Modulation of Rat Chorda Tympani NaCl Responses and Intracellular Na⁺ Activity in Polarized Taste Receptor Cells by pH

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ABSTRACT Mixture interactions between sour and salt taste modalities were investigated in rats by direct measurement of intracellular pH (pH_i) and Na^+ activity ($[Na^+]_i$) in polarized fungiform taste receptor cells (TRCs) and by chorda tympani (CT) nerve recordings. Stimulating the lingual surface with NaCl solutions adjusted to pHs ranging between 2.0 and 10.3 increased the magnitude of NaCl CT responses linearly with increasing external pH (pH_o). At pH 7.0, the epithelial sodium channel (ENaC) blocker, benzamil, decreased NaCl CT responses and inhibited further changes in CT responses induced by varying pH_o to 2.0 or 10.3. At constant pH_o, buffering NaCl solutions with potassium acetate/acetic acid (KA/AA) or HCO_3^-/CO_2 inhibited NaCl CT responses relative to CT responses obtained with NaCl solutions buffered with HEPES. The carbonic anhydrase blockers, MK-507 and MK-417, attenuated the inhibition of NaCl CT responses in HCO_3^-/CO_2 buffer, suggesting a regulatory role for pH_i. In polarized TRCs step changes in apical pH_o from 10.3 to 2.0 induced a linear decrease in pH_i that remained within the physiological range (slope = 0.035; $r^2 = 0.98$). At constant pH_o, perfusing the apical membrane with Ringer's solutions buffered with KA/AA or HCO_3^-/CO_2 decreased resting TRC pH_i, and MK-507 or MK-417 attenuated the decrease in pH_i in TRCs perfused with HCO_3^-/CO_2 buffer. In parallel experiments, TRC $[Na^+]_i$ decreased with (a) a decrease in apical pH, (b) exposing the apical membrane to amiloride or benzamil, (c) removal of apical Na^+, and (d) acid loading the cells with NH_4Cl or sodium acetate at constant pH_o. Diethylpyrocarbonate and Zn^{2+}, modification reagents for histidine residues in proteins, attenuated the CO_2-induced inhibition of NaCl CT responses and the pH_i-induced inhibition of apical Na^+ influx in TRCs. We conclude that TRC pH_i regulates Na^+-influx through amiloride-sensitive apical ENaCs and hence modulates NaCl CT responses in acid/salt mixtures.

KEY WORDS: salt taste • sour taste • intracellular pH • taste mixture interaction • epithelial sodium channel

INTRODUCTION

During a meal taste receptors cells (TRCs)* are normally stimulated by mixtures of taste stimuli. Since individual TRCs generally respond to more than one class of stimuli (Gilbertson et al., 2001), their outputs reflect the interplay of intracellular processes that link the various transduction mechanisms in complex nonlinear ways. Such interactions are the source of modifications in perceived intensity and quality arising in the peripheral taste organs. Mixture interactions may give rise to either mixture suppression or enhancement. The rat chorda tympani (CT) nerve responses to Na^+ salts are suppressed when presented together with potassium benzoate (Sato and Beidler, 1979) or potassium gluconate (Stewart et al., 1996). In contrast, CT responses to NaCl are enhanced in mixtures of mannitol or cellobiose (Lyall et al., 1999). In foods and beverages hu-

man TRCs are exposed to pHs ranging from 2.3 to 8.6 (Feldman and Barnett, 1995). Protons are not only the primary sour stimulus source (DeSimone et al., 1995, 2001a; Lyall et al., 2001, 2002), but also may play a regulatory role in the transduction of other taste modalities. Beidler (1954) and Ogawa (1969) reported that rat CT nerve responses to NaCl are inhibited at pHs below 3–4, suggesting that taste mixture interactions occur between sour taste and salt taste modalities. However, at present the physiological mechanisms involved in mixture interactions between sour taste and salt taste modalities have not been delineated.

Amiloride inhibits a significant part of the rat CT responses to NaCl, indicating that in fungiform TRCs amiloride-sensitive epithelial sodium channels (ENaCs) contribute most to the NaCl-induced increase in CT response (Lindemann, 1996, 2001; Herness and Gilbertson, 1999; Kretz et al., 1999; Lin et al., 1999; Stewart et al., 1997). Several studies indicate that protons regulate amiloride-sensitive ENaCs in a variety of epithelial cells (Lyall and Biber, 1995; Lyall et al., 1995; Garty and Palmer, 1997; Chalfant et al., 1999; Zeiske et al., 1999; Konstas et al., 2000; Howard et al., 2001). The α, β, and γ subunits of cloned ENaC expressed in *Xenopus laevis*

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Abbreviations used in this paper: ASIC, acid-sensing ion channel; CPC, cetylpyridinium chloride; CT, chorda tympani; ENaC, epithelial sodium channel; TRC, taste receptor cell; VSI, voltage sensitivity index.

oocytes demonstrated decreased Na$^+$ current when intracellular pH (pH$_i$) was decreased, but not when extracellular pH (pH$_o$) was decreased (Chalfant et al., 1999). A reduction in pH$_i$ reduced the single-channel open probability of ENaC without altering single channel conductance (Chalfant et al., 1999; Zeiske et al., 1999). We have shown previously that weak organic acids as well as fully dissociated strong acids produce a sustained decrease in TRC pH$_i$ (DeSimone et al., 2001a; Lyall et al., 2001, 2002). Given that acid stimulation decreases pH$_i$, it is reasonable to hypothesize that intracellular protons also modulate the activity of TRC ENaC over a wide range of pH. If TRC ENaC is also subject to regulation by H$^+$ ions, changes in pH could modulate CT responses to NaCl. Intracellular second messengers, cAMP and Ca^{2+}, regulate salt taste (Gilbertson et al., 1993; Lin et al., 1999; Alam et al., 2002; Russell et al., 2002) and sour taste (Gilbertson et al., 1993; Lyall et al., 2002) modalities. It is likely that changes in one or more second messengers (Liu and Simon, 2001) during acid stimulation may also participate in mixture interactions and alter salt responses in bimodal TRCs.

In this paper we investigated mixture interactions between sour taste and salt taste modalities by monitoring CT responses to NaCl under lingual voltage-clamp conditions and the temporal changes in intracellular Na$^+$ ([Na$^+$]$_i$) and pH$_i$ in polarized fungiform TRCs over a wide range of pHs. Our results demonstrate that changes in pH$_i$ regulate apical Na$^+$ influx via amiloride-sensitive ENaCs in TRCs and hence modulate NaCl CT responses.

MATERIALS AND METHODS

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. Body temperatures were maintained at 36–37°C with a circulating water heating pad. The left CT nerve was exposed laterally as it exited the tympanic bulla (Ye et al., 1993, 1994; DeSimone et al., 1995; Stewart et al., 1998) 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 of 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 Soltec (model 3314) chart recorder and also captured on disk using Labview software and analyzed off-line (DeSimone et al., 2001b; Lyall et al., 2001, 2002). Stimulus solutions were injected into a Lucite chamber (3 ml; 1 ml/s) 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

TABLE I
Rinse and Stimulus Solutions for CT Experiments

Set 1

Solution	KCl	NaCl[a]	HEPES	Mannitol	HCl	Tris-base	pH
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	
R7	10	0	10	200	0	0	7.0
S7	10	100	10	0	0	0	7.0
R2	10	0	0	200	10	0	2.0
S2	10	100	0	0	10	0	2.0
R10.3	10	0	0	200	0	10	10.3
S10.3	10	100	0	0	0	10	10.3

Set 2

Solution	KA	NaCl	HEPES	Mannitol	AA	pH
	mM	*mM*	*mM*	*mM*	*mM*	
HRH6.1	525	0	30	200	0	6.1
HSH6.1	525	100	30	0	0	6.1
HRAA6.1	525	0	0	200	30	6.1
HSAA6.1	525	100	0	0	30	6.1

Set 3

Solution	KA	NaCl	HEPES	Mannitol	AA	pH
	mM	*mM*	*mM*	*mM*	*mM*	
LRH6.1	175	0	10	200	0	6.1
LSH6.1	175	100	10	0	0	6.1
LRAA6.1	175	0	0	200	10	6.1
LSAA6.1	175	100	0	0	10	6.1

Set 4

Solution	KCl	NaCl	HEPES	Mannitol	KHCO$_3$	%CO$_2$/%O$_2$	pH
	mM	*mM*	*mM*	*mM*	*mM*		
RH7.4	72	0	10	200	0	0	7.4
SH7.4	72	100	10	0	0	0	7.4
RCO$_2$7.4	0	0	0	200	72	10/90	7.4
SCO$_2$7.4	0	100	0	0	72	10/90	7.4

R, rinse solution; S, NaCl stimulus solution; H, HEPES; AA, acetic acid; KA, potassium acetate.
[a]In some solutions the NaCl was replace with 100 mM KCl or NH$_4$Cl.

permitted the recording of neural responses with the chemically stimulated receptive field under 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 voltage sensitivity index (VSI) was calculated as the difference between the CT response at −60 and 60 mV (CT$_{-60 mV}$-CT$_{60 mV}$) relative to 0CC voltages (Ye et al., 1993, 1994). The anterior lingual surface was stimulated with a rinse solution containing KCl and with a salt stimulus solution containing KCl + NaCl (Table I). Amiloride (100 μM) or benzamil (5 μM) was used to block Na$^+$ ion entry via the apical Na$^+$ channels (Ye et al., 1993, 1994; Stewart et al., 1998).

Solutions

The composition of various rinse and stimulus solutions used in the CT experiments is given in Table I. The solutions listed in set 1 (Table I) were used to investigate the effect of pH$_o$ on NaCl CT responses at constant osmolarity. The solution osmo-

TABLE II

Solutions Used in In Vitro Experiments

Set 1

Solution	NaCl	KCl	CaCl$_2$	MgCl$_2$	Glucose	HEPES	HCl	Tris-base	NMDGCl[a]	Na-pyruvate	pH
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	
R	140	5	1	1	10	10	0	0	0	10	7.4
RC	150	5	1	1	10	10	0	0	0	0	7.4[b]
RHCl	150	5	1	1	10	0	10	0	0	0	2.0
RTris	150	5	1	1	10	0	0	10	0	0	10.3
R0Na$^+$	0	5	1	1	10	10	0	0	150	0	7.4[b]

Set 2

Solution	NaCl	KCl	CaCl$_2$	MgCl$_2$	Glucose	HEPES	NaHCO$_3$	%CO$_2$/%O$_2$	pH
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*		
RC	150	5	1	1	10	10	0	0	7.4
RCO$_2$	78	5	1	1	10	0	72	10/90	7.4

Set 3

Solution	NaCl	KCl	CaCl$_2$	MgCl$_2$	Glucose	HEPES	NH$_4$Cl	NaA	pH
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	
RC	150	5	1	1	10	10	0	0	7.4
RNH$_4$Cl	100	5	1	1	10	10	50	0	7.4
RNaA	100	5	1	1	10	10	0	50	7.4

Set 4

Solution	NaCl	KCl	CaCl$_2$	MgCl$_2$	Glucose	MOPS	NaA	Mannitol	AA	pH
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	
RCM	150	5	1	1	10	30	0	0	0	7.4
RNaA1	0	5	1	1	10	30	150	0	0	6.3
RNaA2	0	5	1	1	10	30	150	0	30	6.3
RMOPS1	0	5	1	1	10	30	150	600	0	6.3
RMOPS2	0	5	1	1	10	0	450	0	30	6.3

Set 5

Solution	NaCl	KCl	CaCl$_2$	MgCl$_2$	Glucose	HEPES	Nigericin	pH
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	*μM*	
CS	4.6	140	2	1	10	10	10	6.5–8.0

R, Ringer's solution; RC, control Ringer's solution; NaA, sodium acetate; H, HEPES; CS, pH calibrating solutions.

[a]In some solutions NaCl was replaced by 150 mM Tris-HCl.

[b]In some experiments the pHs of RC and R0Na$^+$ were adjusted to 4.4.

larity was adjusted with mannitol. Mannitol by itself at these concentrations does not evoke a neural response in the rat CT (Lyall et al., 1999). The solutions listed in Table I (sets 2 and 3) were used to investigate the effect of intracellular acid loading on NaCl CT responses at constant pH$_o$ and osmolarity. Some experiments were performed at high osmolarity with solutions containing 525 mM KA + 30 mM AA (pH 6.1; Table I, set 2) and others at lower osmolarity with solutions containing 175 mM KA + 10 mM AA (pH 6.1; Table I, set 3). The solutions listed in set 4 (Table I) were used to investigate the effect of CO$_2$ on NaCl CT responses at constant pH$_o$ and osmolarity. In some experiments both HEPES- and HCO$_3$/CO$_2$-buffered solutions contained 50 mM MK-507 or MK-417 (Merck), membrane-permeable inhibitors of carbonic anhydrases (Fanous et al., 1999; Lyall et al., 2001) or 20 mM diethylpyrocarbonate (DEPC; Sigma-Aldrich), a modification reagent for histidine residues in proteins (Mankelow and Henderson, 2001; Seebungkert and Lynch, 2001).

Data Analysis

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., 1999, 2001, 2002; DeSimone et al., 2001a). The changes in the area under the integrated NaCl CT response curves under different conditions were normalized to the responses observed in each animal to 300 mM NH$_4$Cl (CT$_{NaCl}$/CT$_{NH4Cl}$) and were expressed as the mean $\pm$ SEM of N; where N represents the number of animals in each group; M $\pm$ SEM (N). Student's *t* test was employed to analyze the differences between sets of data.

In Vitro Studies

pH_i measurement. Rats were anesthetized with isoflurane and killed by cervical dislocation. The tongues were rapidly removed and stored in ice-cold Ringer's solution (R; Table II, set 1). The lingual epithelium was isolated by collagenase treatment (Lyall et al., 1997; Stewart et al., 1998). 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; DeSimone et al., 2001a). The tissue was intermittently perfused with Ringer's solution (R; Table II, set 1) containing $25 \mu M$ of the pH-sensitive fluoroprobe BCECF-AM (Molecular Probes) at $4°C$ for 2 h. Before the experiment was started, the tissue was perfused on both sides with control solution for 15 min. The control solution was Ringer's solution without Na-pyruvate (RC; Table II, set 1). The tissue was continuously perfused at the rate of 1 ml/min and the solution changes in the apical or basolateral compartment were made using three way miniature LFAA solenoid valves (The Lee Company). The TRCs in the taste bud were visualized from the basolateral side through a $40\times$ 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) attached to an image intensifier (VS4–1845; Videoscope), an epifluorescent light source (TILL Photonics Polychrome IV), a 515 nm dichroic beam splitter (Omega Optical), 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 10-s intervals. Small regions of interest (ROIs) in the taste bud (diameter 2–3 μm) were chosen in which the changes in FIR (fluorescence intensity ratio; F_{490}/F_{440}) were analyzed using TILLvisION v3.1 imaging software. Each ROI contained 2–3 receptor cells. Thus, the fluorescence intensity recorded for a ROI represents the mean value from 2–3 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 six ROIs ($\sim$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 bilateral perfusion of high K^+ calibrating solutions containing $10 \mu M$ nigericin (CS; Table II, set 5) adjusted to pHs between 6.5 and 8.0 (DeSimone et al., 2001b; Lyall et al., 2001, 2002).

[Na^+]_i measurement. We monitored relative changes in intracellular sodium activity ($[Na^+]_i$) in polarized TRCs by loading the tissue with the sodium-sensitive fluoroprobes sodium green or SBFI (both from Molecular Probes). Tissues were loaded with sodium green AM ($30 \mu M$) at $4°C$ for 2 h. The tissue was loaded with SBFI-AM ($10 \mu M$) in the presence of 0.15% pluronic at room temperature for 4 h. Before the experiment was started, the tissue was perfused on both sides with control solution (RC) for 15 min. In sodium green–loaded cells the changes in $[Na^+]_i$ were monitored by exciting the cells at 490 nm and the emitted light was imaged at 535 nm at 10-s intervals. Sodium green is ideal for studying the effects of amiloride, which emits strongly when excited with UV light. Since it is a single wavelength dye its emission can be affected by factors such as dye bleaching, dye leakage from the cells, changes in focal plane, and variations in cell volume (Xu et al., 1995). The SBFI-loaded TRCs were alternately excited at 340 and 380 nm and imaged at 10 s intervals. The emitted light was detected with a set up containing a 430 nm dichroic beam splitter (Omega Optical), and a 510 nm emission filter (20 nm band pass; Omega Optical). The changes in FIR are independent of dye bleaching, dye leakage from the cells, changes in focal plane, and variations in cell volume. The fluorescence of SBFI (Diarra et al., 2001) and sodium green (Xu et al., 1995) is pH independent. All experiments were done at room temperature ($\sim$22°C).

Data analysis. The changes in TRC pH_i were expressed as the mean $\pm$ SEM of N; where N represents the number of ROIs within the taste bud; M $\pm$ SEM (N). In TRCs loaded with sodium green the changes in $[Na^+]_i$ were expressed relative to the fluorescence intensity (F_{490}) under control conditions. The F_{490} under control conditions for each ROI was taken as 100%. For TRCs loaded with SBFI, the relative changes in FIR (F_{340}/F_{380}) were compared between different ROIs under different conditions. The data were also presented as the mean $\pm$ SEM from different tissue preparations. In this case N represented the number of taste buds. Student's *t* test was employed to analyze the differences between sets of data.

Solutions

The composition of various Ringer's solutions used in the in vitro experiments is given in Table II. In some experiments the apical or basolateral perfusion solutions contained either MK-417 or MK-507 (Merck), membrane-permeable inhibitors of carbonic anhydrases (Fanous et al., 1999; Lyall et al., 2001). The drugs were used at lower concentrations when applied from the basolateral side.

R E S U L T S

In Vivo Studies

Effect of external pH (pH_o) on NaCl CT responses Under physiological conditions TRCs are exposed to wide variations in pH (Feldman and Barnett, 1995). We investigated if changes in pH alter neural responses to NaCl stimulation. The CT responses were monitored while the tongue was stimulated with 100 mM NaCl solutions adjusted to pH 2.0, 7.0, and 10.3. In Fig. 1 the lingual surface was perfused with a rinse solution R7 (pH 7; Table I, set 1) and the CT activity was taken as baseline. Perfusing the tongue with NaCl stimulus solution S7 (pH 7; Table I, set 1) increased CT activity (upward deflection in the CT trace; a and b). Replacing S7 with a similar NaCl stimulus solution S2, containing 10 mM HCl (pH 2.0; Table I, set 1) decreased the CT response (b and c). Upon reperfusing S7 the CT activity increased to its original steady-state level (c and d) and rinsing with R7 returned the CT activity to baseline (d and e). In the next step, replacing R7 with a similar rinse solution R2, containing 10 mM HCl (pH 2.0, Table I, set 1) reversibly increased the CT activity (e–g). This increase in CT response is due to the presence of HCl in the rinse solution, R2 (DeSimone et al., 1995; Stewart et al., 1998; Lyall et al., 2001, 2002). The NaCl CT responses were normalized to the response observed in each animal to 300 mM NH_4Cl (CT_{NaCl}/CT_{NH4Cl}). In five experiments, replacing S7 with S2 decreased the magnitude of the NaCl CT response from 0.71 ± 0.03 to 0.60 ± 0.04 ($\Delta = -0.11 \pm 0.01$), a decrease of $16.1 \pm 1.9\%$ ($P < 0.01$) with respect to its activity in S7. In the same experiments replacing R7 with R2 increased CT activity by 0.17 ± 0.01 ($n = 5$). If there
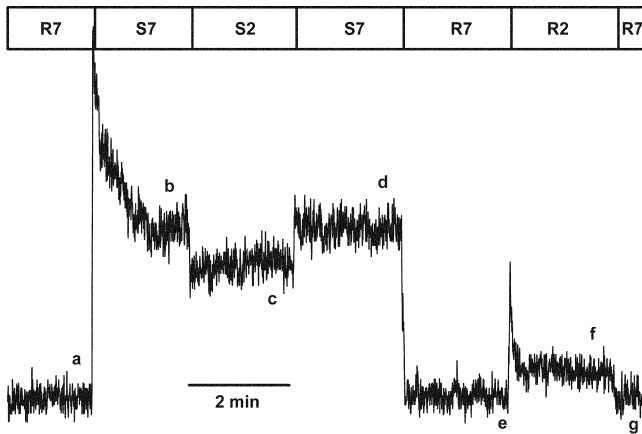
FIGURE 1. Effect of pH_o on the NaCl CT responses. The integrated CT responses were recorded under open-circuit conditions (i.e., under zero current clamp; 0CC). The tongue was initially perfused with rinse (R7) solution containing 10 mM KCl + 200 mM mannitol and then with the NaCl stimulus solution (S7) containing 10 mM KCl + 100 mM NaCl (a and b). Both R7 and S7 were buffered to pH 7.0 with HEPES. In the second step, S7 was replaced with the NaCl stimulus solution S2 (b and c). In the final step, R7 was replaced with the rinse solution R2 (e and f). Both R2 and S2 were adjusted to pH 2.0 with 10 mM HCl (without HEPES).
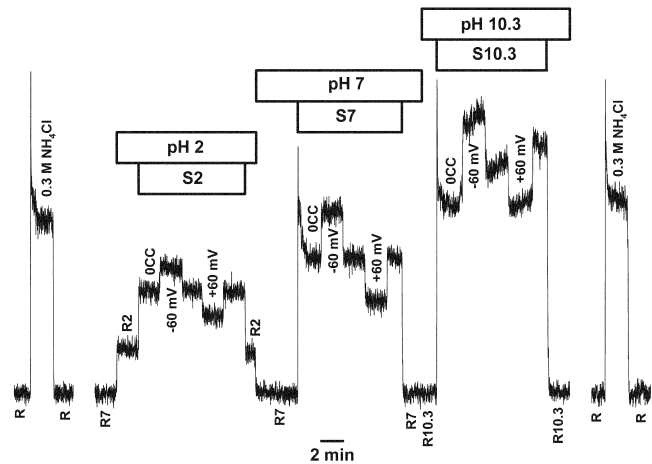

FIGURE 2. Effect of pH_o on the voltage sensitivity of the NaCl CT responses. The lingual surface was perfused with rinse (R) solutions containing 10 mM KCl + 200 mM mannitol and stimulus (S) solutions containing 10 mM KCl + 100 mM NaCl adjusted to pH 2.0 (R2 and S2; left), 7.0 (R7 and S7; middle), and 10.3 (R10.3 and S10.3; right). Integrated CT responses were recorded under open-circuit conditions (i.e., under zero current clamp; 0CC), at −60 mV, and at 60 mV lingual voltage-clamp. The NaCl CT responses were voltage-sensitive. The CT responses were enhanced at −60 mV and suppressed at 60 mV voltage-clamp relative to 0CC. Also shown are the CT responses to 0.3 M NH_4Cl test pulses before and after the experiment.

was no interaction between HCl and NaCl at the level of TRCs, one would predict that replacing S7 with S2 would produce the same magnitude of increase in the CT response as observed by replacing R7 with R2. However, under these conditions there was an overall decrease in the magnitude of the CT response (b and c). In additional experiments, the magnitude of the CT response to S10.3 (pH 10.3; Table I, set 1) was also decreased upon switching from S10.3 to S7 or by replacing S10.3 with S2 (unpublished data). However, under these conditions, it is difficult to estimate the actual pH-induced inhibition in the CT response because at low pH there is an upward shift in the baseline.

Fig. 2 shows the effect of pH on the NaCl CT responses under 0CC, at −60 mV, and at 60 mV lingual voltage-clamp. The tongue was stimulated with 100 mM NaCl solutions adjusted to pH 2.0, 7.0, and 10.3. In Fig. 2 (middle) the lingual surface was perfused with a rinse solution R7 and the CT activity was taken as baseline. Perfusing the tongue with NaCl stimulus solution S7 increased CT activity under 0CC. Consistent with previous studies (Ye et al., 1993, 1994; Stewart et al., 1998; DeSimone et al., 2001b; Lyall et al., 2001, 2002) the NaCl CT responses were voltage sensitive, the CT responses were enhanced at −60 mV and suppressed at 60 mV voltage-clamp relative to 0CC. The numerical value of the CT response was expressed as the area under the integrated CT response curve and normalized in each animal to the 300 mM NH_4Cl response (CT_{NaCl}/CT_{NH4Cl}) as described in MATERIALS AND METHODS. Fig. 3 summarizes data from several animals. At pH 7.0,

the mean normalized CT response to 100 mM NaCl at 0CC was 0.68 ± 0.02 (N = 7; black bar 1). In five animals the CT responses were also recorded under lingual voltage-clamp conditions. The mean normalized CT response at 0CC (0.68 ± 0.03) increased to 0.90 ± 0.04 (black bar 2) at −60 mV (ΔCT = 0.22 ± 0.02; P < 0.01) and decreased to 0.46 ± 0.03 at 60 mV (ΔCT = −0.22 ± 0.02; P < 0.01; paired; N = 5; black bar 3). At pH 7.0 the VSI ($CT_{−60 mV}$-$CT_{60 mV}$) was 0.44 ± 0.02.

Also shown in Fig. 2 (left) are the CT responses when both the rinse and the NaCl stimulus were applied at pH 2.0. Replacing the rinse solution R7 (pH 7.0; Table I, set 1) with the rinse solution R2 (pH 2.0; Table I, set 1) increased the CT response. This increase in CT response is due to the presence of HCl in R2 (DeSimone et al., 1995; Stewart et al., 1998; Lyall et al., 2001, 2002). Subsequently, perfusing the tongue with the NaCl stimulus solution S2 (pH 2.0; Table I, set 1) produced a further increase in CT response under 0CC. If there was no interaction between HCl and NaCl at the level of TRCs, one would predict that the CT responses would be additive. However, the magnitude of the CT response (S2-R2 at 0CC) was decreased by 50% relative to the CT response at pH 7.0 (S7-R7 at 0CC). At pH 2.0, the NaCl CT response was significantly less voltage-sensitive relative to pH 7.0 (compare middle panel). The data from several animals are also summarized in Fig. 3. At pH 2.0 the mean normalized CT response to 100
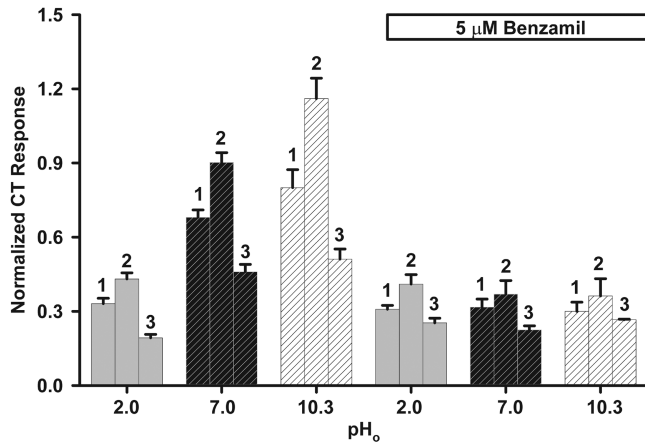
FIGURE 3. Effect of benzamil on the pH_o-induced changes in NaCl CT responses. The lingual surface was perfused with rinse (R) solutions (10 mM KCl + 200 mM mannitol) and stimulus (S) solutions (10 mM KCl + 100 mM NaCl) adjusted to pH 2.0, 7.0, and 10.3 in the presence and absence of 5 μM benzamil. Integrated CT responses were recorded under 0CC (1), at −60 mV (2), and at 60 mV (3) lingual voltage clamp. The area under the CT response curve was taken as the magnitude of the CT response. In each animal the NaCl CT responses were normalized to the CT response obtained with 300 mM NH_4Cl (CT_{NaCl}/CT_{NH4Cl}). The values are presented as M ± SEM of N (see text for details).

FIGURE 4. Relationship between pH_o versus NaCl CT responses and between pH_o and TRC pH_i. The area under the CT response curve was taken as the magnitude of the CT response. The NaCl CT responses were normalized to the response observed in each animal to 300 mM NH_4Cl (CT_{NaCl}/CT_{NH4Cl}). The values are presented as M ± SEM of N, where N represents the number of observations at each pH_o (in each case N was between 2 and 9). The NaCl CT responses were linearly related to pH_o with a slope of 0.06 (filled circles; $r^2 = 0.99$). The changes in TRC pH_i were also linearly related to pH_o with a slope of 0.035 (open circles; intercept = 6.92; $r^2 = 0.95$). TRC pH_i values are presented as mean ± SEM of N, where N represents the number of observations at each pH_o (in each case N was between 4 and 6).

mM NaCl at 0CC was 0.35 ± 0.02 (N = 6; gray bar 1). This value is significantly less than the corresponding value (0.68 ± 0.02) at pH 7.0 (P < 0.001; unpaired). In five animals the CT responses were also recorded under lingual voltage-clamp conditions. The mean normalized CT response at 0CC (0.33 ± 0.02) increased to 0.43 ± 0.02 at −60 mV (ΔCT = 0.10 ± 0.01; P < 0.025; gray bar 2) and decreased to 0.19 ± 0.01 at 60 mV (ΔCT = −0.14 ± 0.02; P < 0.025; paired; N = 5; gray bar 3). Thus lowering the NaCl stimulus solution pH from 7.0 to 2.0 decreased mean normalized NaCl CT response by 48.5% and decreased the VSI from 0.44 ± 0.02 to 0.24 ± 0.02, a decrease of 45.4 ± 4.5% (P < 0.001; N = 5).

Also shown in Fig. 2 (right) are the CT responses when both the rinse and the stimulus solutions were applied at pH 10.3. Replacing rinse solution R7 (pH 7.0; Table I, set 1) with rinse solution R10.3 (pH 10.3; Table I, set 1) did not alter the baseline CT response. Subsequently, perfusing the tongue with the NaCl stimulus solution S10.3 (pH 10.3; Table I, set 1) increased the CT response above baseline under 0CC. However, the magnitude of the CT response (S10.3-R10.3 at 0CC) was increased by 50% relative to CT response at pH 7.0 (S7-R7 at 0CC). At pH 10.3, the NaCl CT response demonstrated greater voltage-sensitivity relative to pH 7.0 (compare middle panel). The data from several animals are also summarized in Fig. 3. At pH 10.3 the mean normalized CT response to 100 mM NaCl at 0CC
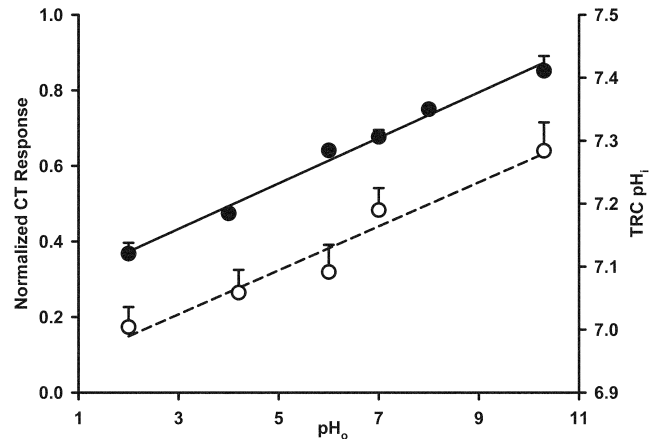
was 0.82 ± 0.06 (N = 5; hatched bar 1) and was significantly greater than the corresponding value at pH 7.0 (0.68 ± 0.02; P < 0.01; unpaired). In four animals the CT responses were also recorded under lingual voltage-clamp conditions. The mean normalized CT response (0.80 ± 0.07) increased to 1.16 ± 0.08 at −60 mV (hatched bar 2) and decreased to 0.51 ± 0.04 at 60 mV (hatched bar 3). Thus increasing the pH from 7.0 to 10.3 increased mean NaCl CT response by 20.6% and increased the VSI from 0.44 ± 0.02 (N = 5) to 0.65 ± 0.05 (N = 4), an increase of 47.7%. When the NaCl CT responses were monitored over the entire range of pH between 2.0 and 10.3, the normalized NaCl CT responses demonstrated a linear relationship with pH_o (Fig. 4, filled circles; slope = 0.06; $r^2 = 0.99$).

We further investigated if changes in pH_o also affect CT responses to KCl and NH_4Cl. The CT responses were recorded under 0CC while the tongue was stimulated with 100 mM NaCl, 100 mM KCl, and 100 mM NH_4Cl solutions adjusted to pH 2.0, 7.0, and 10.3 (Table I, set 1). In a representative experiment (unpublished data), at pH 2.0, 7.0, and 10.3, the mean normalized CT responses to NaCl increased linearly with increasing pH and were 0.46, 0.62, and 0.77, respectively. The responses to 100 mM KCl at the same pHs were 0.35, 0.34, and 0.34, respectively. And the responses to 100 mM NH_4Cl at the same pHs were 0.70, 0.68, and

0.67, respectively. These results indicate that unlike NaCl, the KCl- and NH₄Cl-induced CT responses are pH insensitive.

As described in detail previously (Ye et al., 1993, 1994), we tested the responses to two reference stimuli, namely, 0.3 M NaCl and NH₄Cl before and after the experiment. The data from an experiment were accepted if the CT responses to the above two reference stimuli did not differ by >10% before and after the experiment. In the data shown in Fig. 2, the responses to 0.3 M NH₄Cl before and after stimulating the tongue repeatedly with NaCl solutions buffered to pH 2, 7, and 10.3 did not differ by >3%.

Effect of Benzamil on NaCl CT Responses

Consistent with our previous studies (Stewart et al., 1998), the addition of 5 μM benzamil to R7 (pH 7.0) did not alter the baseline CT activity. Subsequent perfusion of S7 + benzamil produced an increase in NaCl CT response. However, the magnitude of the response in the presence of benzamil was significantly smaller as compared with control. The effect of benzamil on several animals is also summarized in Fig. 3. At pH 7.0, the mean normalized CT response to 100 mM NaCl at 0CC in the presence of benzamil was 0.32 ± 0.03 (N = 4; black bar 1). The CT response increased to 0.37 ± 0.06 at -60 mV (black bar 2) and decreased to 0.22 ± 0.02 at $+60$ mV (P > 0.05; black bar 3). The VSI was 0.15 ± 0.04. Thus at pH 7.0, benzamil decreased the mean normalized NaCl CT response by 53.4% and decreased the VSI by 67.4% relative to control. These effects of benzamil are consistent with its known role as a blocker of ENaCs (Lyall et al., 1993, 1995; Lyall and Biber, 1995; Stewart et al., 1998).

Data shown in Fig. 5 demonstrate that at pH 7.0, the benzamil-induced inhibition of NaCl CT response (left) are comparable in magnitude to the inhibition observed by decreasing the pH of the NaCl stimulus solution from 7.0 to 2.0 in the absence of benzamil in the same animal (right). The mean normalized CT responses at pH 2.0 under 0CC (0.31 ± 0.02) were not significantly different from CT responses at pH 7.0 in the presence of benzamil (0.32 ± 0.03; P > 0.05; N = 4). Similarly, the mean VSI at pH 2.0 (0.16 ± 0.03) was not significantly different from its corresponding value (0.14 ± 0.04) at pH 7.0 in the presence of benzamil. Data summarized in Fig. 3 also show that in the continuous presence of benzamil, applying the stimulus at pH 2.0 (switching from R2 to S2) did not demonstrate any further suppression of the NaCl CT response. Furthermore, in the presence of benzamil, no significant changes were observed in the VSI at pH 2.0. In additional experiments, 100 μM amiloride inhibited the NaCl CT response at pH 7.0 and in the continuous presence of amiloride, applying the stimulus at pH 2.0,
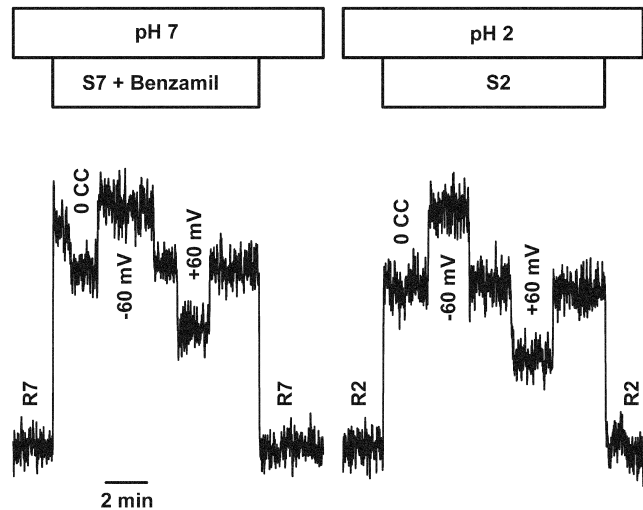

FIGURE 5. Comparative effects of benzamil and pH_o on NaCl CT responses. The lingual surface was perfused with rinse (R) solution and the stimulus (S) solution at pH 7.0 containing 5 μM benzamil (left) or with both rinse (R2) and stimulus (S2) solutions adjusted to pH 2.0 without benzamil (right) in the same animal. Integrated CT responses were recorded under 0CC, at -60 mV, and at 60 mV lingual voltage-clamp. The area under the CT response curve was taken as the magnitude of the CT response.

did not produce further suppression in the NaCl CT response (unpublished data). These results indicate that a decrease in pH inhibits most of the amiloride- and benzamil-sensitive component of the NaCl CT response. The data further indicate that the amiloride-insensitive component of the CT response is pH-insensitive.

In three animals, in the presence of benzamil, the mean normalized NaCl CT responses at pH 10.3 under 0CC, -60 mV, and 60 mV were 0.30 ± 0.04, 0.36 ± 0.07, and 0.27 ± 0.01, respectively (Fig. 3). These values are close to those obtained with benzamil at pH 2.0 or 7.0. The data indicate that in the presence of benzamil increasing the pH from 7.0 to 10.3 induced no enhancement in the NaCl CT response. In addition, no enhancement of the VSI was observed at pH 10.3 in the presence of benzamil. Together, these results suggest that the effects of pH_o on the NaCl CT responses are due to the modulation of ENaC activity by H^+ ions.

Effect of Membrane Permeable Weak Acids on NaCl CT Responses

To investigate if changes in NaCl CT responses are due to the changes in TRC pH_i, further experiments were done in which both the rinse and stimulus solutions were maintained at constant pH_o by buffering the solutions with weak organic acids, acetic acid (AA; Table I, sets 2 and 3) or CO_2 (Table I, set 4) and their corresponding potassium salts, potassium acetate (KA) or $KHCO_3$ (Lyall et al., 2001).
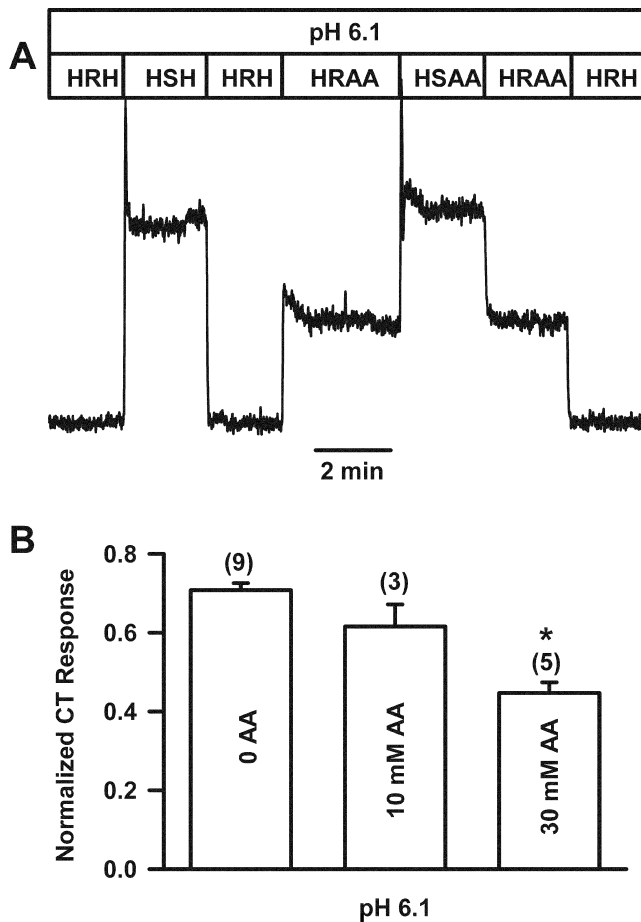
FIGURE 6. Effect of intracellular acid loading with acetic acid on NaCl CT responses. (A) CT responses were recorded while the lingual surface was perfused with rinse (R) solutions containing 525 mM KA + 200 mM mannitol and with stimulus (S) solutions containing 525 mM KA + 100 mM NaCl. Both rinse (HRH) and stimulus (HSH) solutions contained, in addition, 30 mM HEPES and the pH was adjusted to 6.1 with KOH. In the second part of the experiment, both the rinse (HRAA) and the stimulus (HSAA) solutions were adjusted to pH 6.1 by adding 30 mM AA. (B) Summarizes data from several animals. The area under the CT response curve was taken as the magnitude of the CT response. In each animal the NaCl CT responses were normalized to the CT response obtained with 300 mM NH$_4$Cl (CT$_{NaCl}$/CT$_{NH4Cl}$). The values are presented as M ± SEM of N. The value of N is given in parentheses. Asterisk indicates P < 0.005 (paired).

Studies with Acetic Acid

Data summarized in Fig. 6 A show that switching from the rinse solution HRH6.1 (pH 6.1; Table I, set 2) to the NaCl stimulus solution HSH6.1 (pH 6.1; Table I, set 2) increased the NaCl CT response. For this experiment the area under the NaCl CT response curve for a time interval of 1 min measured from the end of the 2-min stimulation period was taken as 100%. In the second step, switching from HRH6.1 to HRAA6.1 (pH 6.1; Table I, set 2), increased the CT response. This in-

crease in CT response is presumably due to the passive influx of membrane-permeable undissociated AA in the rinse solution across the apical membranes of TRCs (Lyall et al., 2001). Subsequently, stimulating with the NaCl stimulus solution HSAA6.1 (pH 6.1; Table I, set 2) increased the CT response further. This increase in CT response is due to the presence of NaCl in HSAA6.1. However, the magnitude of the NaCl CT response in the presence of AA (HSAA6.1-HRAA6.1) was ~40% less than the corresponding NaCl CT response in the presence of HEPES (HSH6.1-HRH6.1). Some experiments were also performed at a lower AA concentration. In this case both the rinse and stimulus solutions contained 175 mM KA and the solutions were buffered to pH 6.1 with either 10 mM HEPES (LRH6.1 and LSH6.1) or 10 mM AA (LRAA6.1 and LSAA6.1) (Table I, set 3). The data from several animals are summarized in Fig. 6 B. In the presence of 175 mM KA + 10 mM AA (pH 6.1) the mean normalized NaCl CT responses decreased from 0.71 ± 0.02 (N = 9) to 0.62 ± 0.06 (N = 3), an inhibition of 12.7 ± 4.0% of control. However, in the presence of 525 mM KA + 30 mM AA (pH 6.1) the NaCl CT response decreased to 0.45 ± 0.03, an inhibition of 36.3 ± 3.8% of control (P < 0.005; N = 5; paired). These results indicate that AA induces a dose-dependent inhibition of NaCl CT responses.

Studies with CO$_2$

Data summarized in Fig. 7 A show that switching from the rinse solution RH7.4 (pH 7.4; Table I, set 4) to the NaCl stimulus solution SH7.4 (pH 7.4; Table I, set 4) increased the NaCl CT response. For this experiment, the area under the NaCl CT response curve for a time interval of 1 min measured from the end of the 2-min stimulation period was taken as 100%. In the second step, switching from the rinse solution RH7.4 to the rinse solution RCO$_2$7.4 (pH 7.4; Table I, set 4) increased the CT response. This increase in CT response most probably involves the passive diffusion of dissolved CO$_2$ across the apical membranes of TRCs and the generation of intracellular H$_2$CO$_3$ in a reaction catalyzed by carbonic anhydrases (Lyall et al., 2001). Subsequently, stimulating with the NaCl stimulus solution SCO$_2$7.4 (pH 7.4; Table I, set 4) increased the CT response further. This increase in CT response is due to the presence of NaCl in SCO$_2$7.4. However, the magnitude of the NaCl CT response in the presence of CO$_2$ (SCO$_2$7.4-RCO$_2$7.4) was ~40% less than the corresponding NaCl CT response in the presence of HEPES (SH7.4-RH7.4). The coupling between pH$_i$ and the CT responses to NaCl is, therefore, seen to be independent of the acid used, pH$_o$, and osmolarity.

Studies with Carbonic Anhydrase Blockers

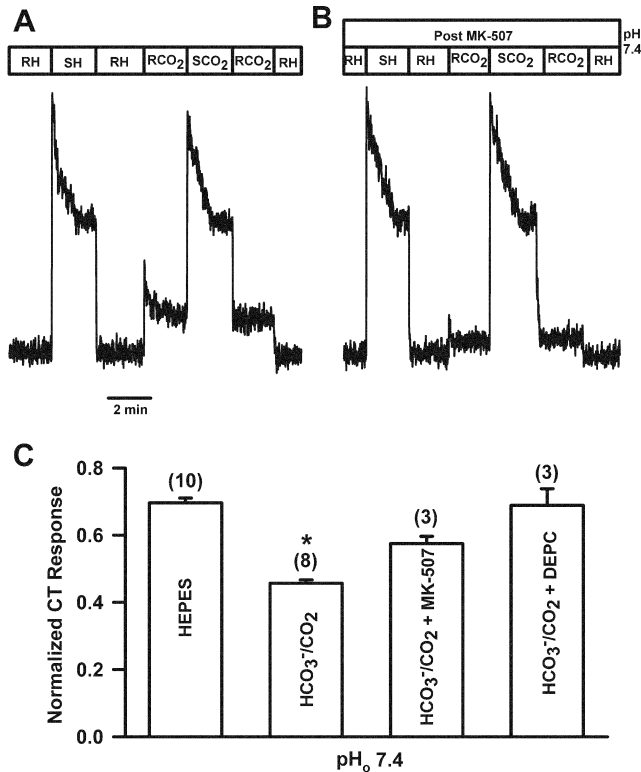We applied 50 mM MK-507 (dissolved in the rinse solution RH7.4; Table I, set 4), a specific blocker of car-

FIGURE 7. Effect of intracellular acid loading with CO_2 on NaCl CT responses. (A) CT responses were recorded while the lingual surface was perfused with rinse (R) solutions containing 72 mM KCl + 200 mM mannitol and with stimulus (S) solutions containing 72 mM KCl + 100 mM NaCl. Both rinse (RH) and stimulus (SH) solutions contained, in addition, 10 mM HEPES and the pH was adjusted to 7.4 with KOH. In the second part of the experiment, both the rinse (RCO_2) and the stimulus (SCO_2) solutions were buffered to pH 7.4 with 72 mM $KHCO_3$/10% CO_2. (B) The lingual surface was treated with 50 mM MK-507 for 15 min, and the CT responses were recorded using the same protocol as in A. (C) Summarizes data from several animals. The area under the CT response curve was taken as the magnitude of the CT response. In each animal the NaCl CT responses were normalized to the CT response obtained with 300 mM NH_4Cl (CT_{NaCl}/CT_{NH4Cl}). The values are presented as M ± SEM of N. The value of N is given in parentheses. Asterisk indicates $P < 0.01$ (unpaired).

bonic anhydrases (Fanous et al., 1999), topically to the lingual surface for 15 min. Consistent with our previous studies with another carbonic anhydrase blocker, MK-417 (Lyall et al., 2001), MK-507 inhibited the dissolved CO_2-induced CT response in $RCO_2$7.4 by ~50% (Fig. 7 B). Subsequently stimulating with $SCO_2$7.4 produced significantly smaller inhibition in NaCl CT responses relative to control (before MK-507 treatment). The data from several animals are summarized in Fig. 7 C. In NaCl solutions buffered with HEPES the mean normalized CT response was 0.66 ± 0.02 (N = 9). In NaCl solutions buffered in HCO_3^-/CO_2 the normalized CT response was 0.44 ± 0.02 (N = 8). This represents a 33% inhibition relative to CT response in HEPES

buffer ($P < 0.025$; paired). However, in the presence of MK-507 the normalized CT response was 0.57 ± 0.02 (N = 3). This represents a 13% inhibition relative to CT response in HEPES buffer ($P < 0.025$). Similar results were obtained with another carbonic anhydrase blocker, MK-417 (unpublished data). Thus, at constant pH_o, presenting the NaCl stimulus with a membrane-permeable weak organic acid (CO_2) inhibits NaCl CT responses. However, treating the tissue with carbonic anhydrase blockers, significantly attenuated the CO_2-induced decrease in NaCl CT response ($P < 0.01$; paired), suggesting a primary role for pH_i in regulating ENaC activity.

Studies with DEPC and Zn^{2+}

Histidine residues in the cytoplasmic NH_2 terminus of the α-rENaC have been suggested to be potential candidates for its pH-sensitivity (Chalfant et al., 1999). To investigate if changes in pH_i exert their effects by interacting directly with histidine residues in TRC ENaC, we tested the effect of DEPC and Zn^{2+}, modification reagents for histidine residues in proteins (Mankelow and Henderson, 2001; Seebungkert and Lynch, 2001), on NaCl CT responses. Fig. 8 A shows that, similar to the data in Fig. 7 A, stimulating the tongue with 100 mM NaCl solution buffered with HCO_3^-/CO_2 (pH 7.4) produced NaCl CT response ($SCO_2$7.4-$RCO_2$7.4) that was ~40% smaller than the corresponding CT response obtained with stimulating the tongue with NaCl solution buffered with HEPES (SH7.4-RH7.4). After this, we applied 20 mM DEPC (dissolved in the rinse solution RH7.4) topically to the lingual surface for 10 min. DEPC had no effect on the NaCl CT response obtained by stimulating the tongue with NaCl solution buffered with HEPES (SH7.4-RH7.4; Fig. 8 B). However, in the continuous presence of DEPC, stimulating the tongue with NaCl solutions buffered with HCO_3^-/CO_2 did not show any inhibition of NaCl CT response, i.e., the CO_2 response ($RCO_2$7.4-RH7.4) and the NaCl response ($SCO_2$7.4-$RCO_2$7.4) were additive (Fig. 8 B). The effects of DEPC were completely reversible. Perfusing the tongue with RH7.4 without DEPC for 10 min (Fig. 8 C) again produced NaCl CT response in HCO_3^-/CO_2 buffer ($SCO_2$7.4-$RCO_2$7.4) that was ~40% smaller than the corresponding response (SH7.4-RH7.4) in HEPES buffer. Similar results were obtained with 20 mM $ZnCl_2$ (unpublished data). The data from several animals are also summarized in Fig. 7 C. In three animals the magnitude of the CT responses obtained by stimulating the tongue with NaCl solutions buffered to pH 7.4 with HEPES were not different ($P > 0.05$) from corresponding CT responses obtained by stimulating with NaCl solutions buffered with HCO_3^-/CO_2 after DEPC treatment. These results suggest that agents that modify histidine residues in TRC ENaC sub-
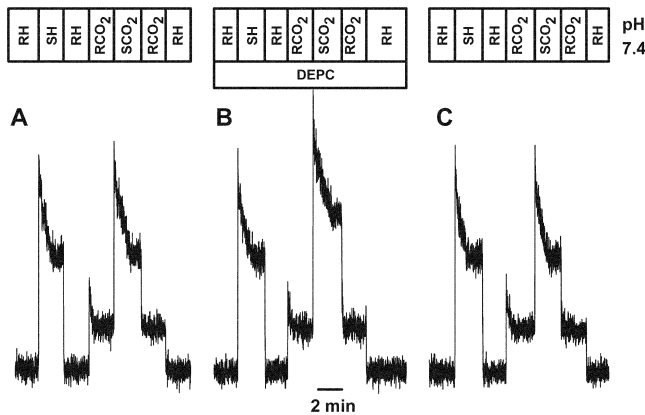
FIGURE 8. Effect of DEPC on CO_2-induced NaCl CT responses. (A) CT responses were recorded while the lingual surface was perfused with rinse (RH) solution containing 72 mM KCl + 200 mM mannitol and with stimulus (SH) solution containing 72 mM KCl + 100 mM NaCl. Both the rinse (RH7.4) and stimulus (SH7.4) solutions contained, in addition, 10 mM HEPES and the pH was adjusted to 7.4 with KOH. In the second part of the experiment, both the rinse (RCO$_2$7.4) and the stimulus (SCO$_2$7.4) solutions were buffered to pH 7.4 with 72 mM KHCO$_3$/10% CO$_2$. (B) The lingual surface was treated with 20 mM DEPC for 10 min and the CT responses were recorded using the same protocol as in A. (C) The lingual surface was perfused with rinse solution without DEPC (RH7.4) for 10 min and the CT responses were recorded using the same protocol as in A.

units prevent the CO_2-induced decrease in NaCl CT responses.

In the above experiment the tongue was preadapted to HCO_3^-/CO_2 buffer (pH 7.4) subsequent to stimulating the tongue with the NaCl solutions also buffered to pH 7.4 with HCO_3^-/CO_2 buffer. However, during a meal rapid changes in pH$_i$ may occur due to the presence of weak organic acids in food. Thus, under physiological conditions, pH$_i$ variations occur without any intermediate preadaptation steps. Therefore, we further examined if a decrease in the NaCl CT response also occurs if CO_2 and NaCl are coapplied at a fixed pH$_o$ (7.4) without preadapting the tongue to CO_2 first. These results are shown in Fig. 9. As shown above, replacing the rinse solution RH7.4 with the NaCl stimulus solution SH7.4 increased the CT response above baseline under 0CC (a and b). Subsequently, perfusing the tongue with the NaCl stimulus solution buffered to pH 7.4 with HCO_3^-/CO_2 (SCO$_2$7.4) decreased CT response relative to the CT response in SH7.4 (b and c). Upon reperfusing the tongue with SH7.4 the CT response increased to its original steady-state level (c and d) and perfusing with RH7.4 returned the CT activity to baseline (d and e). In the final step, replacing RH7.4 with RCO$_2$7.4 reversibly increased the CT activity (e–g). In four experiments, replacing SH7.4 with SCO$_2$7.4 decreased the magnitude of the NaCl CT response from 0.77 ± 0.03 to 0.63 ± 0.05 ($\Delta = -0.14 \pm 0.03$), a de-
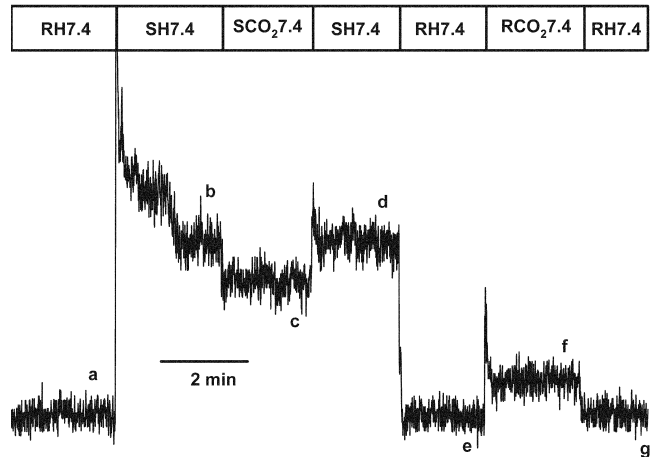

FIGURE 9. Effect of CO_2 on the NaCl CT responses without pre-adaptation. The integrated CT responses were recorded under open-circuit conditions (i.e., under zero current-clamp; 0CC). The tongue was initially perfused with the rinse solution RH7.4 and then with the NaCl stimulus solution SH7.4 (a-b). Both RH7.4 and SH7.4 were buffered to pH 7.4 with HEPES. In the second step, SH7.4 was replaced with the NaCl stimulus solution SCO$_2$7.4 (b-c). In the final step, RH7.4 was replaced with RCO$_2$7.4 (e and f). Both RCO$_2$7.4 and SCO$_2$7.4 were buffered to pH 7.4 with HCO_3^-/CO_2.

crease of $18.7 \pm 3.9\%$ relative to its response in SH7.4 ($P < 0.05$; N = 4). In the above experiments, replacing RH7.4 with RCO$_2$7.4 increased the CT activity by 0.17 ± 0.02. From these results it follows that if the effects of CO_2 and NaCl stimulation were independent then replacing SH7.4 with SCO$_2$7.4 should have increased CT activity by the same magnitude as observed by replacing RH7.4 with RCO$_2$7.4. However, replacing SH7.4 with SCO$_2$7.4 produced an overall decrease in NaCl CT activity. The data further indicate that the actual decrease in NaCl CT activity (SH7.4-SCO$_2$7.4) is greater than the increase in CT activity induced by decreasing pH$_i$ alone (RCO$_2$7.4-RH7.4).

In Vitro Studies

To correlate pH dependence of the NaCl CT responses with changes in pH$_i$, we further tested if the above conditions that induce alteration in NaCl CT responses also cause parallel changes in resting TRC pH$_i$.

Effect of Acid Stimulation on TRC pH$_i$

Studies with HCl. Fig. 10 A shows that in polarized TRCs perfused with control Ringer's solution (RC; pH 7.4; Table II, set 1), decreasing the apical solution pH to 2.0 (RHCl; Table II, set 1) reversibly decreased resting pH$_i$ from 7.46 ± 0.01 to 7.10 ± 0.02 ($\Delta pH_i = -0.35 \pm 0.03$; N = 5; P < 0.001, paired) and an increase in the apical solution pH from 7.4 to 10.3 (RTris; Table II, set 1) reversibly increased pH$_i$ (Fig. 10 B) from 7.34 ± 0.01 to

A

7.4 | 2.0 | 7.4 | Ap$_{pH}$

M±SEM (5)

2 min

TRC pH$_i$

B

7.4 | 10.3 | 7.4 | Ap$_{pH}$
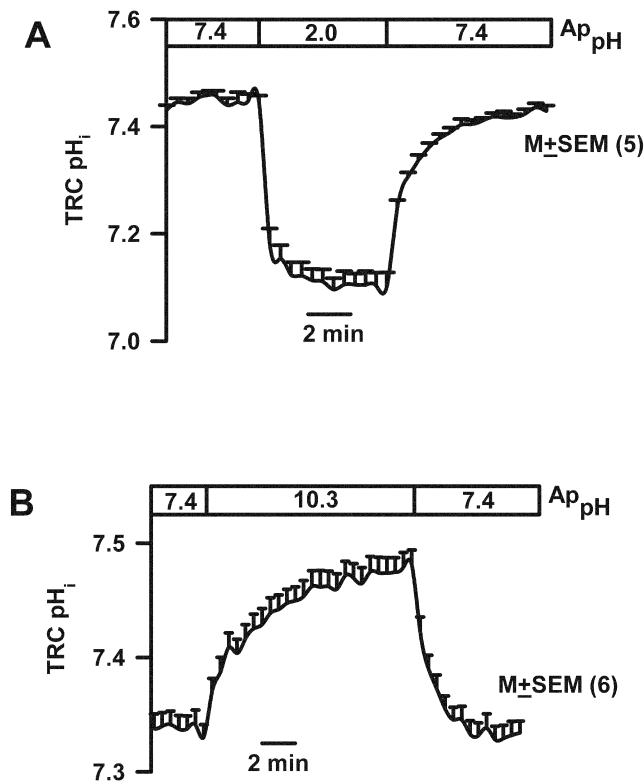
M±SEM (6)

2 min

TRC pH$_i$

FIGURE 10. Effect of pH$_o$ on TRC pH$_i$. The pH$_i$ was monitored while the polarized TRCs were initially perfused on both sides with control Ringer's solution (pH 7.4) and when the apical membrane was perfused with a similar solution at pH 2.0 (A) or with a similar solution at pH 10.3 (B). The pH$_i$ values are presented as mean ± SEM of six ROIs within the taste bud.

RH | RCO$_2$ | RH | RCO$_2$ | RH | pH 7.4
| | | 10 MK-417 | | (mM)

M±SEM (6)

2 min

TRC pH$_i$

FIGURE 11. Effect of CO$_2$ on TRC pH$_i$. The pH$_i$ was monitored while the polarized TRCs were initially perfused on both sides with control Ringer's solution (pH 7.4) and when the apical membrane was perfused with a similar solution buffered with 72 mM NaHCO$_3$/10% CO$_2$ at pH 7.4 (a and b). In the second part of the figure, the basolateral cell membrane was perfused with control Ringer's solution containing 10 mM MK-417 (c and d). In the continuous presence of basolateral MK-417 the apical solution was once again switched from a HEPES-buffered solution to a similar solution buffered with HCO$_3^-$/CO$_2$ at pH 7.4 (d and e). The pH$_i$ values are presented as mean ± SEM of six ROIs within the taste bud.

7.48 ± 0.01 (ΔpH$_i$ = 0.14 ± 0.02; N = 6; P < 0.001). Varying the pH$_o$ between 10.3 and 2.0 decreased TRC pH$_i$ from 7.35 ± 0.01 to 7.05 ± 0.01 (N = 6), indicating that during acid taste transduction, changes in TRC pH$_i$ remain within the physiological range. The mean changes in TRC pH$_i$ in several tissues induced by variations in pH$_o$ are also plotted in Fig. 4 (slope = 0.035; r^2 = 0.95). The data show that the NaCl CT responses are linearly related to changes in TRC pH$_i$.

The mean change in TRC pH$_i$ for a given change in pH$_o$ (ΔpH$_i$/ΔpH$_o$) was 0.065 for the data shown in Fig. 10 A and 0.049 for the data shown in Fig. 10 B. The slope of the relationship between pH$_o$ and pH$_i$ was 0.035 (Fig. 4). These results indicate that both apical cell membrane and the paracellular shunt pathway restrict H$^+$ entry such that a large change in apical pH is translated into a relatively small change in TRC pH$_i$ that remains within the physiological range (Lyall et al., 2001, 2002). In parallel experiments, a step change in the rinse solution pH from 7.0 to 10.3 did not induce changes in CT responses (Fig. 2, right); however, a step change in rinse solution pH from 7.0 to 2.0 induced an increase in CT nerve activity (Fig. 2, left).
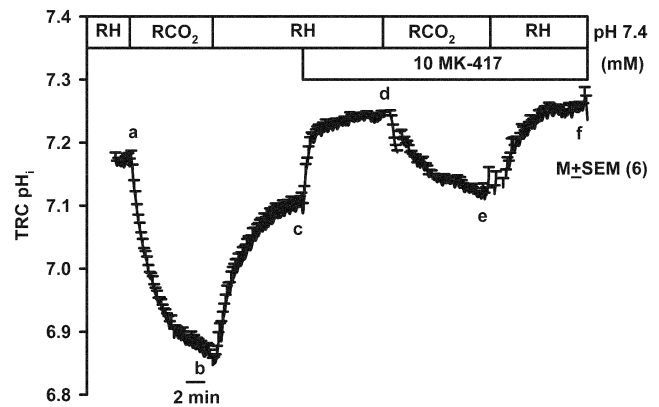
Studies with CO$_2$

Fig. 11 shows that in polarized TRCs initially perfused with Ringer's solution buffered to pH 7.4 with HEPES, switching to a similar solution buffered with NaHCO$_3$/CO$_2$ (RCO$_2$; pH 7.4; Table II, set 2) in the apical compartment induced a rapid and reversible decrease in TRC pH$_i$ (a and b) from 7.16 ± 0.01 to 6.85 ± 0.01 (ΔpH$_i$ = 0.31 ± 0.01; N = 6; P < 0.001; paired). In three polarized TRC preparations CO$_2$ decreased TRC pH$_i$ by 0.26 ± 0.03 pH unit (P < 0.025; paired). In the experiment shown in Fig. 11, perfusing the basolateral side with 10 mM MK-417, a membrane permeable blocker of carbonic anhydrases, increased TRC pH$_i$ (c and d) from 7.11 ± 0.01 to 7.24 ± 0.01 (ΔpH$_i$ = 0.13 ± 0.03; P < 0.025). However, in the continuous presence of MK-417, switching from HEPES-buffered solution (RC) to the HCO$_3$/CO$_2$-buffered solution (RCO$_2$) decreased pH$_i$ (d and e) from 7.24 ± 0.01 to 7.11 ± 0.01 (ΔpH$_i$ = 0.13 ± 0.01; N = 6; P < 0.001). Thus MK-417 inhibited the dissolved CO$_2$-induced decrease in TRC pH$_i$ by 61.6%. Since in our in vivo studies (compare Fig. 7 B) the drug was applied topically to the lingual surface, we also tested if the apical application of MK-417 also inhibits CO$_2$-induced decreases in TRC pH$_i$. In another tissue, switching from HEPES-buffered solution to the HCO$_3$/CO$_2$-buffered solution in the apical compartment decreased pH$_i$ from 7.17 ± 0.002 to 6.94 ± 0.002 (ΔpH$_i$ = 0.23 ± 0.002; N = 6; P < 0.001). After this, the apical membrane was perfused with

HEPES-buffered solution (pH 7.4) containing 40 mM MK-417 for 15 min. Upon switching to a similar solution buffered with HCO_3/CO_2 (pH 7.4; also containing MK-417) in the apical compartment decreased TRC pH_i from 7.07 ± 0.003 to 6.98 ± 0.001 ($\Delta pH_i = 0.09 \pm 0.003$; N = 6; P < 0.001). Thus, in the presence of apical 40 mM MK-417, the dissolved CO_2-induced decrease in TRC pH_i was inhibited by 60%. Similar results were obtained with MK-507 (unpublished data).

Studies with Acetic Acid

The polarized TRCs were initially perfused on the apical side with a solution containing 150 mM NaA and buffered to pH 6.3 with MOPS (RNaA1; Table II, set 4) while the basolateral side was perfused with a control Ringer's solution (pH 7.4; RCM). Replacing the apical solution with a similar solution containing 150 mM NaA buffered to pH 6.3 with AA (RNaA2; Table II, set 4) induced a small but significant decrease in TRC pH_i (Fig. 12, a and b). In the second step replacing 150 mM NaA + 600 mM mannitol (Man; RMOPS1; Table II, set 4) with a similar solution (RMOPS2; Table II, set 4) containing 450 mM NaA (both solutions were buffered to pH 6.3 with AA) induced an approximately twofold greater decrease in TRC pH_i (d and e). It is interesting to note that increasing the osmolarity of the 150 mM NaA solution with 600 mM mannitol induced a small increase in TRC pH_i (c and d). It is likely that this increase in pH_i is related to the cell shrinkage-induced activation of the basolateral Na^+-H^+ exchanger (unpublished data).

In parallel experiments (compare Fig. 6), giving the NaCl stimulus with AA inhibited NaCl CT responses (HSAA6.1-HRAA6.1) relative to control (HSH6.1-HRH6.1). Both the magnitude of AA-induced inhibition of the NaCl CT response and the magnitude of the AA-induced decrease in TRC pH_i were greater at higher AA concentrations. These data are consistent with the hypothesis that at constant pH_o the presence of weak acids in the apical solution induces a decrease in TRC pH_i, which then acts as a modifier of cell function. To correlate pH dependence of NaCl CT responses with changes in pH_i, we further tested if the above conditions that induce alteration in NaCl CT responses and pH_i also cause parallel changes in resting $[Na^+]_i$ in polarized TRCs.

SBFI Loading

Fig. 13 A shows the transmitted image of a fungiform papilla containing a single taste bud mounted in the microscopy chamber. The taste bud was viewed from the basolateral side with a 40× water immersion objective. Fig. 13, B and C, show the image of the same taste bud excited at 340 and 380 nm, respectively. The resulting ratio image (F_{340}/F_{380}) is shown in Fig. 13 D. Fig. 13
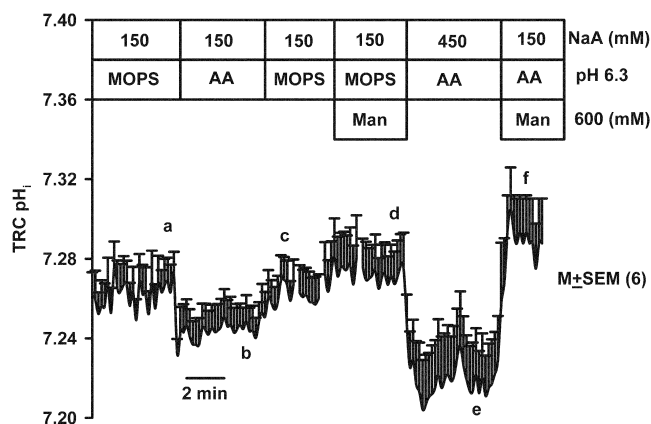

FIGURE 12. Effect of acetic acid (AA) on TRC pH_i. The pH_i was monitored while the polarized TRCs were initially perfused on the basolateral side with control Ringer's solution (pH 7.4) and the apical side was perfused with a solution containing 150 mM NaAc (150 NaAc replacing 150 mM NaCl) buffered to pH 6.3 with MOPS. In the first step, the apical solution was switched to a similar solution buffered to pH 6.3 with AA (a–c). In the second step, the apical solution containing 150 mM NaAc + 600 mM mannitol (Man) was replaced with an isosmotic solution containing 450 mM NaAc (both solutions were buffered to pH 6.3 with AA; d–f). The pH_i values are presented as mean ± SEM of five ROIs within the taste bud.

shows that SBFI is specifically taken up by the TRCs within the papilla and is excluded from the surrounding epithelial cells. However, squamous epithelial cells on the papillary periphery also absorb the dye. Measurements of F_{340}/F_{380} changes were made exclusively from the dye-loaded TRCs and reflect relative changes in TRC $[Na^+]_i$. Similar to SBFI, the single wavelength dye, sodium-green, was also specifically taken up by the TRCs within the papilla (unpublished data). We have demonstrated previously that the pH-sensitive fluoroprobe, BCECF, is also taken up specifically by the TRCs within the fungiform papilla (Lyall et al., 2001, 2002). Therefore, both changes in $[Na^+]_i$ and pH_i reflect changes within TRCs.

Effect of Amiloride and Benzamil on TRC $[Na^+]_i$

We monitored changes in resting $[Na^+]_i$ in polarized TRCs loaded with SBFI. Fig. 14 A shows that in TRCs perfused with Ringer's solution, decreasing apical Na^+ to zero (150 mM NaCl was replaced with an equivalent amount of Tris-HCl; R0Na$^+$; Table II, set 1) reversibly decreased FIR (F_{340}/F_{380}), indicating a reversible decrease in TRC $[Na^+]_i$. Similar results were obtained when NaCl was replaced with NMDG-Cl (compare Fig. 20). In TRCs loaded with sodium-green (Fig. 14 B), perfusing the apical membrane with control Ringer's solution (RC; Table II, set 1) containing amiloride decreased fluorescence intensity, F_{490}, indicating a decrease in resting $[Na^+]_i$ (a and b). In the continuous
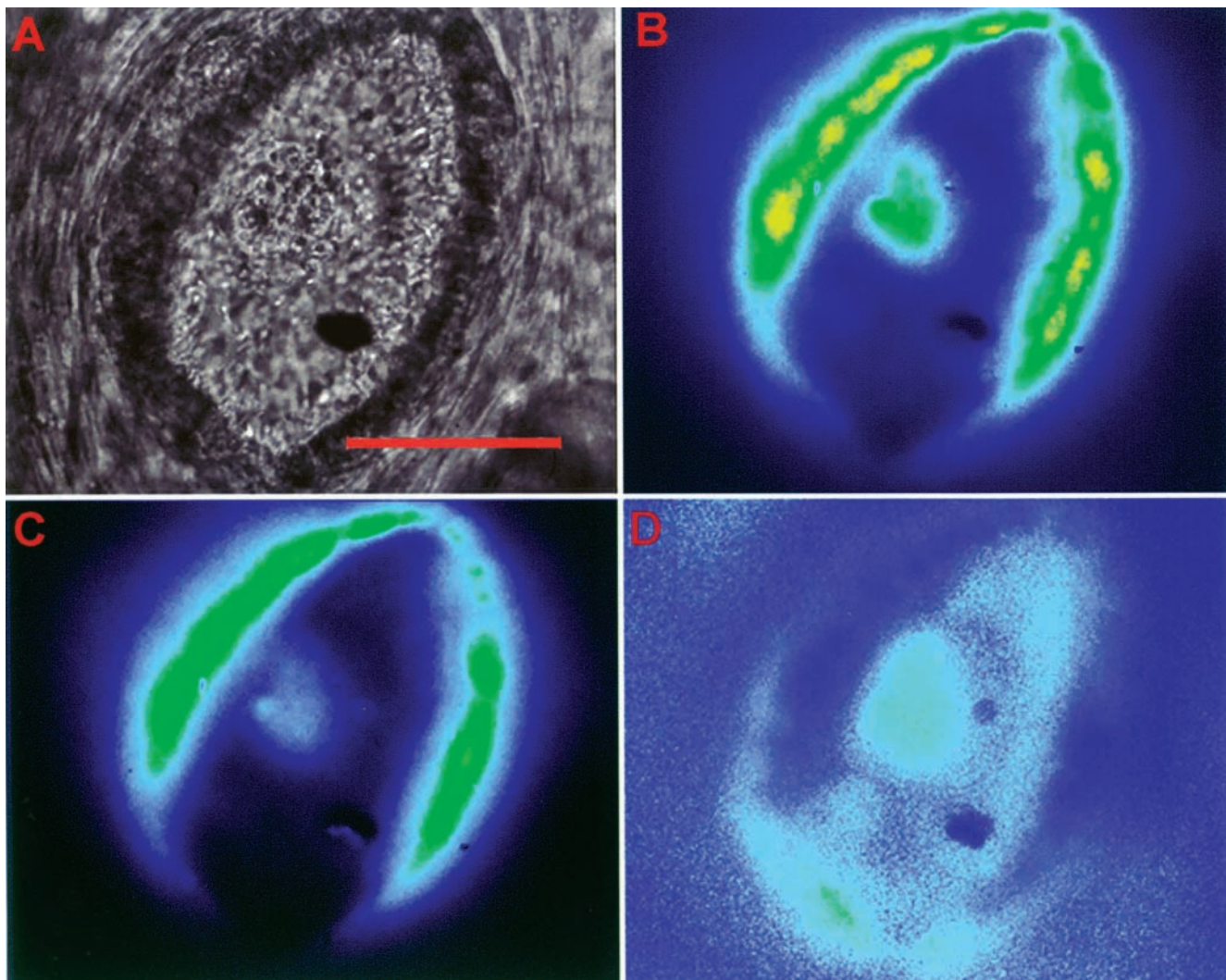
FIGURE 13. SBFI loading of TRCs. An isolated piece of rat anterior lingual epithelium containing a single fungiform papilla was mounted in a special microscopy chamber and was perfused with control solution containing SBFI-AM. The taste bud was viewed from the basolateral side with a 40× magnification. Bar, 10 μm. The figure shows the transmitted image of the taste bud (A), and the fluorescence image of the same taste bud excited at 340 nm (B) and excited at 380 nm (C). The fluorescence intensity ratio image, F_{340}/F_{380}, is shown in D.

presence of amiloride, decreasing the apical Na^+ concentration from 150 mM to zero (150 mM NaCl was replaced with an equivalent amount of NMDG-Cl; R0Na$^+$; Table II, set 1) further decreased F_{490} (Fig. 14 B, b and c). Returning to 150 mM apical Na^+ in the presence of amiloride restored $[Na^+]_i$ to its previous value (c and d), and removing amiloride restored $[Na^+]_i$ to its original value (d and e). Similar results were obtained with apical 5 μM benzamil, another potent blocker of TRC ENaC (Stewart et al., 1998). The amiloride-induced decrease in resting TRC $[Na^+]_i$ (a and b) reflects the inhibition of Na^+ entry via ENaC. A further decrease in TRC $[Na^+]_i$ by lowering the external Na^+ to zero in the presence of amiloride (b and c) reflects Na^+ efflux via an amiloride-insensitive pathway (Doolin and Gilbertson, 1996; DeSimone et al., 2001b).

Effect of Acid Stimulation on TRC $[Na^+]_i$

Fig. 15 shows that in TRCs loaded with sodium green and perfused with control Ringer's solution (RC), decreasing apical pH from 7.4 to 3.0 (RHCl; Table II, set 1) reversibly decreased F_{490}. Similarly, in TRCs loaded with SBFI, increasing the apical pH from 7.4 to 10.3 (RTris; Table II, set 1) increased and decreasing pH from 7.4 to 3.0 decreased FIR (F_{340}/F_{380}; unpublished data). These results indicate that extracellular alkalinization increases and extracellular acidification decreases resting TRC $[Na^+]_i$.

To further investigate if changes in pH$_i$ modulate resting TRC $[Na^+]_i$ we monitored temporal changes in $[Na^+]_i$ after intracellular acid loading with membrane-permeable weak organic acids at constant pH$_o$.
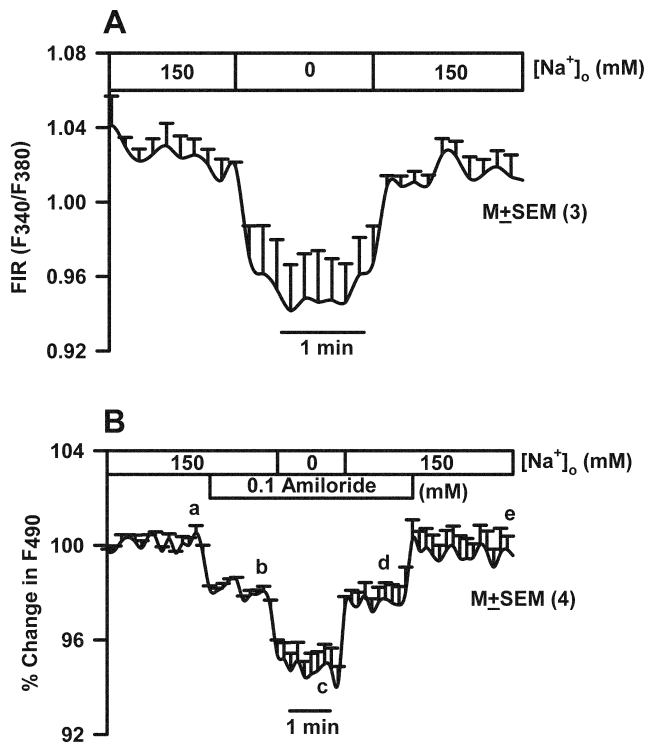
FIGURE 15. Effect of pH_o on TRC $[Na^+]_i$. The relative changes in $[Na^+]_i$ were monitored in polarized TRCs loaded with sodium green while they were initially perfused on both sides with control Ringer's solution (pH 7.4) and when the apical membrane was perfused with a similar solution at pH 3.0 (1 mM HCl). The relative changes in $[Na^+]_i$ are presented as mean ± SEM of the percentage change in fluorescence intensity (F_{490}) of four ROIs within the taste bud.

FIGURE 14. Effect of apical Na^+ and amiloride on TRC $[Na^+]_i$. (A) The relative changes in $[Na^+]_i$ were monitored in polarized TRCs loaded with SBFI while they were initially perfused on both sides with control Ringer's solution (pH 7.4) and when the apical membrane was perfused with a Na^+-free solution (150 mM NaCl was replaced with an equivalent amount of Tris-HCl). The relative changes in $[Na^+]_i$ are presented as mean ± SEM changes in FIR (F_{340}/F_{380}) of three ROIs within the taste bud. (B) The relative changes in $[Na^+]_i$ were monitored in polarized TRCs loaded with sodium green while they were initially perfused on both sides with control Ringer's solution (pH 7.4) and when the apical membrane was perfused with (i) control Ringer's solution containing 100 µM amiloride (a and b) and (ii) a Na^+-free solution (150 mM NaCl was replaced with an equivalent amount of NMDG-HCl) also containing 100 µM amiloride (b and c). The relative changes in $[Na^+]_i$ are presented as mean ± SEM of the percentage change in fluorescence intensity (F_{490}) of four ROIs within the taste bud.

Effect of Intracellular Acid Loading on TRC $[Na^+]_i$

Studies with basolateral NH_4Cl pulses. Fig. 16 shows the effect of a short basolateral side NH_4Cl pulse on TRC $[Na^+]_i$. In polarized TRCs loaded with SBFI (Fig. 16 A), immediately after the application of basolateral 50 mM NH_4Cl (RNH$_4$Cl; Table II, set 3) there was a rapid increase in FIR (F_{340}/F_{380}; a and b) followed by a slow decline toward baseline (b and c). Upon NH_4Cl washout, FIR decreased rapidly and became lower than its resting value (c and d). Qualitatively, similar changes in F_{490} were observed in TRCs loaded with sodium green (Fig. 16 B). Fig. 16 C shows the effect of a similar NH_4Cl pulse on TRC pH_i in a parallel experiment in another tissue. Consistent with our previous studies
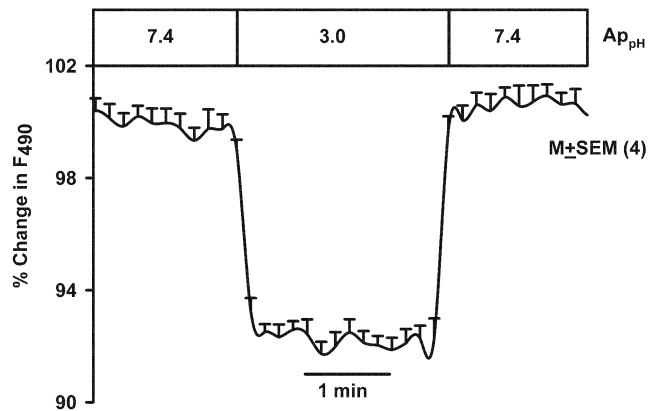
(Lyall et al., 2002), after the application of NH_4Cl TRC pH_i rapidly alkalinized (a and b), presumably due to the entry of NH_3 and conversion of free intracellular H^+ to NH_4^+ (Roos and Boron, 1981). This was followed by a slow decline of pH_i toward baseline (b and c), presumably reflecting NH_4^+ entry or pH compensation mechanism in TRC membranes (Roos and Boron, 1981). On NH_4Cl washout, TRC pH_i acidified and became lower than its resting value (c and d). This is due to the rapid exit of NH_3 from cells and the conversion of NH_4^+ to H^+. The spontaneous slow recovery of TRC pH_i upon NH_4Cl washout is due to the presence of a basolateral membrane Na^+-H^+ exchanger (Lyall et al., 2002). Together, the data presented in Fig. 16 indicate that at constant pH_o, the intracellular alkalinization phase of the NH_4Cl pulse is associated with an increase and the intracellular acidification phase of the NH_4Cl pulse is associated with a decrease in resting TRC $[Na^+]_i$.

Studies with Basolateral Na-acetate Pulses

Fig. 17 A shows the effect of a short basolateral side NaA pulse on TRC $[Na^+]_i$. In polarized TRCs loaded with sodium green, after the application of basolateral 50 mM NaA (RNaA; Table II, set 3) there was a rapid decrease in F_{490} (a and b). This was followed by a slow partial recovery toward baseline (b and c). Upon NaA washout, F_{490} increased and became higher than its resting value (c and d) and then slowly returned toward baseline (d and e). Fig. 17 B shows the effect of a similar NaA pulse on TRC pH_i in a parallel experiment in another tissue. Immediately after the application of NaA TRC pH_i rapidly acidified (a and b), presumably due to the entry of membrane-permeable undissociated AA and its conver-
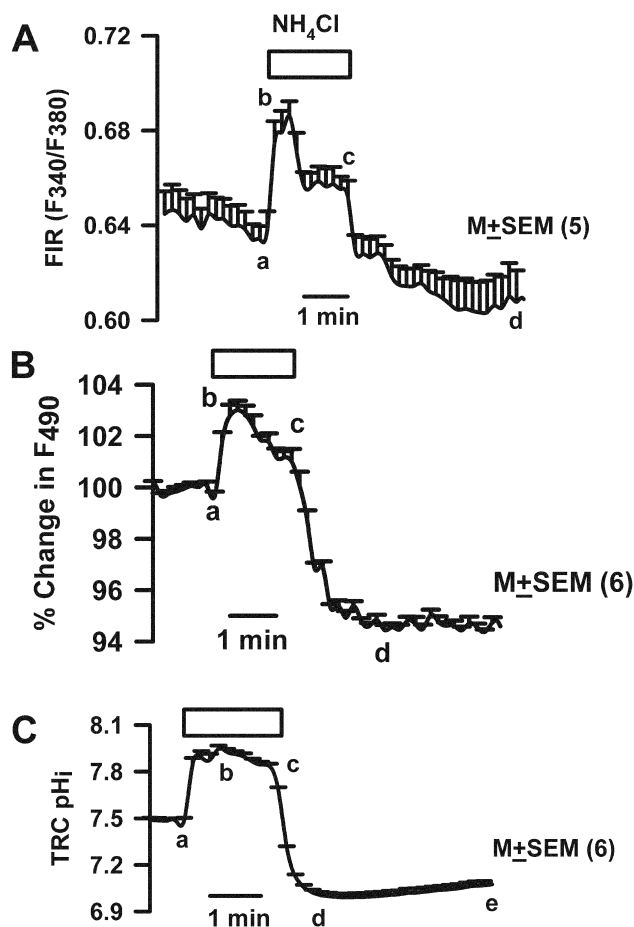
FIGURE 16. Effect of basolateral NH_4Cl pulse on TRC $[Na^+]_i$ and pH_i. The relative changes in $[Na^+]_i$ were monitored in polarized TRCs loaded with SBFI (A) or with sodium green (B). The TRCs were initially perfused on both sides with control Ringer's solution (pH 7.4). At the time period indicated by the top horizontal bar the basolateral membrane was perfused with a similar solution containing 50 mM NH_4Cl (NH_4Cl replacing 50 mM NaCl in the solution). The relative changes in $[Na^+]_i$ are presented as mean $\pm$ SEM change in FIR (F_{340}/F_{380}) of five ROIs or as a percentage change in F_{490}, respectively, of six ROIs within the taste bud. (C) The relative changes in pH_i were monitored in polarized TRCs loaded with BCECF. The TRCs were initially perfused on both sides with control Ringer's solution (pH 7.4). At the time period indicated by the top horizontal bar the basolateral membrane was perfused with a similar solution containing 50 mM NH_4Cl. The pH_i values are presented as mean $\pm$ SEM of six ROIs within the taste bud.

FIGURE 17. Effect of basolateral Na-acetate pulse on TRC $[Na^+]_i$ and pH_i. (A) The relative changes in $[Na^+]_i$ were monitored in polarized TRCs loaded with sodium green while they were initially perfused on both sides with control Ringer's solution (pH 7.4). At the time period indicated by the top horizontal bar the basolateral membrane was perfused with a similar solution containing 50 mM Na-acetate (Na-acetate replacing 50 mM NaCl in the solution). The relative changes in $[Na^+]_i$ are presented as percentage mean $\pm$ SEM change in F_{490} of four ROIs within the taste bud. (B) The relative changes in pH_i were monitored in polarized TRCs while they were initially perfused on both sides with control Ringer's solution (pH 7.4). At the time period indicated by the top horizontal bar the basolateral membrane was perfused with a similar solution containing 50 mM Na-acetate. The pH_i values are presented as mean $\pm$ SEM of seven ROIs within the taste bud.

sion to free intracellular H^+ and acetate anion (Roos and Boron, 1981). The intracellular acidification was transient and was followed by a spontaneous recovery of pH_i (b and c) presumably indicating pH_i recovery due to the presence of a basolateral Na^+-H^+ exchanger (Lyall et al., 2002). Upon NaA washout, TRC pH_i alkalinized and became higher than its resting value (c and d). This is due to the rapid exit of undissociated AA from cells and decrease in intracellular H^+. The spontaneous
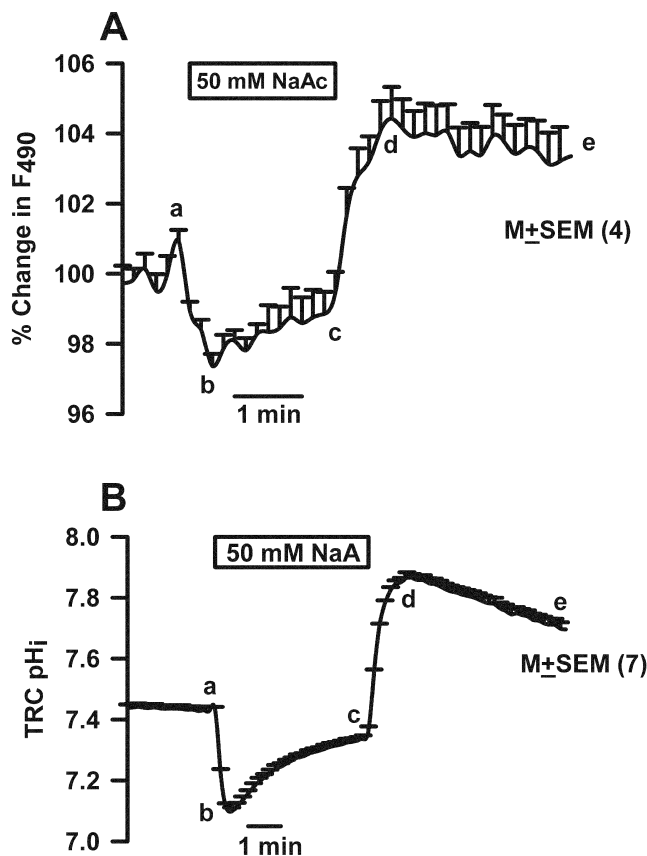
recovery of alkaline pH_i toward baseline (d and e) reflects the presence of an as yet unknown pH recovery mechanism(s) in TRC membranes that allows base (OH^-) exit or entry of acid equivalents at alkaline pH_i. Together, the data presented in Fig. 17 indicate that the intracellular acidification phase of the NaA pulse is associated with a decrease and the intracellular alkalinization phase of the NaA pulse is associated with an increase in resting TRC $[Na^+]_i$.

Studies with DEPC

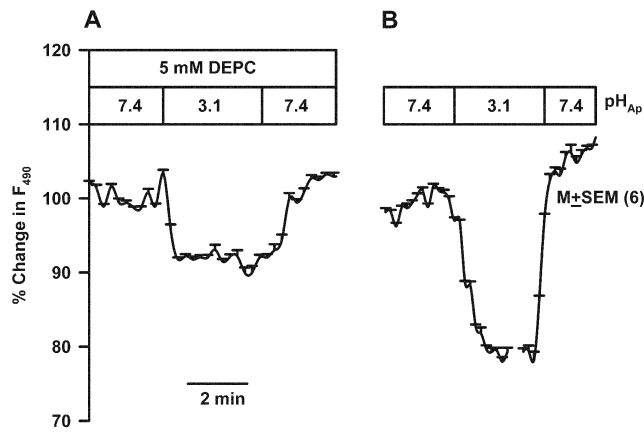Data presented in Fig. 18 show the effect of DEPC on pH_o-induced changes in TRC $[Na^+]_i$. Treating the ba-

FIGURE 18. Effect of DEPC on apical pH_o-induced changes in TRC $[Na^+]_i$. The relative changes in $[Na^+]_i$ were monitored in polarized TRCs loaded with sodium green. (A) The tissue was initially perfused on the basolateral side with control Ringer's solution containing 5 mM DEPC (pH 7.4) and on the apical side with control Ringer's solution without DEPC. A decrease in apical solution pH from 7.4 to 3.1 decreased F_{490}. (B) The tissue was perfused with control Ringer's solution without DEPC on both sides and the apical solution pH was decreased from 7.4 to 3.1. The relative changes in $[Na^+]_i$ are presented as percentage mean ± SEM changes in fluorescence at 490 nm (F_{490}) of six ROIs within the taste bud.

solateral membrane of polarized TRCs with 5 mM DEPC (Fig. 18 A) significantly inhibited the decrease in TRC $[Na^+]_i$ induced by lowering the apical solution pH from 7.4 to 3.1 relative to control (Fig. 18 B). Data presented in Fig. 19 show that treating the basolateral membrane with 5 mM DEPC significantly attenuated the NH_4Cl-induced changes in TRC $[Na^+]_i$ (Fig. 19 B, d–f) relative to control (Fig. 19 A, a–c). In three tissues treated with DEPC there was a 50% decrease in pH_o- or NH_4Cl-induced changes in TRC $[Na^+]_i$. Similar results were obtained in polarized TRCs loaded with SBFI (unpublished data).

Effect of pH and DEPC on Na⁺ Influx Across the Apical Membranes of TRCs

We further investigated if the Na^+ influx across the apical membranes of polarized TRCs was affected by pH and DEPC. The relative changes in $[Na^+]_i$ were monitored in TRCs loaded with sodium green during a unilateral step change in apical Na^+ concentration from 150 mM to 0 (Fig. 20 A). The zero Na^+ solution contained 150 mM NMDG-Cl ($R0Na^+$; Table II, set 1). In this experiment the basolateral membrane was continuously perfused with Ringer's solution containing 150 mM NaCl (Table II, set 1, RC). At pH 7.4 decreasing the apical Na^+ concentration from 150 mM to zero produced a reversible decrease in F_{490} (a–c). Decreasing the apical pH from 7.4 to 4.4 (i.e., perfusing RC at pH 4.4) decreased F_{490} and this magnitude (c and d) was smaller
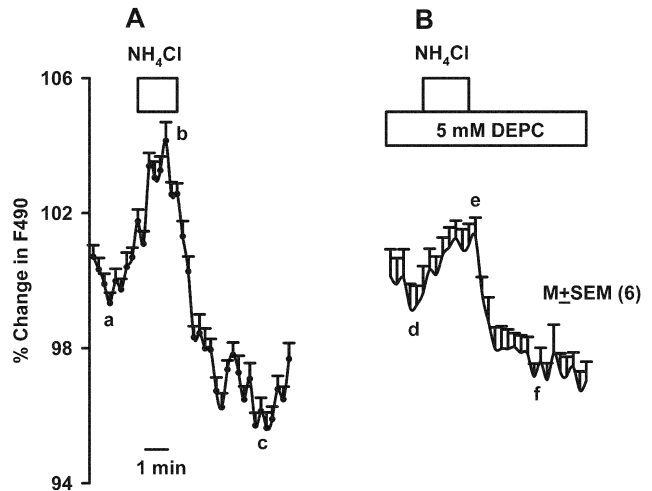

FIGURE 19. Effect of DEPC on basolateral NH_4Cl-induced changes in TRC $[Na^+]_i$. The relative changes in $[Na^+]_i$ were monitored in polarized TRCs loaded with sodium green. (A) TRCs were initially perfused on both sides with control Ringer's solution (pH 7.4). At the time period indicated by the top horizontal bar the basolateral membrane was exposed to a similar solution containing 50 mM NH_4Cl (NH_4Cl replacing 50 mM NaCl in the solution) and then was perfused again with control Ringer's solution. (B) TRCs were initially perfused on the apical side with control Ringer's solution and on the basolateral side with control Ringer's solution containing 5 mM DEPC. At the time period indicated by the top horizontal bar the basolateral membrane was exposed to a similar solution containing 50 mM NH_4Cl + 5 mM DEPC and then was perfused with control Ringer's solution containing 5 mM DEPC. The relative changes in $[Na^+]_i$ are presented as percentage mean ± SEM changes in fluorescence at 490 nm (F_{490}) of six ROIs within the taste bud.

than that induced by decreasing apical Na^+ concentration to zero at pH 7.4 (a and b). However, upon decreasing the apical Na^+ concentration from 150 mM to 0 at pH 4.4 (i.e., perfusing $R0Na^+$ at pH 4.4) induced a further decrease in F_{490} that was completely reversible (d–f). In three animals the magnitude of the decrease in F_{490} produced by lowering apical pH from 7.4 to 4.4 was 74.0 ± 3.4% relative to the decrease in F_{490} induced by reducing apical Na^+ concentration from 150 mM to 0 at pH 7.4. These data suggest that similar to the case with amiloride and benzamil (compare Fig. 14 B), a decrease in pH blocks only a part of the apical Na^+ entry that presumably occurs via the AS-ENaCs. However, a significant part of the apical Na^+ entry is pH insensitive. In the second part of the experiment (Fig. 20 B), the basolateral membrane was perfused with Ringer's solution containing 5 mM DEPC (pH 7.4) for 10 min and apical Na^+ influx was monitored again using solutions containing 0 and 150 mM NaCl adjusted to pH 4.4 (g–i). After DEPC treatment, the magnitude of change in F_{490} induced by apical Na^+ removal was 78% greater than the corresponding changes at pH 4.4 before DEPC treatment (d–f). In four experiments, after DEPC treatment the mag-
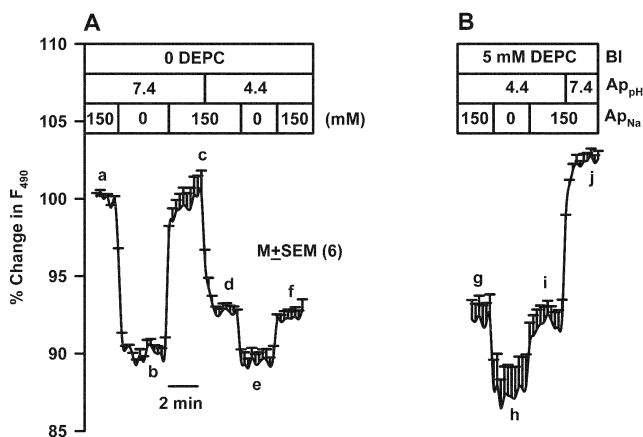
FIGURE 20. Effect of DEPC and pH on Na^+ influx across the apical membrane of TRCs. In polarized TRCs loaded with sodium green the Na^+ influx across the apical cell membranes was measured as relative changes in $[Na^+]_i$ during a unilateral step change in apical Na^+ concentration from 150 mM to zero. (A) The Na^+ influx was measured using 0 and 150 mM Na^+-solutions adjusted to pH 7.4 (a–c) and to pH 4.4 (d–f). In the second part of the experiment (B) the basolateral membrane was perfused with Ringer's solution containing 5 mM DEPC (pH 7.4) for 10 min and apical Na^+ influx was estimated again using 0 and 150 mM Na^+-solutions adjusted to pH 4.4 (g–i). The relative changes in $[Na^+]_i$ are presented as percentage mean ± SEM changes in fluorescence at 490 nm (F_{490}) of six ROIs within the taste bud.
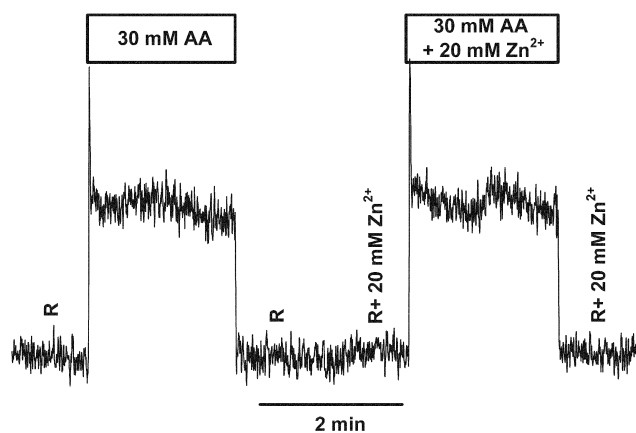
FIGURE 21. Effect of Zn^{2+} on CT response to AA stimulation. The tongue was initially rinsed with the rinse solution R (10 mM KCl) and then stimulated with 10 mM KCl + 30 mM AA. In the second part of the experiment, the tongue was rinsed with 10 mM KCl + 20 mM $ZnCl_2$ and stimulated with 10 mM KCl + 30 mM AA + 20 mM $ZnCl_2$.

nitude of the changes in F_{490} induced by apical Na^+ removal at pH 4.4 increased by 71.5 ± 5.3% (P < 0.01; paired) relative to before DEPC treatment. These results suggest that in the presence of DEPC the pH-induced inhibition of apical Na^+ influx is partially reversed.

Effect of Zn^{2+} and DEPC on weak organic acid entry across the apical membranes of TRCs. Data presented in Figs. 8 and 16–20 show that DEPC and Zn^{2+} uncouple pH_i-induced inhibition of Na^+ influx via AS-ENaCs. However, it is possible that DEPC and Zn^{2+} could also inhibit acid entry across the apical membranes of TRCs. A reduced apical acid entry will attenuate the acid-induced decrease in TRC pH_i and result in less inhibition of the Na^+ influx via the AS-ENaCs. We tested this possibility by monitoring the CT responses to 30 mM AA in the absence and presence of 20 mM $ZnCl_2$. As shown in Fig. 21, topical lingual application of 20 mM $ZnCl_2$ did not inhibit CT responses to AA. These data are consistent with the effect of topical application of 20 mM DEPC (compare Fig. 7 B), which also did not have an effect on CT responses to CO_2 stimulation. Together, the data suggest that DEPC and Zn^{2+} do not affect the apical entry of weak organic acid but relieve the acid-induced inhibition of apical Na^+ flux in TRCs.

D I S C U S S I O N

The data presented in this study demonstrate that the variations in rat NaCl CT responses in vivo under differ-

ent physiological conditions are related to the changes in Na^+ and H^+ intracellular activities measured in vitro in polarized fungiform TRCs. Our results show that weak organic acids (AA or CO_2) in the form of neutral molecules as well as strong acids (HCl) as free H^+ ions gain access into TRCs via the apical cell membrane. However, H^+ ions may also permeate the paracellular pathway and enter TRCs across the basolateral cell membrane, decreasing resting pH_i. The acid-induced decrease in TRC pH_i serves as a proximate stimulus for sour taste (DeSimone et al., 1995, 2001a; Lyall et al., 2001, 2002). However, when both sour and salt stimuli are presented together, a decrease in pH_i suppresses NaCl CT responses. In contrast, intracellular alkalinization, which by itself does not increase baseline CT nerve activity, enhances NaCl CT responses. The taste mixture interactions occur at the level of TRCs and involve a direct effect of intracellular H^+ ions on the apical ENaCs.

Relationship between TRC pH_i and NaCl CT Responses

Our studies demonstrate that the NaCl CT responses are linearly related to pH_o (Fig. 4). Presenting the NaCl stimuli at $pH_o < 7.0$ inhibited NaCl CT responses and presenting the NaCl stimulus at $pH_o > 7.0$ enhanced CT responses to NaCl. Since changes in TRC pH_i are also linearly related to pH_o, it follows that the changes in NaCl CT responses are also linearly related to pH_i. Two key observations indicate that changes in TRC pH_i modulate NaCl CT responses via its effect on the apical ENaCs. First, benzamil (a specific blocker of ENaC) inhibited NaCl CT responses at pH 7.0, and in the continuous presence of benzamil, no further changes in NaCl CT responses were observed upon decreasing pH to 2.0 or increasing pH to

10.3 (Fig. 3). Second, equivalent inhibition of the NaCl CT responses was observed with benzamil and by decreasing the stimulus solution to pH 2.0 in its absence in the same animal (Fig. 5). Reports of decreased rat responses to NaCl at pHs below 3–4 are consistent with the regulatory pattern of pH on ENaC activity (Beidler, 1954; Ogawa, 1969). However, in these and other studies the expected increase in CT response at higher pHs was not observed (Beidler, 1954; Ogawa, 1969; Elliott and Simon, 1990). This may be due to the narrow range of pH values investigated and the use of a saturating concentration level of NaCl (Elliott and Simon, 1990).

Inside the TRCs dissolved CO_2 is converted to H_2CO_3 in a reaction catalyzed by intracellular carbonic anhydrases and yields free H^+ and HCO_3^- ions (Lyall et al., 2001). This suggests that it is the CO_2-induced decrease in TRC pH_i that inhibits NaCl CT responses (Fig. 7 A) relative to CT responses in HEPES buffer (pH 7.4). This is supported by the observations that the carbonic anhydrase blockers, MK-417 or MK-507, attenuate the CO_2-induced decrease in TRC pH_i and the CO_2-induced decrease in NaCl CT response (Fig. 7, B and C).

Effect of pH on the Amiloride-insensitive NaCl CT Response

The data further suggest that the amiloride-insensitive part of the CT responses is pH insensitive. DeSimone et al. (2001b) have recently demonstrated that the amiloride-insensitive part of NaCl CT response is cetylpyridinium chloride (CPC) sensitive and probably represents a nonspecific cation channel that is permeable to Na^+, K^+, and NH_4^+ ions. In our preliminary studies, the CT responses to KCl and NH_4Cl stimulation were also insensitive to changes in pH_o. Both KCl and NH_4Cl CT responses are also amiloride insensitive (Kloub et al., 1997; DeSimone et al., 2001b). Single-unit recordings demonstrate that the amiloride-sensitive response is associated with N fibers that respond specifically to Na salts and can be ascribed to TRCs containing ENaC. In contrast, the amiloride-insensitive responses are associated with the H fiber type and respond to Na^+, K^+, NH_4^+, and other cations (Frank et al., 1983). The generalist characteristics of the H fibers may be ascribed to the amiloride-insensitive CPC-sensitive nonspecific apical cation channel (DeSimone et al., 2001b, 2002). The ENaC pathway represents a high-capacity, low-affinity system and the amiloride-insensitive CPC-sensitive pathway is a low-capacity high-affinity system similar respectively to the N- and H-fiber types (DeSimone et al., 2001b). Thus, at low pH, the H-fibers may still respond to cation stimulation.

Relationship between NaCl CT Responses and TRC [Na⁺]ᵢ

It is well established for several mammalian species that NaCl CT responses are composed of two parts, an amiloride- and benzamil-sensitive component and an amiloride- and benzamil-insensitive component (Fig. 3) (Lindemann, 1996, 2001; Stewart et al., 1997; Herness and Gilbertson, 1999; Lin et al., 1999; Kretz et al., 1999; DeSimone et al., 2001b, 2002). In our polarized preparations, the resting TRC $[Na^+]_i$ concentration was decreased when (a) Na^+ concentration was decreased in the apical compartment; (b) the apical membrane was treated with either amiloride or benzamil in the presence of Na^+; and (c) the magnitude of $[Na^+]_i$ decrease was significantly attenuated upon decreasing apical Na^+ concentration in the presence of amiloride. These results suggest that there are also two pathways for Na^+ influx across the apical membranes of TRCs, an amiloride-sensitive and amiloride-insensitive pathway. It is well established that the apical ENaCs are the transducers for the Na^+-specific taste in fungiform TRCs (Stewart et al., 1997; Lindemann, 1996, 2001; Herness and Gilbertson, 1999; Kretz et al., 1999; Lin et al., 1999; DeSimone et al., 2001b, 2002). Our data suggest that the amiloride- and benzamil-insensitive component is also present in the apical membranes of TRCs. In our previous studies (DeSimone et al., 2001b) amiloride + 2 mM CPC completely inhibited the NaCl CT responses to baseline and decreased resting TRC $[Na^+]_i$ below its resting level (DeSimone et al., 2002). Our studies indicate that stimulating the tongue with NaCl solutions induces an influx of Na^+ ions via the above two apical Na^+ entry pathways and results in an increase in TRC $[Na^+]_i$. Since amiloride or benzamil block $\sim$75% of the NaCl response, it indicates that the Na^+ flux via apical ENaCs contributes most to the CT response. However, at present the relationship between TRC $[Na^+]_i$ and the magnitude of NaCl CT responses has not been established.

Relationship between TRC pHᵢ and [Na⁺]ᵢ

Our results demonstrate that a decrease in TRC pH_i inhibits ENaC activity, decreasing apical Na^+ influx and thus resting TRC $[Na^+]_i$. In contrast, an increase in pH_i activates ENaC activity, increasing apical Na^+ influx and thus resting TRC $[Na^+]_i$ (Figs. 15–20). This relationship between TRC pH_i is observed not only with changes in pH_o but is also seen with intracellular acid loading at constant pH_o using basolateral NaA and NH_4Cl pulses. Although in our studies the changes in pH_i and $[Na^+]_i$ were not determined in the same TRCs simultaneously, the data shown in Figs. 16 and 17 show that $[Na^+]_i$ changes are temporally related to changes in pH_i. The changes in TRC $[Na^+]_i$ are sensitive to even small changes in pH_i since spontaneous recovery in TRC pH_i following basolateral NaA pulses were reflected in changes in F_{490} in cells loaded with sodium-green (Fig. 17 A). These results are consistent with the effects of apical or basolateral NH_4Cl pulses in cultured A6 cells (Zeiske et al., 1999) in which an intracellular

alkalinization increased and intracellular acidification decreased short-circuit current (I_{sc}, a measure of amiloride-sensitive apical Na^+ influx). The changes in pH_i and I_{sc} demonstrated very similar time course, indicating a probable causal relationship between pH_i and the magnitude of I_{sc}.

In other studies, intracellular alkalinization increased and acidification decreased TRC volume (unpublished data). During intracellular alkalinization, e.g., immediately after an NH_4Cl pulse (Fig. 16) or after basolateral NaA washout (Fig. 17), an increase in cell volume is expected to dilute the dye resulting in a decrease in F_{490}. Similarly, upon intracellular acidification, a decrease in cell volume would be expected to increase dye concentration and hence an increase in F_{490}. The data clearly show that F_{490} changes do not reflect changes in cell volume, but follow changes in TRC pH_i. The data further suggest that the actual changes in F_{490} must be significantly greater under these conditions but are attenuated due to changes in cell volume.

Our data demonstrate that changes in TRC pH_i are linearly related to NaCl CT responses (Fig. 4) and to $[Na^+]_i$ (Figs. 16 and 17). A linear relationship between pH_i and I_{sc} and apical membrane potential was also reported in the principal cells of frog skin (Lyall et al., 1993, 1995; Lyall and Biber, 1995), in A6 cells exposed to apical or basolateral NH_4Cl pulses (Zeiske et al., 1999), and in cultured M-1 mouse cortical collecting duct cells during changes in P_{CO2} (Howard et al., 2001). The cloned ENaC expressed in oocytes also demonstrated steep pH-dependence of amiloride-sensitive currents between pH_i of 7.0 and 6.4 (Konstas et al., 2000).

Temporal Relationship between NaCl CT Responses, TRC pH_i, and $[Na^+]_i$

As discussed previously (Lyall et al., 2001, 2002), it is important to note that in our studies CT nerve activity in anesthetized rats was monitored at normal physiological temperatures, and the stimuli were applied to the tongue at the rate of 1 ml/s at room temperature. In contrast, our pH_i and $[Na^+]_i$ measurements were made in a small microscopy chamber in which the maximum flow rate was limited to 1 ml/min, and the measurements were made at room temperature (see MATERIALS AND METHODS). However, we made some CT recordings with stimulus and rinse applied at 1 ml/min (Lyall et al., 2001). For acids, the phasic part of the CT response was strongly influenced by the flow rate, however, the magnitude of the pseudo-steady-state of the CT response was unaffected by the flow rate. At the flow rate of 1 ml/min the HCl- or the CO_2-induced CT response profiles, i.e., the rising phase of the response following stimulation and the rate of decline of the response after washout were similar to the TRC pH_i changes observed in vitro (Lyall et al., 2001).

In the in vivo studies the tongue was perfused with the rinse and stimulus solutions also at room temperature. Lundy and Contreras (1997) compared the relative CT responses to mineral salts at 25°C and 35°C 30 s after the stimulus on-set and reported that taste nerve responses varied with adaptation/stimulus temperature. However, only the response magnitudes to Na-acetate and the NH_4^+ salts were found to be statistically smaller at 25°C than at 35°C. Their studies further showed that the amiloride-suppression of CT nerve responses to NaCl was greater at 25°C than at 35°C by ~15%. These data indicate that only quantitative differences in the neural responses to mineral salts are observed when stimulus solutions are presented at room temperature. Therefore, under our experimental conditions the in vitro measurements conducted at room temperature can be directly related to the neural responses. In our recent studies (Lyall et al., 1999, 2001, 2002), the in vitro measurements of pH_i, cell volume, Na^+-H^+ exchange activity, effect of ion substitution, and the effect of second messengers (cAMP and Ca^{2+}) on polarized TRCs have demonstrated remarkably good correlation with the CT responses measured under similar experimental conditions.

These data suggest that under our experimental conditions changes in TRC pH_i are correlated with the pseudo-steady-state of the NaCT responses in vivo when stimuli are applied at low flow rate or with the time-average CT responses when stimuli are applied at high flow rate at room temperature. In our present studies, the changes in pH_i were also temporally related to changes in TRC $[Na^+]_i$ (Figs. 16 and 17). Therefore, it follows that under our experimental conditions the pH_i-induced changes in the relative concentration of TRC $[Na^+]_i$ can also be related to pH_i-induced alterations in NaCl CT responses observed in vivo.

Possible Mechanisms for H^+ Effects on ENaC

Protons modulate the activity of amiloride-sensitive ENaCs in a variety of epithelial cells (Lyall and Biber, 1995; Lyall et al., 1995; Garty and Palmer, 1997; Chalfant et al., 1999; Zeiske et al., 1999; Awayda et al., 2000; Konstas et al., 2000; Nakhoul et al., 2001). The cloned α, β, and γ subunits of ENaC expressed in *Xenopus* oocytes demonstrated decreased Na^+ currents when pH_i was decreased but not when pH_o was decreased (Chalfant et al., 1999). Acidification reduced and alkalinization increased channel activity by a voltage-independent mechanism. A reduction in pH_i reduced single-channel open probability, reducing single-channel open time, and increased single-channel closed time without altering single-channel conductance. By expressing various combinations of α, β, and γ subunits of ENaC it was further demonstrated that the α-ENaC subunit is regulated directly by pH_i (Chalfant et al., 1999). In *Xenopus*

laevis oocytes expressing either wild-type $\alpha\beta\gamma$-ENaC or Liddle $\alpha\beta$(R564stop)γ-ENaC cytosolic, but not extracellular acidification, substantially reduced the amiloride-sensitive inward currents (Konstas et al., 2000). The ENaC currents demonstrated a steep pH dependence between pH_i 7.2 and 6.4. The inhibition of wild-type ENaC and Liddle ENaC by cytosolic acidification was independent of so-called sodium-feedback inhibition, since it was not associated with a concomitant increase in intracellular Na^+ concentration. This is also the case in TRCs, since a decrease in pH_i is consistently associated with a decrease in $[Na^+]_i$ (Figs. 15–17).

The specific amino acid residues within the ENaC that interact with H^+ ions have not been identified as yet. However, COOH-terminal deletions in the α or γ subunit or in all three subunits of ENaC did not abolish the inhibitory effect of cytosolic acidification, indicating that ENaC's pH-sensitivity is not mediated by its cytoplasmic COOH terminus (Konstas et al., 2000). Histidine residues are potential pH-sensitive residues. Since COOH-terminal deletions preserve pH-sensitivity in Liddle's syndrome, histidine 568 in the β-subunit and histidine 648 in the α-subunit are most likely not involved in pH-sensing. The lack of sensitivity of ENaC to pH_o suggests that histidine residues at positions 6, 7, and 8 in the extracellular loops of the α, β, and γ-subunits, respectively, also do not contribute to the pH-sensitivity of ENaC. However, it is suggested that histidine residues in the cytoplasmic NH_2 terminus of α-rENaC may serve as potential candidates for its pH sensitivity (Chalfant et al., 1999). Our data (Figs. 7 and 18–20) suggest that possible modification of histidine residues with DEPC or Zn^{2+} makes the ENaC activity unresponsive to changes in TRC pH_i. It is likely that similar to the case of the ROMK channel other amino acids such as lysine and/or arginine residues may also be involved in pH sensitivity (Choe et al., 1997). However, in another study (Awayda et al., 2000) the cloned ENaC expressed in oocytes was not appreciably affected by changes in pH_o. The reason for the lack of pH sensitivity of ENaC in that study is not clear.

In 100 mM NaCl solutions buffered at pH 7.0 with HEPES, both DEPC and Zn^{2+} had no effect on NaCl CT responses (compare Fig. 7), which is consistent with the presumed action of these agents on the pH regulatory site of ENaC. However, in other studies (unpublished data) at the lower NaCl concentration of 30 mM, topical lingual application of Zn^{2+} enhanced NaCl CT responses relative to control. These effects of Zn^{2+} are consistent with its role in relaxing the sodium-feedback inhibition of ENaC (Lyall et al., 1995; Garty and Palmer, 1997; Gilbertson and Zhang, 1998; Konstas et al., 2000). The sulfhydryl reagent, p-hydroxymercuribenzoate, reversibly removed sodium feedback inhibition (Gilbertson and Zhang, 1998). Since Zn^{2+} has

a high affinity for sulfhydryl groups (Lyall et al., 1995), it is likely that Zn^{2+} also enhances NaCl responses at low NaCl concentrations by modification of cysteine residues in TRC ENaCs. In frog skin epithelial cells Cd^{2+} and Zn^{2+} increased pH_i and increased I_{sc} (Lyall et al., 1995). However, in our studies, apical application of $ZnCl_2$ did not produce significant changes in TRC pH_i (unpublished data).

In A6 cells changes in pH_i were positively correlated with Na^+ current through the native ENaC and the apically dominated transepithelial conductance (Zeiske et al., 1999). The pH_i affected the open Na^+ channel density by altering the open probability of the channel, and/or the total channel number. In contrast, Awayda et al. (2000) reported that in A6 cells, decreasing the apical pH_o from 7.4 to 6.4 or to 5.4 over a period of minutes caused a slow stimulation of the amiloride-sensitive I_{sc}. The effects on I_{sc} were blocked by buffering intracellular Ca^{2+} with BAPTA. The observed variations in the sensitivity of ENaC to pH suggest that the role of pH_i in regulating ENaCs may differ among tissues and may depend on additional Na^+ transport pathways, such as, Na^+-K^+-ATPase, Na^+-H^+ exchangers, NaCl cotransport, and Na^+-K^+-$2Cl^-$ cotransport. The overall changes in $[Na^+]_i$ may reflect a combined effect of pH on these pathways (Nakhoul et al., 2001). Similar to the case in other epithelial cells (Lyall et al., 1995; Garty and Palmer, 1997; Howard et al., 2001), in TRCs, the activation of ENaC was only observed at alkaline pH_o (Figs. 2 and 3).

It is likely that other factors may also come into play during mixture interactions. In a recent study by Liu and Simon (2001), stimulation of rat fungiform TRCs with acid revealed two distinct responses. Type I cells responded with an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$), and type II cells responded with a decrease in $[Ca^{2+}]_i$. In our preliminary studies an increase in $[Ca^{2+}]_i$ in ionomycin-treated fungiform TRCs inhibited rat NaCl CT responses (Alam et al., 2002). Therefore, it is likely that in a subset of TRCs, changes in other intracellular second messengers during acid stimulation can further modify CT responses to NaCl. The CT responses to Na^+ salts are also affected by the anion. The CT responses to Na^+ salts such as, gluconate and acetate, which are less permeable than Cl^- ions, gave lower CT responses relative to NaCl (Elliott and Simon, 1990; Ye et al., 1994; Kloub et al., 1997). These data suggest that transepithelial potentials generated by the less permeable anions across tight junctions also affect CT responses to Na^+ salts. However, at present it is not clear if changes in pH_o or pH_i also affect anion permeability across the paracellular shunt pathway.

The main conclusions of the paper and the proposed mechanisms for mixture interaction between sour taste and salt taste modalities are summarized in a schematic
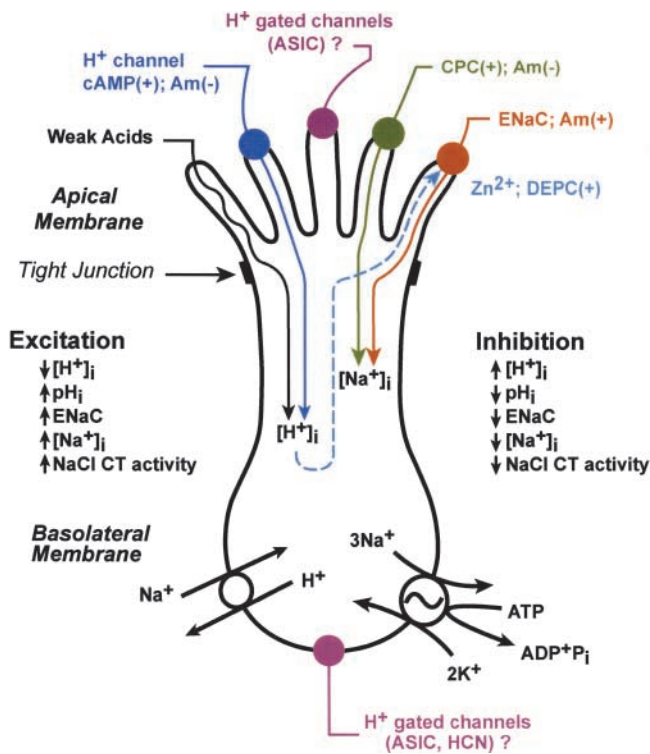
FIGURE 22. Proposed mechanism for taste mixture interactions between sour taste and salt taste modalities. The apical membrane of fungiform TRCs contains pathways for the entry of Na^+ and H^+ ions. The Na^+ influx occurs, in part, via the amiloride- and benzamil-sensitive ENaCs (Am(+); red circle) and also through an amiloride-insensitive (Am(−)), CPC-modulated (CPC(+)) cation channel that is permeable to Na^+, K^+, and NH_4^+ ions (green circle). The Na^+ exits from the TRCs across the basolateral membrane via the ouabain-sensitive Na^+-K^+-ATPase ($\sim$). Weak organic acids enter TRCs across the apical cell membrane as neutral molecules and strong acids gain access into TRCs via an H^+ entry pathway that is amiloride- and Ca^{2+}-insensitive, but is activated by cAMP (cAMP(+); Am(−); blue circle). Acid entry decreases TRC pH_i. TRC pH_i recovery occurs in part due to the activation of basolateral Na^+-H^+ exchanger. However, both ASIC and hyperpolarization-activated (HCN) channels in the basolateral membranes of TRCs could also be activated if H^+ ions can cross the tight junctions and decrease pH_i in the basolateral compartment. When both acid stimuli and NaCl stimuli are presented together, the acid-induced decrease in TRC pH_i inhibits apical Na^+ influx via amiloride- and benzamil-sensitive ENaCs (dotted line) and subsequently inhibits CT responses to NaCl. On the other hand, an increase in pH_i enhances apical Na^+ influx via ENaCs and enhances CT responses to NaCl. Since Zn^{2+} and DEPC inhibit pH_i-induced effects on NaCl CT responses, the data suggest that protons act directly on histidine residues in ENaC subunits and modify its activity.

(Fig. 22). Na^+ ions enter the fungiform TRCs across the apical cell membranes via two distinct pathways, one that is amiloride- and benzamil-sensitive, and the other that is insensitive to both drugs (Ye et al., 1993, 1994; Lindemann, 1996, 2001; Stewart et al., 1997; Herness and Gilbertson, 1999; Kretz et al., 1999; Lin et al., 1999; DeSimone et al., 2001b). The amiloride- and ben-

zamil-sensitive ENaCs are the principal receptors for the Na^+-specific taste. The amiloride- and benzamil-insensitive pathway is a CPC-sensitive cation channel that is permeable to Na^+, K^+, and NH_4^+ ions (DeSimone et al., 2001b, 2002). The Na^+ exit from TRCs occurs via the basolateral ouabain-sensitive Na^+-K^+-ATPase (DeSimone et al., 1981). The apical cell membranes of TRCs also contain acid entry mechanisms. The weak organic acids, such as AA and dissolved CO_2, enter across the apical membranes of TRCs as neutral molecules and decrease pH_i (Lyall et al., 2001). There is also an H^+ entry mechanism in the apical membranes of TRCs that is amiloride- and Ca^{2+}-insensitive, but is activated by cAMP (Lyall et al., 2002). The H^+ exit from TRCs occurs in part, via the basolateral 5-(N-methy-N-isobutyl)-amiloride (MIA)-sensitive Na^+-H^+ exchanger (Lyall et al., 2002). 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 (HCN) channels (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.

Acid-induced decrease in TRC pH_i inhibits apical Na^+ influx through amiloride- and benzamil-sensitive ENaCs. This inhibition is not observed in the presence of Zn^{2+} or DEPC. It has been suggested that intracellular H^+ ions can directly modulate Na^+ currents through the α-subunit of the expressed ENaC (Chalfant et al., 1999) and involves the interaction of protons with histidine residues. In our studies the amiloride-insensitive component of the CT response was not affected by changes in pH_o or pH_i.

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