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Differential Transduction Mechanisms Underlying NaCl- and KCl-induced Responses in Mouse Taste Cells

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Abstract

The transduction mechanism of salt-induced responses of mouse taste cells was investigated using the patch clamp and the local stimulation techniques under quasi-natural conditions. Apically applied NaCl induced a voltage-independent current, which was partially suppressed by amiloride and Cd^{2+}. In contrast, apically applied 0.5 M KCl induced an inwardly rectifying current (KCl-induced I_{ir}). The KCl-induced I_{ir} was unaffected by amiloride. The I_{ir} was suppressed not only by external Ba^{2+} and Cs^{+}, but also by a Cl^{-} channel blocker, niflumic acid. The E_r of the KCl-induced response was independent of the apical ionic concentration, but rather was close to the equilibrium potential of Cl^{-} (E_{Cl}) at the basolateral membrane. The KCl-induced I_{ir} displayed a fast run-down under the conditions of the conventional whole cell clamp method, but not under the perforated patch conditions. Immunohistochemical localization of an inwardly rectifying Cl^{-} channel protein, ClC-2, was observed in taste bud cells of the fungiform papillae. It is concluded that the transduction mechanism of NaCl-induced responses is completely different from that of KCl-induced responses in mouse taste cells.

Introduction

Amiloride-sensitive Na^{+} channels are thought to be the main contributor to transduction of the salt-induced response because a diuretic, amiloride, greatly suppresses the salt-induced response recorded from the chorda tympani nerve in the mammalian gustatory system [rat (Heck *et al.*, 1984; Brand *et al.*, 1985; Formaker and Hill, 1988); hamster (Herness, 1987); mouse (Ninomiya *et al.*, 1989); monkey (Hellekant *et al.*, 1988)] and the extracellularly recorded action potentials induced in NaCl from fungiform taste cells (Avenet and Lindemann, 1991). This fact correlates with the results in pioneering works using microelectrode impalement, that depolarizing receptor potentials are associated with an increase in membrane conductance in response to NaCl (Ozeki, 1971; Sato and Beidler, 1982).

In contrast to NaCl-induced responses, the chorda tympani nerve responses induced by K salts are, in general, known to be insensitive to amiloride (Ye *et al.*, 1994; Lindemann, 1996; Ninomiya *et al.*, 1996), whereas responses induced by lower concentrations of K and NH_4 salts are only partially suppressed by amiloride in the rat chorda tympani nerve (Kloub *et al.*, 1997; Lundy *et al.*, 1997). Apically applied K^{+} channel blockers, TEA, Ba^{2+} and Cs^{+}, never suppress chorda tympani responses induced by K and NH_4 salts (Ye *et al.*, 1994; Kloub *et al.*, 1997), whereas a membrane permeant K^{+} channel blocker, 4-aminopyridine (4-AP) (Hille, 1992), partially blocks K salt responses (Kim and Mistretta, 1993), suggesting that 4-AP penetrating through a trans- and/or paracellular pathway inhibits K^{+} channels at the basolateral membrane (Stewart *et al.*, 1997). Therefore, transduction of responses induced by K and NH_4 salts is thought to be mainly generated at the basolateral membrane of rat taste cells (Lindemann, 1996; Stewart *et al.*, 1997). Thus, the diffusion model, which is similar to that proposed for the amiloride-insensitive component of the NaCl-induced response (Simon *et al.*, 1993; Ye *et al.*, 1993), can be applied to the KCl-induced response: KCl can pass through tight junctions into the interstitial fluid of the taste buds and the consequent increase in external K^{+} concentration depolarizes the basolateral membrane of taste cells (Lindemann, 1996; Stewart *et al.*, 1997). This model is supported by the fact that less mobile anions than Cl^{-}, such as gluconate^{-} and acetate^{-}, lower the chorda tympani responses (Ye *et al.*, 1994; Kloub *et al.*, 1997).

However, both the NaCl and KCl responses in NaCl-best fibers of the chorda tympani nerve are suppressed by amiloride, but not those in HCl-best fibers in the rat (Ninomiya and Funakoshi, 1988) and hamster (Hettinger and Frank, 1990). All the taste cells in the fungiform papillae display several kinds of K^{+} channels and ~50% of taste cells express amiloride-sensitive Na^{+} channels (Miyamoto *et al.*, 1999). According to the diffusion model, all taste cells responsive to NaCl must also be responsive to KCl and only NaCl

responses are amiloride-sensitive. Therefore, with the diffusion model it is difficult to explain how the gustatory system discriminates NaCl taste from KCl taste.

In the present study we have examined the mechanism underlying the NaCl- and KCl-induced responses using a combination of a localized stimulation method with non-dissociated taste cells and the patch clamp technique. We report that apically applied 0.5 M KCl induces an amiloride-insensitive inwardly rectifying current, suggesting that the mechanism of generation of the KCl-induced response is completely different from that of the NaCl-induced response, which is mainly mediated by amiloride-sensitive and amiloride-insensitive cation conductances.

Materials and methods

Non-dissociated cell preparation and experimental set-up

Mouse strains C57BL/6 and BALB/c, ranging in weight from 20 to 35 g, were anesthetized by i.p. injection of pentobarbital (30 mg/kg) and killed by dislocating the cervical vertebrae. The tongue was then quickly removed, washed with normal extracellular solution (NES) and 1.5–2.0 mg/ml elastase (Boehringer Mannheim, Germany) dissolved in NES was injected into the tongue (0.2–0.4 ml/tongue) from the cut end. After the tongue had been incubated in NES for 30 min at 26°C, the epithelial sheet was peeled free from the rest of the tongue.

The epithelial sheet was pinned serosal side up on silicone rubber molded on the bottom of a laboratory dish and was washed three times with a divalent cation-free extracellular solution containing 2 mM EDTA to exclude divalent cations in NES. Individual taste buds with an epithelial brim were obtained by sucking fungiform papilla from the inside with a pipette (~100 μm in diameter) after incubation in divalent cation-free extracellular solution for 20 min in a refrigerator (4°C) and washing with NES. The experimental set-up for the combination of the whole cell clamp technique and the localized chemical stimulation procedure to apply taste stimuli to the apical membrane of taste cells (see insets in figures) was as previously described (Miyamoto *et al.*, 1996).

Using this technique quasi-natural conditions of gustatory stimulation were attained, although the apical and basolateral membranes were not completely isolated. Delivery of the taste stimulus was regulated by a microinjector system (IM-200; Narishige, Japan). Because the background flow was slowed down in the taste pore region due to the epithelial brim, the stimulus ejected from the microinjector at constant pressure was maintained for 10 s or longer. Liquid junction potentials at the pipette tip were corrected using a utility application of pCLAMP software (Axon Instruments, USA).

Electrical recording and data analysis

The whole cell configuration of the patch clamp technique (Hamill *et al.,* 1981) was mainly used. Pipettes were fabricated from borosilicate glass capillaries with a microfilament inside (Clark Electromedical Instruments, UK) using an electrode puller (PP-83; Narishige). The electrode tip was heat polished using a microforge (PF-83; Narishige) so that the resistance was between 10 and 20 MΩ when filled with the high Cl^- K^+ solution. Whole cell currents were measured with a patch clamp amplifier (EPC-7; List, Germany) and the current signals were filtered at 1 kHz, digitized at 125 kHz and analyzed with pCLAMP, Axograph (Axon Instruments) and Origin (Microcal Software Inc., USA) software. Averaged values of experimental data were expressed as means ± SEM. Differences between means were tested using a paired or unpaired *t*-test (StatView software; SAS Institute Inc., USA). All experiments were performed at 22–24°C.

Solutions

NES contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES–Tris, pH 7.4. In the divalent cation-free extracellular solution $CaCl_2$ and $MgCl_2$ were replaced by 2 mM EDTA. The high Cl^- K^+ pipette solution (high Cl^- K^+ solution) contained 120 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, 10 mM HEPES–Tris, pH 7.2. The 120 mM KCl in the high Cl^- K^+ solution was replaced by 20 mM KCl and 100 mM potassium gluconate in the low Cl^- high K^+ intracellular solution (low Cl^- K^+ solution) and by 120 mM CsCl in the high Cs^+ intracellular solution (high Cl^- Cs^+ solution). A pipette solution containing 250 μg/ml amphotericin B (Sigma, USA) (Rae *et al.*, 1991) was employed to obtain perforated patches. In the Na^+-free extracellular solution (Na^+-free solution), *N*-methyl-D-glucamine$^+$ ($NMDG^+$) was substituted for Na^+. The salt stimuli, NaCl and KCl, were dissolved in deionized water. Stock solutions of 10 mM amiloride (Sigma) were prepared in deionized water and diluted with one of the bathing solutions. These stock solutions were diluted with one of the bathing solutions immediately before use. Niflumic acid (Sigma) was dissolved in external solutions immediately before use.

Immunohistochemistry

The peeled lingual epithelia were fixed for 1 h at 4°C with periodic acid–lysine–paraformaldehyde (PLP) solution (McLean and Nakane, 1974) containing 0.075 M L-lysine monohydrochloride, 2% paraformaldehyde and 0.01 M sodium periodate in 0.0375 M phosphate-buffered saline, pH 7.4. The fixed epithelia were rinsed in 0.1 M phosphate buffer, pH 7.4, and preincubated for 1 h at room temperature with 1% bovine serum albumin (BSA) in phosphate-buffered saline, pH 7.4, containing 0.1% Triton X-100 (PBS/TX). The epithelia were then incubated with antibody to ClC-2 or ClC-3 (1:100 dilution in PBS/TX; Alomone Laboratories Ltd, Israel) for 48 h at 4°C. The epithelia were rinsed with PBS/TX containing BSA, followed by incuba-

tion with protein A–gold (6 nm; Upstate Biotechnology, USA) for 2 h at room temperature. The site of immunogold labeling was then visualized with a silver enhancement kit (British BioCell International, UK). The tissues were embedded in Epon-Araldite and cut into 2 μm sections, which were counterstained lightly with toluidine blue. The immunoreactivity of the tissues was detected and photographed with an optical microscope (Axiophoto; Zeiss, Germany). As immunohistochemical controls, epithelia treated with the dilution solution (PBA/TX containing BSA) and antibodies absorbed with each antigen in place of the primary antibody were processed simultaneously.

Results

Membrane properties of non-dissociated taste cells

In the present experiment we have recorded stable NaCl- and KCl-induced responses from a total of 152 of 348 cells obtained from taste buds of the fungiform papillae. As has been shown previously, taste cells obtained from mouse fungiform papillae display voltage-gated Na^+ and K^+ currents and sometimes spontaneous firing was observed (Miyamoto *et al.*, 1996; Furue and Yoshii, 1997). The voltage-gated currents, spontaneous firing and membrane potential were strongly affected by Na^+ removal from the external solution. Therefore, taste cells were identified by the presence of voltage-gated Na^+ and/or K^+ currents and by an elongated shape revealed by intracellular injection of a fluorescent dye, lucifer yellow (Bigiani and Roper, 1993; Miyamoto *et al.*, 1996). When high or low Cl^- K^+ solution was used as the internal solution, 55% of all taste bud cells (348 cells) examined displayed voltage-gated Na^+ inward currents and the rate of appearance of cells displaying inward currents was slightly increased (62% of 151 cells) when the K^+ outward current was suppressed by the Cs^+ internal solution.

The taste cells of the fungiform papillae showed a wide range of resting potentials [zero current potential (V_0)], from –21 to –80 mV with a mean of –43 ± 2 mV (n = 68), in NES, as previously reported (Miyamoto *et al.,* 1996). The input resistance measured from the minimum slope conductance (R_m) of the current evoked by a voltage ramp from –120 to 60 mV (180 mV/s) was 2.5 ± 0.2 GΩ (n = 68) and the membrane capacitance (C_m) of these cells was 6.6 ± 0.3 pF (n = 64). Seventy-five percent of taste cells responded to apically applied 0.5 M NaCl (Miyamoto *et al.*, 1998). The V_0 (–35 ± 2 mV, n = 34), R_m (1.8 ± 0.2 GΩ, n = 34) and C_m (7.4 ± 0.4 pF, n = 31) values of taste cells responsive to 0.5 M NaCl were significantly different from the V_0 (–54 ± 5 mV, n = 18), R_m (2.9 ± 0.5 GΩ, n = 18) and C_m (5.0 ± 0.5 pF, n = 17) values of taste cells not responsive to 0.5 M NaCl ($P < 0.0001$, $P < 0.02$ and $P < 0.01$, respectively). On the other hand, 94% of taste cells responded to 0.5 M KCl, so that the V_0 (–46 ± 4 mV, n = 18), R_m (3.4 ± 0.7 GΩ, n = 18) and C_m (6.7 ± 0.7 pF, n = 16) values of 0.5 M KCl-responsive taste cells were nearly equal to the average value of all taste cells examined.

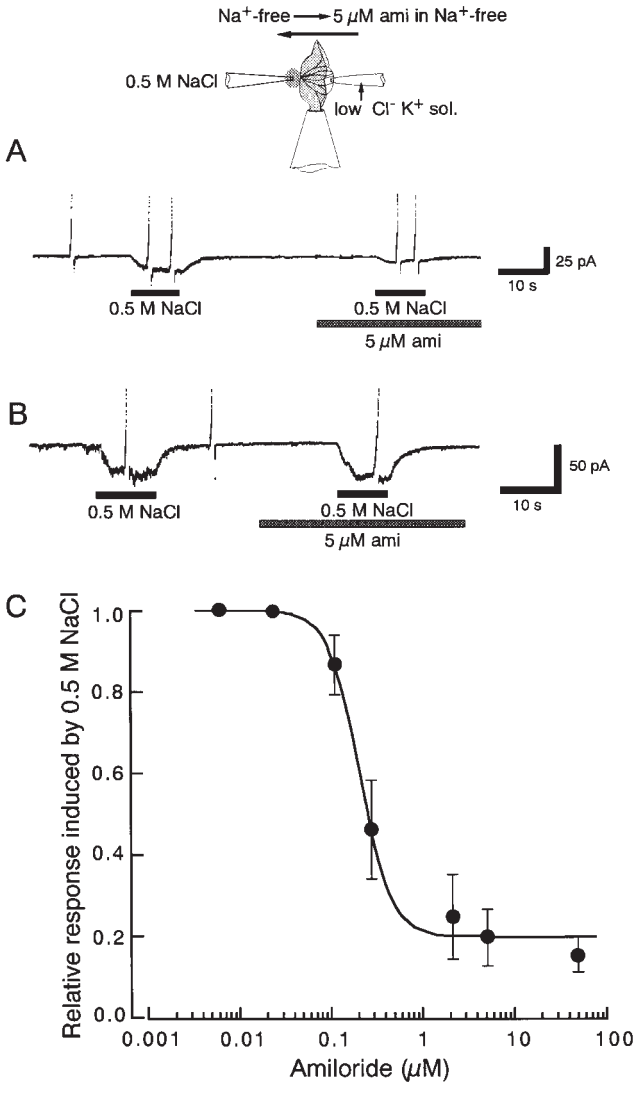

Figure 1 Inhibitory effect of amiloride on the 0.5 M NaCl-induced responses in taste cells. **(A)** Suppression of the 0.5 M NaCl-induced current response at –80 mV by 5 μM amiloride (ami) in an amiloride-sensitive taste cell. C57BL/6 mouse. **(B)** No suppression of the 0.5 M NaCl-induced current response at –80 mV by 5 μM amiloride (ami) in an amiloride-insensitive taste cell. BALB/c mouse. **(C)** Dose–response curve for amiloride inhibition of voltage responses induced by 0.5 M NaCl. The relative magnitudes of the responses with amiloride to those without amiloride were plotted against amiloride concentrations. Data were obtained from amiloride-sensitive cells of a C57BL/6 mouse. Each point represents the average (± SEM) of 3–16 cells. The solid line is a fit of the data using a sigmoidal binding equation, with a K_i of 0.2 μM. Insets in this and the other figures show configurations for stimulation, recording and perfusion.

Properties of NaCl-induced responses

Some NaCl-induced responses were suppressed by a diuretic, amiloride (amiloride-sensitive responses), but other NaCl-induced responses were not affected by amiloride (amiloride-insensitive responses). The fungiform papillae of

strain C57BL/6 contained more amiloride-sensitive taste cells than those of strain BALB/c, as has been previously reported (Miyamoto *et al.*, 1999). Figure 1 shows examples of NaCl-induced current responses obtained from an amiloride-sensitive taste cell (Figure 1A) and an amiloride-insensitive taste cell (Figure 1B). The mean magnitudes of the amiloride-sensitive current response induced by 0.5 M NaCl at a holding potential of –80 mV before and during 5 μM amiloride application were –14 ± 3 and –6 ± 1 pA (n = 13), respectively. The mean magnitudes of the amiloride-insensitive current responses induced by 0.5 M NaCl at a holding potential of –80 mV with and without 5 μM amiloride were –18 ± 4 and –15 ± 5 pA (n = 3), respectively, and no significant suppression by a 5 μM concentration of amiloride was observed.

In most of the amiloride-sensitive taste cells a large part of the 0.5 M NaCl-induced response was suppressed by a 5 μM concentration of amiloride with a K_i of 0.2 μM (Figure 1C). However, suppression was not complete even with application of 50 μM amiloride, which was the maximum dose used, with ~20% of depolarizing responses remaining even after 50 μM amiloride (Figure 1C). Because no gradual increase in response was observed during apical application of 0.5 M NaCl after amiloride adaptation, the residual response after amiloride application is not due to washing out of the amiloride by the stimulus application. Because the bathing solution was usually an Na^+-free solution, a depolarizing response mediated by influx of external Na^+ could not be generated at the basolateral membrane during NaCl stimulation.

The *I–V* relationships of amiloride-sensitive current responses were obtained from currents evoked by a voltage ramp from –100 to 60 mV (100 mV/s) before and during 0.5 M NaCl stimulation with or without amiloride (Figure 2A). The V_0 shifted from –70 mV before stimulation to –48 mV during stimulation and returned to –66 mV during stimulation with 5 μM amiloride in this case. These changes in V_0 roughly correspond to the membrane potential changes before and during stimulation with or without amiloride under current clamp conditions (data not shown).

The current responses induced by 0.5 M NaCl in a Na^+-free solution consisted of amiloride-sensitive and amiloride-insensitive components as described above. Figure 2B shows an example of the *I–V* relationships of the total, amiloride-sensitive and amiloride-insensitive current components estimated from differences in *I–V* relationships in the absence and presence of amiloride and with and without 0.5 M NaCl stimulation. The extrapolated E_r of the amiloride-sensitive current component was 103 ± 5 mV (n = 4) when the pipettes were filled with high Cl^- Cs^+ solution. The permeability ratio of Na^+ to Cs^+ estimated on the basis of the Goldman–Hodgkin–Katz equation (Hille, 1992) was 14.1. On the other hand, the E_r of the amiloride-insensitive component (22 ± 15 mV, n = 4) was close to the equilibrium potential of cations (E_{cat} = 36.0 mV) at the apical membrane. Thus, the E_r of the total current response induced by apically applied 0.5 M NaCl was 32 ± 4 mV (n = 10), which is a value between the E_r values of the amiloride-sensitive and amiloride-insensitive components.

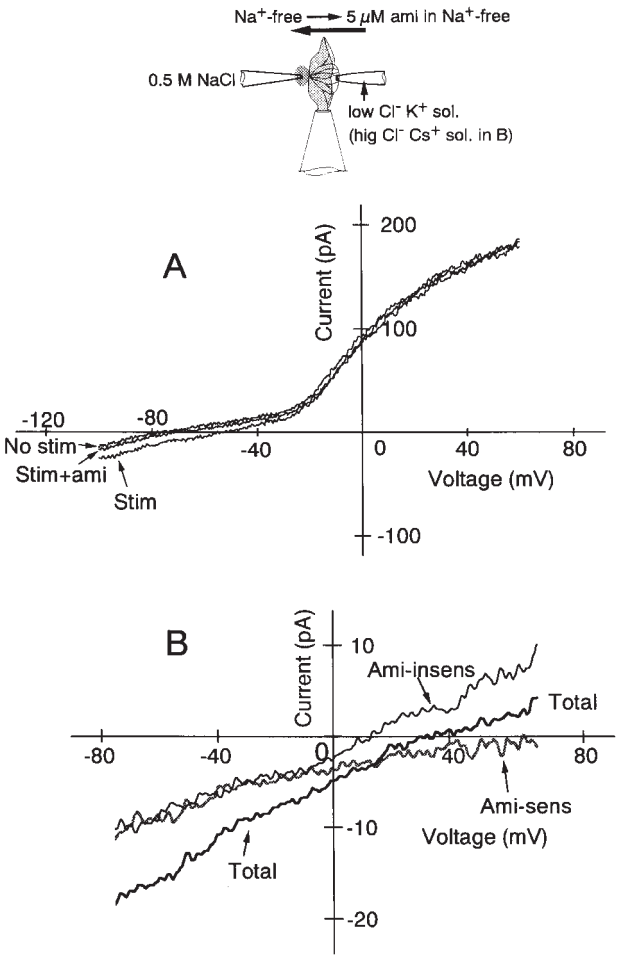

Figure 2 Amiloride-sensitive responses induced by 0.5 M NaCl in taste cells. **(A)** *I–V* relationships of currents induced by a voltage ramp from –100 to 60 mV (100 mV/s) before (no stim) and during 0.5 M NaCl stimulation in the absence (Stim) and presence of 5 μM amiloride (ami) (Stim + ami). C57BL/6 mouse. **(B)** *I–V* relationships of total (thick line), amiloride-sensitive (Ami-sens, dashed line) and amiloride-insensitive (Ami-insens) currents induced by 0.5 M NaCl. *I–V* relationships for these three components were estimated from differences in *I–V* relationships obtained before and during 0.5 M NaCl stimulation in the absence and presence of 5 μM amiloride. Liquid junction potentials are corrected in *I–V* relationships of this and the other figures. C57BL/6 mouse.

Suppression of the NaCl-induced response by Cd^{2+}

An inorganic cation channel blocker, Cd^{2+} (1 mM), partially suppressed the 0.5 M NaCl-induced responses in 60% of the taste cells examined. This suppression was fully reversible (Figure 3A–C). The magnitudes of the current response at a holding potential of –80 mV were –13 ± 1 (n = 5) and –6 ± 2 pA (n = 5) before and during application of 1 mM Cd^{2+}, respectively (Figure 3D).

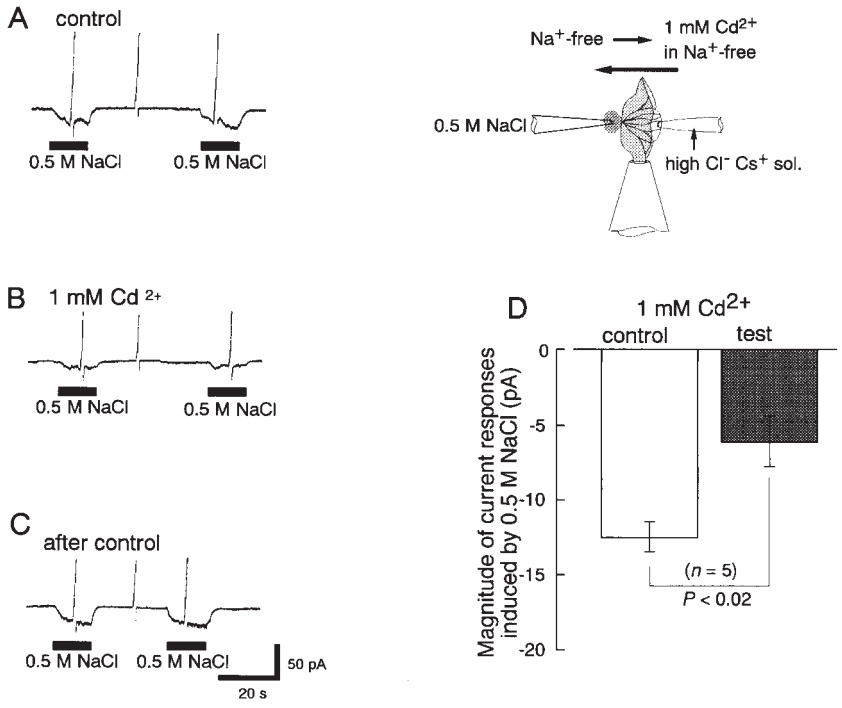

Figure 3 Effect of Cd^{2+} on 0.5 M NaCl-induced current responses in taste cells. (A–C) Current responses induced by 0.5 M NaCl at –80 mV before **(A)**, during **(B)** and after **(C)** 1 mM Cd^{2+}. **(D)** The magnitude of suppression at –80 mV. C57BL/6 mouse.

The V_0 was –54 ± 3 mV (n = 5) before 0.5 M NaCl stimulation, –36 ± 2 mV (n = 5) during stimulation and hyperpolarized to –42 ± 3 mV (n = 5) during application of 0.5 M NaCl plus 1 mM Cd^{2+}. Although the E_r of the total current during the response was 26 ± 19 mV (n = 5), that of the Cd^{2+}-sensitive component was 28 ± 9 mV (n = 5), and the permeability ratio of Na^+ to Cs^+ (P_{Na}/P_{Cs}) was calculated to be 0.73, indicating that the 0.5 M NaCl-induced Cd^{2+}-sensitive currents may be mediated by a type of non-selective cation channel.

Properties of KCl-induced responses

In contrast to the 0.5 M NaCl-induced responses, suppression of the 0.5 M KCl-induced responses by amiloride was never observed in the mouse taste cells examined (n = 8). Some taste cells had a deep resting potential and very high excitability under current clamp conditions, as seen in Figure 4A. As described above, most of these taste cells did not respond to 0.5 M NaCl but responded to 0.5 M KCl with strong depolarization and initiation of transient repetitive firing, both of which were unaffected by 5 μM amiloride (Figure 4A). Under voltage clamp conditions, 0.5 M KCl evoked inward current responses whose mean magnitude was –42 ± 6 pA (n = 16) at a holding potential of –80 mV and which were also unaffected by 5 μM amiloride (Figure 4B).

Taste cells in general display an inwardly rectifying K^+ current (I_{Kir}) (Kinnamon and Roper, 1988; Miyamoto *et al.*, 1991; Sun and Herness, 1996; Kolesnikov and Margolskee, 1998). Most of the mouse taste cells also display an I_{Kir} (no stim in Figures 5C and 6B and D). This current was greatly suppressed by submillimolar external Ba^{2+} (Figure 6D) without suppression of the outward current.

To compare the response to 0.5 M NaCl with that to 0.5 M KCl in the same cell, we attempted dual stimulation to the apical surface of a taste bud (see Figure 5, inset) and observed the effects of amiloride. Amiloride (10 μM) suppressed both the voltage and current responses induced by 0.5 M NaCl, but not the responses induced by 0.5 M KCl (right traces in Figure 5A and B). The I–V relationship of the responses to 0.5 M KCl was completely different from that of 0.5 M NaCl (Figure 5C). The I–V relationship of the inward current simply shifted to the right in response to 0.5 M NaCl, meaning that a shift of V_0 to a positive potential caused the voltage-independent increase in inward current during the response to 0.5 M NaCl (Figure 5C).

When 0.5 M KCl was apically applied as the taste stimulus, the inward-rectifying current (KCl-induced I_{ir}) was greatly potentiated with a positive shift of V_0 from –80 to –60 mV in the taste cell (Figure 5C). On average, V_0 was depolarized by apically applied KCl from –83 ± 3 (n = 9) to –66 ± 5 mV (n = 9) when Na^+-free solution was employed as the external solution and the patch pipette was filled with low Cl^- K^+ solution. A large part of the KCl-induced I_{ir} was also suppressed by submillimolar concentrations of external Ba^{2+} (Figure 6B and D) without suppression of the outward current.

The E_r of the KCl-induced I_{ir} was estimated from the

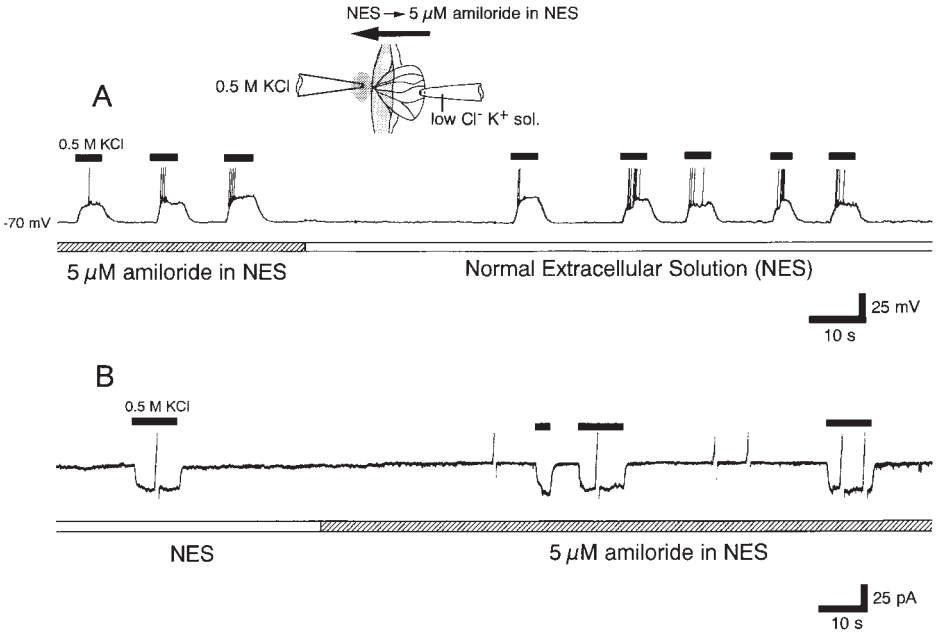

Figure 4 Effect of amiloride on the 0.5 M KCl-induced current responses in taste cells. **(A)** No effect of 5 μM amiloride on voltage responses with repetitive spikes induced by 0.5 M KCl under current clamp conditions. Action potentials were induced by depolarizing responses induced by 0.5 M KCl. C57BL/6 mouse. **(B)** No effect of 5 μM amiloride on current responses induced by 0.5 M KCl at –80 mV under voltage clamp conditions. C57BL/6 mouse.

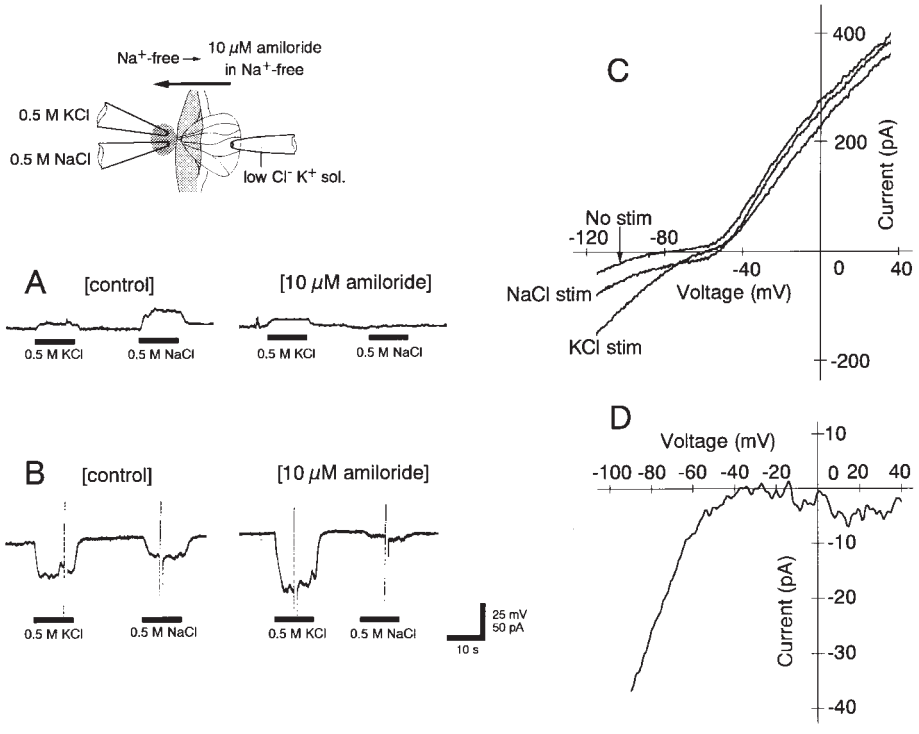

Figure 5 Comparison of KCl-induced responses with NaCl-induced responses in single taste cells. Differential effect of 10 μM amiloride on 0.5 M KCl- and 0.5 M NaCl-induced responses under current clamp **(A)** and under voltage clamp conditions at –80 mV **(B)**. The data in (A) and (B) were obtained from different cells. **(C)** *I–V* relationships of currents induced by a voltage ramp from –140 to 60 mV (200 mV/s) before (no stim) and after 0.5 M NaCl (NaCl-stim) or 0.5 M KCl stimulation (KCl-stim). The data was obtained from the same cell as in (B). **(D)** *I–V* relationships of the 0.5 M KCl-induced current estimated from differences in *I–V* relationships obtained before and during 0.5 M KCl stimulation. The data in (C) and (D) were obtained from different cells. The E_r is close to E_{Cl} at the basolateral membrane (–43.9 mV). C57BL/6 mouse.

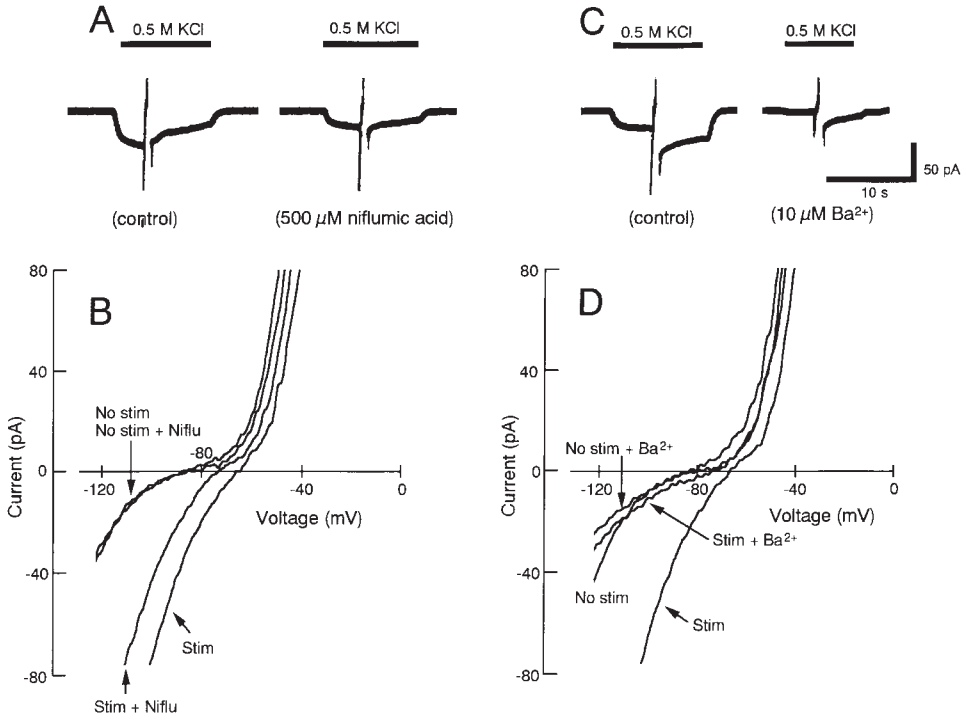

Figure 6 Comparison between the inhibitory effects of niflumic acid and Ba^{2+} on KCl-induced responses. **(A)** Suppression of the KCl-induced response by 500 μM niflumic acid. C57BL/6 mouse. **(B)** *I–V* relationships before (No stim) and during (Stim) 0.5 M KCl stimulation in the absence and presence of 500 μM niflumic acid (No stim + Niflu and Stim + Niflu). **(C)** Suppression of the KCl-induced response by 10 μM Ba^{2+}. C57BL/6 mouse. **(D)** *I–V* relationships before (No stim) and during (Stim) 0.5 M KCl stimulation in the absence and presence of 10 μM Ba^{2+} (No stim + Ba^{2+} and Stim + Ba^{2+}). C57BL/6 mouse.

difference between the *I–V* relationships before and during KCl stimulation. The E_r values with low Cl^- K^+ solution and with high Cl^- K^+ solution were –46 ± 4 (n = 9) and –9 ± 5 mV (n = 3), respectively, when taste buds were perfused with Na^+-free solution. As seen in Figure 5D, these E_r values were very close to E_{Cl} at the basolateral membrane (–43.9 mV with low Cl^- K^+ pipette solution; –4.2 mV with high Cl^- K^+ pipette solution), suggesting the involvement of Cl^- conductance (I_{Clir}) at the basolateral membrane of taste cells in the generation of the KCl-induced I_{ir}.

Suppression of the KCl-induced response by niflumic acid

A Cl^- channel blocker, niflumic acid, partially suppressed the KCl-induced I_{ir} (Figure 6A and B), resulting in preservation of the inward current, whose V_0 is close to E_K (–80 mV) at the basolateral membrane. These results suggest that opening of another functional channel which is sensitive to niflumic acid plays an important role in generation of the 0.5 M KCl-induced depolarization. In addition, the 0.5 M KCl-induced response was very sensitive to external Ba^{2+}. Suppression of the 0.5 M KCl-induced response by Ba^{2+} and the *I–V* relationships before and during 0.5 M KCl stimulation with and without application of external 10 μM Ba^{2+} are shown in Figure 6C and D. External Ba^{2+} suppressed not only the inward current induced by 0.5 M KCl but also the I_{Kir} in the absence of KCl stimulation (Figure 6D), whereas niflumic acid suppressed only the KCl-induced I_{ir} (Figure 6B). The KCl-induced I_{ir} was almost completely suppressed by 10 μM Ba^{2+}, whereas 500 μM niflumic acid showed only partial suppression (Figure 6), which was of the same degree as suppression by 5 μM Ba^{2+} (Figure 7).

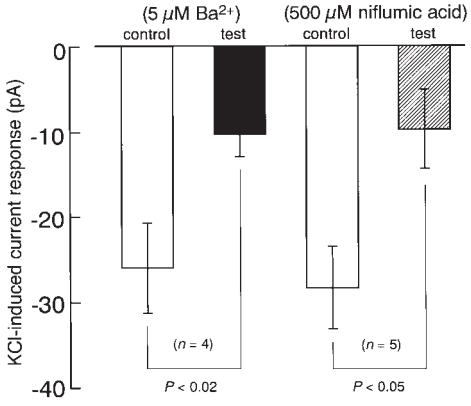

Figure 7 Comparison between the inhibitory magnitudes of 5 μM Ba^{2+} and 500 μM niflumic acid. Data were obtained from taste cells of a C57BL/6 mouse.

Run-down of the KCl-induced I_{ir}

Using the conventional whole cell clamp method, the 0.5 M KCl-induced response decreased time dependently, as seen

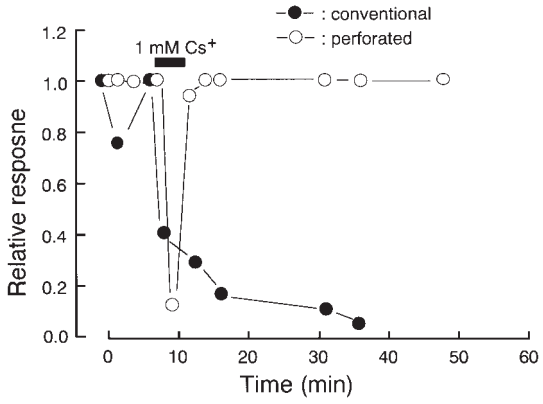

Figure 8 Run-down of the KCl-induced response. The 0.5 M KCl-induced I_{ir} spontaneously diminished, especially after inhibition by externally applied 1 mM Cs^+, using a conventional whole cell clamp method (filled circles), but not using the perforated patch technique (open circles). C57BL/6 mouse.

in Figure 8. The magnitude of the KCl-induced I_{ir} at a holding potential of –80 mV was retained for the first 10 min, but never recovered on washing after suppression by 1 mM Cs^+. On the other hand, the magnitude of the KCl-induced I_{ir} recorded using the perforated patch technique fully recovered after removal of Cs^+ and the magnitude remained at this level for at least 50 min in this cell. Run-down of the 0.5 M KCl-induced response was also observed without Cs^+ treatment. The response induced by 0.5 M NaCl was unaffected by both the conventional and perforated patch whole cell methods. These facts suggest that the mechanism of generation of the KCl-induced response strongly depends on some internal factors which are readily lost on internal dialysis, but that of the NaCl- induced response does not.

The presence of ClC-2 protein in lingual epithelium

ClC-2 protein, whose gene was first cloned from rat brain, is known to generate an inwardly rectifying Cl^- conductance in various type of cells, including epithelial cells and neurons (Thiemann *et al.*, 1992; Begenisich and Melvin, 1998). We observed that immunoreactivity for ClC-2 protein is present in the taste bud cells of the lingual epithelium (Figure 9). However, immunoreactivity for ClC-2 protein was clearly recognized on the serosal side but not in the mucosal region. Similar immunostaining for ClC-2 protein was observed in epithelial cells in the basal region of the lingual epithelium as well as taste bud cells. In contrast, we failed to find immunoreactivity for ClC-3 protein, which generates an outwardly rectifying Cl^- channel and contributes to volume regulation in several cells (Duan *et al.*, 1997), in either taste bud or surrounding epithelial cells of the lingual epithelium.

Discussion

Signal transduction for salty taste has been mainly examined by analysis of gustatory neural responses, but a high concentration of NaCl or KCl as the salt stimulus is

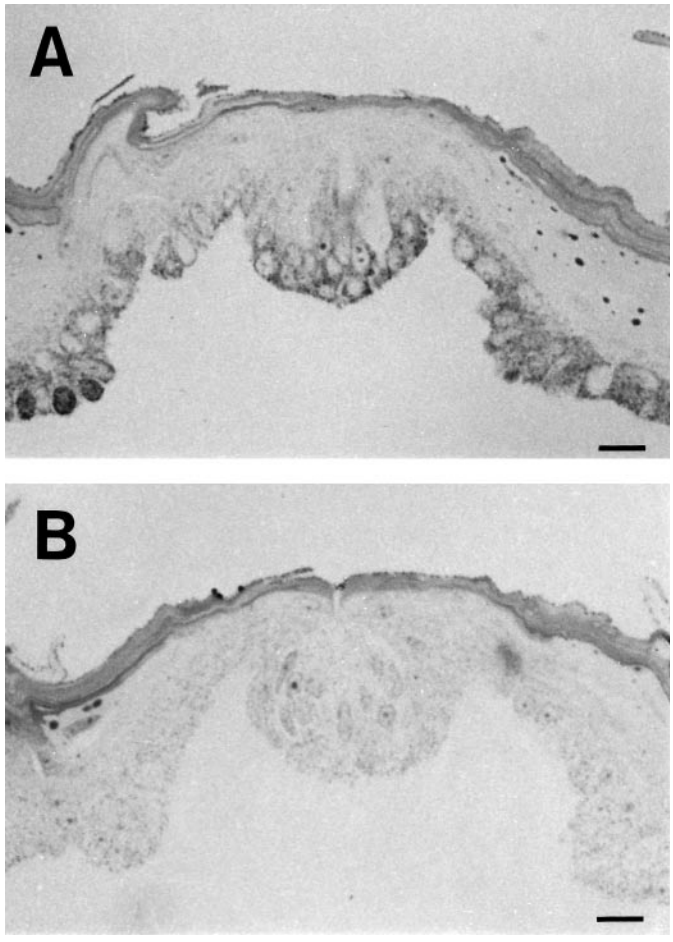

Figure 9 Immunohistochemical localization of an inwardly rectifying Cl^- channel protein, ClC-2, in taste bud cells of fungiform papillae. **(A)** Positive labeling of ClC-2 was detected in the basal region of the taste bud. Significant immunoreactivity was also present in the basal region of surrounding epithelial cells. **(B)** Control, no primary antibody. Scale bars 10 μm. C57BL/6 mouse.

harmful to isolated taste cells due to hypertonicity. We developed a localized stimulation method with non-dissociated taste cells (Miyamoto *et al.*, 1996), where the salt stimulus never affects the basolateral membrane directly due to a barrier of epithelial brim, excluding as much as possible any artificial effects generated by a salt stimulus containing a high concentration of NaCl or KCl. The procedure enabled us to constantly and repeatedly record 0.5 M NaCl- or KCl-induced current or voltage responses from non-dissociated taste cells.

Transduction mechanism of NaCl-induced responses

In the present experiment it has been demonstrated that the 0.5 M NaCl-induced responses consist of at least two different components, an amiloride-sensitive Na^+ conductance and a Cd^{2+}-sensitive non-selective cation conductance. These two components may contribute to the generation

of NaCl-induced responses of different degrees from cell to cell.

The absence or presence of the amiloride-sensitive component depended on the taste cell: fungiform taste cells were categorized into amiloride-sensitive and amiloride-insensitive cells (Miyamoto *et al.,* 1998, 1999). Amiloride inhibition of NaCl-induced responses showed a K_i of 0.2 μM, which is consistent with previous reports on mammalian taste cells (Avenet and Lindemann, 1991; Doolin and Gilbertson, 1996). However, NaCl-induced responses were not completely suppressed even by 50 μM amiloride, indicating that both amiloride-sensitive and amiloride-insensitive components exist simultaneously in a single taste cell.

More than 90% of taste cells responded to Na^+ removal due to a decrease in the Na^+-permeable conductance, but <60% of cells were amiloride-sensitive in the rat (Doolin and Gilbertson, 1996) and mouse (Miyamoto *et al.*, 1999). These facts indicate that the more dominant and common component in NaCl-responsive taste cells in the mouse is the amiloride-insensitive, non-selective cation conductance, as has been reported in frog taste cells (Miyamoto *et al.*, 1989; Fujiyama *et al.*, 1993; Okada *et al.*, 1998). The presence of a stationary cation conductance including amiloride-sensitive and amiloride-insensitive conductances may be the reason why NaCl-responsive cells display a small resting potential and low membrane resistance in NES. The amiloride-sensitive component in responses induced by 0.5 M NaCl displayed a high selectivity for Na^+ (P_{Na}/P_{Cs} = 14.1), which is roughly consistent with previous reports (Miyamoto *et al.*, 1996; Gilbertson and Fontenot, 1998).

In the present experiment we have shown that Cd^{2+}-sensitive cation channels, which display similar permeability for Na^+ and Cs^+ (P_{Na}/P_{Cs} = 0.73), possibly contribute to the generation of NaCl-induced responses other than amiloride-sensitive Na^+ channels. The amiloride-sensitive Na^+ and the Cd^{2+}-sensitive non-selective cation conductances may be located at the apical receptive membrane because the E_r values were much closer to the equilibrium potential of Na^+ (E_{Na}) and of cations (E_{cat}), respectively, at the apical receptive membrane than those at the basolateral membrane during 0.5 M NaCl stimulation.

Transduction mechanism of KCl-induced responses

More than 90% of taste cells in the mouse fungiform papillae responded to apically applied 0.5 M KCl, whereas only 75 and 50% of taste cells responded to 0.5 M NaCl and 25 mM citric acid, pH 3.0 (Miyamoto *et al.*, 1998). In contrast to the NaCl-induced responses, all the 0.5 M KCl-induced responses were insensitive to 5 or 10 μM amiloride. This result is consistent with the fact that responses induced by K salts are insensitive to amiloride (Ye *et al.*, 1994; Lindemann, 1996; Ninomiya *et al.*, 1996).

The chorda tympani responses induced by K and NH_4 salts are never suppressed by apically applied K^+ channel blockers, such as TEA, Ba^{2+} and Cs^+ (Ye *et al.*, 1994; Kloub *et al.*, 1997), other than the membrane permeant K^+ channel blocker 4-AP (Hille, 1992), which partially blocks K salt responses (Kim and Mistretta, 1993). Thus, it has been believed that transduction of responses induced by KCl is mainly generated at the basolateral membrane of taste cells (Lindemann, 1996; Stewart *et al.*, 1997), although a contribution of conductance at the apical membrane was not necessarily ruled out.

It has been reported that mammalian taste cells display an inwardly rectifying K^+ current (I_{Kir}) (Miyamoto *et al.*, 1996; Sun and Herness, 1996). In the present experiment we have found that apically applied 0.5 M KCl enhances the inwardly rectifying current (I_{ir}) with a shift of E_r which is roughly equal to E_{Cl} at the basolateral membrane. The 0.5 M KCl-induced response is blocked by submillimolar concentrations of external Ba^{2+} and Cs^+, suggesting that the KCl-induced I_{ir} seems to be a I_{Kir}. However, the KCl-induced I_{ir} is also suppressed by a Cl^- channel blocker, niflumic acid. Both Ba^{2+} and niflumic acid blocked the KCl-induced I_{ir}. Ba^{2+} suppressed not only the KCl-induced I_{ir} but also the basal I_{Kir} in the absence of KCl stimulation, however niflumic acid suppressed only the KCl-induced I_{ir}. In fact, 10 μM Ba^{2+} completely suppressed the KCl-induced I_{ir}, whereas 500 μM niflumic acid showed only partial suppression, which was approximately of the same degree as the suppression by 5 μM Ba^{2+}. These results indicate that the KCl-induced I_{ir} involves both inwardly rectifying K^+ current (I_{Kir}) and inwardly rectifying Cl^- current (I_{Clir}) components. The latter may be mediated by novel inwardly rectifying Cl^- channels, which are blocked by Ba^{2+} as well as niflumic acid. Recently the inwardly rectifying Cl^- conductance, which is identical to a cloned inwardly rectifying Cl^- channel protein ClC-2 (Thiemann *et al.*, 1992; Begenisich and Melvin, 1998), has been reported to be inhibited by Ba^{2+} much more strongly than known Cl^- channel blockers such as 5-nitro-2-(3-phenylpropylamino)benzoic acid and 4,4′-diisothiocyanostilbene-2,2′-di-sulphonic acid in porcine choroid plexus epithelial cells (Kajita *et al.*, 2000). An immunohistochemical experiment demonstrated the presence of ClC-2 protein in taste bud cells. These results suggest that ClC-2 contributes to the KCl-induced I_{ir}.

The KCl-induced I_{ir} readily underwent run-down with the conventional whole cell clamp method but was preserved for a long time (>30 min) using the perforated patch technique with amphotericin B. The E_r of the KCl-induced I_{ir} was independent of ionic concentrations at the apical receptive membrane. A preliminary result showed that a G protein activator, GTPγS, induced a similar I_{ir} and an adenylate cyclase inhibitor, SQ22536 suppressed the KCl-induced I_{ir}, suggesting that the transduction mechanism of the KCl-induced responses requires G_s-coupled adenylate cyclase to activate inwardly rectifying Cl^- channels localized at the basolateral membrane (Miyamoto *et al.*, 2000). The inwardly rectifying Cl^- channels in porcine choroid plexus

have been reported to be activated by intracellular protein kinase A (Kajita *et al.*, 2000).

Both KCl- and NaCl-induced responses are simultaneously observed in some cells, where NaCl-induced responses are amiloride-sensitive but KCl-induced responses are amiloride-insensitive. However, there is no evidence that KCl stimulation induces activation of non-selective cation channels, which may play an important role in the NaCl-induced responses as described above, resulting in the hypothesis that non-selective cation channels at the apical membrane are gated by Na^+ but not by K^+.

The E_r of the 0.5 M NaCl-induced responses showed a positive potential of ~32 mV, whereas the E_r of the 0.5 M KCl-induced responses showed a negative potential of ~–46 mV, indicating that 0.5 M KCl induces a hyperpolarizing response in taste cells, which have a resting membrane potential more positive than –45 mV, whereas 0.5 M NaCl is always able to induce a depolarizing response if a taste cell possesses a negative resting potential. NaCl-responsive taste cells possess a significantly more positive resting potential than KCl-responsive taste cells.

There may be two groups of taste cells contributing to salt transduction. One group of taste cells, which have a more positive resting potential than –46 mV, cannot produce a depolarizing response to KCl stimulation because the reversal potential of I_{Kir} is –46 mV. The other group of taste cells, which have a more negative resting potential than –46 mV, can produce a depolarizing response to both NaCl and KCl stimulation. Given that these two groups of taste cells are connected to different gustatory nerve fibers, the former group of taste cells can transmit only NaCl-induced responses, while the latter can transmit both. Thus, the difference in taste quality between NaCl and KCl can be discriminated by a difference in the resting membrane potential of a single taste cell. The response to KCl in a NaCl-best fiber is known to be much weaker than that to NaCl in the chorda tympani nerve (Ninomiya and Funakoshi, 1988; Hettinnger and Frank, 1990). Therefore, amiloride-sensitive taste cells connected to NaCl-best fibers may possess a relatively more positive resting potential caused by amiloride-insensitive cation conductance, resulting in a smaller KCl-induced depolarization than the NaCl-induced one.

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