Intracellular pH Modulates Taste Receptor Cell Volume and the Phasic Part of the Chorda Tympani Response to Acids

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The relationship between cell volume and the neural response to acidic stimuli was investigated by simultaneous measurements of intracellular pH (pHi) and cell volume in polarized fungiform taste receptor cells (TRCs) using 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) in vitro and by rat chorda tympani (CT) nerve recordings in vivo. CT responses to HCl and CO2 were recorded in the presence of 1 M mannitol and specific probes for filamentous (F) actin (phalloidin) and monomeric (G) actin (cytochalasin B) under lingual voltage clamp. Acidic stimuli reversibly decrease TRC pHi and cell volume. In isolated TRCs F-actin and G-actin were labeled with rhodamine phalloidin and bovine pancreatic deoxyribonuclease-1 conjugated with Alexa Fluor 488, respectively. A decrease in pHi shifted the equilibrium from F-actin to G-actin. Treatment with phalloidin or cytochalasin B attenuated the magnitude of the pHi-induced decrease in TRC volume. The phasic part of the CT response to HCl or CO2 was significantly decreased by preshrinking TRCs with hypertonic mannitol and lingual application of 1.2 mM phalloidin or 20 μM cytochalasin B with no effect on the tonic part of the CT response. In TRCs first treated with cytochalasin B, the decrease in the magnitude of the phasic response to acidic stimuli was reversed by phalloidin treatment. The pHi-induced decrease in TRC volume induced a flufenamic acid–sensitive nonselective basolateral cation conductance. Channel activity was enhanced at positive lingual clamp voltages. Lingual application of flufenamic acid decreased the magnitude of the phasic part of the CT response to HCl and CO2. Flufenamic acid and hypertonic mannitol were additive in inhibiting the phasic response. We conclude that a decrease in pHi induces TRC shrinkage through its effect on the actin cytoskeleton and activates a flufenamic acid–sensitive basolateral cation conductance that is involved in eliciting the phasic part of the CT response to acidic stimuli.

INTRODUCTION

Sour taste is a primary taste modality and is uniquely associated with acidic stimuli. It evokes an innate rejection response to extremely acidic or sour stimuli, limiting the ad libitum ingestion of acid (Beauchamp et al., 1991; Scott and Plata-Salaman, 1991). In this regard, along with the lungs and kidneys, which are the primary organs for acid secretion, acid sensing taste receptor cells (TRCs) in the oral cavity, by restricting the in-}

Recent studies indicate that the proximate signal for sour taste transduction is an acid-induced decrease in pHi in a subset of TRCs (Lyall et al., 2001). In the case of strong acids, an apical adenosine 3’5’-cyclic monophosphate (cAMP)–sensitive, but Ca2+-insensitive H+ conductance allows H+ entry and the subsequent decrease in TRC pHi (Lyall et al., 2002a; 2004a). However, H+-gated channels, such as the acid sensing ion channel (ASIC) in the apical membrane and both ASIC (Ugawa et al., 1998; Lin et al., 2002) and hyperpolarization-activated channels (HCN) (Stevens et al., 2001) in the basolateral membrane of TRCs, and TASK-2, a two pore domain K+ channel (Lin et al., 2004; Richter et al., 2004a) may also play a role in sour taste transduction.

Abbreviations used in this paper: BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N’,N’’’-tetraacetic acid tetrakis(acetoxymethyl ester); BCECF, 2’,7’-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; Bz, benzamil; cAMP, adenosine 3’5’-cyclic monophosphate; CPC, cetylpyridinium chloride; CT, chorda tympani; DAPI, 4’,6-diamidino-2-phenilindole; FIR, fluorescence intensity ratio; G, monomeric; NHE-1, Na+–H+ exchanges-1; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoxa- zole; ROI, region of interest; RVD, regulatory volume decrease; RVI, regulatory volume increase; SANSCC, shrinkage-activated nonselective cation channel; TRC, taste receptor cell; TRP, transient receptor potential; VGCC, voltage-gated Ca2+ channel.
Effect of TRC pH and Volume on CT Acid Responses

...transduction... How is the decrease in pH only. However, at present a major unsolved problem in events related to the tonic part of the neural response sequent activation of basolateral NHE-1 are specific... 16 Effect of TRC pH and Volume on CT Acid Responses

...effect of the phasic neural response. Treating rat tongue... Rhod-2 calcium imaging dye probes (DeSimone et al., 2005). Some of the data have appeared in abstract form (De-Simone et al., 2005).

**MATERIALS AND METHODS**

**In Vitro Studies**

**Simultaneous Measurement of TRC Volume and pH, Using BCECF.** The animals were housed in the Virginia Commonwealth University animal facility in accordance with institutional guidelines. All in vitro and in vivo animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Commonwealth University. Female Sprague-Dawley rats (150–200 g) were anesthetized by exposing them to the inhalation anesthetic, isofluorane (1.5 ml), in a desiccator. When rats were fully unconscious, a midline incision was made in the chest wall and the aorta severed. The tongues were then rapidly removed and stored in ice-cold Ringer’s solution (R; Table 1). The lingual epithelium was isolated by collagenase treatment. A small piece of the anterior lingual epithelium containing a single fungiform papilla was mounted in a special microscopy chamber as described earlier (Lyall et al., 2001).

Simultaneous measurement of cell volume changes and pH were made using the pH-sensitive dye BCECF and recording at both the pH-sensitive and pH-insensitive (isosbestic) wavelengths...
FIR measurements were made in an optical plane in the taste receptor cells within the ROI. In a typical experiment, the intensity recorded for an ROI represents the mean value from two to three receptor cells. Thus the fluorescence intensity ratio (FIR; \( F_{490}/F_{440} \)) was essentially similar to that used earlier for measuring \([\text{Na}^+]_i\) changes with SBFI (Lyall et al., 2002b, 2005a).

\( \text{Ca}^{2+} \) and \( \text{pH} \) in vitro experiments is given in Table I. However, in some experiments, the control solution (C) was made hypertonic by increasing the \( \text{NaCl} \) concentration from 150 to 500 mM. In some experiments, the \( \text{NH}_4\text{Cl} \) concentration was varied between 0 and 25 mM. To maintain constant osmolarity, an equivalent amount of \( \text{NaCl} \) or \( \text{Cl}^- \) solution was continuously bubbled with 10% CO₂. The stock solutions were then mixed with appropriate solutions to achieve the final concentration of the drugs used in the experiments.

**Data Analysis.** Changes in TRC \( \text{pH} \) were expressed as the mean ± SEM of \( n \) where \( n \) represents the number of ROIs within the taste bud, \( M = \text{SEM} \). The changes in fluorescence intensity at 440 nm were expressed relative to the fluorescence intensity under control conditions. The \( F_{490} \) and \( F_{440} \) of DNase-1 conjugated with different tissue preparations (N). In this case \( N \) represented the number of polarized lingual preparations studied. Student’s \( t \) test was employed to analyze the differences between sets of data.

**Labeling of F- and G-actin.** Fungiform taste bud fragments were harvested from isolated lingual epithelia as described before (Vinnikova et al., 2004). The isolated taste buds were placed on a CellTak (Sigma-Aldrich)-coated coverslip that formed the bottom of a perfusion chamber and were perfused with Ringer’s solution (R; Table I; pH 7.4). The isolated TRCs were loaded with bovine pancreatic deoxyribonuclease (DNase-I) conjugated with Alexa Fluor 488, 4',6-diamidino-2-phenylindole (DAPI), and rhodamine phalloidin (all from Molecular Probes) at room temperature. DNase-1 binds to monomeric G-actin, phalloidin selectively labels polymerized F-actin, and DAPI selectively binds DNA and is a nuclear stain. TRCs were treated with 0.5 mM DAPI, 10 \( \mu \text{M} \) DNase 1, 0.3 \( \mu \text{M} \) DAPI, and 10 \( \mu \text{M} \) rhodamine phalloidin and were dissolved in DMSO. The stock solutions were then mixed with appropriate solutions to achieve the final concentration of the drugs used in the experiments.

**TABLE I**

| R       | C       | \( \text{NH}_4\text{Cl} \) | NMDGCl | NMDGCl/NH\(_4\text{Cl} \) | HCl       | CO\(_3^−/\text{HCO}_3^− \) | HK*       |
|---------|---------|-----------------------------|--------|---------------------------|-----------|---------------------------|-----------|
| mM      | mM      | mM                          | mM     | mM                        | mM        | mM                        | mM        |
| NaCl    | 140     | 150                         | 135    | 150                       | 78        | 4.6                       | 140       |
| KCl     | 5       | 5                           | 5      | 5                         | 5         | 5                         | 5         |
| CaCl\(_2\) | 1   | 1                            | 1      | 1                         | 1         | 1                         | 2         |
| MgCl\(_2\) | 1   | 1                            | 1      | 1                         | 1         | 1                         | 1         |
| NaPy*   | 10      | 10                          | 10     | 10                        | 10        | 10                        | 10        |
| HEPES   | 10      | 10                          | 10     | 10                        | 10        | 10                        | 10        |
| Glucose | 10      | 10                          | 10     | 10                        | 10        | 10                        | 10        |
| NMDGCl  |        | 150                         | 135    |                           |           |                           |           |
| \( \text{NH}_4\text{Cl}^\circ \) | 15      | 15                          |        |                           |           |                           |           |
| HCl*    |        |                              |        |                           | 1         |                           |           |
| NaHCO\(_3\) | 72  |                              |        |                           |           |                           |           |
| pH      | 7.4     | 7.4                         | 7.4    | 7.4                       | 3.0       | 7.4                       | 6.5–8.0   |

*NaPy, sodium pyruvate; R, Ringer’s solution; C, control solution; \( \text{NH}_4\text{Cl} \), solution containing \( \text{NH}_4\text{Cl} \); NMDG-Cl, \( \text{Na}^+ \)-free solution containing \( \text{NH}_4\text{Cl} \); HCl, solution containing HCl.

\( *15 \text{ mM Na-acetate replaced } 15 \text{ mM } \text{NH}_4\text{Cl}. \)

\( ^{58.3} \text{ mM acetic acid was used instead of } 1 \text{ mM } \text{HCl} (\text{pH 3.0}); 50 \text{ mM KCl replaced } 50 \text{ mM NaCl; } \text{Cl}^- \)-free solution contained gluconate salts of Na³⁻, K⁺, Ca²⁺, and Mg²⁺, and control solution was adjusted to pH 7.8 or 6.7.

\( ^{4} \text{CO}_3^−/\text{HCO}_3^− \) solution was continuously bubbled with 10% CO₂.

\( ^{+} \text{HK, high K}^+ \) solutions containing 10 \( \mu \text{M} \) nigericin.

(Muallem et al., 1992). TRCs within the taste bud were loaded with BCECF. The detailed method for the measurement of TRC \( \text{pH} \), using BCECF imaging has been described earlier (Lyall et al., 2001). Small regions of interest (ROIs) in the taste bud (diameter 2–3 \( \mu \text{m} \)) were chosen in which changes in the fluorescence intensity ratio (FIR; \( F_{490}/F_{440} \)) were analyzed using imaging software (TILLvision v 4.0.7.2; TILL Photonics). Each ROI contained two to three receptor cells. Thus the fluorescence intensity recorded for an ROI represents the mean value from two to three receptor cells within the ROI. In a typical experiment, the FIR measurements were made in an optical plane in the taste bud containing at least four ROIs (~8–12 cells). The background and autofluorescence at 490 and 440 nm were corrected from images of a taste bud without the dye. The changes in TRC \( \text{pH} \) were calibrated by bilateral perfusion of high \( K^+ \) solutions (HK; Table I) containing 10 \( \mu \text{M} \) nigericin adjusted to pH values between 6.5 and 8.0. The relative changes in TRC volume were monitored at the isosbestic wavelength 440 nm. The fluorescence intensity at this wavelength is independent of pH and reflects the dye concentration inside the cell. Dye loss and bleaching was significantly reduced by establishing BCECF loading conditions in intact taste buds so that images at 490 and 440 nm can be acquired between 10 and 50 ms, respectively, and taking paired images at 490 and 440 nm at 15-s intervals.

**Measurement of TRC \([\text{Ca}^{2+}]_i\).** Relative changes in \([\text{Ca}^{2+}]_i\) were measured in polarized TRCs by loading the tissue with Fura-2-AM (Molecular Probes). The method for loading Fura-2 and recording temporal changes in FIR (\( F_{360}/F_{390} \)) was essentially similar to that used earlier for measuring \([\text{Na}^+]_i\), changes with SBFI (Lyall et al., 2002b, 2005a).

**Solutions.** The composition of the various solutions used in the in vitro experiments is given in Table I. However, in some experiments, the control solution (C) was made hypertonic by increasing the \( \text{NaCl} \) concentration from 150 to 500 mM. In some experiments, the \( \text{NH}_4\text{Cl} \) concentration was varied between 0 and 25 mM. To maintain constant osmolarity, an equivalent amount of \( \text{NaCl} \) or NMDGCl was replaced with \( \text{NH}_4\text{Cl} \). Some solutions contained the following drugs: ionomycin (a \( \text{Ca}^{2+} \)-ionophore; 10 \( \mu \text{M} \)), nigericin (a \( \text{K}^+/\text{H}^+ \) exchanger; 10 \( \mu \text{M} \)), phalloidin (F-actin modifier; 10 \( \mu \text{M} \)), cytochalasin B (G-actin modifier; 20 \( \mu \text{M} \)). All drugs were purchased from Sigma-Aldrich and were dissolved in DMSO. The stock solutions were then mixed with appropriate solutions to achieve the final concentration of the drugs used in the experiments.
Alexa 488 was made in a solution containing 20 mM Tris (pH 7.6) + 50 mM NaCl + 50% glycerol + 1 mM dithiothreitol and 0.1 mg/ml bovine serum albumin and stored at −20°C. The stock solution was then mixed with control solution to give the final concentration of 0.3 μM. The labeled nuclei, F-actin, and G-actin were imaged using an LSM 510 Meta confocal microscope (Carl Zeiss MicroImaging, Inc.). For DAPI the excitation wavelength was 405 nm and the emitted light was detected using a bandpass filter (520–530 nm). For rhodamine phalloidin, the excitation wavelength was 405 nm and the emitted light was detected using a bandpass filter (550–600 nm). For DNase-I conjugated with Alexa 488 the excitation wavelength was 488 nm and the emitted light was detected using a bandpass filter 505–530 nm. The isolated taste bud fragments were visualized by taking optical sections in the Z-axis when perfused with control solution at pH 7.4 and 6.7 (C; Table I), and in the presence of 10 μM cytochalasin B (pH 7.4) or 500 mM mannitol (pH 7.4).

In Vivo Studies

CT Nerve Recordings. Female Sprague-Dawley rats (150–200 g) were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg), and supplemental pentobarbital (20 mg/kg) was administered as necessary to maintain surgical anesthesia. The animal’s corneal reflex and toe-pinch reflex were used to monitor the depth of surgical anesthesia. Body temperatures were maintained at 37°C with a Deltaphase Isothermal PAD (Model 39 DP; Braintree Scientific, Inc.). The left CT nerve was exposed laterally as it exited the tympanic bulla and placed onto a 32G platinum/iridium wire electrode. An indifferent electrode was placed in nearby tissue. Neural responses were differentially amplified with an optically coupled Isolated Bio-Amplifier (ISO-80; World Precision Instruments). For display, responses were filtered using a band pass filter with cutoff frequencies 40 Hz–3 kHz and fed to an oscilloscope. Responses were then full-wave rectified and integrated with a time constant of 1 s. Integrated neural responses and current and voltage changes were recorded on a chart recorder and also captured on disk using Labview software (National Instruments) and analyzed offline. Stimulus solutions were injected into a Lucite chamber (3 ml; 1 ml/s) affixed by vacuum to a 28 mm² patch of anterior dorsal lingual surface. The chamber was fitted with separate Ag-AgCl electrodes for measurement of current and potential and served as inputs to a voltage-current clamp amplifier that permitted the recording of neural responses with the chemically stimulated receptive field under zero current clamp or voltage clamp. The clamp voltages were referenced to the mucosal side of the tongue. Neural responses were recorded and analyzed as described before (Lyall et al., 2005a,b).

Solutions. The composition of the various rinse and stimulating solutions used in the CT experiments is given in Table II. CT responses to 300 mM NH₄Cl, 300 mM NaCl, 100 mM NaCl, 1 M mannitol (M), 10 mM quinine (Q), and 20 mM HCl were recorded with reference to 10 mM KCl rinse. Body temperatures were maintained at 37°C with a Deltaphase Isothermal PAD (Model 39 DP; Braintree Scientific, Inc.). The left CT nerve was exposed laterally as it exited the tympanic bulla and placed onto a 32G platinum/iridium wire electrode. An indifferent electrode was placed in nearby tissue. Neural responses were differentially amplified with an optically coupled Isolated Bio-Amplifier (ISO-80; World Precision Instruments). For display, responses were filtered using a band pass filter with cutoff frequencies 40 Hz–3 kHz and fed to an oscilloscope. Responses were then full-wave rectified and integrated with a time constant of 1 s. Integrated neural responses and current and voltage changes were recorded on a chart recorder and also captured on disk using Labview software (National Instruments) and analyzed offline. Stimulus solutions were injected into a Lucite chamber (3 ml; 1 ml/s) affixed by vacuum to a 28 mm² patch of anterior dorsal lingual surface. The chamber was fitted with separate Ag-AgCl electrodes for measurement of current and potential and served as inputs to a voltage-current clamp amplifier that permitted the recording of neural responses with the chemically stimulated receptive field under zero current clamp or voltage clamp. The clamp voltages were referenced to the mucosal side of the tongue. Neural responses were recorded and analyzed as described before (Lyall et al., 2005a,b).

The osmotically induced transient decrease in volume (Fig. 1 A, a–b) occurred without a significant change in TRC pHᵢ (g–h; solid line). However, the RVI
Lyall et al. 19

(b–c) was accompanied by an increase in pHi (h–i). In the initial 2-min period both pHi and F440 changed linearly with time (r^2 = 0.99 ± 0.02; n = 6). Similarly, a decrease in osmolarity induced a transient increase in volume (c–d) without a significant change in TRC pHi (i–j). However, the RVD (d–e) was accompanied by a decrease in pHi (j–k). In the initial 1-min period, both pHi and F440 changed linearly with time (r^2 = 0.98 ± 0.03; n = 6). These results suggest that pH regulatory mechanisms in TRC membranes are involved in both RVI and RVD. In two additional polarized TRC preparations, hypertonic NaCl produced a similar relationship between pHi and cell volume. Essentially similar results were obtained with hypertonic mannitol (unpublished data). These results are consistent with our previous studies in which TRC volume was monitored using the cell volume probe, calcein (Lyall et al., 1999) or the Na^+-sensitive dye, sodium-green (Lyall et al., 2005a). These results indicate that in BCECF-loaded TRCs, changes in F440 and Fir (F490/F440) reliably report changes in volume and pHi, respectively.

Effect of Weak Organic Acids and Bases on TRC pHi and Volume. At constant pHb and osmolarity, exposing the basolateral membrane of TRCs to weak organic acids or bases induces transient changes in pHi that recover spontaneously (Vinnikova et al., 2004). We hypothesize that at constant pHb and osmolarity, changes in TRC pHi will be accompanied by parallel changes in cell volume. Changes in pHi were induced by exposing the basolateral membrane of polarized TRCs to short pulses of NH4Cl, Na-acetate, or CO2.

Exposing the basolateral membrane to 15 mM NH4Cl (NH4Cl; Table I; pH 7.4) produced a rapid intracellular alkalinization (Fig. 1 B, a–b, solid line). NH3 diffuses across the basolateral membrane into TRCs and binds to free intracellular H^+ to form NH4^+ and increases pHi. This was followed by a spontaneous decrease in pHi (b–c) due to the slower entry of NH4^+ across the basolateral membrane and its dissociation to NH3 + H+. In addition, one or more pH compensatory mechanisms in TRC membranes may be involved in assisting the recovery from alkaline pHi. Upon NH4Cl washout, pHi decreased below its resting value (Fig. 1 B, c–d). This is due to the rapid exit of intracellular NH3 and the conversion of intracellular NH4^+ to NH3 + H+. The loss of NH3 from the cells results in the accumulation of excess H^+ inside the cells. The decrease in pHi was transient and recovered spontaneously toward its resting value (Fig. 1 B, d–e). The alkalinization phase of the NH4Cl pulse (a–b) was associated with a decrease in pHi to below its resting value (Fig. 1 B, c–d). This is due to the rapid exit of intracellular NH3 and the conversion of intracellular NH4^+ to NH3 + H+. The loss of NH3 from the cells results in the accumulation of excess H^+ inside the cells. The decrease in pHi was transient and recovered spontaneously toward its resting value (Fig. 1 B, d–e).
pH$_i$ and F$_{440}$ in five additional TRC preparations (see also Fig. 1 D and Fig. 2 A below).

Exposing the basolateral membrane to 15 mM Na-acetate (Table I, Na-acetate, pH 7.4) produced a rapid intracellular acidification (Fig. 1 C, a–b, solid line), due to the entry of the membrane-permeable undissociated acetic acid and its subsequent dissociation to free intracellular H$^+$ and acetate anion. Intracellular acidification was transient and demonstrated spontaneous recovery (Fig. 1 C, b–c), presumably, due to the activation of basolateral NHE-1 (Vinnikova et al., 2004). Upon Na-acetate washout, pH$_i$ alkalinized and became higher than its resting value (Fig. 1 C, c–d) due to the rapid exit of the undissociated acetic acid from cells and a decrease in intracellular H$^+$. The spontaneous recovery of alkaline pH$_i$ toward baseline (Fig. 1 C, d–e) reflects the presence of an as yet unknown pH recovery mechanism(s) in TRC membranes that allows base (OH$^-$) exit or entry of acid equivalents at alkaline pH$_i$.

Basolateral Na-acetate caused a rapid increase in F$_{440}$ (f–g, dotted line). In the initial 30-s period both pH$_i$ and F$_{440}$ changed linearly with time ($r^2 = 0.82 \pm 0.05$; n = 6). This was followed by a partial recovery of both pH$_i$ (b–c) and F$_{440}$ toward baseline (g–h). Upon Na-acetate washout, there was a rapid transient decrease in F$_{440}$ (h–i) followed by partial recovery in cell volume (i–j). Upon Na-acetate washout, in the initial 1-min period, both pH$_i$ and F$_{440}$ changed linearly with time ($r^2 = 0.97 \pm 0.04$; n = 6). In two additional TRC preparations Na-acetate pulses produced a similar relationship between pH$_i$ and F$_{440}$. Similarly, a near linear relationship between TRC pH$_i$ and F$_{440}$ was observed during basolateral CO$_2$ pulses (unpublished data).

### Effect of Basolateral Na$^+$ Removal on TRC pH$_i$ and Volume.
We hypothesize that at constant pH$_i$ and osmolarity, a decrease in basolateral Na$^+$ concentration will produce a decrease in TRC pH$_i$ (Vinnikova et al., 2004) accompanied by a parallel decrease in cell volume.

Perfusing the basolateral membrane with Na$^+$-free solution (NMDGCl, Table I, pH 7.4) produced a decrease in TRC pH$_i$ (Fig. 1 D, a–b, solid line) due to the reversal of basolateral NHE-1 (Vinnikova et al., 2004). In two additional TRC preparations basolateral Na$^+$-free solution also acidified pH$_i$ and increased F$_{440}$. In the absence of Na$^+$, exposing the basolateral membrane to a short NH$_4$Cl pulse (NMDG/NH$_4$Cl, Table I, pH 7.4) produced similar changes in pH$_i$ as shown in Fig. 1 B. However, no spontaneous pH$_i$ recovery occurred in the absence of Na$^+$ (Fig. 1 D, d–e). This is because in the absence of Na$^+$, NHE-1 is inhibited. Subsequently, perfusing with control solution (C; Table I; pH 7.4) produced a rapid increase in pH$_i$ to its control value (e–f) due to the activation of NHE-1 (Vinnikova et al., 2004).

![Figure 2](image-url)

**Figure 2.** Effect of basolateral NH$_4$Cl concentration and pH on TRC pH$_i$ and volume. (A) A lingual epithelium loaded with BCECF was initially perfused on both sides with Na$^+$-free solution containing 150 mM NMDG-Cl (pH 7.4). All basolateral solutions contained, in addition, 10 mM BaCl$_2$, and 2 mM CPC was added to the apical solution. Temporal changes in TRC pH$_i$ and volume were monitored as changes in FIR (F$_{490}$/F$_{440}$, solid line) and fluorescence intensity of the isosbestic wavelength 440 nm (F$_{440}$, dotted line) following exposure of the basolateral membrane to 25, 20, 15, 10, 5, and 1 mM NH$_4$Cl (equivalent amount of NMDG-Cl replaced NH$_4$Cl in the basolateral solutions). The F$_{440}$ under control conditions for each ROI was taken as 100%. Inset, relationship between TRC pH$_i$ and percent change in F$_{440}$ relative to 0 NH$_4$Cl. Line of best fit was represented by the following equation: % change in F$_{440} = -36.3 \times$ pH$_i + 345.9; r^2 = 0.99$. Values are presented as mean ± SEM of n, where n = number of ROIs within the taste bud. (B) A lingual epithelium loaded with BCECF was initially perfused on both sides with control solution containing 150 mM NaCl (pH 7.4). During the time period shown by the top horizontal bar the basolateral membrane was perfused with control solution at pH 7.8, 6.7, and 7.4. Temporal changes in TRC pH$_i$ and volume were monitored as changes in FIR (F$_{490}$/F$_{440}$, solid line) and fluorescence intensity of the isosbestic wavelength 440 nm (F$_{440}$, dotted line), respectively. The F$_{440}$ under control conditions for each ROI was taken as 100%. Inset, relationship between TRC pH$_i$ and percent change in F$_{440}$ in the initial 100 s between pH 7.6 and 7.1. Line of best fit was represented by the following equation: % change in F$_{440} = -14.5 \times$ pH$_i + 203.2; r^2 = 0.99$. Values are presented as mean ± SEM of n, where n = number of ROIs within the taste bud.
A decrease in pH, induced by basolateral Na⁺ removal increased F₄₄₀ (Fig. 1 D, g–h, dotted line). Following Na⁺ removal, in the initial 1-min period, both pHᵢ and F₄₄₀ changed linearly with time (r² = 0.90 ± 0.01; n = 6), indicating that a decrease in pHᵢ causes cell shrinkage. In the absence of Na⁺, basolateral NH₄Cl pulse initially produced a rapid decrease in F₄₄₀ (h–i) and, upon NH₄Cl washout, increased F₄₄₀ (j) above its value just before the pulse (h). In the absence of Na⁺, no recovery was observed in either pHᵢ (d–e) or F₄₄₀ (j–k). Na⁺ addition produced pHᵢ recovery (e–f) and a decrease in F₄₄₀ (k–l). In the initial 2-min period, both pHᵢ and F₄₄₀ changed linearly with time (r² = 0.92 ± 0.04; n = 6), indicating that an increase in pHᵢ causes cell swelling.

**Effect of Step Changes in Basolateral NH₄Cl Concentration on TRC pHᵢ and Volume.** We hypothesize that at constant pHᵢ and osmolarity, step changes in basolateral NH₄Cl concentration will produce graded changes in TRC pHᵢ (Vinnikova et al., 2004) accompanied by parallel graded changes in cell volume. During step changes in NH₄Cl both the pHᵢ recovery and the NH₄⁺ loss from the cells were minimized. pHᵢ recovery was inhibited by perfusing the tissue with Na⁺-free solution (compare Fig. 1 D). NH₄⁺ loss through apical VR-1 variant cation channels and basolateral K⁺ channels was minimized by adding 2 mM cetylpyridinium chloride (CPC) and 10 mM BaCl₂ to the apical and basolateral solution, respectively (Vinnikova et al., 2004).

Exposing the basolateral membrane to 25 mM NH₄Cl (NMDG/NH₄Cl, Table I, pH 7.4) induced a rapid increase in mean pHᵢ from 6.58 to 6.98 (Fig. 2 A, solid line, point a) and decreased F₄₄₀ by ~8%, indicating cell swelling. Decreasing the NH₄Cl concentration in a stepwise manner produced a graded decrease in pHᵢ (at time points b, c, d, e, and f) with minimal spontaneous pHᵢ recovery and a rapid and graded increase in F₄₄₀. At time points a, b, c, d, e, and f there was a linear relationship between pHᵢ and F₄₄₀ (Fig. 2, inset; r² = 0.98; n = 8). There was also a linear relationship between pHᵢ and F₄₄₀ at the time points represented by l, m, n, o, p, and q, during which small, but significant, spontaneous pHᵢ recovery was observed. This pHᵢ recovery is due to one or more Na⁺-independent pH recovery mechanisms in TRC membranes. Similar results were obtained in two additional TRC preparations.

**Effect of pHᵢ on TRC pHᵢ and Volume.** We hypothesize that changes in either apical or basolateral pH will induce changes in TRC pHᵢ (Lyall et al., 2001, 2002b) accompanied by parallel changes in cell volume. Elevating basolateral pH from 7.4 to 7.8 (Fig. 2 B) increased TRC pHᵢ (a–b, solid line) and cell volume (c–e, dotted line). Lowering basolateral pH from 7.8 to 6.7 decreased pHᵢ (b–c) and decreased cell volume (f–g). In the initial 100 s, changes in TRC pHᵢ between 7.6 and 7.1 produced a linear change in F₄₄₀ (Fig. 2 B, inset; r² = 0.99 ± 0.01; n = 4). It should be noted that changes in basolateral pHᵢ induced sustained changes in pHᵢ and cell volume (see also Fig. 6).

The relationship between basolateral pHᵢ, TRC pHᵢ, and cell volume shown in Fig. 2 B was not affected by replacing 50 mM NaCl with 50 mM KCl in the basolateral solution (unpublished data). An increase in basolateral K⁺ concentration is expected to depolarize the membrane potential, suggesting that the relationship between pHᵢ and cell volume is indifferent to changes in membrane potential. The relationship between basolateral pHᵢ, TRC pHᵢ, and cell volume was also not altered in the absence of Cl⁻ (unpublished data).
Similarly, during calibration of the pH using high K+ solutions plus nigericin (HK; Table I), changes in pH, between 6.7 and 7.8 also demonstrated a linear relationship with changes in F440 ($r^2 = 0.96 \pm 0.04$; $n = 6$). In the presence of high K+ membrane potential depolarizes to 0 mV and nigericin causes equilibration of pH with pHe. Under these conditions there is no pH gradient, and no pH or volume regulatory mechanisms are operative.

Perfusing the apical membrane with HCl (HCl, Table I, pH 3) induced a sustained decrease in resting TRC pH, (Fig. 3 A, a–b) and a sustained decrease in cell volume (d–e). In the initial 100 s, changes in TRC pH produced a linear change in F440 ($r^2 = 0.97 \pm 0.01$; $n = 7$). Changes in pH, (b–c) and volume (e–f) recovered upon reperfusing the apical membrane with control solution (pH 7.4). Similar results were obtained with acetic acid, pH 3.0 (unpublished data).

In a lingual epithelium initially perfused on both sides with HEPES-buffered control solution (C, Table I, pH 7.4), switching to a similar solution buffered with CO2/HCO3− (CO2/HCO3−, Table I, pH 7.4) on the apical side reversibly decreased TRC pH, (Fig. 3 B, a–b–c). A decrease in TRC pH (Fig. 3 B, a–b) was accompanied by an increase in F440 (d–e), indicating cell shrinkage. Thus both strong and weak acids decrease pH and decrease TRC volume independent of the stimulus pH.

**Effect of Changes in [Ca2+]i on TRC pH and Volume.** Changes in [Ca2+]i, modulate TRC pH, (Lyall et al., 2002a; 2004a). We hypothesize that changes in TRC [Ca2+]i will also induce parallel changes in cell volume. In a polarized TRC preparation loaded with Fura-2, basolateral ionomycin (10 μM) produced a reversible increase in FIR (Fig. 4 A, $F_{490}/F_{380}$, a–b) and a reversible increase in [Ca2+]i. Ionomycin alkalinized resting TRC pH, (Fig. 4 B, a–b, solid line) and decreased F440 (d–e, dotted line), indicating cell swelling. In the initial 100 s following ionomycin treatment there was a linear relationship between the increase in pH, and the decrease in F440 ($r^2 = 0.95 \pm 0.05$; $n = 6$). Similarly, upon ionomycin washout, pH decreased with an increase in F440, indicating cell shrinkage. Ionomycin produced similar effects in two additional TRC preparations. Ionomycin induced intracellular alkalinization by increasing the rate of pH, recovery from an NH4Cl pulse (Fig. 4 C). Under control conditions, the mean pH, recovery rate was 0.038 ± 0.003 pH/min (c–d) and was significantly increased to 0.11 ± 0.003 pH/min (g–h) after the ionomycin treatment (P < 0.01, n = 10, paired). An increase in [Ca2+]i activates NHE-1 (Lyall et al., 2002a, 2004a).

**Effect of pH on F- and G-actin in Isolated Taste Bud Fragments.** Major cell cytoskeletal proteins have been localized in TRCs (Hofer and Drenckhahn, 1999; Ohishi et al., 1999; Sekerkova et al., 2004). We hypothesize that one of the ways that changes in pHi can modulate cell volume rapidly is by interacting with the cytoskeleton and altering cell structure. The actin cytoskeleton of TRCs was visualized using specific fluorescent probes for both monomeric (G)-actin and filamentous (F)-actin. Fig. 5 (A–E) shows a confocal image of an optical plane through an isolated fungiform taste bud fragment perfused with control solution (C, Table I, pH 7.4). The figure shows F-actin binding to rhodamine phalloidin (A, red), transmitted image (B); G-acting binding to pancreatic DNase 1 conjugated with Alexa 488 (C, green);
staining of TRC nuclei with DAPI (D, blue), and an overlay of all images (E). In individual TRCs both F-actin and G-actin are present in the apical region, basolateral region, and the soma surrounding the nuclei. Lowering pH from 7.4 to 6.7 for 1 min (Fig. 5, F–J) produced a decrease in F-actin label (A versus F) and increased G-actin labeling (C versus H). The increase in G-actin was more pronounced in the soma surrounding the cell nuclei. However, it should be pointed out that the increase in G-actin label at pH 6.7 was observed only in a subset of TRCs. This suggests that a decrease in pHi induces changes in cell volume by interacting with the actin cytoskeleton in a subset of TRCs. Similar results were obtained in two additional fungiform taste bud fragments. Treating the taste bud fragments with 20 μM cytochalasin B decreased F-actin (unpublished data). In addition, in taste bud fragments pretreated with 20 μM cytochalasin B or 10 μM phalloidin, a decrease in basolateral pH from 7.4 to 6.7 did not produce a decrease in F-actin or an increase in G-actin label (unpublished data). We hypothesize that changes in the actin cytoskeleton of TRCs will inhibit pHi-induced changes in cell volume.

In Vivo Studies
A decrease in TRC pH is the proximate stimulus for sour taste transduction (Lyall et al., 2001). Since changes in TRC pH demonstrate a linear relationship with cell volume (compare Figs. 1–4 and 6), we hypothesize that a decrease in cell volume is an essential intracellular intermediate signaling event during acid taste transduction. To test this hypothesis, CT responses to acidic stimuli were monitored in the presence of hypertonic mannitol and F- and G-actin modifiers.

Effect of Hypertonic Mannitol on the CT Response to Acidic Stimuli. We preshrank TRCs by superfusing the tongue with the rinse solution (R) containing 1 M mannitol. Replacing the rinse with an isosmotic and iso-pH CO₂/HCO₃⁻ buffered solution (Table II; pH 7.4) elicited a CT response that is composed of two components with distinct temporal characteristics. A rapid transient increase in the phasic neural response (Fig. 7 A, a–b), which declined to a quasi-steady state after ~2–5 s (b–c), defined as the tonic phase of the CT response (c–d). Repeatedly stimulating the tongue with dissolved

**Figure 5.** Labeling of F- and G-actin in an isolated fungiform taste bud fragment at pH 7.4 and 6.7. A confocal image of an optical plane of a fungiform taste bud fragment showing F-actin binding to rhodamine phalloidin (A and F, red), transmitted image (B and G), G-actin binding to pancreatic DNase 1 conjugated with Alexa 488 (C and H, green), staining of TRC nuclei with DAPI (D and I, blue), and an overlay of all images (E and J) are shown while the taste bud was perfused with control solution at pH 7.4 (A–E) or pH 6.7 (F–J).
CO₂ produced almost identical CT response profiles. Replacing the rinse (R) with another rinse containing 1 M mannitol (R/M; pH 7.4) produced a transient phasic response (e–f) that spontaneously decreased to rinse level. Replacing R/M with dissolved CO₂ solution containing mannitol (M/CO₂; pH 7.4) produced a CT response in which the phasic part of the neural response relative to the tonic part (h–i) was 65% smaller relative to control (b–c). For comparison, a set of transient phasic responses to CO₂ stimulation in the presence and absence of mannitol are shown in Fig. 7 B in an extended time scale. The magnitude of the peak phasic response was normalized to the phasic response elicited by stimulation with 300 mM NH₄Cl. In the second part of the experiment the CT responses were recorded with both rinse (R/M) and the CO₂ solution (M/CO₂) containing, in addition, 1 M mannitol (M). The arrows indicate the time when the tongue was superfused with different solutions.

M+CO₂ produced almost identical CT response profiles. Similar results were obtained with 10 mM acetic acid (Table II; pH 6.1) (unpublished data).

Stimulating the tongue with 20 mM HCl + 1 M mannitol (R+M+HCl) also elicited a CT response in which the phasic part of the CT response relative to the tonic part (Fig. 7 C, b–i, R+M+HCl) was decreased by 78% relative to its value in the absence of mannitol (Fig. 7 C, b–c, R+HCl). Mannitol produced only minimal effects on the tonic part of the HCl CT response (i–j) relative to control (c–d). These results indicate that the phasic part of the CT response to both strong and weak acid...
stimulation is attenuated by increasing the osmolarity of the stimulating solution with mannitol.

**Effect of Hypertonic Mannitol on the CT Response to NaCl and Quinine.** Stimulating the tongue with 10 mM KCl rinse solution containing 1 M mannitol (R+M; Table II) and then with the NaCl stimulating solution containing 10 mM KCl + 100 mM NaCl + 1 M mannitol (R+N+M) elicited a CT response (Fig. 8 A) in which both the phasic part of the response (g–h) and the tonic part of the response (i–j) were greater relative to their respective values (a–b) and (c–d) in the absence of mannitol. Repeatedly stimulating the tongue with R+N+M produced almost identical CT response profiles. Similar results were obtained with hypertonic cellobiose (Lyall et al., 1999). In contrast, hypertonic urea did not affect CT responses to NaCl (Lyall et al., 1999) or to acidic stimuli (unpublished data). These results indicate that in contrast to acidic stimuli, hypertonic mannitol increases the phasic and tonic components of the CT response to NaCl.

Hypertonic mannitol had no effect on either the phasic or the tonic component of the CT response to 100 mM NaCl + 5 µM Bz (N+Bz+M) relative to its value in the absence of mannitol (N+Bz) (Fig. 8 B). Thus mannitol specifically increases both the phasic and the tonic component of the Bz-sensitive NaCl CT response derived from Na⁺ flux through apical ENaC (Lyall et al., 1999). In contrast, mannitol does not affect the Bz-insensitive NaCl CT response derived from Na⁺ flux through the TRPV1 variant salt taste receptor (Lyall et al., 2004b). Hypertonic mannitol had no effect on either the phasic or the tonic component of the CT response to 10 mM quinine (Q+M) relative to its values in the absence of mannitol (Q), indicating that the transduction mechanism for quinine does not involve cell shrinkage. Thus an increase in osmolarity specifically decreases the magnitude of the phasic response to acidic stimuli through cell shrinkage.

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**TABLE II**

*Composition of Solutions Used in In Vivo Experiments*

| Rinse (R) | Stimulus solution (S) |
|----------|-----------------------|
| mM       | mM                   |
| 10 KCl   | 300 NH₄Cl, 300 NaCl  |
| 10 KCl   | 10 KCl + 100 NaCl*    |
| 10 KCl   | 10 KCl + 20 HCl      |
| 10 KCl   | 10 KCl + 10 quinine  |
| 72 KCl + 10 HEPES, pH 7.4 | 72 KHCO₃ + 10% CO₂, pH 7.4 |
| 175 KCl + 10 HEPES, pH 6.1 | 175 K-acetate + 10 acetic acid, pH 6.1 |

*In some experiments 5 µM Bz was added to the NaCl stimulating solutions. In some experiments 0.5 or 1.0 M mannitol was added to both rinse and stimulating solutions.*

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**Figure 8.** Effect of hypertonic mannitol on the CT response to NaCl, NH₄Cl, and quinine. (A) The CT responses to 100 mM NaCl + 10 mM KCl (R+N) were recorded relative to the rinse solution, R (10 mM KCl). In the second part of the experiment the CT responses were recorded with both rinse (R+M) and the NaCl solution (R+N+M) containing, in addition, 1 M mannitol (M). (B) The CT responses to 100 mM NaCl + 10 mM KCl + 5 µM Bz (N+Bz), 10 mM KCl + 10 mM quinine (Q), or 0.3 M NH₄Cl were recorded relative to the rinse solution, R (10 mM KCl) in the absence and presence of 1 M mannitol (M). In the second part of the experiment CT responses to the above stimuli were again recorded in the same animal after the topical lingual application of 20 µM cytochalasin B for 20 min. The arrows indicate the time when the tongue was superfused with different solutions.

**Effect of F- and G-actin Probes on the CT Response to Acidic Stimuli.** Cytochalasin B shifts the equilibrium from F-actin to G-actin and inhibits the pH-induced decrease in cell volume (Fig. 6 B). Superfusing the tongue with dissolved CO₂ solution containing 1 M mannitol decreased the magnitude of the phasic response relative to the tonic response (Fig. 9 A, f–g) compared with control (b–c). Cytochalasin B (Fig. 9 A, post-cytochalasin B) inhibited the phasic part of the CT response by >80% (j–k) relative to control (b–c). Cytochalasin B produced no effect on the tonic part of the CT response (k–l versus c–d). After cytochalasin B treatment, increasing the osmolarity of the CO₂ solution with 1 M mannitol (M+CO₂) completely eliminated the phasic part of the CT response (n–o) relative to control (f–g). Cytochalasin B also diminished the response to rinse + mannitol (R+M) relative to rinse alone (R). This indicates that
interfering with the cell cytoskeleton also attenuates the osmotically induced decrease in TRC volume.

Phalloidin binds to F-actin and stabilizes the cell cytoskeleton and inhibits the pH-induced decrease in cell volume (Fig. 6 A). In three animals, treating the lingual surface with 1.2 mM phalloidin for 45 min also inhibited the phasic part of the CT response to CO2 (Fig. 9 A) 20 μM cytochalasin B for 20 min or (B) 1.2 mM phalloidin for 30 min. The CT responses were recorded also with both rinse (R+M) and the CO2 solution (M+CO2) containing, in addition, 1 M mannitol (M). The arrows indicate the time when the tongue was superfused with different solutions. In B, the peak CT response data were normalized to the tonic 300 mM NH4Cl response in each animal (as described in the MATERIALS AND METHODS section) and are presented as the mean ± SEM of the relative peak phasic response from three animals. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 9. Effect of cytochalasin B and phalloidin on the CT response to CO2. The CT responses to dissolved CO2 (72 mM KHCO3 + 10%/90% CO2/O2; pH 7.4) were recorded relative to the rinse solution, R (72 mM KCl + 10 mM HEPES; pH 7.4) before and after the topical lingual application of (A) 20 μM cytochalasin B for 20 min or (B) 1.2 mM phalloidin for 30 min. The CT responses were recorded also with both rinse (R+M) and the CO2 solution (M+CO2) containing, in addition, 1 M mannitol (M). The arrows indicate the time when the tongue was superfused with different solutions. In B, the peak CT response data were normalized to the tonic 300 mM NH4Cl response in each animal (as described in the MATERIALS AND METHODS section) and are presented as the mean ± SEM of the relative peak phasic response from three animals. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 10. Effect of cytochalasin B and phalloidin on the CT response to HCl. (A) The CT responses to 20 mM HCl + 10 mM KCl (R+M+HCl) were recorded relative to the rinse solution, R (10 mM KCl). CT responses were also recorded with both rinse (R+M) and the HCl solution (R+M+HCl) containing, in addition, 500 mM mannitol (M). CT responses were recorded under control conditions (Control), after topical lingual application of 20 μM cytochalasin B for 20 min (Post-cyto B), and after topical lingual application of 1.2 mM phalloidin for 30 min (Post-cyto B and phalloidin). (B) Summary of the effect of cytochalasin B and phalloidin on the CT response to HCl. The relative peak phasic responses (a–b, c–d, e–f, g–h, i–j, and k–l) from three animals were normalized to tonic 500 mM NH4Cl in each individual animal and presented as mean ± SEM for control (black bar), post-cytochalasin (white bar), and post-cytochalasin B and phalloidin treatment (hatched bar). *, P < 0.05; **, P < 0.01; ***, P < 0.001.

CT responses to 20 mM HCl were recorded under control conditions, after topical lingual application of 20 μM cytochalasin B for 20 min and then after treating the tongue with 1.2 mM phalloidin for additional 40 min. Cytochalasin B inhibited the phasic part of the HCl CT response relative to the tonic part (Fig. 10 A, peak [f] to tonic level) relative to control (peak [b] to tonic level). In the presence of 1 M mannitol (R+M+HCl), the post-cytochalasin B phasic response to HCl (peak [h] to tonic level) was almost eliminated relative to control (peak [d] to tonic level). Subsequently treating the tongue with phalloidin, both the magnitude of the phasic part of the CT response to HCl in the absence (Fig. 10 A; peak [j] to tonic level) and presence of mannitol (peak [l] to tonic level) increased to near control levels. The data from three such experiments are summarized in Fig. 10 B and indicate that phalloidin partially reverses the attenuation in the phasic part of the
CT response to HCl alone and to stimulation with M+HCl (hatched bars) caused by cytochalasin B pretreatment (open bars). Thus mannitol and the decrease in pH produce additive effects on TRC volume.

Cytochalasin B had no effect on the CT responses to 100 mM NaCl + 5 μM Bz (N+Bz), 10 mM quinine (Q), or 300 mM NH4Cl relative to control (Fig. 8B). This indicates that changes in cytoskeleton specifically modulate the phasic part of the CT response to acidic stimuli and do not affect other taste modalities.

Relationship between pH Decrease, Changes in Cell Cytoskeleton, Cell Shrinkage, and Membrane Conductance. We hypothesize that pH-induced changes in the cell cytoskeleton activate a membrane conductance in TRCs that is involved in the phasic response to acidic stimuli. To detect the presence of a membrane conductance activated during the phasic response, CT responses to HCl were monitored during lingual voltage clamp. Just before superfusing the tongue with 20 mM HCl, during the rinse (10 mM KCl) a transepithelial voltage of −60 or +60 mV was applied across the tongue (Fig. 11A, arrows). We reasoned that any conductance that is activated during pH-induced cell shrinkage should be voltage sensitive, and hence, should affect the phasic part of the CT response to acid stimulation. Second, in TRCs that are preshrunken with hypertonic mannitol, this conductance will either not be activated by acid stimulation or its activation will be significantly reduced. Imposing a −60 mV transepithelial potential across the tongue (Fig. 11A) did not affect the magnitude of the phasic response (−60 mV) relative to zero current clamp (0 mV). In contrast, imposing a +60 mV transepithelial potential enhanced the magnitude of the phasic part of the CT response (+60 mV) by almost twofold relative to 0 mV (0 mV). In the presence of 1 M mannitol, the phasic responses at 0, −60, and +60 mV were attenuated relative to control. In addition, hypertonic mannitol (M+HCl) almost completely inhibited the increase in the magnitude of the phasic part of the CT response at +60 mV applied transepithelial potential (Fig. 11A). It should be noted that both the applied transepithelial potential and the hypertonic mannitol produced only minimal effects on the tonic part of the CT response to HCl. The magnitude of the peak phasic response to HCl was monitored for a range of voltages between −80 and +80 mV in three animals. The results demonstrate that the membrane conductance becomes activated at positive voltages (Fig. 11B, ○). Preshrinking TRCs with hypertonic mannitol inhibited the membrane conductance at all voltages (Fig. 11B, ○). This suggests the possibility that the membrane conductance activated during acid transduction is most likely activated due to pH-induced cell shrinkage.

Several epithelial as well as nonepithelial cells contain shrinkage-activated nonselective cation channels (Koch and Korbmacher, 1999, 2000). These channels discriminate poorly between NH4+, Na+, K+, and Li+ ions but are not permeable to divalent cations and can be blocked specifically by flufenamic acid. Most importantly, modifying the cytoskeleton with cytochalasin D and taxol inhibited the nonselective cation channels (Koch and Korbmacher, 2000). Therefore, we tested the possibility that flufenamic acid–sensitive nonselective cation channels are activated by pH-induced TRC shrinkage.

Topical lingual application of flufenamic acid (40 μM) for 20 min (Fig. 12) inhibited the phasic part of the CT response to HCl relative to the tonic part (l-m) compared with control (b-c). In the presence of 1 M mannitol, the post-flufenamic acid phasic response to

![Image 325x444 to 541x726]
HCl (r–s) was significantly smaller relative to control (h–i). This treatment affected neither the phasic nor the tonic responses to 0.3 M NH₄Cl (unpublished data). This suggests that at this concentration, flufenamic acid most likely does not uncouple gap junctions and does not produce nonspecific effects (Srinivas and Spray, 2003). Thus the effect of flufenamic acid inhibition of the phasic responses is modality specific. The observation that a longer time exposure to flufenamic acid is required to inhibit the membrane conductance suggests that this conductance is most likely localized in the basolateral membrane of TRCs.

Applying a +60 mV transepithelial voltage enhanced the phasic response to HCl and flufenamic acid inhibited the phasic CT response at 0 and +60 mV (Fig. 13 B) relative to control (Fig. 13 A). Peak phasic response to HCl was monitored for a range of voltages between −80 and +80 mV in three animals before and after flufenamic acid treatment. The data demonstrate that the channel becomes activated at positive voltages (Fig. 13 C, ○). Flufenamic acid inhibited the membrane conductance at all applied voltages (Fig. 13 C, □).

**Effect of Chelating TRC [Ca²⁺] with BAPTA on the CT Responses to Acidic Stimuli.** Shrinkage-activated flufenamic acid–sensitive cation conductance was reported to be indifferent to changes in [Ca²⁺], (Koch and Korbmacher, 2000). Therefore, if this channel is linked to sour taste transduction, then the phasic CT response to acids should also be indifferent to changes in TRC [Ca²⁺]. We loaded TRCs in vivo with BAPTA-AM. BAPTA-AM is membrane permeable, and once inside the cell, the -AM group is hydrolyzed by intracellular nonspecific esterases, and free acid is released. BAPTA-acid chelates free intracellular Ca²⁺ and decreases resting TRC [Ca²⁺]. In addition, any increase in [Ca²⁺] during taste transduction, due either to the release of Ca²⁺ from intracellular stores or the influx of Ca²⁺ through membrane voltage-gated Ca²⁺ channels (VGCCs) in TRC membranes, is buffered by intracellular BAPTA. CT responses to 20 mM HCl were recorded before and after topical lingual application of 30 mM BAPTA-AM. BAPTA completely inhibited the tonic

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**Figure 12.** Effect of flufenamic acid on the CT response to HCl. CT responses to 20 mM HCl were monitored in the absence and presence of 1 M mannitol (M) before (A) and after (B) treating the tongue with 40 μM flufenamic acid for 20 min. The arrows represent the time periods at which the tongue was superfused with different solutions.

**Figure 13.** Effect of applied voltage on the phasic response to HCl stimulation in the presence and absence of flufenamic acid. The CT responses to 20 mM HCl were recorded relative to the rinse (R = 10 mM KCl) before (A) and after (B) treating the tongue with 40 μM flufenamic acid for 20 min. Just before superfusing the tongue with HCl, during perfusion of the tongue with the rinse solution a transepithelial voltage of either −60 or +60 mV was applied across the receptive field. The arrows represent the time periods at which the tongue was superfused with HCl. (C) The CT responses to 20 mM HCl were recorded relative to the rinse (R = 10 mM KCl) before and after treating the tongue with 40 μM flufenamic acid for 20 min. Just before superfusing the tongue with HCl, during perfusion of the tongue with the rinse solution, a transepithelial voltage between −80 or +80 mV was applied across the receptive field. For each voltage step the magnitude of the peak phasic response was calculated. The peak CT response data were normalized to the tonic 300 mM NH₄Cl response in each animal (as described in the MATERIALS AND METHODS section) and are presented as the mean ± SEM of the relative peak phasic response from three animals.
phase of the CT response to HCl stimulation (Fig. 14, post-BAPTA) relative to control without affecting the transient phasic response. In three such experiments after BAPTA-AM treatment, the tonic CT response to HCl was not different from baseline. Similar results were obtained with CO$_2$ and acetic acid stimulation after BAPTA treatment (unpublished data). This indicates that the phasic CT response to acidic stimuli is indifferent to changes in TRC [Ca$^{2+}$], while the tonic phase is sustained by an increase in TRC Ca$^{2+}$.

**DISCUSSION**

In this study, we present new evidence that suggests that following a decrease in pH$_i$, the proximate sour stimulus, the downstream transduction mechanisms for the phasic and tonic components of the CT response to acid stimulation follow distinct and separate pathways. Our results demonstrate a strong relationship between the acid-induced decrease in TRC pH$_i$, actin cell cytoskeleton, cell shrinkage, flufenamic acid–sensitive membrane cation conductance and the phasic part of the CT response to acidic stimuli. The relationship between pH$_i$ and each of the above events as they relate to the phasic part of the CT response to acid stimulation and to the overall sour taste transduction is discussed below.

**Relationship between Osmotically Induced Changes in TRC Volume and pH$_i$**

In many cell types, osmotic changes in cell volume are accompanied by changes in pH$_i$. Typically, osmotic cell swelling results in a decrease and osmotic shrinkage in an increase in pH$_i$ (Jakab et al., 2002). Consistent with this, osmotic shrinkage of TRCs also resulted in cell alkalization during RVI, and osmotic swelling produced a decrease in pH$_i$ during RVD (Fig. 1 A). In the nominal absence of HCO$_3^-$, basolateral NHE-1 serves as a major pH recovery mechanism in TRCs (Vinnikova et al., 2004). Since both pH$_i$ and cell volume recovery from an intracellular acid load were dependent upon the presence of Na$^+$ in the basolateral compartment (Fig. 1 D), it suggests that following osmotic shrinkage, both pH$_i$ recovery and RVI are due, in part, to the activation of NHE-1 (Su et al., 2003). However, in the absence of external Na$^+$, step changes in basolateral NH$_4$Cl concentration produced a graded decrease in pH$_i$ (Fig. 2 A). At each step, the decrease in pH$_i$ demonstrated a small but significant pH$_i$ recovery. This indicates that besides NHE-1, Na$^+$-independent pH regulatory mechanisms also operate in TRC membranes and participate in pH$_i$ and cell volume recovery. Osmotic shrinkage is an important activator of the Na$^+$-H$^+$ exchanger (Su et al., 2003). The intracellular signaling mechanisms by which shrinkage activates the exchanger have not been fully elucidated. However, it is likely that additional pH regulatory mechanisms, membrane channels and transporters, and shrinkage-activated pathways, such as myosin light chain kinase and other signaling pathways, may also be involved in RVD (Lang et al., 1998; Putney et al., 2002; Wehner et al., 2003).

**Relationship between TRC pH$_i$ and Cell Volume**

Osmotic cell swelling and shrinkage produce changes in TRC pH$_i$ (Figs. 1–6). However, the notion that at constant pH$_o$ and osmolarity a primary change in pH$_i$ can produce a change in cell volume has only been recognized recently. Fraser et al. (2005) were the first to demonstrate that a primary increase in the acidity of resting frog skeletal muscle cells decreases cell volume. Here we demonstrate that at constant pH$_o$ and osmolarity, a primary change in TRC pH$_i$ modulates cell volume. This relationship was observed irrespective of the method used to produce changes in pH$_i$. Although the rate of pH$_i$ recovery due to the activation of pH recovery mechanisms from intracellular acidosis or alkalosis was relatively slower than rapid transient changes in pH$_i$ produced by basolateral NH$_4$Cl, Na-acetate, or CO$_2$ pulses, even under these conditions, pH$_i$ recovery demonstrated a near linear relationship with TRV volume. These results indicate that cell volume changes occur at the rate at which TRC pH$_i$ is varied.

At present the exact mechanism by which changes in pH$_i$ modulate TRC volume is not known. In the nominal absence of HCO$_3^-$, changes in pH$_i$ induced by the NH$_4$Cl pulse produce parallel changes in TRC volume in the absence of external Na$^+$ (Fig. 1 D and Fig. 2 A). In addition, the relationship between basolateral pH$_o$, TRC pH$_i$, and volume was not altered by external K$^+$ or Cl$^-$ (unpublished data). This suggests that pH$_i$ induc...
neous pH$_i$ recovery from an intracellular acid or alkaline load involves Na$^+$, K$^+$, Cl$^-$, or HCO$_3^-$-dependent transporters (Putnam et al., 2004). Recent studies suggest that an increase in intracellular H$^+$ titrates the mean charge (z$_e$) of the intracellular membrane-impermeant anions (X$^{-}$) and causes cellular volume decrease (Fraser et al., 2005). This is consistent with the well-known properties of polyelectrolytes in solution. Polyelectrolytes, composed of similarly charged monomers, exist in a volume-expanded state while the corresponding neutralized molecule will encompass a smaller volume (Rice and Nagasawa, 1961). As might be expected by analogy with Donnan potentials, a decrease in the mean charge on the impermeable anion by titration also leads to membrane potential depolarization. Our results suggest that changes in pH$_i$ can also alter cell volume by shifting the equilibrium of F- and G-actin toward G-actin, thus altering cell structure and function. Whether charge titration is also associated with the F-to G-actin transformation is, however, presently unknown.

**Regulation of TRC pH$_i$ and Cell Volume by [Ca$^{2+}$]$_i$**

Changes in both pH$_i$ and TRC volume are regulated by [Ca$^{2+}$]$_i$ through the activation of the basolateral NHE-1 (Fig. 4). Changes in [Ca$^{2+}$]$_i$ produced similar results in rat alveolar type II cells (Murao et al., 2005). Treating Ehrlich ascites tumor cells with lysophosphatidic acid increased [Ca$^{2+}$]$_i$, and produced initial cell shrinkage due to the activation of Ca$^{2+}$-dependent K$^+$ and Cl$^-$ efflux, followed by activation of Na$^+$-H$^+$ exchanger and RVI, resulting in cell swelling (Pedersen et al., 2000). This suggests that during acid taste transduction, acid-induced increase in TRC [Ca$^{2+}$]$_i$ (Liu and Simon, 2001; Lyall et al., 2003; Richter et al., 2003) will activate basolateral NHE-1 and facilitate cell volume recovery, resulting in neural adaptation (Lyall et al., 2002a, 2004a).

**Relationship between pH$_o$, TRC pH$_i$, and Volume**

Cell volume changes were also induced by alterations in pH$_o$, (Fig. 2 B and Figs. 3 and 6). Varying the basolateral pH between 7.8 and 6.7 (Fig. 2 B) induced an almost linear change in TRC pH$_i$ with a mean slope of 0.7 (Lyall et al., 2001). Increasing basolateral pH induced intracellular alkalization and increased cell size. Conversely, a decrease in basolateral pH induced intracellular acidification and decreased cell size. This relationship between pH$_o$ and volume was linear between pH 6.9 and 7.5. Under these conditions no spontaneous pH$_i$ recovery was observed. This is due to the fact that NHE-1 activity is differentially regulated by pH$_o$ and pH$_i$. At constant pH$_i$, a decrease in pH$_o$ activates NHE-1. On the contrary, a decrease in basolateral pH$_i$ inhibits NHE-1 (Vinnikova et al., 2004). In the absence of pH$_i$ recovery, spontaneous RVI is not observed (Fig. 1 D). We also did not observe spontaneous pH$_i$ recovery at alkaline basolateral pH. It is likely that the pH regulatory mechanisms involved in lowering alkaline pH$_i$ to baseline are active at the physiological pH (Fig. 1, A and C) but are inhibited at more alkaline basolateral pH. During stimulation of the apical membrane with acid stimuli there was also a direct relationship between a decrease in pH$_i$ and cell volume (Fig. 3). Under these conditions no spontaneous pH$_i$ recovery was also observed. This is due to the inhibition of the basolateral NHE-1. It is suggested that a change in apical pH$_o$ induces inhibition of NHE-1 through changes in the cell cytoskeleton (Lyall et al., 2004a).

**Relationship between Cytoskeletal Elements, TRC pH$_i$, and Volume**

Several studies suggest a link between cell cytoskeleton and cell volume. Changes in cellular F-actin content and/or distribution have been reported in cells treated with hypo- or hypertonic solutions (Pedersen et al., 2001; Jorgensen et al., 2003; Ebner et al., 2005). Treatment of fungiform papillae with cytochalasin D diminished the positive reactions in the taste pore cells and increased the inner diameter of the ring reactions (Ohishi et al., 1999). Ethanol transiently shrinks TRCs (Lyall et al., 2005a,b) presumably through modifications of F-actin content (Siegmund et al., 2004). Our data suggest that osmotic effects and changes in cytoskeleton produce additive effects on the phasic CT response to acids (Figs. 5, 9, and 10). The CT responses to mannitol alone, mannitol + acidic stimuli (Fig. 10), and mannitol + NaCl (unpublished data) were attenuated after treating the cell with cytochalasin B or phalloidin. It is suggested that changes in cytoskeletal elements may directly regulate the activity of membrane transporters that are involved in volume and pH regulation (Kurashima et al., 1999; Wehner et al., 2003). In our studies, a decrease in pH$_i$ modified the actin cytoskeleton of TRCs by shifting the equilibrium from F-actin to G-actin (Fig. 5). In TRCs pretreated with cytochalasin B or phalloidin, changes in pH$_i$ did not produce the shift from F-actin to G-actin (unpublished data). However, treating TRCs with phalloidin or cytochalasin B (Fig. 6) did not alter the magnitude of the pH$_i$-induced decrease in pH$_o$ but specifically attenuated the pH$_i$-induced cell shrinkage. This indicates that in TRCs, phalloidin or cytochalasin B decouple the linear relationship between pH$_i$ and cell volume.

**Role of pH$_o$, Actin Cytoskeleton and Cell Volume in the Phasic Part of the CT Response to Acidic Stimuli**

Increasing the osmolarity of the rinse solution with mannitol (Figs. 7–9), cellobiose, or ethanol induced cell shrinkage and elicited transient phasic CT re-
responses (Lyall et al., 1999, 2005a,b). The above osmolytes give a transient phasic CT response because they alter TRC osmotic pressure, which is an affect quite apart from any further interaction they may or may not have with a specific taste receptor. The results suggest that cell shrinkage is an intracellular signal for the phasic part of the CT response to hypertonic stimuli. Cell shrinkage is accompanied by changes in membrane conductance. In the case of NaCl (Fig. 8 A), osmotic cell shrinkage increased the magnitude of both the phasic (g–h) and tonic (i–j) part of the CT response. This is consistent with the observations that cell shrinkage activates apical amiloride-sensitive ENaC, resulting in a greater apical Na+ flux and an enhanced CT response to NaCl (Lyall et al., 1999). In contrast, hypertonic solutions of urea or ethanol, which readily permeate cell membranes, do not alter cell volume or the CT response to NaCl (Lyall et al., 2005a).

In contrast to its effect on the NaCl response, osmotic shrinkage of TRCs in vivo inhibited the phasic CT responses to acidic stimuli without affecting the tonic part of the CT response (Figs. 7 and 9–11). The results suggest that for the phasic response, the downstream intracellular signal after a decrease in pH, is a decrease in cell volume. The pH induces a decrease in cell volume by altering the equilibrium of the actin cytoskeleton from F-actin to G-actin (Fig. 5). This is supported by the observations that the phasic response to acidic stimuli is blocked by osmotic cell shrinkage, cytochalasin B, or phalloidin treatment, and that the osmotic effects are additive with cytochalasin B or phalloidin treatment. While cytochalasin B shifts the equilibrium of the actin cytoskeleton from F-actin to G-actin, phalloidin binds to F-actin and stabilizes the cell cytoskeleton. Therefore, both these treatments tend to attenuate pH-induced changes in cell volume (Fig. 6). Data shown in Fig. 10 demonstrate that phalloidin can partially reverse the F-actin to G-actin shift. This is demonstrated by the observation that phalloidin partially reversed the inhibition of the phasic response induced by cytochalasin B pretreatment. This is an important observation and strengthens the notion that a decrease in pH induces changes in volume through the actin cytoskeleton that modulates the phasic part of the CT response to acids.

Changes in Cytoskeleton and Cell Volume Are Coupled to the Activation of a Flufenamic Acid-sensitive Membrane Conductance

In many cell types, changes in pH result in either depolarization or hyperpolarization of the membrane potential (Lyall and Biber, 1994). The changes in potential depend upon the activation or the inhibition of specific membrane conductances. It is likely that changes in the cell cytoskeleton are associated with alterations in TRC membrane permeability. It is suggested that in amphibian skeletal muscle fibers changes in pH induce cell shrinkage by altering the activity of intracellular membrane-impermeant osmolytes and the mean charge of the impermeant osmolytes (Fraser et al., 2005). In TRCs containing apical amiloride-sensitive ENaCs, pH-induced inhibition of the channel will result in hyperpolarization of the apical membrane potential (Lyall et al., 2002b). In cell membranes in which the major membrane conductance is contributed by K+ channels, pH-induced inhibition of K+ channels will depolarize the potential across the membrane (Lyall et al., 1992). In addition, H+ sensitivity of the leak K+ channel (TASK-2) in TRCs may be an important determinant in setting resting potential and regulating the excitability of TRCs (Lin et al., 2004).

Data summarized in Figs. 11–13 suggest that pH-induced changes in actin cytoskeleton and the resulting decrease in cell volume activate a membrane conductance. The channel is activated at positive voltages. Examples of such channels include the transient receptor potential (TRP) channels (Nilius et al., 2004; Voets et al., 2004). This channel is most likely a stretch-activated channel since its activity is inhibited by preshrinking cells with hypertonic mannitol (Figs. 11 and 12). Stretch-activated conductance may involve either anion or cation channels. TRCs have been shown to contain stretch-activated Cl− channels. Their activity is enhanced by osmotic cell swelling and inhibited by NPPB (Gilbertson, 2002). It was reported that in mouse TRCs, acid stimulation activates an NPPB-sensitive Cl− channel (Miyamoto et al., 1998), suggesting a direct involvement of a Cl− channel in acid taste transduction. However, in mouse taste cells, NPPB applied to the basolateral side suppressed the citric acid-induced responses but the apically applied NPPB only slightly suppressed the citric acid respond. This suggests that most likely the NPPB-sensitive Cl− channels are localized in the basolateral membrane (Miyamoto et al., 1998). However, in our studies, topical application of 50 mM NPPB, an inhibitor of swelling activated Cl− channels, inhibited the phasic part of the CT response to HCl, and the osmotic effects of cell shrinkage were additive with NPPB treatment (DeSimone et al., 2005). At this concentration, NPPB most likely produces nonspecific effects on membrane conductances. This suggests that swelling activated Cl− channels do not have a significant role during the phasic part of the CT response to HCl stimulation.

Recently, flufenamic acid-sensitive nonselective cation channels activated by cell shrinkage have been described (Koch and Korbmacher, 2000). A flufenamic acid-sensitive cation channel was recently demonstrated in frog taste cell membranes (Sato et al., 2004). The role for NPPB-insensitive poorly selective cation
conductance in mouse taste cells in sour taste transduction has been suggested (Miyamoto et al., 1998). Data summarized in Figs. 12 and 13 strongly support the conclusion that an acid-induced decrease in pH lowers TRC volume, resulting in the activation of shrinkage-activated flufenamic acid-sensitive cation channels. The resulting increase in channel conductance is involved in the phasic part of the CT response to acid stimulation. The channel conductance is regulated by the actin cytoskeleton and is inhibited in the presence of hypertonic mannitol. At present, the identity of this putative F-actin– and pH-induced cell shrinkage–sensitive channel is not known. It has been demonstrated that cell shrinkage activates a nonselective cation channel in a variety of cells. Treating the cells with cytochalasin D inhibited the current response through this nonselective cation channel induced by cell shrinkage (Koch and Korbmacher, 2000). Additional support that this is most likely a cation channel comes from its voltage sensitivity at positive voltages (Figs. 11 and 12). Nonselective cation channels, including TRP channels, demonstrate similar sensitivity at positive voltages (Nilius et al., 2004; Voets et al., 2004). Anion channels have also been described that are regulated by actin cytoskeleton and cell volume (Gilbertson, 2002). However, those channels are activated by cell swelling.

Role of $[Ca^{2+}]_{i}$ in Acid Taste Transduction

As stated above, following a decrease in pH, the downstream signaling event in acid taste transduction is an increase in $[Ca^{2+}]_{i}$, in a subset of TRCs (Liu and Simon, 2001; Lyall et al., 2003; Richter et al., 2003). The increase in $[Ca^{2+}]_{i}$, and the subsequent activation of basolateral NHE-I is linked to neural adaptation of CT response to acid stimulation (Lyall et al., 2002a, 2004a). The neural adaptation is related to the tonic part of the CT response to acidic stimuli. Chelating TRC $[Ca^{2+}]_{i}$ in vivo with BAPTA completely inhibited the tonic phase of the CT response to HCl without affecting the transient phasic part of the CT response (Fig. 14). These results suggest that the transduction events leading to the transient phasic response are indifferent to changes in the $Ca^{2+}$ concentration in the cytosolic compartment of TRCs. We speculate that if an increase in $Ca^{2+}$ concentration in the TRC synaptic regions is required for the initiation of CT nerve activity during the phasic response, these synaptic subregions are not accessible to BAPTA. This would suggest that cytosolic $Ca^{2+}$ and synaptic region $Ca^{2+}$ exist in isolated cell compartments. In this regard it is interesting to note that the flufenamic acid-sensitive shrinkage-activated cation conductance was reported to be insensitive to changes in $[Ca^{2+}]_{i}$ (Koch and Korbmacher, 2000). This suggests
that the transduction mechanism for the phasic part of the CT response is quite distinct from the tonic part of the neural response to acid stimulation.

The main conclusions of the paper and the proposed mechanisms for the phasic and the tonic CT response to acidic stimuli are summarized in Fig. 15. Fig. 15 A shows the proposed acid transporters in TRC membranes. Acid equivalents enter TRCs across the apical membrane by at least two mechanisms and decrease pH. For fully dissociated strong acids, H⁺ ions permeate the apical membrane through an amiloride- and Ca²⁺-insensitive, but cAMP-sensitive pathway (Lyall et al., 2002a). Weak acids on the other hand cross the apical membrane as neutral molecules and generate intracellular acid equivalents. At present it is not clear if H⁺-gated channels (ASIC, HCN, and TASK-2 K⁺ channels) also play a role in sour taste transduction (Ugawa et al., 1998; Stevens et al., 2001; Lin et al., 2002; Richter et al., 2004b). In Fig. 15 B, an acid-induced decrease in pH in a subset of TRCs alters the cell cytoskeleton by shifting the F- to G-actin equilibrium toward G-actin and by titrating the mean charge of the intracellular membrane-impermeant anions, which could include elements of the cytoskeleton (Fraser et al., 2005), resulting in cell shrinkage. The pH₂-induced decrease in cell volume activates a flufenamic acid–sensitive shrinkage-activated nonselective cation channel (SANSCC) in the basolateral membrane of TRCs. The entry of one or more monovalent cations through SANSCC depolarizes the receptor potential and is the basis for the phasic (P) component of the CT response to acidic stimuli. The phasic part of the CT response to acids seems to be indifferent to changes in cytosolic Ca²⁺ should they occur during this phase. Fig. 15 C shows that in acid-sensing TRCs, the decrease in pH and cell depolarization activate voltage-gated Ca²⁺ channels (VGCCs) or the capacitative Ca²⁺ entry through store-operated Ca²⁺ channels (SOCs) (Pérez et al., 2003), resulting in an increase in TRC [Ca²⁺]. Increase in [Ca²⁺], in turn, activates basolateral NHE-1, which is responsible for pH₂ and cell volume recovery and for the neural adaptation (tonic response [T]) in the CT response to acid stimuli. The accompanying Na⁺ ions exit TRCs across the basolateral membrane via the ouabain-sensitive Na⁺-K⁺-ATPase. Although NHE-3 is present in the apical membrane of TRCs, under the experimental conditions examined so far, it is quiescent and does not participate in pH₂ regulation (Vinnikova et al., 2004).

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