Presynaptic (Type III) cells in mouse taste buds sense sour (acid) taste

Yijen A. Huang[1], Yutaka Maruyama[1], Robert Stimac[1] and Stephen D. Roper[1,2]

[1]*Department of Physiology and Biophysics and* [2]*Program in Neuroscience, University of Miami School of Medicine, Miami, FL 33136, USA*

Taste buds contain two types of cells that directly participate in taste transduction – receptor (Type II) cells and presynaptic (Type III) cells. Receptor cells respond to sweet, bitter and umami taste stimulation but until recently the identity of cells that respond directly to sour (acid) tastants has only been inferred from recordings *in situ,* from behavioural studies, and from immunostaining for putative sour transduction molecules. Using calcium imaging on single isolated taste cells and with biosensor cells to identify neurotransmitter release, we show that presynaptic (Type III) cells specifically respond to acid taste stimulation and release serotonin. By recording responses in cells isolated from taste buds and in taste cells in lingual slices to acetic acid titrated to different acid levels (pH), we also show that the active stimulus for acid taste is the membrane-permeant, uncharged acetic acid moiety (CH_3COOH), not free protons (H^+). That observation is consistent with the proximate stimulus for acid taste being intracellular acidification, not extracellular protons *per se*. These findings may also have implications for other sensory receptors that respond to acids, such as nociceptors.

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Corresponding author S. Roper: Department of Physiology and Biophysics, University of Miami School of Medicine, 1600 NW 10th Ave., Miami, FL 33136, USA. Email: sroper@med.miami.edu

Sour is a basic taste quality alongside sweet, bitter, salty and umami. Sourness is elicited by acids and is generally an aversive taste for humans and non-human animals. Presumably, sourness functions to signal rancidity in potential sources of food as well as to protect against acid/base unbalances that might follow consumption of excessive acid. Several molecular transduction mechanisms for sour taste have been put forth over the decades, including ASIC channels, HCN channels, and a matrix of KCNK channels in taste bud cells (reviewed by Roper, 2007). Recently, the TRP-like channels PKD2L1 and PKD1L3 have also been proposed as sour taste transducers. Taste cells express PKD2L1 and PKD1L3 (LopezJimenez *et al.* 2006) and when these channels are expressed in heterologous cells, they confer acid sensitivity (Ishimaru *et al.* 2006). Further, mutant mice lacking the taste cells that express PKD2L1 channels do not sense acid taste (Huang *et al.* 2006). PKD2L1 appears to be expressed selectively in one class of taste bud cells, Type III cells (Kataoka *et al.* 2008). Despite these findings, a definitive explanation for sour taste is lacking and there is no consensus yet regarding its underlying mechanism(s). In part, this is due to the lack of definitive information about which cells in the taste bud are the actual sour receptor cells and what the proximate stimulus for acid taste is.

Taste buds consist of several different categories of cells, generally classified as Types I, II, III and IV. Functional characterization of taste cells indicates that one of these categories, Type II, represents receptor cells (Clapp *et al.* 2004; DeFazio *et al.* 2006). These cells express G protein coupled taste receptors (T1Rs, T2Rs) and their downstream effectors, phospholipase C subtype $\beta 2$ (PLC $\beta 2$) and IP_3 receptor subtype 3. Consequently, receptor cells are directly stimulated by sweet, bitter and umami taste compounds (Tomchik *et al.* 2007). In response to taste stimulation, receptor cells secrete ATP, a taste neurotransmitter, via an unconventional mechanism – gap junction hemichannels composed of the pannexin 1 gap junction protein (Huang *et al.* 2007; see Romanov *et al.* 2007). Receptor (Type II) cells, however, may not directly participate in sour taste. Other taste cells, possibly Type III cells, appear to be acid sensitive, although this has only been tested to date *in situ* (Richter *et al.* 2003; Tomchik *et al.* 2007). Type III cells form morphologically identifiable synapses with postsynaptic structures and have been shown to express molecules associated with vesicular exocytosis (Yee *et al.* 2001; DeFazio *et al.* 2006). Type III cells also express a candidate acid transducer channel, PKD2L1 (Kataoka *et al.* 2008). These cells have been termed presynaptic cells to underscore the observation that they possess synapses and express synapse-related proteins (DeFazio *et al.* 2006). When stimulated, presynaptic (Type III) cells release serotonin (5-HT) in a calcium-dependent manner, consistent with

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vesicular exocytosis at synapses (Huang *et al.* 2005, 2007).

The present work was undertaken (a) to explore which taste cell(s) are ultimately responsible for acid sensitivity, (b) to investigate how the cells respond to acid stimulation, and (c) to identify the neurotransmitter(s) involved in sour taste. By using single, isolated taste cells free of any indirect excitation that might occur *in situ*, the present findings confidently establish that only presynaptic (Type III) cells respond to acid stimulation with Ca^{2+} influx and transmitter (5-HT) secretion. Curiously, other taste cells, including receptor (Type II) also are affected by the presence of acids but in a fundamentally different manner from presynaptic cells, not involving Ca^{2+} influx and not associated with transmitter secretion.

Methods

Ethical approval

Mice were killed following National Institutes of Health guidelines, as approved by the University of Miami Animal Care and Use Committee. All experiments were conducted following the guidelines of these two regulatory bodies.

Animals

Adult C57BL/6J mice of both sexes were used in this study ($n = 59$). Mice were killed by exposure to 100% CO_2 until they were unconscious, and remained in the chamber until clinical death was assured (∼1–2 additional minutes). This procedure minimizes distress (NIH Office of Animal Care and Use, http://oacu.od.nih.gov/ARAC/EuthCO2.pdf). Cervical dislocation followed CO_2 exposure and tongues were then removed for further dissection (next).

Isolated taste cells

We removed the lingual epithelium containing taste papillae from the tongue by injecting 1 mg ml^{-1} collagenase A (Roche), 2.5 mg ml^{-1} dispase II (Roche), and 1 mg ml^{-1} trypsin inhibitor (Sigma) directly under the epithelium surrounding taste papillae. The peeled epithelium was bathed in Ca^{2+}-free Tyrode solution for 30 min at room temperature and isolated taste cells were drawn into fire-polished glass micropipettes with gentle suction. Taste cells were transferred to a shallow recording chamber having a glass coverslip base. The coverslip base was coated with Cell-Tak (BD Biosciences) to hold taste cells firmly attached. Taste cells were superfused with Tyrode solution (in mM: 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 10 glucose, 10 sodium pyruvate, 5 $NaHCO_3$, pH 7.4, 310–320 mosmol l^{-1}). For nominally Ca^{2+}-free Tyrode solution, $MgCl_2$ was substituted for $CaCl_2$ (in mM: 140 NaCl, 5 KCl, 3 $MgCl_2$, 10 Hepes, 10 glucose, 10 sodium pyruvate, 5 $NaHCO_3$, pH 7.4, 310–320 mosmol l^{-1}).

Lingual slice preparation

We prepared lingual slices containing the vallate papilla and loaded taste cells with a calcium indicator dye as previously described (Caicedo *et al.* 2000, 2002; Tomchik *et al.* 2007). Briefly, Calcium Green-1 dextran (CaGD; 1 mM in H_2O, molecular weight 3000 kDa; Invitrogen, Carlsbad, CA, USA) was injected iontophoretically through a fire-polished glass micropipette into the crypt surrounding the vallate papilla. Sections of 100 μm of the dye-loaded tissue were prepared with a vibrating microtome (VT1000S; Leica, Nussloch, Germany) and mounted in a recording chamber. Lingual sections were superfused with Tyrode solution (30°C) at a rate of 1 ml min^{-1}. 'Puffer' pipettes (2 μm tip diameter) were used to deliver taste stimuli directly to the taste pores of taste buds in the lingual slice. Stimuli were ejected for 2 s with air pressure (1–5 p.s.i.) (Picospritzer; Medical Systems, Greenvale, NY, USA). Bathing solutions were as described above.

Ca^{2+} imaging

For isolated, Fura-2-loaded taste cells, sequential images were recorded at 40× with a band pass emission filter (510 ± 80 nm) when excited at 340 nm followed by 380 nm (e.g. Huang *et al.* 2007). Images were processed with Indec Workbench v5 software. Data shown are the ratios, F_{340}/F_{380}, indicating relative changes in $[Ca^{2+}]_i$. For lingual slices containing dye-loaded taste cells, taste buds were viewed with a scanning laser confocal microscope using argon and krypton lasers (Fluoview; Olympus Optical, Thornwood, NY, USA). We measured fluorometric signals as relative fluorescence change, $\Delta F/F_o$, and corrected for photobleaching when necessary (Caicedo *et al.* 2000; Tomchik *et al.* 2007).

Stimulation

Isolated taste cells were stimulated by bath-perfusion of KCl (50 mM substituted equimolar for NaCl), taste mix (10 μM cycloheximide, 2 mM saccharin, 0.1 mM SC45647, 1 mM denatonium), sodium acetate (20 mM), or acetic acid (10 mM). All stimuli were made up in Tyrode solution and applied at pH 7.2 except for acetic acid. A series of acetic acid solutions with pH 5.0–7.0 was prepared by titrating 10 mM acetic acid with 1 N NaOH. HCl taste stimulus solutions of pH 1.5, 3 and 5 were prepared by titrating Tyrode buffer with 1 N HCl. In all cases, Na^+ concentration in the external bath was kept constant between stimulus solutions by making appropriate ion substitutions (e.g. 20 mM sodium acetate substituted for 20 mM NaCl).

In lingual slices, acetic acid taste stimuli were delivered with focal pipettes positioned adjacent to the taste pore of taste buds. Acetic acid was titrated with NaOH or

HCl to achieve specific concentrations of the protonated uncharged moiety (CH_3COOH, hereafter, HOAc), according to the Henderson–Hasselbalch equation (using pK_a of acetic acid of 4.76). For example, at pH 5.0, 37% of the aqueous solution of acetic acid consists of HOAc, and thus a solution of 10 mM acetic acid, titrated with NaOH to pH 5 will contain 3.7 mM HOAc (e.g. see Fig. 5*A*). To vary HOAc but keep pH constant, solutions of increasing initial acetic acid concentrations were titrated to pH 5.0. For example, 173 mM acetic acid titrated to pH 5.0 contains 64 mM HOAc (i.e. 37% of 173 mM, e.g. see Fig. 5*B*). NaCl was added to the acetic acid taste stimuli to yield equi-osmolar solutions (316 mosmol l^{-1}). Lucifer yellow, 500 μM, was added to the stimulus solutions as a tracer to determine the distribution and dilution of stimulus at the taste pore (Richter *et al.* 2003). No further attempt was made to buffer the acetic acid taste stimuli. Acetic acid taste solutions were applied with a constant air pressure ('puffer pipette') for sufficient duration (2 s) to bathe the underlying taste pore entirely and uniformly with the stimulus concentration contained in the pipette.

Biosensor cells

CHO cells coexpressing 5-HT_{2c} receptors and P2x2/P2x3 receptors (hereafter, 'dual biosensor cells') were prepared and loaded with Fura-2 as described in Huang *et al.* (2007). An aliquot of suspended biosensor cells preloaded with Fura-2 was transferred to a recording chamber containing taste cells and tested for sensitivity to 5-HT (3 nM) or ATP (300 nM). Selected biosensor cells were drawn and held to a fire-polished glass micropipette with gentle suction for use in testing transmitter release from taste cells. In separate experiments we verified that biosensor cells do not respond to bath-applied KCl, taste mix (10 μM cycloheximide, 2 mM saccharin, 0.1 mM SC45647, 1 mM denatonium), acetic acid (10 mM; pH 5.0), or sodium acetate (20 mM; pH 7.2) (see Huang *et al.* 2005). To test for 5-HT secretion, dual biosensor cells were preincubated with 500 μM ATP for 30 min to desensitize purinoceptors for the duration of the experiment. Conversely, to test for ATP secretion, dual biosensor cells were preincubated for 30 min with 10 μM 5-HT, which rendered the serotonergic receptors refractory throughout the experiment.

Results

Taste buds contain separate populations of sensory cells believed to participate directly in taste transduction – receptor (Type II) cells and presynaptic (Type III) cells. Only presynaptic cells form morphologically distinct synapses with gustatory afferent nerve terminals (Yee *et al.* 2001). Receptor cells specifically and selectively respond to sweet and bitter taste stimulation whereas presynaptic cells respond to KCl depolarization (DeFazio *et al.* 2006). We isolated individual taste cells, loaded them with Fura-2, and tested responses to a bath-applied mixture of sweet and bitter taste stimuli and to potassium chloride. This allowed us to reliably and unambiguously identify and distinguish receptor and presynaptic cells (Huang *et al.* 2007; Tomchik *et al.* 2007). Isolated receptor *versus* presynaptic cells, identified in this manner, were subjected to acid taste stimulation.

Receptor and presynaptic cells respond to acetic acid stimulation

When superfused with the sour taste stimulus acetic acid (10 mM, pH 5.0), isolated receptor and presynaptic cells alike exhibited pronounced Ca^{2+} transients. Surprisingly, applying a mineral acid, HCl, at the same or even more acidic pH, e.g. 10 mM HCl (pH 3), evoked small to negligible intracellular Ca^{2+} changes (Fig. 1*A* and *B*). (At pH 1.5, HCl did stimulate Ca^{2+} responses in taste cells. However, these responses were not repeatable and were likely to have represented cell damage.) To determine the source of Ca^{2+} in acid-evoked taste cell responses, we removed extracellular Ca^{2+} from the bath. Removing bath calcium significantly reduced acetic acid-induced Ca^{2+} responses in presynaptic (Type III) cells but had little effect on acid-evoked responses from receptor (Type II) cells (Fig. 1). These findings indicate that acetic acid elicits an increase in $[Ca^{2+}]_i$ in presynaptic cells by Ca^{2+} influx, but in receptor cells presumably by release from intracellular Ca^{2+} stores. This conclusion was tested in greater detail (below).

The proximate stimulus for sour taste is believed to be intracellular acidification (Lyall *et al.* 2001, reviewed by Roper, 2007). Thus, we tested the effects of taste stimulation designed to lower intracellular pH while leaving extracellular pH unaffected. When cells are bathed in sodium acetate at neutral pH, this effectively acidifies the cytosol (e.g. Slotki *et al.* 1993; Speake & Elliott, 1998; see discussion in Roper, 2007). Further, intracellular acidification elicits IP_3-mediated Ca^{2+} release from intracellular stores (Slotki *et al.* 1993). Accordingly, we tested whether sodium acetate stimulated intracellular Ca^{2+} release in isolated taste cells. Bathing Fura-2-loaded taste cells in 20 mM sodium acetate (substituted for NaCl, pH 7.2), a protocol that lowers cytosolic pH in other tissues by ~0.3 pH units (Speake & Elliott, 1998), evoked robust Ca^{2+} responses in receptor and presynaptic cells alike. Importantly, under these conditions, Ca^{2+} responses in both cell types were unaffected by removing Ca^{2+} from the bath (Fig. 2). This indicates that Ca^{2+} responses evoked by sodium acetate are produced by intracellular store release for receptor and presynaptic taste cells alike. (Note, Na^+, or 'salty' taste, was not a stimulus in these experiments

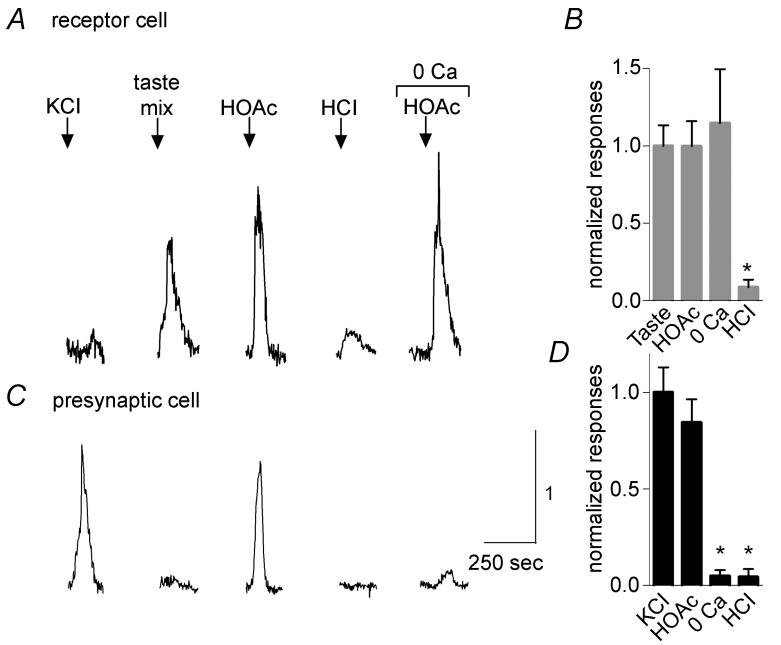

Figure 1. Acetic acid taste stimulation evokes Ca^{2+} responses in receptor (Type II) cells and presynaptic (Type III) cells
A, examples of receptor cell responses to bath applied KCl (50 mM), taste mix (10 μM cycloheximide, 2 mM saccharin, 1 mM denatonium and 0.1 mM SC45647), acetic acid (10 mM, pH 5), HCl (10 mM, pH 3), and acetic acid when Ca^{2+} in the bathing solution had been exchanged with Mg^{2+}. *B*, summary of data for receptor cells. *C*, example of responses from a presynaptic cell to the same sequence of stimuli as in *A*. *D*, summary of data for presynaptic cells. In *B* and *D*, bars show Ca^{2+} response amplitudes (means ± S.E.M.). All responses were normalized to the (pooled) mean response to taste mix (receptor cells) or to KCl depolarization (presynaptic cells). Data in *B* and *D* are from 22 experiments ($n = 32$ cells). $^*P < 0.05$. HOAc, acetic acid.

because sodium acetate was substituted equimolar for NaCl. Thus, there was no change in $[Na^+]_o$.)

Sodium acetate triggers Ca^{2+} release from intracellular stores

Intracellular Ca^{2+} store release in gustatory receptor (Type II) cells via a pathway involving phospholipase C subclass $\beta 2$ (PLC$\beta 2$) and IP_3 has been thoroughly documented for sweet, bitter and umami taste stimulation (e.g., Gilbertson *et al.* 2000; Tomchik *et al.* 2007). However, considerably less is known about Ca^{2+} store release in presynaptic (Type III) cells and how/whether it is triggered by intracellular acidification. Thus, we explored whether Ca^{2+} transients in presynaptic cells evoked by sodium acetate (i.e. by presumed intracellular acidification) were abolished by thapsigargin, a sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase inhibitor that depletes intracellular Ca^{2+} stores. Ca^{2+} transients evoked by sodium acetate as well as Ca^{2+} signals produced by stimulating P2Y receptors were significantly reduced by incubating isolated taste cells with thapsigargin (1 μM, 5–10 min) (Fig. 3). In marked contrast, this treatment did not affect Ca^{2+} influx stimulated by KCl depolarization (not shown) or by acetic acid taste stimulation (10 mM, pH 5).

We also tested whether incubating taste cells with a broad spectrum PLC blocker affected Ca^{2+} transients evoked by sodium acetate. Indeed, U73122 (10 μM, 6 min) significantly reduced sodium acetate-evoked Ca^{2+} responses in presynaptic cells. In contrast, Ca^{2+} influx into presynaptic cells stimulated by 10 mM acetic acid (pH 5) was unaltered by U73122 (Fig. 3). Collectively, the findings suggest that intracellular acidification in presynaptic cells produced by sodium acetate stimulates PLC/IP_3 release of Ca^{2+} from intracellular stores. This clearly differs from the frank Ca^{2+} influx in these cells stimulated by acetic acid at pH 5.

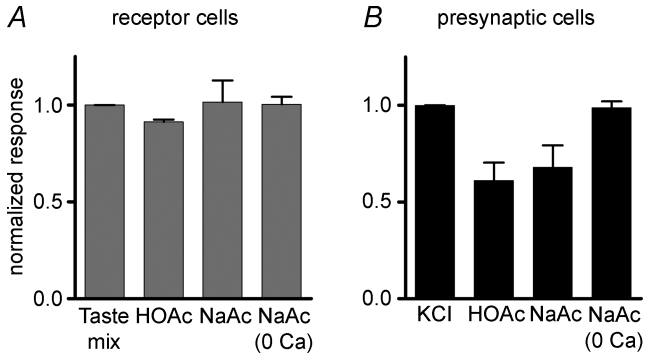

Figure 2. Intracellular acidification by sodium acetate evokes Ca^{2+} responses in receptor cells and in presynaptic cells
A, receptor cells: Ca^{2+} responses evoked by bath-applied taste mix (as in Fig. 1*A*), acetic acid (10 mM, pH 5), and sodium acetate (20 mM, pH 7.2). Bars show means ± S.E.M. of responses. Responses were normalized to the (pooled) mean response evoked by taste mix ($n = 3$). *B*, presynaptic cells: similarly, Ca^{2+} responses evoked by KCl depolarization, acetic acid and sodium acetate ($n = 9$). As in *B*, responses were normalized to the (pooled) mean response to KCl depolarization. HOAc, acetic acid; NaAc, sodium acetate.

Threshold for acid-stimulated Ca^{2+} influx in presynaptic cells

Next, we investigated at which point Ca^{2+} responses make the transition from store release to Ca^{2+} influx in presynaptic cells as the acid stimulus strength increases. This threshold is important because transmitter

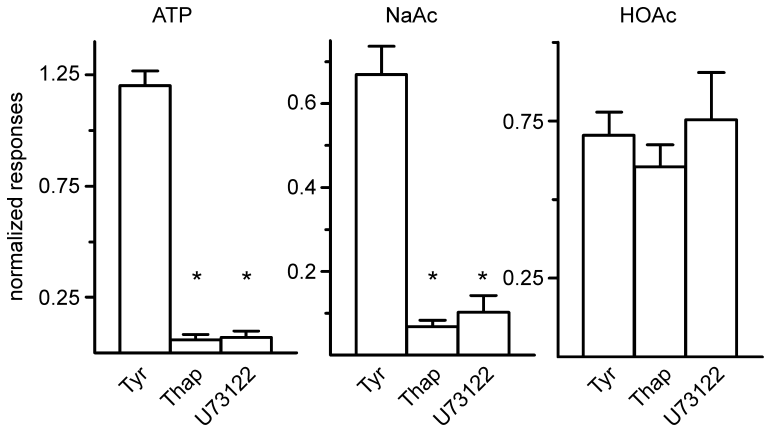

Figure 3. Intracellular Ca^{2+} release in presynaptic (Type III) cells is via a phospholipase C-mediated pathway
Data show Ca^{2+} responses evoked by ATP (1 μM), sodium acetate (NaAc, 20 mM, pH 7) and acetic acid (HOAc, 10 mM, pH 5). ATP and NaAc stimulate intracellular Ca^{2+} release via P2Y receptor activation and cytosolic acidification, respectively (Fig. 1; see Slotki *et al.* 1993; Speake & Elliott, 1998). In constrast, HOAc elicits Ca^{2+} signals via Ca^{2+} influx (see Fig. 1*C* and *D*). Treating presynaptic cells with thapsigargin (1 μM) significantly reduced Ca^{2+} responses evoked by ATP and NaAc, consistent with Ca^{2+} store release mechanisms for these stimuli. However, thapsigargin did not significantly alter HOAc-evoked Ca^{2+} responses, as expected for acid-stimulated Ca^{2+} influx. U73122 (5 μM) reduced Ca^{2+} signals stimulated by ATP and NaAc, but not by HOAc, suggesting that intracellular Ca^{2+} store release initiated by ATP or NaAc involves a phospholipase C. Bars show mean amplitudes ± S.E.M. of responses. Responses are normalized to the (pooled) mean response to KCl depolarization in the same cells (ATP, $n = 8$; NaAc, $n = 10$; HOAc, $n = 10$). Abbreviations: Tyr, Tyrode buffer; Thap, thapsigargin; NaAc, sodium acetate; HOAc, acetic acid. $^*P < 0.05$

(serotonin, 5-HT) secretion from presynaptic cells depends on Ca^{2+} influx, not intracellular Ca^{2+} release (Huang *et al.* 2005; see below). Establishing a threshold for Ca^{2+} influx might support a link between acid taste mechanisms and transmitter release. We applied 10 mM acetic acid titrated to different pH levels and measured Ca^{2+} responses in presynaptic cells in the presence and absence (replacement by Mg^{2+}) of extracellular Ca^{2+}. (In receptor, Type II, cells, acetic acid at all pH levels stimulated Ca^{2+} store release only.) Concentration–response curves showed that Ca^{2+} responses evoked by acetic acid titrated to pH 7 (i.e. sodium acetate) were solely generated by intracellular release. As the pH of the taste stimulus became more acidic, the Ca^{2+} response amplitude declined, but more importantly, a greater portion of the response was generated by Ca^{2+} influx (Fig. 4). The threshold for stimulating Ca^{2+} influx in isolated presynaptic cells appears to be between pH 6.0 and pH 6.5. By pH 5.0 there was negligible Ca^{2+} store release; nearly the entire acid-evoked Ca^{2+} signal was generated by Ca^{2+} influx.

Intracellular acidification is the proximate stimulus for acid taste in taste buds in lingual slices

To further test the concept of intra- *versus* extracellular acidification as the proximate stimulus of sour taste, we recorded responses in intact taste buds in lingual slices of vallate papillae in response to stimuli with varying acidity. This preparation preserves sensory epithelial polarity and allows recordings under more physiologically relevant conditions (Caicedo *et al.* 2000, 2002; Richter *et al.* 2003; Tomchik *et al.* 2007). Taste cells were loaded with Calcium Green dextran and taste stimuli were delivered to the apical

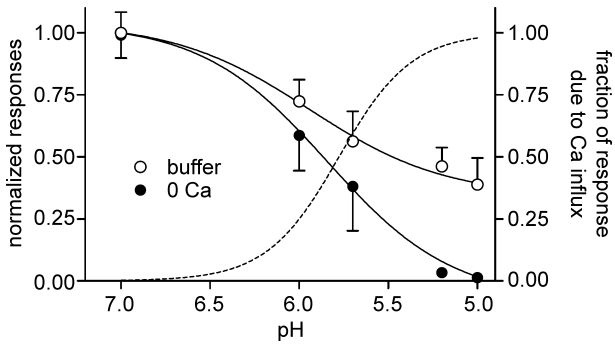

Figure 4. Acetic acid taste stimulation of presynaptic (Type III) taste cells varies with pH
Symbols are means ± S.E.M. of Ca^{2+} responses evoked by 10 mM acetic acid titrated with 1 N NaOH to different pH levels (for example, acetic acid titrated to pH 7 is equivalent to sodium acetate). Left ordinate (continuous lines) data are normalized to Ca^{2+} responses evoked by 10 mM sodium acetate, pH 7.0. Responses were recorded from cells bathed in Tyrode buffer (○) and in buffer in which Ca^{2+} was replaced by equimolar Mg^{2+} (●) to determine the approximate proportion contributed by Ca^{2+} influx. The difference between responses with and without Ca^{2+} is plotted as a dashed line and normalized to responses at pH 7.0, thus showing the fraction of response that is due to Ca^{2+} influx (right ordinate). Each point represents data from 3–11 cells.

tips of taste buds via focal micropipettes. Concentrations of acid stimuli were measured at the taste pore by observing the dilution of a known concentration of the fluorescent tracer included in the taste stimulus.

Focal application of 20 mM sodium acetate, pH 7.2, did not evoke Ca^{2+} responses in taste cells in the lingual slice preparation (data not shown), unlike the situation when this stimulus was bath-applied to isolated taste cells. Presumably this is due to the healthier condition and more intact intracellular buffering capacity of taste cells in the lingual slice preparation. Furthermore, focal application of sodium acetate in the lingual slice preparation reaches considerably less exposed surface of taste cells – only the apical tips of the taste cells penetrate into the taste pore. However, as shown previously (Richter *et al.* 2003), titrating sodium acetate to pH 5 (i.e. stimulating with acetic acid) evokes robust responses in a subset of taste cells. Next, we tested whether acid-evoked taste cell responses varied with the concentration of extracellular proton in the stimulating solution (i.e. with pH) or with the concentration of protonated acetic acid (HOAc). We stimulated taste buds with solutions consisting of equal concentrations of the uncharged, protonated acetic acid moiety (HOAc) but varying in extracellular proton concentration (i.e. pH), and compared these findings with those when taste buds were stimulated with solutions of equal pH (i.e. equal proton concentration) but varying $[HOAc]_o$ (see Methods). The results clearly showed that the effective stimulus was the uncharged acetic acid moiety, not extracellular protons; responses varied with increasing [HOAc], not with pH (Fig. 5). The threshold for acid-evoked responses using focal taste stimulation in the lingual slice preparation appears to be ~30 mM HOAc. (This compares with the threshold of 2–5 mM HOAc when isolated presynaptic taste cells were bathed in acetic acid, i.e. 10 mM acetic acid at pH 6.0–6.5, Fig. 4.) These findings emphasize that the proximate stimulus for the acid-evoked responses is not extracellular H^+ but intracellular acidification, consistent with the data from the isolated taste cells.

Acid taste stimulation elicits serotonin release from presynaptic cells

We previously reported that isolated taste buds secrete serotonin (5-HT) in response to acid taste stimulation (Huang *et al.* 2005) and subsequently that presynaptic cells specifically are the cells of origin for 5-HT secretion (Huang *et al.* 2007). Here we used biosensor cells to test whether acetic acid stimulated 5-HT release from isolated presynaptic cells. 5-HT biosensor cells were drawn onto a glass micropipette with gentle suction and positioned next to isolated presynaptic cells to measure acid-evoked release of the monoamine. We confirmed that the 5-HT biosensor itself was not directly stimulated by acetic acid at concentrations used in this report. Bath application of acetic acid (10 mM, pH 5.0) evoked Ca^{2+} transients in the presynaptic cell, as before, and also resulted in rapid and repeatable 5-HT biosensor responses, demonstrating

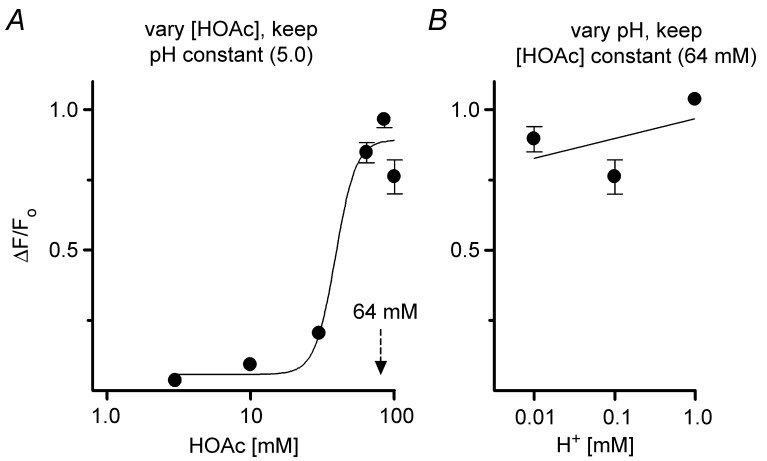

Figure 5. The amplitude of acid-evoked Ca^{2+} responses varies with [HOAc] but not with $[H^+]$ (i.e. pH)
Acetic acid solutions of varying pH and acid concentration were focally applied to the taste pore in the lingual slice preparation. Each data point is the mean ± S.E.M. ($n = 3$–22). *A*, responses evoked by acetic acid solutions of a constant $[H^+]$ (pH 5) but varying [HOAc] from 3 to 100 mM. *B*, responses obtained when [HOAc] was maintained at 64 mM but $[H^+]$ was varied from pH 5–3. (Note that this situation contrasts with the experiment shown in Fig. 4. In Fig. 4 the proportion of the protonated species in the stimulus, HOAc, was not maintained at a constant value but allowed to increase as the pH dropped, as per Henderson–Hasselbalch relations, see Methods.) The deviation of the slope from zero in *B* is not significant ($P < 0.23$) ($r^2 = 0.20$). These data indicate that acid taste responses vary with the concentration of the membrane-permeant acid (HOAc) but not with the membrane-impermeant proton (H^+), supporting the interpretation that intracellular *not* extracellular acidification evokes sour taste.

stimulus-evoked 5-HT release from presynaptic cells (Fig. 6*A*).

We next tested whether 5-HT release evoked by acid stimulation was Ca^{2+} dependent. Mg^{2+} (3 mM) was substituted for Ca^{2+} (2 mM) in the bath, and isolated presynaptic cells were stimulated with acetic acid, as before. Replacing bath Ca^{2+} with Mg^{2+} rapidly and reversibly reduced or eliminated acid-evoked Ca^{2+} transients in presynaptic cells, consistent with acid-evoked Ca^{2+} influx into presynaptic cells as shown above (Fig. 1), and also reduced or eliminated 5-HT release (Fig. 6). Our findings strongly suggest that 5-HT release from presynaptic cells evoked by acetic acid stimulation is triggered by Ca^{2+} influx, consistent with vesicular exocytosis at synapses.

In contrast to presynaptic cells, which release 5-HT, gustatory receptor (Type II) cells secrete ATP in response to taste stimulation (Huang *et al.* 2007; Romanov *et al.* 2007). Thus we tested whether acetic acid also stimulates ATP secretion from receptor cells. As with 5-HT biosensors and presynaptic (Type III) cells, above, we positioned ATP biosensors against isolated receptor cells. We consistently recorded robust Ca^{2+} transients in receptor cells and ATP secretion in response to taste stimulation (with a mixture of 10 μM cycloheximide, 2 mM saccharin, 1 mM denatonium and 0.1 mM SC45647), confirming taste-evoked ATP secretion (Huang *et al.* 2007). Surprisingly, despite the presence of large, acid-evoked Ca^{2+} transients in receptor cells, in no case did we observe ATP secretion evoked by acetic acid stimulation (Fig. 7).

Because acetic acid stimulates Ca^{2+} release from intracellular stores in receptor (Type II) cells (Fig. 1), these data indicate store-released Ca^{2+} *per se* is inadequate to trigger transmitter (ATP) secretion. This contrasts with acetic acid-evoked Ca^{2+} influx into, and subsequent 5-HT release from, presynaptic cells. Thus, we tested whether Ca^{2+} release from intracellular stores stimulated by intracellular acidification (with sodium acetate) could either trigger 5-HT release from presynaptic (Type III) taste cells or ATP secretion from receptor (Type II) cells. We bath-applied sodium acetate (20 mM, pH 7.2) to individual receptor cells and to presynaptic cells in parallel to produce intracellular acidification in these cells, as described in the previous experiments. However, in this case we also tested for transmitter secretion using 5-HT biosensors for presynaptic cells (Fig. 6*B*) and ATP biosensors for receptor cells (Fig. 7*B*). The results indicated that although sodium acetate triggers large Ca^{2+} transients due to store release of Ca^{2+} in receptor and presynaptic cells alike, this did not stimulate ATP or 5-HT secretion. This was the case even though the Ca^{2+} transients evoked by store release were as large if not larger than those produced by Ca^{2+} influx in presynaptic cells.

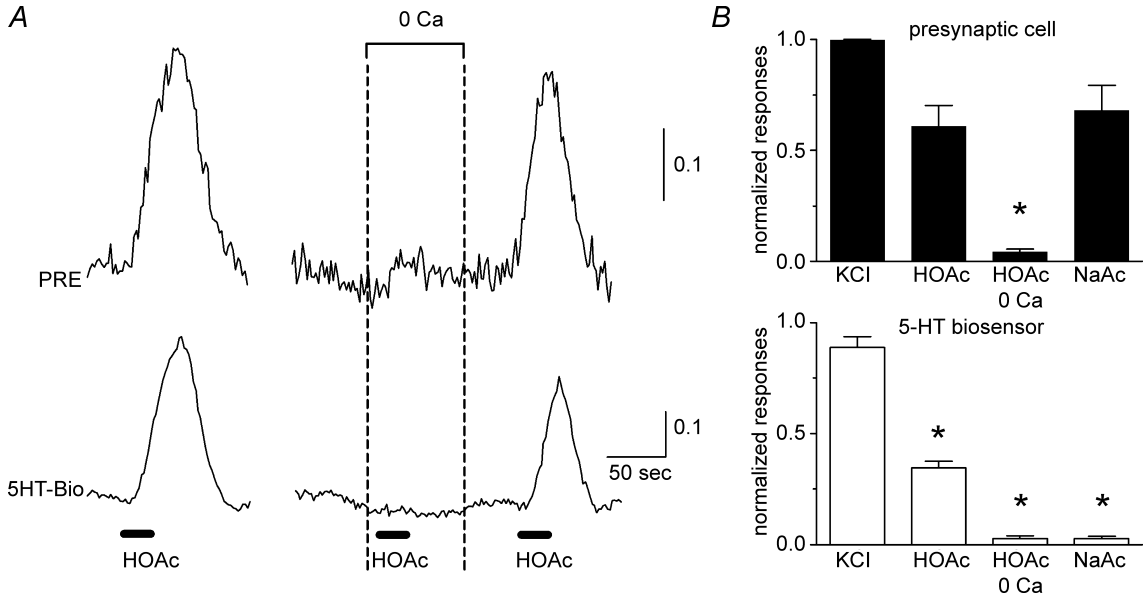

Figure 6. Acid taste stimulation evokes 5-HT release from presynaptic (Type III) taste cells
A, recording of Ca^{2+} responses in a presynaptic cell (PRE) and from a closely apposed 5-HT biosensor cell (5-HT Bio). Bath-applied acetic acid (HOAc, bar at bottom of traces, 10 mM, pH 5.0) evoked Ca^{2+} responses in the presynaptic cell (top trace) and, after a brief delay, in the biosensor cell (bottom trace), indicating 5-HT secretion. Responses in the presynaptic and 5-HT biosensor cells alike were abolished when Ca^{2+} was replaced with Mg^{2+} in the bathing solution (0 Ca, dashed lines). *B*, summary of data. Bars represent means ± S.E.M. of individual responses. All responses were normalized to the (pooled) mean KCl response for all experiments in the series ($n = 11$). Filled bars, data from presynaptic cells. Open bars, corresponding data from the apposed 5-HT biosensor cells. $^*P < 0.05$

Table 1. Summary of taste cell responses to sodium acetate and acetic acid stimulation

	Sodium acetate, 20 mM, pH 7.2	Acetic acid, 10 mM, pH 5.0
Taste receptor cells	Ca^{2+} store release No transmitter secretion	Ca^{2+} store release No transmitter secretion
Presynaptic cells	Ca^{2+} store release No transmitter secretion	Ca^{2+} influx 5-HT release

Taken together, the data indicate that mild intracellular acidification (e.g. bath-applied sodium acetate, pH 7) triggers intracellular Ca^{2+} release in receptor and presynaptic cells alike, but that this does not stimulate transmitter release in either cell type. Stronger acidic stimulation (10 mM acetic acid, pH 5) also elicits Ca^{2+} store release in receptor (Type II) cells but without transmitter secretion. In marked contrast, acid stimulation triggers Ca^{2+} influx into and 5-HT secretion from presynaptic cells. These data are summarized in Table 1.

Discussion

The present study was undertaken to investigate mechanisms underlying sour (acid) taste transduction in mouse taste buds, namely, to identify confidently which cells are directly sour responsive, to discover what is the proximate stimulus for acid taste stimulation, and to identify what neurotransmitter(s) sour-responsive taste cells release. The essential findings are that a specific sour-responsive subset of taste bud cells, namely presynaptic (Type III) taste bud cells, responds to intracellular acidification with Ca^{2+} influx and serotonin secretion. Only presynaptic cells show Ca^{2+} *influx* and serotonin secretion with acid taste stimulation in the range that elicits sour taste in humans and aversive behaviour in rodents. These transduction mechanisms for acid taste differ fundamentally from taste transduction for sweet, bitter and umami, which involve GPCR activation, Ca^{2+} store release, and ATP secretion via pannexin hemichannels from receptor (Type II), not presynaptic (Type III), taste cells (Huang *et al.* 2007). Our findings reinforce and provide further clarification of the longstanding, though puzzling observation that organic acids such as acetic acid are more intensely sour than mineral acids such as HCl at the same pH (Harvey, 1920). Organic acids acidify the cytosol more readily than do mineral acids and thereby more effectively stimulate sour-responsive taste cells.

DeSimone and colleagues have postulated that the proximate stimulus for acid taste is intracellular

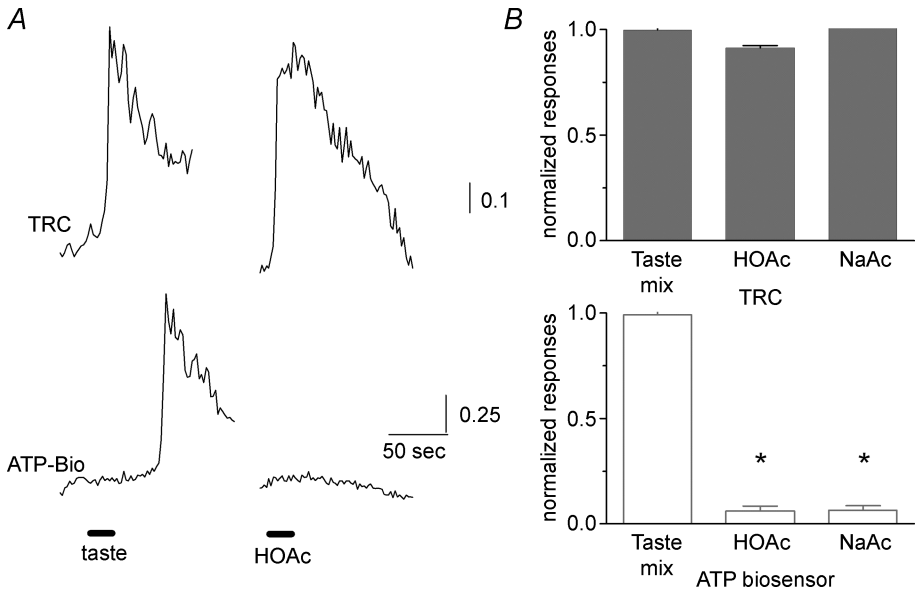

Figure 7. Acid taste stimulation does not evoke ATP secretion from receptor (Type II) cells
A, Ca^{2+} responses in a taste receptor cell (TRC) and from an apposed ATP biosensor cell (ATP-Bio). Bath-applied taste mix and acetic acid (10 mM, pH 5.0) (bars at bottom of traces) evoked Ca^{2+} responses in the receptor cell (top traces). However, only taste stimulation led to a response from the biosensor cell, indicating ATP secretion (bottom trace). *B*, summary of data. Bars represent means ± S.E.M. normalized to responses evoked by taste mix ($n = 4$). Filled bars, responses from receptor cells. Open bars, corresponding results from the adjacent ATP biosensor. $^*P < 0.05$.

acidification (Lyall *et al.* 2001, 2006). They applied acetic acid to the serosal or mucosal surfaces of isolated sheets of epithelium mounted in an Ussing chamber and recorded changes in intracellular pH, though without distinguishing taste cell types. Richter *et al.* (2003) extended these findings by showing that citric acid, applied to the mucosal surface of lingual slices, rapidly permeates the epithelium and acidifies the cytosol of all cells in the epithelium. However, only a subset of taste bud cells responded to cytosolic acidification with a transient influx of Ca^{2+}. The present data identify these acid-responding cells as presynaptic (Type III) taste bud cells and show that the Ca^{2+} influx leads to the release of serotonin. Taste cells did not respond well to extracellular acidification alone, such as by bath applied HCl. Nor did taste cells show a concentration–response relationship for extracellular $[H^+]$. Instead, taste cells responded in a concentration-dependent manner to the membrane-permeant, uncharged acetic acid moiety (HOAc), consistent with intracellular acidification. HOAc produces an intracellular acidification by diffusing into the cytosol. Once HOAc is inside the cell, it dissociates and delivers H^+ to the cytosol, acidifying the intracellular milieu:

$$\underbrace{H^+ + OAc^- \rightleftharpoons HOAc}_{\text{extracellular}} \rightarrow [\text{plasma membrane}] \rightarrow \underbrace{HOAc \rightleftharpoons H^+ + OAc^-}_{\text{intracellular}}$$

These findings are entirely consistent with the long-established psychophysical findings in human taste research that at equal pH values, organic acids such as acetic and citric acid are more effective taste stimuli (sour) than mineral acids such as HCl (Harvey, 1920). Indeed, counterintuitively, HCl is not nearly as sour as acetic (or citric) acid at equi-pH solutions; the sour threshold for solutions of HCl is much more acidic (Harvey, 1920). Of course, at sufficiently high enough concentration (i.e. low pH), HCl indeed evokes sour taste. The effectiveness of acetic and citric acids as sour tastants reflects the much higher membrane permeability of the protonated moieties of acetic and citric acids relative to protons, and thus the ability of the organic acids to deliver protons into the cell interior.

The implication of these findings for acid taste transduction mechanisms is that candidate sour taste transducer proteins such as ASIC channels (Ugawa *et al.* 1998; Richter *et al.* 2004), HCN channels (Stevens *et al.* 2001), or PKD2L1/PKD1L3 channels (LopezJimenez *et al.* 2006; Ishimaru *et al.* 2006; Huang *et al.* 2006) are likely to be gated by intracellular acidification instead of (or in addition to) extracellular protonation. Key proton-binding sites are likely to be in the intracellular domains of sour taste transduction molecules. To date, intracellular proton-binding sites have not been explored in detail with the proposed acid taste transduction channels. Parenthetically, a similar situation might hold for sensory transduction in inflammatory pain, where tissue acidification from lactic and carbonic acids is likely to generate local intracellular acidification in nociceptors.

It is interesting to note that intracellular acidification produced by sodium acetate triggers intracellular Ca^{2+} release in receptor and presynaptic cells alike. Intracellular Ca^{2+} release evoked by cytosolic acidification has been reported for other tissues (Slotki *et al.* 1993; Speake & Elliott, 1998). In presynaptic taste cells, further acidification (i.e. increased cytosolic acidification) ultimately triggers Ca^{2+} influx. This was not observed in taste receptor (Type II) cells. Furthermore, in presynaptic cells there even appears to be a concurrent suppression of intracellular Ca^{2+} release with increased cytosolic acidification (Figs 1*C* and 4). That is, there is a transition from Ca^{2+} store release to Ca^{2+} influx during increasingly stronger acid taste stimulation. Suppression of intracellular Ca^{2+} store release by sufficiently strong intracellular acidification may occur due to IP_3 receptor inhibition at pH values ~6 and lower (Mourey *et al.* 1990; Lopez-Colome & Lee, 1996).

Our results show that sour taste stimulation elicits Ca^{2+} influx and 5-HT release from presynaptic (Type III) cells, but does not stimulate transmitter (ATP) secretion receptor (Type II) cells, despite the presence of robust Ca^{2+} signals. The lack of transmitter secretion from receptor cells may be explained by the fact that ATP secretion from these cells is via pannexin 1 (Px1) hemichannels (Huang *et al.* 2007). Px1 hemichannels are gated open by intracellular Ca^{2+}, but intracellular acidification inhibits them (Locovei *et al.* 2006) and prevents ATP secretion (Huang *et al.* 2007). The blockage of gap junction hemichannels by intracellular acidification may similarly explain why the robust Ca^{2+} responses elicited by sodium acetate fail to trigger ATP secretion from receptor cells. It would be interesting to test whether sodium acetate or acid taste stimuli are able to reduce gustatory single fibre responses to sweet, bitter or umami tastants. To our knowledge, those experiments have not been conducted. Unlike its actions on gap junction hemichannels, intracellular acidification apparently has less effect on voltage-gated Ca^{2+} channels and thus upon depolarization-stimulated Ca^{2+} influx in presynaptic (Type III) cells. Hence, acid taste stimulation does not inhibit, but instead triggers, 5-HT release from presynaptic cells. A full explanation for these mechanisms awaits detailed intracellular pH measurements during sodium acetate and acetic acid taste stimulation under the conditions in our experiments.

Important next steps will include to resolve what is the role of 5-HT released by presynaptic cells when they are stimulated by acid tastants. Is 5-HT a synaptic transmitter onto sensory afferent fibres or a paracrine transmitter acting within taste buds (Kaya *et al.* 2004)? Recent studies indicate that taste thresholds are altered in human subjects when tissue 5-HT levels are manipulated by monoamine

reuptake inhibitors (Heath *et al.* 2006) but precise sites and mechanisms of 5-HT actions in taste buds are not yet known.

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