Proton Currents through Amiloride-sensitive Na Channels in Hamster Taste Cells

Role in Acid Transduction

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ABSTRACT The activity of taste cells maintained in the intact hamster tongue was monitored in response to acid stimulation by recording action currents from taste receptor cells with an extracellular "macro" patch pipette: a glass pipette was pressed over the taste pore of fungiform papillae and perfused with citric acid, hydrochloric acid, or NaCl. Because this technique restricted stimulus application to the small surface area of the apical membranes of the taste cells, many nonspecific, and potentially detrimental, effects of acid stimulation could be avoided. Acid stimulation reliably elicited fast transient currents (action currents of average amplitude, 9 pA) which were consistently smaller than those elicited by NaCl (29 pA). The frequency of action currents elicited by acid stimuli increased in a dose-dependent manner with decreasing pH from a threshold of about pH 5.0. Acid-elicited responses were independent of K^+, Na^+, Cl^-, or Ca^{2+} at physiological (salivary) concentrations, and were unaffected by anthracene-9-carboxylic acid, tetraethylammonium bromide, diisothiocyanate-stilbene-2,2′-disulfonic acid, vanadate, or Cd^{2+}. In contrast, amiloride (≤ 30 μM) fully and reversibly suppressed acid-evoked action currents. At submaximal amiloride concentrations, the frequency and amplitude of the action currents were reduced, indicating a reduction of the taste cell apical conductance concomitant with a decrease in cell excitation. Exposure to low pH elicited, in addition to transient currents, an amiloride-sensitive sustained d.c. current. This current is apparently carried by protons instead of Na^+ through amiloride-sensitive channels. When citric acid was applied while the taste bud was stimulated by NaCl, the action currents became smaller and the response resembled that produced by acid alone. Because of the strong interdependence of

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the acid and salt (NaCl) responses when both stimuli are applied simultaneously, and because of the similarity in the concentration dependence of amiloride block, we conclude that amiloride-sensitive Na^+ channels on hamster taste receptor cells are permeable to protons and may play a role in acid (sour) taste.

INTRODUCTION

Recent evidence indicates the acid taste may be transduced by a number of different mechanisms. The source of these differences is not presently clear. In salamanders, protons directly block apically localized K^+ channels by decreasing their open probability (Kinnamon, Dionne, and Beam, 1988; Kinnamon and Roper, 1988; Teeter, Sugimoto, and Brand, 1989; Cummings and Kinnamon, 1992). In frog, Miyamoto, Okada, and Sato (1988) concluded that protons gate a Ca^{2+} conductance that is responsible for taste cell depolarization. Depolarizations linked to an increase of the membrane conductance of taste cells have also been reported (rat: Ozeki, 1971). In dog, paracellular current flow in lingual epithelium has been shown to change ion selectivity from cationic to anionic in response to low pH, causing excitatory current loops (Simon and Garvin, 1985). Finally, in humans, a change in taste cell intracellular pH due to permeation of the undissociated form of weak acids has been suggested to be involved in acid detection (Ganzevles and Kroeze, 1987). Thus, a variety of mechanisms, reflecting a wide range of possible pH effects, may play a role in acid transduction.

One of the major approaches used to investigate taste transduction has been to record activity in primary gustatory afferents in response to taste stimuli applied to the tongue. Gustatory afferents receive input directly from taste receptor cells. However, a number of studies have suggested that the taste bud may not be merely a passive transducer of the taste signal. In vertebrates, both electrical synapses between taste receptor cells (West and Bernard, 1978; Teeter, 1985; Yang and Roper, 1987; Sata and Sato, 1989) and chemical synapses between receptor cells and basal cells have been demonstrated (Kinnamon, Taylor, Delay, and Roper, 1985; Delay, Kinnamon, and Roper, 1986; Kinnamon, 1987; Delay and Roper, 1988). A recent report by Ewald and Roper (1992) has demonstrated functional synaptic connections between vertebrate taste receptor cells and basal cells. The presence of such synaptic machinery within the taste bud suggests that there may be significant processing of taste signals before they are transmitted to the primary gustatory afferents. Because comparatively little is known about how the taste receptor cells themselves directly respond to chemical stimuli, we have performed experiments where taste receptor cell activity may be monitored noninvasively in situ during chemostimulation.

Taste receptor cells have been shown to generate action potentials in response to taste stimuli (Avenet and Lindemann, 1987; Kinnamon and Roper, 1987; Béhé, DeSimone, Avenet, and Lindemann, 1990), while surrounding lingual epithelial cells do not possess this property (Kinnamon and Roper, 1988). Avenet and Lindemann (1991) recently described a recording technique whereby action potentials in taste cells can be recorded noninvasively in the intact tongue in response to focal application of taste stimuli. Taste receptor cell activity is manifest as fast current transients (action currents) driven across the apical membrane of the taste receptor cells by potential changes that occur during action potentials. In this report, we used this technique to investigate the response of hamster fungiform taste cells to acid

stimulation. We present evidence here that protons can directly permeate amiloride-sensitive Na^+ channels, causing taste receptor cell depolarization and subsequent generation of action potentials in response to acid stimulation.

Part of these results have appeared in abstract form (Gilbertson, Avenet, Kinnamon, and Roper, 1991).

MATERIALS AND METHODS

The method and recording devices have been described in detail previously (Avenet and Lindemann, 1991). Briefly, 4–10-wk-old hamsters were killed with CO_2 and the tongue was

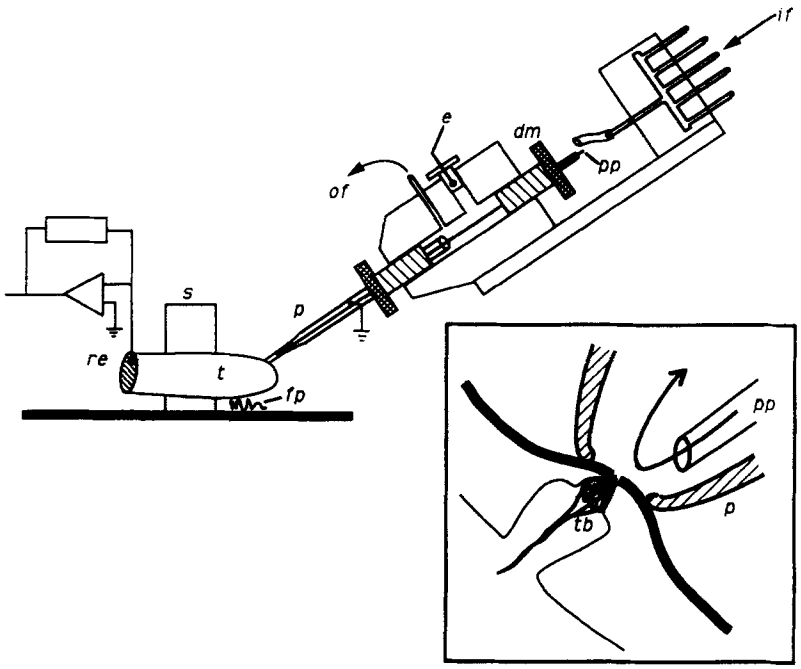

FIGURE 1. Recording apparatus. After removal from the hamster, the tongue (*t*) was placed in a Sylgard chamber (*s*) that contained a 5-mm-diam channel and mounted onto a glass slide. In the posterior chamber, the recording electrode (*re*) was placed in the cut end of the tongue. In the anterior chamber, the tongue rested on filter paper (*fp*) to facilitate blotting of the tongue. Individual fungiform papillae were easily distinguished in the anterior chamber and the macro patch pipette (*p*), with gentle suction, formed a loose "seal" over the papillae containing taste bud(s) (*tb, inset*). The interior of the pipette was grounded by means of a reference electrode (*e*). Up to eight different solutions could be perfused via inflow tubes (*if*) through a capillary perfusion pipette (*pp*) positioned within 50 μm of the patch pipette opening by means of a distance micrometer (*dm*). Solutions were carried out of the pipette through a single outflow tube (*of*) connected to a suction pump. Adapted from Avenet and Lindemann (1991).

removed and rinsed with distilled water. The tongue was immediately mounted in a bipartitioned Sylgard recording chamber containing a channel 5 mm in diameter and 8 mm in length connecting two open compartments (Fig. 1). The tongue was positioned such that its anterior half protruded in one compartment and could be viewed with a dissecting microscope at ×5–50 with glass fiber illumination. Fungiform papillae were easily distinguished at the tongue surface

and a pipette with a 100-μm fire-polished tip (pulled on a model PP-83 patch pipette puller; Narishige USA, Inc., Greenvale, NY) was positioned over the taste pore by means of a Huxley-type 3-D micromanipulator. In the anterior compartment, the tongue rested on a cushion of filter paper for continuous blotting. An agar bridge connected to a Ag/AgCl pellet was placed into an incision in the posterior half of the tongue in the posterior compartment.

The pipette assembly (Avenet and Lindemann, 1991) allowed perfusion of taste stimuli in the pipette through an internal quartz capillary (Fig. 1, *inset*). The quartz capillary (244 μm o.d., 100 μm i.d.; Polymicron Technologies, Inc., Phoenix, AZ) was pulled in a flame to a tip diameter of 30–50 μm. The inner capillary tip was adjusted to within 50 μm of the pipette opening with a distance micrometer. Using a completely closed (air-tight) perfusion system, up to eight different solutions could be applied in a single experiment and were fed from pressurized reservoirs (100 kPa). Bacterial filters (0.22 μm; Millipore Corp., Bedford, MA) were inserted into the perfusion lines that connected the stimulus reservoirs to the pipette assembly.

Electrical Recording

The Ag/AgCl pellet connected to the perfusate in the pipette assembly was grounded, whereas the Ag/AgCl electrode inserted into the tongue tissue (posterior chamber) was connected to the input stage of a patch-clamp amplifier with a 100-MΩ feedback resistor. Recording instability and changes in the d.c. current offsets during perfusion were minimized by this arrangement of active and ground electrodes. Negative pressure applied to the pipette by mouth through connecting tubing resulted in a 50-MΩ "seal" over the taste papillae after fluid at the tongue surface was removed by gently blotting the tongue with filter paper. For recording, the amplifier was placed in voltage-clamp mode and the pipette potential (V_{hold}) was set at 0 mV. The low seal resistance (50 MΩ) permitted much of the transcellular current to be shunted through the pipette–papillae junction. Consequently, the pipette potential (0 mV) had minimal effects on the apical membrane potential of the taste receptor cells. Perfusion of the recording/stimulus pipette was initiated and maintained by negative pressure supplied by a vacuum pump. The perfusate was collected in a reservoir in series with the suction. The pH of the perfusate was routinely monitored to ensure the stability of each of the stimulating solutions. The rate of accumulation of a solution in the reservoir monitored the perfusion. Perfusion rates were typically 0.2–0.5 ml/min.

Using this technique, we were limited to recording from only those cells that generated action potentials during chemostimulation (cf. Avenet and Lindemann, 1991). Taste cells that produced subthreshold responses to chemostimulation would not be detected with this technique. Furthermore, because of the low seal values between pipette and papillae, d.c. (i.e., steady-state) currents across the apical membrane could not be quantitatively assessed during chemostimulation due to large junction potentials generated when the ionic strength or ionic composition of the perfusing solution was changed. However, it was possible to measure changes in d.c. current when changes in the ionic composition of the perfusing solution were limited to very low (micromolar) variations, such as the addition of potent drugs (e.g., Fig. 5 *C*).

Data were stored digitally on a VCR recorder and analyzed off-line. When action currents were recorded, the patch-clamp amplifier was kept in the tracking mode to reduce any d.c. current changes linked to sustained currents elicited during chemostimulation. The relatively constant baseline resulting from this procedure made it possible to analyze the frequency of action currents. To study sustained currents elicited by chemostimulation (e.g., Fig. 5 *C*), tracking was switched off.

Data Processing

To evaluate the frequency of action currents from taste cells, the analog signal from the VCR recorder output was filtered at 300 Hz and redigitized at 1 kHz by a MacAdios II analog-digital

converter (GW Instruments, Somerville, MA) and stored on a Macintosh IIfx computer in samples of 70 s. Superscope software (version 1.5; GW Instruments) allowed the display and analysis of responses. To quantify action potential frequency, action currents were detected by setting a threshold value above the background noise and were converted into events of equal amplitude and summated. The summated responses were displayed as a function of time and instantaneous frequencies were graphically computed as the slope of the summated curve. Values are given ±SD except where noted.

Solutions and Reagents

Artificial hamster saliva (Hettinger, T., personal communication) contained (mM): 6.6 NaCl, 43 $KHCO_3$, 1.5 $CaCl_2$, and 1.3 $MgCl_2$, adjusted to pH 7.4 with HCl.[1] The simplified adapting solution contained (mM): 30 *N*-methyl-D-glucamine chloride (NMDG-Cl) and 5 HEPES, adjusted to pH 7.0 with HCl. For NaCl stimulation, NaCl (50–200 mM) was added to the artificial saliva or to the buffered adapting solution. For acid stimulation, citric acid or HCl (1–3 mM) was added to the artificial saliva or to HEPES-free adapting solution. A titration curve was used to determine the concentration of citric acid or HCl to obtain a given pH. Because citric acid acts as a buffer (pK_a = 3.06, near the pH levels used in these studies), the majority of experiments were done using citric acid as the chemostimulus. The effects of the following chemicals on chemosensory responses were also recorded: 0.1–100 μM amiloride, 8 mM tetraethylammonium bromide (TEA), 100–500 μM diisothiocyanate-stilbene-2,2′-disulfonic acid (DIDS), 100 μM anthracene-9-carboxylic acid (9-AC), and 100 μM Na-ortho-vanadate. All compounds were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Action Currents in Taste Cells

Responses of taste receptor cells to chemical stimuli in these experiments were manifest as transient currents driven across the apical membrane by the potential changes of the cell(s) during action potentials. We have termed these fast transient currents "action currents" to indicate this point. Three pieces of evidence indicate that action currents are generated in the taste receptor cells themselves and not from other sources (cf. Avenet and Lindemann, 1991). First, taste receptor cells are the only cells within the lingual epithelium capable of generating action potentials (Kinnamon and Roper, 1988). Second, action currents closely resemble in both waveform and duration action potentials recorded in isolated taste receptor cells (Béhé et al., 1990). Third, action currents are typically monophasic and of variable amplitude (e.g., Fig. 5*A*), and consequently can be readily distinguished from the uniform, biphasic responses generated by gustatory afferent fibers (cf. Avenet and Lindemann, 1991). No evidence of afferent nerve fiber responses were seen in our experiments, consistent with the fact that the axons of gustatory afferents were not functional in excised tongues or that these signals were below the noise level of the system.

In this study, two parameters of action currents are presented, their amplitude and frequency in response to chemostimulation. The amplitude of the action currents is

[1] Because we have used a closed (air-tight) perfusion system, the loss of bicarbonate as CO_2 in this solution was minimal and, as such, the pH of the artificial saliva solution both with and without acid stimuli did not vary over the time course of these experiments.

directly proportional to the passive ohmic resistance across the apical membrane (Avenet and Lindemann, 1991). If the resistance is decreased due to opening of ion channels and/or larger currents through open channels, then the amplitude of action currents is likewise increased. That is, the greater the apical conductance, the larger the apical currents that can be driven by the action potentials and, hence, the larger the action current amplitude recorded by the extracellular pipette. The presence of multiple amplitudes of action currents within a single recording (e.g., Figs. 4 *B* and 5 *A*) suggests that more than a single cell may be responding. The frequency of action currents, however, is directly related to the intensity of taste cell chemostimulation. The frequency of action currents increases with increasing concentration of the taste stimulus for acid responses (e.g., Fig. 3 *C*) and for NaCl (salt) responses (Avenet and Lindemann, 1991). In this study, an increase in frequency may be attributed to either an increase in excitation of individual taste cells within the taste bud, or to an increase in the number of responding cells, or to a combination of these two phenomena. The in situ technique cannot distinguish between these possibilities. Because the frequency of action currents is related to the intensity of chemostimulation in a dose-dependent manner but the amplitude of action currents is not, we have focused our analyses upon the frequency of the action current response.

Preliminary Observations

We recorded action currents in response to NaCl or citric acid from a total of 76 of 167 fungiform papillae located on the anterior dorsal portion of the hamster tongue. Of the remaining 91 papillae, 84 showed no response to any chemical stimuli and 7 were spontaneously active (see below). Lack of chemosensory responses in the 84 "silent" papillae might be due to inadequate positioning of the pipette over the taste pore; to excess mucus in the taste pore which prevented the stimulating solution from reaching the taste cells; to the absence of a taste bud on the papillae; to the absence of acid- or salt-sensitive taste cells in the taste bud; or to a combination of any of the above.

Spontaneous action currents were rarely observed during perfusion with the adapting solution alone. If they occurred, they were usually observed at the very beginning of the experiment and disappeared after a few minutes of continuous perfusion. This phenomenon was also observed in rat taste buds and may be due to mechanical stimulation during the onset of perfusion (Avenet and Lindemann, 1991). In some experiments (7 of 167 papillae), however, action currents occurred spontaneously for up to 70 min, particularly after prolonged acid stimulation. Since spontaneous firing might reflect cell damage, data from these papillae were not included in further analyses. We avoided prolonged acid stimulation by exposing the taste buds to acid for no longer than 2 min and routinely rinsed with control (adapting) solution for 3–5 min after acid stimulation to allow complete recovery. This protocol gave good reproducibility and allowed us to record from single taste buds for as long as 2.5 h.

Response Characteristics

When the adapting solution was artificial saliva, application of an acid stimulus (8 mM citric acid, pH 2.6) elicited a slowly activating and sustained response (Fig. 2 *A*).

After the delay for the chemostimulus to reach the tip of the pipette, a series of action currents (7–10 pA) at a frequency of 1–2/s was recorded. The response was maintained as long as the acid solution was present, although some adaptation in frequency and/or amplitude typically occurred (see below). Fig. 2 *A* shows the

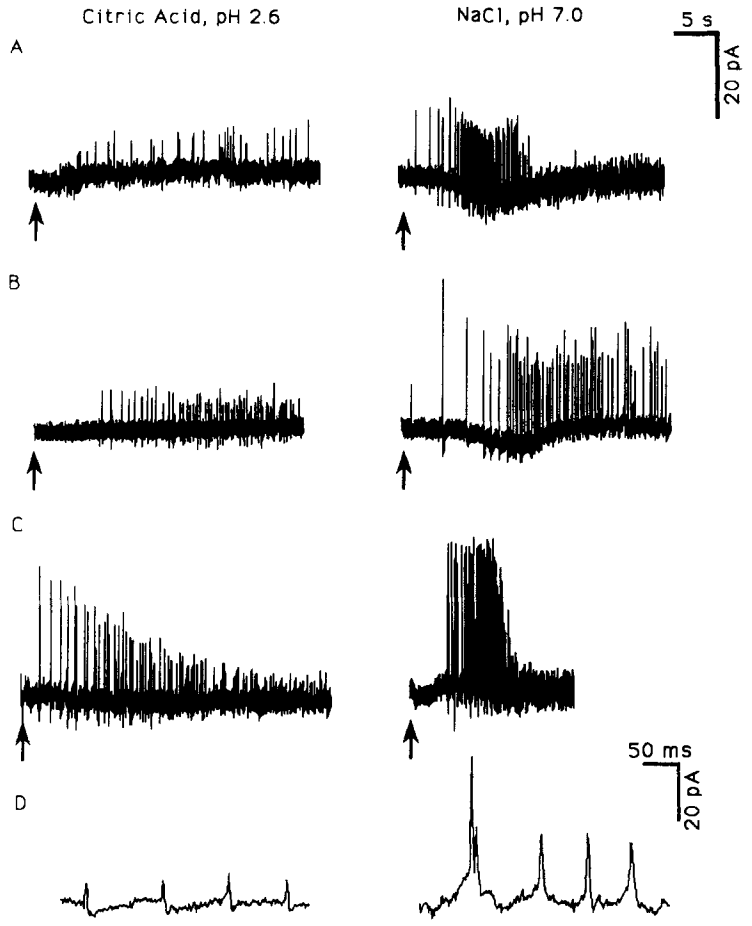

FIGURE 2. Rapid transient currents recorded from single taste buds (*A–C*) in response to 3 mM citric acid (pH 2.6) or 200 mM NaCl (pH 7.0). Responses were recorded in a solution containing artificial saliva (*A*) or in one containing only NMDG-Cl (*B–D*). Responses to these stimuli were typically either sustained (*B*) or adapting (*C*) in nature. In all records, arrows indicate the approximate onset of the stimulus which was maintained for the duration of the illustrated traces. (*D*) Current transients in two different taste buds shown at an expanded time base. Inward current is reflected downward. All records were low pass filtered at 300 Hz.

response on the same taste bud to 200 mM NaCl added to artificial saliva. In cases where both stimuli were tested on the same taste bud (e.g., Fig. 2, *A* and *B*), the amplitude of the action currents elicited by NaCl was almost always larger than for acid stimulation. The mean amplitude of acid-induced action currents was 9.2 ± 4.1

pA at pH 2.6 (n = 48 taste buds), whereas the amplitude of NaCl-induced action currents was significantly larger (29.1 ± 12.4 pA, n = 24 taste buds; $P < 0.05$, unpaired t test). In some cases, the amplitude of action currents in response to acid stimulation tended to decrease with prolonged exposure (Fig. 2 *C*). Furthermore, NaCl responses sometimes adapted completely, whereas complete adaptation was never observed for acid-evoked responses.

To quantify chemoresponses and to compare response patterns in different taste buds, we summated action currents during a response and displayed this as a function of time.[2] Fig. 3 *A* shows the summated responses of a taste bud measured at different pH values. At low intensity stimulation (pH > 3.1), the rate of action currents was nearly constant throughout the duration of the stimulation. At higher

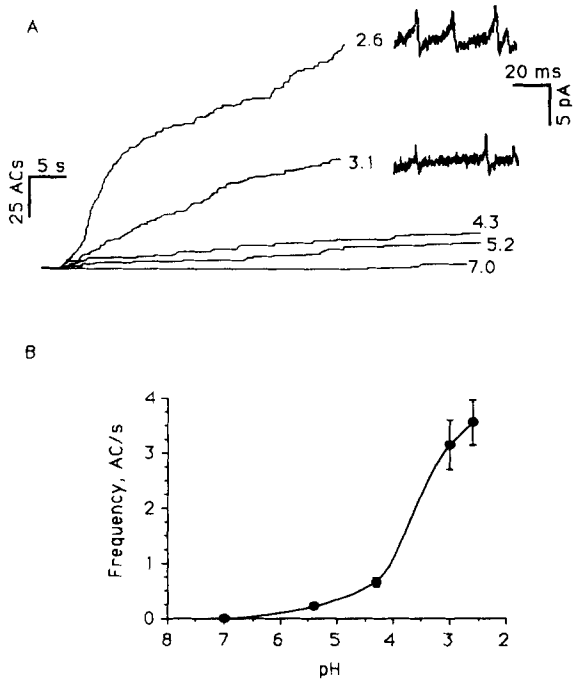

FIGURE 3. (*A*) Summated action currents recorded in a single taste bud in solutions varying in pH. All solutions contained 30 mM NMDG-Cl with 0.1 M citric acid added to adjust pH. Examples of traces are shown for responses recorded in pH 2.6 and pH 3.1 solutions. (*B*) Frequency of action currents as a function of pH. Frequency was determined by measuring the initial slope of summated action currents as shown in *A*. Mean values are given ± SE and represent from 2 to 43 taste buds per point.

intensity stimulation (pH ≤ 3.1) an initial high rate of firing (at pH 2.6; average rate = 3.6 ± 1.9 s^{-1}, n = 25 taste buds) was sometimes observed to decline after a few seconds to a lower rate (at pH 2.6; average rate = 1.8 ± 0.9 s^{-1}, n = 4 taste buds). This is seen as a change in the slope of the summated records (Fig. 3 *A*). Typically, during all phases of the chemosensory response, action currents were also slightly larger (10–20%) for these stronger stimulations, similar to the effect seen for

[2] It should be noted that we cannot distinguish activity of individual taste receptor cells. Therefore, the responses we record reflect activity in all the taste cells that were firing. In rare cases, different amplitudes of action currents were distinguishable, suggesting the presence of different responding cells (e.g., Fig. 4 *B*), but we did not systematically analyze this.

changes in the strength of NaCl stimulation (Avenet and Lindemann, 1991). These differences may be attributed to changes in the current across the apical membrane carried by these stimuli (see above). As mentioned above, complete adaptation of acid-induced action currents was never observed. Fig. 3 *B* shows the dose dependence of the acid response: the maximum frequency (measured by the initial slope of the summated response, as in Fig. 3 *A*) is displayed as a function of the pH of the citric acid solution. The threshold for the response is between pH 5.0 and 6.0. Due to the possibility of nonselective effects or irreversible changes of low pH, stimulation below pH 2.6 was avoided.[3]

Although citric acid was used primarily for acid stimulation because of its ability to buffer solutions near pH 3.0, similar responses were also obtained with HCl. Typically, the frequency of action currents obtained on a single taste bud was slightly less when HCl was used compared with citric acid at the same pH. This difference was not attributable to the presence of the citrate ion because NMDG-citrate (30 mM) at pH 7.0 did not elicit responses (data not shown).

Effects of Ionic Changes and Blockers in the Mucosal Solution

To investigate the mechanism of acid taste transduction, we replaced all cations in the artificial saliva with the impermeant cation, NMDG. Fig. 2, *B* and *C* shows recordings from two taste buds in response to 3 mM citric acid (pH 2.6) and 200 mM NaCl added to NMDG-Cl. The responses were qualitatively similar to the ones observed in artificial saliva with respect to frequency and amplitude.

Though a Ca^{2+} conductance has been reported to be involved in the transduction of acid stimuli (Miyamoto et al., 1988), neither the presence of Ca^{2+} nor any other cation was necessary for the generation of action currents in response to acid stimulation in the hamster. This was further verified by increasing Ca^{2+} concentration to 10 mM or decreasing it to 10^{-9} M by adding 5 mM EGTA. Neither of these two manipulations affected responses to acid stimulation (data not shown). Thus, the presence of permeant cations other than protons was not necessary to elicit acid responses.

In salamanders, acid stimulation causes a block of apical K^+ channels (Kinnamon et al., 1988; Teeter et al., 1989). To determine if K^+ channels were involved in acid responses in the hamster, we first tested for the presence of apical K conductances in those experiments where acid stimuli evoked responses. 0.03–1 M KCl rarely elicited responses (2 of 16 taste buds), and most importantly, taste buds that responded strongly to citric acid did not respond to an increase in KCl concentration.

It is possible that taste cells were rapidly depolarized by KCl such that voltage-gated Na^+ channels were inactivated and consequently action potentials could not be elicited. However, when acid stimuli were applied in solutions containing high KCl, the frequency of responses was not different than that obtained in a solution containing only NMDG-Cl (Fig. 4 *A*), suggesting that the taste receptor cells were still capable of responding. The amplitude of the acid-induced action currents were in some cases affected by high KCl, but in no predictable manner. In some experiments

[3] At this pH, citric acid had a mild sour taste (personal observations). The threshold for human sour taste is about pH 3.5–4.0 for a variety of acids (Berg, Filipello, Hinreiner, and Webb, 1955).

they were reduced (e.g., Fig. 4 *A*), while in others they were increased or unaffected. Additionally, we applied TEA to block apical K conductance, if it existed, and thereby depolarize the taste cells (cf. Kinnamon and Roper, 1988; Roper and McBride, 1989). At 8 mM, a concentration that blocks nearly all the voltage-dependent K^+ conductance in these cells (Cummings and Kinnamon, 1990), TEA never elicited a response. Furthermore, when TEA was applied together with citric acid, the response to citric acid was not modified (Fig. 4 *B*). Based on these experiments, no relationship between K^+ channels and the acid response could be established.

To check for the possible involvement of a Cl^- conductance in the generation of the acid response, as suggested from the work of Simon and Garvin (1985), we varied Cl^- concentration in the mucosal solution by replacing Cl^- with SO_4^-. Responses were

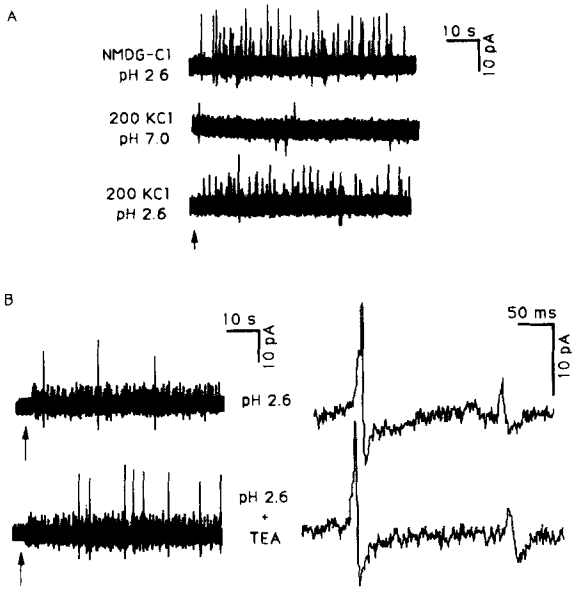

FIGURE 4. Responses to acid stimulation were unaffected by KCl and TEA. (*A*) Sequential recordings from a taste bud stimulated with NMDG-Cl at pH 2.6, KCl at pH 7.0, KCl at pH 2.6, and NMDG-Cl at pH 2.6. 3 mM citric acid was used to adjust the pH to 2.6. The frequency and amplitude of responses to NMDG-Cl and KCl at pH 2.6 did not vary considerably within a taste bud. (*B*) Frequency and amplitude of acid-induced responses were not affected by 8 mM TEA added to the stimulating solution. Right-hand traces were expanded from those shown on the left, illustrating that both large unit and small unit action currents did not change during TEA treatment.

indistinguishable in Cl^--containing and Cl^--free solutions (n = 4 experiments). In other experiments, we increased the Cl^- concentration by adding 90 or 200 mM NMDG-Cl in the control solution. Changes in Cl^- concentration had no effect on responses elicited by citric acid (pH 2.6) (Table I). Furthermore, application of the Cl^- channel blocker 9-AC (100 μM) did not affect the acid response (n = 3 taste buds).

To test for the involvement of apically located ATP-dependent pumps in the acid response, Na-ortho-vanadate (vanadate), which is known to block several classes of active transport mechanisms (Pedersen and Carafoli, 1987), was added to the mucosal solution. While the taste cells were being stimulated with citric acid at pH 2.6, 100 μM vanadate was applied. No effect on the response was detectable even

after 5 min of application (n = 3 taste buds). Similarly, applying the Cl^-/HCO_3^- exchange blocker DIDS, at concentrations from 100 to 500 μM, did not affect the response of taste cells to acid stimulation (n = 5 taste buds).

Because direct application of acid stimuli may affect intracellular pH, we attempted, in five experiments, to alter intracellular pH by exposing taste buds to 20 mM NH_4Cl for 3–5 min (Boron and DeWeer, 1976; Thomas, 1984). Neither during the initial periods of application of NH_4Cl (which should lead to a transient cell alkalinization), nor after removal of NH_4Cl (which should cause cell acidification), were action currents elicited from taste cells, nor were the acid responses of these cells affected. However, because only a small area of the taste receptor cell is exposed in the taste pore, we cannot be sure that NH_4Cl treatment resulted in changes in the intracellular pH. On the other hand, exposing the identical surface to citric acid elicited action currents.

Since the above experiments seem to rule out the involvement of any cations except protons in the acid response, and because neither ATP-dependent pumps/

TABLE I

Effect of Mucosal Cl^- Concentration on Frequency or Amplitude of Acid-induced Action Currents

$[Cl^-]_{mucosal}$	Frequency	n	Amplitude	n
	ACs · s^{-1} (±SE)		*pA (±SE)*	
0	2.9 ± 0.8	4	8.7 ± 0.6	4
30	3.6 ± 0.4	25	9.2 ± 0.6	48
90	2.6 ± 0.3	3	9.3 ± 1.2	3
200	2.3 ± 0.4	4	8.3 ± 1.1	4

Chemosensory stimulus was 3 mM citric acid (pH 2.6) in solutions of 0, 30, 90, or 200 mM NMDG-Cl. For zero Cl^- solutions, citric acid was applied in 30 mM NMDG-SO_4. Frequency was determined by the initial slope of the summated action currents (see Fig. 3 *A*). Amplitude was determined as the mean action current amplitude during the initial phase (~10 s) of the response to compensate for adaptation in action current size (e.g., Fig. 2 *C*).

exchangers nor changes in intracellular pH affected the acid response, we suggest that a direct flux of protons is the initial event responsible for the excitation of the taste cells in response to acid stimulation. One possible mechanism for this proton influx may be through proton-selective channels. If this proton flux occurs through a proton-selective channel, we would expect to be able to block the response with Cd^{2+}, since Cd^{2+} has been shown to block proton channels in other tissues (Byerly, Meech, and Moody, 1984). However, adding Cd^{2+} (0.1–2 mM) to the citric acid solution did not suppress the acid response (data not shown). This suggests that proton flux during acid stimulation occurs through a channel other than a proton-selective one. This was shown to be the case by the following experiments.

Effect of Mucosal Amiloride

Palmer (1982, 1984, 1987) has shown that the amiloride-sensitive Na^+ channel in toad bladder has a high proton permeability, higher than for any other cation.

Therefore, another possible mechanism for proton influx into the taste cells is through amiloride-sensitive Na^+ channels, which have been shown to mediate salt taste transduction (Avenet and Kinnamon, 1991; Avenet and Lindemann, 1991). To test for the involvement of Na^+ channels in the acid response, we applied the diuretic drug amiloride in the presence of citric acid stimuli. In 24 of 26 taste buds tested, the acid response obtained with citric acid was consistently inhibited by amiloride. Fig. 5 *A* illustrates the inhibitory effect of 30 μM amiloride applied during citric acid (pH

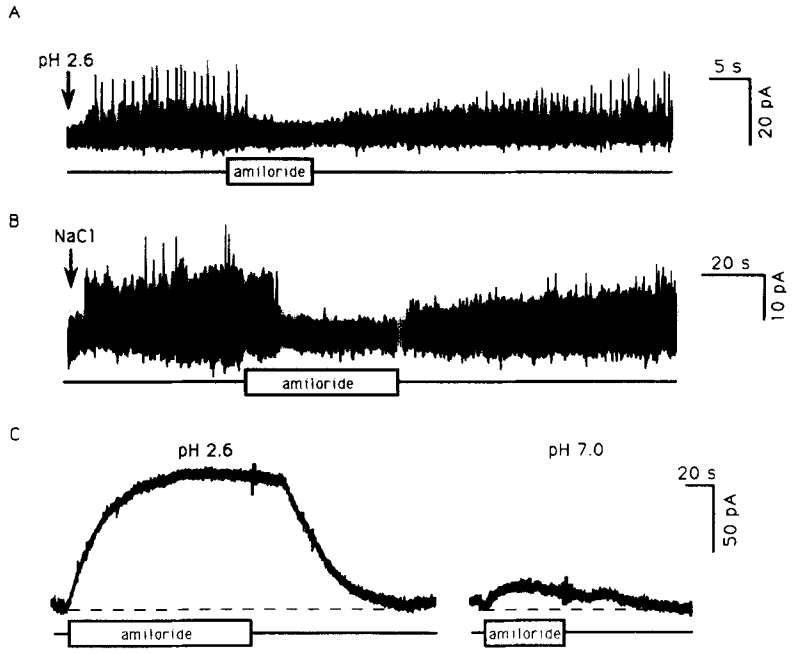

FIGURE 5. Responses to acid stimulation were blocked by amiloride. (*A*) Application of 30 μM amiloride during citric acid (pH 2.6) stimulation completely and reversibly blocked acid-induced action currents. Recovery of the acid response after wash-out of amiloride was characterized by a slow, progressive increase in the amplitude and frequency of action currents. Adapting solution contained only 30 mM NMDG-Cl. (*B*) Application of 10 μM amiloride inhibited action currents elicited by NaCl (100 mM) in a similar manner. (*C*) 30 μM amiloride blocked a larger component of the steady-state current at pH 2.6 than at pH 7.0, suggesting that in response to acid stimulation an amiloride-sensitive current had developed. Adapting solution was 30 mM NMDG-Cl. Steady-state currents were recorded with the tracking mode of the patch-clamp amplifier turned off.

2.6) stimulation. Recovery from amiloride during washout was typically slower than onset and occurred with a slow increase in the amplitude of the action currents. This block and recovery resembled the effect of amiloride on NaCl responses in the hamster (Fig. 5 *B*) and in the rat (cf. Fig. 4 in Avenet and Lindemann, 1991).

If an apical conductance to protons is mediating acid-induced responses, one would expect a d.c. current change across the apical membrane in response to acid stimulation. Due to the low seal value between pipette and papillae, however,

significant junction potentials normally are generated when the ionic composition in the stimulus/recording pipette is changed, i.e., during chemosensory stimulation. This complicates any attempt to measure changes in transcellular d.c. currents elicited by chemosensory stimulation. However, since amiloride acts at concentrations in the micromolar range, adding or deleting amiloride does not alter the ionic composition of the chemical stimulus significantly, and hence does not produce junction potentials. Thus, we were able to measure whether the steady-state transcellular current evoked by acid stimulation was affected by amiloride. Fig. 5 *C* shows that

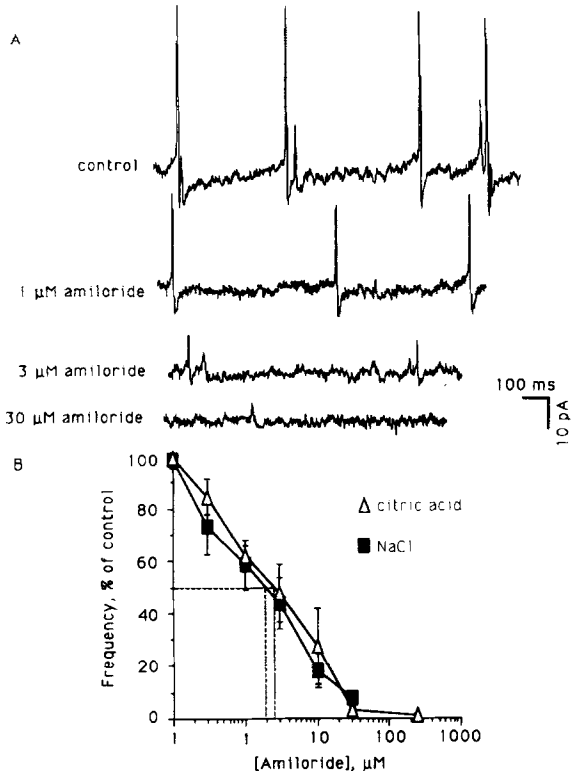

FIGURE 6. Amplitude and frequency of acid-induced action currents were inhibited by amiloride in a dose-dependent manner. (*A*) Increasing the concentration of amiloride in the stimulating solution decreased the amplitude of action currents. Control refers to a solution containing 30 mM NMDG-Cl and 3 mM citric acid (pH 2.6) without added amiloride. This taste bud displayed unusually large action currents in response to acid stimulation. (*B*) Frequency of action currents (relative to control) in response to stimulation with either 3 mM citric acid, pH 2.6 (Δ), or 200 mM NaCl, pH 7.0 (■), decreased as a function of amiloride concentration. Values are given ± SE. The concentration of amiloride producing a half-maximal block (*dotted line*) was 1.9 μM for NaCl responses and 2.4 μM for citric acid responses. Citric acid responses were recorded in NMDG-Cl adapting solution.

a component of the steady-state current can be blocked by amiloride. This amiloride-blockable component of the current is approximately five times larger when measured at pH 2.6 than at pH 7.0 in 30 mM NMDG-Cl adapting solution. The amplitude of the amiloride-sensitive, steady-state current increased from 13.4 ± 6.3 pA (n = 11 taste buds) at pH 7.0 to 68.0 ± 45.3 pA (n = 12) at pH 2.6. Therefore, an amiloride-sensitive inward current developed in response to acid stimulation.

Submaximal concentrations of amiloride decreased the amplitude as well as the frequency of the acid-evoked action currents in a dose-dependent manner (Fig. 6, *A*

and *B*). This indicates that amiloride reduces apical conductance of taste cells (cf. Avenet and Lindemann, 1991). The concentration of amiloride at which the frequency of the acid response was reduced by 50% (K_i) was 2.4 μM. In another series of experiments, we determined the dose–response curve for the effect of amiloride on NaCl responses. The concentration of amiloride at which the frequency of the NaCl

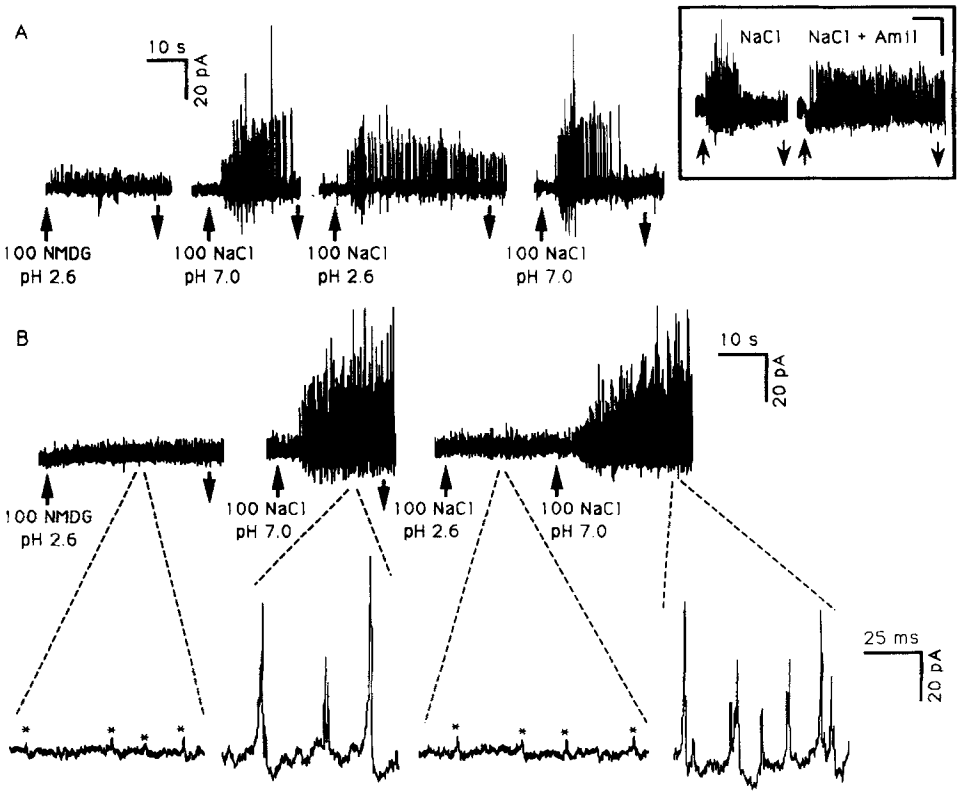

FIGURE 7. Effects of low pH on NaCl responses. (*A*) Adapting NaCl responses became more sustained at pH 2.6. Note that the taste bud stops firing action currents in response to 100 mM NaCl, pH 7.0, before the stimulus is stopped (*downward arrows*). NaCl at pH 2.6 produced a lower frequency response that is maintained throughout its application. Upon returning to pH 7.0, the NaCl response adapts again. This is similar to the effect seen when a low dose of amiloride (1 μM) is applied to a fast adapting NaCl response (*inset*). Scale bar in inset, 10 s, 20 pA. (*B*) Sustained NaCl responses were blocked reversibly at pH 2.6. The response of the taste bud to NaCl at pH 2.6 resembles the response to acid stimulation alone. Asterisks mark small action currents characteristic of the acid response.

response was reduced by 50% was 1.9 μM, which was close to the K_i for acid. Furthermore, the dose–response curves for inhibition of the salt and the acid responses nearly superimpose (Fig. 6 *B*). These data suggest that acid and NaCl responses are mediated by the same conductance mechanism.

Interaction of NaCl and Acid Stimulation

If the same membrane conductance pathway is responsible for both salt and acid taste transduction, one would expect strong interaction when both stimuli are applied simultaneously. Indeed, we observed that NaCl responses were affected by acid stimuli. Two types of NaCl responses were typically observed in rat (Avenet and Lindemann, 1991) and hamster (Fig. 2, *B* and *C*, and Avenet, Kinnamon, and Roper, 1991): a fast adapting response that occurred in about half the taste buds tested and, in the other half, a sustained response that lasted as long as NaCl was applied. Fig. 7 *A* shows that when citric acid was applied simultaneously with NaCl on adapting taste buds, the NaCl response became sustained. If acid and salt transduction mechanisms are independent, we may expect to see small amplitude currents, typical of acid responses, also under these conditions. None were seen; however, they could have been masked by larger NaCl-induced action currents. A similar effect could be observed when amiloride was applied at submaximal concentrations (1 μM) on an adapting taste bud (Fig. 7 *A*, *inset*), suggesting that the transformation of an adapting response to a sustained response occurred by reducing amiloride-sensitive Na^+ conductance. That is, the effect of acid, in this case, was identical to a partial inhibition of apical Na^+ channels by amiloride. When citric acid was applied to taste buds that responded in a sustained manner to NaCl, the NaCl response was blocked and the remaining response closely resembled the response of the taste bud to acid stimulation alone (Fig. 7 *B*). In this case, citric acid stimulation seemed to obscure the salt response completely in favor of an "acid-like" response.

DISCUSSION

This study describes experiments designed to elucidate mechanisms for acid transduction in hamster fungiform papillae using a noninvasive, extracellular recording technique. This technique is particularly well suited to the study of acid taste as it restricts the stimuli to the apical membrane of taste receptor cells, which, by virtue of its small contribution to the total surface area, avoids the many nonspecific and often deleterious effects of low pH. Chemostimulation of fungiform papillae with acid stimuli reliably elicited fast, transient currents whose frequency varied in a dose-dependent manner with proton concentration. This activity was indicative of activity in taste receptor cells, since these are the only cells in the taste bud or in the surrounding epithelia capable of generating action potentials.

Our data suggest that protons can permeate amiloride-sensitive Na^+ channels and the subsequent depolarization causes taste receptor cell excitation. This conclusion is based upon four pieces of evidence. First, no cations other than protons in the mucosal solution were needed to elicit acid responses. Second, amiloride blocks acid-induced action currents in the identical concentration range as for NaCl elicited responses. Third, acid stimulation, like the taste cell response to NaCl, is accompanied by an increase in inward current (pipette to taste pore) that is amiloride sensitive. Fourth, there is a strong interdependence between the citric acid and NaCl responses when both stimuli are applied simultaneously. Preliminary results using patch-clamp recording confirm that amiloride-sensitive proton currents are found in

isolated hamster fungiform taste cells (Gilbertson, Roper, and Kinnamon, 1992) and support this finding.

Responses to acid stimuli were unaffected by KCl and TEA. In only rare instances did cells that responded to citric acid also respond to KCl. These results suggest that the predominant mechanism for acid taste transduction in the hamster does not involve a proton block of apical K^+ channels, as is the case for the mudpuppy (Kinnamon and Roper, 1988). Other experiments ruled out the participation of Cl^- channels, Cd^{2+}-sensitive proton channels, HCO_3^-/Cl^- exchangers, Ca^{2+} channels, ATP-dependent pumps, and intracellular pH changes as being the transduction mechanisms generating action currents in these taste buds. Based upon these results, we were unable to confirm prevailing hypotheses for the mechanism of acid taste transduction. We are aware, however, that we were able to record only from those cells that generated action potentials in response to chemostimulation. If other acid transduction mechanisms occur without associated voltage-dependent phenomena, as is the case for bitter stimuli (Akabas, Dodd, and Al-Awqati, 1988; Hwang, Verma, Bredt, and Snyder, 1990), or with only subthreshold receptor potentials, this noninvasive recording method would not allow us to measure taste cell responses.

An alternative explanation to the present results is that protons have two concurrent effects. The first, as shown in Fig. 7 *A*, is that protons might block Na^+ channels. The second is that protons also bind to a receptor that initiates a cascade of events leading to opening of a basolateral conductance that depolarizes the cell to generate an action potential. In this case, amiloride would prevent one from recording acid-induced responses by blocking an apical Na^+ leak conductance through which action currents reach the recording electrode. We believe this explanation to be unlikely for the following reasons. First, in addition to inhibiting the amplitude of acid responses, amiloride reduces the *frequency* of acid-induced action currents (Fig. 6 *B*). This would not be predicted if we were merely blocking our recording pathway and not the mechanism that generated the action potentials. Second, as shown in Fig. 5 *C*, amiloride blocks a larger component of the apical d.c. current at pH 2.6 than at pH 7.0, suggesting that protons induce an amiloride-sensitive sustained inward current. The explanation we favor, which is consistent with all the results presented, is one in which protons permeate amiloride-sensitive Na^+ channels in the apical membranes of hamster taste cells.

A model consistent with these results is illustrated in Fig. 8. The amiloride-sensitive Na^+ channel (Fig. 8 *A*) is permeable to both Na^+ ions and protons. Under NaCl stimulation (Fig. 8 *B*), Na^+ ions permeate these channels to produce a depolarizing receptor potential. Similarly, when acid stimuli are applied (Fig. 8 *C*), protons permeate the channel to depolarize the taste cell. Because the conductance of Na^+ channels to protons is relatively low, and thus protons carry less current than Na^+ ions through the amiloride-sensitive channel (Palmer, 1987), smaller amplitude action currents are recorded (see Fig. 2). Amiloride inhibits responses to both of these stimuli by blocking the Na^+ channel (Figs. 5 and 6). When both stimuli are applied simultaneously (Fig. 8 *D*), a small amplitude response is recorded (Fig. 7 *B*), similar to the acid response alone. This is explained by the fact that protons bind much more tightly to the anionic selectivity site in the channel than do Na^+ ions (Palmer, 1987), resulting in a reduced current flow. Because of this Na^+/proton

interaction, it might be expected that acids will modify the physiological response to salty stimuli and vice versa (see below).

Implications of Proton Permeability in Apical Na^+ Channels

Action currents are driven through the capacitance and conductance of the taste cell apical membrane by action potentials in the taste receptor cell (Avenet and Lindemann, 1991). Based on their relative amplitudes, one can estimate the relative permeabilities of protons and Na^+ ions. Assuming that the inward current through the taste cell membrane is dependent upon the mucosal concentration of the ion being transported, one would expect transients for a 2.5-mM proton concentration (pH 2.6) to be 80 times smaller than with 200 mM NaCl, if the permeability of

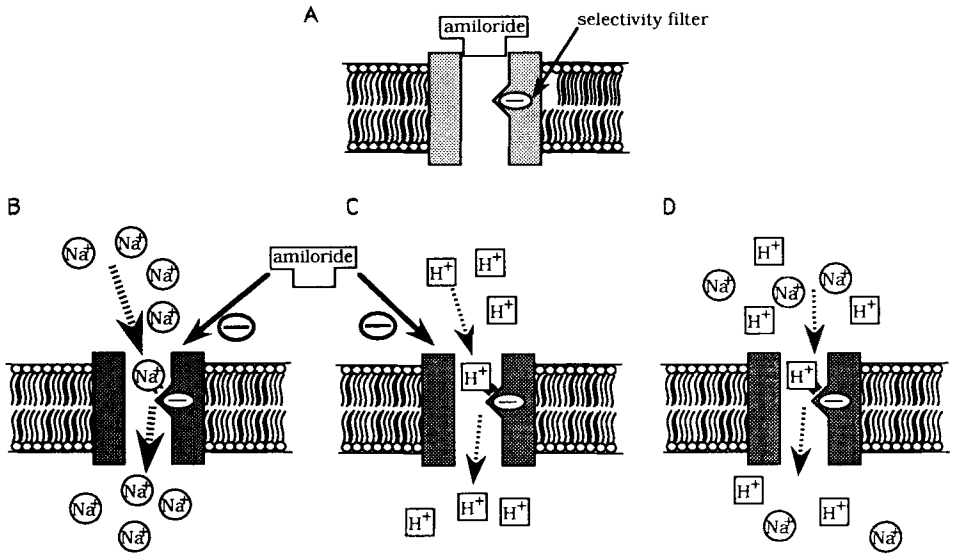

FIGURE 8. Model of the role of the amiloride-sensitive Na^+ channel in acid transduction. The amiloride-sensitive Na^+ channel (*A*) is shown under conditions of salt (NaCl) stimulation (*B*), acid stimulation (*C*), and when both stimuli are simultaneously applied (*D*). Refer to text for details.

protons (P_{H^+}) equals the permeability of Na^+ (P_{Na^+}). Our results showed that the amplitude of NaCl-induced action currents was approximately three times greater than the amplitude of citric acid–induced action currents. From this, one can estimate that P_{H^+}/P_{Na^+} is ~ 25. It is known that proton permeability in the epithelial amiloride-sensitive Na^+ channel is higher than for any other cation (Palmer, 1982, 1984, 1987) and that amiloride inhibits this proton flux through epithelial Na^+ channels (Palmer, 1984). These observations, coupled with reports showing similar amiloride analogue sensitivities (Schiffman, Frey, Suggs, Cragoe, and Erickson, 1990) and ion selectivity (Heck, Persaud, and DeSimone, 1989), support the idea that the channel responsible for salt taste transduction is the same channel found in transporting tissues.

Thus, considering the relatively low salivary Na^+ concentration in hamster (see Materials and Methods), the large electrochemical gradient for protons during acid stimulation (similar to the Ca^{2+} gradients for many neurons) and the apparent high permeability of protons in amiloride-sensitive Na^+ channels, it is conceivable that most of the taste cells possessing apical Na^+ channels, which have been implicated in salt taste transduction, will also be excited under conditions of low pH.

Interaction with NaCl Stimulation

In the presence of high Na^+, the stimulation by protons differs, depending on the type of NaCl response. A taste bud that adapts rapidly to NaCl will develop a sustained response if the pH is lowered below 3.0. One possible explanation for the adapting process is a strong depolarization without repolarization of the taste cell. This may be the case for taste buds having a high Na^+ permeability. For these cells, acid appears to convert the initial high frequency phasic response into sustained tonic activity at a lower frequency, since the same effect can be seen by adding submaximal concentrations of amiloride to NaCl. Coupled with decreases in the action current amplitude, this can be explained in terms of a reduction of the Na^+ permeability by protons, and therefore a reduction in taste cell excitation. This shift of an adapting NaCl response to a sustained response in the presence of acid (or submaximal amiloride) is interesting in light of human psychophysical evidence which shows that while the perceived saltiness of NaCl solutions is decreased by high concentrations of citric acid, it can actually be increased by low (near threshold) concentrations of acid (Pangborn and Trabue, 1967; Helleman, 1992). It is possible that this enhancement by a low concentration of acid in this instance is acting by preventing complete adaptation of the NaCl response. One might expect a similar psychophysical response to submaximal concentrations of amiloride (see Fig. 7 *A*, *inset*).

For cells responding with sustained action currents to NaCl stimulation, inhibition by protons may be complete and appears to be replaced by a proton flux. These responses resemble the proton block described in TTX-sensitive Na^+ channels in nerve (for review, see Hille, 1992) and in amiloride-sensitive Na^+ channels in toad bladder (Palmer, 1984, 1987). This interpretation remains speculative, however, and the elucidation of the mechanism of proton block/permeation in the presence of Na^+ awaits investigation at the single channel level in taste receptor cells.

Physiological Significance

Psychophysical studies have failed to demonstrate a measurable effect of amiloride on sour taste perception (Schiffman, Lockhead, and Maes, 1983). Other studies, though, have shown that amiloride does not even reduce the perceived salt intensity in humans when presented in whole-mouth tests (Desor and Finn, 1989), suggesting that the action of amiloride may be difficult to assess in human psychophysical tests. However, in recordings from whole hamster chorda tympani nerve, Hettinger and Frank (1990) demonstrated that amiloride reduced responses to hydrochloric acid in a reversible manner. Based on our results, we expect an interdependence and cross-adaptation between acid and salt stimuli. Such a dependence has been observed in insects, where salt responses were inhibited at pH values <3.0 (blowfly: Evans and Mellon, 1962; Gillary, 1966). In hamster, there is evidence showing that NaCl

responses are reduced or attenuated in the presence of acid (Hyman and Frank, 1980; Travers and Smith, 1984). McBurney and Bartoshuk (1973), in a human psychophysical study, presented evidence showing that citric acid, in addition to being perceived as sour, tastes somewhat salty. Further, they showed that acid stimuli can cross-adapt some of the saltiness of NaCl. Finally, Van Buskirk and Smith (1981), while recording from hamster parabrachial pontine neurons, found statistically significant correlations between acid (HCl) and NaCl responses in across-neuron comparisons, while none were found between other taste modalities (i.e., salt and sweet). These reports suggest that acid and salt taste are linked in some way. It would be of interest to investigate this interdependence further in mammals.

A recent study by Okada, Miyamoto, and Sato (1991) lends support to our hypothesis that protons permeate the amiloride-sensitive Na^+ channel leading to taste cell excitation. Okada et al. (1991) measured responses from the frog glossopharyngeal nerve elicited by chemical stimuli applied to the tongue. Responses were compared before and after application of arginine^8-vasopressin (AVP), one of a group of hormones known to increase the number of amiloride-sensitive Na^+ channels expressed in epithelial and taste cells (Palmer, Li, Lindemann, and Edelman, 1982; Garty and Benos, 1988; Okada, Miyamoto, and Sato, 1990; Marunaka and Eaton, 1991). After AVP treatment, responses to salty (NaCl) and acid (HCl) stimuli were equally enhanced, whereas responses to bitter and sweet compounds were unaffected. One can interpret this to mean that increasing the number of amiloride-sensitive Na^+ channels increases both the inward Na^+ influx during salt stimulation and the inward proton influx during acid stimulation. This result would be predicted according to our model of acid transduction (Fig. 8).

The presence of a large number of Na^+ channels on the hamster taste cells and their permeability to protons implicate their involvement in acid taste transduction. However, if proton permeability through amiloride-sensitive Na^+ channels were the only mechanism for acid transduction, it would be difficult to explain how acid stimuli could be distinguished from salty stimuli. Additional mechanisms may be present, since any apical conductance mechanism is likely to be affected by protons. We did observe, on rare occasions, the presence of an apical K^+ conductance. It is probable that the cells that have this K^+ conductance will be depolarized by acid stimulation in a manner analogous to proton block shown in *Necturus* (Kinnamon and Roper, 1988; Kinnamon et al., 1988; Cummings and Kinnamon, 1992). Additionally, there may be other taste cells excited by acid stimulation, but not firing action potentials, which would remain undetected with our method. Alternative methods, such as single channel recording of the amiloride-sensitive channel on taste cells or the use of voltage-sensitive or Ca^{2+}-sensitive dyes, would help clarify this point. In conclusion, it is likely that several mechanisms, including the permeation of protons through amiloride-sensitive Na^+ channels, may contribute to acid transduction.

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