Basolateral Na^+–H^+ exchanger-1 in rat taste receptor cells is involved in neural adaptation to acidic stimuli

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The role of basolateral Na^+–H^+ exchanger isoform-1 (NHE-1) was investigated in neural adaptation of rat taste responses to acidic stimuli, by direct measurement of intracellular pH (pH_i) in polarized taste receptor cells (TRCs) and by chorda tympani (CT) taste nerve recordings. In TRCs perfused with CO_2/HCO_3^--free solution (pH 7.4), removal of basolateral Na^+ decreased pH_i reversibly and zoniporide, a specific NHE-1 blocker, inhibited the Na^+-induced changes in pH_i. The spontaneous rate of TRC pH_i recovery from NH_4Cl pulses was inhibited by basolateral zoniporide with a K_i of 0.33 μm. Exposure to basolateral ionomycin, reversibly increased TRC Ca^{2+}, resting pH_i, and the spontaneous rate of pH_i recovery from an NH_4Cl pulse. These effects of Ca^{2+} on pH_i were blocked by zoniporide. In *in vivo* experiments, topical lingual application of zoniporide increased the magnitude of the CT responses to acetic acid and CO_2, but not to HCl. Topical lingual application of ionomycin did not affect the phasic part of the CT responses to acidic stimuli, but decreased the tonic part by 50% of control over a period of about 1 min. This increased adaptation in the CT response was inhibited by zoniporide. Topical lingual application of 8-CPT-cAMP increased the CT responses to HCl, but not to CO_2, and acetic acid. In the presence of cAMP, ionomycin increased sensory adaptation to HCl, CO_2, and acetic acid. Thus, cAMP and Ca^{2+} independently modulate CT responses to acidic stimuli. While cAMP enhances TRC apical H^+ entry and CT responses to strong acid, an increase in Ca^{2+} activates NHE-1, and increases neural adaptation to all acidic stimuli.

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The neural responses to taste stimuli comprise two distinct processes that differ in temporal scale and amplitude (Pfaffmann, 1955; Smith & Bealer, 1975; Smith *et al.* 1978; Lyall *et al.* 2001; Lyall *et al.* 2002*a*; Simon, 2002). Following the application of a salt or acid stimulus, the chorda tympani (CT) taste nerve usually demonstrates a rapid phasic increase in neural activity followed by a slower decrease in response that asymptotically approaches a steady-state, the so-called tonic response level. This slow decrease in neural response during the continuous presence of the stimulus operationally defines taste neural adaptation. While the phenomenon of adaptation in taste is well documented, little is known in taste receptor cells (TRCs) that give rise to it for any of the taste modalities. Recent studies suggest that in the case of sour taste, acid stimuli induce a decrease in intracellular pH (pH_i) in a subset of TRCs (Lyall *et al.* 2001, 2002*a*,*b*) with a parallel increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Liu & Simon, 2001; Richter *et al.* 2003; Lyall *et al.* 2003). The elevated $[Ca^{2+}]_i$ levels in turn activate a specific Ca^{2+}-activated Na^+–H^+ exchanger (NHE) in TRC membranes that appears to be a functional adaptation mechanism for CT responses to HCl (Lyall *et al.* 2002*a*). The NHEs constitute a gene family consisting of six isoforms each of which possesses distinct characteristics and serves a specialized function (Josette & Pouysségur, 1995; Orlowski & Grinstein, 1997; Wakabayashi *et al.* 1997; Ritter *et al.* 2001). At present, the identity of the specific NHE isoform involved in neural adaptation has not been determined. Secondly, it remains to be established, if this adaptation mechanism occurs during normal sour taste transduction, and applies to both strong and weak acid stimulation.

We have recently demonstrated the presence of the mRNA for the Na^+–H^+ exchanger isoform 1 (NHE-1) in TRCs by RT-PCR, and have localized NHE-1 to the basolateral membranes of TRCs by means of specific

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NHE-1 antibodies (Vinnikova *et al.* 2004). The studies further demonstrated that in the nominal absence of CO_2/HCO_3^-, the basolateral NHE-1 is functional and is a major pathway for pH_i regulation in TRCs. In this paper, we examined the role of basolateral NHE-1 in the neural adaptation to acid stimulation using a recently discovered potent and selective blocker of NHE-1, with high aqueous solubility, zoniporide (Guzman-Perez *et al.* 2001). The effect of zoniporide was investigated on the pH_i regulation in polarized rat fungiform TRCs using pH imaging *in vitro*, and on the neural adaptation, by monitoring rat CT taste nerve responses to acid stimulation *in vivo*. The results demonstrate that in polarized TRCs, NHE-1 is selectively blocked by basolateral application of zoniporide with a mean K_i of 0.33 μM. In parallel *in vivo* experiments, topical lingual application of zoniporide, increased the CT taste nerve responses to acetic acid and CO_2 stimulation relative to control. In *in vitro* experiments, the basolateral NHE-1 was activated by an increase in TRC Ca^{2+} and this increase in activity was blocked by zoniporide. In anaesthetized rats, topical lingual application of a Ca^{2+} ionophore, ionomycin, increased TRC Ca^{2+}, and increased CT taste nerve adaptation to acid stimulation. In contrast, topical lingual application of zoniporide inhibited NHE-1 activity, and completely eliminated the ionomycin-induced increase in neural adaptation to the acidic stimuli. The data demonstrate conclusively that the TRC basolateral NHE-1 is involved in neural adaptation of the CT responses to acid stimulation, and thus plays an important role in modulating sour taste transduction.

Methods

pH imaging

For the *in vitro* experiments, rats were anaesthetized with isoflurane and killed by cervical dislocation. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Commonwealth University. The tongues were rapidly removed and stored in ice-cold Ringer solution (R; Table 1). The lingual epithelium was isolated by collagenase treatment (Béhé *et al.* 1990). A small piece of the anterior lingual epithelium containing a single fungiform papilla was mounted in a special microscopy chamber (Chu *et al.* 1995) as described before (Lyall *et al.* 2001, 2002*a*,*b*; DeSimone *et al.* 2001*a*). The TRCs within the taste bud were loaded with the pH-sensitive dye BCECF and perfused on both sides with control Ringer solution (RC; Table 1). The detailed method for the measurement of pH_i using BCECF has been described before (Lyall *et al.* 2001, 2002*a*,*b*; DeSimone *et al.* 2001*a*; Vinnikova *et al.* 2004). In brief, TRCs in the taste bud were visualized from the basolateral side through a 40 × objective (Zeiss; 0.9 NA) with a Zeiss Axioskop 2 plus upright fluorescence microscope and imaged with a set-up consisting of a cooled CCD camera (Imago, TILL Photonics, Applied Scientific Instrumentation, Eugene, OR, USA) attached to an image intensifier (VS4-1845 Videoscope, Washington, DC, USA), an epifluorescent light source (TILL Photonics Polychrome IV), a 515 nm dichroic beam splitter, and a 535 nm emission filter (20 nm band pass; Omega Optical). The cells were alternately excited at 490 and 440 nm and imaged at 15 s intervals. In the chamber, the taste bud is orientated along its *z*-axis with its apical membrane facing down and the basolateral membrane up facing the objective. The fluorescence measurements are made in an *x*–*y* plane perpendicular to the *z*-axis. Since under these conditions the complete spindle-shaped profile of an individual taste cell is not known, it is difficult to separate cells just by imaging the soma of TRCs. Therefore, small regions of interest (ROIs) in the taste bud (diameter 2–3 μm^2) were chosen in which the changes in the FIR (fluorescence intensity ratio; F_{490}/F_{440}) were analysed using TILLvisION v3.1 imaging software. Each ROI contained two to three receptor cells. Thus the fluorescence intensity recorded for a 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 six ROIs (approximately

Table 1. Solution used in *in vitro* experiments (mm)

	R	RC	R0Na	RNH_4Cl	RNaA	RCO_2	RCO_2NH_4Cl
NaCl	140	150	—	120	120	114	84
KCl	5	5	5	5	5	5	5
$CaCl_2$	1	1	1	1	1	1	1
$MgCl_2$	1	1	1	1	1	1	1
NaPy	10	—	—	—	—	—	—
Hepes	10	10	10	10	10	—	—
Glucose	10	10	10	10	10	10	10
NMDGCl	—	—	150	—	—	—	—
NH_4Cl	—	—	—	30	—	—	30
NaA	—	—	—	—	30	—	—
$NaHCO_3$	—	—	—	—	—	36	36
$^*CO_2$ (%)	—	—	—	—	—	5	5
pH	7.4	7.4	7.4	7.4	7.4	7.4	7.4

R, Ringer solution; RC, control Ringer solution; R0Na, 0 Na^+ Ringer solution; RCO_2, CO_2/HCO_3^- Ringer solution; RNH_4Cl, 30 mM NH_4Cl Ringer solution; RNaA, 30 mM sodium acetate Ringer solution; RCO_2NH_4Cl, CO_2/HCO_3^- Ringer solution containing 30 mM NH_4Cl; NaPy, sodium pyruvate; NaA = sodium acetate; Hepes, *N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulphonic acid].$^*CO_2$, 5/95% (CO_2/O_2).

12–18 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 pH_i were calibrated by perfusing the epithelium on both sides with high K^+ calibrating solutions containing 10 μM nigericin adjusted to pHs between 6.5 and 8.0.

Solutions

The composition of the solutions used in the *in vitro* experiments is given in Table 1. The control solution (RC; Table 1) was Ringer solution without sodium pyruvate (Lyall *et al.* 2002*a*,*b*). In some experiments, the control solutions contained cariporide (HOE-642; Aventis Pharma, Germany) or zoniporide, both specific blockers of the NHE-1 (Scholz *et al.* 1995; Guzman-Perez *et al.* 2001). Zoniporide was a generous gift from Pfizer Inc. (Groton, CT, USA). Some experiments were also performed with amiloride (Sigma), a non-specific blocker of NHEs. In some experiments, in control Ringer solution 30 mM NaCl was replaced with either 30 mM NH_4Cl (RNH_4Cl; Table 1) or 30 mM sodium acetate (RNaA; Table 1). In some experiments, the basolateral membrane of polarized TRCs was perfused with a control solution containing the Ca^{2+} ionophore ionomycin (15 μM) or the membrane-permeant form of the cAMP, 8-(4-chlorophenylthio)adenosine 3′:5′-cyclic monophosphate (8-CPT-cAMP; 250 μM) (both from Sigma) (Lyall *et al.* 2002*a*).

Data analysis

Within a single taste bud, pH_i values were expressed as the mean ± standard error of the mean of *n*; where *n* represents the number of ROIs within the taste bud; M ± S.E.M. (*n*). The data were also presented as the mean ± standard error of the mean from different tissue preparations (*N*). In this case *N* represented the number of polarized lingual preparations studied. Under each experimental condition the data are averaged from at least three lingual preparations. We have previously shown that in TRCs perfused with a nominally HCO_3^--free Ringer solution, the intrinsic buffering capacity (β_1) is related to TRC pH_i, and is expressed as: $\beta_1 = -36.9 \times pH_i + 288.6$ mM pH^{-1} (Vinnikova *et al.* 2004). The values of β_1 were used to calculate the net H^+ flux ($J_{H^+} = \beta_1 \times \delta pH_i\ min^{-1}$) associated with the basolateral NHE-1 activity under different experimental conditions. Student's *t* test was employed to analyse the differences between sets of data.

Chorda tympani (CT) nerve recordings

Female Sprague-Dawley rats (150–200 g) were anaesthetized by intraperitoneal injection of pentobarbital (60 mg kg^{-1}) and supplemental pentobarbital (60 mg kg^{-1}) was administered as necessary to maintain surgical anaesthesia. All procedures for the *in vivo* experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Commonwealth University. Body temperatures were maintained at 36–37°C with a circulating water heating pad. The left CT taste nerve was exposed laterally as it exits 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 a custom-built, optically coupled isolation amplifier. For display, responses were filtered using a band-pass filter with cutoff frequencies 40 Hz to 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 records were recorded on a chart recorder and also captured on disk using Labview software and analysed off-line (Lyall *et al.* 2001, 2002*a*,*b*). Stimulus solutions were injected into a Lucite chamber (3 ml; 1 ml s^{-1}) affixed by vacuum to a 28 mm^2 patch of anterior dorsal lingual surface. The chamber was fitted with separate Ag–AgCl electrodes for measurement of current and potential. These electrodes served as inputs to a voltage-current clamp amplifier that permitted the recording of neural responses with the chemically stimulated receptive field under current (zero current-clamp, 0cc) or voltage-clamp (Ye *et al.* 1993, 1994). The clamp voltages were referenced to the mucosal side of the tongue.

The anterior lingual surface was stimulated with rinse solutions (R) and with acidic stimuli (S): HCl, acetic acid or CO_2. The composition of the rinse solutions and the corresponding stimulating solutions is given in Table 2. In some experiments ionomycin (150 μM), 8-CPT-cAMP (20 mM) or zoniporide (100–500 μM) was dissolved directly in dimethyl sulfoxide (DMSO) and applied topically to the lingual surface for 45 min. In experiments with ionomycin, the rinse solution and the stimulating solution contained, in addition, 10 mM $CaCl_2$ (Lyall *et al.* 2002*a*). DMSO alone had no effect on CT responses as previously shown (Lyall *et al.* 1999).

In isolated lingual preparations 15 μM ionomycin, 250 μM 8-CPT-cAMP and zoniporide (1–50 μM) were applied on the basolateral side *in vitro* and induced their effects within minutes. However, in the *in vivo*

Table 2. Rinse and stimulus solutions for CT experiments

	KCl (mM)	Hepes (mM)	HCl (mM)	$KHCO_3$ (mM)	KA (mM)	$*CO_2$ (%)	AA (mM)	pH
R_{HCl}	10	—	—	—	—	—	—	—
S_{HCl}	—	20	—	—	—	—	—	1.7
R_{AA}	175	10	—	—	—	—	—	6.1
S_{AA}	—	—	—	—	175	—	10	6.1
R_{CO_2}	72	10	—	—	—	—	—	7.4
S_{CO_2}	—	—	—	72	—	10	—	7.4

R, rinse solution; S, stimulating solution; KA, potassium acetate; AA, acetic acid; Hepes, *N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulphonic acid]; $*CO_2/O_2$ (10/90%). In experiments with ionomycin all rinse solutions and stimulating solutions contained, in addition, 10 mM $CaCl_2$.

experiments, ionomycin (150 μM) and zoniporide (100–500 μM) were necessarily applied topically to the lingual surface at approximately 10-fold higher concentrations for 45 min. In preliminary experiments topical application of 5 mM or 10 mM 8-CPT-cAMP did not affect the CT responses to HCl. Cyclic AMP increased CT responses at 15 mM and the maximum enhancement was observed at 20 mM concentration (data not shown). The data presented in this study were obtained with 20 mM 8-CPT-cAMP. The fact that higher concentrations of these drugs and longer exposure times were required to observe significant effects on CT responses is consistent with the presence of a significant diffusion barrier in the taste pore region (Lyall *et al.* 2001).

Data analysis

Consistent with our previous studies (Lyall *et al.* 2002*a*), in the presence of ionomycin + $CaCl_2$, following stimulation with HCl, acetic acid and CO_2 the CT responses demonstrated rapid adaptation within the first 30 s of stimulus application (see Figs 7, 9 and 11). However, the initial rate of decrease in the CT response and its magnitude varied with the acid stimuli. Therefore, the numerical value of an integrated CT response was obtained in the quasi-steady-state part of the response as the area under the integrated CT response curve for a time interval of 1 min measured from the end of a typical 2 min stimulation period (Lyall *et al.* 2002*b*). The changes in the area under the integrated CT responses to acid stimulation under different conditions were normalized to the responses observed in each animal to 300 mM NH_4Cl and were expressed as the mean ± S.E.M. of N; where N represents the number of animals in each group; M ± S.E.M. (N). Under each experimental condition the data were averaged from at least three animals. Student's t test was employed to analyse the differences between sets of data.

Results

In vitro studies

Effect of external Na^+ and zoniporide on TRC pH_i. In polarized TRCs perfused on both sides with control Ringer solution (RC; Table 1), unilaterally switching to a Na^+-free Ringer solution (R0Na; Table 1) in the basolateral compartment, decreased mean TRC pH_i (Fig. 1; a–b) from 7.15 ± 0.01 to 6.66 ± 0.01 ($\Delta pH_i = -0.49$ pH unit; $n = 6$). Re-perfusing the control solution (RC) in the basolateral compartment increased pH_i to near its original level (b–c). In three polarized TRC preparations, perfusing Na^+-free Ringer's solution in the basolateral compartment decreased resting TRC pH_i by 0.62 ± 0.03 pH unit ($P < 0.001$; $N = 3$). These results indicate that TRC pH_i is dependent upon the Na^+ concentration in the basolateral compartment (Vinnikova *et al.* 2004).

Data presented in Fig. 1 also shows that decreasing basolateral Na^+ concentration from 150 mM (RC; Table 1) to zero in the presence of 10 μM zoniporide (i.e switching to R0Na + 10 μM zoniporide; Table 1) inhibited the changes in TRC pH_i relative to control (d–e *versus* a–b). The initial rate of change in TRC pH_i was measured for the first 2 min following a change in the basolateral Na^+ concentration. As shown before, the intrinsic buffering capacity (β_1) is related to TRC pH_i as follows: $\beta_1 = -36.9 \times pH_i + 288.6$ mM pH^{-1} (Vinnikova *et al.* 2004). Taking the mean pH_i value at the midpoint of the first 2 min between points a and b (pH 7.07) gives the mean β_1 value of 27.6 mM pH^{-1}. Thus, in the absence of zoniporide, lowering the basolateral Na^+ concentration from 150 mM to 0 decreased TRC pH_i at the mean rate of –0.086 ± 0.003 pH unit min^{-1} (a–b), and decreased J_{H^+} by 2.37 ± 0.08 mM min^{-1}. Re-perfusing with control Ringer solution (RC; Table 1) increased pH_i at the mean rate of 0.101 ± 0.008 pH unit min^{-1} (b–c; $n = 6$). Again, taking the mean pH_i value at the midpoint of the first 2 min between points b and c (pH 6.75) gives the mean β_1 value of 39.6 mM pH^{-1} and the value of J_{H^+} as 4.0 ± 0.32 mM min^{-1}. In contrast, in the presence 10 μM zoniporide, the corresponding rates of J_{H^+} upon Na^+ removal (d–e) and its addition (e–f) were –0.58 ± 0.13 mM min^{-1} (75.5% inhibition) and 0.51 ± 0.10 mM min^{-1} (87.2% inhibition), respectively ($P < 0.001$; paired). Similar results were obtained in 2 additional experiments (data not shown). Similar effects were also observed with another specific NHE-1 blocker, cariporide, and a non-specific NHE blocker, amiloride (Vinnikova *et al.* 2004).

Effect of intracellular acid loading and zoniporide on TRC pH_i

Studies with sodium acetate (NaA). Figure 2 shows the effect of a short basolateral NaA pulse on the temporal changes in TRC pH_i. Immediately following the perfusion of Ringer solutions containing 30 mM NaA (RNaA; Table 1) in the basolateral compartment, pH_i rapidly acidified (a–b), due to the entry of the membrane-permeant undissociated acetic acid and its conversion to free intracellular H^+ ions and acetate anion (Roos & Boron, 1981). The intracellular acidification was transient and was followed by a spontaneous recovery of pH_i (b–c). Upon NaA washout, TRC pH_i alkalinized and became higher than its resting value (c–d). This occurs due to the rapid exit of the undissociated acetic acid from the cells resulting in a decrease in intracellular H^+ ions. The spontaneous recovery of alkaline pH_i towards baseline (d–e) reflects the presence of as yet unidentified pH recovery mechanism(s) in TRCs that allow base (OH^-) exit or entry of acid equivalents at alkaline pH_i.

The initial rate of change in pH_i was measured during the first 2 min of spontaneous TRC pH_i recovery following the NaA pulse (b–c). Taking the mean pH_i value at the midpoint of the first 2 min between points b and c (pH 7.01) gives the mean β_1 value of 29.9 mM pH^{-1}. Thus under control conditions, the spontaneous mean initial pH_i recovery rate (b–c; 0.084 ± 0.003 pH unit min^{-1}; $n = 6$) represents a mean J_{H^+} of 2.51 ± 0.09 mM min^{-1}. Similarly, in the presence of 10 μM zoniporide, using a mean pH_i value at the midpoint of the first 2 min between points g and h (pH 6.78) gave a mean β_1 value of 38.5 mM pH^{-1}. Thus in the presence of zoniporide, the spontaneous mean initial pH_i recovery rate (g–h; 0.030 ± 0.001 pH unit min^{-1}; $N = 6$) represents a mean J_{H^+} of 1.15 ± 0.04 mM min^{-1}. This represents a 54.2% ($P < 0.01$) inhibition of J_{H^+} compared to control. However, zoniporide did not affect the exit of undissociated acetic acid from cells (h–i) and the subsequent spontaneous recovery of alkaline pH_i towards baseline (i–j). These data indicate that zoniporide specifically inhibits pH_i recovery from an intracellular acid load and does not affect mechanisms involved in base (OH^-) exit or entry of acid equivalents at alkaline pH_i. Similar results were obtained with cariporide and amiloride (Vinnikova *et al.* 2004).

Studies with NH_4Cl. Data presented in Fig. 3 show the effect of a short basolateral NH_4Cl pulse on the temporal

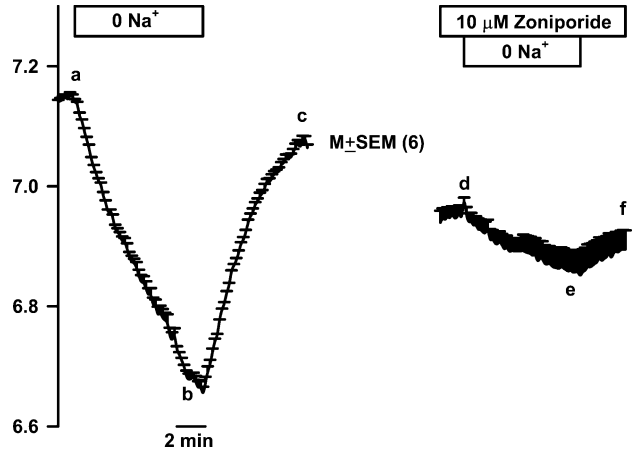

Figure 1. Effect of basolateral Na^+ removal on TRC pH_i
A lingual epithelial preparation was perfused on both sides with control solution containing 150 mM NaCl (RC; pH 7.4; Table 1). At the time period shown by the top horizontal bar the basolateral membrane solution was switched to a Na^+-free solution (0 Na^+) containing 150 mM NMDG-Cl (R0Na; pH 7.4; Table 1). Under control conditions removal of Na^+ decreased TRC pH_i (a–b) and upon re-perfusing the control solution increased pH_i to near its control value (b–c). In the second half of the experiment, the basolateral membrane was treated with 10 μM zoniporide. In the presence of zoniporide, the changes in pH_i upon removal (d–e) and re-addition (e–f) of Na^+ were significantly attenuated. The pH_i values are presented as mean ± S.E.M. of *n* (number of ROIs within the taste bud).

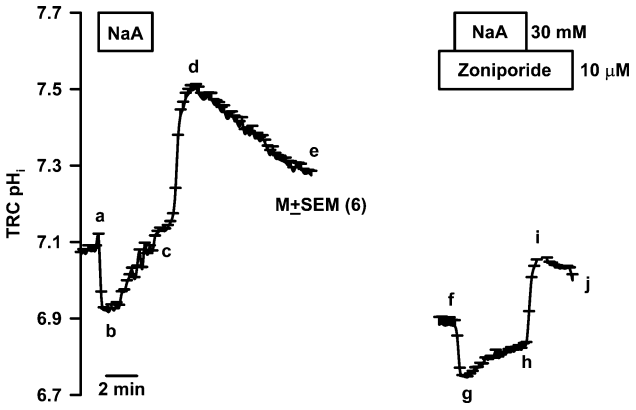

Figure 2. Effect of zoniporide on the spontaneous TRC pH_i recovery following intracellular acid loading with sodium acetate (NaA)
A lingual epithelial preparation was initially perfused on both sides with control solution containing 150 mM NaCl (RC; pH 7.4; Table 1). Temporal changes in TRC pH_i were monitored following the exposure of the basolateral membrane to short sodium acetate (RNaA; Table 1) pulses under control conditions (a–b–c–d–e) and in the presence of 10 μM zoniporide in the basolateral compartment (f–g–h–i–j). The pH_i values are presented as mean ± S.E.M. of *n* (number of ROIs within the taste bud).

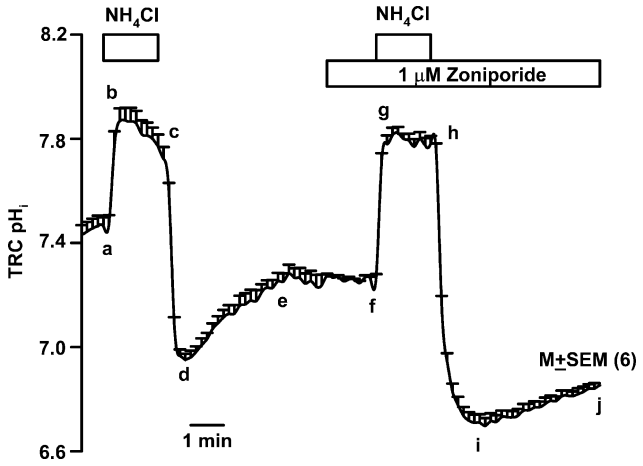

Figure 3. Effect of zoniporide on the spontaneous TRC pH_i recovery following acid loading with NH_4Cl

A lingual epithelial preparation was initially perfused on both sides with a control solution containing 150 mM NaCl (RC; pH 7.4; Table 1). Temporal changes in TRC pH_i were monitored following a short basolateral NH_4Cl pulse (RNH_4Cl; Table 1) under control conditions (a–b–c–d–e) and in the presence of 1 μM zoniporide in the basolateral compartment (f–g–h–i–j). The pH_i values are presented as mean ± S.E.M. of *n* (number of ROIs within the taste bud).

changes in TRC pH_i. Immediately following NH_4Cl perfusion (RNH_4Cl; Table 1), TRC pH_i rapidly alkalinized (a–b), due to the entry of NH_3 and conversion of free intracellular H^+ ions to NH_4^+ ions (Roos & Boron, 1981).

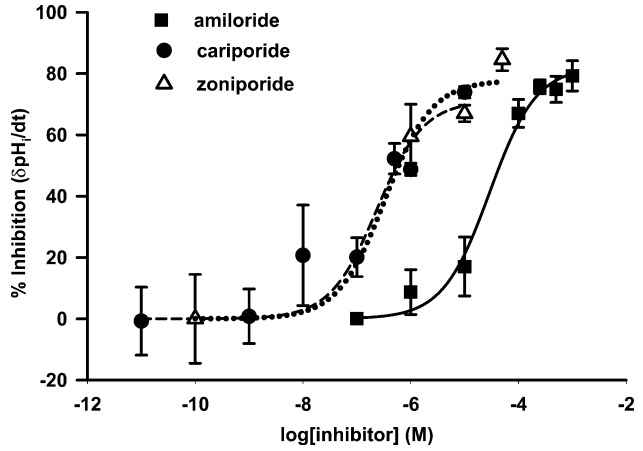

Figure 4. Effect of NHE-1 blockers on the spontaneous TRC pH_i recovery from basolateral NH_4Cl pulses

Lingual epithelial preparations were initially perfused on both sides with control solution containing 150 mM NaCl (RC; pH 7.4; Table 1). Temporal changes in TRC pH_i were monitored following exposure of the basolateral membranes to short NH_4Cl pulses (RNH_4Cl; Table 1), in the presence of increasing concentrations of amiloride (■), cariporide (●), and zoniporide (△). Each drug was tested individually in a separate lingual preparation. In each case, the spontaneous rate of pH_i recovery in the absence of the drug was taken as 100%. The spontaneous pH_i recovery rates (δpH_i min^{-1}) are presented as means ± S.E.M. of number of lingual epithelial preparations, *N*, where $N = 3$.

This was followed by a slow decline of pH_i towards baseline (b–c), presumably, reflecting NH_4^+ entry or pH compensation mechanisms in TRC membranes (Roos & Boron, 1981). Upon replacing NH_4Cl solution (RNH_4Cl; Table 1) with control Ringer solution (RC; Table 1) in the basolateral compartment, TRC pH_i acidified (c–d), and became lower than its resting value due to the combined effect of rapid NH_3 exit from the cells and the conversion of NH_4^+ to NH_3 + free H^+ ions. This decrease in pH_i was transient and was followed by spontaneous pH_i recovery towards its control value (d–e). The initial spontaneous rate of change in TRC pH_i was measured for the first 2 min following the NH_4Cl pulse. In the presence of 1 μM zoniporide in the basolateral compartment, the mean pH_i recovery rate (i–j; 0.045 ± 0.001 pH unit min^{-1}; $n = 6$) was significantly reduced (58.9% inhibition) compared to control (d–e; 0.11 ± 0.003 pH unit min^{-1}). This was equivalent to a decrease in J_{H^+} from 2.08 ± 0.06 to 1.44 ± 0.03 mM min^{-1} (30.8% inhibition; $P < 0.01$) in the presence of 1 μM zoniporide. Although not shown in the figure, increasing zoniporide concentration to 10 μM, decreased the mean pH_i recovery rate to 0.036 ± 0.003 pH unit min^{-1} (67.3% inhibition) and J_{H^+} to 1.18 ± 0.10 (43.3% inhibition; $P < 0.01$) relative to control.

Zoniporide inhibited the spontaneous rate of pH_i recovery after the NH_4Cl pulse in a dose-dependent manner (Fig. 4; open triangles and dotted line). The data were fitted to a Michaelis–Menten-type equation. In three polarized TRC preparations, the mean K_i value (a concentration that inhibits the spontaneous rate of pH_i recovery by 50%) for zoniporide was 0.33 μM (range = 0.17–0.64 μM). Cariporide, another specific blocker of NHE-1, also inhibited the spontaneous rate of pH_i recovery after the NH_4Cl pulse in a dose-dependent manner (Fig. 4; filled circles and dashed line) with a K_i of 0.23 μM (range = 0.14–0.40 μM). Amiloride, a non-specific blocker of NHEs, also inhibited the spontaneous rate of pH_i recovery in a dose-dependent manner (Fig. 4; filled squares and continuous line) with a mean K_i value of 29.0 μM (range = 23–36 μM) (Vinnikova *et al.* 2004). The data summarized in Fig. 4, also show that at zoniporide, cariporide and amiloride concentrations greater than 50 times the value of K_i, the mean maximal inhibition of the spontaneous pH_i recovery rate was around 80%. These data suggest that in the nominal absence of CO_2/HCO_3^-, about 80% of the pH_i recovery from an intracellular acid load occurs via the basolateral NHE-1. The remaining 20% of the pH_i recovery must involve additional pH regulatory mechanisms, yet to be identified in TRCs (Vinnikova *et al.* 2004).

Effect of ionomycin and zoniporide on TRC pH_i

Data summarized in Fig. 5 show that perfusing control Ringer solution (RC; Table 1) containing 10 μM zoniporide in the basolateral compartment decreased the mean resting TRC pH_i (a–b) from 7.26 ± 0.01 to 7.17 ± 0.02 ($P < 0.01$; $n = 8$). This indicates that under control conditions, there is a basal NHE-1 activity in the basolateral membranes of TRCs. Following a short basolateral NH_4Cl pulse (b–c–d), no spontaneous pH_i recovery (e–f) was observed in the presence of zoniporide. In the continuous presence of zoniporide, treating the basolateral membrane with 15 μM ionomycin produced a small but significant ($P < 0.01$; $n = 8$) increase in TRC pH_i (f–g) from 6.65 ± 0.03 to 6.77 ± 0.04 but increased the spontaneous initial J_{H^+} from 0.26 ± 0.18 mM min^{-1} (e–f) to 3.55 ± 0.45 mM min^{-1} (h–i). Thus in the presence of zoniporide, ionomycin increased J_{H^+} by 13.6 ± 1.7-fold ($P < 0.01$; $n = 8$). In the final step, perfusing the basolateral membrane with Ringer solution (RC; Table 1) containing ionomycin, but without zoniporide, increased TRC pH_i (i–j) from 6.80 ± 0.07–7.34 ± 0.01 ($P < 0.001$), and increased the J_{H^+} from the NH_4Cl pulse (k–l) to 10.70 ± 0.84 mM min^{-1} (a 41.1 ± 3.2 fold increase relative to zoniporide alone; e–f; $P < 0.001$). Similar results were obtained in two additional TRC preparations (data not shown). These results indicate that ionomycin alkalinizes resting TRC pH_i and activates basolateral NHE-1, and zoniporide inhibits the ionomycin-induced increase in the basolateral NHE-1 activity.

In vivo studies

Effect of zoniporide on the CT responses to acidic stimuli.

The above results (cf. Fig. 5) indicate that under physiological conditions the basolateral NHE-1 maintains a basal activity that is involved in maintaining steady-state pH_i in TRCs. We reasoned that during acid stimulation NHE-1 must be involved in pH_i recovery. If this is the case, then inhibiting the basal NHE-1 activity with zoniporide should enhance CT responses to acid stimulation. To test this hypothesis, we monitored CT responses to 20 mM HCl (S_{HCl}; Table 2; pH 1.7), 10 mM acetic acid adjusted to pH 6.1 with potassium acetate (S_{AA}; Table 2), and dissolved CO_2 (S_{CO_2}; pH 7.4; Table 2), before and after the topical lingual application of 250 μM zoniporide. The numerical value of an integrated CT response to a particular acid stimulus was obtained in the quasi-steady-state part of the response (i.e. during the adaptation phase), as the area under the integrated CT response curve for a time interval of 1 min, measured from the end of a typical 2 min stimulation period (see Methods). Stimulating the tongue with HCl, acetic acid, and CO_2 increased CT responses (Fig. 6*A*) relative to the corresponding rinse solutions: R_{HCl}, R_{AA}, and R_{CO_2}, respectively (Table 2) (Lyall *et al.* 2001, 2002*a*,*b*). As predicted from *in vitro* studies, the topical application of 250 μM zoniporide enhanced the CT responses to CO_2 by 45% (Fig. 6*A*, lower panel) and acetic acid by 154% (Fig. 6*A*, middle panel). However, no changes in the CT response to HCl were observed after zoniporide treatment (Fig. 6*A*, top panel). The mean data from four individual animals are summarized in Fig. 6*B*. The topical lingual application of zoniporide increased the normalized CT responses to acetic acid and CO_2 by $111 \pm 19\%$ ($P < 0.01$) and $57.0 \pm 8.6\%$ ($P < 0.01$; paired; $N = 4$), respectively, relative to control. No significant changes in HCl CT responses were observed after zoniporide treatment.

An increase in TRC $[Ca^{2+}]_i$ alkalinizes resting pH_i, accelerates the spontaneous pH_i recovery rate from a basolateral NH_4Cl pulse (cf. Figure 5), and increases neural adaptation to HCl stimulation (Lyall *et al.* 2002*a*). We reasoned that if NHE-1 is involved in neural adaptation, topical application of zoniporide should inhibit the Ca^{2+}-induced increase in neural adaptation to acid stimulation.

Effect of ionomycin on CT responses to acidic stimuli

Consistent with our previous studies (Lyall *et al.* 2002*a*), topical lingual application of ionomycin + 10 mM $CaCl_2$

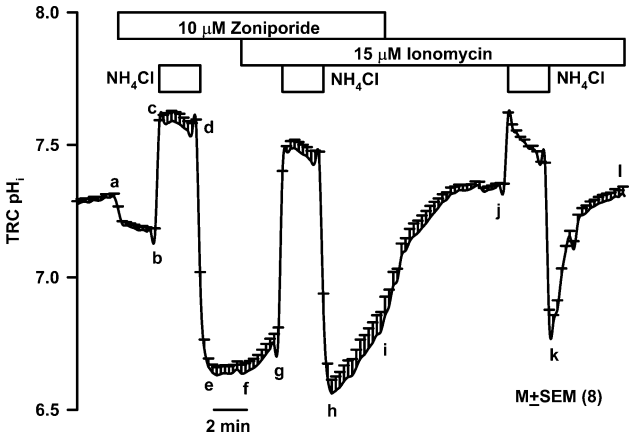

Figure 5. Effect of ionomycin and zoniporide on TRC pH_i
A lingual epithelial preparation was initially perfused on both sides with control Ringer solution (RC; Table 1). Temporal changes in TRC pH_i were monitored following short basolateral NH_4Cl pulses (RNH_4Cl; Table 1) in the presence 10 μM zoniporide (b–c–d–e–f); 10 μM zoniporide + 15 μM ionomycin (g–h–i); and 15 μM ionomycin (j–k–i). The pH_i values are presented as mean ± S.E.M. of number of ROIs within the taste bud, *n*.

(Fig. 7, lower panel, middle trace) did not affect the phasic part of the CT response to 20 mM HCl (e–f *versus* a–b), but it decreased the tonic part of the CT response to 50% of its initial level within 1 min (f–g *versus* b–c). In order to investigate, if ionomycin affects the tonic phase of the CT responses to strong acids only, or if its effects can be generalized also to weak organic acids, CT responses were also recorded with CO_2 and acetic acid.

In Fig. 7 (upper panel) stimulating the tongue with dissolved CO_2 at pH 7.4 (S_{CO_2}; Table 2) increased the CT response (a–b–c) relative to the rinse solution (R_{CO2}; pH 7.4; Table 2). CO_2 diffuses across the apical membranes of TRCs, and once inside the cells, is converted to H_2CO_3 by carbonic anhydrases in TRCs, which dissociates into $H^+ + HCO_3^-$. Thus at constant external pH (pH_o), the generation of acid equivalents inside the cells elicits a CT response. This is consistent with the observation that topical lingual application of the membrane-permeant blockers of carbonic anhydrases, inhibit CT responses to CO_2 (Lyall *et al.* 2001, 2002*a*). Topical lingual application of ionomycin + 10 mM $CaCl_2$ (Fig. 7, upper panel, middle trace) did not affect the initial CT response to dissolved CO_2 (e–f *versus* a–b) but the response declined to 30% of its initial level within 1 min (f–g *versus* b–c).

In Fig. 7 (middle panel) stimulating the tongue with acetic acid (AA) solution (S_{AA}, pH 6.1; Table 2) increased the CT response (a–b–c) relative to the rinse solution (R_{AA}; Table 2). The undissociated acetic acid diffuses across the apical membranes of TRCs, and once inside the cells, dissociates into H^+ + acetate anion. Thus at constant pH_o, the generation of acid equivalents inside the cells elicits a CT response. Topical lingual application of ionomycin + 10 mM $CaCl_2$ (middle panel, middle trace) did not affect the initial CT response to acetic acid (e–f *versus* a–b), but it declined to 50% of its initial level within 1 min (f–g *versus* b–c).

The ionomycin effects were completely reversible (Lyall *et al.* 2002*a*). Upon suffusing the tongue with rinse solution without ionomycin for 5 min, control level responses to HCl, CO_2, and acetic acid were restored (data not shown). Taken together, the above results indicate that ionomycin

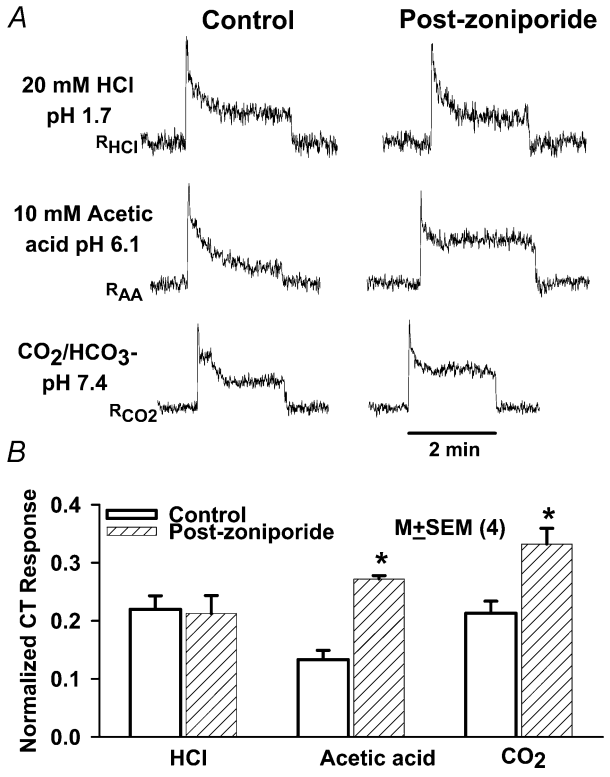

Figure 6. Effect of zoniporide on the CT responses to HCl, CO_2 and acetic acid

A, the CT responses were recorded at zero current-clamp (0cc) under control conditions (control), and after the topical lingual application of 250 μM zoniporide (Post-zoniporide). The tongue was stimulated with 20 mM HCl (S_{HCl}), 10 mM acetic acid titrated to pH 6.1 with potassium acetate (S_{AA}), and CO_2/HCO_3^- buffer, pH 7.4 (S_{CO_2}). The corresponding rinse solutions were: R_{HCl}, R_{AA}, and R_{CO_2}, respectively (Table 2). *B* summarizes mean data from 4 individual experiments. The CT responses to acidic stimuli were normalized to 300 mM NH_4Cl stimulation in each experiment. The open bars represent normalized CT responses under control conditions and hatched bars represent the corresponding CT responses postzoniporide treatment. The normalized CT responses are presented as mean ± S.E.M. values from 4 animals (*N*). $^*P < 0.01$.

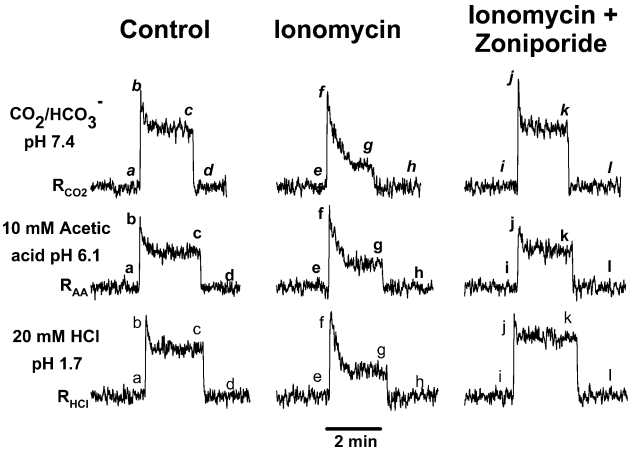

Figure 7. Effect of ionomycin and zoniporide on the CT responses to HCl, CO_2 and acetic acid

The CT responses were recorded at zero current-clamp (0cc) under control conditions (Control), after the topical lingual application of 150 μM ionomycin alone (Ionomycin), and following treatment with 500 μM zoniporide + 150 μM ionomycin (Zoniporide). All rinse (R) and test solutions (S) contained, in addition, 10 mM $CaCl_2$. The tongue was stimulated with 20 mM HCl (S_{HCl}), 10 mM acetic acid titrated to pH 6.1 with potassium acetate (S_{AA}), and CO_2/HCO_3^- buffer, pH 7.4 (S_{CO_2}). The corresponding rinse solutions were: R_{HCl}, R_{AA}, and R_{CO_2}, respectively (Table 2). See text for details.

reversibly increases the neural adaptation to both strong and weak acid stimuli, and that the ionomycin effects are independent of the pH of the acidic stimuli.

Effect of zoniporide on the CT responses to acidic stimuli

To test if ionomycin induces its effects via the activation of the basolateral NHE-1, we further investigated the effect of ionomycin on the CT responses to acid stimulation in the presence of zoniporide. Ionomycin (150 μM) was topically applied to the tongue together with varying concentrations of zoniporide (0–500 μM) for 45 min. Data summarized in Fig. 7 also show that when 150 μM ionomycin was applied together with 500 μM zoniporide, ionomycin failed to produced the observed neural adaptation in the CT responses to CO_2 (upper panel; right trace), acetic acid (middle panel; right trace), and HCl (bottom panel; right trace) stimulation. Thus, in the presence of zoniporide, the inhibition of the NHE-1 activity results in the CT responses to HCl, CO_2 and acetic acid, which were not different from control (i–j–k *versus* a–b–c). Stated another way, in the presence of zoniporide, the ionomycin-induced changes in the CT responses recover to near control levels. The effect of zoniporide was dose dependent (Fig. 8). The percentage recovery of the tonic part of the CT response to acids, in the presence of zoniporide, was calculated as follows:

$$[(CT_Z - CT_I)/(CT_C - CT_I)] \times 100$$

where, CT_C, CT_I and CT_Z are the normalized CT responses to a particular acid stimulation under control conditions (C), after topical application of ionomycin alone (I), and after ionomycin + zoniporide treatment (Z), respectively. At a zoniporide concentration of 100, 250 and 500 μM the tonic part of the CT responses to HCl recovered by $19.2 \pm 15.0\%$ ($P > 0.05$), $45.3 \pm 8.4\%$ ($P < 0.05$) and $117.4 \pm 16.0\%$ ($P < 0.01$; $N = 3$; paired), respectively, relative to ionomycin alone (Fig. 8*A*). The corresponding recoveries for CT responses to CO_2 were $10.7 \pm 14.6\%$ ($P > 0.05$), $44.0 \pm 4.9\%$ ($P < 0.01$) and $127.9 \pm 23.1\%$ ($P < 0.05$; $N = 3$; paired), respectively, relative to ionomycin alone (Fig. 8*B*). The corresponding recoveries for CT responses to acetic acid were $-1.7 \pm 6.4\%$ ($P > 0.05$), $64.0 \pm 13.5\%$ ($P < 0.05$) and $119.3 \pm 3.7\%$ ($P < 0.01$; $N = 3$; paired), respectively, relative to ionomycin alone (Fig. 8*C*). These results demonstrate that at the zoniporide concentration that causes maximum inhibition of the NHE-1 activity, neural adaptation to acid stimulation is abolished completely.

Independence of cAMP and Ca^+ effects on the CT responses to acid stimulation

In polarized TRCs, stimulating the apical membrane with HCl produced a decrease in pH_i, and treating TRCs with cAMP, increased the magnitude of the HCl-induced decrease in pH_i (Lyall *et al.* 2002*a*). In parallel *in vivo* experiments, stimulating the tongue with HCl elicited CT responses at zero current-clamp (0cc) that were voltage-insensitive. The topical lingual application of the membrane-permeant form of the cAMP (8-CPT-cAMP) increased the magnitude of the CT responses to HCl relative to control and the post-cAMP CT responses to HCl demonstrated significant voltage sensitivity (Lyall *et al.* 2002*a*). However, at present it is not clear if (i) cAMP also enhances the CT responses to weak organic acids, (ii) cAMP and Ca^{2+} act independently to modulate the phasic

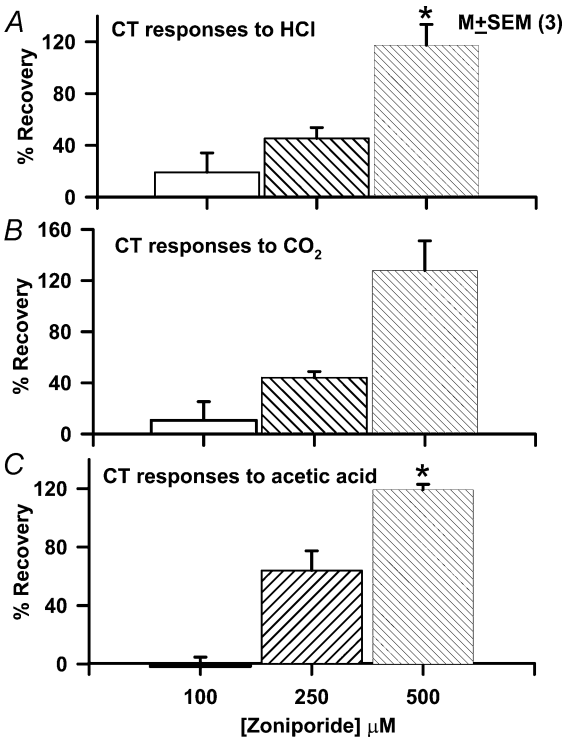

Figure 8. Effect of varying zoniporide concentration on the ionomycin-induced changes in CT responses to acid stimulation
The CT responses to acidic stimuli were recorded under control conditions (Control), after the topical lingual application of 150 μM ionomycin (Ionomycin), and following topical lingual application of zoniporide (100, 250, and 500 μM) + 150 μM ionomycin (Zoniporide). All rinse (R) and test solutions (S) contained, in addition, 10 mM $CaCl_2$. The percentage recovery of the tonic part of the normalized CT response to 20 mM HCl (*A*), CO_2 (*B*), and 10 mM acetic acid (*C*) was calculated at each zoniporide concentration for each individual animal (see text for details). The percentage recovery was expressed as the mean ± S.E.M. of 3 animals (*N*) at each zoniporide concentration. *$P < 0.01$ (paired).

and tonic part of the CT response to acids, and (iii) cAMP and Ca^{2+} have antagonistic effects on the neural adaptation to acid stimulation.

Data summarized in Fig. 9 show that under zero current-clamp (0cc), topical lingual application of 8-CPT-cAMP enhanced the CT response to 20 mM HCl by approximately twofold, but had no effect on the CT responses to CO_2 (pH 7.4) and acetic acid (pH 6.1) relative to control. These results indicate that cAMP specifically modulates the H^+ flux across the apical membranes of TRCs. In contrast to fully dissociated strong acids (e.g. HCl), cAMP does not affect the passive flux of the undissociated form of the weak organic acids (acetic acid and CO_2) across the apical membranes of taste cells. In addition, cAMP had no effect on the tonic component of the CT responses to HCl, acetic acid or CO_2.

We monitored CT responses to 20 mM HCl under zero current-clamp (0cc) and between –90 mV and +90 mV lingual voltage-clamp conditions before and after cAMP treatment. As reported before (Lyall *et al.* 2002*a*), HCl CT responses did not demonstrate voltage sensitivity at ±60 mV. However, at ±90 mV the CT responses demonstrated small, but significant, voltage sensitivity, increasing at –90 mV and decreasing at +90 mV relative to 0cc (0 mV; Fig. 10*A*). The topical lingual application of 8-CPT-cAMP enhanced the CT responses to HCl relative control at 0cc, and the post-cAMP HCl CT responses became significantly voltage-sensitive at ±30, ±60 mV and ±90 mV. Figure 10*B* summarizes data from three individual animals in which the voltage sensitivity of the CT responses was investigated between –90 mV and +90 mV, before and after cAMP treatment. The data demonstrate that under control conditions (pre-cAMP), the CT responses to HCl do not demonstrate voltage sensitivity between –60 and +60 mV applied voltage-clamp. The voltage sensitivity was only observed when the clamp voltage was increased to ±90 mV. At –90 mV the HCl CT responses increased and at +90 mV voltage-clamp decreased relative to 0cc. The post-cAMP CT responses to HCl were enhanced at 0cc relative to control and demonstrated a near linear relationship between –90 and +90 mV applied voltage-clamp and CT activity. The data demonstrate that cAMP activates an apical H^+ conductive pathway in TRCs. This apical H^+ conductive pathway is both amiloride- and Ca^{2+}-insensitive (Lyall *et al.* 2002*a*).

Similar to the case under control conditions (cf. Fig. 7), data summarized in Fig. 11 show that post-cAMP CT responses to HCl, CO_2 and acetic acid are also subject to regulation by TRC $[Ca^{2+}]_i$. Topical lingual application

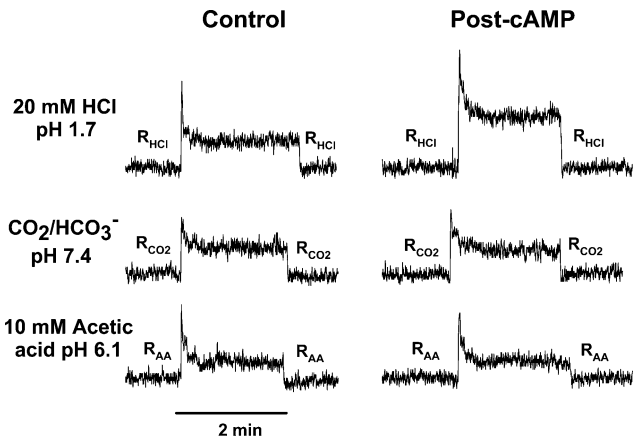

Figure 9. Effect of 8-CPT-cAMP on the CT responses to HCl, CO_2 and acetic acid
The CT responses to 20 mM HCl (pH 1.7), CO_2 (pH 7.4) and 10 mM acetic acid (pH 6.1) were recorded under open-circuit conditions (0cc) before (Control) and after the topical lingual application of 20 mM 8-CPT-cAMP for 45 min (Post-cAMP).

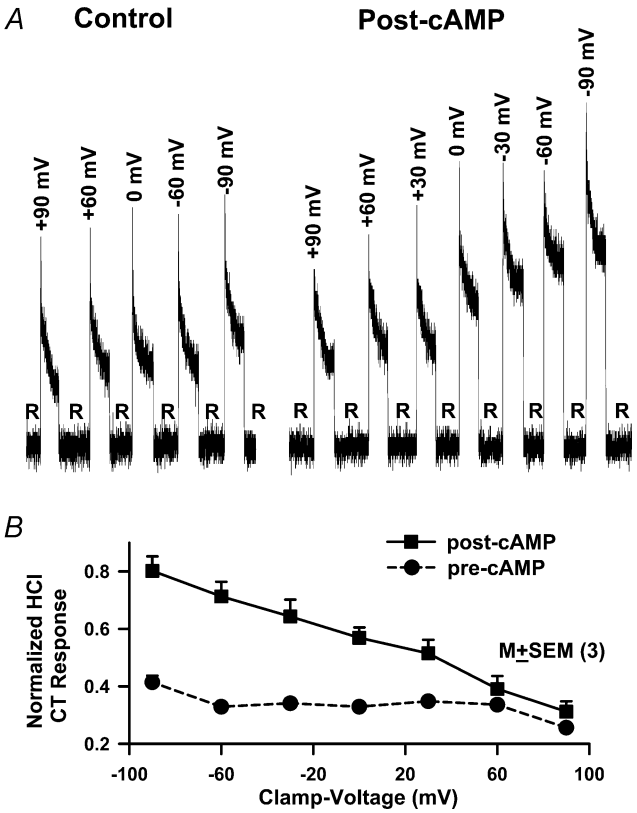

Figure 10. Effect of lingual voltage-clamp on HCl CT responses
A, the CT responses to 20 mM HCl were recorded under zero current-clamp conditions (0cc), at ±30 mV, ±60 mV and ±90 mV. Rinse (R) = 10 mM KCl. *B*, the CT responses to 20 mM HCl were recorded before (precAMP; ●) and after the topical lingual application of 8-CPT-cAMP (postcAMP; ■) under lingual voltage-clamp conditions between –90 mV and +90 mV. Values are presented as means ± S.E.M. of 3 animals (*N*). R = rinse solution.

of 8-CPT-cAMP + ionomycin, induced a rapid neural adaptation of CT responses to HCl, CO_2, and acetic acid relative to post-cAMP alone. Similar results were obtained in two additional animals (data not shown). These data suggest that both cAMP and $[Ca^{2+}]_i$ modulate CT responses to acidic stimuli independently. While cAMP activates H^+ entry across the apical membranes of TRCs, and enhances the phasic part of the CT response to HCl, an increase in $[Ca^{2+}]_i$ activates basolateral NHE-1, and increases neural adaptation to both strong and weak acid stimulation.

Discussion

The CT response profiles for acidic stimuli are composed of a phasic and a tonic response. While several factors may be involved in determining the overall CT response profile to acidic stimuli, the data presented in this study demonstrate that the phasic and tonic parts of the CT response are largely determined by the entry of acid equivalents across the apical membrane and the exit of H^+ across the basolateral membrane of TRCs via the NHE-1, respectively. The H^+ conductive pathway in the apical membrane and the basolateral NHE-1 activity are regulated differentially by second messengers (cAMP or Ca^{2+}) and pharmacological agents (zoniporide), and thus can be studied as separate entities.

In polarized TRCs, stimulating the apical membrane with acidic stimuli induced sustained decreases in pH_i (Lyall *et al.* 2001, 2002*a*,*b*). Thus both strong acids and weak organic acids gain entry into TRCs across the apical cell membrane and induce a decrease in pH_i. Weak organic acids permeate the apical membrane as neutral molecules, and strong acids via an H^+ entry pathway that is both amiloride- and Ca^{2+}-insensitive, but is activated by cAMP (Lyall *et al.* 2001, 2002*a*,*b*; DeSimone *et al.* 2001*b*). D er

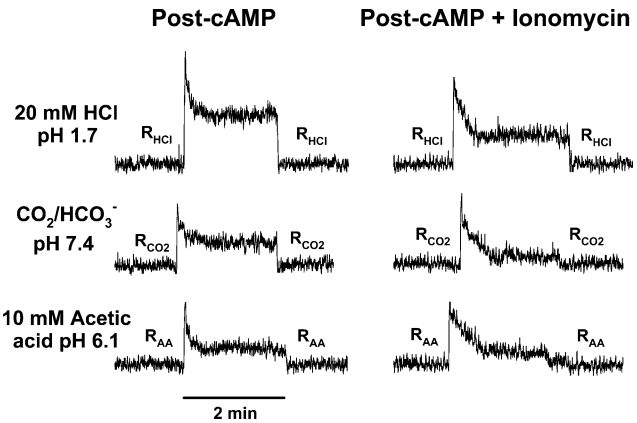

Figure 11. Effect of cAMP and ionomycin on the CT responses to HCl, CO_2 and acetic acid
The CT responses to 20 mM HCl (pH 1.7), CO_2 (pH 7.4) and 10 mM acetic acid (pH 6.1) were recorded under open-circuit conditions (0cc) after the topical lingual application of 20 mM 8-CPT-cAMP (Post-cAMP) and following treatment with 20 mM 8-CPT-cAMP + 150 μM ionomycin (Post-cAMP + Ionomycin). All rinse solutions (R) and the acidic stimuli (S) contained, in addition, 10 mM $CaCl_2$ (Table 2).

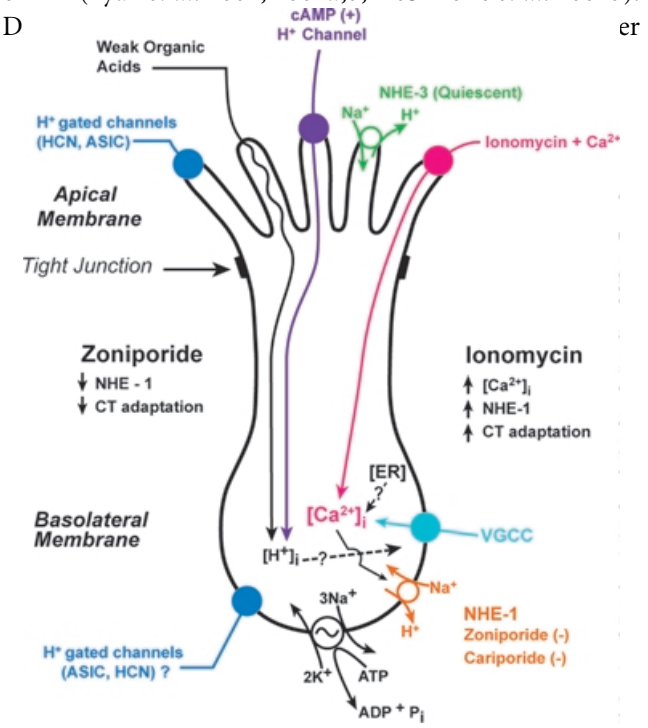

Figure 12. Proposed model for the mechanism of neural adaptation to acidic stimuli
Our data suggest that the basolateral NHE-1 is involved in neural adaptation during sour taste transduction. Acidic stimuli applied to the lingual surface induce a decrease in TRC pH_i as a result of the entry of acid equivalents across the apical cell membranes of TRCs. The apical membranes of TRCs exhibit an amiloride- and Ca^{2+}-insensitive, cAMP-dependent H^+ conductive pathway for strong acids (purple). In contrast, weak organic acids permeate across the apical membranes passively as undissociated molecules. The pH_i recovery occurs, in part, due to the presence of zoniporide- and cariporide-sensitive basolateral NHE-1. Although NHE-3 is present in the apical membranes of TRCs, under our experimental conditions the NHE-3 seems to be quiescent, and does not participate in pH_i regulation in TRCs (green). In the model the pathways for the apical entry and the basolateral exit of acid equivalents are shown in a single TRC. However, within a taste bud, TRCs are heterogeneous, and it is quite likely that not all of the above components are present in all cells. The favourable Na^+ gradient for the Na^+–H^+ exchangers is maintained by the basolateral Na^+–K^+-ATPase. An increase in TRC $[Ca^{2+}]_i$ induced by ionomycin, activates basolateral NHE-1, increasing the neural adaptation in CT responses to acidic stimuli. Zoniporide, a specific blocker of NHE-1, increased the magnitude of the CT responses to acidic stimulation under control conditions, and completely reversed the ionomycin-induced increase in neural adaptation to acidic stimuli. We conclude that during sour taste transduction the basolateral NHE-1 plays an important role in determining the adaptation (tonic) phase of the CT taste nerve responses to acidic stimuli.

than a decrease in pH_o, is the stimulus intensity variable that correlates specifically with increased CT taste nerve activity. Since inhibiting acid-induced TRC acidification also inhibits the acid-evoked CT response (Lyall *et al.* 2001, 2002*b*), it indicates that a decrease in TRC pH_i is the proximate stimulus for sour taste.

The data further demonstrate that TRC pH regulatory mechanisms play an important role in the neural adaptation to acidic stimuli (Lyall *et al.* 2002*a*; Vinnikova *et al.* 2004). The functional characteristics of the basolateral NHE-1, as they relate to TRC pH_i regulation and its role in sour taste transduction are discussed below.

Role of basolateral NHE-1 in TRC pH_i regulation

Our pH- and Na^+-imaging studies (Vinnikova *et al.* 2004) demonstrated that in TRCs, basolateral NHE-1 is functional in the nominal absence of CO_2/HCO_3^-. At the physiological pH, NHE-1 activity is low. Thus, under control conditions, i.e. in the absence of an acid stimulus, TRCs regulate their pH_i by maintaining a balance between the generation of intracellular H^+ due to metabolic activity and the exit of intracellular acid equivalents from the cells, in part, by the basolateral NHE-1 (Vinnikova *et al.* 2004). At constant pH_o, it is activated by intracellular acidification and by the Na^+ concentration in the basolateral compartment. However, changes in pH_o have an opposite effect on the basolateral NHE-1. An increase in basolateral pH enhanced, and a decrease in basolateral pH attenuated, the NHE-1 activity. Here, we demonstrate that NHE-1 activity is inhibited by zoniporide, a novel specific blocker of NHE-1 (cf. Figs 1–5). Cariporide (HOE 642), another specific blocker of NHE-1 activity, and amiloride, a non-specific blocker of NHEs, also inhibit NHE-1 activity in TRCs (Vinnikova *et al.* 2004).

Similar to the case in other tissues (Josette & Pouysségur, 1995; Ritter *et al.* 2001), in TRCs the basolateral NHE-1 activity is regulated by $[Ca^{2+}]_i$. Treating the basolateral membrane of polarized TRCs with ionomycin, a Ca^{2+} ionophore, increased $[Ca^{2+}]_i$, alkalinized resting pH_i, and increased the spontaneous recovery of pH_i from an NH_4Cl pulse. Both the ionomycin-induced alkalinization and the increase in pH_i recovery rate were blocked by zoniporide (Fig. 5).

Role of basolateral NHE-1 during acid stimulation

The topical lingual application of zoniporide enhanced the CT response to CO_2 and acetic acid by 57% and 111%, respectively, relative to control (Fig. 6*B*). In contrast, HCl responses were not affected by zoniporide. The fact that zoniporide only enhanced the CT responses to CO_2 and acetic acid, but not to HCl, is related to the pH of the acidic stimuli. The tongue was stimulated with CO_2 solutions at the physiological pH of 7.4, and with acetic acid solutions that were adjusted to pH 6.1. In contrast, 20 mM HCl stimulating solutions were at a pH of 1.7. We have previously shown that a decrease in pH_o inhibits NHE-1 activity (Vinnikova *et al.* 2004). Similar dependence of NHE activity on pH_o has been reported in other cells (Vaughan-Jones & Wu, 1990). Upon acidification of the basolateral compartment, protons bind to the external H^+ binding site on the NHE-1 and inhibit its activity. Similarly, a decrease in apical pH inhibits the NHE-1 activity; however, the mechanism of inhibition is more complex, and most likely involves changes in cell volume and/or changes in cytoskeleton of the cell (Vinnikova *et al.* 2004). Thus, it follows that, during HCl stimulation at pH_o 1.7, the basolateral NHE-1 activity is already inhibited and zoniporide treatment does not produce additional inhibition of the exchanger. In contrast, at pH 7.4 or 6.1, the basolateral NHE-1 still retains its basal activity, which can be blocked by zoniporide, eliciting increased CT responses to CO_2 and acetic acid stimulation relative to control.

Effect of NHE-1 activation on CT responses to acid stimulation

The Ca^{2+}-induced increase in NHE-1 activity has important consequences for both TRC pH_i regulation *in vitro* and the CT responses during acid stimulation *in vivo* (Lyall *et al.* 2002*a*). Stimulating the apical membrane with HCl induced a sustained decrease in TRC pH_i. However, upon NHE-1 stimulation with ionomycin, the HCl-induced decrease in TRC pH_i was transient and demonstrated rapid recovery towards its baseline value (Lyall *et al.* 2002*a*). Consistent with these *in vitro* results, topical lingual application of ionomycin, did not alter the initial magnitude of the CT responses to HCl (the phasic part of the CT response) but greatly accelerated the adaptation phase (tonic phase) of the neural response (Figs 7 and 8). The data further demonstrate that these effects of ionomycin can be generalized to strong acids, as well as to weak organic acids, such as CO_2 and acetic acid. The ionomycin-induced enhanced adaptation in the CT response was independent of pH_o and the accompanying anion. Although, these data are sufficient to demonstrate that the activation of a pH recovery mechanism in TRCs results in the rapid adaptation of the neural response to acid stimulation, they do not, by themselves,

prove that the basolateral NHE-1 is involved in this process.

The additional proof that it is indeed the activation of basolateral NHE-1 that brings about the neural adaptation comes from our studies with zoniporide. Under control conditions, topical lingual application of zoniporide, increased CT responses to CO_2 and acetic acid. In addition, zoniporide pretreatment reversed the ionomycin-induced increase in the adaptation phase of the CT responses to HCl, CO_2 and acetic acid, in a dose-dependent manner. At a zoniporide concentration of 500 μM, the CT responses to all three acidic stimuli, following ionomycin treatment, were at or exceeded control level responses. Thus at zoniporide concentrations that cause complete inhibition of the basolateral NHE-1 activity, an increase in TRC $[Ca^{2+}]_i$ failed to enhance its activity and abolished high $[Ca^{2+}]_i$-induced neural adaptation.

Recent studies indicate that acid responses occur in a subset of TRCs that participate in sour taste transduction. There are significant variations in the resting pH_i values in individual ROIs within a taste bud. TRCs can be separated into two distinct populations based on their initial value of resting pH_i. One subset of TRCs demonstrated a normal distribution with a mean pH_i of around 7.2. The second subset demonstrated a skewed distribution with a mean pH_i of around 7.45 (Vinnikova *et al.* 2004). It is suggested that TRCs having relatively more alkaline resting pH_i values, and consequently lower buffer capacity (β_1) values, will produce greater decreases in pH_i when stimulated with acids.

In polarized taste bud preparations, stimulating with apical acetic acid or HCl at pH 3.0, produced significantly different regional changes in TRC pH_i. At the same pH acetic acid produced a greater mean decrease in TRC pH_i compared to HCl. The changes in pH_i varied widely within different ROIs after treatment with the two acids. After HCl treatment 34.5% of ROIs responded with a decrease in $pH_i > 0.3$ pH unit. It is likely that TRCs in the ROIs that respond with the greatest decrease in pH_i participate most in sour transduction. In contrast, after acetic acid treatment, 91.4% of ROIs responded with a decrease in $pH_i > 0.3$ pH unit (Lyall *et al.* 2001). The data suggest that HCl-induced CT responses are elicited by a sub-population of TRCs contained in different ROIs within the taste bud that contain an apical H^+ entry mechanism. This conclusion is further supported by the observations that cAMP increases H^+ conductive entry across the apical membranes of TRCs (Fig. 10) and enhanced CT responses to HCl stimulation without affecting responses to acetic acid or CO_2 (Fig. 9). In contrast, for acetic acid, the influx of acid equivalents into TRCs is augmented by a significant flow of unionized acetic acid that results in a greater CT response relative to HCl (Lyall *et al.* 2001).

Although stimulating the apical membrane with acid stimuli decreases pH_i in a significant number of TRCs within the taste bud, only a subset of TRCs respond with a decrease in pH_i and a concomitant increase in $[Ca^{2+}]_i$ (Liu & Simon, 2001; Richter *et al.* 2003; Lyall *et al.* 2003). Since an increase in $[Ca^{2+}]_i$ is a prerequisite for the release of neurotransmitter, it is suggested that this subset of TRCs participate in sour taste transduction within the taste bud. Thus, it is likely that an acid-induced increase in TRC $[Ca^{2+}]_i$ subsequently activates the basolateral NHE-1 and brings about pH_i recovery and serves as a source of neural adaptation.

In a subset of TRCs in which an acid-induced decrease in pH_i is not accompanied by a concomitant increase in $[Ca^{2+}]_i$, changes in pH_i may participate in mixture interactions and/or in modulating responses to other taste modalities. We have previously shown that in TRCs containing ENaC, changes in pH_i modulate amiloride-sensitive apical Na^+ entry and the rat CT responses to NaCl (Lyall *et al.* 2002*b*).

Our studies further suggest that cAMP and $[Ca^{2+}]_i$ modulate CT responses to acids independently (Figs 9–11). Cyclic AMP enhanced the phasic part of the CT responses to HCl, but had no effect on the CT responses to CO_2 and acetic acid. The observed voltage sensitivity of the post-cAMP CT responses to HCl suggest that cAMP activates an apical H^+ conductance. However, cAMP does not affect the passive apical entry of the undissociated weak organic acids. In contrast, changes in $[Ca^{2+}]_i$ modulate the tonic part of the CT response to both strong and weak organic acids. Since in the presence of cAMP, topical lingual application of ionomycin enhanced neural adaptation to HCl stimulation, we conclude that the effects of $[Ca^{2+}]_i$ are independent of those due to cAMP. This is consistent with the concept that the sites of action of $[Ca^{2+}]_i$ and cAMP are different, non-interacting cellular entities, i.e. the basolateral NHE1 and an apical H^+ conducting pathway, respectively.

The main conclusions of the paper and the proposed mechanisms for the regulation of neural adaptation are summarized in a schematic diagram (Fig. 12). The apical cell membranes of TRCs contain acid entry mechanisms. In the case of fully dissociated strong acids, H^+ ions enter across the apical membrane of TRCs via an amiloride- and Ca^{2+}-insensitive, but cAMP-sensitive H^+ pathway (Lyall *et al.* 2002*a*). Weak organic acids, such as acetic acid and dissolved CO_2, enter across the apical membrane as neutral

molecules and decrease pH_i by generating intracellular acid equivalents. In the case of CO_2, the dissolved gas is converted to H_2CO_3 by intracellular carbonic anhydrases, subsequently, yielding $H^+ + HCO_3^-$. However, there is some evidence that H^+-gated channels, such as the acid-sensing ion channel (ASIC) in the apical membrane of TRCs (Ugawa *et al.* 1998; Lin *et al.* 2002), and both ASIC (Ugawa *et al.* 1998; Lin *et al.* 2002) and hyperpolarization-activated channels (HCN) (Stevens *et al.* 2001) in the basolateral membranes of TRCs may also play a role in sour taste transduction. These channels in the basolateral membrane could be activated if H^+ ions can cross the tight junctions and decrease pH_i in the basolateral compartment.

The H^+ exit from the cells occurs in part, via the zoniporide-sensitive basolateral NHE-1. The accompanying Na^+ ions exit TRCs via the basolateral ouabain-sensitive Na^+–K^+-ATPase. We have previously demonstrated the presence of mRNA transcripts for NHE-3 in TRCs and the specific binding of NHE-3 antibodies to the apical membranes of TRCs; however, under the experimental conditions examined so far, the apical NHE-3 appears to be quiescent and does not contribute to pH_i regulation in TRCs (Vinnikova *et al.* 2004). Although the model depicts the presence of acid entry and exit pathways in a single TRC, it must be emphasized that within a taste bud, TRCs are heterogeneous, and it is likely that not all of the above mechanisms are present in all taste cells. Topical lingual application of ionomycin + $CaCl_2$, increases TRC $[Ca^{2+}]_i$ and results in the activation of basolateral NHE-1. The activation of NHE-1 results in an acid-induced decrease in pH_i that is transient, i.e. recovers spontaneously, leading to a rapid adaptation in the CT responses to acid stimulation. However, topical lingual application of zoniporide, a specific NHE-1 blocker, reverses the observed adaptation in CT responses to acid stimulation. Taken together, the data indicate that in TRCs, a basolateral NHE-1 is involved in neural adaptation to acidic stimuli. Recent, studies (Liu & Simon, 2001; Richter *et al.* 2003; Lyall *et al.* 2003) suggest that in a subset of TRCs an acid-induced decrease in pH_i is accompanied by an increase in TRC $[Ca^{2+}]_i$. It is likely that an increase in TRC $[Ca^{2+}]_i$ activates basolateral NHE-1 and increases neural adaptation.

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