Chem. Senses 33: 231–241, 2008
doi:10.1093/chemse/bjm082
Advance Access publication December 21, 2007

Immunohistochemical Localization of Cystic Fibrosis Transmembrane Regulator and Clara Cell Secretory Protein in Taste Receptor Cells of Rat Circumvallate Papillae

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Abstract

Taste receptor cells (TRCs) are the sensory cells of taste transduction and are organized into taste buds embedded in the epithelium of the tongue, palate, pharynx, and larynx. Several studies have demonstrated that TRCs involved in sweet as well as bitter and umami responses express α-gustducin, an α-subunit of the G-protein complex. It has been further demonstrated that this typical taste protein is a potent marker of chemosensory cells located in several tissues, including gastric and pancreatic mucosa and the respiratory apparatus. We recently observed that α-gustducin and phospholipase C beta 2–immunoreactive cells were colocalized in the airways with cystic fibrosis transmembrane regulator (CFTR) and Clara cell–specific secretory protein of 10 (CC10) and 26 kDa (CC26). This finding suggests that TRCs might themselves express secretory markers. To test this hypothesis, we investigated the expression of CFTR, CC10, and CC26 in rat circumvallate papillae using reverse transcriptase–polymerase chain reaction analysis, immunohistochemistry, and confocal laser microscopy. The results showed that secretory markers such as CFTR, CC10, and CC26 are present in taste cells of rat circumvallate papillae, and their immunoreactivity is expressed, to a different extent, in subsets of taste cells that express α-gustducin. The presence of CFTR, CC10, and CC26 in taste bud cells and their coexpression pattern with α-gustducin confirms and extends our previous findings in airway epithelium, lending further credence to the notion that chemoreception and secretion may be related processes.

Key words: α-gustducin, CFTR, chemoreception, Clara cell–specific secretory protein, immunohistochemistry, taste

Introduction

Taste receptor cells (TRCs), the sensory cells of taste transduction, are organized into end organs called taste buds, which are embedded in the epithelium of the soft palate and in distinct regions on the tongue, pharynx, and larynx (Miller 1995). In rats, lingual taste buds are distributed in diverse papillae, which differ in their morphology, location on the tongue, and gustatory sensitivities. The circumvallate papillae, located on the posterior tongue, are innervated by glossopharyngeal nerve fibers that are predominantly responsive to acid and bitter stimuli (Frank 1991). Typical TRCs extend from base to apex of the bud and make contact with the oral cavity through the apical taste pore, which is strategically situated so as to be bathed by the fluid of the oral cavity and enriched by saliva secreted by von Ebner's glands (Matsuo 2000). TRCs protrude into taste pores with short microvilli on the apical surface, where receptors and channels involved in taste transduction are presumably located. A large class of membrane receptors, the G-protein–coupled receptors (GPCRs), have been implicated in the signaling mechanisms of the different tasting modalities (Hoon et al. 1999; Chandrashekar et al. 2000; Chaudhari et al. 2000; Li et al. 2002).

Several study results now suggest that the taste-sensing mechanism has a broader function than was originally thought, going beyond food evaluation (Meyerhof 2005; Sbarbati and Osculati 2005). Lower vertebrates utilize taste buds to explore their external environment (Cinar and Senol 2005); in mammals, the presence of cells expressing components of taste-signaling pathways in tissues outside the oral cavity has been documented in numerous studies carried out in a variety of species (Hofer et al. 1996; Adler et al. 2000; Wu et al. 2002). Generally, these cells are well suited to performing a chemoreceptor function because they are localized

in epithelia of endodermic origin (i.e., digestive and respiratory apparatuses) in which broad mucosal surfaces communicating with the external environment are continually exposed to the chemical components of the luminal contents. In particular, expression of α-gustducin, an α-subunit of the G-protein complex involved in sweet as well as bitter (Wong et al. 1996) and umami responses (Ruiz et al. 2003), has been identified in chemosensory cells scattered throughout the epithelial cells lining the gastric and pancreatic mucosa (Hofer et al. 1996; Hofer and Drenckhahn 1998; Dyer et al. 2005), suggesting that a taste-related chemoreceptor mechanism also exists in the gastrointestinal apparatus. In the respiratory apparatus, epithelial cells that include taste transduction components have been found in recent studies (Finger et al. 2003; Sbarbati, Merigo, Benati, Tizzano, Bernardi, Crescimanno, et al. 2004). Chemosensory cell clusters and solitary chemosensory cells expressing molecules typical of TRCs such as α-gustducin, phospholipase C beta 2 (PLCβ2) and type III inositol 1,4,5-trisphosphate receptor (IP3R3) have been found throughout the airways (Sbarbati, Merigo, Benati, Tizzano, Bernardi, Osculati 2004; Merigo et al. 2005).

The presence of chemosensory mechanisms in the respiratory apparatus is surprising and could be clinically important because it suggests the possibility of modulating airway function (e.g., secretion) by acting on specific chemoreceptors. We recently used immunohistochemistry to show that airway epithelial cells express molecules of both the taste and secretory pathways (Merigo et al. 2007). In particular, we observed that the Clara cell–specific secretory protein (CCSP) of 10 and 26 kDa (CC10 and CC26, respectively) and cystic fibrosis transmembrane regulator (CFTR) were colocalized with α-gustducin or PLCβ2 in a subset of epithelial cells at all levels of the airway. This finding raised the intriguing possibility of immunohistochemical similiarities between the TRCs of rat circumvallate papillae and airway epithelial cells, leading to the hypothesis that TRCs might also express secretory markers. To test this hypothesis, we selected CFTR, CC10, and CC26 as secretory markers because they are not only molecules typical of the airways but also widely expressed in a variety of tissues.

In the present study, we investigated the expression of CFTR, CC10, and CC26 in rat circumvallate papillae using reverse transcriptase–polymerase chain reaction (RT-PCR) analysis and immunohistochemistry. In a second step, in order to more exactly identify the immunoreactive cell type, we examined the expression patterns of CFTR, CC10, and CC26 in comparison to α-gustducin using double-label immunofluorescence and confocal laser microscopy.

Materials and methods

Total RNA isolation and RT-PCR

Four adult Wistar rats were used to perform this experiment. The animals were anesthetized with ether and killed by vertebrae dislocation. The total RNA was isolated from circumvallate papillae, the esophagus, trachea, and lung by using Trizol reagent (Invitrogen, Life Technologies, Milan, Italy) according to the manufacturer's instructions. Following spectrophotometric determination of total RNA content, samples of RNA (about 1 μg of the total RNA) from each tissue were digested with RNase-free DNase I Amp Grade (Invitrogen), reverse transcribed to cDNA, and amplified with gene-specific primers by using the SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen). The following primer sequences were used: Glyceraldehyde-3 phosphate dehydrogenase (GAPDH): 5′-ACTGGC GTCTTCACCACCAT-3′ (forward) and 5′-ATCCACA GTCTTCTGGGTGG-3′ (reverse); product size, 273 bp; CFTR: 5′-GCGATGCTTTGTCTGGAGATT-3′ (forward) and 5′-CCACTTGTAAAGGAGCAATCCATA-3′ (reverse); product size, 403 bp; CC10: 5′-TTACAACATCAGCCCA CATCTACA-3′ (forward) and 5′-TGTGATGCCGATCT TCATGGT-3′ (reverse); product size, 70 bp; and CC26: 5′-AAGCTACCATTTCCCATCATCGACG-3′ (forward) and 5′-TTGCCAGATGGGAGCTCTTTGG-3′ (reverse); product size, 477 bp. Expression of GAPDH was used as the internal standard. Polymerase chain reaction amplification was performed in an Eppendorf Mastercycler gradient at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 15–30 s for 25–35 cycles. The PCR products were identified on the basis of their size, determined by gel electrophoresis in 1.5% agarose gels.

Tissue preparation

The study was conducted on 14 adult Wistar rats of both sexes (150–200 g; Morini Company, Reggio Emilia, Italy) kept at the departmental animal facility. The rats were handled in accordance with the guidelines for animal experimentation as established by Italian law. The animals were anesthetized with ether and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The tongue was removed and further fixed by immersion in the same fixative for 2 h at 4 °C. After rinsing in 0.1 M PB, the tissues were put into 30% sucrose overnight and cut (40 μm thickness) on a freezing microtome (Reichert-Jung, Vienna, Austria). Sections from 5 animals were processed both for immunoperoxidase and single immunofluorescence labeling and sections from 9 animals for double immunofluorescence labeling. Two or 3 sections per each animal were used for each immunohistochemical experiment.

Primary antibodies

For this study, we used the following rabbit polyclonal primary antibodies: anti-CC10 (Santa Cruz Biotechnology, Inc., Heidelberg, Germany, cat #sc-25555) raised against amino acids 1–96 representing full length of CC10 of mouse origin; anti-CC26 (Chemicon International Inc., Temecula, CA, cat #AB3700) gel purified native nonselenium

glutathione peroxidase from rat lung, identified as CC26; anti-α-gustducin (Santa Cruz Biotechnology, cat #sc-395) raised against a peptide fragment containing amino acids 93–113 of rat α-gustducin; and anti-CFTR (Alomone Labs Ltd, Jerusalem, Israel, cat #ACL-006) raised against a peptide (C)KEETEEEVQDTRL, corresponding to amino acid residues 1468–1480 of cytoplasmic, C-terminal part of human CFTR.

Immunohistochemistry

Sections were blocked for 1 h in 0.3% Triton X-100, 1% bovine serum albumin (BSA), and 1% normal swine serum in 0.1 M phosphate buffered solution (PBS) (blocking solution); the same solution was used to dilute the antibodies. Immunohistochemical staining was performed using the avidin-biotin complex (ABC) technique. Briefly, endogenous peroxidase was quenched by immersion in a solution of 0.3% hydrogen peroxide in methanol for 30 min. After washing in 0.05 M Tris-HCL buffer, pH 7.6, the sections were treated with 5% normal swine serum for 20 min. Subsequently, sections were incubated overnight at 4 °C with anti–α-gustducin (1:400) or anti-CFTR (1:2000), or anti-CC10 (1:2000) or anti-CC26 (1:2000), diluted with blocking solution. After 3 washes, sections were then reacted with biotinylated swine anti-rabbit immunoglobulins (DAKO, Milan, Italy) diluted 1:400 for 2 h. The immunoreaction was detected using a Vectastain Elite ABC kit (Vector, Burlingame, CA) and then visualized with 3.3′-diaminobenzidine tetrahydrochloride (DAKO) for 5–10 min. Finally, sections were collected on polylysine-coated slides and mounted with DAKO Faramount Aqueous Mounting Medium. Control sections were prepared by preabsorbing the α-gustducin or CFTR antibody with the corresponding peptide (5 μg/1 ml of antibody, Santa Cruz Biotechnology or Alomone Labs Ltd, respectively) or by omitting the primary antibody. No controls exhibited immunolabeling. Sections were observed under an Olympus BX51 photomicroscope equipped with a KY-F58 CCD camera (JVC). Electronic images were analyzed and stored using Image-ProPlus software (Media Cybernetics, Silver Springs, MD).

Immunofluorescence

Single labeling

Free-floating sections were blocked for 1 h in 0.3% Triton X-100, 1% BSA, and 1% normal goat serum in 0.1 M PBS (blocking solution); the same solution was used to dilute the antibodies. Subsequently, sections were incubated overnight in anti-CFTR (1:200), anti-CC10 (1:200), or anti-CC26 (1:200) antiserum at 4 °C. After washes, sections were reacted with a secondary rhodamine (TRITC)–conjugated goat anti-rabbit IgG antibody (Jackson Laboratories, Inc., West Grove, PA; 1:200) for 1 h at room temperature. The sections were washed and mounted with fluorescent mounting medium (DAKO). Control sections were prepared by preabsorbing the anti-CFTR antibody with the corresponding peptide at 5 μg/ml (Alomone Labs Ltd) and/or by omitting the primary antibody. The controls did not exhibit immunolabeling.

Double labeling

We utilized a method which relied on the use of secondary monovalent Fab fragments (Lewis et al. 1993; Negoescu et al. 1994) because all primary antibodies are raised in the same species. Briefly, free-floating sections (40 μm thick) were blocked for 1.5 h in blocking solution. Subsequently, sections were incubated overnight at 4 °C with the following first primary antibodies: anti-α-gustducin (1:100), anti-CFTR (1:200), anti-CC10 (1:200), or anti-CC26 (1:200). After washes, they were reacted with Cy3-conjugated Affinity Pure Fab Fragment Goat anti-Rabbit IgG (Jackson Laboratories, Inc; 1:100) at room temperature for 1 h. Unoccupied antigen-binding sites in the primary or secondary antibody were then blocked by incubation with normal rabbit serum (nrs; DAKO; 1:50) followed by an excess of unconjugated Affinity Pure Fab Fragment Goat anti-Rabbit IgG (Jackson Laboratories, Inc.; 1:20) for 4 h. After washes, the blocking solution was again applied for 1.5 h followed by incubation overnight with the second primary antibody. Finally, sections were exposed to FITC-conjugated Affinity Pure Fab fragment Goat anti-Rabbit IgG (Jackson Laboratories, Inc; 1:100) for 1 h.

Control sections were prepared by one of the following methods: 1) adding the protein (5 μg/1 ml of antibody) to the primary α-gustducin or CFTR antiserum; 2) omitting the primary antibody; 3) changing the sequence of primary antibody application; 4) replacing the second primary antibody with nrs; or 5) changing the sequence of secondary antibody application. No controls exhibited immunolabeling.

Specimens were examined under a Zeiss LSM 510 confocal microscope equipped with argon (488 nm) and helium/neon (543 nm) excitation beams. Sequential acquisition, for instance, one color at a time, was utilized on double-label tissues to avoid side-band excitation of the inappropriate fluorophore. All images were composed using Adobe Photoshop software (version 6.0; Adobe Systems, Mountain View, CA), adjusting only brightness and contrast.

Results

CFTR mRNA and protein expression in TRCs

In RT-PCR analysis, the amplified product of the expected size for CFTR was observed in the circumvallate papillae, trachea, and lung (Figure 1a). No product was detected from mRNA obtained from the esophagus, which was used as a negative control. GAPDH standard product was expressed in all the tissues (Figure 1d).

In immunohistochemistry analysis, as positive controls for taste cells of rat circumvallate papillae, we used antibody

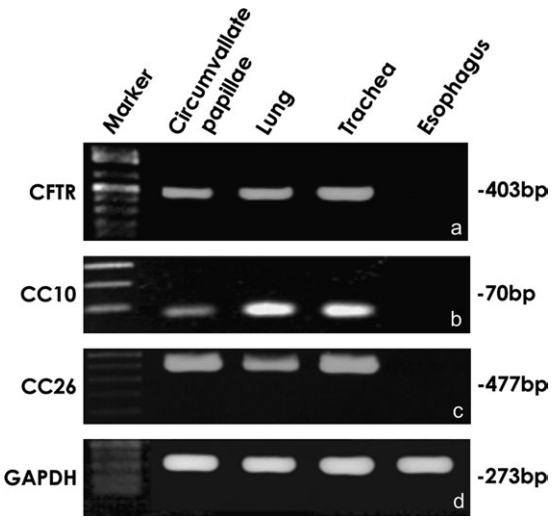

Figure 1 Semiquantitative RT-PCR demonstrated transcriptional expression of CFTR **(a)**, CC10 **(b)**, and CC26 **(c)** in circumvallate papillae, lung, trachea, and esophagus homogenates. Esophagus tissue was used as negative control. GADPH standard product was used as internal standard **(d)**.

against α-gustducin, which is an α-subunit of a heterotrimeric G-protein expressed in a subset of TRCs (Boughter et al. 1997).The α-gustducin is a protein predominantly associated with the inner surface of the plasma membrane (Gilman 1987), but it is also distributed within the cytoplasm.

As expected, α-gustducin immunostaining was present in many TRCs with a predominantly cytoplasmic labeling, extending in some cells from the apical to the basal cell pole (Figure 2a,b). To assess CFTR protein expression, we used immunoperoxidase (Figure 2c,d) and immunofluorescence experiments (Figure 2e,f). CFTR staining was detected in elongated TRCs with round or oval nuclei. Reactivity was mainly localized in the basolateral region (below and around the nucleus) and/or sometimes in the apical process of the cells. CFTR was not detected in the surrounding epithelia devoid of taste buds. The specificity of the CFTR antibody was tested on lung sections, which showed strong staining of bronchiolar epithelial cells. No specific labeling was seen when α-gustducin and CFTR antibodies were preincubated with the corresponding antigen peptide.

Coexpression of CFTR with α-gustducin in TRCs

To determine the cell type expressing CFTR immunoreactivity, we examined the expression pattern of CFTR compared with α-gustducin, using double-label immunofluorescence. Confocal laser scanning microscope images revealed that the number of CFTR-positive cells appeared to be higher than that of α-gustducin and many CFTR-positive cells also expressed α-gustducin expression (Figure 3a–c). Coexpression of labeling was mainly observed in the perinuclear cytoplasm or in the apical cell pole; occasional cells showed complete coincidence of labeling. In addition, a subset of

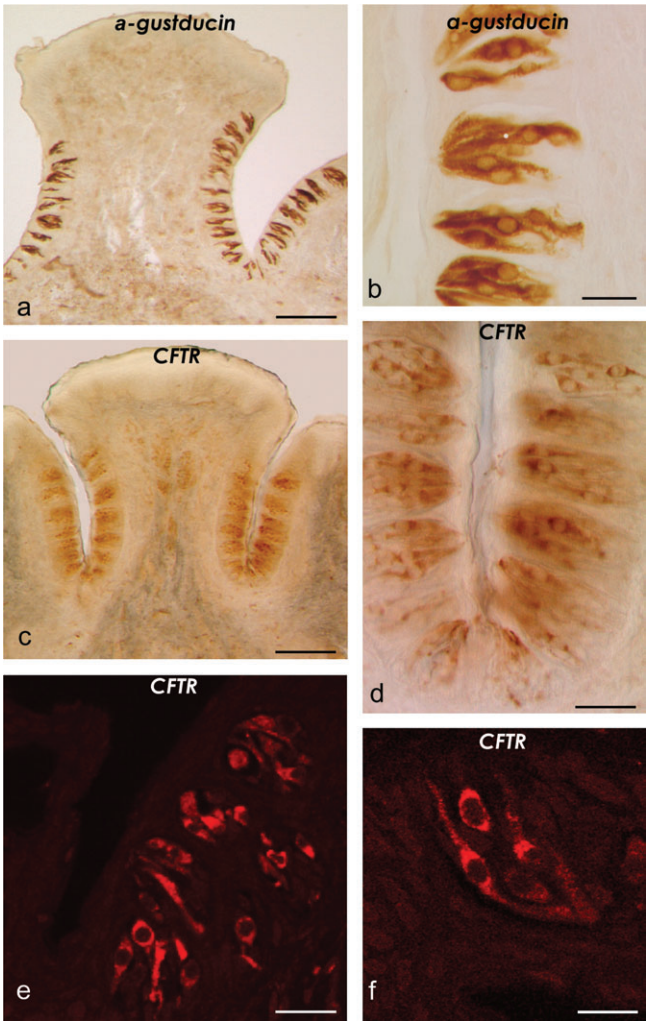

Figure 2 Immunoperoxidase **(a–d)** and immunofluorescent **(e, f)** staining of rat circumvallate papillae showing gustducin and CFTR localization in TRCs. The taste pore is toward the left in the photomicrograph (b, e, f). Scale bar, 10 μm (b, f), 30 μm (d, e), 125 μm (a, c).

α-gustducin–positive cells lacked CFTR expression and other cells were only CFTR positive. No specific double labeling was observed when the second primary antibody was replaced with nrs (Figure 3d–f) or when the anti-CFTR antibody was preincubated with the corresponding antigen peptide (Figure 3g–i).

Although a rigorous morphometric count was not carried out, in three 40-μm-thick sections from 3 animals, we counted the number of cells that were immunoreactive for CFTR only, for α-gustducin only, and those showing colocalization of both antigens. On a total of 432 positive cells, 395 cells were positive for CFTR only, 247 cells were positive for α-gustducin only, and 210 cells showed colocalization of both antigens (Table 1). We estimated that the double-labeled cells ($CFTR^+/\alpha\text{-gustducin}^+$) represented 53% of all CFTR-expressing cells and 85% of all α-gustducin–expressing cells.

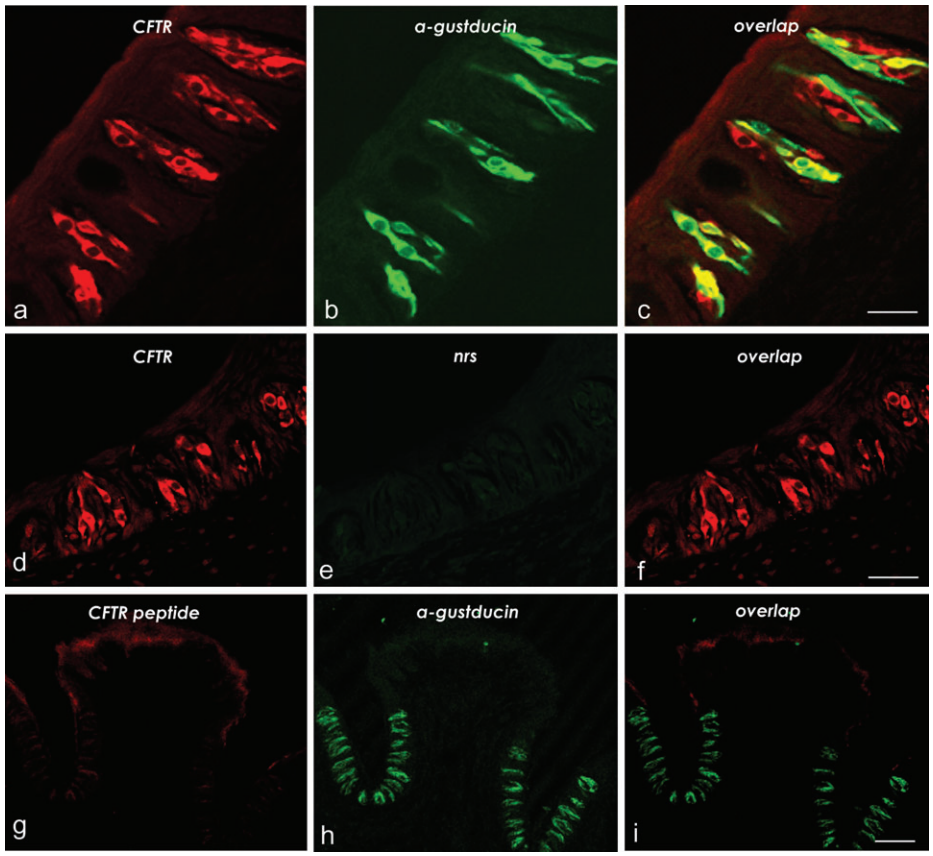

Figure 3 Double immunofluorescence by laser scanning confocal microscopy for CFTR and gustducin in TRCs of rat circumvallate papillae **(a–c)**. Coexpression pattern was observed in the perinuclear cytoplasm or in the apical cell pole. Occasional cells showing a complete coincidence of immunostaining were observed. Only gustducin-positive cells or only CFTR-positive cells were present. The specificity of the double-labeling procedure was demonstrated by absence of labeling when the second primary antiserum was replaced with normal rabbit serum **(**nrs, **d–f)** or when the anti-CFTR antibody was preabsorbed with its specific peptide **(g–i)**. Left: First primary antibody. Middle: Second primary antibody. Right: Overlay. Scale bar, 20 μm (c), 30 μm (f), 100 μm (i).

Clara cell secretory protein mRNA and protein expression in TRCs

In RT-PCR, CC10, and CC26, mRNA expressions were detected in the circumvallate papillae, lung, and trachea (Figure 1b,c). No transcripts were detected from mRNA obtained from the esophagus.

In immunohistochemistry analysis, the specificity of CC10 and CC26 antibodies was tested on lung sections, which confirmed strong staining of bronchiolar epithelial cells. Using both immunoperoxidase and single immunofluorescence, we showed that CC10 and CC26 immunoreactivities were present in the cells of taste buds. CC10 immunostaining was primarily perinuclear or in the apical process of spindle-shaped cells, with a finely granulated pattern (Figure 4a–c), whereas the labeling pattern for CC26 appeared to be localized, with a punctate pattern, primarily along the apical and basolateral membrane of many TRCs (Figure 4d–f). In some cells, CC26 labeling was also seen in the perinuclear region or throughout the cytoplasm of the entire cell or along the apical process. CC10 and CC26 were not detected in the surrounding epithelia devoid of taste buds. No specific labeling was seen when CC10 or CC26 antiserum was replaced with blocking solution.

Table 1 Immunolabeled TRCs in rat circumvallate papillae

Total labeled TRCs	Single-labeled cells			Double-labeled cells				
	Number			Number		Percentage		
	CFTR	α-gustducin	CC10	CFTR/α-gustducin	CC10/α-gustducin	CFTR	α-gustducin	CC10
432	395	247		210		53	85	
562		231	502		171		74	34

Coexpression of Clara cell secretory protein with α-gustducin in TRCs

In order to clarify which taste cell types express CC10 and CC26, we performed double immunostaining to compare these proteins to α-gustducin and to observe the relationships

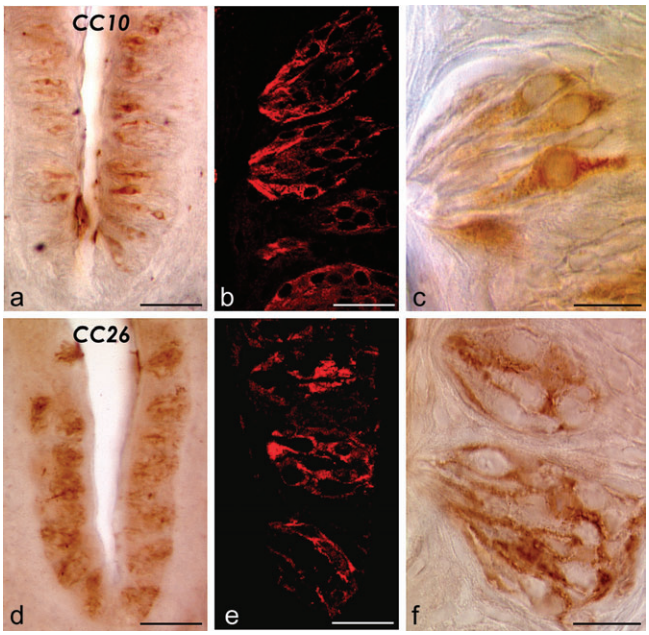

Figure 4 Immunoperoxidase **(a**, **c**, **d**, **f)**, and immunofluorescent **(b**, **e)** staining of rat circumvallate papillae showing CC10 (a–c) and CC26 (d–f) localization in TRCs. The taste pore is toward the left in the photomicrograph (b, c, e, f). Scale bar, 10 μm (c, f), 20 μm (b, e), 30 μm (a, d).

among themselves. Using laser scanning confocal microscopy, no specific double labeling was seen when the second primary antibody was replaced with nrs (Figure 5a–c). Dual immunofluorescence analysis of CC10 and α-gustducin showed that these proteins were colocalized, with a denser and more granular labeling, in the perinuclear cytoplasm or along the apical cell process in a subset of TRCs (Figure 5d–i). More taste cells expressed CC10 than α-gustducin and not all α-gustducin–immunoreactive cells were CC10 positive. As well as CFTR, the immunoreactive cells of 3 papillae sections from 3 animals were counted. On a total of 562 immunoreactive cells, 231 cells were positive for α-gustducin only, 502 cells were positive for CC10 only, and 171 cells showed colocalization of both antigens (Table 1). We estimated that the double-labeled cells ($CC10^+$/α-$gustducin^+$) represented 74% of all α-gustducin–expressing cells and 34% of all CC10-expressing cells.

Dual immunofluorescence analysis of CC26 and α-gustducin demonstrated that these proteins were coexpressed within TRCs, but in many cells their intracellular localization appeared to be distinct: CC26 showed more peripheral immunoreactivity, whereas α-gustducin staining was mainly localized within the cytoplasm of TRCs (Figure 5j–l). In some cells, the colocalization pattern was observed in the perinuclear cytoplasm or restricted to spots, which appeared somewhat too small to permit us to quantify the immunoreactive cells. Furthermore, some CC26-immunoreactive cells lacked α-gustducin expression and other cells were only α-gustducin immunopositive.

Because TRCs expressed both CC10 and CC26, it remained to be shown whether CC10 and CC26 were coexpressed. By dual immunofluorescence analysis, the labeling pattern for CC10 appeared distinct from that observed for CC26. CC10 immunoreactivity was located more within the cytoplasm, whereas CC26 labeling was distributed along the apical and basolateral membrane (Figure 5m–o). The colocalization pattern was mainly restricted to the apical process or to small spots.

Discussion

Summary of results

The present study shows, by RT-PCR and immunohistochemistry, that CFTR, CC10, and CC26, which are secretory markers typical of the airways, are expressed in TRCs. Additionally, we found that their immunoreactivity is also colocalized with that of α-gustducin, suggesting that CFTR, CC10, and CC26 localization occurs in cells expressing molecules of the taste-signaling cascade.

CFTR expression in TRCs

CFTR is a cAMP-regulated Cl^- channel, which allows transepithelial salt absorption as well as secretion in many epithelial tissues (Cheng et al. 1980; Bear et al. 1992). It has been extensively investigated due to its involvement in cystic fibrosis. CFTR is predominantly located at the apical epithelial surface associated with the cell membrane, but it was also found in the basolateral membrane as well as in many intracellular membranes (Bradbury 1999).

In our study by RT-PCR analysis, CFTR was detected in the circumvallate papillae as well as in the trachea and lung, which are well known sites of CFTR localization. Morphologically, CFTR was expressed in bipolar or pear-shaped cells of the rat circumvallate papillae, with intense immunoreactivity in the apical process or in the basal region of the cell, mainly localized around the nucleus. In addition, CFTR was observed in a substantial number of cells expressing α-gustducin, even though approximately one-half of the cells that were CFTR positive did not display α-gustducin, suggesting that presumably G-protein α-subunits other than α-gustducin might be involved with CFTR. Conversely, few α-gustducin–immunoreactive cells lacked CFTR expression.

To our knowledge, this study is the first to demonstrate CFTR expression in TRCs. This finding is not surprising because sensory cells, like other epithelia, possess several types of chloride channels, such as olfactory receptor neurons (Delay et al. 1997) and TRCs (Herness and Sun 1999; Miyamoto et al. 2001). Recently, a chloride channel, ClC-4, has been shown in a subset of mouse taste cells expressing IP_3, a second messenger of taste transduction (Huang et al. 2005). Their function in these cells is not well understood, but it has been suggested that they could likely be involved

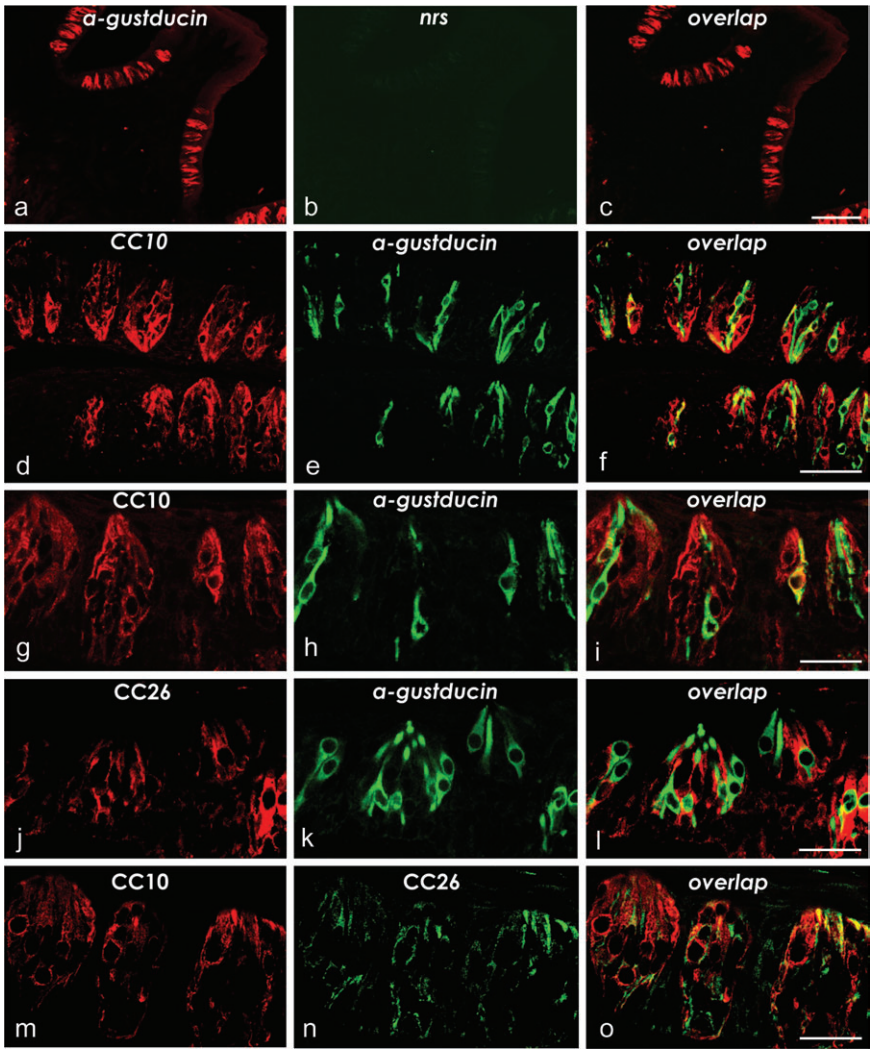

Figure 5 Double immunofluorescence by laser scanning confocal microscopy for CC10 or CC26 with gustducin and for CC10 with CC26 in TRCs of rat circumvallate papillae. The specificity of the double-labeling procedure was demonstrated by the absence of labeling when the second primary antiserum was replaced with normal rabbit serum **(**nrs, **a–c)**. The coexpression pattern of CC10 with gustducin was visible in the perinuclear cytoplasm or along the apical cell process in a substantial number of TRCs **(d–i)**. The coexpression pattern of CC26 with gustducin was visible in the perinuclear cytoplasm or restricted to small spots in a subset of TRCs **(j–l)**. The coexpression pattern of CC10 with CC26 was mainly restricted to the apical process or to small spots in a subset of TRCs **(m–o)**. Left: First primary antibody. Middle: Second primary antibody. Right: Overlay. The taste pore is toward the top in the photomicrographs **(g–o)**. Scale bar, 20 μm (i, l, o), 30 μm (f), 100 μm (c).

in taste transduction (Herness and Sun 1999), playing a role similar to that of chloride currents in olfactory transduction (Kurahashi and Yau 1994).

Our finding of CFTR expression in TRCs is not in itself sufficient to prove a connection between CFTR and taste function; however, on the basis of experimental findings in other epithelia and of recent advances in taste transduction, more than one mechanism, not mutually exclusive, might explain the occurrence of CFTR in taste cells. Recent data suggest that in TRCs, basolateral channels, like apical channels, serve as receptors for small stimuli such as inorganic ions, which can diffuse through tight junctions and reach ion channels on the basolateral membrane, independently of G-protein–mediated transduction (Lindemann 1996; Herness and Gilbertson 1999; Bigiani et al. 2003). This view is supported by the finding that bitter-tasting quinine caused taste cell depolarization by activating Cl^- secretion across the basolateral membrane in frog taste cells (Okada et al. 1988). Thus, it could be that the stimulation of the CFTR channel located apically or basolaterally is involved in bitter-induced responses.

Another possible mechanism, in agreement with the colocalization pattern of CFTR and α-gustducin, could be a link between CFTR activity and the G-protein–signaling pathway. In the bitter transduction pathway, the binding of tastants to the T2Rs family of GPCRs activates heterotrimeric G-proteins which dissociate into α- and βγ-subunits, which generate 2 separate different responses. The α-gustducin

subunit–mediated response induces the activation of a phosphodiesterase that is commonly associated with decreased levels of cAMP (McLaughlin et al. 1992, 1993; Kusakabe et al. 2000). The βγ-subunits promote the activation of a taste-specific PLCβ2 enzyme which determines an increase in second messengers IP3 and DAG (Yan et al. 2001), leading to Ca^{2+} release from internal stores (Clapp et al. 2001; Perez et al. 2002). Changes in intracellular calcium levels activate the transient receptor potential channel M5 (TRPM5), which leads to membrane depolarization due to influx of cation (Perez et al. 2002; Hofmann et al. 2003; Liu and Liman 2003).

In this complex context, the mechanism responsible for the G-protein–mediated activation of CFTR in several epithelia, such as intestinal epithelium, alveolar epithelium, and sweat glands has not been unequivocally identified (Tilly et al. 1991; Gadsby et al. 1995; Kemp and Oliver 1996; Reddy, Sun, et al. 2001; Reddy and Quinton 2001).

Another possible role of CFTR in TRCs may be consistent with its function as a regulator of other channels, including aquaporins (Nilius and Droogmans 2003). Aquaporins are involved in the transmembrane movement of water in many epithelia, and generally, their expression is strongly correlated with the distribution of CFTR, suggesting that water movement is coupled with electrolyte secretion (Burghardt et al. 2003). Three types of aquaporin channels (AQ1, AQ2, and AQ5) have recently been observed in rat taste buds (Watson et al. 2007). Therefore, it could be speculated that the presence of CFTR in TRCs could be related to the regulation of aquaporin water channels, as in other epithelia.

Although these observations provide possible support for the role of CFTR in TRCs, further functional investigations aiming to clarify the contribution of CFTR to taste transduction could lead to a more detailed classification of TRCs and provide new insights into the function of the CFTR channel in the taste transduction pathway.

Clara cell secretory protein expression in TRCs

CC10 and CC26 are primarily expressed by nonciliated cells along the tracheobronchial epithelium, with a greater density in the terminal bronchioles. (Bedetti et al. 1987; Lund et al. 1988). These proteins are also found in a variety of tissues and secretions but never described in TRCs. Not a single exclusive activity has been ascribed to them, and, also for this reason, they are referred to in the literature by various names.

Despite the fact that these proteins appear to be closely related because they are identified as a secretory product of Clara cells, studies employing knockout mice with CCSP deficiency have revealed a coexpression of CC10 and CC26 within Clara cells but with distinct intracellular distribution and independent mechanisms of secretion (Stripp et al. 2002).

CC10 is a member of the secretoglobin family assigned to subgroup 1A1, known by many other names such as Clara cell secretory protein 16 (CC16 or CCSP), uteroglobin, urine protein-1, human protein 1, and polychlorinated biphenyl–binding protein. CC10 has also been detected in the prostate, endometrium, and kidney (Hermans and Bernard 1999) as well as in biological fluids including alveolar fluid, sputum, urine, and serum (Singh et al. 1985). Despite its abundance in the lung, the function of CC10 is still poorly understood in vivo (Singh and Katyal 2000). CC10 deficiency is associated with increased sensitivity to microorganisms (Harrod et al. 1998) and to lung epithelial damage induced by ozone (Mango et al. 1998) or hyperoxia (Johnston et al. 1997), which indicates its protective effect against inflammatory response and oxidative stress. However, CC10 is considered a "multifunctional protein" because it plays many roles including its capacity to bind hydrophobic molecules and to inhibit phospholipase A_2 (PLA_2), which controls the production of arachidonic acid (AA).

In this study, as RT-PCR and immunohistochemical analyses revealed, TRCs of rat circumvallate papillae express CC10. CC10-positive cells were more numerous than the α-gustducin–expressing cells, and approximately one-third of the cells that were CC10 positive displayed double-labeled expression with α-gustducin. In the past, analogies between secretory molecules present in taste organs and CCSPs have been occasionally described; surfactant-like molecules have been identified in taste organs (Sbarbati et al. 1991). Our finding strengthens the analogy between gustatory and respiratory epithelium, as suggested in our previous studies (Sbarbati and Osculati 2005; Merigo et al. 2005), and raises the possibility that chemoreceptive cells can respond to the luminal environment with a secretory function. The finding in the gut that anti-inflammatory molecules are expressed in cell types from various tissues (Haller 2006) has led to the new concept that the defense responses induced by luminal content are generated by a cross talk among various cell types. In support of this view, it has recently been shown that in CCSP knock-out mice, CCSP deficiency was closely associated with altered posttranslation modification of annexin 1, a protein with immunomodulatory properties, both in ciliated cells and macrophages of the conducting airways, suggesting an interaction of these cell types with secretory cells, probably due to a paracrine mechanism (Reynolds 2007). In this context, CCSP functions as an essential link between airway epithelium cells and immune system cells that can generate integrated responses of host defense mechanisms. The presence of CC10 in TRCs suggests that TRCs might participate in the defense mechanisms of gustative epithelium inducing appropriate responses to external stimuli by a possible paracrine effect on other cell types.

CC26 is highly expressed in the olfactory and respiratory epithelium, mainly located at the apical cell pole and mucus layer (Novoselov et al. 1999; Hofman et al. 2002). It has been isolated from rat lung lavage (Power and Nicholas 1999) and

also detected in lamellar body and cytosolic fractions (Akiba et al. 1998). CC26 is also known by other names such as peroxiredoxin 6 and 1-Cys peroxiredoxin (Manevich and Fisher 2005). It performs both nonselenium glutathione peroxidase (Shichi and Demar 1990) and Ca^2-independent PLA_2 activities (Chen et al. 2000). Through its peroxidase activity, CC26 protects cells against oxidative stress, being able to reduce H_2O_2, short-chain hydroperoxides, fatty acid hydroperoxides, and phospholipid hydroperoxides (Fisher et al. 1999) into corresponding alcohols using reduced glutathione as a physiological electron donor (Manevich et al. 2004). Through its PLA_2 activity, also called acid Ca^2-independent phospholipase A_2 ($aiPLA_2$), CC26 participates in the intracellular release of AA from the phospholipid membrane. This activity has been studied mainly in the lung, providing evidence of its role in the metabolism of the lung surfactant phosphatidylcholine.

In the present study, CC26 was expressed in the taste buds, with immunoreactivity localized more along the apical and basolateral membrane of TRCs. CC26 immunoreactivity was only partly colocalized with α-gustducin pattern owing to their different localization within TRCs. Similarly, a comparison of CC10 with CC26 provided evidence of the distinct intracellular distribution of these 2 molecules, consistent with their independent expression previously observed in other tissues.

By analogy with its functions in other tissues, CC26 in TRCs may have a dual role: it may protect taste cells and enzymes from reactive oxygen species and also regulate the phospholipid metabolism through its PLA_2 activity. Recently, a member of the PLA_2 family (PLA_2-IIA) was found in a subset of rat TRCs expressing PLCβ2. Additionally, some PLA_2-IIA–positive cells were also α-gustducin immunoreactive (Oike, Matsumoto, et al. 2006). Here, we achieved comparable results using CC26, an $aiPLA_2$ enzyme of the PLA_2 family. The localization of PLA_2 enzymes in TRCs and their coexpression with molecules of the taste-signaling pathway might suggest a possible role of AA in taste. A recent experiment was designed to study the role of AA in TRCs by investigating the expression of enzymes involved in the AA cascade, such as monoglyceride lipase (MGL), cyclooxygenase-2 (COX-2), and PLA_2-IIA (Oike, Wakamori, et al. 2006). Interestingly, MGL, COX-2, and PLA_2-IIA immunoreactivities were observed in taste cells expressing TRPM5, a channel closely linked to α-gustducin and PLCβ2 in bitter transduction (Perez et al. 2002). Electrophysiological experiments in the same study showed that AA was able to activate TRPM5 in heterologously expressed cultured cells, providing strong evidence for the AA contribution to TRC function.

Taken together, our data emphasize the expression of Clara cell markers in taste buds, which further characterizes the phenotype of taste cells, indicating that these cells have additional functions apart from their implication in the transduction of taste stimuli. The most plausible role of Clara cell proteins in TRCs, in analogy with the above findings from other tissues, might be a defense role against infection, inflammation, or oxidative injury of the gustative mucosa. Clearly, further studies are required to understand the functional significance of these proteins in taste cells.

Conclusion

In conclusion, our data point out important similarities between taste bud cells and airway epithelium. The presence of CFTR, CC10, and CC26 in taste bud cells and their coexpression pattern with α-gustducin may have important functional implications. It can be hypothesized that the chemoreceptive capability of TRCs could drive ion transport processes that modify the tuning of chemoreceptive elements, changing either the microenvironment around taste pores or intracellular ion concentrations, which in turn affect other intracellular targets. The molecular link between chemoreception and secretion could open up new strategies in specific therapies.

Acknowledgements

The authors wish to thank Christine Harris for revising the manuscript.

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Accepted November 16, 2007