DEPARTMENT OF

Neurosciences, Biomedicine and Movement Sciences

GRADUATE SCHOOL OF

Natural Sciences and Engineering

DOCTORAL PROGRAM IN

Nanoscience and Advanced Technologies

XXIX° Cycle /2013

TITLE OF THE DOCTORAL THESIS

"SWEET SENSING IN THE CILIATED CELLS OF MURINE TRACHEA"

S.S.D. BIO/16

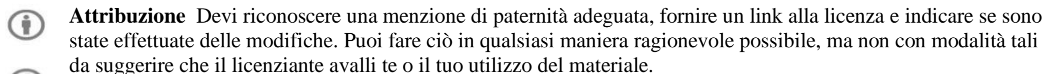
Coordinator: Prof. Franco Tagliaro

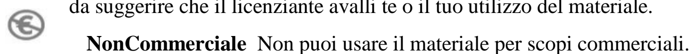
Tutor: Dott.ssa Maria Paola Cecchini

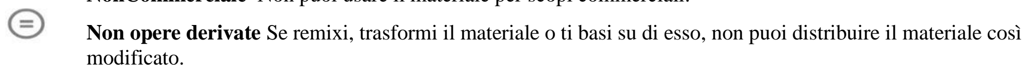
Doctoral Student: Dott.ssa Chiara Lasconi

Quest'opera è stata rilasciata con licenza Creative Commons Attribuzione – non commerciale
Non opere derivate 3.0 Italia . Per leggere una copia della licenza visita il sito web:

http://creativecommons.org/licenses/by-nc-nd/3.0/it/


Attribuzione Devi riconoscere una menzione di paternità adeguata, fornire un link alla licenza e indicare se sono state effettuate delle modifiche. Puoi fare ciò in qualsiasi maniera ragionevole possibile, ma non con modalità tali da suggerire che il licenziante avalli te o il tuo utilizzo del materiale.


NonCommerciale Non puoi usare il materiale per scopi commerciali.


Non opere derivate Se remixi, trasformi il materiale o ti basi su di esso, non puoi distribuire il materiale così modificato.

Sweet sensing in the ciliated cells of murine trachea
Chiara Lasconi
Tesi di Dottorato
Verona,
ISBN

"SWEET SENSING IN THE CILIATED CELLS OF MURINE TRACHEA"

ABSTRACT

Sweet and bitter are two basic taste sensations perceivable thanks to taste receptor cells (TRCs) in the oral cavity. Interestingly, T1R3 sweet receptor and members of the bitter T2Rs family are also localized in different extra-oral tissues (e.g. airways system). In the context of this diffuse extra-oral chemosensory system, airways ciliated cells represent an interesting cellular population. Preliminary studies reported that their cilia exhibit bitter and sweet receptors and some elements of the chemoreception transduction pathway. To the best of our knowledge, no physiological data regarding ciliated cells sweet response in the airways have been reported in the literature. Therefore, to investigate the functional role of sweet receptors within the airways, we chose to explore the physiological behavior of murine tracheal ciliated cells stimulating them with sweet compounds by means of calcium-imaging technique. This method allows to follow the changes in intracellular calcium concentration thus showing possible calcium-mediated sweet responses. We planned to work with acute slices of mice tracheas, instead of using cells cultures, to better preserve the native conditions, avoiding excessive cells handling and artefacts introduction.

We decided also to stimulate the ciliated cells with a bitter compound, yet investigated in cultured cells, to see the cellular behavior towards the two different stimulation qualities (sweet and bitter). In addition, we adopted immunohistochemical analysis to verify that the ciliated cells, used for the physiology experiments, retained the phenotypic expression of molecules of the taste transduction pathway normally expressed in native ciliated cells.

Our experiments showed that some ciliated cells respond to sweet compounds. In particular, combining two different stimuli, glucose (sweet) first and then bitter (denatonium), 36% of the ciliated cells responded to both stimulations while 37% were responsive only to bitter, 2% only to sweet and 25% of the cells were unresponsive to both stimulations. Another important finding is that, when stimulating the cells with two artificial sweeteners (acesulfame K and sucralose), we observed that 31% of the cells responded to both the sweeteners, 24% only to acesulfame K and 5% only to sucralose. 40% of the cells were irresponsive to both

the artificial sweeteners. Combining the artificial stimulus acesulfame K to the bitter one denatonium, 65% of the cells responded to both stimuli, 7% only to acesulfame K while 5% only to denatonium. Moreover, the immunohistochemical results confirmed the presence of α-gustducin and PLCβ2, two markers of taste signaling pathway, in the ciliated cells.

The above described data are interesting and clearly demonstrate that the ciliated cells of murine trachea are able to perceive and respond to sweet compounds, natural and artificial, with possible implications in the glucose sensing mechanisms in the airways, especially in relationship with respiratory infections. Moreover, it is clear that the ciliated cells exhibit a different pattern of response suggesting their heterogeneity, according with the previous literature. These data might be important for considering this cellular population a new cellular model of extra-oral chemoreception investigation. Future research could unravel the roles of airways ciliated cells in health and pathological conditions with a possible therapeutic aim.

1. INTRODUCTION **8**

1.1 “CANONICAL” GUSTATORY TASTE RECEPTORS 8

1.2 BITTER AND SWEET TASTE TRANSDUCTION 10

1.2.1 Sweet Receptors 10

1.2.2. Bitter Receptors 13

1.2.3. Calcium-mediated mechanism of taste transduction 15

1.3 EXTRA ORAL TASTE TRANSDUCTION 16

1.4 THE AIRWAYS EPITHELIUM 17

1.5 CILIATED CELLS AND THEIR ROLE IN THE CONDUCTING AIRWAYS 21

2. MATERIALS AND METHODS **24**

2.1 DISSECTION 24

2.2 CALCIUM IMAGING 24

2.2.1 Preparation of acute slices of mouse trachea 24

2.2.2 Fluorescence imaging and data analysis 26

2.2.3 Stimuli and perfusion system 28

3.2 IMMUNOHISTOCHEMISTRY 29

2.3.1 Immunohistochemistry on tracheal slices after calcium imaging experiments 29

3. RESULTS **31**

3.1 CALCIUM IMAGING EXPERIMENTS FROM ACUTE MICE TRACHEAL SLICES 31

3.1.1 Tracheal ciliated cells respond to glucose and denatonium 33

3.1.2 Tracheal ciliated cells respond to artificial sweeteners 35

3.2 IMMUNOHISTOCHEMISTRY EXPRESSION OF TASTE TRANSDUCTION COMPONENTS IN MICE TRACHEAL CILIATED CELLS 39

4. DISCUSSION 41

ACKNOWLEDGEMENTS 47

REFERENCES 48

1. INTRODUCTION

Sweet and bitter are two basic taste sensations perceivable thanks to taste receptor cells (TRCs) in the mouth. Sweet is a taste related with the recognition of high-caloric food for energy intake as well as for the hedonic reward system, whereas, bitter alerts the organism of potentially harmful substances which have to be refused and expelled (Yarmolinsky et al., 2009). Thus, sweet and bitter are not just simple sensations; they are triggers of innate behaviors resulting in the acceptance or in the rejection of substances. TRCs are capable of detecting sweet and bitter compounds because of the expression of sweet and bitter receptors respectively. These taste ceptors have also been found outside the oral cavity (Sbarbati et al., 2010; Finger & Kinnamon, 2011), arousing curiosity about their role and corroborating the idea that sweet and bitter are involved in other systems beside the gustatory one.

1.1 "CANONICAL" GUSTATORY TASTE RECEPTORS

TRCs are found in the dorsal surface of the tongue, soft palate, pharynx, and the upper part of the esophagus arranged in taste buds (Purves et al., 2001). Taste buds are onion-shaped structures specialized end organ of gustation; they are composed of 50-100 cells embedded within the stratified lingual epithelium; they have an apical pore (taste pore) which allows the contact with the tastants, and at the base, they form chemical synapses with gustatory afferent nerves (Kinnamon & Cummings, 1992; Lindemann, 2001). In humans, there are around 5,000 taste buds with similar functions (Chaudhari & Roper, 2010). Most of the taste buds are housed within the papillae: the fungiform papillae on the anterior two thirds of the tongue, the circumvallate papillae in an arc of approximately nine papillae on the posterior tongue just anterior to the lingual tonsil and the foliate papillae on the posterior lateral edges of the tongue. In mouse and rat there is only one circumvallate papilla on the posterior tongue. Other taste buds are isolated and dispersed on the soft palate (Yarmolinsky et al., 2009). The receptor taste cells are not neural cells. They are specialized epithelial cells with some neural properties but they lack an axon (Gilbertson & Kinnamon, 1996; Smith & Margolskee, 2001). Taste cells have a short

life span (around 10 days) and they are replaced continuously throughout the bud life. Inside a taste bud, there are elongate epithelial cells and few basal cells. The basal cells are small and round proliferative cells located at the base of the bud, while the elongate epithelial cells (taste cells) are bipolar cells extending from the taste pore to the base of the bud (Delay et al., 1986; Kinnamon & Cummings, 1992). Taste cells can be classified in three different types (Figure 1.1): type I cells have a putative glial function; type II express all the elements of the taste transduction cascade for sweet, bitter and umami (the “amino acids taste”) and type III are characterized by morphologically identifiable synaptic contacts with the gustatory nerve fibers, so these cells are thought to be involved in transmission of information to the nervous system (Finger, 2005). Type II are named the taste receptor cells (TRCs) (DeFazio et al., 2006) because each of them expresses taste receptors specific for a taste quality only (Nelson et al., 2001). Therefore, a given taste receptor cell is “tuned” to sweet, bitter, or umami.

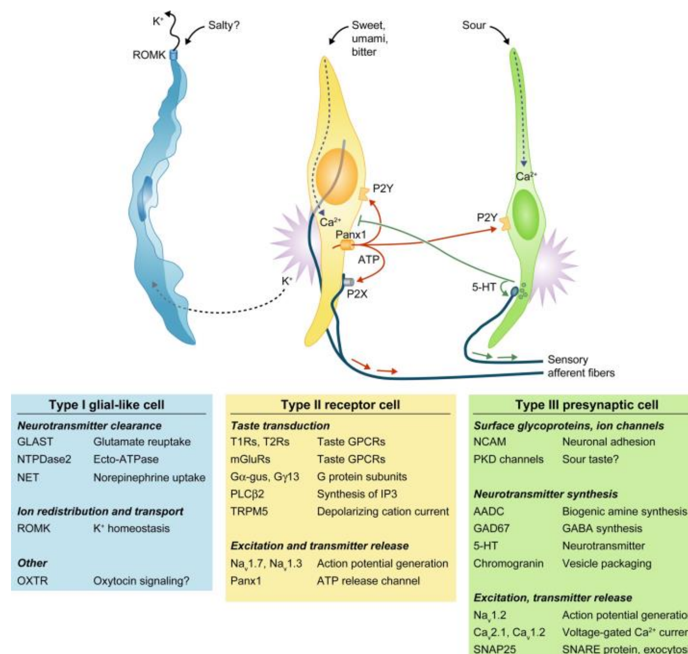


Figure 1.1. The three major classes of taste cells. This classification incorporates ultrastructural features, patterns of gene expression, and the functions of each of Types I, II (receptor), and III (presynaptic) taste cells. Type I cells (blue) degrade or absorb neurotransmitters. They also may clear extracellular K^+ that accumulates after action

potentials (shown as bursts) in receptor (yellow) and presynaptic (green) cells. K^+ may be extruded through an apical K channel such as ROMK. Salty taste may be transduced by some Type I cells, but this remains uncertain. Sweet, bitter, and umami taste compounds activate receptor cells, inducing them to release ATP through pannexin1 (Panx1) hemichannels. The extracellular ATP excites ATP receptors (P2X, P2Y) on sensory nerve fibers and on taste cells. Presynaptic cells, in turn, release serotonin (5-HT), which inhibits receptor cells. Sour stimuli (and carbonation, not depicted) directly activate presynaptic cells. Only presynaptic cells form ultrastructurally identifiable synapses with nerves. Tables below the cells list some of the proteins that are expressed in a cell type–selective manner. (from Chaudhari & Roper, 2010)

1.2 BITTER AND SWEET TASTE TRANSDUCTION

Taste transduction begins when chemical stimuli reach the taste bud pores and interact with TRCs apical membranes; here reside sweet and bitter taste receptors. These interactions lead to a membrane conductance change, an increase in cytoplasmic calcium and a strong depolarization with possible action potential initiation. The outcome is the release of ATP, and possibly other molecules, into the extracellular space of the activated TRCs (Kinnamon & Cummings, 1992; Chaudhari & Roper, 2010).

1.2.1 Sweet Receptors

The sweet taste receptors belong to a family of three Class C G protein-coupled receptors (C-GPCRs): the type1, member1 (T1R1), type 1, member 2 (T1R2) and type 1, member 3 (T1R3) receptors. They are characterized by a large N-terminal extracellular domain and multiple ligand-binding sites (Max et al., 2001). They can combine forming the umami taste receptor (T1R1-T1R3), the heterodimeric sweet taste receptors (T1R2-T1R3) (Nelson et al., 2001; Li et al., 2002; Zhang et al., 2003; Zhao et al., 2003; Chaudhari & Roper, 2010; Laffitte et al., 2014) and the homodimeric low-affinity sweet taste receptor (T1R3-T1R3) (Nelson et al., 2001; Zhao et al., 2003; Laffitte et al., 2014). T1R3 is a major player in sweet sensation. Its gene, Tas1r3, was identified within the coding sequence of the saccharin preference (Sac) locus; a single chromosomal region, in mice, which largely determines the preference and

the intake of sugars. Tas1r3 polymorphisms determine sweetener preference phenotypes in mice (Bachmanov et al., 2001a). T1R3 knockout mice have a dramatic reduction in multiple sweet preference and intake (Damak et al., 2003; Zhao et al., 2003; Lemon & Margolskee, 2009). Sac non-taster mice that showed weak preference for sugars, fully recovered their taste deficit when transfected with T1R3 gene sequence of Sac taster mice (Nelson et al., 2001). Furthermore T1R3 is expressed in around 30% of the taste buds and every T1R2-positive TRC, co-expresses T1R3 (Nelson et al., 2001). The heterodimeric T1R2-T1R3 receptor (Figure 1.2) is the primary sweet taste receptor. Both T1R2 and T1R3 are necessary for sweetness detection (Nie et al., 2005), although T1R3-T1R3 receptors can respond to high concentrations of sugars and to artificial sweeteners (Nelson et al., 2001; Zhao et al., 2003; Laffitte et al., 2014).

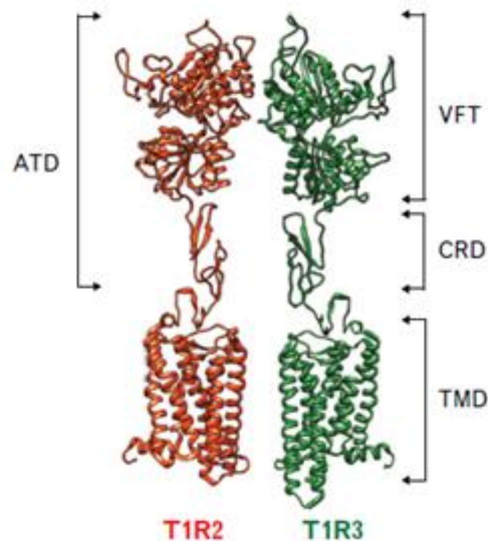


Figure 1.2. Schematic model of the sweet taste receptor. The sweet taste receptor is composed of two subunits, T1R2 and T1R3. The two subunits belong to the class C GPCRs. T1R2 and T1R3 possess a large aminoterminal domain (ATD) that includes a Venus flytrap domain (VFT) connected to a helical transmembrane domain (TMD) (characteristic of GPCRs) by a short cysteine-rich domain (CRD). The VFT is composed of two lobes separated by a large cleft, in which most sweeteners bind. (from Laffitte et al., 2014).

T1R2-T1R3 recognizes a wide variety of chemically distinct sweet tasting molecules: simple sugars (e.g. glucose, sucrose, lactose, fructose), artificial sweeteners (e.g. acesulfame-K, sucralose, dulcin, saccharin), D-amino acids (e.g. alanine, threonine, D-tryptophan, D-histidine), intensely sweet peptides or proteins (e.g. monellin, thaumatin) and even some alcohols (e.g. glycerol, sorbitol, and xylitol) (Li et al., 2002; Yarmolinsky et al., 2009; Laffitte et al., 2014). Although the sugars are chemically similar, it is challenging to think about any common feature within the broad range of sweet taste receptor ligands. The most quoted hypothesis is that all sweet substances have a glucophore consisting in two electronegative groups, an acid (AH) and a base (B) respectively. This glucophore forms a double hydrogen-bound with a similar AH-B structure in the taste receptor binding site (Shallenberger & Acree, 1967; Birch, 1987; Atta-ur-Rahman, 1995; Roper, 2007). Another possible explanation to the vast responsiveness capability of the T1R2-T1R3 sweet taste receptor is that it has multiple binding pockets with different selectivity, located in different regions (Figure 1.3). In the extracellular domain there is a Venus flytrap (VFT) motif, which might bind sugars, sweet amino acids, and other sweet compounds, inducing conformational change in the "trap" (Morini et al., 2005). Key binding sites for sugars and sweet-tasting proteins are located in the N-termini of both T1R2 and T1R3 (Nie et al., 2006). The cyclamate-binding pocket, as well as the antagonists binding site, are burrowed into the membrane (Jiang et al., 2005). T1R2-T1R3 and T1R3-T1R3 receptors are low-affinity receptors; they are activated by sweet substances at millimolar concentrations. In this way, during food intake, they can detect relevant concentrations of nutritive substances without reaching saturation. (Nelson et al., 2001; Damak et al., 2003; Zhao et al., 2003).

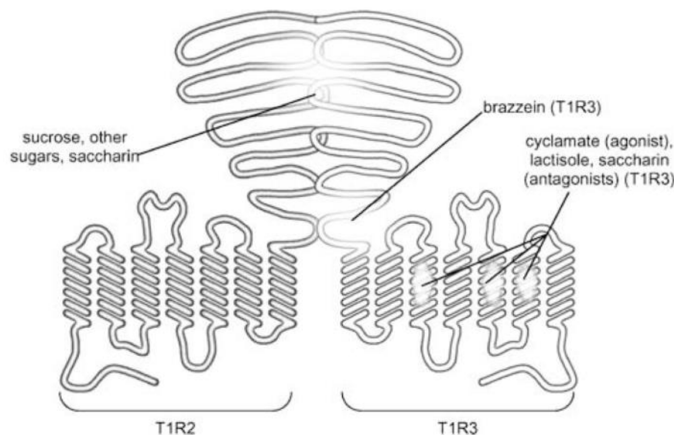


Figure 1.3 Schematic drawing of the sweet GPCR dimer T1R2+T1R3, showing the multiple ligand binding sites. T1R2 is shown on the left, T1R3 is on the right. Details of the interactions, if any, between the extensive N termini of T1R2 and T1R3 are not known. The N termini possess one or more binding pocket(s) for saccharin, sucrose, and other sugars. Another ligand-binding pocket, located in T1R3 near the first transmembrane region, exists for certain sweet-tasting proteins such as brazzein. A third ligand pocket, embedded in the transmembrane (TM) regions of T1R3, is comprised of portions of TM3, 5, and 6. This site binds the artificial sweetener, cyclamate, but also is the binding site for antagonists such as saccharin (which at high concentrations is a sweet receptor antagonist) and lactisole (from Roper, 2007)

1.2.2. Bitter Receptors

Bitter taste is innate and triggers stereotypical behaviors of rejection. This taste quality likely evolved as a warning alarm to avoid food intoxication, although there is not a clear correlation between bitterness and toxicity (Glendinning, 1994). The diversity of bitter compounds is astounding; a complete inventory does not exist, but they are estimated to be tens of thousands. Many bitter substances are natural; they derive from plants, animals or bacteria products, others are chemically synthetized and others are generated during food processing and aging (e.g. fermentation) (Meyerhof et al., 2010). Not only their origins, but also their structures are diverse: amides, alkaloids, amino acids, urea, fatty acids, phenols, amines, esters and many others (Roper, 2007; Meyerhof et al., 2010). This huge variability of ligands

might be one evolutional reason behind the approximately 30 different genes encoding for bitter taste receptors in human and 40 in mice. They encode for GPCRs of the T2Rs family (Figure 1.4) (Adler et al., 2000; Bufe et al., 2002; Bachmanov et al., 2014). T2Rs share 30%-70% of amino acid sequence identity, with the short extracellular N-domain showing the highest divergence, probably to be able to recognize such many structurally different ligands (Adler et al., 2000). The diversity, the genetics, the expression profile and functional validations support the role of T2Rs as bitter taste receptors. T2Rs genes cluster in a few chromosomal locations implicated in bitter sensation; chromosomes 5, 7, and 12 in human and chromosomes 2, 6, and 15 in mice (Bachmanov et al., 2014). All the members of the T2R family are found within the taste buds, with different patterns and levels of expression. On average each TRC displays an overlapping subset of 4-11 T2Rs (Behrens et al., 2007) and can functionally discriminate among bitter compounds (Caicedo & Roper, 2001). Several T2Rs are broadly tuned to detect stimuli of different chemical classes, whereas others appear to be more specific, activated by one or a few ligands (Meyerhof et al., 2010). The T2Rs have an high affinity for their ligands; their detection threshold is in the range of µM (Chandrashekar et al., 2000; Roper, 2007; Meyerhof et al., 2010). Intriguingly, some T2Rs show plasticity in their ligands sensitivity. The hT2R46, for example, is the bitter receptor for strychnine and brucine, two structurally very similar bitter compounds with different toxicity level; the first one is highly toxic (LD= 5-10 mg) the latter one is nontoxic (LD=100 mg). Remarkably, the hT2R46 recognizes strychnine with a sensitivity about 100 times greater (threshold = 0.1 µM) than brucine (threshold= 10 µM), matching almost perfectly the difference in their toxicity (Meyerhof et al., 2010). Thus, T2Rs are responsible for bitterness; they can detect very small amounts of possible harmful substances and alert the organism, discriminating between bitter compounds with just unpleasant taste and bitter toxic compounds.

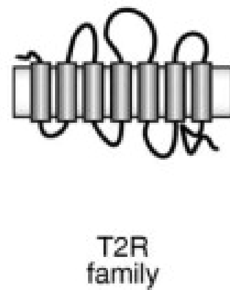


Figure 1.4. Diagrammatic representation of bitter-taste GPCRs T2Rs. T2Rs are integral membrane proteins that contain seven trans-membrane domains. They consist of around 300–330 amino acids and have a short extracellular N-terminus. T2Rs are broadly tuned to detect stimuli of different chemical classes, or more specifically activated by few agonists (from Boughter Jr. & Munger, 2004)

1.2.3. Calcium-mediated mechanism of taste transduction

Bitter (T2Rs) and sweet receptors (T1R2-T1R3) use at least one common signaling pathway to transduce tastes (Zhang et al., 2003). When they bind their ligands, taste GPCRs activate heterotrimeric guanine nucleotide-binding regulatory proteins (G-proteins). The G-proteins are composed of a GTP-binding α-subunit and a βγ-subunit (Hepler & Gilman, 1992). In most of the taste cells receptors the G protein involved is α-Gustducin (McLaughlin & McKinnon, 1992). Upon activation Gβγ subunits interacts with a phospholipase, phospholipase-C-beta-2 (PLCβ2). In turn, PLCβ2 promotes the synthesis of inositol-3-phosphate (IP$_3$), which opens IP3R3 ion channels in the endoplasmic reticulum, inducing calcium release in the cytosol (Chaudhari & Roper, 2010). The increased intracellular calcium activates a taste selective cation channel transient-receptor-potential-cation-channel-subfamily-M-member 5, (TRPM5), causing membrane depolarization, action potential generation and release of ATP through CALHM1 channel (Figure 1.5 and 1.6) (Romanov et al., 2007; Huang & Roper, 2010a; Kinnamon, 2012; Taruno et al., 2013). α-Gustducin, PLCβ2 and TRPM5 are expressed in TRCs, together with T2Rs or T1Rs. Knockout of α-Gustducin or PLCβ2 or TRPM5 strongly impairs responses

to most bitter and sweet taste stimuli (Wong et al., 1996; Lindemann, 2001; Zhang et al., 2003; Caicedo et al., 2003; Huang & Roper, 2010b; Kinnamon, 2012), indicating that the calcium-mediated mechanism of transduction is the main, though not the only, pathway used by TRCs to decode bitter and sweet taste sensations.

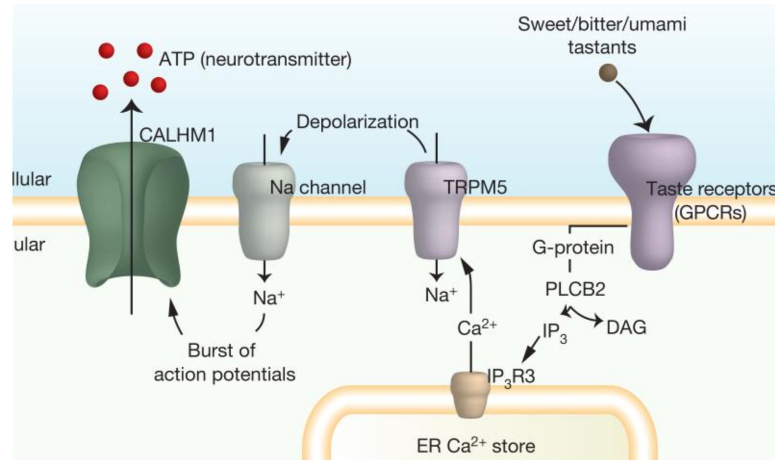


Figure 1.5. Calcium-mediated sweet and bitter taste transduction mechanisms (from Taruno et al., 2013)

1.3 EXTRA ORAL TASTE TRANSDUCTION

Mechanisms underlying bitter and sweet transduction in the oral cavity are known and well described. Interestingly, T1Rs, T2Rs and elements of the taste transduction-signaling pathway are also reported in strategic points of extra-oral tissues, expanding the canonical idea of taste receptors as mere taste transducers. Sweet/bitter taste transduction elements were found in different tissues e.g. upper airways, gastrointestinal tract, testis and some brain areas (Figure 1.6) (Herrera Moro Chao et al., 2016)

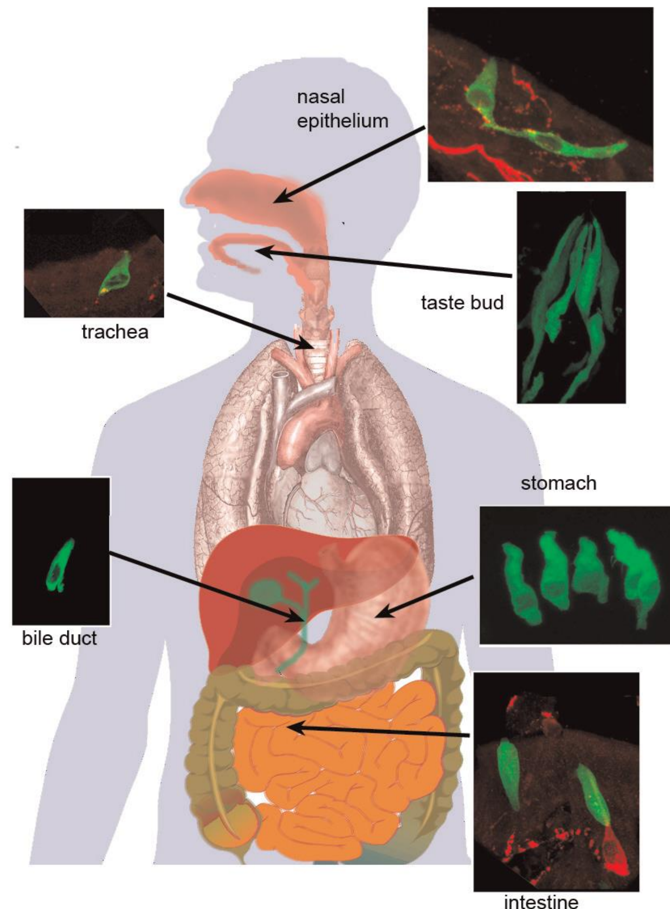


Figure 1.6. TRCs in taste buds and examples of chemosensory cells in non-taste tissues (Finger & Kinnamon, 2011).

These extra-oral chemosensory cells, not aggregated in buds, are of different types: some of them are innervated, others are endocrine cells and the morphology is not the same. Despite these differences, these chemosensory cells are generally found within the epithelia of endodermic organs to have access to the luminal content of each organ. Since many years, it was assumed that these chemosensory cells are part of a diffuse chemosensory system (DCS) distributed in different areas of the body (Sbarbati, Osculati, 2005; Sbarbati et al., 2010).

1.4 THE AIRWAYS EPITHELIUM

The airways can be divided into two different structural and functional parts: the conducting airways and the respiratory airways. While the latter ones are

responsible for gas exchange and are located within the lungs (respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli), the conducting airways clean, warm, moisten and conduct the air. They are composed by nose, pharynx, larynx, trachea, bronchi, and bronchioles. Luminal surfaces of the airways are covered by a continuous epithelium, which vary in cells types composition and distribution, depending on the function of the airway region. Particularly, the conducting airways are lined by a pseudostratified columnar ciliated epithelium that gradually turns first into a simple columnar then into a cuboidal epithelium (Ganesan et al., 2013). The pseudostratified epithelium, typical of the trachea and the majority of the upper respiratory tract, is mainly composed by three cell types: ciliated cells, non-ciliated mucus secretory cells (goblet cells), and basal cells (Figure 1.7).

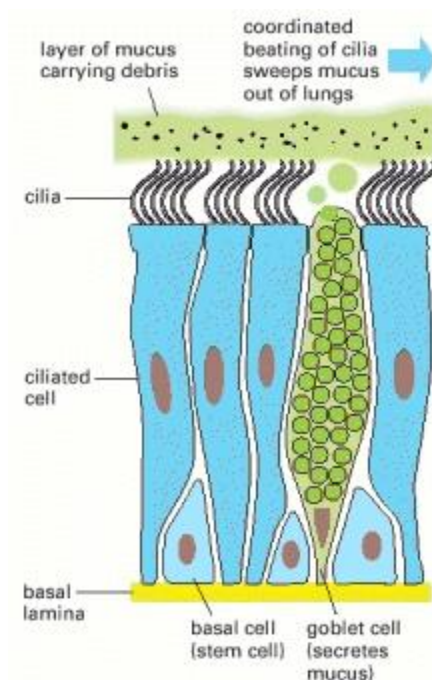


Figure 1.7. Respiratory epithelium (Alberts et al., 2002)

Ciliated cells are the 50%–80% of epithelial cells lining the airways; therefore, they are the predominant cell type within the airways. They have microtubule based cell organelles, protruding from apical cell surface to the lumen. Cilia membranes are contiguous with cell membranes and express receptors and ion channel proteins to regulate motility, in response to mechanical or chemical stimuli. The ciliated cells

beat in an ATP-dependent coordinated metachronous way moving mucus from the lower airways to the upper airways (Yaghi & Dolovich, 2016).

Goblet cells are identified by their apical secretory granules. They take part in airway first-line defense by secreting high molecular weight mucus glycoproteins (mucins). These viscous mucins entrap external and possible harmful irritants, particles and micro-organisms, favoring their transport and expulsion. To overcome chronic insult, the goblet cells increase in number with a resulting higher amount of secreted mucins (Rogers, 1994).

Basal cells are thought to be the stem cells of the airway conductive epithelium. They are firmly connected with the basement membrane, anchoring ciliated and goblet cells to the basal lamina. They regulate inflammatory response (Knight & Holgate, 2003).

The airways epithelium has been described as an inert barrier between the external environment and the inner tissues. However, it is now generally accepted that this epithelium is not just a protective covering. (Knight & Holgate, 2003). The canonical function of the airways conductive epithelium is to ensure the air passage to and from the alveoli (Crystal et al., 2008). Since the inhaled air is dirty, full of debris and microorganism, airways epithelium has evolved innate defense mechanisms to prevent them to reach the lungs. The first line defense mechanism is the mucociliary escalator: a coordinated work between the cilia and the goblet cells. The goblet cells secrete different type of mucins, which can entrap virtually all particles, while the cilia beat fast and unidirectionally from the distal airways to the proximal airways, transporting the mucus and all the entrapped debris till the larynx (Knowles & Boucher, 2002). Here the mucus is either swallow or expelled through caught. The efficient clearance of the airways depends on the efficacy of ciliary beat as well as on the hydration of mucus; an hydrated mucus is less viscous and facilitate the cilia movement.

The airways epithelium not only remove potentially harmful and pathogenic particles, it also takes part in the luminal glucose homeostasis reducing the risk of infections. In normal conditions the glucose concentration in the mucus layer of the

airways is up to 12 times lower than the circulating levels (Kalsi et al., 2008, 2009; Gill et al., 2016). Such low levels of glucose help preventing infections; indeed patients with high glucose levels have more probability to be colonized or infected by pathogenic drugs resistant bacteria (Philips et al., 2005). Glucose concentration depend on trans-epithelial movement of glucose into the lumen and on removal of glucose from the lumen (Figure 1.8) (Baker, 2011). Movement of glucose from the blood and interstitial fluid into the airway lumen occurs via passive paracellular pathways. This process could be affected by the permeability of epithelial tight junctions and by the concentration gradient (Garnett et al., 2012a). On the other side, facilitative glucose transporters(GLUTs) mediate glucose removal from the lumen (Kalsi et al., 2008; Pezzulo et al., 2011). These transporters, namely GLUT 1, 2 and GLUT10, are expressed in the apical and the basolateral membrane of airways epithelial cells (Kalsi et al., 2008; Pezzulo et al., 2011; Garnett et al., 2012c; Merigo et al., 2012; Garnett et al., 2012a). Experiments conducted on airways epithelial monolayer culture showed that the application of phloretin, a GLUT inhibitor, leads to an increased glucose concentration in the apical compartment (Kalsi et al., 2008). Once the glucose is taken from the lumen into the cells, it is rapidly metabolized. In this way glucose cannot accumulate within the cells and the concentration gradient is preserved (Garnett et al., 2012a).

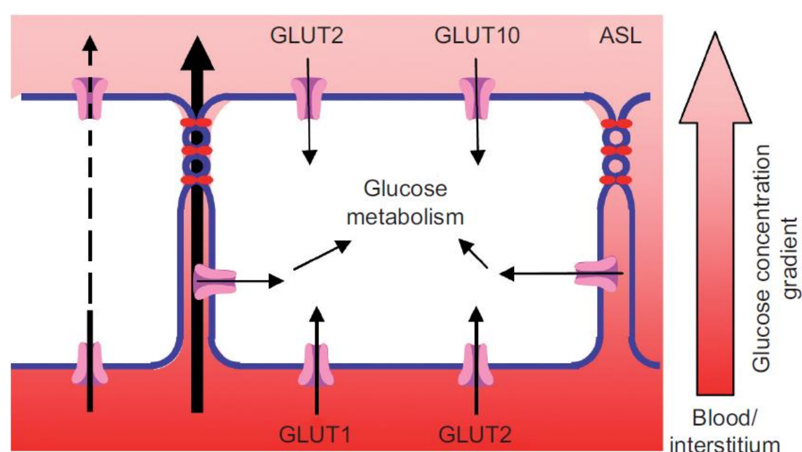


Figure 1.8. Current model of the mechanisms controlling glucose concentrations in the airways (Garnett et al., 2012a)

1.5 CILIATED CELLS AND THEIR ROLE IN THE CONDUCTING AIRWAYS

Traditionally, cilia are broadly divided in immotile sensory cilia and motile cilia. Immotile cilia are generally short and are specialized to sense fluid flow, light, odorants or signaling molecules. This class of cilia includes the primary cilium, expressed in almost all non-cycling mammalian cells and required for signaling transduction during morphological development, as well as specialized sensory cilia involved in vision (retinal photoreceptor connecting cilium), audition (ear kinocilia) and olfaction (olfactory neuron multicilia) (Choksi et al., 2014). In contrast with immotile sensory cilia, the motile cilia are long and possess dynein arms, which move in an ATP-dependent manner. Motile cilia serve principally a mechanical function: the flagellum, a single motile mono-cilium, beats in a wavelike fashion allowing sperm cells to swim; the nodal monocilia rotates, generating an asymmetric leftward-directed fluid flow necessary for the correct positioning of asymmetric inner organs during embryonic development. Airway epithelial cells as well as ependymal cells of the central nervous system, own between two and hundreds of motile cilia. These ciliated cells beat in a coordinated fashion to clear the airways or to circulate cerebrospinal fluid (Choksi et al., 2014; Walentek & Quigley, 2017).

In addition to their motile function, it is known that ciliated cells can exhibit an array of sensory functions. Motile cilia of the mammalian oviduct and of the airway epithelium express TPRV4, a member of transient receptor potential cation channels family, sensitive to osmotic force, mechanical stimuli and temperature. This receptor allows cilia to sense mucus viscosity so that they can increase their beating rate if the mucus became more viscous (Bloodgood, 2010; Jain et al., 2012). Moreover, the ciliated cells of the mammalian oviduct express both progesterone and estrogen receptors. The cilia respond to the sex hormones regulating their rate of beating, in order to ensure that the oocyte will move through the Fallopian tubes and reach the uterus only when the uterine mucosa is ready for implantation (Jain et

al., 2012). Finally, motile cilia express Vangl2, a planar cell polarity (PCP) protein necessary to orient and adjust the direction of cilia movements (Jain et al., 2012). Motile cilia of the airways epithelium are an example of cilia that, beyond their mechanic role in the airways clearance, exhibit also sensory functions. In particular, it was showed that these cells express, within the cilia, several members of the bitter taste receptors family, T2Rs and can sense potentially noxious agents (Shah et al., 2009). Bitter agonists stimulate calcium-mediated responses and lead to an increase in ciliary beating rate, so motile cilia can also be considered as "chemosensory organelles" (Shah et al., 2009). In addition, even sinonasal ciliated cells express T2Rs and respond to bacterial compounds increasing beat frequency and releasing nitric oxide (NO) (Lee & Cohen, 2014; Lee et al., 2014). Furthermore, Merigo and collaborators showed that the ciliated cells of rat trachea express the sweet receptor T1R3 (Merigo et al., 2012). In particular, the T1R3 was found in the apical surface beneath the cilia, in some spots along the cilia, and on the basolateral membrane (Figure 1.09). The expression of the T1R3 on the cilia is strikingly; it is a strategic position to easily access the luminal content and it is the same pattern observed in human T2Rs-ciliated cells (Shah et al., 2009). T1R3 is also co-expressed with GLUT2 in the apical membrane of some ciliated cells, giving insights about a role of T1R3 in glucose regulation. (Figure 1.10) (Merigo et al., 2012).

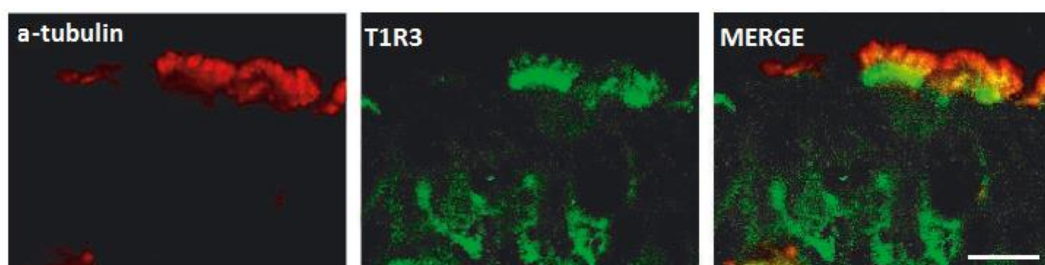


Figure 1.09. Double-immunofluorescent confocal microscopy showing expression of a-tubulin (red) with T1R3 (green) in tracheal cells. Scale bar: 10 μm (Merigo et al., 2012)

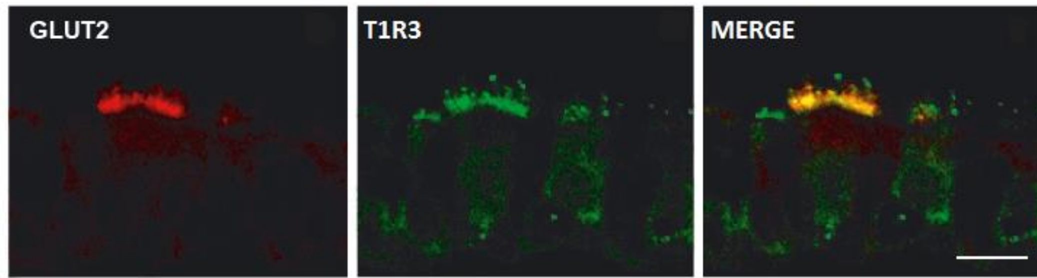


Figure 1.10. Double-immunofluorescent confocal microscopy showing expression of GLUT2 (red) with T1R3 (green) in tracheal cells. Scale bar: 20 μm. (Merigo et al., 2012)

.

To our knowledge, no physiological data are reported to date in the literature regarding ciliated cells sweet response in the airways. Therefore, on the basis of the previous findings described, we chose to investigate the physiological behavior of the airways ciliated cells stimulated with sweet compounds using calcium-imaging technique. This method allows us to follow the changes in intracellular calcium concentration; thus, it enables the recognition of possible sweets calcium-mediated responses. We decided to work with acute slices of mice tracheas, instead of using cells cultures, to better preserve the native conditions, avoiding excessive cells handling and artefacts introduction.

2. MATERIALS AND METHODS

2.1 DISSECTION

Experiments were performed on 1- to 2-month-old C57BL/6 mice. Animals were handled in accordance with the guidelines of the Italian Animal Welfare Act and European Union guidelines on animal research. Mice were sacrificed by exposure to carbon dioxide followed by cervical dislocation. The trachea was removed and put in cold Ringer solution containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4. All tissues attached outside the trachea were removed under a stereoscope (Olympus SZ30), and the tracheal lumen was flushed with mammalian Ringer solution to clear any remaining mucus and blood.

2.2 CALCIUM IMAGING

2.2.1 Preparation of acute slices of mouse trachea

The calcium imaging technique (Bootman et al., 2013) was used to monitor cytosolic calcium in tracheal cells using an acute slice preparation.

The trachea was cut transversally, then the proximal portion was embedded in 2% low melting point agarose prepared in Ringer solution. Tracheal slices of 100–200 μm were cut with a vibratome (Vibratome 1000 Plus Sectioning System) and kept in cold Ringer solution until use (Figure 2.1). The murine tracheal slice preparation maintained the cross-sectional structure and individual cells could be distinguished by their morphology (see Figures 3.1).

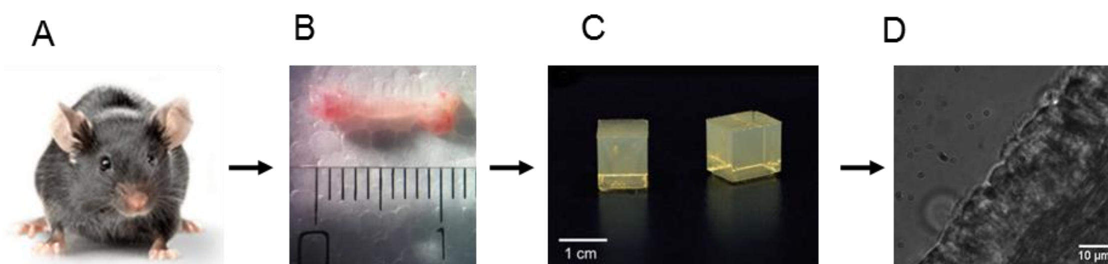


Figure 2.1. Schematic representation of murine tracheal slices preparation for calcium imaging acquisitions. Trachea was dissected (A-B), included in 2% agarose in Ringer solution (C, modified from Shimazaki et al., 2006), and cut with a vibratome to obtain 100-150 µm thick slices (D).

Fluo-4 acetoxymethyl ester (AM) (Thermo Fisher Scientific) was prepared as 1 mM stock solution in DMSO and added to fresh Ringer solution to a final concentration of 20 µM (2% DMSO). To help the dye uptake, Pluronic F-127 (Molecular Probes) was added to a final concentration of 0.02%; 100 µM bromsulphthalein (BSP; Sigma), an inhibitor that prevents extrusion of the indicator by organic anion transporters, was included to aid retention of Fluo-4 by the cells (Delmotte & Sanderson, 2006). The slices were incubated in dye solution for 90 minutes at room temperature, then washed and kept in fresh Ringer solution until use (Figure 2.2).

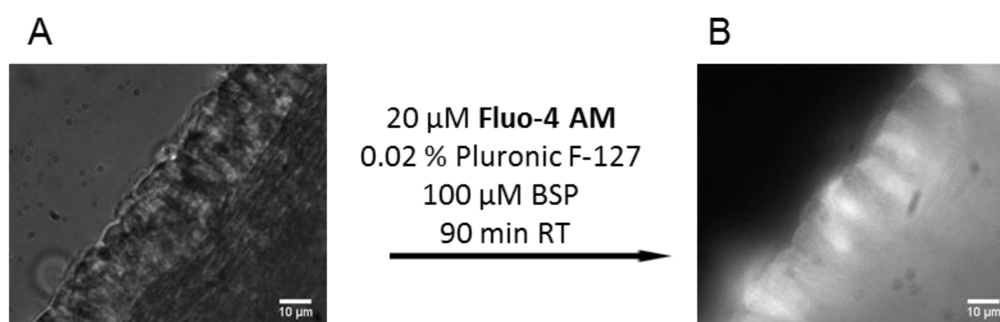


Figure 2.2. Loading tracheal cells with the calcium indicator Fluo-4. (A) Several cells could be clearly distinguished by their morphology in a tracheal acute slice. The slice was incubated in the indicated solution to load the calcium indicator Fluo-4. (B) A fluorescent image of the same slice loaded with Fluo-4.

2.2.2 Fluorescence imaging and data analysis

The non-ratiometric green-fluorescent calcium indicator Fluo-4 was used to measure changes in intracellular calcium concentrations. Inside the cells, the membrane permeant Fluo-4 AM is cleaved by cytosolic esterases, and the fluorescent indicator Fluo-4 is entrapped inside the cells, where it binds free intracellular calcium ions. Ca^{2+} binding leads to an increase in Fluo-4 fluorescence intensity, which can be measured using appropriate wavelength settings (Figure 2.3 A-B).

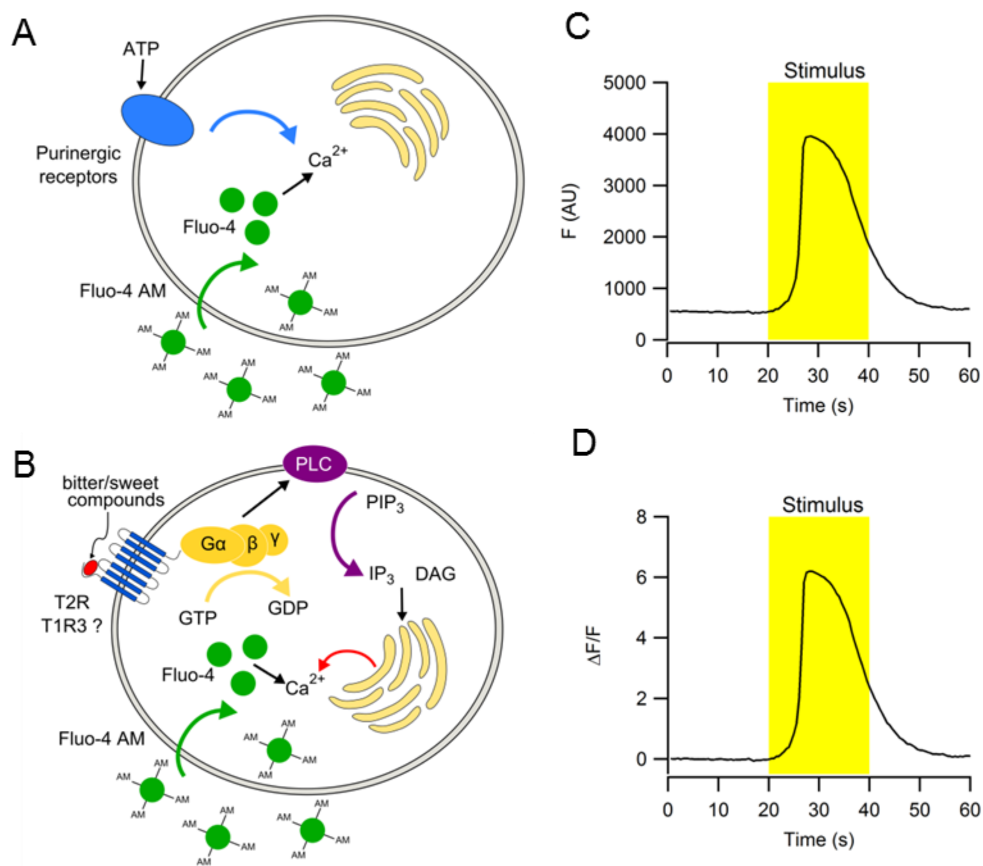


Figure 2.3. Schematic representation of Fluo-4AM mechanism of action.

(A-B) Fluo-4 AM can cross the cell membrane and enter the cytosol. In the cytosol, esterases remove the AM ester group allowing Fluo-4 to bind to intracellular free calcium. Calcium binding results in an increase in emitted fluorescence intensity. In (A), intracellular calcium increase is caused by ATP activation of purinergic receptors. In (B), calcium increase is due to activation of G-protein coupled receptors. (C) Representative

time course of the measurement of fluorescence intensity in arbitrary units (AU), and (D) normalized change of fluorescence (ΔF/F) upon cell stimulation.

Tracheal slices loaded with Fluo-4 were mounted in a home-made perfusion chamber equipped with a perfusion system (see 2.2.3). Imaging experiments were performed using an upright fluorescence microscope (Olympus, BX51WI) with a 60X water immersion objective (NA 0.9). A mercury lamp and an appropriate fluorescence filter cube (Olympus, UMNB2), with excitation filter BP 470-490, dichroic mirror DM 500 and emission filter LP 500 were used. The emitted fluorescence (> 500 nm) was detected with a CCD camera (SensiCam 12 bit; PCO AG) controlled by software (PCO AG, Figure 2.4). Images were acquired at 1.6 - 5 Hz and analyzed with ImageJ and IgorPro software.

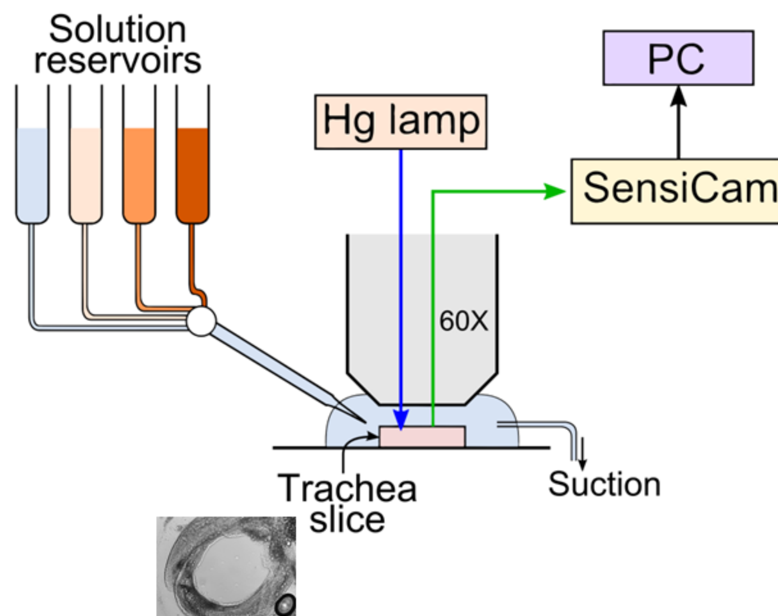


Figure 2.4. Schematic representation of the experimental set-up for calcium imaging recordings. A tracheal slice is mounted in a perfusion chamber on the stage of a fluorescence microscope. The slice is continuously perfused by a multichannel perfusion system switching from a solution to another without stopping the flow. A mercury lamp and appropriate filter are used for fluorescence excitation of Fluo-4 with wavelengths between 470 and 490 nm. The emitted fluorescence signals (> 500 nm) are collected by a CCD camera.

To evaluate changes in fluorescence in individual ciliated cells, a region of interest was drawn around the cell. The basal fluorescence level (F) was calculated as the mean fluorescence intensity in the 10 seconds previous to the stimulation (Figure 2.3 C-D). Changes in fluorescence are reported as relative changes in fluorescence intensity normalized to baseline fluorescence according to the formula $\Delta F/F = [F(t)-F]/F$, where F(t) is the fluorescence at time t and F is the mean basal fluorescence level (Figure 2.3 C-D). A stimulus was considered to elicit a response only if the change in $\Delta F/F$ after the stimulation overcame a threshold defined as the mean basal fluorescence plus 3 times the value of standard deviation (threshold=F+3*SD). To check the viability of the cells at the end of the experiment, a solution containing 30 µM ATP was used to elicit a cytosolic increase of calcium. Only cells that responded to ATP were included in the analysis.

2.2.3 Stimuli and perfusion system

Tracheal slice were continuously perfused with mammalian Ringer solution. A gravity-driven multichannel perfusion system (AutoMate Scientific) was used to deliver the test solutions directly at the apical surface of ciliated cells through a tip with 360 µm internal diameter. This system allows switching from a solution to another in about 300 ms, without interrupting the solutions flow. The following stimuli compounds were added to the Ringer solution at the indicated concentrations: 10 mM glucose, 10 mM acesulfame K, 10 mM sucralose, 5 mM denatonium benzoate, or 30 µM ATP. Stock solutions of acesulfame K (100 µM), sucralose (100 mM), denatonium (100 mM) and ATP (300 µM) were prepared in Ringer solution and stored at -20°C, then diluted in Ringer solution just before the experiments. Glucose was freshly dissolved in Ringer solution. Chemicals, unless otherwise stated, were purchased from Sigma.

3.2 IMMUNOHISTOCHEMISTRY

Tracheas were immersed in a fixative solution (4% paraformaldehyde, PFA) for 2 hours at 4°C. Then, the organs were rinsed with PBS and put in 30% sucrose solution overnight at 4°C for cryoprotection. The following day, organs were rinsed with PBS and frozen in Optimal Cutting Temperature compound (OCT) at -80°C. Organs were cut into 10-18 μm thick sections with a cryostat, collected on microscope slides and kept at -80°C for further use.
Sections were rehydrated with PBS, incubated for 15 minutes with 1% SDS in PBS for antigen retrieval, then incubated in blocking solution 2% BSA-TPBS (PBS + 0.02 % Tween-20) for 1 hour. After rinsing with PBS, sections were incubated overnight at 4°C with the following primary antibodies: α-Gustducin 1:200 (SantaCruz, sc-395), PLCβ2 1:200 (SantaCruz, sc-398996), and acetylated-tubulin (Sigma, T7451). Sections were then rinsed in PBS and incubated for 1 hour at room temperature with the following fluorescent labeled-secondary antibodies: FITC goat-anti-mouse; FITC chicken-anti-rabbit; TRITC chicken-anti-rabbit, or TRITC donkey-anti-goat. All the secondary antibodies were used at 1:500 and were purchased from Thermo Fisher. The sections were rinsed in T-PBS and incubated for 30 minutes at room temperature with DAPI (1:50) to stain the nuclei. Finally the sections were washed and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Images were visualized by Nikon Eclipse Ti confocal microscope, acquired using NIS Elements software at 1024x1024 pixels resolution and analyzed with ImageJ software.
Chemicals, unless otherwise stated, were purchased from Sigma.

2.3.1 Immunohistochemistry on tracheal slices after calcium imaging experiments

As described in the previous section, the calcium imaging technique was performed on acute slices from the trachea to evaluate the functional activity of ciliated cells. Immunostaining with acetylated-tubulin was performed on some slices after calcium imaging acquisition. Slices were washed in PBS, fixed for 20 minutes with

4% PFA and treated with 1% SDS for 15 minutes. Slices were then incubated with primary and secondary antibodies following the same protocol used for the immunofluorescence assays on cryosections, as described previously .

3. RESULTS

3.1 CALCIUM IMAGING EXPERIMENTS FROM ACUTE MICE TRACHEAL SLICES

The expression of several components of the taste transduction cascade in airway ciliated cells of the trachea leaded to investigate if bitter and sweet compounds were able to induce a physiological response in these cells. Therefore, we decided to perform Ca^{2+}-imaging assay on acute slices of 100-200 µm thickness of murine trachea. To test if the histological organization of the tissue was preserved after the cutting procedure and the Ca^{2+}-Imaging recordings, we performed immunohistochemical analysis using acetylated-tubulin (Ac-Tubulin) to stain the cilia. The pattern of expression and the morphology of the ciliated cells were not affected by the procedures; indeed many intact cilia still protrude from apical membranes toward the lumen (Figure 3.1).

Tracheal slices were loaded with the Ca^{2+} indicator dye Fluo-4 AM (20 µM for 90 min at room temperature), fixed at the bottom of a recording chamber, and stimulated with various compounds using a multichannel perfusion system. Since previous data have shown that most of the tracheal ciliated cells isolated from different species (Zhang et al., 2003; Ma et al., 2006) (Li et al., 2012); responded to ATP stimulation, we used ATP (30 µM) as a positive control to assess the viability of the cells and to verify that the solutions were reaching the cells. Figure 3.2A shows two fluorescence images of a mouse tracheal slice. Two ciliated cells were highlighted by drawing a region of interest around each of them (blue and red). The top image was taken at rest, while the bottom image was recorded during ATP stimulation. Measurements of normalized fluorescence intensities ($\Delta F/F$) in the two selected regions show that the application of ATP for 30 sec induced a significant increase of the intracellular Ca^{2+} concentration that was reversible after the removal of ATP. In all recordings, Ringer solution was perfused to verify possible artifacts due to slice movements during the solution switching, discarding from the analysis the cells showing a change of Fluo-4 signal.

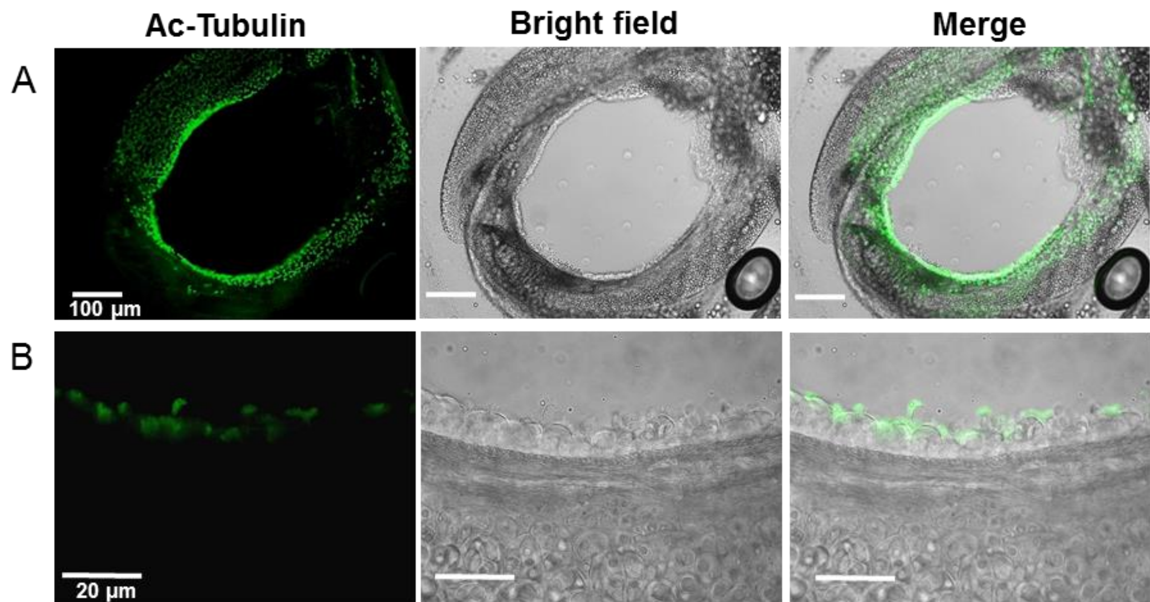


Figure 3.1 Histological organization of the tracheal slices used for Ca^{2+}-recordings. The figure shows the expression of the Ac-tubulin (green) in an entire tracheal ring, previously used for Ca^{2+}-recordings (A). The ciliated cells line the tracheal lumen and the cilia were intact (B).

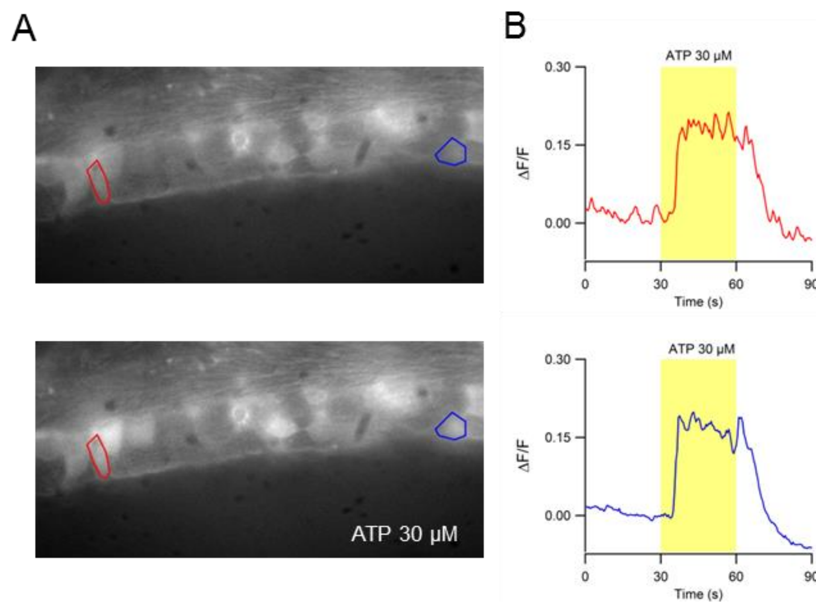


Figure 3.2 ATP evokes calcium transients in tracheal ciliated cells (A) Fluorescence images taken at rest in the absence of stimulation (top panel) and near the peak fluorescence (bottom panel) during 30 s stimulation with ATP. (B) Time course of normalized fluorescence signal for two representative ciliated cells highlighted in red and blue in (A). The yellow box shows the time of application of 30 μM ATP.

3.1.1 Tracheal ciliated cells respond to glucose and denatonium

Since previous data from rat showed the localization of T1R3 sweet receptor and glucose transporters GLUT2 in the cilia of tracheal airway epithelium (Merigo et al., 2012; 2016), we tested if glucose could induce a Ca^{2+} increase in tracheal ciliated cells. The left column of Figure 3.3 shows responses of two representative cells to 10 mM glucose. The time course of the glucose-stimulated fluorescence showed a transient increase in intracellular Ca^{2+} concentration for one cell (Figure 3.3A), whereas did not present a significant variation for the other cell (Figure 3.3B). The percentage of ATP-responding ciliated cells responding to glucose was 37% (37 of 99 cells) (see Table 3.1).

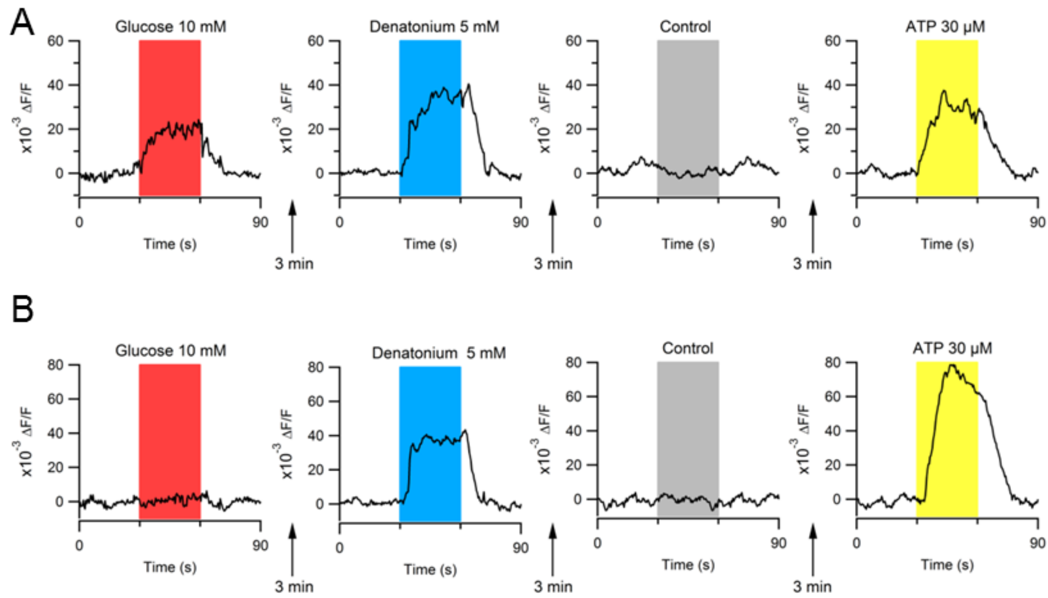


Figure 3.3 Responses to glucose and/or denatonium of tracheal ciliated cells. Time course of calcium-mediated responses recorded from two tracheal ciliated cells from the same slice. Cells were stimulated with glucose (10 mM, red boxes), followed by denatonium (5 mM, blue boxes), Ringer (grey boxes) as negative control, and finally with ATP (ATP 30 μM, yellow boxes) to assess the cells viability. (**A**) A ciliated cell responding to both glucose and denatonium. (**B**) A ciliated cell not responding to glucose (red box), but responding to denatonium (blue box). In both ciliated cells, the negative control does not elicit any calcium increase, while the positive control evokes a response.

Since previous experiments reported that human ciliated cells express several members of the bitter taste receptor family, T2Rs, and respond to various bitter compounds, such as denatonium (Shah et al., 2009), we investigated the responses of murine tracheal slice to denatonium. Figure 3.3 shows results of a typical experiment, in which the first stimulus was 10 mM glucose, followed by 5 mM denatonium. We waited at least 3 minutes between the different stimulations to avoid possible adaptation/desensitization of the cells. Figure 3.3A shows that one ciliated cell responded both to glucose and denatonium, whereas Figure 3.3B shows another ciliated cell that could be activated only by denatonium. The percentage of ATP-responding ciliated cells responding to denatonium was 73% (72 of 99 cells) (see Table 3.1).

Taken together, these experiments show that tracheal ciliated cells have heterogenous response profiles. Among the 99 cells that responded to ATP and did not show a mechanical response to the application of the Ringer solution, 36% responded both to glucose and denatonium, 37% only to denatonium, 2% only to glucose, and 25% were unresponsive both to glucose and denatonium stimulation (Table 3.1)

	N° of cells	%
Glucose and Denatonium	35	36
Only Glucose	2	2
Only Denatonium	37	37
No response	25	25
Total	99	

Table 3.1 Tracheal ciliated cells responses to glucose and denatonium. The table shows the summary of the different responsiveness profile of tracheal ciliated cells to glucose and denatonium.

3.1.2 Tracheal ciliated cells respond to artificial sweeteners

Sweet taste receptors can recognize a great variety of ligands, including the artificial sweeteners acesulfame K (AK) and sucralose (Laffitte et al., 2014). Therefore, using the same approach described above, we tested if these two structurally unrelated artificial sweeteners could induce a Ca^{2+} increase in tracheal ciliated cells. Figure 3.4 shows a representative cell responding both to stimulation with 10 mM AK or 10 mM sucralose.

Interestingly AK and sucralose induced responses with different kinetics, indeed AK application leads to a prolonged response whereas sucralose induces a short response with a fast increase of the fluorescence emission followed by a similar rapid decrease of the signal, during the presence of the stimulus. Moreover, ciliated cells showed a heterogeneous sensitivity to these two artificial sweeteners, indeed about 30 % of the ATP-responding ciliated cells responded both to AK and sucralose, 24% only to AK and 5 only to sucralose (Table 3.2)

Thus, ciliated cells lining the trachea can detect and respond to both natural and artificial sweeteners.

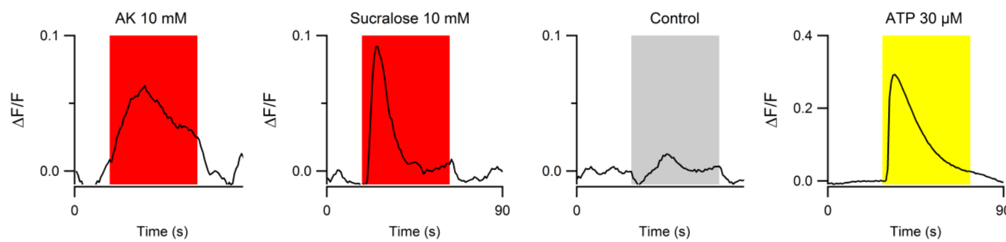


Figure 3.4 Responses to the artificial sweeteners of tracheal ciliated cells. Time course of calcium-mediated responses recorded from a tracheal ciliated cell. The cell was stimulated with AK (10 mM, red box), sucralose (10 mM, red box), Ringer (grey box) as negative control, and finally with ATP (ATP 30 μM, yellow box) to assess the cells viability.

	N° of cells	%
AK and Sucralose	20	31
Only AK	16	24
Only Sucralose	3	5
No response	26	40
Total	65	

Table 3.2. Tracheal ciliated cells responses to the artificial sweetener AK and sucralose. The table shows the summary of the different responsiveness profile of tracheal ciliated cells to AK and sucralose.

In the previous experiments, we found that 94% of the glucose-responsive ciliated cells were also sensitive to the bitter compound denatonium (35 of 37 cells). Therefore, we tested if also the artificial sweetener AK could activate the cells sensitive to denatonium. Figure 3.5 shows two representative cells stimulated first

by 5 mM denatonium and then by 10 mM AK. Among the 86 cells that responded to ATP (and did not show a mechanical response to the application of the Ringer solution), 65% responded both to AK and denatonium, 5% only to denatonium, 7% only to AK, and 23% were unresponsive both to AK and denatonium stimulation (Table 3.3). Moreover, similarly to results with glucose and denatonium, 90 % of the AK-responsive ciliated cells were also activated by denatonium (56 of 62 cells, Table 3.3).

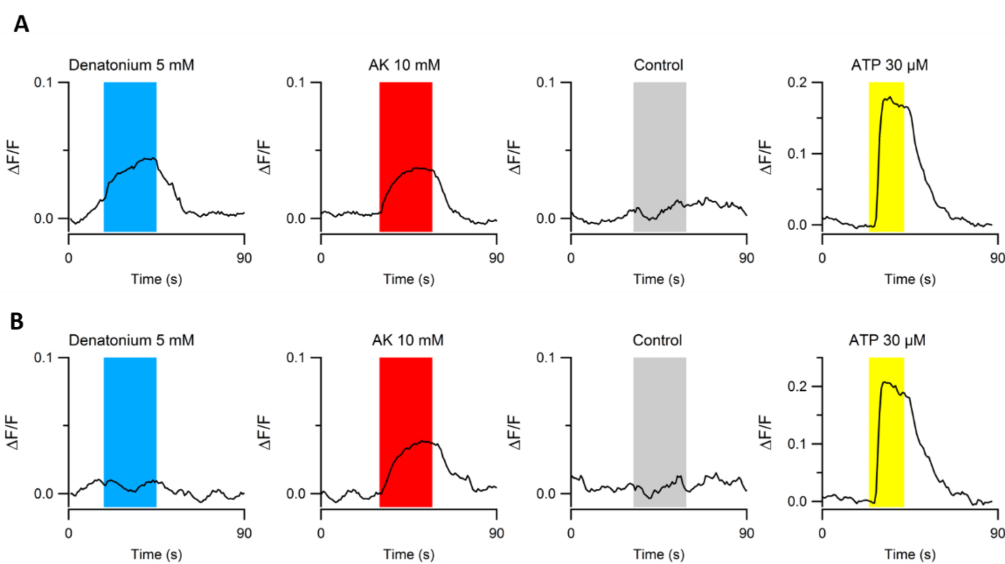


Figure 3.5 Responses to the artificial sweetener AK and/or denatonium of tracheal ciliated cells. Time course of calcium-mediated responses recorded from two tracheal ciliated cells from the same slice. Cells were stimulated with denatonium (5 mM, blue boxes), AK (10 mM, red boxes), Ringer (grey boxes) as negative control, and finally with ATP (ATP 30 μM, yellow boxes) to assess the cells viability. (**A**) A ciliated cell responding to both AK and denatonium. (**B**) A ciliated cell not responding to denatonium (blue box), but responding to AK (red box). In both ciliated cells, the negative control does not elicit any calcium increase, while the positive control evokes a response.

	N° of cells	%
Denatonium and AK	56	65
Only AK	6	7
Only Denatonium	4	5
No response	20	23
Total	86	

Table 3.3. Tracheal ciliated cells responses to the artificial sweetener AK and denatonium. The table shows the summary of the different responsiveness profile of tracheal ciliated cells to AK and denatonium.

3.2 IMMUNOHISTOCHEMISTRY EXPRESSION OF TASTE TRANSDUCTION COMPONENTS IN MICE TRACHEAL CILIATED CELLS

In mice tracheas we found that α-Gustducin was expressed in solitary chemosensory cells and in the apical portion of a subpopulation of ciliated cells (Figure 3.6 A and B) while PLCβ2 was expressed both in solitary chemosensory cells and in the apical membrane of a subpopulation of ciliated cells (Figure 3.7 A and B). Some cells did not express neither α-Gustducin nor PLC β2 (Figure 3.6 C and 3.7 C).

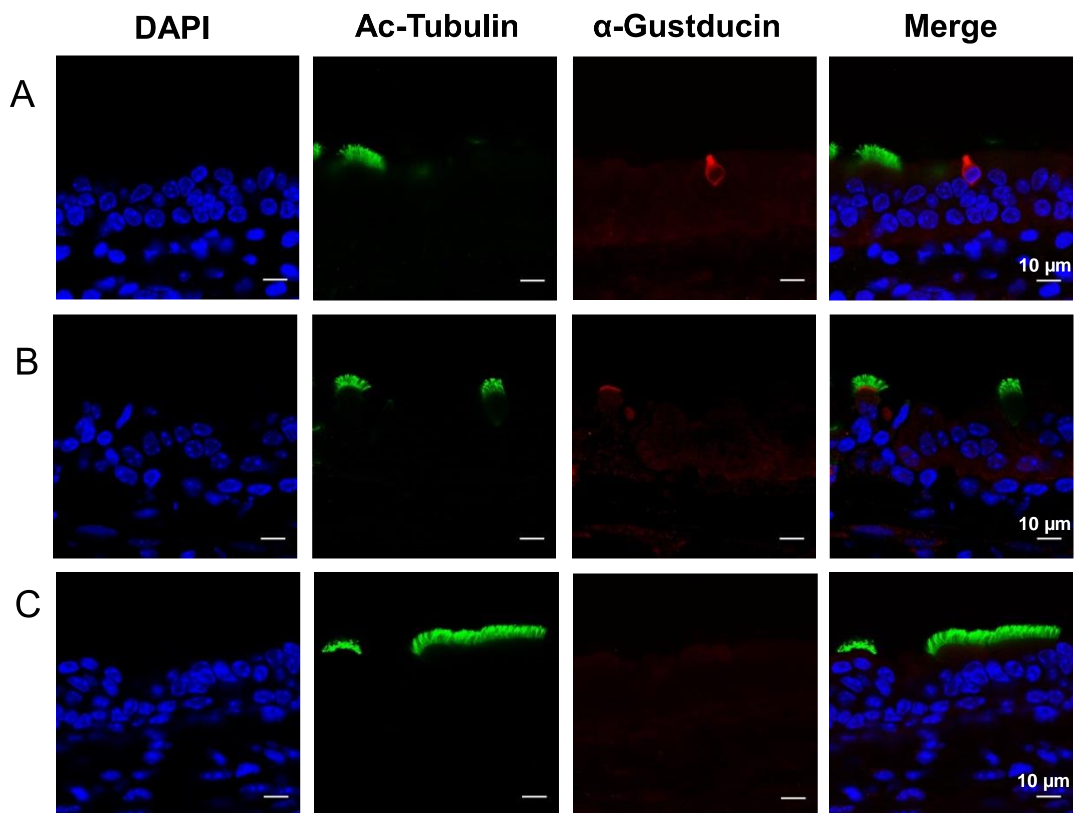


Figure 3.6 α-Gustducin is selectively expressed in solitary chemosensory cells and in a subpopulation of ciliated cells from the mouse. Double-immunofluorescence of acetylated-tubulin (Ac-Tubulin) and α-Gustducin showing the expression in solitary chemosensory cells (A) and in a subpopulation of ciliated cells (B). Some ciliated cells did not express α-Gustducin (C) Cells nuclei are stained with DAPI (blue).

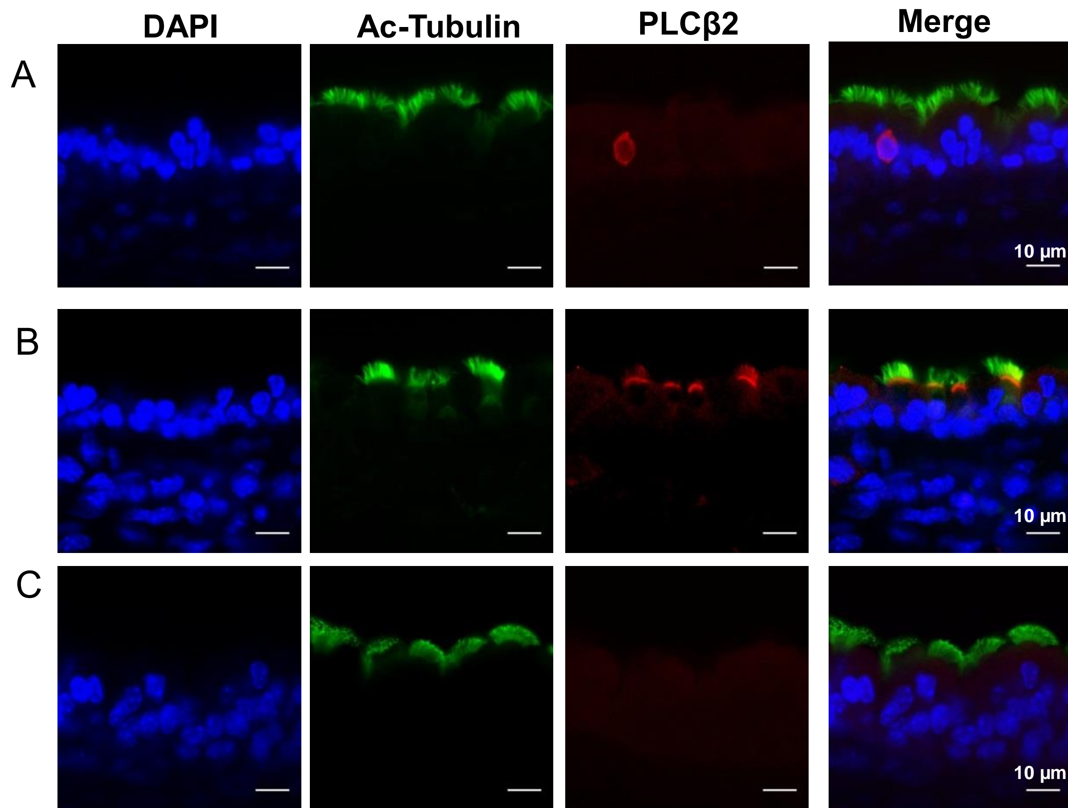


Figure 3.7 PLCβ2 is selectively expressed in solitary chemosensory cells and in a subpopulation of mouse ciliated cells. Double-immunofluorescence of acetylated-tubulin. Ac-Tubulin and PLCβ2 showing the expression of PLCβ2 in solitary chemosensory cells (A) and in a subpopulation of ciliated cells (B). Some ciliated cells did not express PLCβ2 (C) Cells nuclei are stained with DAPI (blue).

4. DISCUSSION

In the present work, by means of the calcium imaging technique, we showed that some mouse tracheal ciliated cells respond to natural and artificial sweet compounds.

We conducted this investigation with the following schedule:

- For the first time, to the best of our knowledge, we collected the cellular physiological data using acute tracheal slices instead of cell cultures. This procedure reflects conditions similar to those of the native epithelium and prevents possible artifacts. In particular, it permits to maintain a cohesive epithelium and to preserve the paracrine cellular communication of the different cell types.
- We stimulated the ciliated cells with glucose (10 mM), a natural sugar, with acesulfame K (AK, 10 mM) and sucralose (10 mM), two artificial sweeteners.
- We stimulated the ciliated cells also with denatonium (5 mM), a bitter substance, to compare the responses of ciliated cells towards the two different stimulation qualities (sweet and bitter).
- We used the immunohistochemical analysis to verify that the epithelial cells of acute slices used for Ca^{2+}-imaging recording, retained the phenotypic expression of molecules of the taste transduction pathway which are expressed in native ciliated cells.

The results obtained with the present work are interesting and can be summarized in the following way:

- First, we found that ciliated cells respond to glucose.
- When we applied two different stimuli, first glucose and then denatonium, we observed that 36% of the ciliated cells responded to both stimulations. A similar percentage (37%) of ciliated cells were only responsive to denatonium, while a small percentage (2%) was only responsive to glucose. 25% of the cells were unresponsive to both stimulations.

- When we tested the responsiveness of the ciliated cells to AK and sucralose, two artificial sweeteners, we observed that 31% of the cells responded to both the sweeteners, 24% only to AK and 5% only to sucralose. 40% of the cells were unresponsive to both the artificial sweeteners.
- When we applied the denatonium followed by the artificial sweetener AK, 65% of the cells responded to both stimuli, 7% only to AK while 5% only to denatonium. 23% of the cells were unresponsive to both stimulations.
- Double-immunofluorescence analysis performed after Ca^{2+}-imaging recordings showed PLCβ2 expression beneath some ciliated cells.

These overall findings indicate that tracheal ciliated cell population exhibits different patterns of responses: indeed, in every stimulation design adopted with the different substances, the ciliated cells responded with different proportion to sweet and/or to bitter stimuli.
In particular, they reacted in part only to sweet, only to bitter, to both of them or to none of them. This physiological datum is important and support the previous data of the work of Merigo and co-workers in which, by means of immunohistochemistry and immunoelectron microscopy, it was pointed out that chemosensory airways cells are not identical and not all ciliated cells expressed the chemoreception markers, suggesting that ciliated cells are a heterogeneous population with phenotypically different subsets (Merigo et al., 2012).
The above described data clearly demonstrate that the ciliated cells of murine trachea are able to perceive and respond to sweet-taste compounds. To the best of our knowledge, this is the first demonstration that the ciliated cells have a sensory capacity for sweetness. This datum strongly argues that the ciliated cells, like the chemosensory cells, may be involved in the mechanisms that regulate the glucose-sensing in trachea and it might be important for improved evaluations on the regulation of glucose homeostasis in airways surface liquid (ASL). In general, the chemoreceptive epithelia respond to local sugar changes by regulating uptake through the modulation of glucose transporter expression. For example, the detection of glucose in the intestinal lumen contributes to its regulation by adjusting the uptake of glucose through the modulation of sweet taste receptor and glucose

transporters expression in the epithelium, or through the activation of multiple signaling pathways (i.e. hormonal, neural, paracrine), involving the cross-talk between different cell types of the epithelium (Young et al., 2013). Similarly, the detection of glucose in the tracheal lumen can activate multiple regulating events that could request the integration of functions (such as epithelial secretion, glucose absorption, frequency of the ciliary beat) carried out by different cell types. In this regard, many stimulating roles of chemoreception are emerging from various studies in airways. For example, it has been demonstrated that hyperosmolar saline solution or sucrose solution significantly increased, in a dose–related fashion, mucin secretion in the cat trachea (Peatfield et al., 1986); a similar finding was also observed with mannitol solution in the excised ferret trachea, suggesting that the secretagogue effect is probably independent by central reflexes and could be due to a direct activity of secretory cells or to the release of mediators that in turns trigger the secretion (Kishioka et al., 2003). Moreover, it has been established that inhalation or application of bitter compounds induced secretive responses in the rat airway (Boschi et al., 2008), decreased airway obstruction in a mouse model of asthma (Deshpande et al., 2010), and stimulated ciliary activity in human airway epithelium (Shah et al., 2009). The demonstration in the present work of the capacity of the ciliated cells to detect sugars could be physiologically important because it suggests that ciliated cells may have the possibility to integrate both chemosensory and mechanical properties. Their chemosensory function could be important to promote ciliary beating and consequently mucociliary clearance. Regarding this, there is evidence from *in vitro* and animal studies that mannitol (natural monosaccharide, sugar alcohol) may increase mucociliary clearance, including improving hydration of airway secretions and reducing sputum viscosity (Bye & Elkins, 2007). In addition, a study of Robinson and collaborators stated that inhaled mannitol appears to increase mucociliary clearance in people with cystic fibrosis, even though the exact mechanism by which it is achieved it is not yet known (Robinson et al., 1999). Indeed, inhaled dry powder mannitol is currently available for the treatment of cystic fibrosis and there is the license for using it in adults in the European Union and for adults and children over the age of six years in Australia (Nolan et al., 2015).

Previous studies have demonstrated that elements associated with sweet taste transduction pathway, such as α-Gustducin, T1R3 and GLUT2 transporter, are expressed in ciliated cells of rat tracheal epithelium (Merigo et al., 2012). The presence of these molecules has been interpreted as an evidence of the involvement of ciliated cells in the regulation of glucose homeostasis. Our physiological datum, together with our immunohistochemical findings confirming the presence of α-Gustducin and PLCβ2 in ciliated cells, reinforces these suggestions and can be a good indicator of the role of ciliated cells in controlling glucose homeostasis

Moreover, another important result of this study is certainly to have shown that a percentage of ciliated cells respond also to synthetic compounds like AK and sucralose (31%). In this regard, it is well known that species differences do exist in mammals (Vigues et al., 2009). Interestingly, rodents do not prefer several artificial sweeteners (Hellekant & Danilova, 1996), but it has been demonstrated by an electrophysiological study on chorda tympani nerve, which innervates the fungiform taste buds of the tongue, that mice respond to saccharin, AK, sucralose, and dulcin (Bachmanov et al., 2001b). The ability of ciliated cells to respond to AK and sucralose stimuli here suggests that the mechanism implicated in sweet taste sensation in the gustatory epithelium also operates in the trachea.

Finally, we have also shown that ciliated cells of the murine trachea respond to bitter stimuli, even when they are administered after a sweet stimulus. In particular, 36% of ciliated cells respond to natural sugar glucose and bitter, and 65% respond to AK and bitter. As expected, this finding confirms that ciliated cells are equipped with the machinery required to detect both the stimuli. Indeed bitter taste receptors have been previously described on human airway epithelial cells, localized on motile cilia (Shah et al., 2009). However, the bimodal response (bitter and sweet) that emerged in some ciliated cells is a novel intriguing finding. It suggests that these two chemosensory modalities might be linked also in this cellular population of the airways, as it was previously hypothesized for the solitary chemosensory cells of the nasal cavity (Lee & Cohen, 2014). Regarding this, Lee and Cohen proposed the idea that the T1Rs sweet receptors found in solitary chemosensory cells of the nasal cavity, may function to desensitize the T2Rs to bitter compounds secreted by some bacteria throughout low-level colonization in healthy subjects. However,

during infection, bacterial consumption of ASL glucose might reduce ASL glucose concentration and allow T2Rs to activate an appropriate defense response. Thus, the two chemosensory modalities could be physiologically correlated also in some tracheal ciliated cells, justifying the presence of a cellular defense mechanism in the overall airways system.

The role of glucose in the airways is interesting, especially in terms of its relationship with respiratory infections. Glucose is normally a component of the ASL and glucose transport plays a key role in maintaining a correct glucose concentration in the ASL, restricting the growth of respiratory pathogens. ASL glucose was found elevated in patients with viral colds, cystic fibrosis, chronic obstructive pulmonary disease (COPD) and asthma (Baker et al., 2007; Garnett et al., 2012)

Airways ciliated cells could be involved in different clinical conditions. For example, it is important to mention that in our recently published work (Merigo et al., 2016) it was found that T1R3 expression pattern in tracheal ciliated cells was reduced in obese rats and the tracheal epithelium of obese animals showed poorly differentiated cells. This altered epithelial morphology seemed to impair the expression of glucose homeostasis molecules. Following this line of reasoning and on the basis of these premises, it could be interesting to investigate the sweet ciliated cells response in the airways of obesity animal models where the reported impairment of the cilia might reflect on the defense mechanism in the airways.

In addition, the airways ciliated cells sweet responses, supported by the immunohistochemical data, might be important for the future pharmacological research. Indeed, concerning bitter taste receptors, recent studies have suggested a drug target role for human bitter airways smooth muscle receptors. In particular, these muscle chemosensory receptors are under investigation for asthma and airways obstructive pathology and it was hypothesized that agonists to these receptors might represent a new class of bronchodilators drugs (Deshpande et al., 2010; Robinett et al., 2011; An & Liggett, 2017). Similarly, sweet epithelial cells receptors of the airways could be considered a potential target for novel drugs aimed to regulate the glucose level in the airways. This might be potentially intriguing for respiratory infections in particular for clinical conditions at risk of developing

airways infections (e.g. mechanical ventilated patients, immunodeficiency syndromes, diabetes). Regarding this, the relationship between airways chemosensory receptors and innate immunity, previously hypothesized by Cohen and co-workes and mentioned above, is interesting. Moreover, it is also important to mention that the genetic variation of sweet receptor genes could alter the response to sweet substances (Fushan et al., 2009; Bachmanov et al., 2014). In the same way, this genetic variability might play a role in susceptibility to respiratory infections (Lee & Cohen, 2014). This idea might partially explain the old evidence that there is a genetic basis to respiratory infections (Greisner & Settipane, 1996; Hamilos, 2007). Thus, also genetic variability features of these chemosensory receptors will be useful for future drug research.

Taken together, our results and the data reported in the literature, show that the airways chemosensory ciliated cells represent one cellular model of chemoreception integrated in a tricky communication cellular network. Future investigations could unravel their roles in health and pathological conditions with a possible therapeutic aim.

ACKNOWLEDGEMENTS

I would like to thank Professor Andrea Sbarbati for introducing me in the surprising word of chemosensation and for giving me the opportunity to begin my journey into scientific research. Thank you to Doctor Maria Paola Cecchini and Doctor Flavia Merigo for believing in me and walking next to me in this journey, giving me advices and small life lessons, support and critics. A special thanks to Professor Anna Menini and her group; you all made me fall in love with research again and you all inspired me to be a better version of myself. No experience would make any sense without precious persons and felines to share it with; thank you to my family and friends that, despite the distance or the time, have always listen to my nonsense, motivating me or making fun of me. A final thanks to my "partner in crimes" soon to be PhD Doctor Maria Rosaria Marinozzi for trusting me and for all our *"über-scientific"* moments.

REFERENCES

Adler E., Hoon M.A., Mueller K.L., Chandrashekar J., Ryba N.J. & Zuker C.S. 2000. A novel family of mammalian taste receptors. Cell. 100: 693–702.

Alberts B., Johnson A., Lewis J., Raff M., Roberts K. & Walter P. 2002. The Airways and the Gut. .

An S.S. & Liggett S.B. 2017. Taste and smell GPCRs in the lung: Evidence for a previously unrecognized widespread chemosensory system. Cell. Signal. .

Atta-ur-Rahman 1995. Bioactive Natural Products (Part E). Elsevier, 595 pp.

Bachmanov A.A., Bosak N.P., Lin C., Matsumoto I., Ohmoto M., Reed D.R. & Nelson T.M. 2014. Genetics of Taste Receptors. Curr. Pharm. Des. 20: 2669–2683.

Bachmanov A.A., Li X., Reed D.R., Ohmen J.D., Li S., Chen Z., Tordoff M.G., de Jong P.J., Wu C., West D.B., Chatterjee A., Ross D.A. & Beauchamp G.K. 2001a. Positional cloning of the mouse saccharin preference (Sac) locus. Chem. Senses. 26: 925–933.

Bachmanov A.A., Tordoff M.G. & Beauchamp G.K. 2001b. Sweetener preference of C57BL/6ByJ and 129P3/J mice. Chem. Senses. 26: 905–913.

Baker E.H. 2011. Inflammation, infection and airway glucose homeostasis. Proc. Physiol. Soc. Proc Physiol Soc 23: .

Behrens M., Foerster S., Staehler F., Raguse J.-D. & Meyerhof W. 2007. Gustatory expression pattern of the human TAS2R bitter receptor gene family reveals a heterogenous population of bitter responsive taste receptor cells. J. Neurosci. Off. J. Soc. Neurosci. 27: 12630–12640.

Birch G.G. 1987. Chemical Aspects of Sweetness. pp. 3–13. In: FRCPath J.D.Ds. FRCP (eds), Sweetness, Springer London,

Bloodgood R.A. 2010. Sensory reception is an attribute of both primary cilia and motile cilia. J Cell Sci. 123: 505–509.

Bootman M.D., Rietdorf K., Collins T., Walker S. & Sanderson M. 2013. Ca2+-sensitive fluorescent dyes and intracellular Ca2+ imaging. Cold Spring Harb. Protoc. 2013: 83–99.

Boschi F., Nicolato E., Benati D., Marzola P. & Sbarbati A. 2008. Drug targeting of airway surface liquid: a pharmacological MRI approach. Biomed. Pharmacother. Biomedecine Pharmacother. 62: 410–419.

Boughter Jr. J.D. & Munger S.D. 2004. Taste Receptors A2 - Lennarz, William J. pp. 158–161. In: Lane M.D. (eds), Encyclopedia of Biological Chemistry, Elsevier, New York.

Bufe B., Hofmann T., Krautwurst D., Raguse J.-D. & Meyerhof W. 2002. The human TAS2R16 receptor mediates bitter taste in response to beta-glucopyranosides. Nat. Genet. 32: 397–401.

Bye P.T.P. & Elkins M.R. 2007. Other mucoactive agents for cystic fibrosis. Paediatr. Respir. Rev. 8: 30–39.

Caicedo A., Pereira E., Margolskee R.F. & Roper S.D. 2003. Role of the G-protein subunit alpha-gustducin in taste cell responses to bitter stimuli. J. Neurosci. Off. J. Soc. Neurosci. 23: 9947–9952.

Chandrashekar J., Mueller K.L., Hoon M.A., Adler E., Feng L., Guo W., Zuker C.S. & Ryba N.J. 2000. T2Rs function as bitter taste receptors. Cell. 100: 703–711.

Chaudhari N. & Roper S.D. 2010. The cell biology of taste. J. Cell Biol. 190: 285–296.

Choksi S.P., Lauter G., Swoboda P. & Roy S. 2014. Switching on cilia: transcriptional networks regulating ciliogenesis. Dev. Camb. Engl. 141: 1427–1441.

Crystal R.G., Randell S.H., Engelhardt J.F., Voynow J. & Sunday M.E. 2008. Airway Epithelial Cells. Proc. Am. Thorac. Soc. 5: 772–777.

Damak S., Rong M., Yasumatsu K., Kokrashvili Z., Varadarajan V., Zou S., Jiang P., Ninomiya Y. & Margolskee R.F. 2003. Detection of sweet and umami taste in the absence of taste receptor T1r3. Science. 301: 850–853.

DeFazio R.A., Dvoryanchikov G., Maruyama Y., Kim J.W., Pereira E., Roper S.D. & Chaudhari N. 2006. Separate populations of receptor cells and presynaptic cells in mouse taste buds. J. Neurosci. Off. J. Soc. Neurosci. 26: 3971–3980.

Delay R.J., Kinnamon J.C. & Roper S.D. 1986. Ultrastructure of mouse vallate taste buds: II. Cell types and cell lineage. J. Comp. Neurol. 253: 242–252.

Delmotte P. & Sanderson M.J. 2006. Ciliary beat frequency is maintained at a maximal rate in the small airways of mouse lung slices. Am. J. Respir. Cell Mol. Biol. 35: 110–117.

Deshpande D.A., Wang W.C.H., McIlmoyle E.L., Robinett K.S., Schillinger R.M., An S.S., Sham J.S.K. & Liggett S.B. 2010. Bitter taste receptors on airway smooth muscle bronchodilate by localized calcium signaling and reverse obstruction. Nat. Med. 16: 1299–1304.

Finger T.E. 2005. Cell Types and Lineages in Taste Buds. Chem. Senses. 30: i54–i55.

Finger T.E. & Kinnamon S.C. 2011. Taste isn't just for taste buds anymore. F1000 Biol. Rep. 3: 20.

Fushan A.A., Simons C.T., Slack J.P., Manichaikul A. & Drayna D. 2009. Allelic polymorphism within the TAS1R3 promoter is associated with human taste sensitivity to sucrose. Curr. Biol. CB. 19: 1288–1293.

Ganesan S., Comstock A.T. & Sajjan U.S. 2013. Barrier function of airway tract epithelium. Tissue Barriers. 1: .

Garnett J.P., Baker E.H. & Baines D.L. 2012a. Sweet talk: insights into the nature and importance of glucose transport in lung epithelium. Eur. Respir. J. 40: 1269–1276.

Garnett J.P., Baker E.H. & Baines D.L. 2012b. Sweet talk: insights into the nature and importance of glucose transport in lung epithelium. Eur. Respir. J. 40: 1269–1276.

Garnett J.P., Nguyen T.T., Moffatt J.D., Pelham E.R., Kalsi K.K., Baker E.H. & Baines D.L. 2012c. Proinflammatory mediators disrupt glucose homeostasis in airway surface liquid. J. Immunol. Baltim. Md 1950. 189: 373–380.

Gilbertson T.A. & Kinnamon S.C. 1996. Making sense of chemicals. Chem. Biol. 3: 233–237.

Gill S.K., Hui K., Farne H., Garnett J.P., Baines D.L., Moore L.S.P., Holmes A.H., Filloux A. & Tregoning J.S. 2016. Increased airway glucose increases airway bacterial load in hyperglycaemia. Sci. Rep. 6: 27636.

Glendinning J.I. 1994. Is the bitter rejection response always adaptive?. Physiol. Behav. 56: 1217–1227.

Greisner W.A. & Settipane G.A. 1996. Hereditary factor for nasal polyps. Allergy Asthma Proc. 17: 283–286.

Hamilos D.L. 2007. Chronic rhinosinusitis patterns of illness. Clin. Allergy Immunol. 20: 1–13.

Hellekant G. & Danilova V. 1996. Species differences toward sweeteners. Food Chem. 56: 323–328.

Hepler J.R. & Gilman A.G. 1992. G proteins. Trends Biochem. Sci. 17: 383–387.

Herrera Moro Chao D., Argmann C., Van Eijk M., Boot R.G., Ottenhoff R., Van Roomen C., Foppen E., Siljee J.E., Unmehopa U.A., Kalsbeek A. & Aerts J.M.F.G. 2016. Impact of obesity on taste receptor expression in extra-oral tissues: emphasis on hypothalamus and brainstem. Sci. Rep. 6: 29094.

Huang Y.A. & Roper S.D. 2010a. Intracellular Ca(2+) and TRPM5-mediated membrane depolarization produce ATP secretion from taste receptor cells. J. Physiol. 588: 2343–2350.

Huang Y.A. & Roper S.D. 2010b. Intracellular Ca2+ and TRPM5-mediated membrane depolarization produce ATP secretion from taste receptor cells. J. Physiol. 588: 2343–2350.

Jain R., Javidan-Nejad C., Alexander-Brett J., Horani A., Cabellon M.C., Walter M.J. & Brody S.L. 2012. Sensory functions of motile cilia and implication for bronchiectasis. Front. Biosci. Sch. Ed. 4: 1088–1098.

Jiang P., Cui M., Zhao B., Snyder L.A., Benard L.M.J., Osman R., Max M. & Margolskee R.F. 2005. Identification of the cyclamate interaction site within the transmembrane domain of the human sweet taste receptor subunit T1R3. J. Biol. Chem. 280: 34296–34305.

Kalsi K.K., Baker E.H., Fraser O., Chung Y.-L., Mace O.J., Tarelli E., Philips B.J. & Baines D.L. 2009. Glucose homeostasis across human airway epithelial cell monolayers: role of diffusion, transport and metabolism. Pflugers Arch. 457: 1061–1070.

Kalsi K.K., Baker E.H., Medina R.A., Rice S., Wood D.M., Ratoff J.C., Philips B.J. & Baines D.L. 2008. Apical and basolateral localisation of GLUT2 transporters in human lung epithelial cells. Pflugers Arch. 456: 991–1003.

Kinnamon S.C. 2012. Taste receptor signalling - from tongues to lungs. Acta Physiol. Oxf. Engl. 204: 158–168.

Kinnamon S.C. & Cummings T.A. 1992. Chemosensory transduction mechanisms in taste. Annu. Rev. Physiol. 54: 715–731.

Kishioka C., Okamoto K., Kim J.-S. & Rubin B.K. 2003. HYperosmolar solutions stimulate mucus secretion in the ferret trachea*. Chest. 124: 306–313.

Knight D.A. & Holgate S.T. 2003. The airway epithelium: structural and functional properties in health and disease. Respirol. Carlton Vic. 8: 432–446.

Knowles M.R. & Boucher R.C. 2002. Mucus clearance as a primary innate defense mechanism for mammalian airways. J. Clin. Invest. 109: 571–577.

Laffitte A., Neiers F. & Briand L. 2014. Functional roles of the sweet taste receptor in oral and extraoral tissues. Curr. Opin. Clin. Nutr. Metab. Care. 17: 379–385.

Lee R.J., Chen B., Redding K.M., Margolskee R.F. & Cohen N.A. 2014. Mouse nasal epithelial innate immune responses to Pseudomonas aeruginosa quorum-sensing molecules require taste signaling components. Innate Immun. 20: 606–617.

Lee R.J. & Cohen N.A. 2014. Bitter and sweet taste receptors in the respiratory epithelium in health and disease. J. Mol. Med. Berl. Ger. 92: 1235–1244.

Lemon C.H. & Margolskee R.F. 2009. Contribution of the T1r3 taste receptor to the response properties of central gustatory neurons. J. Neurophysiol. 101: 2459–2471.

Li X., Staszewski L., Xu H., Durick K., Zoller M. & Adler E. 2002. Human receptors for sweet and umami taste. Proc. Natl. Acad. Sci. U. S. A. 99: 4692–4696.

Lindemann B. 2001. Receptors and transduction in taste. Nature. 413: 219–225.

Ma W., Korngreen A., Weil S., Cohen E.B.-T., Priel A., Kuzin L. & Silberberg S.D. 2006. Pore properties and pharmacological features of the P2X receptor channel in airway ciliated cells. J. Physiol. 571: 503–517.

Max M., Shanker Y.G., Huang L., Rong M., Liu Z., Campagne F., Weinstein H., Damak S. & Margolskee R.F. 2001. Tas1r3, encoding a new candidate taste receptor, is allelic to the sweet responsiveness locus Sac. Nat. Genet. 28: 58–63.

McLaughlin S.K. & McKinnon P.J. 1992. Gustducin is a taste-cell-specific G protein closely related to the transducins. Nature. 357: 563–569.

Merigo F., Benati D., Cristofoletti M., Amarù F., Osculati F. & Sbarbati A. 2012. Glucose transporter/T1R3-expressing cells in rat tracheal epithelium. J. Anat. 221: 138–150.

Merigo F., Boschi F., Lasconi C., Benati D. & Sbarbati A. 2016. Molecules implicated in glucose homeostasis are differentially expressed in the trachea of lean and obese Zucker rats. Eur. J. Histochem. EJH. 60: 2557.

Meyerhof W., Batram C., Kuhn C., Brockhoff A., Chudoba E., Bufe B., Appendino G. & Behrens M. 2010. The molecular receptive ranges of human TAS2R bitter taste receptors. Chem. Senses. 35: 157–170.

Morini G., Bassoli A. & Temussi P.A. 2005. From small sweeteners to sweet proteins: anatomy of the binding sites of the human T1R2_T1R3 receptor. J. Med. Chem. 48: 5520–5529.

Nelson G., Hoon M.A., Chandrashekar J., Zhang Y., Ryba N.J. & Zuker C.S. 2001. Mammalian sweet taste receptors. Cell. 106: 381–390.

Nie Y., Hobbs J.R., Vigues S., Olson W.J., Conn G.L. & Munger S.D. 2006. Expression and purification of functional ligand-binding domains of T1R3 taste receptors. Chem. Senses. 31: 505–513.

Nie Y., Vigues S., Hobbs J.R., Conn G.L. & Munger S.D. 2005. Distinct contributions of T1R2 and T1R3 taste receptor subunits to the detection of sweet stimuli. Curr. Biol. CB. 15: 1948–1952.

Nolan S.J., Thornton J., Murray C.S. & Dwyer T. 2015. Inhaled mannitol for cystic fibrosis. Cochrane Database Syst. Rev. CD008649.

Peatfield A.C., Richardson P.S. & Wells U.M. 1986. The effect of airflow on mucus secretion into the trachea of the cat. J. Physiol. 380: 429–439.

Pezzulo A.A., Gutiérrez J., Duschner K.S., McConnell K.S., Taft P.J., Ernst S.E., Yahr T.L., Rahmouni K., Klesney-Tait J., Stoltz D.A. & Zabner J. 2011. Glucose depletion in the airway surface liquid is essential for sterility of the airways. PloS One. 6: e16166.

Philips B.J., Redman J., Brennan A., Wood D., Holliman R., Baines D. & Baker E.H. 2005. Glucose in bronchial aspirates increases the risk of respiratory MRSA in intubated patients. Thorax. 60: 761–764.

Purves D., Augustine G.J., Fitzpatrick D., Katz L.C., LaMantia A.-S., McNamara J.O. & Williams S.M. 2001. The Organization of the Taste System. .

Robinett K.S., Deshpande D.A., Malone M.M. & Liggett S.B. 2011. Agonist-promoted homologous desensitization of human airway smooth muscle bitter taste receptors. Am. J. Respir. Cell Mol. Biol. 45: 1069–1074.

Robinson M., Daviskas E., Eberl S., Baker J., Chan H.K., Anderson S.D. & Bye P.T. 1999. The effect of inhaled mannitol on bronchial mucus clearance in cystic fibrosis patients: a pilot study. Eur. Respir. J. 14: 678–685.

Rogers D.F. 1994. Airway goblet cells: responsive and adaptable front-line defenders. Eur. Respir. J. 7: 1690–1706.

Romanov R.A., Rogachevskaja O.A., Bystrova M.F., Jiang P., Margolskee R.F. & Kolesnikov S.S. 2007. Afferent neurotransmission mediated by hemichannels in mammalian taste cells. EMBO J. 26: 657–667.

Roper S.D. 2007. Signal transduction and information processing in mammalian taste buds. Pflugers Arch. 454: 759–776.

Sbarbati A., Bramanti P., Benati D. & Merigo F. 2010. The diffuse chemosensory system: exploring the iceberg toward the definition of functional roles. Prog. Neurobiol. 91: 77–89.

Shah A.S., Ben-Shahar Y., Moninger T.O., Kline J.N. & Welsh M.J. 2009. Motile Cilia of Human Airway Epithelia Are Chemosensory. Science. 325: 1131–1134.

Shallenberger R.S. & Acree T.E. 1967. Molecular theory of sweet taste. Nature. 216: 480–482.

Smith D.V. & Margolskee R.F. 2001. Making sense of taste. Sci. Am. 284: 32–39.

Taruno A., Vingtdeux V., Ohmoto M., Ma Z., Dvoryanchikov G., Li A., Adrien L., Zhao H., Leung S., Abernethy M., Koppel J., Davies P., Civan M.M., Chaudhari N., Matsumoto I., Hellekant G., Tordoff M.G., Marambaud P. & Foskett J.K. 2013. CALHM1 ion channel mediates purinergic neurotransmission of sweet, bitter and umami tastes. Nature. 495: 223–226.

Vigues S., Dotson C.D. & Munger S.D. 2009. The receptor basis of sweet taste in mammals. Results Probl. Cell Differ. 47: 187–202.

Walentek P. & Quigley I.K. 2017. What we can learn from a tadpole about ciliopathies and airway diseases: Using systems biology in Xenopus to study cilia and mucociliary epithelia. Genes. N. Y. N 2000. 55: .

Wong G.T., Gannon K.S. & Margolskee R.F. 1996. Transduction of bitter and sweet taste by gustducin. Nature. 381: 796–800.

Yaghi A. & Dolovich M.B. 2016. Airway Epithelial Cell Cilia and Obstructive Lung Disease. Cells. 5: .

Yarmolinsky D.A., Zuker C.S. & Ryba N.J.P. 2009. Common sense about taste: from mammals to insects. Cell. 139: 234–244.

Young R.L., Chia B., Isaacs N.J., Ma J., Khoo J., Wu T., Horowitz M. & Rayner C.K. 2013. Disordered Control of Intestinal Sweet Taste Receptor Expression and Glucose Absorption in Type 2 Diabetes. Diabetes. 62: 3532–3541.

Zhang Y., Hoon M.A., Chandrashekar J., Mueller K.L., Cook B., Wu D., Zuker C.S. & Ryba N.J.P. 2003. Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. Cell. 112: 293–301.

Zhao G.Q., Zhang Y., Hoon M.A., Chandrashekar J., Erlenbach I., Ryba N.J.P. & Zuker C.S. 2003. The receptors for mammalian sweet and umami taste. Cell. 115: 255–266.