctional actin-bundling proteins that are highly enriched in the microvilli of certain chemosensory and mechanosensory cells, where they are believed to regulate the integrity and/or dimensions of the parallel-actin-bundle cytoskeletal scaffold. We have determined that, in rats and mice, affinity purified espin antibody intensely labels the lingual and palatal taste buds of the oral cavity and taste buds in the pharyngo-laryngeal region. Intense immunolabeling was observed in the apical, microvillar region of taste buds, while the level of cytoplasmic labeling in taste bud cells was considerably lower. Taste buds contain tightly packed collections of sensory cells (light, or type II plus type III) and supporting cells (dark, or type I), which can be distinguished by microscopic features and cell type-specific markers. On the basis of results obtained using an antigen-retrieval method in conjunction with double immunofluorescence for espin and sensory taste cell-specific markers, we propose that espins are expressed predominantly in the sensory cells of taste buds. In confocal images of rat circumvallate taste buds, we counted 21.5 $\pm$ 0.3 espin-positive cells/taste bud, in agreement with a previous report showing 20.7 $\pm$ 1.3 light cells/taste bud when counted at the ultrastructural level. The espin antibody labeled spindle-shaped cells with round nuclei and showed 100% colocalization with cell-specific markers recognizing all type II [inositol 1,4,5-trisphosphate receptor type III (IP_3R_3), α-gustducin, protein-specific gene product 9.5 (PGP9.5)] and a subpopulation of type III (IP_3R_3, PGP9.5) taste cells. On average, 72%, 50%, and 32% of the espin-positive taste cells were labeled with antibodies to IP_3R_3, α-gustducin, and PGP9.5, respectively. Upon sectional analysis, the taste buds of rat circumvallate papillae commonly revealed a multi-tiered, espin-positive apical cytoskeletal apparatus. One espin-positive zone, a collection of $\sim$3 μm-long microvilli occupying the taste pore, was separated by an espin-depleted zone from a second espin-positive zone situated lower within the taste pit. This latter zone included espin-positive rod-like structures that occasionally extended basally to a depth of 10–12 μm into the cytoplasm of taste cells. We propose that the espin-positive zone in the taste pit coincides with actin bundles in association with the microvilli of type II taste cells, whereas the espin-positive microvilli in the taste pore are the single microvilli of type III taste cells.

Introduction

A large variety of chemosensory and mechanosensory cells detect stimuli via actin-based, finger-like protrusions that include microvilli and their derivatives, such as hair cell stereocilia. The numbers, dimensions and arrangement of hair cell stereocilia are precisely regulated (Tilney *et al.*, 1992), and it is possible that this type of regulation extends to the microvillar specializations of other sensory cells. The sizes, shapes, placement and physical properties of structures like microvilli and stereocilia are thought to be determined in large part by the parallel-actin-bundle scaffold at their core (Bartles, 2000; DeRosier & Tilney, 2000). This cytoskeletal scaffold consists of actin filaments of uniform polarity cross-linked by actin-bundling proteins (Bartles, 2000).

Espins are high-affinity actin-bundling proteins that are enriched in a variety of structures that contain parallel actin bundles (Bartles, 2000). They are present at high levels in the stereocilia of hair cells in the inner ear (Zheng *et al.*, 2000; Sekerková *et al.*, 2004) and are the target of mutations that cause deafness and vestibular dysfunction in mice and humans (Zheng *et al.*, 2000; Naz *et al.*, 2004; Donaudy *et al.*, 2006). The single espin gene encodes several isoforms that are expressed in a cell- or tissue-specific manner and differ significantly in their ability to bind certain ligands, such

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as the profilins, SH3 proteins and phosphatidylinositol 4,5-bisphosphate (Sekerková *et al.*, 2004). Different transcriptional start sites distinguish four major espin isoform size classes, which range from ~110 kDa to ~25 kDa in apparent molecular mass and are designated 1–4, in order of decreasing size; splice variants are further specified alphabetically (Sekerková *et al.*, 2004). Beyond bundling actin filaments in a Ca^{2+}-resistant fashion (Bartles *et al.*, 1998; Chen *et al.*, 1999), the espins appear to function in part to regulate the length of microvilli and stereocilia by increasing the steady-state length of the dynamic parallel-actin-bundle scaffold at their core (Loomis *et al.*, 2003; Rzadzinska *et al.*, 2005). Recently, we presented immunocytochemical evidence that espins are present at high levels in the microvilli of several other classes of sensory cell besides hair cells— including vomeronasal sensory neurons, Merkel cells, and solitary chemoreceptor cells— and suggested that espins might confer special properties on the microvillar specializations of sensory cells (Sekerková *et al.*, 2004). In that study, we noticed intense immunolabeling of the microvillar region of taste buds using pan-espin antibody. Moreover, using isoform-specific espin antibody, we tentatively identified the espins of taste bud microvilli as espin 3 or espin 4 isoforms (Sekerková *et al.*, 2004).

Taste buds, the peripheral receptors of the vertebrate gustatory system, are distributed in multiple specific locations in the oral and pharyngo-laryngeal epithelium. They contain tightly packed collections of ~50– 100 slender, polarized taste cells and supporting cells and a small number of basal cells that are believed to be pluripotent progenitor cells (Farbman, 1980; Delay *et al.*, 1986). Using ultrastructural criteria, mammalian taste bud cells historically were classified as dark, supporting (type I) cells and light, sensory (type II) cells (Farbman, 1965; Murray & Murray, 1967; Pumplin *et al.*, 1997). Recently, however, taste buds have been shown to house a much more diverse population of cell types. The sensory cells are now further categorized on the basis of microscopic features and cell type-specific markers into type II and type III cells (Murray, 1986; Yee *et al.*, 2001), while the supporting cells remain designated as type I cells. Type II cells are specialized taste receptor cells that are believed to recognize bitter, sweet and umami stimuli (Clapp *et al.*, 2001, 2004; Miyoshi *et al.*, 2001; Nelson *et al.*, 2001; Zhang *et al.*, 2003). Type III cells, which are also considered to be taste receptor cells, were first identified in foliate papillae in the rabbit (Murray, 1986) and later in the rhesus monkey (Farbman *et al.*, 1985), mouse (Takeda, 1976; Takeda *et al.*, 1989) and rat (Yee *et al.*, 2001). Type III cells represent ~5– 15% of the taste bud cell population, display a single blunt apical microvillus, exhibit serotonin immunoreactivity and show synaptic innervation (Murray *et al.*, 1969; Royer & Kinnamon, 1988; Yee *et al.*, 2001). Type I, type II and type III cells all project their microvillar specializations into the highly confined space of the taste pit or pore. Therefore, we investigated which types of cells were responsible for the intense espin antibody labeling we observed in the microvillar region of taste buds. In the present study, we examined the generality of espin antibody labeling in taste buds and more rigorously identified their espin-containing cells.

Materials and methods

EXPERIMENTAL ANIMALS

Adult ($n = 10$) and 1-day-old (P1) ($n = 2$) Sprague-Dawley rats and adult C57BL/6 mice ($n = 3$), of either sex, were used in this study. All experiments conformed to protocols approved by the Northwestern University Animal Care and Use Committee and followed guidelines issued by the National Institutes of Health. Animals were deeply anesthetized by i.p. injection of sodium pentobarbital (60 mg/kg body weight) and perfused through the ascending aorta with saline, followed by 4% freshly depolymerized paraformaldehyde in 0.12 M phosphate buffer, pH 7.4. The tongue, soft palate and pharyngeal region were dissected and postfixed for 10 min and stored in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). The tongues and soft palate of adult rats and mice were cryoprotected in 30% sucrose in PBS containing 0.008% NaN_3 for 48 h at 4°C, mounted in OCT, and sectioned on a cryostat.

WHOLE-MOUNT IMMUNOSTAINING

The tongue, pharynx and soft palate of adult and P1 rats were stained as whole-mount preparations. To block nonspecific binding and facilitate better antibody penetration, the tissues were pretreated with a mixture of dimethylsulfoxide (DMSO), 30% H_2O_2 and methanol (1:1:4, by volume) for 1 h and then incubated in 3% normal goat serum (NGS), 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS; 100 mM Tris-HCl, 150 mM NaCl, pH 7.6) with 2% Triton X-100 and 0.2% DMSO for 1 h. In spite of these pretreatments, the immunostaining of adult tongue whole-mounts was lighter than expected on the basis of the immunostaining intensities observed in sections of adult tongue. The specimens were incubated overnight with affinity purified rabbit anti-espin antibody (0.5 μg/ml) or preimmune IgG at room temperature. The espin antibody, which we raised against purified recombinant rat espin 2B and affinity purified on columns of rat espin 2B-Sepharose 4B, is known to react with all espin isoforms and should be considered a pan-espin antibody (Sekerková *et al.*, 2003, 2004). The next day specimens were incubated with biotinylated donkey anti-rabbit IgG secondary antibody (1:500; Amersham, Piscataway, NJ) overnight at 4°C. On the third day, the specimens were incubated with avidin-biotin complex (ABC) at 4°C (4.5 μl of solution A + 4.5 μl of solution B/ml of ABC Elite kit, mixed 1 h prior to use; Vector Laboratories, Burlingame, CA). The antibodies and the ABC components were diluted in TBS containing 1% NGS, 1% BSA, 2% Triton X-100, and 0.2% DMSO. After each overnight incubation, the samples were thoroughly washed (6– 7 washes, 1 h each) in TBS with 0.2% Triton X-100 at room

temperature. The antibody was visualized by reaction with diaminobenzidine (DAB).

BRIGHT-FIELD MICROSCOPY

Coronal and sagittal sections of adult rat and mouse tongue, 20– 25 μm-thick, were treated with 0.6% H_2O_2 and 10% methanol in TBS for 30 min and then with a mixture of 3% NGS and 1% BSA in TBS with 0.2% Triton X-100 for 1 h to block nonspecific binding. Thereafter, the sections were incubated with rabbit anti-espin antibody (0.5 μg/ml) or preimmune IgG for 2 days at 4°C. The bound antibody was detected by the avidin-biotin amplification method using biotinylated donkey anti-rabbit IgG (1:500), the ABC Elite kit and DAB as the chromogen (see above). The sections were dehydrated, cleared and mounted in Entellan (Merck, Darmstadt, Germany).

FLUORESCENCE MICROSCOPY

Thus far, a large number of immunocytochemical markers for taste cells have been identified. Unfortunately, none of them, except for the recently described synaptobrevin-2 (Pumplin & Getschman, 2000; Yang *et al.*, 2004), encompasses the entire population of type II and type III cells (Nelson & Finger, 1993; Pumplin *et al.*, 1999; Yang *et al.*, 2000; Clapp *et al.*, 2001, 2004; Ueda *et al.*, 2003; Yee *et al.*, 2001, 2003; Takeda *et al.*, 2004). We chose to examine the colocalization of espin with inositol 1,4,5-trisphosphate receptor type III (IP_3R_3), α-gustducin and neuronal protein gene product 9.5 (PGP9.5), as they are commonly used markers that are relatively broadly distributed throughout the cytoplasmic compartment, and their immunolabeling proved to be highly reproducible and not adversely affected by antigen-retrieval procedure needed to better reveal cytoplasmic espin. There is currently a scarcity of immunocytochemical markers for the type I cells of taste buds. A promising candidate, human blood group antigen H (Pumplin *et al.*, 1999), has also been detected in some type II and type III cells (Udea *et al.*, 2003).

To enhance cytoplasmic immunostaining, antigen retrieval was carried out on the sections by placing them into a 10 mM sodium citrate solution at 80°C for 30 min. This treatment was followed by the standard NGS-BSA blocking and Triton X-100 treatment (see above). Sections from adult rats and mice were incubated in a mixture of rabbit anti-espin antibody (1 μg/ml) and either mouse monoclonal anti-IP_3R_3 antibody (1:100; BD Transduction Labs, San Jose, CA) or mouse monoclonal anti-PGP9.5 antibody (1:50; Biogenesis, Kingston, NH) overnight at 4°C. When examining rat tissue, we preferred to use the rabbit polyclonal rabbit PGP9.5 antibody (Biogenesis; see more below), because the PGP9.5 monoclonal antibody showed a confounding punctate cytoplasmic labeling in taste cells (not shown) similar to the Golgi complex-like staining described in taste cells labeled with antibodies to syntaxin-1, SNAP-25 and VAMP-2/synaptobrevin (Pumplin & Getschman, 2000). Goat anti-rabbit IgG and goat anti-mouse IgG secondary antibodies labeled with Alexa 488 or 596 (Molecular Probes, Eugene, OR) were used. For one group of sections, espin was visualized with Alexa 488-labeled goat anti-rabbit IgG secondary antibody, and nuclei were labeled by 7-amino-actinomycin (7AAD; Molecular Probes) nuclear dye.

For double labeling with two different primary antibodies derived from rabbit, we used sequential staining (Jackson ImmunoResearch Laboratories, West Grove, PA) with $F(ab')_2$ fragment. The sections were first reacted with rabbit polyclonal antibody for 1 h and then incubated overnight at 4°C with saturating levels of rhodamine Red-X conjugated affinity purified goat anti-rabbit $F(ab')_2$ fragment (1:100; Jackson ImmunoResearch Laboratories). After six 20-min washes with TBS at room temperature, the sections were reacted with the second primary rabbit polyclonal antibody, which was visualized by Alexa 488-conjugated goat anti-rabbit IgG. The following polyclonal rabbit primary antibodies, in all possible combinations, were used: anti-espin (1 μg/ml), anti-α-gustducin (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-PGP9.5 (1:50; Biogenesis). None of the combinations showed evidence of crossreactivity. As an example compare Fig. 2B with Fig. 2D, which was stained with same antibodies but in a reverse order. Control sections processed without one or both of the primary antibodies were free of immunostaining in their corresponding color channel.

QUANTITATIVE ANALYSIS

For quantitative analyses, we used images of taste buds from the circumvallate papillae of adult rats ($n = 2$ rats for each parameter) captured using a Nikon PCM2000 confocal system and its Simple PCI Program.

To estimate the percentage of espin— positive ($espin^+$) cells in the whole taste bud, we used every 5th coronal section, 25 μm-thick, double-labeled with espin antibody and 7AAD nuclear dye. With this sampling, we assured that only one section/taste bud was counted. Confocal images were captured from sections cut along the longitudinal axis of the taste bud encompassing the central region of a taste bud (e.g., see Fig. 3A). Grazing/lateral sections of taste buds were not included. A 60X oil-immersion objective and additional 2X digital magnification were used. Nuclei of $espin^+$ and espin-negative ($espin^-$) taste bud cells were counted. This sampling method allowed a determination of the percentage of $espin^+$ taste bud cells but not their total numbers in the taste buds.

The percentages of cell-specific $marker^+$ taste cells within the $espin^+$ cell population in taste buds were estimated as follows: The numbers of $espin^+$ and cell-specific marker double-labeled cells were counted in 20 μm-thick serial sections cut along the transverse axis of the taste cells. Every 5th section was sampled to avoid counting any given taste bud more than once. We obtained 6– 7 transverse sections with taste bud regions per papilla. Generally, 4 of these sections each contained 60– 90 countable taste bud profiles, while the remaining sections each had only ~6– 10. We examined taste bud profiles from the 40– 50-μm wide mid-region of the taste bud that showed clear cytoplasmic labeling and included nuclei (see Fig. 3B– E). Cross-sectional profiles of the apical (taste pit/pore) region of the taste bud were excluded from the analysis, because they included only the narrow apical portion of the cells and the intensely labeled $espin^+$ microvilli. Typically, 3– 6 confocal fields containing 6– 50 taste buds each were obtained from each section. No taste buds were counted twice. The same confocal setting was used for each image taken from a given section. Z-stacks including 5– 7 optical sections (1 μm thick) were acquired from a chosen field. Projections

consisting of the clearest consecutive 2– 3 optical sections were transferred to Adobe Photoshop (e.g., see Fig. 3B). One-third of the taste bud profiles in these images were sampled, and the espin$^+$/cell-specific marker$^+$ and espin$^+$/cell-specific marker$^-$ cells in these taste buds were counted. On the basis of visual perception it was possible to categorize the espin antibody labeled taste bud cells into two categories: one with moderate cytoplasmic labeling (e.g., see Fig. 3C_1, yellow asterisks) relative to the intense apical labeling of the microvillar region and another with light cytoplasmic labeling (e.g., see Fig. 3C_1, white asterisks). We counted both groups as espin$^+$ taste bud cells, because they were clearly visible on the computer screen, and there was no evidence of background staining in the surrounding tissue. The percentages of double-labeled cells from the total number of the espin$^+$ cells were calculated for each individual taste bud, and the frequency distributions of the percentages among taste buds were plotted on graphs. All data are shown with the standard error of mean (S.E.M.).

PHOTOGRAPHY

Whole-mount images were captured with a Nikon digital DN100 camera mounted on an Olympus SZH10 stereomicroscope. Images from sections were captured with a Spot RT CCD video camera (Diagnostics Instruments) mounted on a Nikon Eclipse 800 microscope using the Spot RT Software 3.5.8. For confocal images, we used the Nikon PCM2000 system and Simple PCI Program. All images were stored and processed in Adobe Photoshop CS. Brightness and contrast were adjusted.

Results

Espin immunolabeling was detected in taste buds throughout the oral cavity and pharyngo-laryngeal region (Fig. 1A–I). This included the taste buds of all three types of lingual papilla, fungiform (Fig. 1A–C), foliate (Fig. 1D) and circumvallate (Fig. 1E); the *Geschmackstreifen* of the soft palate (Fig. 1F); the nasoincisory taste buds (not shown); and the scattered taste buds in the pharynx (Fig. 1G–I), larynx (Fig. 1G and H) and epiglottis (Fig. 1G and I). The locations of the espin$^+$ taste buds were the same in P1 animals and adults. In addition to labeling taste buds, the espin antibody labeled Merkel cells in the hard palate (arrowheads in Fig. 1A) and scattered brush cells in the pharyngo-laryngeal region (small stained objects in Fig. 1H and I). The identity of these structures was verified by sectional analysis (data not shown; see also Sekerková *et al.*, 2004). The brush cells will be the subject of a separate study.

ESPIN IMMUNOFLUORESCENCE LABELING OF TASTE BUD CELLS IN CIRCUMVALLATE PAPILLAE

Sectional analysis was performed on the taste buds of lingual circumvallate papillae of the rat and mouse. In both species, we observed a similar overall pattern of espin antibody labeling, which was characterized by extensive labeling of apical processes in the taste pit and in the pore region, accompanied by a weaker, yet specific cytoplasmic labeling for a number of cells within the taste bud (Figs. 2A, C_1, D_1, E_1, and 3A_1). With this method we also observed a light nuclear labeling in these cells. Confocal analysis of the apical region revealed the presence of a multi-tiered espin$^+$ cytoskeletal apparatus. An espin$^+$ zone was present near the base of the taste pit and appeared connected to espin$^+$ rod-like structures that extended basally to a depth of 10–12 μm into the cytoplasm of the taste bud cells (arrowheads in Figs. 2A, B, C_1, D_1, E_1, and 3A_1). Given their location and the precedent for espins being associated with parallel actin bundles (Bartles, 2000; Zheng *et al.*, 2000; Sekerková *et al.*, 2004), these rod-like structures likely represent the actin filament-rich microvillar core bundles that extend rootlets deep into taste cells (Murray & Murray, 1967; Takeda *et al.*, 1989). Occasionally, as shown by open arrowheads in Fig. 2B and 2C_1, these core bundles extended even deeper into the cells and appeared discontinuous. A second $\sim$3 μm-long espin$^+$ zone was found in the taste pore (arrows in Figs. 2A, B, C_1, D_1, E_1, and 3A_1). It appeared as if the entire taste pore was filled with espin antibody labeled microvillar structures. Remarkably, in many instances, the region between these two espin$^+$ zones, which measured $\sim$3 μm in length, was depleted of espin immunolabeling (Figs. 2E_1 and 3A_1). This pattern was especially evident in the taste buds of rat circumvallate papillae. When we took images at high gain to better visualize light cytoplasmic immunostaining of the taste bud cells, the fluorescence signal from the apical region of the taste bud became oversaturated and obscured the stratified arrangement of the espin$^+$ microvilli (e.g., Fig. 2C_1). In rare instances we found cells with espin$^+$ projections situated in unusual positions, more basally within the taste bud (crossed arrow in Fig. 2E_1).

DOUBLE IMMUNOFLUORESCENCE FOR ESPIN AND CELL-SPECIFIC MARKERS

Careful inspection of the taste buds sectioned longitudinal to the long axis of the taste cells revealed that the espin antibody labeled only a subset of cells in circumvallate taste buds. The espin$^+$ cells were often spindle-shaped with round nuclei (Fig. 2B, C_1, D_1, and E_1), a distinctive feature of sensory (or light) taste cells. To confirm that these cells were indeed sensory cells, we employed double immunofluorescence using the espin antibody and antibodies to cell-specific markers.

IP_3R_3 and α-gustducin colocalize in a subset of type II cells, while IP_3R_3 antibody labels the majority of the type II cell population and a subset of type III cells (Yang *et al.*, 2000; Clapp *et al.*, 2001, 2004). We found that all $IP_3R_3^+$ cells (white asterisks in Fig. 2C) and all α-gustducin$^+$ cells (white asterisk in Fig. 2D) were double-labeled with espin antibody, while not all

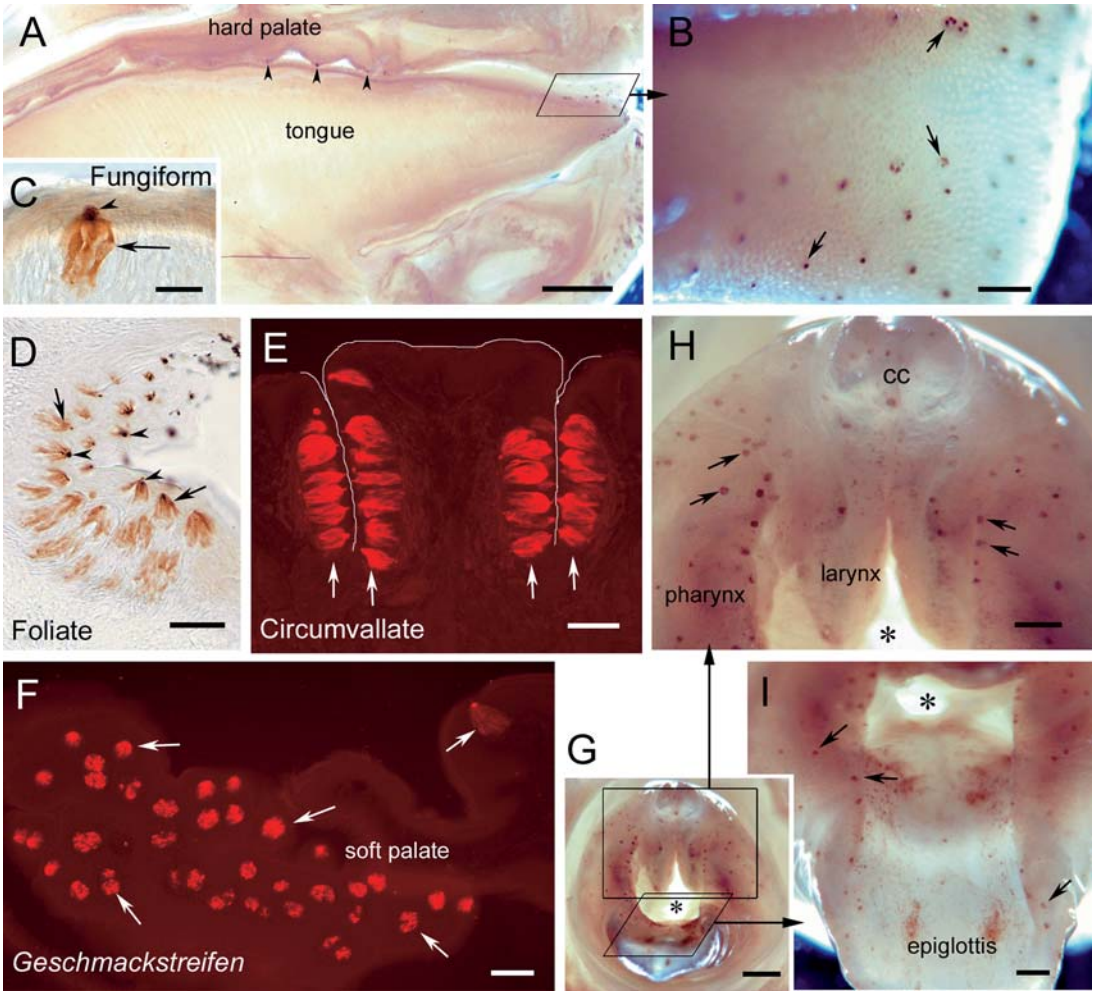


Fig. 1. Espin immunolabeling in the oral cavity and pharyngo-laryngeal region. (A and B) Espin immunoperoxidase-labeled whole-mount of P1 rat tongue shows immunostained clusters of presumptive taste cells in the fungiform papillae (arrows in B) at the anterior portion of the tongue (box in A). Merkel cells in the hard palate are also labeled (arrowheads in A). (C and D) Sectional views of espin immunoperoxidase-labeled taste buds in the fungiform (C) and foliate (D) papillae of an adult rat. Note the intense apical espin immunolabeling (arrowheads) and lighter cytoplasmic immunolabeling of the taste bud cells (arrows). (E) Sectional view of espin immunofluorescence-labeled taste buds in the circumvallate papilla of an adult mouse. Arrows point to the rows of taste buds lining the "crevices" of the circumvallate papilla. The boundaries of the papilla are highlighted by the white line, which traces the surface of the lingual epithelium. (F) Sectional view of espin immunofluorescence-labeled *Geschmackstreifen* in the soft palate of an adult rat. Arrows point to individual taste buds. (G-I) Espin immunoperoxidase-labeled whole-mount preparation of the pharyngo-laryngeal region of an adult rat. Numerous scattered taste buds are labeled with espin antibody (arrows). Small objects labeled by espin antibody in the laryngeal region and epiglottis are brush cells. Asterisks, laryngeal aditus; cc, corniculate cartilage. Scale bars = 1 mm in A; 200 μm in B, H, I; 20 μm in C; 50 μm in D, E, F; and 500 μm in G.

espin$^+$ taste bud cells were labeled with IP_3R_3 antibody (blue asterisk in Fig. 2C) or α-gustducin antibody (blue asterisk in Fig. 2D). As an additional marker, we used antibody to PGP9.5 (Fig. 2E), which is present in a population of type II cells that does not contain α-gustducin (Fig. 2G; see also Yee *et al.*, 2001), as well as in a small subpopulation of type III cells (Yee *et al.*, 2001). While we observed espin$^+$/PGP9.5$^+$ cells (Fig. 2E and F, white asterisks) and intensely PGP9.5$^+$ nerve fibers (blue arrows in Fig. $2E_2$ and F_2), a greater number of espin$^+$/PGP9.5$^-$ cells was detected (Fig. 2E and F, blue asterisks). Notably, the espin$^+$/PGP9.5$^+$ cells often showed light espin antibody labeling in their cytoplasm, which could be discerned clearly only by careful examination of the confocal images (see Fig. 2F). These double-labeled cells could be identified more readily in transverse taste bud sections (see below).

QUANTITATIVE ANALYSIS OF THE ESPIN$^+$ AND DOUBLE-LABELED CELLS

Using the nuclear marker 7AAD and espin immunolabeling, the ratio of the espin$^+$ and espin$^-$ taste bud

Table 1. The numbers of espin$^+$ cells and the numbers of espin$^+$ and cell-specific marker$^+$ double-labeled cells in the circumvallate taste buds of adult rats.

Cell-specific marker	Number of taste buds	Espin$^+$ cells/ taste bud	Espin$^+$ and marker$^+$ taste cells/taste bud	Double-labeled taste cells (as a% of espin$^+$ cells)
IP_3R_3	206	22.0 ± 0.5	15.9 ± 0.4	71.5 ± 0.7
α-gustducin	187	22.6 ± 0.6	11.2 ± 0.3	49.5 ± 0.7
PGP9.5	119	19.1 ± 0.6	6.1 ± 0.3	31.6 ± 0.9
Total	512	21.5 ± 0.3		

The data are shown as mean ± S.E.M.

cells was determined in longitudinal sections (Fig. 3A). We found that 62 ± 1% ($n = 98$ taste buds) of the cells within the taste buds show espin antibody labeling.

The single-labeled espin$^+$ cells and the double-labeled, espin$^+$ and cell specific marker$^+$ cells were counted in transverse sections of the taste buds (Fig. 3B–E). Counting taste bud cells in transverse sections had two advantages. First, all cells within the taste bud could be counted, since the cytoplasmic markers clearly revealed individual cells even if their nuclei were not included in a given plane of section (see examples in Fig. 3C–E). Second, by not trying to image the microvillar region, the confocal gain could be increased to allow greater distinctions in the degree of cytoplasmic antibody labeling for the different markers. In total, we counted 512 taste buds from 2 adult rats and found an average of 21.5 ± 0.3 espin-labeled cells/taste bud (Table 1), with a range from 3 to 44 espin$^+$ cells/taste bud. This wide range primarily reflected the variability in the taste bud size (e.g., note the small taste bud among average-sized taste buds in Fig. 3B, arrow). While all three cell-specific markers used for the quantitative analysis showed 100% colocalization with espin for each marker, the number of the espin antibody labeled cells was greater than the number of double-labeled cells (Fig. 3C–E). We found that, on average, 72%, 50%, and 32% of the espin$^+$ taste bud cells were labeled with antibody to IP_3R_3, α-gustducin, and PGP9.5, respectively (Table 1). The differences among individual taste buds were relatively large, as is evident in the histograms shown in Fig. 4. Since the espin$^+$ cells represented only 62% of the total number of cells in taste buds, we could calculate that in taste buds, on average, $IP_3R_3^+$, α-gustducin$^+$, and PGP9.5$^+$ cells represented 45%, 31% and 19% of the total number of cells in taste buds, respectively.

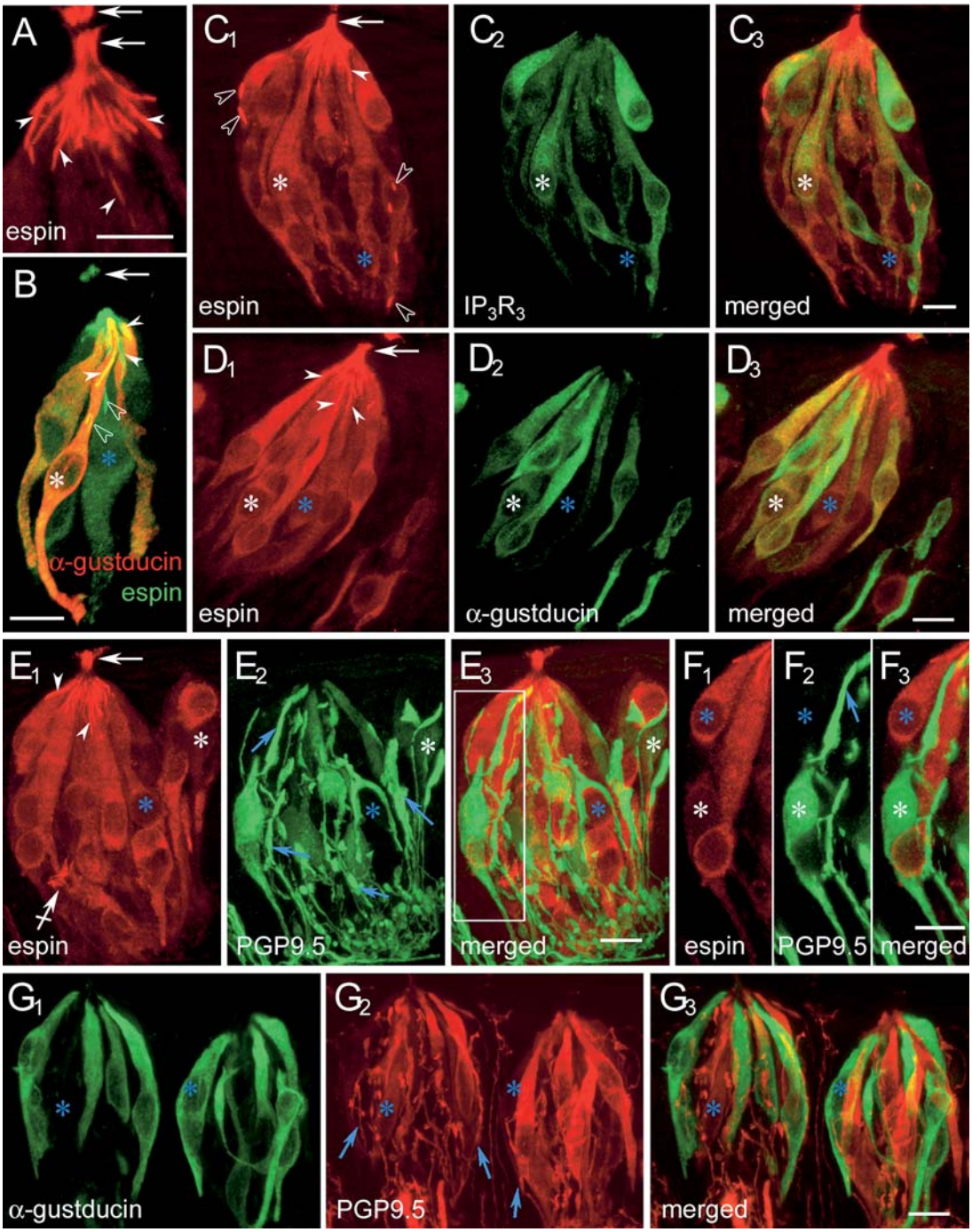
The qualitative analysis of transverse sections clearly revealed two intensity categories of cytoplasmic espin immunolabeling in taste bud cells, suggesting that the espin$^+$ taste bud cells contained different levels of cytoplasmic espin proteins. A moderate cytoplasmic espin immunolabeling (relative to the apical staining) was detected in the $IP_3R_3^+$ cells (Fig. 3C, yellow asterisks), which correspond to type II cells, including

Fig. 2. Laser scanning confocal images of longitudinally cut circumvallate taste buds of the rat (A-F) and mouse (G). (A) Intense espin immunolabeling of the apical taste bud region. Arrows point to collections of ~3 μm-long espin antibody labeled microvilli in two apposing taste pores. The upper arrow points to the labeled taste pore region of a taste bud that is situated across the crevice of the papilla. Situated lower in the apical region of the taste bud, in association with the base of the taste pit, espin$^+$ rod-like structures, which are likely to represent the microvillar core actin bundles and their rootlets, are also labeled with espin antibody (arrowheads). (B) Taste bud double labeled with antibodies to espin (green) and α-gustducin (red). Long espin$^+$ core bundles (arrowheads) in the taste pit region often reach deep within the cells. Note the long core bundle in the espin$^+$/α-gustducin$^+$ taste cell labeled with a white asterisk. Espin$^+$ objects that appear to be bundle fragments can also be seen deeper within the taste cells (open arrowheads). Arrow indicates an espin antibody labeled microvillar collection in the taste pore. Blue asterisk indicates an espin$^+$/α-gustducin$^-$ taste bud cell. (C– G) Examples of taste buds double labeled with antibodies to espin and IP_3R_3 (C), espin and α-gustducin (D), espin and PGP9.5 (E and F), or α-gustducin and PGP9.5 (G). In the apical region of taste buds, espin antibody (C_1, D_1, and E_1) intensely labels the taste pore (arrows) and the long core bundles in association with the taste pit (filled arrowheads), while the cytoplasm of these cells shows lighter immunolabeling. An espin-deficient zone between the espin$^+$ taste pore microvilli and the espin$^+$ taste pit core bundles is clearly evident in E_1 and E_3 (see also Fig. 3A). Espin immunolabeling is present in all taste cells labeled with antibodies directed against IP_3R_3, α-gustducin or PGP9.5 (e.g., white asterisks in C– G) and also in a subgroup of taste bud cells that are not labeled with antibodies to these cell-specific markers (e.g., blue asterisks in C– G). The images in $E_{1–3}$ are 14-μm-thick confocal projections, while those in $F_{1–3}$ depict a thinner, 3-μm-thick, optical section from the boxed area in E_3. α-gustducin and PGP9.5 do not colocalize in the taste cells (e.g., blue asterisks in G). The blue arrows in E_2, F_2, and G_2 point to nerve fibers, which are intensely labeled with the PGP9.5 antibody. The open arrowheads in C_1 point to additional examples of apparent espin$^+$ core bundle fragments deep within the cytoplasm of taste cells. The crossed arrow in E_1 points to a rare example of a cell situated in a basal location of the taste bud that appears to have a collection of espin$^+$ microvilli at one pole. Scale bars = 10 μm.

those which are α-gustducin$^+$ (Fig. 3D, yellow asterisks), as well as a subset of the type III cells. In contrast, the espin$^+$/IP$_3$R$_3^-$ cells showed a lighter cytoplasmic labeling with espin antibody (see Fig. 3C, white asterisks). The espin$^+$/α-gustducin$^-$ taste bud cells showed either moderate (Fig. 3D, magenta asterisks) or light staining with espin antibody (Fig. 3D, white asterisks). The espin$^+$/PGP9.5$^-$ cells (Fig. 3E, magenta and white asterisks) and the espin$^+$/PGP9.5$^+$ cells (Fig. 3E, yellow and blue asterisks) showed a range of moderate to light cytoplasmic espin immunolabeling intensities.

Discussion

We have shown that espin antibody specifically labels taste buds throughout the oral cavity and pharyngolaryngeal region and that the espins of taste buds are enriched in the specialized microvillar projections of taste cells. Our results confirm that sensory cells contain high levels of espin proteins in their microvilli and raise a number of questions regarding the possible roles of espins in the organization and function of taste cell microvilli and in taste perception.



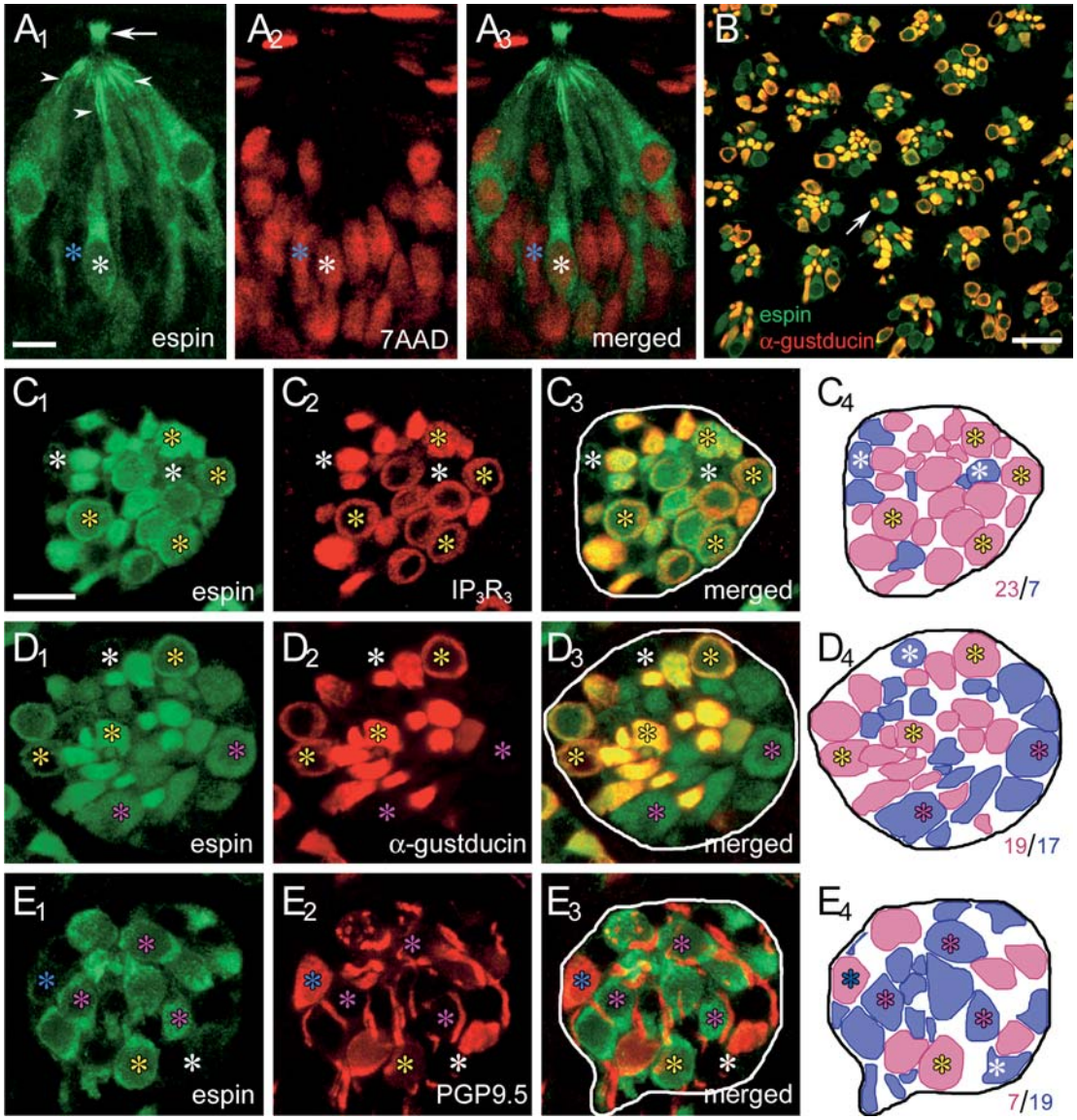


Fig. 3. Laser scanning confocal images of rat circumvallate taste buds. (A) An example of a taste bud in longitudinal section labeled with espin antibody (A_1 and A_3) and 7AAD nuclear dye (A_2 and A_3). In the apical region, the taste pore microvilli (arrow) and the core bundles in association with the base of the taste pit (arrowheads) show intense espin immunolabeling, while an espin-free zone is present between these two espin$^+$ structures, in the mid-region of the taste pit. White and blue asterisks show the nuclei of espin$^+$ and espin$^-$ taste bud cells, respectively. (B) Transverse section through a field of taste buds double-labeled with antibodies to espin (green) and α-gustducin (red). Note a small-sized taste bud (arrow) among the more typical, mid-sized taste buds. (C–E) Examples of taste buds cut in tranverse section labeled with antibodies to espin and IP_3R_3 (C), espin and α-gustducin (D), or espin and PGP9.5 (E). All $IP_3R_3^+$ (C) and α-gustducin$^+$ (D) taste cells show moderate espin immunolabeling (yellow asterisks). Light espin immunolabeling is detected in $IP_3R_3^-$ taste bud cells (white asterisks in C). The α-gustducin$^-$ taste bud cells (D) show both moderate (magenta asterisks) and light espin immunolabeling (white asterisks). In specimens double labeled with antibodies to espin and PGP9.5 (E), four categories of taste bud cells can be recognized: moderate espin$^+$/light PGP9.5$^+$ (yellow asterisk), light espin$^+$/intense PGP9.5$^+$ (blue asterisks), moderate espin$^+$/PGP9.5$^-$ (magenta asterisks), and light espin$^+$/PGP9.5$^-$ (white asterisk) taste bud cells. Panels C_4, D_4, and E_4 illustrate the assignments for espin$^+$/cell-specific marker$^+$ double-labeled cells (dark pink profiles) and espin$^+$ single-labeled cells (violet profiles) used for counting. Scale bars = 10 μm in A, C–E, and 25 μm in B.

ESPINS ARE PRESENT IN THE SENSORY CELLS OF TASTE BUDS

On the basis of their numbers, morphological features and content of cell-specific markers, we conclude that the espin$^+$ cells in the taste buds of rat circumvallate papillae are predominantly the sensory cells. The espin$^+$ cells, which accounted for more than half of the cells in taste buds, were present in

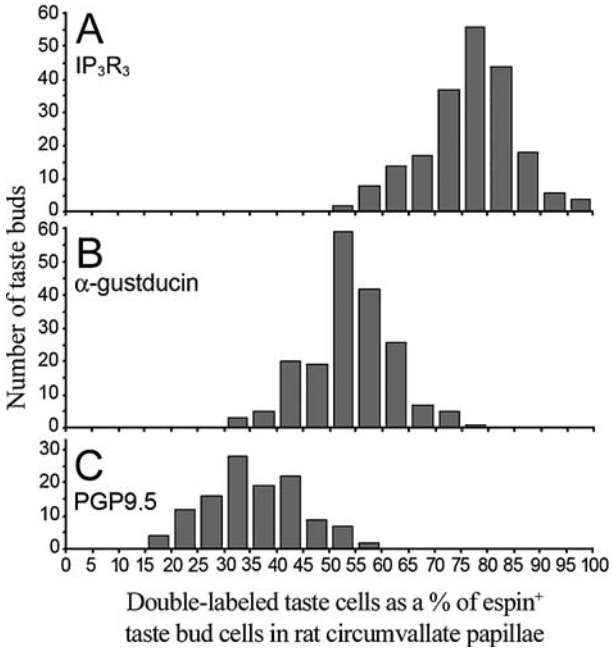


Fig. 4. Frequency distribution of the espin$^+$ taste bud cells in rat circumvallate taste buds that are also labeled with antibody to IP_3R_3 (A), α-gustducin (B) or PGP9.5 (C). The percentages of double-labeled cells from the total number of the espin$^+$ cells were tabulated for each individual taste bud, and the frequency distributions of these percentages among taste buds are plotted as histograms. Note that the distribution of the double-labeled cells shows considerable variability for each of the cell-specific markers.

numbers similar to those of the relatively electron-lucent light cells. Pumplin and Getschman (2000) counted 20.7 $\pm$ 1.3 light cells/circumvallate taste bud, a number that agrees with our number of 21.5 $\pm$ 0.3 espin$^+$ cells/circumvallate taste bud. In addition, the labeled cells were of the expected spindle shape with round nuclei, had their espin$^+$ collections of microvilli positioned in expected locations in the taste pit and pore, and were labeled with antibodies to commonly used cell-specific markers of type II and type III taste cells. We found complete colocalization of IP_3R_3, α-gustducin, and PGP9.5 with espin in taste cells. The fractions of cells we observed with these three cell-specific markers were similar to those of earlier studies showing that $\sim$33% of taste bud cells are α-gustducin$^+$ (Boughter *et al*., 1997; McLaughlin, 1992; Pumplin & Getschman, 2000; Takeda *et al*., 2004), while another $\sim$23% contain PGP9.5 (Takeda *et al*., 2004).

IP_3R_3, which is believed to be part of a downstream signaling cascade involving tastant-mediated activation of phospholipase Cβ_2, has been implicated in the transduction of bitter, sweet and umami taste (Lindemann, 2001; Zhang *et al*., 2003; Scott, 2004). A subset of the $IP_3R_3^+$ type II cells expresses α-gustducin, which is closely related to the transducins of retinal photoreceptors and involved in the transduction of bitter and sweet taste (McLaughlin *et al*., 1992; Chandrashekar *et al*., 2000; Yan *et al*., 2001; Margolskee, 2002). Accordingly, we found that IP_3R_3 was present in the majority of the espin$^+$ cells, whereas a lower percentage of espin$^+$ cells contained α-gustducin. Espin antibody labeling was also detected in all PGP9.5$^+$ cells, which comprise subgroups of both the type II and the type III cells. Notably α-gustducin and PGP9.5 were never colocalized and, thus, likely define two subclasses of type II cells (Fig. 2G, see also Yee *et al*., 2001). The presence of IP_3R_3, α-gustducin, and PGP9.5 in the espin$^+$ cells is a strong indication that espins are expressed in type II cells, which are involved in sensory processing of bitter, sweet, and umami taste stimuli.

Our data also suggest that, in addition to type II cells, espins are present in type III sensory cells, as IP_3R_3 and PGP9.5 are known to be present in a subset of type III cells (Clapp *et al*., 2001, 2004; Yee *et al*., 2001). In addition, the espin immunolabeling that we observed within the taste pore likely corresponds to the unusual microvillar projections of type III cells (see more below). In our study we noted that the espin$^+$/$IP_3R_3^-$ cells (on average, $\sim$27% of espin$^+$ cells) showed only light cytoplasmic labeling. These lightly stained cells might represent the type III cells. This notion is supported by the fact that most of the PG9.5$^+$ cells were also lightly labeled by espin antibody, and it was reported that PGP9.5 is present in a different subgroup of type III cells from IP_3R_3 (see diagram in Yang *et al*., 2004). It is possible that the different intensities of cytoplasmic espin immunolabeling we observed are a hallmark of functional differences between type II and type III cells. We have noticed similar differences when comparing the cytoplasmic immunolabeling of inner and outer hair cells in the cochlea (unpublished data).

Although our data support the conclusion that type II and type III taste cells contain espin, we cannot rule out the possibility that some type I cells accumulate espin in their microvilli. Precise cell-type identifications in our case are complicated by the low cytoplasmic and intense microvillar nature of the espin immunolabeling, the paucity of markers for type I cells (Ueda *et al*., 2003) and the tightly packed cellular organization of taste buds. In addition, taste buds exhibit continuous turnover (Farbman, 1980; Delay *et al*., 1986), and therefore differences in cell cycle or differentiation state could also contribute to differences in immunolabeling. Interestingly, at P1 we observed intense espin immunostaining in clusters of cells at the locations of fungiform papillae. This suggest that espins may already be expressed at high levels in taste cells before mature taste bud structures are present (Kossel *et al*., 1997), and could, therefore, be an early marker for differentiating taste cells. Similarly, α-gustducin$^+$ cells are known to be present in early postnatal lingual papillae

(Sbarbati *et al.*, 1999). In a recent study, we showed that espins are also expressed early in differentiating hair cells (Sekerková *et al.*, 2006).

ESPINS ARE ENRICHED IN THE SPECIALIZED APICAL CYTOSKELETAL STRUCTURES OF TASTE CELLS

One noteworthy aspect of our espin immunolabeling was the detection of two different espin$^+$ cytoskeletal structures at the apical pole of taste buds. One labeled structure, which was present in the lower half of the taste pit, fits the ultrastructural description of the core actin bundles of type II cell microvilli. The microvilli of type II cells, which are usually shorter and thicker than those of the surrounding type I cells, are confined to the lower half of the taste pit and include core actin bundles that extend rootlets deep into the taste cells, sometimes up to half way down the length of the cell (Murray & Murray, 1967; Takeda *et al.*, 1989). At the level of resolution afforded by the confocal microscope, it was not possible to discern the individual microvilli within this espin$^+$ zone in the lower portion of the taste pit. However, the long core actin bundles associated with this zone were readily visible in our images. We showed previously that espins are present at high levels in the parallel actin bundle at the core of microvillus-like projections in a variety of other cell types, including hair cells, solitary chemoreceptor cells and vomeronasal sensory neurons (Bartles *et al.*, 1998; Bartles, 2000; Zheng *et al.*, 2000; Loomis *et al.*, 2003; Sekerková *et al.*, 2004). Moreover, we have determined that espins can cause a concentration-dependent elongation of microvillar parallel actin bundles in transfected epithelial cells and that the levels of espin protein are positively correlated with stereociliary parallel actin bundle length in hair cells at different positions along the cochlear spiral *in situ* (Loomis *et al.*, 2003; Sekerková *et al.*, 2006). In fact, the long, sturdy actin bundles observed in light taste cells caused Murray and Murray (1967) to compare these bundles to those of hair cell stereocilia. Type II cells also bear a resemblance to brush cells and solitary chemoreceptor cells in the digestive and respiratory tracts (Höfer & Drenckhahn, 1999; Finger *et al.*, 2003; Sbarbati & Osculati, 2005). Like a subset of type II taste cells, brush cells and solitary chemoreceptor cells express α-gustducin (Höfer & Drenckhahn, 1998; Finger *et al.*, 2003; Sbarbati *et al.*, 2004; Sekerková *et al.*, 2004) and possess short apical microvilli with core actin bundle rootlets that reach deep into the cell (Höfer *et al.*, 1999). We showed previously that solitary chemoreceptor cells in the vomeronasal organ contain espin and α-gustducin (Sekerková *et al.*, 2004), and we have also detected espin$^+$ brush cells in a variety of other locations (our unpublished results). These observations further strengthen the relationship between the type II taste cells and brush cells, as proposed by Sbarbati and Osculati (2005).

In addition to labeling the espin$^+$ microvillar core bundles of type II cells, we observed an espin$^+$ structure that filled the taste pore. Remarkably, this structure often appeared disconnected from the microvillar core bundles of the type II cells in the taste pit, giving the intense espin immunolabeling observed in the apical regions of taste buds a multi-layered appearance. Our best candidate for these espin$^+$ structures in the taste pores are the microvilli of the type III taste cells. We know from our double immunolabeling experiments that type III cells contain espins (see above). Moreover, the type III cell has been reported to display an apical microvillar specialization that differs significantly from the microvilli of other taste bud cell types and consists of a single, blunt microvillus that extends into the taste pore (Takeda, 1976; Murray, 1986; Takeda *et al.*, 1989; Yee *et al.*, 2001).

We found an $\sim$3-μm region between the espin$^+$ microvilli of the type II cells in taste pit and the espin$^+$ microvilli of the type III cells in the taste pore to be depleted of espin immunolabeling. On the basis of earlier ultrastructural studies, this space—extending from the upper half of the taste pit to the taste pore—is expected to contain the microvilli of type I cells (Murray & Murray, 1967). Thus, even though their microvilli contain parallel actin bundles that stain with heavy meromyosin (Takeda *et al.*, 1989) and antibody to β-actin (Höfer & Drenckhahn, 1999), type I cells appear to contain little or no espin, based on our results. This would make the situation in taste buds similar to those in the inner ear and vomeronasal organ, where the microvillar specializations of the sensory cells contain high levels of espins, while those of the neighboring supporting cells show little or no espin (Zheng *et al.*, 2000; Sekerková *et al.*, 2004). This is yet another illustration of the concept that different cell types use different actin-bundling proteins in their parallel actin bundle-containing structures (Bartles, 2000; Höfer *et al.*, 2000). Additional immunocytochemical labeling experiments carried out at the electron-microscopic level will be required to test the various aspects of this model. However, our findings raise a number of questions about the roles of espins in taste receptor cell microvilli that will be addressed in part through an analysis of the effects of espin mutations on the taste receptor cell structure and function.

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