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Identification of cholinergic chemosensory cells in mouse tracheal and laryngeal glandular ducts

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ABSTRACT

Specialized epithelial cells in the respiratory tract such as solitary chemosensory cells and brush cells sense the luminal content and initiate protective reflexes in response to the detection of potentially harmful substances. The majority of these cells are cholinergic and utilize the canonical taste signal transduction cascade to detect "bitter" substances such as bacterial quorum sensing molecules. Utilizing two different mouse strains reporting expression of choline acetyltransferase (ChAT), the synthesizing enzyme of acetylcholine (ACh), we detected cholinergic cells in the submucosal glands of the murine larynx and trachea. These cells were localized in the ciliated glandular ducts and were neither found in the collecting ducts nor in alveolar or tubular segments of the glands. ChAT expression in tracheal gland ducts was confirmed by *in situ* hybridization. The cholinergic duct cells expressed the brush cell marker proteins, villin and cytokeratin-18, and were immunoreactive for components of the taste signal transduction cascade (Gα-gustducin, transient receptor potential melastatin-like subtype 5 channel = TRPM5, phospholipase $C_{\beta 2}$), but not for carbonic anhydrase IV. Furthermore, these cells expressed the bitter taste receptor Tas2r131, as demonstrated utilizing an appropriate reporter mouse strain. Our study identified a previously unrecognized presumptive chemosensory cell type in the duct of the airway submucosal glands that likely utilizes ACh for paracrine signaling. We propose that these cells participate in infection-sensing mechanisms and initiate responses assisting bacterial clearance from the lower airways.

1. Introduction

In the last years, specialized cholinergic chemosensory ("taste") epithelial cells were identified in several organs outside of the gustatory system, e.g. urethra, thymus, thyroid gland, heart, auditory tube, airways and lung [1–7]. In mucosal epithelia, these chemosensory cells express one or more members of the taste receptor family 1 (Tas1R) (sweet and umami) and Tas2R (bitter) that are coupled to various G-proteins (e.g. α-gustducin), phospholipase beta 2 (PLCβ2), and the transient receptor potential channel M5 (TRPM5) [8–10]. In the respiratory and urogenital tract they are important for the sensing of potentially dangerous substances including bacteria [1,11–13]. Activation of the chemosensory cells results in initiation of protective responses such as aversive respiratory reflexes in the trachea and nose [11,14] and micturition in the urethra [1]. Local inflammatory responses and mucosal swelling were detected in the vomeronasal duct and nose [13,15,16]. Based upon their characteristic morphological feature, i.e. an apical, brush-like tuft of rigid, villin-containing microvilli and the presence of cytokeratin 18 filaments [17,18], cholinergic chemosensory cells of the trachea, urethra, and auditory tube were classified as brush cells.

Brush cells and solitary chemosensory cells in the nose are considered to be part of a large family of chemosensory cells in different organs [19]. Brush cells were identified either in clusters or disseminated in the mucosa of the mouse gastrointestinal tract [20,21]. In the mouse gall bladder epithelium, brush cells were found almost 40 years ago [22] and were later also identified in the rat pancreatic [23] and bile ducts [24,25]. In the normal human bronchial tree, glands are present in the submucosa of the airways with cartilage in the wall [26–28]. In mice, submucosal glands are very sparse and located only at the border between the trachea and larynx [29,30]. Ducts from these glands pass through the lamina propria to empty onto the epithelial surface [29].

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Three segments can be discriminated in human bronchial glands: ciliated duct, collecting duct and secretory tubules [31]. Although the ciliated duct is lined by respiratory epithelium, the presence of brush cells in this tissue has not yet been described. The airway lining fluid covering the epithelium in large airways is critical for the maintenance of normal respiratory function. Water, electrolytes, and a mixture of compounds with antimicrobial, antiinflammatory, and antioxidant properties are secreted from the serous glands [32]. Inhaled particulates, bacteria and viruses get entrapped into the mucus and float on the serous secretions [33]. To colonize mucosal surfaces and invade the host, microbes typically have to first penetrate the airway lining fluid, and then either attach to the apical surface of epithelial cells or release toxins that disrupt epithelial integrity [34]. Here, we show that cholinergic brush cells are present in the duct of airway glands. These cells potentially detect chemical constituents at the gland entrance and—through local release of non-neuronal ACh—may prevent access of bacteria or other potential hazards to the secretory parts of the glands and thus help to maintain normal gland secretion.

2. Material and methods

2.1. Animals

Analyses were performed on C57Bl/6 mice (n = 4), two different transgenic mouse strains expressing enhanced green fluorescence protein (eGFP) under control of the promotor for choline acetyltransferase (ChAT-eGFP; [35,36]) (n = 8), and a gene-targeted mouse strain with tauGFP expression in cells expressing the bitter receptor Tas2r131 (n = 4, Tas2r131Cre/Rosa26tauGFP, short Tas2r131-tauGFP;4). Animals were between 8 and 16 weeks old and of either sex. All strains were kept under specified pathogen free conditions. All animal procedures were conducted in accordance with the EU Directive 2010/63/EU for animal experiments, the German Animal Protection Law, and protocols approved by the county administrative government in Gießen, or by the local veterinarian.

2.2. Tissue preparation

Tas2r131-tauGFP mice were anesthetized with pentobarbital (Merial, Halbergmoos, Germany) and transcardially perfused with PBS followed by 4% ice-cold paraformaldehyde (PFA) in PBS. Tissues were removed, post-fixed for 2 h and incubated overnight in 30% sucrose solution before being frozen in Isopentane (2-methylbutane) cooled by liquid nitrogen. C57BL/6 and ChAT-eGFP animals were killed by overdose of isoflurane (Abbott, Wiesbaden, Germany). Tissue used for immunohistochemistry was fixed by transcardiac perfusion of the circulation with either Zamboni solution (2% PFA/15% saturated picric acid in 0.1 M phosphate buffer, pH 7.4) or 4% PFA that followed an initial perfusion with a rinsing solution containing heparin (2 ml/l; 10,000 U; Ratiopharm, Ulm, Germany) and procaine hydrochloride (5 g/l; Merck, Darmstadt, Germany), pH 7.4. Airways were dissected and kept for an additional 1 h in Zamboni fixative/4% PFA depending on the perfusion fixative at room temperature. The tissue was extensively washed for 3 days with 0.1 M phosphate buffer, pH 7.4. Tissue blocks were rinsed in 18% sucrose (Merck) for at least 1 day until the tissue sank to the bottom of the vessel and were then frozen. For *in situ* hybridization (ISH), tissue was immediately placed into TissueTek O.C.T. Compound (Sakura, Alphen aan den Rijn, Netherlands), frozen as described above and stored at −80 °C until further use.

2.3. Antibodies

Sources and working dilutions of the primary antibodies used for immunohistochemistry were as follows: Anti-CK18 (cytokeratin 18) raised in rabbit, monoclonal (clone SP69) SPB-M3694, dilution 1:200, Spring Bioscience, Pleasanton, CA, USA; Anti-eGFP raised in chicken, polyclonal NB 100-1614, dilution 1:1600, Novus Biologicals, Littleton, CO, USA; Anti-Gα-gustducin, raised in rabbit, polyclonal sc-395, dilution 1:3000, Santa Cruz Biotechnology, Heidelberg, Germany; Anti-PLCβ2, raised in rabbit, polyclonal sc-206, dilution 1:800, Santa Cruz Biotechnology; Anti-TRPM5 Rabbit, polyclonal, dilution 1:4000 [37]; Anti-human villin, raised in rabbit, polyclonal V2121-95, dilution 1:50, US Biological, Salem, MA, USA; Anti-chicken villin, raised in rabbit, polyclonal, dilution 1:6400 [38]; Anti-carbonic anhydrase IV (CA IV), raised in rabbit, polyclonal HPA017258, dilution 1:400, Sigma-Aldrich, Steinheim, Germany; Anti-ChAT, raised in goat, polyclonal AB144P, dilution 1:250, Chemicon, Temecula, CA, USA; Anti-β-tubulin IV antibody, raised in mouse, monoclonal (clone ONS1A6) MU178-UC dilution 1:250, Biogenex, The Hague, Netherlands; Anti-α-smooth muscle actin (SMA) directly Cy3-conjugated antibody, raised in mouse, monoclonal (clone 1A4) C6198, dilution 1:500, Sigma-Aldrich.

2.4. Immunohistochemistry

Serial frontal cryosections (10 μm) from mouse larynx and trachea were air-dried and incubated for 1 h with blocking solution containing 10% normal horse serum, 0.5% Tween 20 and 0.1% bovine serum albumin in PBS, pH 7.4. Sections from ChAT-eGFP and Tas2r131-tauGFP mice were first incubated with the anti-eGFP antibody overnight, followed by incubation with FITC- or Cy3-conjugated donkey anti-chicken IgY antibody for 1 h, both at room temperature and then processed for incubation with primary antibodies to GFP, villin, α-gustducin, PLCβ2 and TRPM5. In addition, sections from ChAT-eGFP mice were incubated with an anti-CA IV antibody. For identification of the cell type of ChAT-eGFP + cells, sections of ChAT-eGFP mice without any immunolabeling were coverslipped with carbonate-buffered glycerol (pH 8.6) and evaluated microscopically, and then rinsed with PBS and cooked in a microwave oven in 0.01 M citrate acid, pH 6.0, for 15 min until the GFP signal was lost. After a microwave step, slides were washed in PBS, then in 0.1% Triton X-100 for 10 min, then washed in PBS again, then unspecific protein-binding sites were blocked for 1 h with PBS, 1% BSA, and 0.1% NaN_3 followed by overnight incubation with a mixture from an anti-cytokeratin-18 and an anti-SMA/Cy3-conjugated antibody. All primary antibodies were diluted in 0.005 M phosphate buffer containing 0.01% NaN_3 and 4.48 g/l NaCl and applied consecutively or in combination overnight at room temperature.

After a washing step, Cy3-conjugated donkey anti-rabbit IgG (Chemicon, 1:2000) or Alexa 488-conjugated donkey anti-goat IgG (Dianova, Hamburg, Germany, 1:800) antibodies were applied for 1 h. For CA IV labeling, sections from trachea and kidney were incubated for 1 h at room temperature with biotin-conjugated donkey anti-sheep/goat Ig (1:400) followed by Cy3-conjugated streptavidin (1:5000; GE Healthcare Life Sciences, Pittsburgh, USA). Anti-β-tubulin IV antibody was directly labeled using a Zenon Alexa Flour 633 Mouse IgG1 Labeling Kit (Life Technologies, Darmstadt, Germany) according to the manufacturer's protocol and incubated on the sections for 1 h at room temperature. Subsequently, sections were rinsed again, postfixed for 10 min in 4% PFA and coverslipped with carbonate-buffered glycerol (pH 8.6). Slides were evaluated with an epifluorescence microscope (Zeiss, Jena, Germany) using appropriate filter sets. Controls for the specificity of antibodies used were tested previously [1] for anti-gustducin and anti-PLCβ2 antibody by preincubation of these antibodies with the corresponding peptides (100 g/ml, Santa Cruz) for 1 h at room temperature before use, and [2] for TRPM5 with the preimmune serum instead of the primary antibody (Krasteva et al., 2012). Controls for the specificity of the secondary reagents were performed by omitting the primary antibody.

2.5. In situ hybridization

Serial 14 μm thick sections were cut with a cryostat and mounted on silanized glass slides. A complementary RNA probe for the