

**Acid-sensitive two-pore domain potassium (K₂P)
channels in mouse taste buds**

Trevor A. Richter¹, Gennady A. Dvoryanchikov¹, Nirupa Chaudhari^{1,2} and Stephen D. Roper^{1,2}

¹Dept of Physiology and Biophysics, and ²Neuroscience Program, University of Miami School of Medicine, 1600 NW 10th Ave, Miami, FL 33136, USA

Key words: Taste, sour, acid, pH, KCNK channels

Running title: 2-pore domain K channels in taste buds

7 figures

2 tables

Corresponding author:

S. Roper

Dept of Physiology and Biophysics,
University of Miami School of Medicine,
1600 NW 10th Ave, Miami, FL 33136

Roper@miami.edu

ABSTRACT

Sour (acid) taste is postulated to result from intracellular acidification that modulates one or more acid-sensitive ion channels in taste receptor cells. The identity of such channel(s) remains uncertain. Potassium channels, by regulating the excitability of taste cells, are candidates for acid transducers. Several two-pore domain potassium leak conductance channels (K₂P family) are sensitive to intracellular acidification. We examined for their expression in mouse vallate and foliate taste buds using RT-PCR, and detected TWIK-1 and -2, TREK-1 and -2 and TASK-1. Of these, TWIK-1 and TASK-1 were preferentially expressed in taste cells relative to surrounding nonsensory epithelium. The related TRESK channel was not detected, whereas the acid-insensitive TASK-2 was. Using confocal imaging with pH-, Ca²⁺-, and voltage-sensitive dyes, we tested pharmacological agents that are diagnostic for these channels. Riluzole (500 μM), selective for TREK-1 and -2 channels, enhanced acid taste responses. In contrast, halothane (≤ ~17 mM), which acts on TREK-1 and TASK-1 channels, blocked acid taste responses. Agents diagnostic for other 2-pore domain and voltage-gated potassium channels (anandamide, 10 μM; Gd³⁺, 1 mM; arachidonic acid, 100 μM; quinidine, 200 μM; quinine, 100 mM; 4-AP, 10 mM; and TEA, 1 mM) did not affect acid responses. The expression of 2-pore domain channels and our pharmacological characterization suggest that a matrix of ion channels, including one or more acid-sensitive 2-pore domain K channels, could play a role in sour taste transduction. However, our results do not unambiguously identify any one channel as the acid taste transducer.

INTRODUCTION

Sour taste is caused by protons that are released from acidic stimuli as they permeate the lingual epithelium. This results in the intracellular acidification of all the cells within a taste bud (Lyll *et al.* 2001; Richter *et al.* 2003a) and Ca^{2+} influx in a select subset of the acidified taste cells (Richter *et al.* 2003a). Ca^{2+} influx presumably leads to neurotransmitter release at synapses between sensory afferent neurons and taste cells. A complete description of the mechanisms by which cytoplasmic acidification stimulates Ca^{2+} influx in the acid-sensitive taste receptor cells remains to be elucidated. Our previous findings indicate that intracellular acidification opens voltage-gated Ca channels in a fraction of taste cells (Richter *et al.* 2003a), presumably due to depolarizing receptor potentials generated by acid taste stimuli. Thus, a key question of sour taste transduction is how intracellular acidification evokes depolarizing receptor potentials.

Protons are postulated to act on ion channels in the membranes of acid-sensitive taste receptor cells and generate inward current. Taste cells express several proton-gated or proton-permeant cation channels that have been proposed to be sour taste transducer molecules, including cation channels (ENaCs, MDEG1, ASIC-2b: Gilbertson *et al.* 1992; Gilbertson and Gilbertson, 1994; Ugawa *et al.* 1998; Liu and Simon, 2001; Lin *et al.* 2002), hyperpolarization-activated cyclic nucleotide-gated channels (HCNs: Stevens *et al.* 2001), and proton-sensitive chloride channels (Miyamoto *et al.*, 2000). However, even though most of these ion channels are expressed in subsets of taste cells, it is not known whether these cells are the same cells that mediate acid taste. Thus, a direct link has not been firmly established between these various channels and sour taste transduction.

Potassium leak channels establish the membrane potential of all cell types and play

especially important roles in excitable cells. The ion channels that produce leak conductances have an unusual structure, with each subunit possessing four transmembrane helices and two pore-lining domains (Lesage *et al* 1996). As many as 15 mammalian genes, comprising the K₂P family, encode these potassium channels (Talley *et al*, 2003). The K₂P channels are widely expressed, and regulate the resting potential and membrane excitability in neurons and cardiac cells via their sensitivity to physiological signals such as Ca²⁺, cyclic nucleotides, or pH (reviewed, Goldstein *et al.* 2001; Talley *et al.* 2003). Certain members of this family are strongly modulated by intracellular acidification, namely TWIK-1 and -2, TREK-1 and -2, (Lesage *et al* 1996; Chavez *et al* 1999; Maingret *et al* 1999; Lesage *et al* 2000;). Other K₂P channels, namely TASK-1 -2 and -3 (Kim *et al* 1999, 2000) and the distantly related TRESK (Sano *et al* 2003), are modulated strongly by extracellular acid and only weakly (if at all) by intracellular acidification. Acid taste stimuli give rise to intracellular acidification and this is postulated to be the proximate stimulus for acid (sour) taste (Lyll *et al.* 2001). In the present study, we investigated whether 2-pore domain K channels sensitive to intracellular acidification are expressed in mouse taste buds and whether they might be relevant to acid taste transduction. We also tested the effects of a panel of pharmacological agents that have been reported to affect these channels. The results are consistent with the notion that one or more acid-sensitive 2-pore domain K channels may play a role in acid taste transduction. Some of these data have been presented in abstract form (Richter *et al.* 2003b).

METHODS

Animals

All experiments were carried out according to the NIH Guidelines for the Care and Use of Animals and protocols were approved by the University of Miami Animal Care and Use Committee. Adult C57/BL mice (The Jackson Laboratory, Bar Harbor, ME) were killed by exposure to CO₂ followed by cervical dislocation. Tongues were dissected free and transferred to Tyrode's buffer.

RT-PCR

Taste epithelium was delaminated by subepithelial injection of proteases as described previously (Gilbertson *et al.* 1993) and nontaste epithelium surrounding the papilla was trimmed away. The preparation comprises predominantly (but not exclusively) taste buds. Total RNA was isolated from these taste buds or from adjacent non-sensory lingual epithelium using the Absolutely RNA Nanoprep kit (Stratagene, La Jolla, CA). Taste buds from a single papilla were dispersed in 100 μ l lysis buffer containing guanidine thiocyanate and β -mercaptoethanol, RNA from the lysate was captured on a silica-based matrix, treated with DNase I, washed and then eluted in 10 μ l 10 mM Tris-HCl (pH 7.5). Purified taste RNA was denatured and first strand cDNA was synthesized at 42°C for 60 min using SuperScript II Reverse Transcriptase in a 20 μ l final volume. After removing template RNA with RNase H, 1 μ l cDNA was used as template in a 25 μ l PCR. Each cDNA preparation was used to test for expression of the entire set of channels described in Results. All reagents were purchased from Invitrogen (Carlsbad, CA).

We designed PCR primers using published full-length cDNA sequences from mouse for TWIK-1 and -2, TREK-1 and TASK-1 and -2 (Table 1). Because no published mouse cDNA

<Table 1 near here>

sequences were available for TASK-3 or TREK-2, we used full-length cDNAs from rat to identify putative orthologs (> 96 % identity) in the mouse genome. We confirmed that intron locations and exon sizes were identical between the rat genes and the presumed orthologs in mouse. Primers for TASK-3 and TREK-2 were then designed in identified mouse exons. We used a similar strategy starting from published human TRESK full-length cDNA sequence to design primers for mouse TRESK. In all cases, each primer pair spanned at least one intron. Conditions for PCR were: 94°C for 2 min; 25–40 cycles at 94°C for 30 s, 56–65°C for 30 s, 72°C for 45 s; and a final extension at 72°C for 5 min. Annealing temperatures were 56°C for TWIK-1 and -2, TREK-1, TASK-3 and TRESK; 57°C for TASK-2; 59°C for TREK-2 and 65°C for TASK-1. We also amplified two control mRNAs, β -actin (5'-caaccgtgaaaagatgacc-3', and 5'-ctgaaaagagcctcagg-3', 449 bp product) and the taste cell-specific G-protein, gustducin (5'-gcaaccactccattgttct-3', and 5'-agaagagcccacagtctttgag-3', 286 bp product), using primer pairs located in separate exons.

Functional imaging

We used circumvallate taste buds, which have been shown by us and others to contain acid-responsive taste cells (Caicedo *et al.* 2002; Richter *et al.* 2003; Ugawa *et al.* 2003). We imaged changes of Ca^{2+} , pH, and membrane potential in taste cells using one of two preparations: (a) 100 μm -thick slices of circumvallate taste papilla (“lingual epithelium slice”, Richter *et al.* 2003) or (b) isolated circumvallate taste buds and cells. For imaging, the functional indicator dyes listed in Table 2 were obtained from Molecular Probes (Eugene, OR) and were stored as stock solutions at -20°C .

<Table 2 near here>

For Ca^{2+} imaging in lingual slices, calcium green dextran (CGD, 1 mM) was injected iontophoretically as described previously (Caicedo and Roper, 2001; Caicedo et al. 2002). To record changes in pH in lingual epithelial slices, we loaded taste cells using the same method for the pH-sensitive fluorescent indicator dye, BCECF (Richter *et al*, 2003a). It should be noted that we only measured *relative changes* in $[\text{Ca}^{2+}]_i$, pH_i , and membrane potential with this method and were not able to determine the absolute values of $[\text{Ca}^{2+}]$, pH, and mV reached during the experiments.

For recordings on taste cells removed from their epithelial environment, taste buds were gently aspirated from the delaminated lingual epithelium (see above, RT-PCR) and plated onto coverslips coated with Cell-Tak adhesive (BD Biosciences, MA). Such preparations, which typically included elongate taste receptor cells dissociated from the taste buds, were loaded with membrane-permeant (AM esters) indicator dyes by incubating them in the dark at 25°. Dyes and incubation times were: for $[\text{Ca}^{2+}]$, 2 μM OG-AM for 25 min; for pH_i , 100 μM HPTS for 10 min; and for membrane potential, 100 μM ANEPPS for 15 min. (see Table 2).

Taste stimuli

Citric acid (100 mM in Tyrode's buffer, pH ~3), a potent acid taste stimulus in rodents and humans and which, like other weak organic acids, is a more effective tastant than HCl (e.g. Beatty and Craig, 1935), was focally applied with pressure ejection from a puffer micropipette. We measured the concentration of citric acid delivered to the taste cells by including a fluorescent tracer dye in the stimulus solutions (200 μM Lucifer yellow CH; Molecular Probes, Eugene, OR). For some experiments as indicated, we used bath-application of KCl or acidified

Tyrode's buffer (pH adjusted to 7.5 with HCl).

Microscopy, data analysis and statistics

Cell responses in the lingual slice preparation were recorded using scanning confocal microscopy with argon laser excitation (488 nm) combined with an FITC filter set (510 LP) to view cells loaded with CGD (Caicedo *et al.* 2000). Data were captured by scanning a field (~10 μm optical slice) containing 1 or more taste buds every 0.5 to 5.0 seconds. Data were stored for offline analysis using Fluoview v. 2.1 (Olympus). Throughout this analysis, we only included data from taste cells that exhibited Ca^{2+} responses to citric acid with $\Delta F/F > 0.1$ and that could be elicited at least 3 times in succession.

When testing the effects of pharmacological agents on taste cell responses (*i.e.* $\Delta[\text{Ca}^{2+}]$) to citric acid, we included data from cells for which we could collect at least 3 control responses (prior to drug application) and 3 or more responses during drug treatment. Data from cells that did not recover their control response by 30 min after washout were not included. We used the same selection criteria for ΔpH_i responses of taste cells.

All recordings for individual cells are presented as tracings of $\Delta F/F$. Responses were measured as the peak $\Delta F/F$ in a record. Histograms comparing taste cell responses before and after a drug treatment were obtained by taking the mean of 3 or more responses ($\Delta F/F$) before applying a drug and normalizing all responses to that mean. To compare the effects of pharmacological treatments against controls, we used a paired Student's t-test. For all statistics, $P < 0.05$ was considered significant. Curves were fitted using Prism (GraphPad Software, San Diego).

Reagents and solutions

Unless stated otherwise, all reagents for physiological experiments were obtained from Sigma (St. Louis, MO). Solutions were prepared freshly for each experiment by dissolving in Tyrode's buffer (in mM: 135 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 5 NaHCO₃, 10 Hepes, 10 glucose and 10 sodium pyruvate; pH 7.4). The following pharmacological agents were used at concentrations that are at least 10 times the IC₅₀ for 2-pore domain K⁺ channels: anandamide (10 μM), Gd³⁺ (1 mM), arachidonic acid (AA, 100 μM), quinidine (200 μM), quinine (100 mM) and riluzole (500 μM). We also tested the effect of 4-aminopyridine (4-AP, 10mM) and tetraethylammonium (TEA, 1 mM) because although 2-pore domain channels are resistant to these treatments (Lesage 2003), most other potassium channels are not. All test solutions were prepared freshly immediately prior to experimentation by dissolving in Tyrode's buffer. Riluzole was prepared as a 100 mM stock solution in DMSO and stored at -80°C. The stock solution was diluted in Tyrode's buffer to a final concentration of 500 μM (0.1 % final DMSO concentration). Halothane was made up as a saturated solution (~17 mM, pH 7.4, cf. Patel *et al.* 1999). To minimize loss of this volatile anesthetic from the perfusion system, the halothane solution was prepared immediately prior to experimentation and was delivered by gravity from 10 ml syringes sealed with plastic stoppers.

RESULTS

As we have previously shown (Richter *et al.* 2003a), applying citric acid focally to the taste pore of vallate taste buds produces an intracellular acidification throughout the entire taste bud (Fig 1A). In a subset of taste cells, this acidification elicits an increase in $[Ca^{2+}]_i$ (Fig 1B).

<Figure 1 near here>

In electrophysiological studies, acid taste stimulation elicits an inward current and a membrane depolarization in taste receptor cells (Kinnamon and Roper, 1988; Gilbertson *et al.* 1992).

Hence, we postulated that the link between cytoplasmic acidification and the rise in $[Ca^{2+}]_i$ in acid-responsive taste cells is membrane depolarization followed by activation of voltage-gated Ca channels. Thus, we tested whether we could measure depolarizing receptor potentials in acid-sensitive taste cells using the voltage-sensitive fluorescent dye, ANEPPS (Hayashi *et al.* 1996). We first determined whether changes in ANEPPS fluorescence ($\Delta F/F_{ANEPPS}$) reliably measured changes in membrane potential in isolated taste buds. To depolarize cells, we applied Tyrode's buffer with elevated concentrations of KCl (20, 50, 100 mM: substituted for equimolar NaCl) to isolated taste buds pre-loaded with ANEPPS. As expected, KCl application resulted in a concentration-dependent increase in $\Delta F/F_{ANEPPS}$, consistent with membrane depolarization of taste cells (Fig. 1C). To estimate the incidence of taste cells responding to acid with membrane depolarization, we measured responses from single cells loaded with ANEPPS. Applying citric acid (100 mM) to isolated taste cells resulted in a pronounced increase in ANEPPS fluorescence in ~ 40% of the isolated cells, consistent with depolarization in a subset of acid-sensitive taste cells (Fig.1D). Collectively, these findings suggest the model for acid taste transduction shown in Fig.2.

<Figure 2 near here>

Expression of acid-sensitive 2-pore domain K channels in mouse taste buds

Using RT-PCR, we asked whether any of the seven 2-pore domain K channels (Sano *et al.* 2003), that are modulated by intracellular acidification were expressed in taste buds and might play a role in acid taste. Expression of five of these acid-sensitive channels was detected in cDNA prepared from mouse circumvallate taste buds, namely TWIK-1 and -2, TREK-1 and -2, TASK-1. Expression of TASK-3 and TRESK mRNAs was not apparent (Fig. 3). We found

<Figure 3 near here>

an identical pattern of expression for each of these five channels in circumvallate and foliate taste buds (not shown). TASK-1 appeared to be expressed selectively in taste buds when compared to non-taste lingual epithelium. The taste-selective expression pattern of TASK-1 was confirmed in 5 independent preparations of RNA from mouse circumvallate and foliate taste buds and 3 preparations from non-taste lingual epithelium. In contrast, RT-PCR products for TWIK-2, TREK-1 and -2, and TASK-2 were readily detected in nontaste lingual epithelium. TWIK-1 appeared to be expressed at a higher level in taste buds compared to nonsensory lingual tissue, although we have not attempted to quantify this differential expression. We also tested for the expression of a related channel, TASK-2, that is activated by external (but not cytoplasmic) acidification. We detected TASK-2 expression at similar levels in taste and non-taste lingual RNAs. PCR for β -actin and the taste cell-specific G protein, α -gustducin, served to confirm the overall quality and inclusion of taste buds, respectively, in each cDNA preparation.

Although the RT-PCRs in this study were not quantitative, we noted that RT-PCR products corresponding to TWIK-1 and -2 were readily detected after only 30 cycles of amplification, whereas products for the remaining channels required up to 40 cycles to be consistently detected. This suggests that the corresponding mRNAs for TWIK-1 and -2 may be

present at relatively high concentrations in taste buds and nonsensory lingual epithelium.

Pharmacological characterization of acid responses

A number of pharmacological agents known to affect the above acid-modulated 2-pore domain K channels were tested for their effect on acid taste responses in mouse taste cells. For example, both quinine and quinidine selectively inhibit TWIK-1 and TASK-2 (Lesage *et al* 1996). Yet, continuous perfusion of lingual slices with quinidine (200 μ M) had no effect on citric acid-induced responses (Fig. 4A). Similarly, 100 mM quinine did not alter citric acid-induced Ca^{2+} responses (not shown). Parenthetically, other (bitter-sensitive) taste cells did respond to quinine, even at 1mM, as we have shown previously (Caicedo *et al.* 2002). The following agents, which act on various 2-pore domain K channels, did not affect citric acid-induced taste responses (Fig. 4): 10 μ M anandamide (an inhibitor of TASK-1, Maingret *et al* 2001), 1 mM Gd^{3+} (an inhibitor of TREK-1, Maingret *et al* 2000), 100 μ M arachidonic acid (an enhancer of TREK-1, -2, Lesage *et al* 2000). We also tested 4-AP and TEA, which are potent inhibitors of voltage-gated and other types of K channels but are relatively ineffective at 2-pore domain K channels (Lesage 2003). Neither 10mM 4-AP nor 1mM TEA had an effect on citric acid taste responses (Fig. 4E, F).

<Figure 4 near here>

In contrast, we observed that riluzole and halothane consistently altered citric acid-induced Ca^{2+} responses in mouse taste cells. Prolonged application of riluzole depresses TREK-1 and -2 channels (Duprat *et al* 2000; Lesage 2003). In the lingual slice preparation, bath applied-riluzole (500 μ M) *increased* citric acid taste responses by a small, but statistically significant extent (Fig. 5). This effect is consistent with block of a resting (leak) K^+ conductance.

<Figure 5 near here>

Halothane, which activates TREK-1 and -2, TASK-1 and -3 channels (Patel *et al.* 1999; Lesage and Lazdunski 2000; Patel and Honore 2001; Terrenoire *et al.* 2001), significantly *reduced* acid-evoked Ca^{2+} transients in taste cells ($P < 0.05$, $n = 8$; Fig. 6C, D). Nevertheless, halothane did not modify acid-evoked intracellular pH changes in taste cells ($P > 0.05$, $n = 10$; Fig. 6 A,B). This result suggests that halothane affects acid taste responses at a step after cytoplasmic acidification but prior to Ca^{2+} influx. Halothane did not alter responses to a bitter stimulus, cycloheximide ($P > 0.05$, $n = 5$, Fig. 6E, F), demonstrating that this anesthetic does not globally affect taste cell responsiveness.

<Figure 6 near here>

The above results indicated that halothane perturbs a step downstream of cytoplasmic acidification. Hence, we tested whether halothane blocks membrane depolarization in response to acidification, or directly blocks Ca^{2+} influx. To image changes in membrane potential in individual cells, this series of experiments was carried out on isolated taste cells. First, we confirmed that applying halothane did not directly influence either the resting pH_i or resting Ca^{2+} concentration of taste cells in an isolated preparation (Fig. 7A, B), just as in the slice. Halothane

<Figure 7 near here>

alone hyperpolarized a significant fraction of ANEPPS-loaded taste cells (Fig. 7C; 31 % of cells). This is consistent with the halothane-induced hyperpolarization noted in neurons (Sirois *et al.*, 1998). More importantly, halothane also prevented acid-evoked depolarization of isolated taste cells (Fig. 7D). Hence, halothane appears to block the Ca^{2+} influx in response to acid stimulation by stabilizing the membrane potential of taste cells.

DISCUSSION

The present study examined a class of potassium leak channels, the K_2P channels. Specifically, we asked whether members of this group that are sensitive to intracellular acidification are expressed in mouse taste buds and might be involved in sour (acid) taste transduction. We found that TWIK-1 and -2, TREK-1 and -2, and TASK-1 but not TASK-3 or TRESK channels are expressed in mouse vallate and foliate taste buds. Of these, only TWIK-1 and TASK-1 appeared to be expressed preferentially in taste buds relative to surrounding non-taste epithelium. Preliminary reports have also described TWIK-1, TREK-1 and -2, TASK-1 and -2 in taste cells from rats (Burks *et al.* 2003; Lin *et al.* 2003). Although the findings in the rat generally concur with our results in mouse. Burks *et al.* (2003) reported TASK-3 in vallate and foliate (but not fungiform) taste buds and apparently did not detect TWIK-2. The origin and significance of the differences in expression between rat and mouse taste buds regarding TWIK-2 and TASK-3 expression is unclear. Indeed, there is precedence for such species difference in channel expression. For example, the proton-gated cation channel, ASIC-2, is expressed in rat but not mouse taste buds (Richter *et al.*, 2004).

Several acid-sensitive ion channels have been proposed to mediate sour taste perception in mammals, including the cation channels ENaC, MDEG1, ASIC-2, and HCN1 and HCN4 (Gilbertson *et al.* 1992; Gilbertson and Gilbertson, 1994; Ugawa *et al.* 1998, 2003; Liu and Simon, 2001; Lin *et al.* 2002 Stevens *et al.* 2001), and chloride channels (Miyamoto *et al.* 2000).

Downstream of the acid transduction channel(s), a basolateral N^+-H^+ exchanger, NHE-1, is believed to play a significant role in the adaptation of the acid taste response (Lyll *et al.* 2004). Despite the apparent variety of acid-sensitive ion channels expressed by taste cells, none of the aforementioned ion channels has been directly and unequivocally demonstrated to mediate sour

taste. For example, HCN1,4 are expressed in a subset of taste cells and ionic currents produced by the cloned channel resemble those in some taste cells (Stevens *et al.* 2001). Similarly, ASIC-2 is expressed in a subset of rat taste cells and ASIC-like currents were recorded in rat taste cells (Ugawa *et al.* 1998, 2003). In both instances, it is not known whether the cells that express HCN and ASIC2 are those that respond to acid taste stimuli. Further, ASIC-2 is not significantly expressed in mouse taste cells (Richter *et al.* 2004). And, genetic ablation of ASIC-2 in mice does not affect behavioral or Ca^{2+} responses to acid taste stimuli (Kinnamon *et al.* 2000, Richter *et al.* 2004). In short, none of the proposed cation or chloride channels has risen to the fore as a compelling sour taste transducer in mammalian taste buds.

The absence of an unequivocal sour taste receptor was in part the rationale for investigating a role for K_2P channels in acid taste. These potassium leak channels are expressed in many tissues. K_2P channels are constitutively active and play a major role in physiological functions including establishing membrane potential and regulating neuronal and muscular excitability in response to neurotransmitters and hormones (Lesage and Lazdunski 2000). Some members of the K_2P family are regulated by pH changes in the extracellular milieu or in the cytoplasm or both, while others are relatively pH insensitive. We have tested those that are affected by intracellular acidification, which is believed to be the proximate stimulus for acid taste (Lyll *et al.* 2001). One such channel, TASK-1, is a proposed acid sensor in the carotid body (Buckler *et al.* 2000). It may be significant that we found TASK-1 is expressed in taste buds and not in surrounding nonsensory tissue. TASK-1 is blocked by both intra- and extracellular acidification (Kim *et al.* 1999). Thus, acid taste stimulation should depolarize taste cells that express this channel. Indeed, citric acid stimulation was shown to result in depolarizing receptor potentials that originate from a blocked resting conductance (Cummings and

Kinnamom, 1992). In short, TASK-1 might be a good candidate for an acid taste transducer in mouse taste buds. Opposing this conclusion, however, is the lack of action of anandamide (a TASK-1 blocker) on acid taste responses in our experiments (Fig 4B). Further studies employing *in situ* hybridization, immunohistochemistry and more detailed functional analyses would be necessary to definitively establish or refute a role for TASK-1 in sour taste.

The results from the pharmacological testing did not allow us to identify unambiguously any one acid-sensitive K_2P channel as uniquely associated with acid taste responses. Expression alone would suggest TWIK-1 and TASK-1 are the most relevant channels because they are preferentially expressed in taste cells. However, this conclusion was not supported by the pharmacological profile of acid responses in our experiments.

The effects of riluzole on acid responses are consistent with an involvement of TREK-1 and -2, both of which are blocked by riluzole. However, because intracellular acidification *opens* TREK-1 and -2 channels to *hyperpolarize* cells (Lesage and Lazdunski 2000), neither of these channels is a candidate for a primary transducer for acid taste. One would anticipate that pharmacological block of TREK-1 and -2 leak conductances would enhance any acid-evoked depolarizing currents, in line with what we observed for taste cells (Fig. 5). This would suggest that TREK-1 and/or TREK-2 might act as modulatory channels in acid-sensing taste cells, serving to oppose acid-evoked responses and thereby keeping depolarizing receptor potentials in check, or assisting in recovery from depolarization. A major caveat is that although riluzole has been widely used to diagnose K_2P channels in numerous studies, it has been shown to affect other ion channels as well (Cao *et al.* 2002). Similarly, the ability of halothane to depress acid taste responses may result from its known ability to activate TREK-1 and TASK-1 channels. By opening these leak conductances, halothane hyperpolarizes membranes and shunts inward

currents, thus preventing depolarizing receptor potentials. These outcomes readily explain the results in taste cells (Fig. 7C,D). Yet, as with riluzole, the actions of halothane are not completely specific to K₂P channels. For instance, halothane also blocks voltage-gated Ca channels (Kamatchi *et al.* 1999), an action that would cooperate with its hyperpolarizing effect on membrane potential to further reduce any acid-evoked Ca²⁺ influx (Fig. 6C,D).

In aggregate, our findings are generally consistent with, but do not prove a role for K₂P channels in taste in general, and acid taste in particular. Certain of the acid-sensitive K₂P channels may be more likely than others to be involved in sour taste because of their preferential expression in taste buds *versus* nontaste tissue (*i.e.* TWIK-1 and TASK-1) and because riluzole and halothane affected acid taste responses (*i.e.*, TREK-1 and -2 and TASK-1). It is possible that acid taste transduction is the result of the effect of intracellular acidification on a *matrix* of acid-sensitive ion channels in taste cells, some of which would tend to depolarize (e.g. ASIC2, HCN-1 and -4, TASK-1) and others, like TREK-1 and -2 channels, to stabilize the membrane potential, with a net depolarizing receptor potential. If this interpretation is correct, it may be difficult to use knock-out mice to isolate any one contributor to sour taste.

As a footnote, throughout this report the assumption has been that *intracellular* acidification in the taste bud is the proximate stimulus for acid taste responses, as proposed by others (Lyall *et al.* 2001) and as we have also observed (Richter *et al.* 2003a). Yet it should be noted that applying acid taste stimuli to the lingual sensory surface will acidify intra- and extracellular compartments alike. Measurements of pH alterations selectively in the narrow intercellular spaces within taste buds after acid taste stimulation have not been possible to date. This may be important because *extracellular* protons affect different K₂P leak channels than do *intracellular* protons. It might also be noted that ASIC-2, a candidate sour taste transducer in rat

taste cells (Ugawa *et al*, 1998, 2003) is gated by extracellular protons. Further, ENaC channels would require a proton *gradient* across the membrane (*i.e.*, extracellular > intracellular concentration) to generate inward current carried by H⁺. Ambiguity about the actual site of action of protons released from sour tastants only adds to the present uncertainties about sour taste transduction.

Acknowledgements

This study was supported by grants NIH/NIDCD 2R01 DC00374 (SDR), 1R01 DC3013 (NC), and 1R21 DC5500 (NC). Current address for T.A. Richter: Ottawa Health Research Institute, 725 Parkdale Ave., Ottawa, Ontario K1Y 4E9, Canada.

REFERENCES

- Beatty RM and Cragg, LH.** The sourness of acids. *J Am Chem Soc* 57: 2347-51, 1935.
- Buckler KJ, Williams BA, Honore E.** An oxygen-, acid- and anaesthetic-sensitive TASK-like background potassium channel in rat arterial chemoreceptor cells. *J Physiol.* 525:135-42, 2000.
- Burks CA, Hansen DR, Rao S, Lin W, Kinnamon SC, and Gilbertson TA.** Rat taste buds express multiple members of the KCNK family of two-pore domain potassium channels. *Chem. Senses* 28: A75, 2003.
- Caicedo A, Jafri MS, and Roper SD.** *In situ* Ca²⁺ imaging reveals neurotransmitter receptors for glutamate in taste receptor cells. *J Neurosci.* 20: 7978-85, 2000.
- Caicedo A and Roper SD.** Taste receptor cells that discriminate between bitter stimuli. *Science.* 291:1557-60, 2001.
- Caicedo A, Kim KN, and Roper SD.** Individual mouse taste cells respond to multiple chemical stimuli. *J Physiol.* 544:501-9, 2002.
- Cao YJ, Dreixler JC, Couey JJ, and Houamed KM.** Modulation of recombinant and native neuronal SK channels by the neuroprotective drug riluzole. *Eur J Pharmacol.* 449:47-54, 2002.
- Chavez RA, Gray AT, Zhao BB, Kindler CH, Mazurek MJ, Mehta Y, Forsayeth JR, and Yost CS.** TWIK-2, a new weak inward rectifying member of the tandem pore domain potassium channel family. *J Biol Chem.* 274: 7887-92, 1999.
- Cummings TA, and Kinnamon SC** Apical K⁺ channels in Necturus taste cells. Modulation by intracellular factors and taste stimuli. *J Gen Physiol.* 99: 591-613, 1992 .

- Duprat F, Lesage F, Patel AJ, Fink M, Romey G, and Lazdunski M.** The neuroprotective agent riluzole activates the two P domain K(+) channels TREK-1 and TRAAK. *Mol Pharmacol.* 57: 906-12, 2000.
- Gilbertson DM and Gilbertson TA.** Amiloride reduces the aversiveness of acids in preference tests. *Physiol Behav* 56(4): 649-54, 1994.
- Gilbertson TA, Avenet P, Kinnamon SC, and Roper SD.** Proton currents through amiloride-sensitive Na channels in hamster taste cells. Role in acid transduction. *J Gen Physiol* 100: 803-24, 1992.
- Gilbertson TA, Roper SD, and Kinnamon SC.** Proton currents through amiloride-sensitive Na⁺ channels in isolated hamster taste cells: enhancement by vasopressin and cAMP. *Neuron* 10(5): 931-42, 1993.
- Goldstein SA, Bockenhauer D, O'Kelly I, and Zilberberg N.** Potassium leak channels and the KCNK family of two-P-domain subunits. *Nat Rev Neurosci.* 2:175-84, 2001.
- Hayashi Y, Zviman MM, Brand JG, Teeter JH, and Restrepo D.** Measurement of membrane potential and [Ca²⁺]_i in cell ensembles: application to the study of glutamate taste in mice. *Biophys J.* 71:1057-70, 1996.
- Kamatchi GL, Chan CK, Snutch T, Durieux ME, and Lynch C 3rd.** Volatile anesthetic inhibition of neuronal Ca channel currents expressed in *Xenopus* oocytes. *Brain Res.* 831:85-96, 1999.
- Kim Y, Bang H, and Kim D.** TBAK-1 and TASK-1, two-pore K(+) channel subunits: kinetic properties and expression in rat heart. *Am J Physiol.* 277:H1669-78, 1999.
- Kim Y, Bang H, and Kim D.** TASK-3, a new member of the tandem pore K(+) channel family. *J Biol Chem.* 275: 9340-7, 2000.

- Kinnamon SC and Roper SD.** Membrane properties of isolated mudpuppy taste cells. *J Gen Physiol.* 91:351-71, 1988.
- Kinnamon SC, Price MP, Stone LM, Lin W, and Welsh, MJ.** The acid sensing ion channel BNC1 is not required for sour taste transduction. *Internat.Symp.Olfact.Taste XIII:* 80, 2000.
- Lesage F.** Pharmacology of neuronal background potassium channels. *Neuropharmacology* 44(1): 1-7, 2003.
- Lesage F, Guillemare E, Fink M, Duprat F, Lazdunski M, Romey G, and Barhanin J.** TWIK1, a ubiquitous human weakly inward rectifying K⁺ channel with a novel structure. *EMBO J* 15: 1004-11, 1996.
- Lesage F. and Lazdunski M.** Molecular and functional properties of two-pore-domain potassium channels. *Am J Physiol Renal Physiol* 279(5): F793-801, 2000.
- Lesage F, Terrenoire C, Romey G, and Lazdunski M.** Human TREK2, a 2P domain mechano-sensitive K⁺ channel with multiple regulations by polyunsaturated fatty acids, lysophospholipids, and Gs, Gi, and Gq protein-coupled receptors. *J Biol Chem* 275: 28398-405, 2000.
- Lin W, Rao S, Kinnamon SC and Gilbertson T.** Evidence for expression of task-like K⁺ channels in rat taste cells *Chem. Senses* 27: A77, 2002.
- Lin, W, Ogura T, and Kinnamon SC.** Acid-activated cation currents in rat vallate taste receptor cells. *J Neurophysiol* 88: 133-41, 2002.
- Liu L and Simon SA.** Acidic stimuli activates two distinct pathways in taste receptor cells from rat fungiform papillae. *Brain Res* 923: 58-70, 2001.

Lyall V, Alam RI, Phan DQ, Ereso GL, Phan TH, Malik SA, Montrose MH, Chu S, Heck GL, Feldman GM, and DeSimone JA. Decrease in rat taste receptor cell intracellular pH is the proximate stimulus in sour taste transduction. *Am J Physiol Cell Physiol* 281: C1005-13, 2001.

Lyall V, Alam RI, Malik SA, Phan TH, Vinnikova AK, Heck GL, and DeSimone JA.

Basolateral Na⁺-H⁺ exchanger (NHE-1) in rat taste receptor cells is involved in neural adaptation to acidic stimuli. *J Physiol*. [Epub ahead of print] Jan 14 as 10.1113/jphysiol.2003.057745, 2004.

Maingret F, Patel AJ, Lesage F, Lazdunski M, and Honore E. Mechano- or acid stimulation, two interactive modes of activation of the TREK-1 potassium channel. *J Biol Chem* 274: 26691-6, 1999.

Maingret F, Patel AJ, Lesage F, Lazdunski M, and Honore E Lysophospholipids open the two-pore domain mechano-gated K(+) channels TREK-1 and TRAAK. *J Biol Chem* 275: 10128–10133, 2000

Maingret F, Patel AJ, Lazdunski M, and Honore E. The endocannabinoid anandamide is a direct and selective blocker of the background K(+) channel TASK-1. *EMBO J*. 20: 47-54, 2001.

Miyamoto T, Fujiyama R, Okada Y, and Sato T. Acid and salt responses in mouse taste cells. *Prog Neurobiol* 62: 135-57, 2000.

Patel AJ and Honore E. Anesthetic-sensitive 2P domain K⁺ channels. *Anesthesiology*. 95:1013-21, 2001.

Patel AJ, Honore E, Lesage F, Fink M, Romey G, and Lazdunski M. Inhalational anesthetics activate two-pore-domain background K⁺ channels. *Nat Neurosci*. 2:422-6,

1999.

Richter TA, Caicedo A and Roper SD. Sour taste stimuli evoke Ca^{2+} and pH responses in mouse taste cells. *J Physiol* 547: 475-83, 2003a.

Richter TA, Pereira E, and Roper SD. Acid-induced calcium responses in murine taste cells. *Chem Senses*. 28:A82-83, 2003b.

Richter TA, Dvorianchikov G, Chaudhari N and Roper SD. ASIC2 is not necessary for sour taste in mice. *J Neurosci* 24: 4088-91, 2004.

Sano Y, Inamura K, Miyake A, Mochizuki S, Kitada C, Yokoi H, Nozawa K, Okada H, Matsushime H, and Furuichi K A novel two-pore domain K^+ channel, TRESK, is localized in the spinal cord. *J Biol Chem*. 278:27406-12, 2003.

Sirois, JE, Pancrazio JJ, Lynch C, and Bayliss DA. Multiple ionic mechanisms mediate inhibition of rat motoneurons by inhalation anaesthetics. *J. Physiol. (Lond.)* 512, 851-862, 1998.

Stevens DR, Seifert R, Bufe B, Muller F, Kremmer E, Gauss R, Meyerhof W, Kaupp UB, and Lindemann B. Hyperpolarization-activated channels HCN1 and HCN4 mediate responses to sour stimuli. *Nature* 413: 631-5, 2001.

Talley EM, Sirois JE, Lei Q, and Bayliss DA. Two-pore-Domain (KCNK) potassium channels: dynamic roles in neuronal function. *Neuroscientist*. 9: 46-56, 2003.

Terrenoire C, Lauritzen I, Lesage F, Romey G, and Lazdunski M. A TREK-1-like potassium channel in atrial cells inhibited by beta-adrenergic stimulation and activated by volatile anesthetics. *Circ Res* 89: 336-42, 2001.

Ugawa S, Minami Y, Guo W, Saishin Y, Takatsuji K, Yamamoto T, Tohyama M, and Shimada S. Receptor that leaves a sour taste in the mouth. *Nature* 395: 555-6, 1998.

Ugawa S, Yamamoto T, Ueda T, Ishida Y, Inagaki A, Nishigaki M, and Shimada S.

Amloride-insensitive currents of the acid-sensing ion channel-2a (ASIC-2a)/ASIC-2b heteromeric sour-taste receptor channel. *J Neurosci* 23: 3616-22, 2003.

Table 1. RT-PCR primer sequences and GenBank accession numbers for each of the eight K₂P channels examined in the present study. Unless otherwise specified, accession numbers are for mouse sequence.

K Channels	Other names	Gene	Accession Number	Forward Primer	Reverse Primer	Product size (bp)
TWIK-1, K _{2p} 1.1	hOHO	KCNK1	NM_008430	5'-ccgagagctgtacaagatcg-3'	5'-tgcccagggattaaaacc -3'	442
TREK-1, K _{2p} 2.1	TPKC1	KCNK2	U73488	5'-tggaacatctccccacg-3'	5'-ccaatcatcatgctcagaacagc-3'	442
TASK-1, K _{2p} 3.1	TBAK-1, OAT-1	KCNK3	AF065162	5'-cgctcaagccgcacaag-3'	5'-acacgaaaccgatgagcacca-3'	295
TASK-2, K _{2p} 5.1		KCNK5	BC058164	5'-atggtgacagaagaatgga-3'	5'-tgagatacctctccaagc-3'	539
TWIK-2, K _{2p} 6.1	TOSS	KCNK6	AF110521	5'-tgctcactgccagcatcc-3'	5'-gctctgagaaggtctctactgc-3'	486
TASK-3 K _{2p} 9.1		KCNK9	AF391084*	5'-agctggagctgtaatcctg-3'	5'-cggtcaccatgttctccata-3'	303
TREK-2 K _{2p} 10.1		KCNK10	NM_023096*	5'-accctgttctc gactctcc-3'	5'-agatttgctccttcagtgc-3'	435
TRESK --		--	XM_285304	5'-ttctctgctgcacagtgttc-3'	5'-aatctctcaaacagctccacat-3'	375

*Rat cDNA sequences.

Table 2. Summary of fluorescent indicators used in the present study for lingual slices and isolated taste bud cells.

Parameter	Indicator	Abbreviation	Preparation
$[Ca^{2+}]_i$	Calcium Green Dextran	CGD	Lingual epithelium slice
$[Ca^{2+}]_i$	Oregon Green BAPTA AM	OG-AM	Isolated taste buds, cells
pH_i	2',7'-Bis(carboxyethyl)-4 or 5-carboxyfluorescein	BCECF	Lingual epithelium slice
pH_i	8-hydroxypyrene-1,3,6-trisulfonic acid	HPTS	Isolated taste buds, cells
Membrane potential	pyridinium, 4-[2-[6-(dioctylamino)-2-naphthalenyl]ethenyl]-1-(3-sulfopropyl)	ANEPPS	Isolated taste buds, cells

Figure legends

Figure 1. Citric acid stimulation acidifies taste cells and elicits depolarizing receptor potentials and Ca^{2+} responses from taste cells in the lingual slice preparation. **A**, repeated focal application of citric acid to the taste pore (triangles) elicits pH decreases throughout the entire taste bud, measured by the decrease in fluorescence in taste buds loaded with BCECF. All cells showed a decrease in F_{BCECF} during citric acid stimulation (cf. Richter et al. 2003a). **B**, applying citric acid focally to the taste pore elicited pronounced increases in $[\text{Ca}]_i$ in a subset of taste cells (~25%, Richter et al. 2003a), measured by ΔF of calcium green dextran (F_{CGD}). **C**, control experiment to validate using ΔF_{ANEPPS} to measure membrane depolarization in isolated taste buds. ANEPPS-loaded isolated taste buds were perfused with 5 to 100 mM KCl (over 5 mM, substituted equimolar for NaCl) to depolarize cells uniformly. Plot shows mean \pm s.e.m. (n=8 taste buds). **D**, applying citric acid to isolated taste buds in vitro produced a membrane depolarization. Because taste cells are tightly packed within taste buds, it was not possible to resolve individual ANEPPS-loaded cells with confidence in preparations of isolated taste buds. **C**, **D** show ΔF_{ANEPPS} averaged over an entire taste bud.

Figure 2. Proposed model for acid (sour) taste transduction. Acid taste stimuli penetrate the lingual epithelium and acidify all cells in the taste bud. A subset of cells (~25%) is depolarized by acidification. The membrane depolarization activates voltage-gated calcium channels (VGCC) which allows Ca^{2+} influx. This influx presumably leads to neurotransmitter release at synapses between taste cells and afferent nerve fibers.

Figure 3. RT-PCR demonstrates that several acid-sensitive K_2P channels are expressed in mouse taste buds. Templates for the reactions were derived from mouse circumvallate taste buds (CV), non-taste lingual epithelium (NT) or brain (Br). The negative control reaction (H_2O) lacked template during

amplification. The size (in bp) of each expected RT-PCR product is indicated in the leftmost column. Identical aliquots of a single cDNA for each tissue were used as template for the PCR reactions shown. The number of PCR cycles used in each case is indicated in parentheses after the name of the gene. Identical results were obtained with at least three additional preparations of RNA from taste buds and non-taste lingual epithelium.

Figure 4. Ca^{2+} responses produced by focal application of citric acid to the taste pore in lingual slices are unaffected by pharmacological agents that block many K^+ channels. Responses ($\Delta\text{F}/\text{F}_{\text{CGD}}$) of taste cells to citric acid were recorded in the absence or presence of: **A**, 200 μM quinidine; **B**, 10 μM anandamide; **C**, 1mM Gd^{3+} ; **D**, 100 μM arachidonic acid; **E**, 10mM 4-AP; **F**, 1mM TEA. Taste cells were loaded with CGD. In each case, 2 to 3 superimposed traces from a representative taste cell are shown. Symbols (\blacktriangle) indicate focal application of citric acid (CA, 100 mM). Data show responses recorded before (left traces) and during (right traces, horizontal bars) continuous perfusion with the agent indicated. Pharmacological agents were applied for 5 to 40 min. at 10 times the IC_{50} reported for K_2P channels. All calibrations are as labeled in F.

Figure 5. Riluzole enhances citric acid-induced Ca^{2+} responses in taste cells in the lingual slice preparation. **A**, Superimposed traces of Ca^{2+} responses ($\Delta\text{F}/\text{F}_{\text{CGD}}$) to focally-applied citric acid (CA, 100 mM, \blacktriangle), recorded from a taste cell in the absence (left) or presence (right) of 500 μM riluzole. **B**, Responses ($\Delta\text{F}/\text{F}_{\text{CGD}}$) of another single taste cell to increasing concentrations of citric acid, applied focally to the taste pore while the slice was bathed in Tyrode control (\bullet) or 500 μM riluzole (\circ). **C**, Average response (mean $\Delta\text{F}/\text{F}_{\text{CGD}} \pm \text{s.e.m.}$, $n=5$ cells) to focal stimulation with citric acid (100 mM) in the absence (solid bar) or presence (open bar) of riluzole (500 μM). There was a small but statistically significant increase in the magnitude of Ca^{2+} responses in riluzole-treated cells ($* = P < 0.05$).

Figure 6. In the lingual slice preparation, halothane inhibits Ca^{2+} responses elicited by citric acid but cytoplasmic acidification remained unaltered. Taste cells were loaded with BCECF (A,B) or CGD (C-F) and were focally stimulated with citric acid (CA, ▲) or, as a control, cycloheximide (CX, ▲). **A, B,** Cytoplasmic acidification produced by focally applied citric acid is not affected by halothane. **A,** Focal stimulation with citric acid elicits decreases in pH throughout the tastebud before (black traces) or during halothane ($\geq 10\text{mM}$, grey traces). Six superimposed traces from one taste cell are shown. **B,** Average $\Delta\text{F}/\text{F}_{\text{BCECF}}$ ($\pm\text{s.e.m.}$, $n=5$ cells). Con, control; halo, halothane ($\geq 10\text{mM}$). **C,D,** Halothane blocks Ca^{2+} responses elicited by citric acid. **C,** Superimposed traces from a single taste cell focally stimulated with 100mM citric acid before (black traces) or during halothane (grey traces). **D,** Average Ca^{2+} response to focally applied citric acid before and during halothane ($\Delta\text{F}/\text{F}_{\text{CGD}}$, $\pm\text{s.e.m.}$, $n=4$ cells) ($* = P < 0.05$). **E,F,** Halothane does not affect Ca^{2+} responses to a bitter taste stimulus, cycloheximide. **E,** Superimposed recordings from a taste cell stimulated focally with $100\ \mu\text{M}$ cycloheximide before (black traces) or during halothane (grey traces), as in A-D. **F,** Average response from bitter-sensitive taste cells to focal application of cycloheximide before and during halothane ($\Delta\text{F}/\text{F}_{\text{CGD}}$, $\pm\text{s.e.m.}$, $n=4$ cells) Calibrations in A,C, are as labeled in E

Figure 7. Halothane blocks citric acid taste responses and hyperpolarizes isolated taste cells and taste buds. **A,** Halothane does not affect the resting pH_i of taste cells within an isolated taste bud. Superimposed traces are for four cells within a single isolated taste bud, loaded with HPTS. Brief application of halothane ($\geq 10\text{mM}$, grey bar) did not affect pH_i , whereas a similar application of Tyrode's buffer, acidified to pH 1.5 with HCl (open bar), resulted in cytoplasmic acidification. **B.** Halothane does not itself affect basal Ca^{2+} levels or elicit a Ca^{2+} response. Traces in B show superimposed records of responses from four isolated taste buds in the same preparation to bath application of halothane (grey bar) and subsequent focal application of $100\ \text{mM}$ citric acid (triangle). **C,** Halothane hyperpolarizes some taste cells and not others. Membrane potential changes were measured by loading isolated taste cells with

ANEPPS. Records show superimposed traces from isolated taste cells. Bath-applied halothane had no effect on V_m in 69 % of the cells, but hyperpolarized 31 % of taste cells. **D**, Focally applied citric acid depolarizes taste cells and halothane blocks this response. Superimposed traces from three isolated taste cells in the same preparation loaded with ANEPPS. Repeated focal application of citric acid (100 mM) transiently depolarized the cells (left). Subsequently, bath application of halothane hyperpolarized the cells (gray traces, right), shown here by the decrease in F_{ANEPPS} below the baseline (dotted line), and blocked the responses to focally applied citric acid (triangles).

Figure 1 (single column, 3.5" wide)

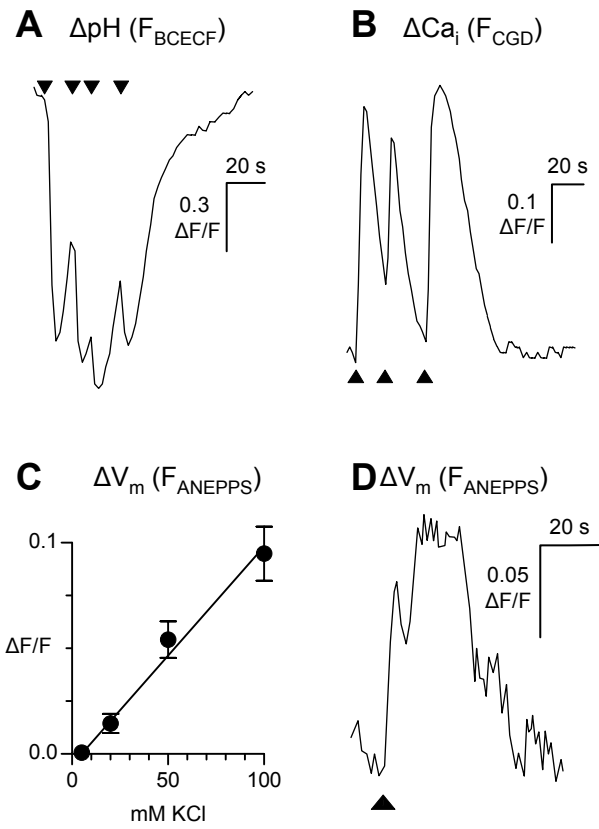


Figure 2 (double column with side legend, 4-5" wide)

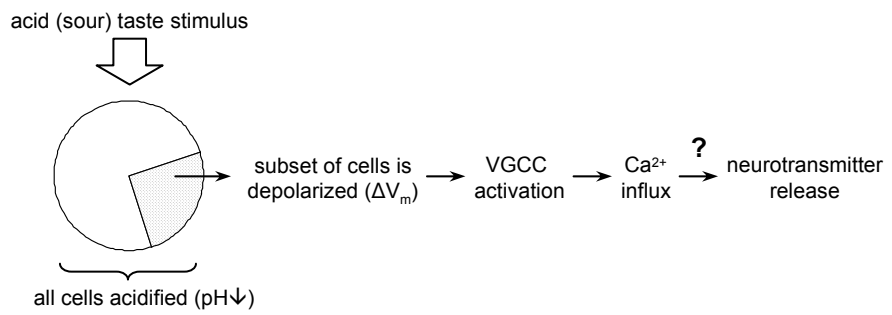


Figure 3 (single column, 3.5" wide)

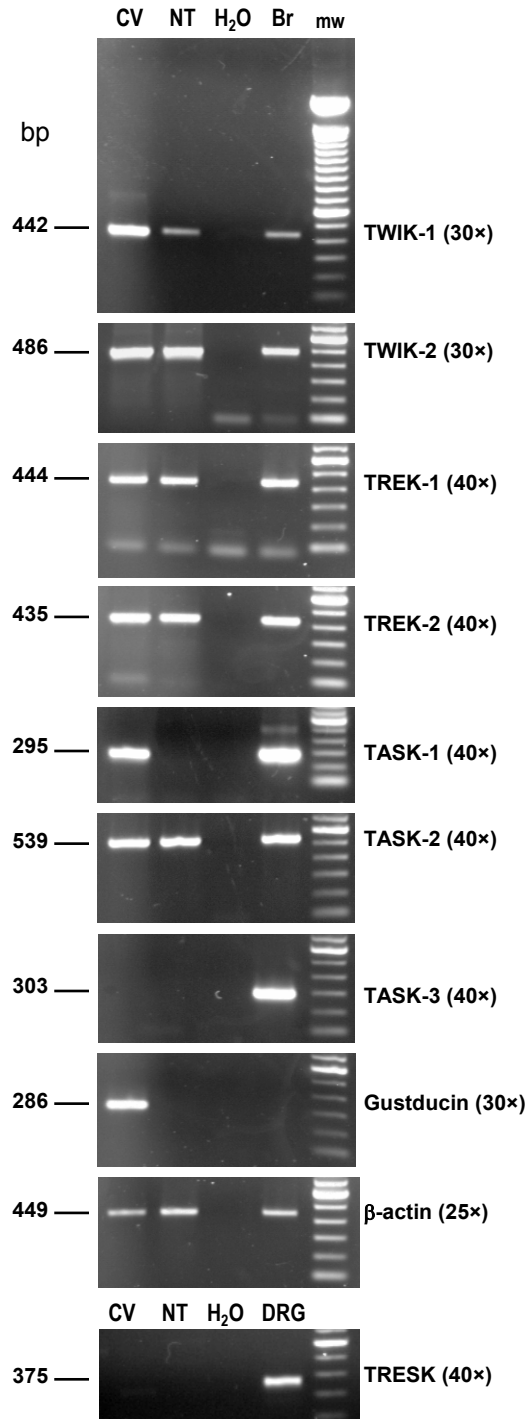


Figure 4 (double column with side legend, 4-5" wide)

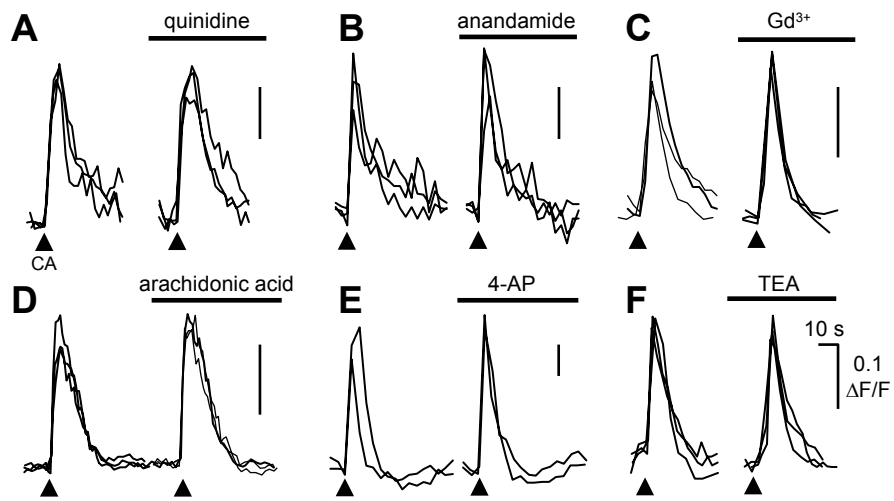


Figure 5 (double column with side legend, 4-5" wide)

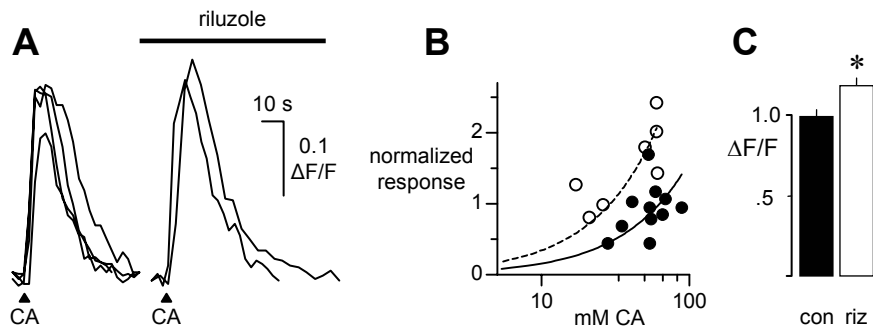


Figure 6 (single column, 3.5" wide)

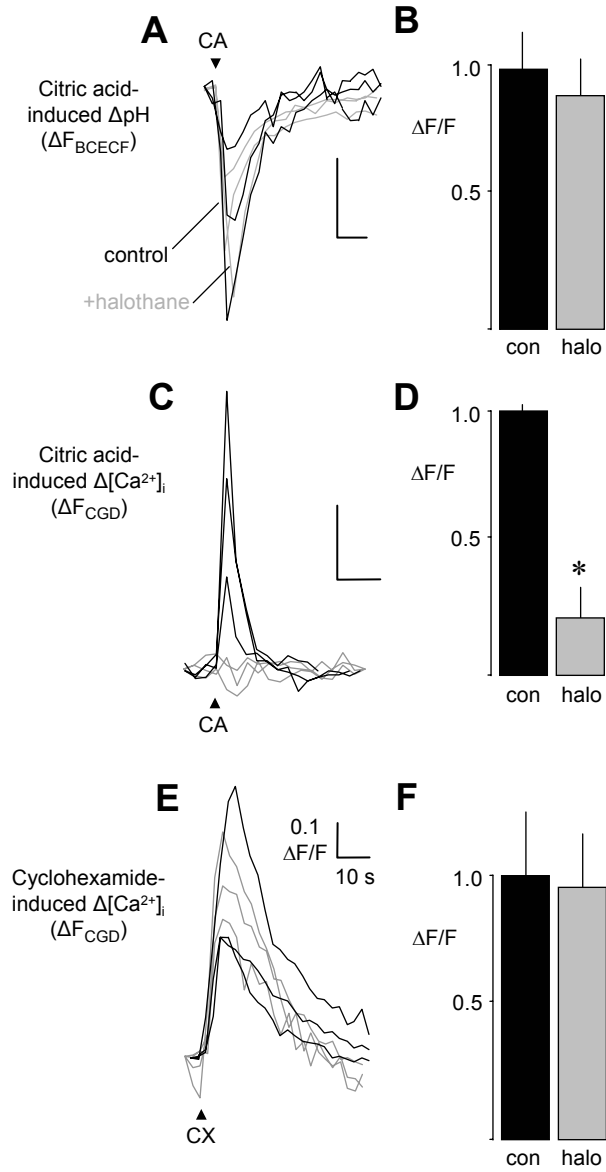


Figure 7 (double column with side legend, 4-5" wide)

