CALHM1 ion channel mediates purinergic neurotransmission of sweet, bitter and umami tastes

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Recognition of sweet, bitter and umami tastes requires the non-vesicular release from taste bud cells of ATP, which acts as a neurotransmitter to activate afferent neural gustatory pathways1. However, how ATP is released to fulfill this function is not fully understood. Here we show that calcium homeostasis modulator 1 (CALHM1), a voltage-gated ion channel2,3, is indispensable for taste-stimuli-evoked ATP release from sweet-, bitter- and umami-sensing taste bud cells. Calhm1 knockout mice have severely impaired perceptions of sweet, bitter and umami compounds, whereas their recognition of sour and salty tastes remains mostly normal. Calhm1 deficiency affects taste perception without interfering with taste bud cell development or integrity. CALHM1 is expressed specifically in sweet/bitter/umami-sensing type II taste bud cells. Its heterologous expression induces a novel ATP permeability that releases ATP from cells in response to manipulations that activate the CALHM1 ion channel. Knockout of Calhm1 strongly reduces voltage-gated currents in type II cells and taste-evoked ATP release from taste buds without affecting the excitability of taste cells by taste stimuli. Thus, CALHM1 is a voltage-gated ATP-release channel required for sweet, bitter and umami taste perception.

Tastes are sensed by dedicated receptor cells in taste buds, which are composed of three anatomically distinct types of cells: types I, II and III. Only type III cells, which sense sour tastes, have neuron-like features, including expression of neurotransmitter biosynthesis enzymes and synaptic vesicles at classical synapses with sensory nerve fibres4. Type II cells, which sense sweet, bitter and umami tastes, share a common signal-transduction pathway. However, they lack classical synaptic structures, yet transmit taste information to gustatory neurons by releasing ATP as a neurotransmitter5. Our work, described below, implicates CALHM1 as a critical component responsible for this ATP release.

Calhm1 (ref. 6) encodes the pore-forming subunit of a voltage-gated, non-selective, plasma membrane ion channel involved in neuronal excitability7 and, potentially, the pathogenesis of Alzheimer’s disease8–10. Calhm1 expression was identified in primate taste buds6, suggesting that CALHM1 may have physiological functions outside the brain. We confirmed that Calhm1 was expressed in mouse taste buds but not in surrounding epithelium (Fig. 1a, b, e–g). To examine CALHM1 function, we generated a constitutive Calhm1+/− mouse and verified loss of Calhm1 expression in taste buds (Fig. 1a, c). Calhm1+/− mice were viable and fertile, with no overt morphological abnormalities in their taste buds nor any altered expression of taste-related marker genes (Fig. 1a and Supplementary Fig. 1). Loss of Calhm1 signal in taste buds of Skn-1a−/− (also known as Pou2f3) knockout mice, from which type II cells are developmentally absent10 (Fig. 1d and Supplementary Fig. 2), and the co-expression of Calhm1 and Trpm5 (Fig. 1h), demonstrated that Calhm1 expression is confined to type II cells. Expression profiling by reverse-transcription PCR of pools of isolated type II and type III cells and individual taste cells also supported the confined expression of Calhm1 to type II cells (Supplementary Fig. 3). Calhm1 expression was observed not only in Tas1r3-expressing sweet and umami taste cells, but also in other type II cells, indicating that Calhm1 is expressed in sweet, bitter and umami taste cells (Fig. 1i).

Figure 1 | CALHM1 is selectively expressed in type II taste bud cells.

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We used two behavioural tests and nerve recordings to evaluate the effect of the loss of CALHM1 expression on taste perception. Two-bottle preference tests revealed that Calhm1−/− mice had a nearly complete loss both of preference for sweet and umami compounds and of avoidance of bitter compounds (Fig. 2a, Supplementary Fig. 4a, c, d and Supplementary Tables 1 and 2). In contrast, there were little or no differences between wild-type and Calhm1−/− mice in their responses to salty and sour compounds (Fig. 2a, Supplementary Fig. 4a and Supplementary Tables 1 and 2). Brief-access taste tests reproduced these phenotypes (Fig. 2b, Supplementary Fig. 4b and Supplementary Table 3), demonstrating that the taste phenotype in Calhm1−/− mice is a sensory defect. Recordings of the whole chorda tympani nerve revealed strongly reduced responses to sweet, bitter and umami compounds in Calhm1−/− mice, whereas responses to NaCl and the acid compounds were not different from wild type (Fig. 2c and Supplementary Fig. 5), indicating that the sensory defect in Calhm1−/− mice was due to the knockout of CALHM1 function in the peripheral taste cell system. Together, these results indicate that CALHM1 has a crucial role in taste-sensing type II cells1,11 (see Supplementary Information for further discussion).

Type II cells signal to the nervous system by non-vesicular ATP release to nearby afferent gustatory nerves. Although connexin hemichannels and pannexin 1 have been proposed, the molecular identity of the ATP-release mechanism remains uncertain. The CALHM1 pore diameter is ~14 Å (ref. 3), similar to that estimated for connexins. We therefore examined the possibility that CALHM1 mediates ATP release. a, Time courses of extracellular ATP levels due to release from mock– and hCALHM1-transfected HeLa cells exposed to normal (1.9 mM) or essentially zero (17 mM) [Ca2+]o. b, Summary of extracellular ATP levels at 20 min in a. c, Low-[Ca2+]o-induced ATP release in CALHM1-expressing cells is abolished by ruthenium red (RuR). d, Effects on low-[Ca2+]o-stimulated ATP release from hCALHM1 cells of 0.5 μg ml−1 brefeldin A (BFA), 10 μM DCPIB, 3 μM A438079, 1 mM 1-heptanol (HEP), 30 mM carbenoxolone (CBX) and 20 μM RuR. e, Time courses of ATP release from hCALHM1 cells induced by various [Ca2+]o. f, ATP levels at 20 min in e plotted against [Ca2+]o and fitted to a Hill equation. g, Depolarization by high [K+]o (117.5 mM K+) induces ATP release specifically from hCALHM1 cells. h, ATP levels at 20 min in g. i, Depolarization-induced ATP release from CALHM1-expressing cells is abolished by RuR. j, Pharmacological sensitivities of depolarization-induced ATP release from hCALHM1 cells: 1 mM HEP, 1 mM probenecid (PROB), 20 μM RuR. Number of wells shown in parentheses throughout. Error bars, s.e. (where visible); *P < 0.01 (Student’s t-test).

Figure 2 | CALHM1 is essential for sweet, bitter and umami taste perception. a, b, Mean preference percentage (taste compound versus water) from 48-h, two-bottle preference tests (a) and brief-access lick scores (b) for indicated compounds in Calhm1−/− mice and wild-type littermates. Error bars, s.e. (8–12 mice per group, 4–6 months old); *P < 0.01 (post hoc Bonferroni’s test (a) and Student’s t-test (b)). c, Summary of responses from whole-chorda tympani nerve recordings stimulated with indicated compounds and normalized to response to NH4Cl from wild-type (n = 7) and Calhm1−/− (n = 8) mice. MSG, monosodium glutamate; IMP, inosine 5’-monophosphate; MCG, monocalcium di-i-glutamate. Error bars, s.e. *P < 0.05 (Student’s t-test).
mediates ATP release. The CALHM1 ion channel can be activated by membrane depolarization or reduction of extracellular Ca²⁺ concentration ([Ca²⁺]o). We first exploited the latter mechanism to activate CALHM1 in heterologous expression systems to determine whether CALHM1 can form an ATP-release channel. Decreasing [Ca²⁺]o from 1.9 mM to essentially zero (17 nM) activated ATP release from human CALHM1 (hCALHM1)-expressing HEpCa cells, whereas little ATP efflux was induced in mock-transfected cells (Fig. 3a, b). Similar low-[Ca²⁺]o-induced ATP release was observed in hCALHM1-expressing COS-1 cells and Xenopus oocytes (Supplementary Fig. 6). Neither CALHM1 expression nor low-[Ca²⁺]o exposure caused cell damage (Supplementary Fig. 7). Involvement of other possible mechanisms was ruled out because ATP release was unaffected by brefeldin A (vesicular release), DCPB (volume-sensitive Cl⁻ channels), A438079 (P2X7 receptors), heptanol (connexin hemichannels) or carbamolxolone (pannexins and connexins at 30 μM) (Fig. 3d). In contrast, ruthenium red (RuR), which inhibits CALHM1 ion currents (2), abolished low-[Ca²⁺]o-evoked ATP release from hCALHM1-expressing cells (Fig. 3c, d). Thus, CALHM1 expression induces a novel ATP permeability. CALHM1 ion channel gating is regulated by [Ca²⁺]o with an apparent half-maximum inhibitory [Ca²⁺]o of IC₅₀ ~ 220 μM and a Hill coefficient of 2.1 (ref. 2). Extracellular Ca²⁺ inhibited ATP release with IC₅₀ = 495 μM and a Hill coefficient of 1.9 (Fig. 3e, f). CALHM1 ion currents are also activated by membrane depolarization (2). At normal levels of [Ca²⁺]o, hCALHM1-expressing, but not mock-transfected, cells released ATP in response to high-[K⁺]o-induced depolarization (Fig. 3g, h). Depolarization-induced ATP release was inhibited by RuR but not by heptanol or probenecid (Fig. 3i, j), which are respectively connexin and pannexin 1 blockers (13). Expression of mouse CALHM1 conferred ATP release with similar properties (Supplementary Fig. 8). Regulation of ATP release in hCALHM1-expressing cells is therefore similar to that of CALHM1 channel gating, indicating that the CALHM1 channel is a conduit for ATP release.

The three types of taste bud cell can be classified on the basis of whole-cell electrophysiological fingerprints (13). We verified these fingerprints by recording whole-cell currents in single isolated taste bud cells from TRPM5–green fluorescent protein (GFP) mice with GFP expressed specifically in type II cells (Supplementary Fig. 9). With tetraethylammonium in the extracellular solution and Ca²⁺ as the major cation in the pipette solution (to block K⁺ currents), the electrophysiological subtypes were identified by the combination of voltage-gated Na⁺ currents (I₅Na) and non-selective, slowly activating outward currents (Islow) with inward tail currents at ~70 mV (Ia) (Fig. 4a–c and Supplementary Fig. 9). To identify CALHM1 ion currents, we recorded whole-cell currents in isolated taste bud cells from wild-type and Calhm1⁻⁻ mice (Fig 4a–c). Loss of CALHM1 substantially reduced the amplitudes of Islow and Ia without affecting the amplitude of I₅Na in type II cells (Fig. 4d–f), whereas no differences were observed in type I and type III cells (Fig. 4b, c, g). The slowly activating outward current in type II cells was inhibited by the non-specific CALHM1 blocker Gd³⁺ but not by probenecid or heptanol, ruling out contributions of pannexin I or connexins (Fig. 4h). These data demonstrate that CALHM1 channel conductance is present in type II cells and contributes the major component of the slowly activating outward current. Notably, the amount of depolarization-induced ATP release from taste cells.

**Figure 4** | CALHM1 is required for taste-evoked ATP release from taste cells.

a–c, Electrophysiological phenotypes of type I, type II and type III cells identified in wild-type (red) and Calhm1⁻⁻ (blue) taste cells. Cells held at −70 mV and pulsed from −80 to −80 mV in 20-mV increments with 1-s duration. Type I current in c recorded from 16 wild-type and 9 Calhm1⁻⁻ cells. d–g, I₅Na (circle; d), Islow at end of pulses (square; d) and Ia (triangle; e) measured for type II cells (n = 9 wild type, n = 10 Calhm1⁻⁻), and I₅Na measured for type III cells (n = 9 wild type, n = 6 Calhm1⁻⁻) (g). Vm, membrane voltage. h, Sensitivities of I₅Na in GFP-positive cells from TRPM5–GFP mice to Gd³⁺ (100 μM), probenecid (1 mM), 1-heptanol (1 mM) (n = 4). i, [Ca²⁺]o, I₅Na evoked by robust [Ca²⁺]o response to a mix of sweet and bitter substances (grey bar). j, k, Basal (j) and taste-evoked (k) responses are comparable in wild-type and Calhm1⁻⁻ cells. KO, knockout; WT, wild type. j, Taste-evoked ATP release from gustatory CVP tissue and non-gustatory lingual epithelium. Bitter mix elicits considerable ATP release from CVP versus lingual epithelium in wild-type mice; this is abolished in Calhm1⁻⁻ mice and by 1 μM tetrodotoxin (TTX). Error bars, s.e.m.; *P < 0.05, **P < 0.01 (Student’s t-test). m, Schematic illustration of signal-transduction cascade in type II taste receptor cells with CALHM1 as the ATP-release pathway.
ATP release from type II cells is correlated with the magnitude of $I_{\text{tole}}$ (ref. 13). Type II cells detect sweet, bitter and umami compounds via G-protein-coupled taste receptors\(^*\) that stimulate a common signal-transduction cascade involving activation of PLCβ2, inositol 1,4,5-trisphosphate-mediated Ca\(^{2+}\) release and Ca\(^{2+}\)-dependent activation of TRPM5 channels\(^1\) that depolarizes the plasma membrane to generate action potentials and subsequent non-vesicular release of ATP. Importantly, no differences in basal Ca\(^{2+}\), and taste-evoked Ca\(^{2+}\) responses were observed between wild-type and Calhm1\(^{-/-}\) type II cells (Fig. 4i–k). Furthermore, Calhm1 deficiency had no effect on $I_{\text{Na}}$ (Fig. 4d), TRPM5 expression in type II cells (Fig. 1a and Supplementary Figs 1i and 2) or the ATP content of taste buds (Supplementary Fig. 10). Strikingly, however, taste-evoked release of ATP from circumvallate papillae was abolished in Calhm1\(^{-/-}\) mice (Fig. 4l and Supplementary Fig. 11). This strongly suggests that CALHM1 contributes to the major ATP-release mechanism in sweet/bitter/umami-sensing taste bud cells (Fig. 4l, m).

Our study sheds light on a novel cellular ATP-releasing mechanism by demonstrating that CALHM1 is a voltage-gated ion channel that mediates tetrodotoxin-sensitive ATP release in taste buds (Fig. 4l) as an essential mechanism of sweet, bitter and umami taste perception. As such, CALHM1 provides a missing link in the signal-transduction cascade in type II cells, connecting taste receptor activation and the generation of Na\(^+\) action potentials to the activation of afferent gustatory neural pathways (Fig. 4m). It has not escaped our attention that other CALHM1 isoforms\(^*\) as well as pannexin 1 and connexins are also present in taste buds\(^9,12,13\) and might also be involved in ATP release in taste, perhaps acting in parallel with or in a complex with CALHM1. Signalling by extracellular ATP is a widespread phenomenon that regulates many physiological activities\(^1^9\), including neurotransmission\(^20,21\), intercellular communication\(^22,23\), vascular tone\(^24\) and sensory transduction\(^25-27\). CALHM1 and its isoforms may participate in physiologically important ATP release elsewhere.

**METHODS SUMMARY**

All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania, the Feinstein Institute for Medical Research, the Monell Chemical Senses Center and the University of Minnesota Duluth.

**Full Methods** and any associated references are available in the online version of the paper.

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**Author Contributions** A.T., V.V., A.L., Z.M., M.O., I.M., H.Z., L.A., S.L., M.A., G.H., G.D. and N.C. designed and performed experiments. P.M. and V.V. generated the Calhm1 knockout mice. J.K. and P.D. designed experiments. M.G.T., M.M.C., P.M. and J.K.F. designed experiments and helped with data interpretation. A.T., J.K.F. and P.M. wrote the manuscript.

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METHODS

Calhm1+/− mice. Calhm1+/− founder mice were generated at genOway (Lyon, France) as described previously. Briefly, Calhm1 exon 1 deletion was performed by homologous recombination in 129sv embryonic stem cells. Positive clones were screened by PCR and confirmed by Southern blot analysis. The resultant embryonic stem cells were injected into blastocysts derived from C57BL/6J mice to obtain chimaeric mice that possessed germline transmission of the targeted Calhm1 locus. Mice on the mixed 129sv × C57BL/6J genetic background were used in this study. Wild-type (+/+) and Calhm1 knockout (−/−) mice of F1 obtained from littermate mice were used for two-bottle preference tests. All other experiments were performed with wild-type and knockout littermates. In some mice, the neomycin cassette was removed and the resulting strain was backcrossed with C57BL/6J mice. Briefly, Calhm1+/− mice were mated with mice bearing an Ella−cre transgene (B6.FVB-Tg (Ella−cre)C345Mldj/F, The Jackson Laboratory) on the C57BL/6J background to remove the neomycin resistance cassette by cre-loxP-mediated excision. The resulting Calhm1−/− pups were further backcrossed to C57BL/6J (The Jackson Laboratory) for at least five generations before being made homozygous. The removal of the neomycin cassette was confirmed by PCR. Backcrossed mice were used for two-bottle preference tests. Loss of CALHM1 expression in Calhm1−/− taste buds was verified by reverse-transcription (RT)–PCR and in situ hybridization.

Laser capture microdissection, RNA amplification and RT–PCR. Circumvallate taste tissue from Calhm1+/+ and Calhm1−/− mice was embedded in cryomolds using OCT freezing medium. Twelve micrometre-thick tissue sections were cut on a Leica CM1850 cryostat (Leica Microsystems), collected on RNA-free membrane slides (Leica) and stained with cresyl violet. Taste buds from the CVP and surrounding lingual epithelium were isolated using a Leica laser microdissection system LM7000 (Leica). Taste buds and surrounding epithelium were pooled from a total of four mice of each genotype. Total RNA from taste bud and lingual epithelium samples was purified using a RNAqueous-Micro Kit (Ambion). Total RNA was amplified using two sequential rounds with MessageAmp II aRNA kit (Ambion), as per the manufacturer’s instructions. One microgram of RNA was transcribed into complementary DNA (cDNA) using Invitrogen’s SuperScript III First-Strand Synthesis System for RT–PCR with random hexamers, according to the supplied protocol. A 50-μl PCR reaction was run with the following final concentrations: 450 ng of each primer (see Supplementary Table 4 for PCR primer sequences), 2 mM MgCl2, 0.3 mM dNTPs, 2.5 μTaq polymerase (Promega GoFlex DNA polymerase) and 1 μl of 10 ng/ml DNA.

PCR cycling conditions used for Calhm1: 95°C for 3 min; 35 cycles of 95°C for 1 min, 65°C for 30 s and 72°C for 30 s; 4°C hold.

In situ hybridization. Oral epithelia containing taste buds were dissected from adult male mice deeply anaesthetized with an overdose of sodium pentobarbitone (Abbott Laboratories) and embedded in the frozen OCT compound (Sakura Finetech). Fresh-frozen sections of 8-μm thickness were prepared using a cryostat (CM3050S, Leica Microsystems). The in situ hybridization procedure was described previously. In brief, digoxigenin- and fluorescein-labeled antisense RNAs prepared using RNA labelling mix (Roche Diagnostics) and an RNA polymerase (Strategene) were used for hybridization after fragmentation under alkaline conditions to a length of about 150 bases. Fresh-frozen sections were fixed with 4% PFA, treated with diethylpyrocarbonate, prehybridized with salmon sperm DNA for 2 h at 65°C and hybridized with antisense riboprobes for 40 h at 65°C. For single labelling of Tas2r108 and Trpm5, however, hybridization and hybridization were carried out at 58°C. After hybridization, the sections were washed in ×2.0 SSC at 58°C or 65°C and blocked with 0.5% blocking reagent (Roche Diagnostics) in Tris-buffered saline. Chromogenic signals, except those from Calhm1, were developed for one day using alkaline phosphatase-conjugated anti-digoxigenin antibody (1:500, Roche Diagnostics) and 4-nitro blue tetrazo- lium chloride/5-bromo-4-chloro-3-indolyl-phosphate as chromogenic substrates. Chromogenic Calhm1 signals were developed for two days to clarify the cells expressing Calhm1 messenger RNA. No Calhm1 signal was observed in Skn-1 or Calhm1 knockout taste tissues after signal development for three days. Stained images were obtained with a Nikon Eclipse 80i microscope (Nikon Instruments Inc.) equipped with a DXM1200C digital camera (Nikon). Fluorescent signals were developed using biotin-conjugated anti-fluorescein antibody (1:500, Vector Laboratories), avidin–biotin complex (Vector Laboratories), tyramide signal amplification biotin system (1:50, PerkinElmer) and Alexa 488-conjugated streptavidin (4 μg ml−1, Invitrogen). Fluorescent images were obtained with a Leica SP2 confocal scanning microscope (Leica). For double labelling of Calhm1 with Trpm5 and Tas2r13, fluorescent signals were first developed and then the fluorescent images were superimposed using Photoshop CS3 (Adobe). RNA probes generated were to nucleotides 1–894 of Tas2r108 (GenBank accession number, AF227148), nucleotides 310–3,491 of Trpm5 (accession number, AF228681), nucleotides 525–2,725 of Tas1r3 (accession number, AF337039), and nucleotides 1–1,407 and 2,148–2,369 of Calhm1 CDNA fragment, which contains 1,047 base pairs of the entire coding sequence and 1,322 base pairs of 3′-non-coding region.

Immunofluorescence staining. For fixed-tissue preparation, mice 12 weeks old or older were anaesthetized with an overdose of sodium pentobarbitone (Abbott Laboratories) and transcardially perfused with ice-cold PBS followed by 4% paraformaldehyde in ice-cold PBS. Tongue epithelia containing CVP were dissected and postfixed with the same fixative at 4°C overnight. Tissue samples were then cryoprotected in 30% sucrose in PBS, embedded in frozen OCT compound (Tissue-Tek OCT Compound, Sakura Finetech) and sectioned using a cryostat (CM1900, Leica Microsystems) at 8 μm. Tissue sections were mounted on tissue-adhesive-coated glass slides (Fisher Scientific) and stored at −80°C until analysed. For immunofluorescence staining, slides were rinsed with PBS, incubated in a preheated target retrieval solution (S1700, Dako) at 80°C for 20 min, allowed to cool to room temperature (20–25°C) for 20 min, still in the target retrieval solution, and washed in PBS for 3 × 10 min. After blocking by 1-h incubation in PBS containing 5% normal donkey serum (PBS-5% NDS) at room temperature, sections were incubated overnight at 4°C with primary antibodies diluted in PBS-5% NDS: 1:3,000 for rabbit anti-TRPM5 (Alomone Labs); 1:500 for rabbit anti-PLCB2 (Santa Cruz Biotechnology); 1:500 for goat anti-KCNQ1 (Santa Cruz). The next day, the slides were washed for 3 × 10 min in PBS at room temperature and incubated for 1 h with Alexa-Fluor-conjugated antibodies diluted in PBS-5% NDS: 1:500 for Alexa Fluor 488 donkey anti-rabbit IgG; 1:500 for Alexa Fluor 568 goat anti-goat IgG (Invitrogen). Finally, slides were washed for 3 × 10 min in PBS at room temperature and mounted in VectaShield with 4′,6-diamidino-2-phenylindole (H-1500, Vector Laboratories). Stained sections were imaged with a confocal scanning microscope (LSM 710, Carl Zeiss).

Single-taste cell analyses. Circumvallate taste epithelium was enzymatically delaminated, taste buds were collected from pooled epithelium and dissociated single-taste cells were collected, all as detailed previously. For patch clamp and fura-2 Ca2+ imaging experiments, isolated cells were placed on polyl-lysine-coated coverslips and allowed to settle for 30–60 min.

Total RNAs isolated from taste buds and from adjacent non-taste epithelium were used as positive and negative controls, respectively. Individual dissociated taste cells from PLCB2–GFP (type II) and GAD1–GFP (type III) transgenic mice were selected for collection on the basis of their expression of GFP, as previously described. Type I taste cells were selected from PLCB2–GFP × GAD1–GFP double-transgenic mice by the absence of GFP expression. Total RNA isolated from taste cells was either subjected to T7 RNA polymerase-based linear amplification (aRNA, Message BOOSTER kit, Epicentre) or was directly converted to cDNA, both as previously described. For aRNA-based analyses, 1% of the resulting cDNA was used as template for PCR. For direct RT–PCR, 15% of cDNA from each cell was used to test for diagnostic messenger RNAs and 30% was used for Calhm1. PCR conditions with HotStarTaq Plus (QIAGEN) were 95°C for 5 min followed by 45 90-s cycles, each of which comprised three 30-s components, one at 94°C, one at the annealing temperature and one at 72°C. PCR primers, annealing temperatures and product sizes were as in Supplementary Table 4. Two different primer sets were used for Calhm1 (Supplementary Table 4). Primers 1 were used for RT–PCR on bulk tissue RNA and primers 2 were used for individual taste cells (Fig. 1a and Supplementary Fig. 3a, b, d). Primers 3 and 4 span an intron, and were used for the single-cell direct RT–PCR to avoid amplifying from genomic DNA (Supplementary Fig. 3c).

The cell type of all single cells and pools of cells was confirmed by RT–PCR for three diagnostic cell-type markers: Enpp2 (type I), Plcb2 (type II) and Snap25 (type III).

Two-bottle preference tests. Calhm1+/− and wild-type control mice were presented for 48 h with two drinking bottles, one containing water and the other a solution supplemented with ascending concentrations of the specific taste compounds to be tested. All solutions were prepared with tap water and served at room temperature. The mice had access to the tap water ad lib (to a standard water bottle from the Rodent Diet 5001, LabDiet, PMI Nutrition International) and drinking solutions. Two independent groups of mice were tested for the following series of taste solutions. The order of testing was as follows: sucrose, saccharin, NaCl, denatonium (group 1); MSG, quinine, HCl (group 2). Sucrose, saccharin, denatonium benzoate, quinine sulphate and MSG were from Sigma-Aldrich. HCl and NaCl were from Thermo Fisher Scientific Inc. The order of stimulus presentation was identical for wild-type and knockout mice. The mice had three days with a single bottle of drinking water between each test series. The two bottles were switched after 24 h to control for a possible effect of the bottle positions. The change in liquid intake was determined by weighing the bottles before and after the test series. The bottles were measured volumetrically to the nearest 0.1 ml. The volumes of water and taste compound solutions consumed were recorded. Solution preference was calculated as the intake of taste solution divided by total liquid intake, and this ratio was expressed as a percentage. The taste compounds were chosen as exemplars of the
was replaced with mice (5 male, 7 female) and 12 wild-type littermate controls. Anaesthesia was maintained with 0.4–0.05.

The bath solution contained (in mM) 150 NaCl, 5 KCl, 38.5 NaHCO3, 1.8 NaH2PO4, 0.6% isoflurane. Body temperature, surgical table temperature, blood oxygen, anaesthesia level and heart rate were continuously monitored. The chorda tympani was dissected free from its junction with the lingual nerve to the tympanic bulla, where the central part of the chorda tympani was cut and the peripheral part then mobilized in the rostral direction so that afferent nerve impulses could be recorded. The nerve impulses were recorded between a silver wire electrode and an indifferent electrode touching the walls of the wound, fed into a custom-made amplifier and monitored over a loudspeaker and an oscilloscope. The nerve impulses were processed by a smoothed absolute value circuit integrator and rectified to a d.c. potential whose amplitude was related to the kind of taste compound used were fed into an oscilloscope and computer. Both the individual nerve impulses and the summed signal were recorded (Gould ES 1000). The stimuli were delivered to the tongue with an open flow system controlled by the computer under conditions of constant flow and temperature (33°C). Each stimulation lasted for 5 s with 50 s rinsing time between stimulations. Care was exercised to make sure that the flow was directed over the fungiform papillae. In each animal, we tried to run the same sequence of stimuli at least three times while we recorded the nerve impulses from the whole chorda tympani nerve. The integrated response during stimulation was calculated by subtracting the area of nerve activity preceding stimulation from that during stimulation. Thus, the data reflect the level of activity during stimulation time. The responses to all compounds were expressed relative to the response to 0.1 M NaCl in each mouse. The average in each animal and group was calculated, the variance determined and the significance between the chorda tympani responses of wild-type and Calhm1 mice determined. P < 0.05 was considered significant.

Cell culture. HeLa cells (American Type Culture Collection) were grown in plastic flasks at 37°C in a humidified incubator in culture medium containing 90% (v/v) DMEM, 10% fetal bovine serum and 1 x Antibiotic-Antimycotic (Invitrogen) with 5% CO2 in-air.

ATP measurements using cultured cells. Extracellular ATP concentration was measured by the luciferin–luciferase reaction as previously described. HeLa cells were seeded onto 96-well microplates (Corning Costar) at a density of 10,000 cells per well one day before transfection. Cells in each well were transfected with 0.2 μg of target plasmid and the sensor plasmid EGFP (pEGFP) or empty plasmid using 0.4 μl Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 20–30 h, media were removed and cells were incubated for 1 h before 75 μl of the 100-μl bath solution containing 2 mM Ca2+ was replaced with an equal volume of test solution to establish final [Ca2+]i and drug concentrations. The plate was immediately placed in a microplate luminometer (Synergy 2, BioTek) and 10 μl of ATP assay solution (FL-AAM and FL-AAB, Sigma-Aldrich) was dispensed into each well. ATP release was measured every 2 min. ATP concentration was calculated from a standard curve created in each plate. Separate standard curves were made in experiments involving all added drugs at the same conditions used. Each bath solution contained (in mM) 150 NaCl, 5 KCl, 10 Hepes and 10 glucose, pH 7.4, adjusted with NaOH. [Ca2+]i values below 90 μM were achieved by mixing CaCl2 and EGTA, and free [Ca2+]i was calculated using WEBMAXC software (http://maxchelator.stanford.edu). When a drug was tested, cells were pre-incubated for 1 h with the drug before being exposed to low-
[Ca\(^{2+}\)]_o\) solution containing the same drug. The high-[K\(^+\)]_o condition (117.5 mM) was established by replacing Na\(^+\) with equimolar K\(^+\).

**ATP measurements using intact taste buds.** Taste-evoked ATP release from intact taste buds was recorded with sheets of lingual epithelium as described previously. The tongue epithelium was cut into pieces containing the CVP or lingual epithelium devoid of taste buds and mounted in a custom Ussing-type chamber that separated the fluid-containing serosal chamber from the apical surface. The apical surface was selectively exposed to a bitter mix (40 mM denu- tonium benzoate, 1 mM cycloheximide, 10 mM HEPES, pH 7.4) for 45 s. The serosal fluid (130 μl) was then collected and added to an equal volume of ATP assay solution (FL-AAM and FL-AAB, Sigma-Aldrich) to determine its ATP content by the luciferin–luciferase assay. The solution on the serosal side contained (in mM) 150 NaCl, 5 KCl, 1.5 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES and 10 glucose, pH 7.4, adjusted with NaOH.

**Single-cell Ca\(^{2+}\) imaging.** Isolated taste cells on coverslips were secured in a perfusion chamber and mounted on the stage of an inverted microscope (Nikon Eclipse TE2000). Cells were loaded with fura-2AM (Molecular Probes; 2.5 μM) for 45 min at room temperature in the bath solution containing (in mM) 150 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES and 10 glucose, pH 7.4, adjusted with NaOH. Cells were continuously perfused with the bath solution and stimulated for 30 s by bath perfusion of taste mix (2 mM saccharin, 100 μM SC45647, 1 mM denato- nium, 10 μM cycloheximide; dissolved in the bath solution at pH 7.4). Fura-2 was alternately illuminated at 340 or 380 nm, and the emitted fluorescence intensity at 510 nm was collected with a PerkinElmer Ultraview imaging system. The background fluorescence was estimated for a region without cells and subtracted during analysis. Changes in [Ca\(^{2+}\)]_o, are presented as changes in fluorescence ratio (F340/F380). Dye calibration was achieved by applying experimentally determined constants to the equation \([\text{Ca}^{2+}]_o = K_D(R - R_{\text{min}})/(R_{\text{max}} - R)\). Macros used for analysis were custom macros written for IGOR PRO (WaveMetrics). Isolated taste bud cells that responded to a taste stimulus by robust [Ca\(^{2+}\)]_o increase were identified as type II cells: 24% of wild-type cells (9 out of 38 cells from three animals) and 39% of Calhm1\(^{-/-}\) cells (12 out of 31 cells from four animals).

**Single-cell electrophysiology.** Whole-cell recordings were made in single taste bud cells isolated from Calhm1\(^{-/-}\) and wild-type littermates and TRPM5–GFP mice with a patch-clamp amplifier (Axopatch 200B, Axon Instruments). TRPM5–GFP mice were a gift from Dr R. F. Margolskee\(^{38}\) and were used for selective recordings from type II cells on the basis of their expression of GFP. The pipette solution contained (in mM) 155 CsF, 2 MgCl\(_2\), 1 CaCl\(_2\), 11 EGTA, 10 HEPES, pH 7.3, adjusted with methanesulphonic acid, ~308 mOsm. The bath solution contained (in mM) 140 Na\(^+\), 5.4 K\(^+\), 20 TEA\(^{-}\), 1.5 Ca\(^{2+}\), 1 Mg\(^{2+}\), 6 Cl\(^{-}\) and 10 HEPES, pH 7.4, adjusted with methanesulphonic acid, ~330 mOsm. Patch pipettes were fabricated from thick-walled borosilicate glass capillaries (PG10150-4, World Precision Instruments) and had a resistance of 5–9 MΩ in the recording condition. Electrode capacitance was compensated electronically, and 70% of series resistance (10–25 MΩ) was compensated with a lag of 20 μs. Currents were low-pass-filtered at 5 kHz with eight-pole Bessel characteristics and sampled at 10 kHz. For testing pharmacological properties of \(I_{\text{slow}}\) in type II cells (Fig. 4h), whole-cell currents were recorded in GFP-positive taste cells isolated from TRPM5–GFP mice and \(I_{\text{slow}}\) was evoked every 5 s by a voltage pulse to +100 mV with 1-s duration from a holding potential of ~70 mV. Current amplitudes were taken at the end of the pulse and normalized to the average of values before drug addition (initial 50 s) for each cell.

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