Functional expression of TMEM16A in taste bud cells

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Key points

- Taste transduction occurs in taste buds in the tongue epithelium.
- The Ca²⁺-activated Cl⁻ channels TMEM16A and TMEM16B play relevant physiological roles in several sensory systems.
- Here, we report that TMEM16A, but not TMEM16B, is expressed in the apical part of taste buds.
- Large Ca²⁺-activated Cl⁻ currents blocked by Ani-9, a selective inhibitor of TMEM16A, are measured in type I taste cells but not in type II or III taste cells.
- ATP indirectly activates Ca²⁺-activated Cl⁻ currents in type I cells through TMEM16A channels.
- These results indicate that TMEM16A is functional in type I taste cells and contribute to understanding the largely unknown physiological roles of these cells.

Abstract  The Ca²⁺-activated Cl⁻ channels TMEM16A and TMEM16B have relevant roles in many physiological processes including neuronal excitability and regulation of Cl⁻ homeostasis. Here, we examined the presence of Ca²⁺-activated Cl⁻ channels in taste cells of mouse vallate papillae by using immunohistochemistry and electrophysiological recordings. By using immunohistochemistry we showed that only TMEM16A, and not TMEM16B, was expressed in taste bud cells where it largely co-localized with the inwardly rectifying K⁺ channel KNCJ1 in the apical part of type I cells. By using whole-cell patch-clamp recordings in isolated cells from taste buds, we measured an average current of −1083 pA at −100 mV in 1.5 μM Ca²⁺ and symmetrical Cl⁻ in type I cells. Ion substitution experiments and blockage by Ani-9, a specific TMEM16A channel blocker, indicated that Ca²⁺ activated anionic currents through TMEM16A channels. We did not detect any Ca²⁺-activated Cl⁻ currents in type II or III taste cells. ATP is released by type II cells in response to various tastants and reaches type I cells where it is hydrolysed by ecto-ATPases. Type I cells also express P2Y purinergic receptors and stimulation of type I cells with extracellular ATP produced large Ca²⁺-activated Cl⁻ currents blocked by Ani-9, indicating a possible role of TMEM16A in ATP-mediated signalling. These results provide a definitive demonstration that TMEM16A-mediated currents are functional.
in type I taste cells and provide a foundation for future studies investigating physiological roles for these often-neglected taste cells.

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**Introduction**

In vertebrates, the process of taste transduction occurs in cells of taste buds that are mainly located in the tongue epithelium. Taste buds are onion-shaped clusters of about 50–100 elongated epithelial cells that extend from the basal lamina to the surface of the tongue, where a pore allows the apical part of taste cells to directly contact chemicals dissolved in the mouth saliva. Taste buds also contain basal cells that will form new taste cells. In several species, at least three main types of elongated taste cells have been identified and named type I, II or III. These cell types can be distinguished based on their ultrastructural morphology, expression of specific proteins and functional properties. Type II and type III cells express taste receptors and respond to tastants, while type I cells mainly have glial-like functions. Type I cells have also been suggested to be involved in salt transduction because they express amiloride-sensitive Na\(^+\) channels (Vandenbeuch et al. 2008; Roper & Chaudhari, 2017; Baumer-Harrison et al. 2020; Yang et al. 2020), but a recent study demonstrated that amiloride-sensitive salt taste is transmitted to the nervous system by a unique cell type that expresses the ATP release channel CALHM1/3 and voltage-gated Na\(^+\) channels, neither of which are found in type I taste cells (Nomura et al. 2020).

Type II cells express G protein-coupled receptors for sweet (TAS1R2–TAS1R3), umami (TAS1R1–TAS1R3) or bitter (TAS2R) detection and use at least one common transduction cascade that involves the G-protein alpha subunit gustducin (GNAT3), phospholipase-C-beta-2 (PLC\(\beta\)2), inositol-3-phosphate (IP3) and Ca\(^{2+}\) release from intracellular stores (Zhang et al. 2003; Hisatsune et al. 2007). The increased intracellular Ca\(^{2+}\) concentration activates TRPM5 and TRPM4, causing membrane depolarization by Na\(^+\) entry and release of ATP through voltage-gated CALHM1/CALHM3 channels (Dutta Banik et al. 2018; Ma et al. 2018).

Type III cells mediate sour detection through the apical ion channel OTOPI that allows entry of H\(^+\) and cell membrane depolarization, which is further amplified by the block of the inwardly rectifier K\(^+\) channel KCNJ2 by low pH (Ye et al. 2016; Tu et al. 2018; Teng et al. 2019). Type III is the only taste cells that form canonical synapses with afferent nerve fibres.

They express the presynaptic synaptosomal-associated protein 25 (SNAP25), the glutamic acid decarboxylase 67 (GAD67) and voltage-gated Ca\(^{2+}\) channels, which trigger the release of neurotransmitters (Yang et al. 2000; DeFazio et al. 2006; Tomchik et al. 2007; Huang et al. 2008; Vandenbeuch et al. 2010).

Type I cells are called ‘glial-like’ cells and are mainly considered to have a supporting role in the taste buds. These cells have projections that wrap around other cells and express proteins involved in removing neurotransmitters, such as the ectonucleotidase TNPase2 (encoded by Entpd2) and the glutamate transporter GLAST (Lawton et al. 2000; Bartel et al. 2006). Moreover, the inwardly rectifier K\(^+\) channel KCNJ1 (also named K\(_{\text{ir}}\)1.1 or renal outer medullary K, ROMK) is expressed at the apical tips of type I cells and may be involved in K\(^+\) homeostasis (Dvoryanchikov et al. 2009).

The three cell types can also be identified by their electrophysiological fingerprint. Indeed, by using voltage-clamp whole-cell recordings in pseudo-physiological solutions, type I cells are characterized by the presence of voltage-gated outward K\(^+\) currents and no inward currents, type II cells by voltage-gated inward Na\(^+\) currents and voltage-gated outward non-selective currents, and type III cells by voltage-gated inward Na\(^+\) and Ca\(^{2+}\) currents and by voltage-gated outward K\(^+\) currents (Medler et al. 2003; Clapp et al. 2006; Romanov & Kolesnikov, 2006; Romanov et al. 2007; Taruno et al. 2013).

Multiple chloride conductances, including Ca\(^{2+}\)-dependent Cl\(^-\) currents, have been recorded in some taste cells (McBride & Roper, 1991; Taylor & Roper, 1994; Wladkowski et al. 1998; Herness & Sun, 1999; Kim et al. 2000; Cherkashin et al. 2016). Kim et al. (2000) clearly demonstrated that a subpopulation of unidentified taste cells had Ca\(^{2+}\)-activated Cl\(^-\) currents and that extracellular ATP could indirectly activate these channels by intracellular Ca\(^{2+}\) increase through activation of P2Y receptors. Ca\(^{2+}\)-activated Cl\(^-\) channels are encoded by two proteins of the TMEM16 family, TMEM16A and TMEM16B (also named Ano1 and Ano2, respectively). They share several electrophysiological properties such as ionic permeability and voltage dependence but can be functionally distinguished by different Ca\(^{2+}\) sensitivity and pharmacological profile (Caputo et al. 2008;
Schroeder et al. 2008; Yang et al. 2008; Stephan et al. 2009; Stöhr et al. 2009; Pifferi et al. 2009a; Scudieri et al. 2012; Pedemonte & Galietta, 2014). Cherkashin et al. (2016) confirmed and extended previous results from Kim et al. (2000) showing that the population of taste cells generating Ca\(^{2+}\)-activated Cl\(^{-}\) currents in response to P2Y agonists largely consisted of type I cells, but that also some type II cells exhibited small Ca\(^{2+}\)-activated Cl\(^{-}\) currents. They indicated that TMEM16B was functional in type I cells, TMEM16A and TMEM16B produced small currents in type II cells, while type III cells did not exhibit any Ca\(^{2+}\)-activated Cl\(^{-}\) current.

Here, by taking advantage of Tmem16a and Tmem16b knockout (KO) mice models (Rock et al. 2008; Zhang et al. 2017) and of a recently discovered specific blocker of TMEM16A, Ani-9 (Seo et al. 2016), we re-examined the functional expression of the two Ca\(^{2+}\)-dependent Cl\(^{-}\) channels of the TMEM16 family in taste cells of vallate papillae by using electrophysiological recordings and immunohistochemistry. Surprisingly, we found that only TMEM16A, and not TMEM16B, is expressed in taste buds. In particular, TMEM16A is largely colocalized with the inwardly rectifier K\(^{+}\) channel KCNJ1 at the apical portion of type I cells. Moreover, by using patch-clamp whole-cell recordings, we functionally measured TMEM16A-mediated currents only in type I and not in type II cells. We then investigated whether TMEM16A-mediated currents in type I cells could be activated by extracellular ATP, a well-known transmitter released by type II cells in taste buds and found that ATP induced Ca\(^{2+}\)-activated Cl\(^{-}\) currents that were blocked by Ani-9, further confirming that they were due to TMEM16A channels. We conclude that TMEM16A, but not TMEM16B, plays a role in taste buds.

Although type I cells make up more than 50% of the cells in each taste bud and are known to have glial-like functions, they have often been overlooked in favour of studies on type II and III cells. A complete knowledge of the functional ion channels of type I cells will help to understand additional roles that these cells may play in taste buds. Although further studies are required to determine the physiological role of TMEM16A in taste buds, we discuss some possible scenarios.

**Methods**

**Animals and ethical approval**

Mice were handled according to the guidelines of the Italian Animal Welfare Act (Decreto legislativo 26/2014/) and European Union guidelines on animal research (2010/63) under a protocol approved by the Italian Ministry of Health. Mice had free access to water and food. Every effort was made to reduce the number of animals used. Adult (> 8 weeks) mice were anaesthetized with CO\(_2\) and decapitated before tongue and nose removal. Mice were transferred to a cage (8 × 10 × 12 cm, height/width/length) and 100% CO\(_2\) was slowly injected into the cage until the animal stopped breathing and no longer displayed pedal reflex (near 3 min, gas flow rate was ∼20% of chamber volume per minute). Young mice, postnatal days 8–10 (P8–P10), were decapitated before tongue removal. Experiments were performed on tissues from both male and female C57BL/6, GAD67-GFP (Oliva et al. 2000), Tmtn16a KO (Rock et al. 2008) or mCherry Tmtn16b KO mice (Zhang et al. 2017). Tmtn16a KO and wild-type (WT) littermates were obtained by breeding heterozygous mice. mCherry Tmtn16b KO mice were kindly provided by Dr Lily Jan (University of California, San Francisco, USA, Zhang et al. 2017). The generated Tmtn16b-deficient mice were engineered by inserting mCherry sequence with a farnesylation signal at the C-terminus (mCherry-F) in frame with the alternative start ATG codon in the third exon of Tmtn16b, and therefore membrane-associated mCherry marks cells that normally express Tmtn16b (see fig. S2C of Zhang et al. 2017).

**Tissue preparation**

The epithelium containing the taste papillae was peeled using an enzymatic–mechanical dissociation protocol slightly modified from those previously reported (Béhé et al. 1990; Bigiani, 2001; Cherkashin et al. 2016). Once removed, the tongue was placed in a Sylgard Petri dish filled with a standard mammalian Ringer solution containing (in mm): 140 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 Hepes, 10 glucose, pH 7.4, with NaOH. Using an insulin syringe, 0.5 ml Ringer solution containing 2 mg ml\(^{-1}\) dispase II (D4693, Sigma, St Louis, MO, USA), 0.2 mg ml\(^{-1}\) elastase (E0127, Sigma) and 0.7 mg ml\(^{-1}\) collagenase B (C9891, Sigma) was injected under the epithelium. The syringe needle was carefully inserted from the posterior to the most anterior part of the tongue. Injection of the enzymatic solution was performed while the needle was gently withdrawn to allow the solution to reach the entire epithelium. After 30 min of incubation, the epithelium was peeled off from the underlying muscle and the vallate papilla and taste cells were isolated as described in the following sections.

**RT-PCR**

The vallate papilla was cut from the peeled tongue epithelium and directly placed in the lysis buffer of the mRNA isolation kit (S1550S, New England Biolabs, Ipswich, MA, USA). The olfactory epithelium was isolated as previously described (Pifferi et al. 2006; Sagheddu et al. 2010). The olfactory turbinates were exposed by bisecting the head along the midline, the epithelium was
carefully removed from turbinates, septum and the roof of the nasal cavity and placed directly in the lysis buffer of the mRNA isolation kit. Vallate papillae mRNA was extracted from four adult C57BL/6 mice and olfactory epithelium mRNA was extracted from two adult C57BL/6 mice. The mRNA isolation kit was used with Oligo d(T)$_{25}$ magnetic beads for mRNA isolation. After extraction, mRNA was further incubated with DNase I (M0303S, New England Biolabs) to remove any residual genomic DNA. cdNA was synthesized using the SMARTER cDNA synthesis kit from 25 ng of mRNA (634860, Takara, Shiga, Japan).

PCR was performed in a thermocycler (ThermaCycler2720, Life Technologies, Carlsbad, CA, USA) using Phusion HS II High-Fidelity DNA Polymerase (F5495, ThermoScientific, Waltham, MA, USA), 0.2 mM for each dNTPs (N0447S, New England Biolabs) and 200 pmol forward/reverse target-specific primers. Cycling parameters were: an initial denaturation step (98°C, 2 min) followed by 38 cycles, each of these cycles including a denaturation step (98°C, 10 s), a primer annealing step (62–64°C, 30 s) and an extension step (72°C, 30 s) step. The reaction was completed by a final extension step at 72°C for 5 min.

The following primer sequences were used to amplify target DNAs: Gapdh fwd 5′-TGCAGATGATGCTGGAGTCT-3′ rev 5′-TCGGGAGCTGACATGGTAC-3′ (Tm = 64°C; 691 bp; GenBank accession no. NM_008084.3); Olfr73 fwd 5′-CCTGGATTAATGACCGTCTTG-3′ rev 5′-CCGTGAGTGGCTGAACCTG-3′ (Tm = 62°C; 272 bp; GenBank accession no. NM_054090.1); Tmem16a fwd 5′-ATTAGGGCAGGTCTTGCAGGAGT-3′ rev 5′-AAACCTTCACTCCAGCAGAATG-3′ (Tm = 62°C; 296 bp; GenBank accession no. NM_178642.6); Tmem16b fwd 5′-ATGACATTTTTCAGGACAAC-3′ rev 5′-GCCGACGCCATCGGGT-3′ (Tm = 62°C; 243 bp; GenBank accession no. NM_001364563.1); Entpd2 fwd 5′-CTCAAGATGGGATGGCTCTGTG-3′ rev 5′-CAGAGACGCCAGACAGGAG-3′ (Tm = 62°C; 834 bp; GenBank accession no. NM_009849.2); Pcl2b fwd 5′-TGAGGTGACAGCTTGGAGGA-3′ rev 5′-GGTGGCAGGAGGCTACTGAAG-3′ (Tm = 62°C; 842 bp; GenBank accession no. NM_001320790.1); Snap25 fwd 5′-TGACACTAGACTTTTGGTATG-3′ rev 5′-GCATCTTTGTTGCGGTCGTTG-3′ (Tm = 62°C; 481 bp; GenBank accession no. NM_001355254.1). The products were visualized following agarose gel electrophoresis (1.5%) and DNA was stained with Midori Green Advance (MG04, Nippon Genetics, Düren, Germany).

Immunohistochemistry

Immunohistochemistry was performed on tongue sections containing the vallate papilla and on olfactory epithelium sections. The dissected tongues were directly fixed in 4% paraformaldehyde in PBS, pH 7.4, for 3–4 h at 4°C. For nose isolation, the lower jaw, the posterior part of the brain and the skinhead were removed. The remaining head, containing the nasal cavity, was fixed in 4% paraformaldehyde in PBS, pH 7.4, for 4–5 h at 4°C and then decalcified in 0.5 m EDTA, pH 8, for 2 days, as previously described (Pifferi et al. 2006, 2009b; Maury & Menini, 2014; Maury et al. 2015; Henriques et al. 2019). For cryoprotection, tongue and olfactory tissues were equilibrated overnight in 30% (w/v) sucrose in PBS at 4°C. Tissues were frozen in optimal cutting temperature compound (Bio-Optica, Milan, Italy) and stored at −80°C before sectioning with a cryostat. Coronal sections (14–16 μm thick) were cut with a cryostat and mounted on Superfrost Plus Adhesion Microscope Slides (ThermoFisher Scientific). Sections were air-dried for 3 h and stored at −80°C for further use. For antigen retrieval, sections were kept in SDS 0.5% (w/v) in PBS for 15 min at room temperature. After pre-treatment, sections were incubated in blocking solution [5% (v/v) FBS or donkey serum and 0.3% (v/v) Triton X-100 in PBS] for 2 h, and then with the primary antibody (diluted in the blocking solution) overnight at 4°C. Slices were then rinsed with PBS and incubated with the chosen fluorophore-conjugated secondary antibody diluted in PBS-T (0.1% Tween 20 in PBS) for 2 h at room temperature. After washing with PBS-T, sections were treated with DAPI (0.2 μg/ml) for 30 min, washed with PBS-T, and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) or Fluoromount-G (ThermoFisher).

The following primary antibodies (dilution; catalogue number, company) were used: polyclonal rabbit anti-TMEM16A (1:100; ab53212, Abcam, Cambridge, MA, USA), polyclonal goat anti-TMEM16A (1:50; sc-69343, Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal rabbit anti-TMEM16B (1:200; 20 647-1-P; Proteintech, Rosemont, IL, USA), polyclonal goat anti-TMEM16B (1:200; sc-206, Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal rabbit anti-NTPDase2 (1:500; mN2-36LI6, from J. Sévigny at Centre de Recherche du CHU de Québec, Université Laval, Quebec http://ectonucleotidases-ab.com), polyclonal rabbit anti-KCNJ2 (1:500; APC-001, Alomone Labs, Jerusalem, Israel), polyclonal goat anti-GNAT3 (1:1000; OAE00418, Aviva Systems Biology, San Diego, CA, USA), polyclonal rabbit anti-PLCβ2 (1:200; sc-206, Santa Cruz Biotechnology), polyclonal chicken anti-GFP (1:200; GTX13970, GeneTex, Irvine, CA, USA), polyclonal rabbit anti-NTPDase2 (1:500; mN2-36LI6, from J. Sévigny at Centre de Recherche du CHU de Québec, Université Laval, Quebec http://ectonucleotidases-ab.com), polyclonal rabbit anti-KCNJ2 (1:500; APC-001, Alomone Labs, Jerusalem, Israel), polyclonal goat anti-GNAT3 (1:1000; OAE00418, Aviva Systems Biology, San Diego, CA, USA), polyclonal rabbit anti-PLCβ2 (1:200; sc-206, Santa Cruz Biotechnology), polyclonal chicken anti-GFP (1:200; GTX13970, GeneTex, Irvine, CA, USA), polyclonal rabbit anti-RFP antibody (1:500, 600-401-379, Rockland).

The following secondary antibodies were used: donkey anti-rabbit Alexa Fluor Plus 594 (1:500; A32754, Life Technologies), donkey anti-goat Alexa Fluor 488 (1:500; A11055, Life Technologies), donkey anti-rabbit Alexa Fluor 488 (1:500; A21206, Life Technologies), donkey anti-goat Alexa Fluor Plus 647 (1:500; A32849, Life Technologies).
Electrophysiological recordings from taste bud cells

The peeled tongue epithelium was placed upside-down in a Sylgard Petri dish, in Ca$^{2+}$-free Ringer solution containing (in mM): 140 NaCl, 5 KCl, 1 MgCl$_2$, 0.5 EDTA, 10 Heps, 10 glucose, pH 7.4, with NaOH. After 30 min of incubation, cells were removed from taste buds of the vallate papilla by gentle suction with a flame-polished glass capillary tube with an opening diameter of 40–50 μm and plated in Petri dishes, pre-coated with 5 mm Concanavalin-A (Type V, Sigma, Milan, Italy), for at least 1 h to favour cell adhesion.

Isolated taste cells were continuously perfused with Ringer solution. Cells were viewed with an inverted microscope (IMT-2 or IX70, Olympus), equipped with 10× and 40× objectives and an additional 1.5× auxiliary lens, and identified by their elongated shape. Whole-cell membrane currents were recorded at room temperature (21–24°C) in the voltage-clamp mode, using an Axopatch 200B or a Multiclamp 700B amplifier controlled by Clampex’s Junction Potential Calculator (based on Barry, 1994). Potential relationships were measured using a ramp protocol from −70 to +100 mV at 0.85 mV ms$^{-1}$.

Extracellular solutions were exchanged through a three-barrel square glass (3SG700-5, Warner Instruments, Hamden, CT, USA) using the gravity-driven perfusion system Fast-Step SF-77B (Warner Instruments). Different solutions were flowing side by side in each glass pipe and a stepper motor moved different pipes, and therefore different solution, in front of the cell. The time course of solution exchange was about 50 ms (see Fig. 9A).

The bath was grounded through a 3 m KCl agar bridge connected to an Ag/AgCl reference electrode. Membrane potentials were not corrected for liquid junction potentials whose values were less than 7 mV as calculated using the Clampex’s Junction Potential Calculator (based on Barry, 1994).

Chemicals, unless otherwise stated, were purchased from Sigma.

Electrophysiological recordings from HEK-293 cells

HEK-293 cells were grown in medium composed of DMEM (Gibco, Italy) supplemented with 10% FBS (Sigma, Italy), 100 IU ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin (Sigma, Italy) at 37°C in a humidified atmosphere of 5% CO$_2$. pEGFP-N1 plasmid containing the cDNA of mouse TMEM16A (version ac, as in Ferrera et al. 2009) was provided by Professor Criss Hartzell (Emory University, USA). HEK-293 cells were transfected with 2 μg of plasmid using the transfection reagent XtremeGENE (Roche Diagnostic, Indianapolis, IN, USA). Twenty-four hours after transfection, the cells were subcultured in 35-mm Petri dishes at a lower density. Electrophysiological recordings were performed between 48 and 72 h after transfection as previously described.
(Pifferi et al. 2009a). TMEM16A-transfected HEK-293 cells were identified by enhanced green fluorescent protein (EGFP) fluorescence using an Olympus IX70 microscope (Olympus, Japan) equipped with the appropriate filter. TMEM16A currents were recorded in the whole-cell configuration in voltage-clamp mode as described for taste bud cells. Cells were kept in mammalian Ringer solution composed of (in mM) 140 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 glucose and 10 Hepes, pH 7.4 with NaOH. The pipette solution contained (in mm): 140 NMDG-Cl, 10 HEDTA, 10 Hepes, 1.24 CaCl$_2$ adjusted to pH 7.3 with NMDG to obtain the final concentration of 0.5 μM free Ca$^{2+}$. The extracellular solution contained (in mm): 140 NMDG-Cl, 2 CaCl$_2$, 1 MgCl$_2$, 10 Hepes, adjusted to pH 7.4 with NMDG. Ani-9 was prepared in DMSO at 10 mM as a stock solution and diluted in the extracellular solution to the final concentration of 1 μM.

### Analysis of electrophysiological data

Igor Pro software (WaveMetrics, Lake Oswego, OR, USA) was used for data analysis and to produce the figures. All averaged data from individual experiments in different cells are presented as mean ± standard deviation and number of cells (n). Statistical analyses of normally distributed data (Shapiro–Wilk test) were performed using unpaired t tests. For not normally distributed data, the Mann–Whitney U-test was used. The homogeneity of the variance was tested using Levene’s test. P values of <0.05 were considered statistically significant.

### Results

#### RT-PCR

To evaluate the expression of *Tmem16a* and *Tmem16b* transcripts in taste buds, we performed RT-PCR analysis on whole mRNA extract from vallate papillae (Fig. 1). To check the integrity and specificity of the mRNA extracts, we amplified markers for the three types of taste bud cells: *Entpd2* for type I, *Plcβ2* for type II and *Snap25* for type III, and for the housekeeping gene *Gapdh* (Fig. 1B). By using primers that span specific and conserved regions from *Tmem16a* and *Tmem16b* sequences, to recognize all isoforms described for both proteins (O’Driscoll et al. 2011; Ponissery Saidu et al. 2013), we amplified *Tmem16a*, but not *Tmem16b* mRNA (Fig. 1A). As lack of *Tmem16b* transcript amplification could be due to problems in the primers themselves, we validated them by performing RT-PCR on whole mRNA extracts from the olfactory epithelium, where expression of *Tmem16a* and *Tmem16b* is well established (Dauner et al. 2012; Maurya & Menini, 2014). RT-PCR from the olfactory epithelium showed the expression of both *Tmem16a* and *Tmem16b* transcripts (Fig. 1C), confirming primer integrity. These results indicate that *Tmem16a*, but not *Tmem16b*, transcripts are expressed in mouse vallate papillae.

#### Expression of TMEM16A in taste buds of vallate papillae

To examine the expression and localization of the TMEM16A and TMEM16B proteins in mouse taste buds from vallate papillae, we performed immunohistochemistry and used KO mice as controls (Figs 2 and 3). Figure 2B shows that TMEM16A was expressed in taste buds from adult mice, with a more intense staining near the apical tip. To verify the specificity of the TMEM16A antibody, we performed control experiments using *Tmem16a* KO mice (Fig. 2C and D). As these mice die a few days after birth (Rock et al. 2008), we could not test adult animals, but we compared results from WT

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and KO mice of about the same age (P8–P10). We found no signal for Tmem16a in KO mice (Fig. 2D), confirming the specificity of the immunostaining for TMEM16A obtained in WT mice.

By contrast, we did not detect immunoreactivity to TMEM16B in taste buds. Figure 3 shows a comparison between confocal micrographs from sections of mouse vallate papilla (top row) and olfactory epithelium (bottom row). TMEM16B immunoreactivity was absent in taste buds (Fig. 3B), although the same antibody revealed the expression of TMEM16B at the apical surface of the olfactory epithelium (Fig. 3E), as previously shown (Maurya et al. 2015; Dibattista et al. 2017). We further investigated the expression of TMEM16B in taste buds by taking advantage of a Tmem16b KO mouse line that was engineered to express mCherry in cells that normally express TMEM1B (Zhang et al. 2017). It is important to note that the use of mCherry Tmem16b KO mice allows the visualization of the entire cells including all membranes that allow farnesylated mCherry to associate with. As a positive control, we used the olfactory epithelium, where TMEM1B is expressed at the apical side of olfactory sensory neurons (Fig. 3E). Figure 3F shows that mCherry was expressed, as expected, in the entire olfactory neurons of KO mice, including cilia, dendrites, somas and axons. By contrast, mCherry was not expressed in taste buds (Fig. 3C), further indicating that TMEM1B is not expressed in mouse vallate papillae.

To identify which cell types express TMEM16A in the taste buds, we performed immunohistochemistry using specific markers for each cell type. Indeed, taste buds are composed of three major cell types, characterized by their morphology and expression of specific proteins (Liman et al. 2014; Roper & Chaudhari, 2017; Kinnamon & Finger, 2019). Type I cells are often identified by NTPDase2 expression (Bartel et al. 2006) and also strongly express at their apical tip the inwardly rectifying potassium channel KCNJ1 (Dvoryanchikov et al. 2009). Type II cells specifically express PLCβ2 (Zhang et al. 2003), while GAD-67 can be used to stain a subset of type III cells (DeFazio et al. 2006; Tomchik et al. 2007). Figure 4A–C shows that TMEM16A was mainly expressed at the apical part of the taste buds and it was difficult to assess co-expression with NTPDase2, PLCβ2 or GAD-67. Lack of co-localization of TMEM16A with these cell markers can be explained because of the different subcellular expression. We took advantage of the specific expression of KCNJ1 at the apical tips of type I cells, and we evaluated the co-localization of this marker with TMEM16A. Figure 4D–F shows that TMEM16A co-localized with KCNJ1 at the apical tip of taste buds, indicating the expression of TMEM16A in type I cells.

Together, these results show that TMEM16A, but not TMEM16B, is expressed in taste buds of vallate papillae, and that TMEM16A is mainly localized at the apical part of type I cells.

Ca$^{2+}$-activated Cl$^-$ currents in taste cells

We investigated whether taste cells have functional Ca$^{2+}$-activated Cl$^-$ channels and compared whole-cell recordings in dissociated cells dialysed with intracellular solutions in the presence or in the absence of 1.5 μM free Ca$^{2+}$.

In a first set of experiments, we evaluated the viability of electrophysiological recordings and the possibility to identify the three types of taste cells using a KCl-based intracellular solution in the absence of Ca$^{2+}$. Indeed, it is difficult to identify the dissociated cell types by their morphology, but their electrophysiological fingerprint can be used to identify them. It has been shown that, in physiological solutions, type I cells are characterized by the presence of only voltage-gated outward K$^+$ currents, type II cells by the combination of voltage-gated inward Na$^+$ and non-selective outward currents, and type III cells by both voltage-gated inward Na$^+$ and outward K$^+$ currents (Bigiani, 2001; Medler et al. 2003; Noguchi et al. 2003; Romanov & Kolesnikov, 2006; Vandenbeuch et al. 2008). Figure 5A shows representative recordings from taste cells displaying three different patterns of currents typical of...
the different cell types: (i) cells with only outward currents (type I, green traces); (ii) cells with small inward and large outward currents (type II, blue traces); and (iii) cells with large inward and outward currents (type III, orange traces), indicating that all three cell types are viable in our preparation of dissociated taste cells.

In a second set of experiments, we modified the intracellular solution to suppress outward K$^+$ currents by replacing K$^+$ ions with NMDG$^+$, a large cation that does not permeate K$^+$ channels. Figure 5B shows that outward currents were eliminated from type I and type III cells, while large voltage-gated outward currents were still present in type II cells as they are due to the activation of CALHM1/CALHM3 channels, which are characterized by a wide pore with very weak ion selectivity and allow the permeation of large ions, including NMDG$^+$ (Taruno et al. 2013; Ma et al. 2016, 2018; Bigiani, 2017). In total, we recorded from 709 viable taste cells and electrophysiologically identified 279 type I (39%), 198 type II (28%) and 232 type III cells (33%).

As our goal was the measurement of Ca$^{2+}$-activated Cl$^-$ currents in electrophysiologically identified cells, we further replaced the external Ringer solution with a one containing only NMDG-Cl and set the resting potential to 0 mV. In these experimental conditions, with NMDG$^+$ as the main intra- and extracellular cation, there is no contribution from inward and outward currents due to voltage-gated Na$^+$ and K$^+$ channels (Fig. 5C). To investigate the presence of Ca$^{2+}$-activated currents in the three types of taste cells, we compared recordings obtained in symmetrical NMDG-Cl solutions with nominally 0 Ca$^{2+}$ or 1.5 $\mu$M free intracellular Ca$^{2+}$. The cell type was determined in external Ringer solution by using the voltage protocol shown at the top of Fig. 5B and then Ringer solution was replaced by NMDG-Cl as in Fig. 5C.

Type II cells showed voltage-gated outward currents slowly activated by depolarization both in the absence and in the presence of 1.5 $\mu$M intracellular free Ca$^{2+}$ (Fig. 6A). Average current amplitudes at +100 mV were not significantly different (Fig. 6B; $P = 0.14$ unpaired $t$ test), indicating that no additional currents were activated by Ca$^{2+}$ in type II cells. To further investigate the presence of Ca$^{2+}$-activated currents that could be masked by the very large CALHM-mediated outward currents, we blocked these currents by adding 200 $\mu$M Gd$^{3+}$, a non-specific ion channel blocker, and verified that also after the blockage the residual outward current amplitudes were not significantly different with 0 or 1.5 $\mu$M Ca$^{2+}$ (Fig. 6A and B; $P = 0.57$ Mann–Whitney $U$-test). As...
a control, we also tested if Gd$^{3+}$ blocks TMEM16A or TMEM16B channels, by recording Ca$^{2+}$-activated Cl$^{-}$ currents in HEK293T cells transfected with TMEM16A or TMEM16B and did not measure any blocking effect by 200 μM GdCl$_3$ on these channels (data not shown).

Type III cells showed negligible currents both in the absence and in the presence of 1.5 μM Ca$^{2+}$, indicating the absence of functional Ca$^{2+}$-activated currents in symmetrical NMDG-Cl solutions (Fig. 6C and D; $P = 0.33$ unpaired $t$ test).

In type I cells, 1.5 μM free Ca$^{2+}$ generated large currents while currents in 0 Ca$^{2+}$ were very small (Fig. 7A and B). Ca$^{2+}$-activated currents had the typical time-dependent features of Ca$^{2+}$-activated Cl$^{-}$ channels, displaying time-dependent deactivation kinetics with hyperpolarizing potentials, activation kinetics with depolarizing potentials, and deactivating inward tail currents when the voltage was stepped to −100 mV at the end of the protocol (Fig. 7A, D and G). The I–V relationship of the steady-state Ca$^{2+}$-activated current at the end of the voltage steps was outwardly rectifying with an average ratio between currents at +100 mV and −100 mV of 1.6 ± 0.7 ($n = 17$).

To examine whether the Ca$^{2+}$-activated current in type I cells was carried by Cl$^{-}$, we replaced extracellular Cl$^{-}$ with MeS$^{-}$, a large impermeant anion and found that the reversal potential shifted toward a positive value (+36 ± 8 mV, $n = 7$), as expected for Cl$^{-}$ channels, indicating that the Ca$^{2+}$-activated current in type I cells is mainly carried by Cl$^{-}$ (Fig. 7D–F). We also tested the blockade of Ca$^{2+}$-activated Cl$^{-}$ currents by Ani-9, a compound that fully blocks TMEM16A in the sub-micromolar range and has a high selectivity for TMEM16A compared with TMEM16B (Seo et al. 2016). Figure 7G and H show that 1 μM Ani-9 caused a strong reversible inhibition of the current in WT type I cells, with an average current block of 75% ± 8% at +100 mV and 90 ± 5% at −100 mV (Fig. 7I), indicating that the current was mainly carried through TMEM16A channels.

We also performed experiments in type I taste cells from Tmem16b KO mice. In agreement with our previous results showing the absence of TMEM16B in taste buds (Figs 1 and 3), we did not find any statistical difference between Ca$^{2+}$-activated currents in type I taste cells from WT and Tmem16b KO (Fig. 7C). Moreover, the shift of the reversal potential upon replacement of Cl$^{-}$ with MeS$^{-}$ and the blocking effect of Ani-9 were similar in type I taste cells from WT and Tmem16b KO (Fig. 7F and I), in agreement with the previous experiments indicating that TMEM16A is the channel responsible for the measured Ca$^{2+}$-activated Cl$^{-}$ currents.

To further investigate the possibility that a small inward current could be activated by Ca$^{2+}$ in type II or III taste cells, we compared recordings in symmetrical NMDG-Cl solutions from the three cell types obtained by holding the cell at −100 mV for several seconds in the presence of 1.5 μM Ca$^{2+}$ in the pipette. Figure 8 further shows that large currents blocked by 1 μM Ani-9 were activated only in type I cells, while type II and III cells had low-amplitude currents that were not blocked by Ani-9 (Fig. 8A, C and E). Average current amplitudes at −100 mV were $-1083 ± 824$ pA ($n = 17$) for type I, $-68 ± 95$ pA ($n = 18$) for type II and $-6 ± 8$ pA ($n = 13$) for type III cells. Cell viability was tested after application of the blocker (Fig. 8B, D and F). A significant blockage by Ani-9 in type I was observed within a few seconds from the application of the
blocker and was very similar to that measured in HEK-293 cells transiently transfected with *Tmem16a* (Fig. 9B).

We could not perform experiments in taste cells from *Tmem16A* KO mice because more than 50% of these mice die within 5 days of birth (Rock *et al.* 2008) and, as shown by Bigiani *et al.* (2002), it is difficult to identify taste buds in very young mice, preventing the possibility of obtaining a number of dissociated cells sufficient for electrophysiological experiments.

Collectively, these data show that intracellular Ca\(^{2+}\) can activate a large Cl\(^{-}\) current in type I (but not in type II or III) taste cells, carried through the TMEM16A channel.

**ATP evokes a Cl\(^{-}\) current in type I taste cells**

Studies by Kim *et al.* (2000) and Cherkashin *et al.* (2016) showed that a subpopulation of taste cells exhibited large Ca\(^{2+}\)-activated Cl\(^{-}\) currents when stimulated with ATP.
P2Y receptor agonists. Perforated patch recordings showed that the subpopulation consisted of type I cells (Cherkashin et al. 2016). Here, we used whole-cell recordings instead of perforated patch to test whether ATP stimulation could activate Ca\(^{2+}\)-activated Cl\(^{-}\) currents in type I cells. We found that 50 \(\mu\)M ATP at the holding potential of −70 mV induced large inward currents in 71% of type I taste cells (89 out of 125) in extracellular Ringer solution (Fig. 10A). ATP-evoked currents were still present when NaCl in the Ringer solution was replaced with NMDG-Cl and 0 K\(^{+}\), indicating that the inward current was not carried by Na\(^{+}\) or K\(^{+}\) (Fig. 10B).

To measure the I–V relationship and to determine the ionic selectivity of the ATP-induced current, we applied voltage ramps before and during ATP stimulation in various ionic conditions (Fig. 10C and D). The I–V relationship of the current activated by ATP was calculated by subtracting the current generated by the voltage ramp before stimulation (Fig. 10E and F). Rectification properties in Ringer varied in different cells from outward to linear rectification, with an average ratio of the current amplitude at +60 mV and −60 mV of 1.3 ± 0.6 (\(n = 18\), Fig. 10G). Different rectifications are probably due to different intracellular Ca\(^{2+}\) concentrations elicited by ATP. The average reversal potential was −6 ± 8 mV (\(n = 20\)) in Ringer solution and shifted to +40 ± 9 mV (\(n = 7\)) upon reduction of the extracellular Cl\(^{-}\) concentration by replacing NaCl with sodium gluconate, indicating that the current was mainly carried by Cl\(^{-}\) (Fig. 10H). To test whether the ATP-induced Cl\(^{-}\) current was due to activation of TMEM16A, we applied the specific blocker Ani-9. Figure 10H shows that Ani-9 caused a sudden reduction of the inward current with a time course similar to that observed in HEK-293 transiently transfected with TMEM16A (Fig. 9B), indicating that the Cl\(^{-}\) current was carried through TMEM16A channels. However, we cannot completely exclude the possibility that a prolonged application of Ani-9 could have a non-specific action and perhaps inhibit ATP-induced Ca\(^{2+}\) signals as observed in some cell lines (Centeio et al. 2020).

Thus, our results in the whole-cell configuration confirm previous data obtained with perforated patch recordings (Kim et al. 2000; Cherkashin et al. 2016) showing that ATP evokes large Ca\(^{2+}\)-activated Cl\(^{-}\) currents in type I cells and extend those results showing that these currents are blocked by Ani-9, consistent with our data indicating that these currents are due to activation of TMEM16A channels.

**Discussion**

In this study, we have demonstrated that a large Ca\(^{2+}\)-activated Cl\(^{-}\) current can be physiologically activated in type I taste bud cells, but not in type II...
Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels in taste bud cells

Our study confirms some data of the previous pioneering reports showing the presence of Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} currents in taste cells and significantly extends and revises them (Cherkashin et al. 2016; Kim et al. 2000). Indeed, Kim et al. (2000) used perforated patch-clamp recordings combined with Ca\textsuperscript{2+} imaging and demonstrated that intracellular Ca\textsuperscript{2+} increase, produced by ionomycin or by ATP, activated Cl\textsuperscript{−} currents in a subpopulation of taste cells, which exhibited no voltage-gated Na\textsuperscript{+} and Ca\textsuperscript{2+} currents. The same authors also showed that the cytosolic Ca\textsuperscript{2+} increase produced by ATP was mediated by P2Y receptors, demonstrating that ATP indirectly activated Cl\textsuperscript{−} channels by causing Ca\textsuperscript{2+} release from intracellular sources.

Figure 7. Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} currents in type I taste bud cells

A, representative whole-cell recordings from type I taste cells measured in symmetrical NMDG-Cl solutions with an intracellular solution containing nominally 0 Ca\textsuperscript{2+} or 1.5 μM Ca\textsuperscript{2+}. The holding potential was 0 mV, and voltage steps from −100 mV to +100 mV with 20 mV increments were applied followed by a step to −100 mV. B and C, scatter dot plots with averages ± SD showing (B) current amplitudes measured at steady state in 0 or 1.5 μM Ca\textsuperscript{2+} (n = 15–17, ***P = 3.5 × 10\textsuperscript{−8}, −100 mV, P = 3.4 × 10\textsuperscript{−9}, +100 mV, Mann–Whitney U-test) and (C) Ca\textsuperscript{2+}-activated currents in cells from WT (n = 15) or Tmem16B KO (n = 7; P = 0.8, −100 mV, P = 0.41, +100 mV, Mann–Whitney U-test) mice. D and G, the same cell was exposed to a control solution containing NMDG-Cl (black traces), or NMDG-MeS (purple traces) or NMDG-Cl with 1 μM Ani-9 (yellow traces), followed by washout in NMDG-Cl (grey traces). E and H, steady-state I–V relationships measured at steady state from the recordings shown in D and G in control condition (squares), MeS (E, circles) or 1 μM Ani-9 (H, diamonds) and after washout (triangles). F and I, scatter dot plots with average ± SD showing (F) ΔE\textsubscript{rev} (mV) after MeS perfusion in cells from WT (n = 11) and Tmem16B KO (n = 7; P = 0.08 unpaired t test) mice, and (I) percentage of current inhibition by 1 μM Ani-9 measured at −100 and +100 mV in cells from WT (n = 5) and Tmem16B KO mice (n = 5; P = 0.27 unpaired t test for −100 mV, P = 0.2 unpaired t test for +100 mV) mice.
stores. Cherkashin et al. (2016) further investigated the distribution of Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} currents in taste cell types using the perforated patch clamp technique. They found clear evidence that type I cells have functional Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels that can be activated by ionomycin or purinergic agonists. By measuring the effect of available blockers for TMEM16A, they found that channels in type I cells were blocked by CaCCinh-A01 but not by T16Ainh-A01. The authors also recorded a very small inward current in type II cells activated by ionomycin or by photorelease of caged Ca\textsuperscript{2+} that could be blocked by T16Ainh-A01. Type III cells exhibited no ion current induced by ionomycin or photorelease of caged Ca. In addition, the same authors found transcripts of Tmem16a and Tmem16b in vallate papillae and confirmed their expression by using immunohistochemistry. They also identified Tmem16a transcripts in type II cells, and Tmem16b transcripts in both type I and type II cells. On the basis of their results, Cherkashin et al. (2016) concluded that type I cells express only TMEM16B, while type II cells express both TMEM16A and TMEM16B channels.

In our study, we used a more recently discovered selective blocker of TMEM16A, Ani-9 (Seo et al. 2016), and we were able to use KO mice for Tmem16a and Tmem16b as a control for the specificity of antibodies. This allowed us to confirm, revise and extend some of the previous discoveries. By using specific antibodies for TMEM16A and TMEM16B validated on tissues from KO mice, we confirmed the expression of TMEM16A in taste buds whereas we did not find expression of TMEM16B. We further investigated the expression of TMEM16B by using the Tmem16b KO mice line engineered by Zhang et al. (2017) to express mCherry under the Tmem16b promoter. We confirmed that mCherry was not expressed in taste buds, whereas it was expressed by olfactory sensory neurons in the olfactory epithelium, further showing that TMEM16B is not expressed in taste buds.

From a functional point of view, we performed patch-clamp experiments in the whole-cell configuration and compared recordings obtained with nominally 0 or 1.5 μM intracellular Ca\textsuperscript{2+} and measured large Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} current in type I cells blocked by Ani-9, a selective blocker of TMEM16A, whereas we could not measure any Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} current in type II and type III cells. Moreover, in type I cells, extracellular...
ATP produced activation of a large Cl\(^-\) current that was inhibited by Ani-9, further demonstrating the functional expression of TMEM16A in type I cells. Recently, Ávalos Padro et al. (2021) reported that the interaction of TMEM16A with KCNE1 switches the gating mode of TMEM16A from Ca\(^{2+}\)-dependent to voltage-dependent, showing that TMEM16A could also be activated in the absence of Ca\(^{2+}\) if it is associated with KCNE1. However, our data show that type II and type III cells lack voltage-gated Cl\(^-\) currents also in nominally 0 intracellular Ca\(^{2+}\) (Fig. 6), further excluding the possibility that TMEM16A could be active in these cell types.

As type II cells are very heterogeneous (Chandrashekar et al. 2006), we cannot exclude the possibility that some rare type II cells could exhibit Ca\(^{2+}\)-activated Cl\(^-\) currents.

### Physiological activators and role of Ca\(^{2+}\)-activated Cl\(^-\) currents in type I taste bud cells

Type I cells comprise about 50% of the cells in each taste bud and are considered to have mainly glial-like functions. Not much is known about their physiological roles, which still need to be extensively investigated. It is well established that ATP is released from type II taste cells and is degraded by NTPDase2 on the surface of type I cells (Bartel et al. 2006; Vandenbeuch et al. 2013). However, type I cells also express P2Y receptors that have been shown to increase cytosolic Ca\(^{2+}\) when activated by ATP (Bystrova et al. 2006; Kim et al. 2000). Immuno-histochemical data showed that P2Y\(_1\) receptors (Kataoka et al. 2004) and P2Y\(_2\) receptors (Bystrova et al. 2006) are expressed in the basolateral membrane of a subset of taste bud cells. Thus, we speculate that ATP released through CALHM1/CALHM3 channels of type II cells reaching the surface of type I cells, where it is degraded by NTPDase2, can also bind to P2Y receptors, and indirectly activate TMEM16A channels causing a flux of Cl\(^-\) according to its electrochemical gradient.

In addition to P2Y receptors, type I cells also express other receptors that, upon activation by their agonists, produce an increase in intracellular Ca\(^{2+}\) and can therefore activate TMEM16A channels. Sinclair et al. (2010) have shown that a subset of type I cells express the oxytocin receptor that upon stimulation by oxytocin causes an intracellular Ca\(^{2+}\) increase similar to that induced by ATP. In another study, Huang & Wu (2018) measured Ca\(^{2+}\) release from intracellular stores in type I cells in response to substance P.

What, then, is the physiological role of Ca\(^{2+}\)-activated Cl\(^-\) channels in type I taste cells? Depending on the Cl\(^-\) equilibrium potential and on the membrane potential, these channels may allow influx or efflux of Cl\(^-\), increasing or decreasing the cytoplasmic Cl\(^-\) concentration and
contributing to cell hyperpolarization or depolarization. At present, estimates of the Cl− concentration inside taste cells are not available and may differ between the basolateral and apical sides. The extracellular Cl− concentration varies depending on apical or basolateral parts of the cell. Indeed, the apical part of taste cells is likely to be immersed in saliva, while the cell body is surrounded by interstitial fluid. Saliva has a naturally low Cl− concentration, being composed of 15 mm NaCl, 22 mm KCl, 3 mm CaCl₂ and 0.6 mm MgCl₂, corresponding to a Cl− concentration of about 44 mm (Breza et al. 2010; Matsuo, 2000), while the interstitial fluid contains about 100−110 mm Cl− (Yunos et al. 2010). However, when salty tastants enter the taste pore, the concentration of Cl− can greatly increase, modifying the Cl− equilibrium potential.

We have shown that TMEM16A largely colocalized with the inwardly rectifying K+ channel KCNJ1 at the apical part of type I cells. KCNJ1 has been proposed to be involved in buffering of basolateral extracellular K+ by mediating the K+ efflux through the apical pore (Dvoryanchikov et al. 2009). As stimulation by tastants of type II cells produces both the local increase of extracellular K+ and release of ATP, we speculate that the activation of TMEM16A by ATP could mediate an influx of Cl− allowing a sustained apical extrusion of K+.

Interestingly, it has been shown that TMEM16A can be activated in the absence of intracellular Ca2+ by low extracellular pH through the titration of glutamic acid 623 (Cruz-Rangel et al. 2017). We speculate that this modulation could be relevant during sour stimulation to be involved in buffering of basolateral extracellular pH through the titration of glutamic acid.

References
Ávalos Padro P, Háfner S, Comoglio Y, Wdziekonski B, Duranton C, Attali B, Barhanin J & Sandoz G (2021). KCNE1 is an auxiliary subunit of two distinct ion channel superfamilies. Cell 184, 534–544.e11.
Barry PH (1994). JPCalc, a software package for calculating liquid junction potential corrections in patch-clamp, intracellular, epithelial and bilayer measurements and for correcting junction potential measurements. J Neurosci Methods 51, 107–116.
Bartel DL, Sullivan SL, Lavoie EG, Sévigny J & Finger TE (2006). Nucleoside triphosphate diphosphohydrolase-2 is the ecto-ATPase of type I cells in taste buds. J Comp Neurol 497, 1–12.
Baumner-Harrison C, Raymond MA, Myers TA, Sussman KM, Rynberg ST, Ugartechea AP, Lauterbach D, Mast TG & Breza JM (2020). Optogenetic stimulation of type I GAD65+ cells in taste buds activates gustatory neurons and drives appetitive licking behavior in sodium-depleted mice. J Neurosci 40, 7795–7810.
Béhé P, DeSimone JA, Avenet P & Lindemann B (1990). Membrane currents in taste cells of the rat fungiform papilla. Evidence for two types of Ca currents and inhibition of K currents by saccharin. J Gen Physiol 96, 1061–1084.
Beidler LM (1953). Properties of chemoreceptors of tongue of rat. J Neurophysiol 16, 595–607.
Bigiani A (2001). Mouse taste cells with glialike membrane properties. J Neurophysiol 85, 1552–1560.
Bigiani A (2017). Calcium homeostasis modulator 1-like currents in rat fungiform taste cells expressing amiloride-sensitive sodium currents. Chem Senses 42, 343–359.
Bigiani A, Cristiani R, Fieni F, Ghioroni V, Bagnoli P & Pietra P (2002). Postnatal development of membrane excitability in taste cells of the mouse vallate papilla. J Neurosci 22, 493–504.
Breza JM & Contreras RJ (2012). Anion size modulates salt taste in rats. J Neurophysiol 107, 1632–1648.
Breza JM, Nikonov AA & Contreras RJ (2010). Response latency to lingual taste stimulation distinguishes neuron types within the geniculate ganglion. J Neurophysiol 103, 1771–1784.
Bystrova MF, Yatzenko YE, Fedorov IV, Rogachevskaja OA & Kolesnikov SS (2006). P2Y isoforms operative in mouse taste cells. Cell Tissue Res 323, 377–382.
Caputo A, Caci E, Ferrera L, Pedemonte N, Barsanti C, Sondo E, Pfeffer U, Ravazzolo R, Zegarra-Moran O & Galietta LJV (2008). TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. Science 322, 590–594.
Centeio R, Cabrita I, Benedetto R, Talbi K, Ousingawat J, Schreiber R, Sullivan JK & Kunzelmann K (2020). Pharmacological inhibition and activation of the Ca2+ activated Cl− channel TMEM16A. Int J Mol Sci 21, 2557.
Chandrashekar J, Hoon MA, Ryba NJP & Zuker CS (2006). The receptors and cells for mammalian taste. Nature 444, 288–294.

Cherkashin AP, Kolesnikova AS, Tarasov MV, Romanov RA, Rogachevskaia OA, Bystrova MF & Kolesnikov SS (2016). Expression of calcium-activated chloride channels Ano1 and Ano2 in mouse taste cells. Pflugers Arch 468, 305–319.

Clapp TR, Medler KF, Damak S, Margolskee RF & Kinnamon SC (2006). Mouse taste cells with G protein-coupled taste receptors lack voltage-gated calcium channels and SNAP-25. BMC Biol 4, 7.

Cruz-Rangel S, De Jesús-Pérez JJ, Aréchiga-Figueroa IA, A Rodriguez-Menchaca AA, Pérez-Cornejo P, Hartzell HC & & Arreola J (2017). Extracellular proton enforces activation of the calcium-dependent chloride channel TMEM16A. J Physiol 595, 1515–1531.

Dauner K, Lissmann J, Jeridi S, Frings S & Möhrle F (2012). Expression patterns of anoctamin 1 and anoctamin 2 chloride channels in the mammalian nose. Cell Tissue Res 347, 327–341.

DeFazio RA, Dvoryanchikov G, Maruyama Y, Kim JW, Pereira E, Roper SD & Chaudhari N (2006). Separate populations of receptor cells and presynaptic cells in mouse taste buds. J Neurosci 26, 3971–3980.

Dibatista M, Pifferi S, Bocaccio A, Menini A & Reisert J (2017). The long tale of the calcium activated Cl⁻ channels in olfactory transduction. Channels (Austin) 11, 399–414.

Dutta Banik D, Martin LE, Freichel M, Torregrossa A-M & Medler KF (2018). TRPM4 and TRPM5 are both required for normal signaling in taste receptor cells. Proc Natl Acad Sci U S A 115, E772–E781.

Dvoryanchikov G, Sinclair MS, Perea-Martinez I, Wang T & Chaudhari N (2009). Inward rectifier channel, ROMK, is localized to the apical tips of glial-like cells in mouse taste buds. J Comp Neurol 517, 1–14.

Ferrera L, Caputo A, Ubby I, Bussani E, Zegarra-Moran O, Ravazzolo R, Pagani F & Galietta LJv (2009). Regulation of TMEM16A chloride channel properties by alternative splicing. J Biol Chem 284, 33360–33368.

Henriquez T, Agostinelli E, Hernandez-Clavijo A, Maurya DK, Rock JR, Harfe BD, Menini A & Pifferi S (2019). TMEM16A calcium-activated chloride currents in supporting cells of the mouse olfactory epithelium. J Gen Physiol 151, 954–966.

Herness MS & Sun XD (1999). Characterization of chloride currents and their noradrenergic modulation in rat taste receptor cells. J Neurophysiol 82, 260–271.

Hisatsune C, Yasumatsu K, Takahashi-Iwanaga H, Ogawa N, Kuroda Y, Yoshida R, Ninomiya Y & Mikoshi k (2007). Abnormal taste perception in mice lacking the type 3 inositol 1,4,5-trisphosphate receptor. J Biol Chem 282, 37225–37231.

Huang AY, Maruyama Y & Roper SD (2008). Norepinephrine is coreleased with serotonin in mouse taste buds. J Neurosci 28, 13088–13093.

Huang AY & Wu SY (2018). Substance P as a putative efferent transmitter mediates GABAergic inhibition in mouse taste buds. Br J Pharmacol 175, 1039–1053.

Huang YA, Maruyama Y & Roper SD (2008). Norepinephrine is coreleased with serotonin in mouse taste buds. J Neurosci 28, 13088–13093.

Kataoka S, Toyonoi T, Seta Y & Ogura T & Toyoshima (2004). Expression of P2Y1 receptors in rat taste buds. Histochem Cell Biol 121, 419–426.

Kim YV, Bobkov YV & Kolesnikov SS (2000). Adenosine triphosphate mobilizes cytosolic calcium and modulates ionic currents in mouse taste receptor cells. Neurosci Lett 290, 165–168.

Kinnamon SC & Finger TE (2019). Recent advances in taste transduction and signaling. F1000Res 8, 2117, 10.12688/f1000research.21099.1.

Lawton DM, Furness DN, Lindemann B & Hackney CM (2000). Localization of the glutamate-aspartate transporter, GLAST, in rat taste buds. Eur J Neurosci 12, 3163–3171.

Liman ER, Zhang YV & Montell C (2014). Peripheral coding of taste. Neuron 81, 984–1000.

Ma Z, Tanis JE, Taruno A & Foskett JK (2016). Calcium homeostasis modulator (CALHM) ion channels. Pflugers Arch 468, 395–403.

Ma Z, Taruno A, Ohmoto M, Jyotaki M, Lim JC, Miyazaki H, Niisato N, Marunaka Y, Lee RJ, Hoff H, Payne R, Demuro A, Parker I, Mitchell CH, Henao-Mejia J, Tanis JE, Matsumoto I, Tordoff MG & Foskett JK (2018). CALHM3 is essential for rapid ion channel-mediated purinergic neurotransmission of GPCR-mediated tastes. Neuron 98, 547–561.e10.

Matsu R (2000). Role of saliva in the maintenance of taste sensitivity. Crit Rev Oral Biol Med 11, 216–229.

Maurya DK, Henriches T, Marini M, Pedemonte N, Galietta LJv, Rock JR, Harfe BD & Menini A (2015). Development of the olfactory epithelium and nasal glands in TMEM16A⁻/⁻ and TMEM16A⁺/⁺ mice. PLoS One 10, e0129171.

Maurya DK & Menini A (2014). Developmental expression of the calcium-activated chloride channels TMEM16A and TMEM16B in the mouse olfactory epithelium. Dev Neurobiol 74, 657–675.

McBride DW & Roper SD (1991). Ca²⁺-dependent chloride conductance in Necturus taste cells. J Membr Biol 124, 85–93.

Medler KF, Margolskee RF & Kinnamon SC (2003). Electrophysiological characterization of voltage-gated currents in defined taste cell types of mice. J Neurosci 23, 2608–2617.

Noguchi T, Ikeda Y, Miyajima M & Yoshii K (2003). Expression of calcium-activated chloride channels TMEM16A and TMEM16B in the mouse olfactory epithelium. Dev Neurobiol 74, 657–675.

Mair DA, Traylor I, Boccaccio A, Menini A & Reisert J (2017). The long tale of the calcium activated Cl⁻ channels in olfactory transduction. Channels (Austin) 11, 399–414.

Pifferi S, Bocaccio A, Menini A & Reisert J (2017). The long tale of the calcium activated Cl⁻ channels in olfactory transduction. Channels (Austin) 11, 399–414.

Pifferi S, Bocaccio A, Menini A & Reisert J (2017). The long tale of the calcium activated Cl⁻ channels in olfactory transduction. Channels (Austin) 11, 399–414.

Pifferi S, Bocaccio A, Menini A & Reisert J (2017). The long tale of the calcium activated Cl⁻ channels in olfactory transduction. Channels (Austin) 11, 399–414.
Pedemonte N & Galietta LJV (2014). Structure and function of TMEM16 proteins (anoctamins). *Physiol Rev* 94, 419–459.

Pifferi S, Dibattista M & Menini A (2009a). TMEM16B induces chloride currents activated by calcium in mammalian cells. *Pflugers Arch* 458, 1023–1038.

Pifferi S, Dibattista M, Sagheddu C, Boccaccio A, Al Qteishat A, Ghirardi F, Tirindelli R & Menini A (2009b). Calcium-activated chloride currents in olfactory sensory neurons from mice lacking bestrophin-2. *J Physiol* 587, 4265–4279.

Pifferi S, Pascarella G, Boccaccio A, Mazzatenta A, Gustincich S, Menini A & Zucchelli S (2006). Bestrophin-2 is a candidate calcium-activated chloride channel involved in olfactory transduction. *Proc Natl Acad Sci U S A* 103, 12929–12934.

Ponissery Saidu S, Stephan AB, Talaga AK, Zhao H & Reisert J (2013). Channel properties of the splicing isoforms of the olfactory calcium-activated chloride channel Anoctamin 2. *J Gen Physiol* 141, 691–703.

Rock JR, Futtner CR & Harle BD (2008). The transmembrane protein TMEM16A is required for normal development of the murine trachea. *Dev Biol* 321, 141–149.

Roebber JK, Roper SD & Chaudhari N (2019). The role of the anion in salt (NaCl) detection by mouse taste buds. *J Neurosci* 39, 6224–6232.

Romanov RA & Kolesnikov SS (2006). Electrophysiologically identified subpopulations of taste bud cells. *Neurosci Lett* 395, 249–254.

Romanov RA, Rogachevskaja OA, Bystrova MF, Jiang P, Margolskee RF & Kolesnikov SS (2007). Afferent neurotransmission mediated by hemichannels in mammalian taste cells. *EMBO J* 26, 657–667.

Roper SD & Chaudhari N (2017). Taste buds: cells, signals and synapses. *Nat Rev Neurosci* 18, 485–497.

Sagheddu C, Boccaccio A, Dibattista M, Montani G, Tirindelli R & Menini A (2010). Calcium concentration jumps reveal dynamic ion selectivity of calcium-activated chloride currents in mouse olfactory sensory neurons and TMEM16b-transfected HEK 293T cells. *J Physiol* 588, 4189–4204.

Schoonder BC, Cheng T, Jan YN & Jan LY (2008). Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. *Cell* 134, 1019–1029.

Scudieri P, Caci E, Bruno S, Ferrera L, Schiavon M, Sondo E, Tomati V, Gianotti A, Zegarra-Moran O, Pedemonte N, Rea F, Ravazzolo R & Galietta LJV (2012). Association of TMEM16A chloride channel overexpression with airway goblet cell metaplasia. *J Physiol* 590, 6141–6155.

Seo Y, Lee HK, Park J, Jeon D-K, Jo S, Jo M & Namkung W (2016). Ani9, a novel potent small-molecule ANO1 inhibitor with negligible effect on ANO2. *PLoS ONE* 11, e0155771.

Sinclair MS, Perea-Martinez I, Dvoryanchikov G, Yoshida M, Nishimori K, Roper SD & Chaudhari N (2010). Oxytocin signaling in mouse taste buds. *PLoS One* 5, e11980.

Stephan AB, Shum EY, Hirsh S, Cygner KD, Reisert J & Zhao H (2009). ANO2 is the ciliary calcium-activated chloride channel that may mediate olfactory amplification. *Proc Natl Acad Sci U S A* 106, 11776–11781.

Stöhr H, Heisig JB, Benz PM, Schönber S, Milenkovic VM, Strauss O, Aartsen WM, Wijnholds J, Weber BHF & Schulz HL (2009). TMEM16B, a novel protein with calcium-dependent chloride channel activity, associates with a presynaptic protein complex in photoreceptor terminals. *J Neurosci* 29, 6809–6818.

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

Taylor R & Roper S (1994). Ca2+-dependent Cl− conductance in taste cells from *Necturus*. *J Neurophysiol* 72, 475–478.

Teng B, Wilson CE, Tu Y-H, Joshi NR, Kinnamon SC & Liman ER (2019). Cellular and neural responses to sour stimuli require the proton channel otop1. *Carr Biol* 29, 3647–3656.e6.

Tizzano M, Grigireit L, Shultz N, Clary MS & Finger TE (2015). Immunohistochemical analysis of human vallate taste buds. *Chem Senses* 40, 655–660.

Tomchik SM, Berg S, Kim JW, Chaudhari N & Roper SD (2007). Breadth of tuning and taste coding in mammalian taste buds. *J Neurosci* 27, 10840–10848.

Tu Y-H, Cooper AJ, Teng B, Chang RB, Artiga DJ, Turner HN, Mulhall EM, Ye W, Smith AD & Liman ER (2018). An evolutionarily conserved gene family encodes proton-selective ion channels. *Science* 359, 1047–1050.

Vandenbeuch A, Anderson CB, Parnes J, Enjyoji K, Robson SC, Finger TE & Kinnamon SC (2013). Role of the ectonucleotidase NTPTase2 in taste bud function. *Proc Natl Acad Sci U S A* 110, 14789–14794.

Vandenbeuch A, Clapp TR & Kinnamon SC (2008). Amiloride-sensitive channels in type I fungiform taste cells in mouse. *BMC Neurosci* 9, 1.

Vandenbeuch A, Zorec R & Kinnamon SC (2010). Capacitance measurements of regulated exocytosis in mouse taste cells. *J Neurosci* 30, 14695–14701.

Wladkowski SL, Lin W, McPheeters M, Kinnamon SC & Mierson S (1998). A basolateral chloride conductance in rat lingual epithelium. *J Membr Biol* 164, 91–101.

Yang R, Crowley HH, Rock ME & Kinnamon JC (2000). Taste cells with synapses in rat circumvallate papillae display SNAP-25-like immunoreactivity. *J Comp Neurol* 424, 205–215.

Yang R, Dzowó YK, Wilson CE, Russell RL, Kidd GJ, Salcedo E, Lasher RS, Kinnamon JC & Finger TE (2020). Three-dimensional reconstructions of mouse circumvallate taste buds using serial blockface scanning electron microscopy: I. Cell types and the apical region of the taste bud. *J Comp Neurol* 528, 756–771.

Yang YD, Cho H, Koo JY, Tak MH, Cho Y, Shim W-S, Park SP, Lee J, Lee B, Kim B-M, Raouf R, Shin YK & Oh U (2008). TMEM16A confers receptor-activated calcium-dependent chloride conductance. *Nature* 455, 1210–1215.
Ye W, Chang RB, Bushman JD, Tu Y-H, Mulhall EM, Wilson CE, Cooper AJ, Chick WS, Hill-Eubanks DC, Nelson MT, Kinnamon SC & Liman ER (2016). The K\(^+\) channel KIR2.1 functions in tandem with proton influx to mediate sour taste transduction. *Proc Natl Acad Sci U S A* **113**, E229–E238.

Yunos NM, Bellomo R, Story D & Kellum J (2010). Bench-to-bedside review: chloride in critical illness. *Critical Care* **14**, 226.

Zhang Y, Hoon MA, Chandrashekar J, Mueller KL, Cook B, Wu D, Zuker CS & Ryba NJP (2003). Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. *Cell* **112**, 293–301.

Zhang Y, Zhang Z, Xiao S, Tien J, Le S, Le T, Jan LY & Yang H (2017). Inferior olivary TMEM16B mediates cerebellar motor learning. *Neuron* **95**, 1103–1111.e4.

**Additional information**

**Data availability statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request. The peer review history is available in the Supporting Information section of this article.

**Author contributions**

D.M.G., K.Y.G.-V. and A.H.-C. designed and performed the research, and analysed the data. A.M. and S.P. designed the research, analysed the data and wrote the paper.

**Competing interests**

The authors declare no conflicts of interests

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**Keywords**

ANO1, anoctamin 1, taste bud

**Supporting information**

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

**Peer Review History**

**Statistical Summary Document**