Distribution and Characterization of Functional Amiloride-sensitive Sodium Channels in Rat Tongue

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ABSTRACT The role of amiloride-sensitive Na^+ channels (ASSCs) in the transduction of salty taste stimuli in rat fungiform taste buds has been well established. Evidence for the involvement of ASSCs in salt transduction in circumvallate and foliate taste buds is, at best, contradictory. In an attempt to resolve this apparent controversy, we have begun to look for functional ASSCs in taste buds isolated from fungiform, foliate, and circumvallate papillae of male Sprague–Dawley rats. By use of a combination of whole-cell and nystatin-perforated patch–clamp recording, cells within the taste bud that exhibited voltage-dependent currents, reflective of taste receptor cells (TRCs), were subsequently tested for amiloride sensitivity. TRCs were held at −70 mV, and steady-state current and input resistance were monitored during superfusion of Na^+-free saline and salines containing amiloride (0.1 μM to 1 mM). Greater than 90% of all TRCs from each of the papillae responded to Na^+ replacement with a decrease in current and an increase in input resistance, reflective of a reduction in electrogenic Na^+ movement into the cell. ASSCs were found in two thirds of fungiform and in one third of foliate TRCs, whereas none of the circumvallate TRCs was amiloride sensitive. These findings indicate that the mechanism for Na^+ influx differs among taste bud types. All amiloride-sensitive currents had apparent inhibition constants in the submicromolar range. These results agree with afferent nerve recordings and raise the possibility that the extensive labeling of the ASSC protein and mRNA in the circumvallate papillae may reflect a pool of nonfunctional channels or a pool of channels that lacks sensitivity to amiloride.

INTRODUCTION

The best-decribed mechanism for the transduction of salts (e.g., NaCl) has been Na^+ influx through amiloride-sensitive sodium channels (ASSCs) leading to a direct depolarization of taste receptor cells (TRCs; for reviews see Avenet, 1992; Gilbertson, 1993). Similar to ASSCs found in other transporting epithelia (Garty and Benos, 1988), ASSCs found in mammalian fungiform TRCs typically have single channel conductances near 5 pS (Avenet, 1992), have inhibition constants (K_i) in the submicromolar amiloride range (Avenet and Lindemann, 1988; Gilbertson et al., 1993), are highly selective for Na^+ over K^+ (DeSimone et al., 1984; Heck et al., 1984), are permeable to protons (Gilbertson et al., 1992, 1993), and are apparently regulated by the same hormones that regulate epithelial ASSCs (Gilbertson et al., 1993).

Although ASSCs are clearly important in the detection of salty stimuli, these channels are apparently not the exclusive transduction mechanism for NaCl. Indeed, afferent nerve responses to NaCl in the mudpuppy (McPheeters and Roper, 1985) and in certain mouse strains (Tonosaki and Funakoshi, 1989) are completely insensitive to amiloride. More typically found in animals is a complex of ASSCs and amiloride-insensitive components. Recordings from the glossopharyngeal nerve (IX) suggest that TRCs in the circumvallate and posterior foliate papillae respond to salts, although at higher concentrations than needed for the more anterior TRCs (Hanamori et al., 1988; Frank, 1991). Furthermore, the salt response recorded in the glossopharyngeal nerve is apparently amiloride insensitive (Hanamori et al., 1988; Formaker and Hill, 1991). Behavioral assays in hamster showing that amiloride, even at high concentrations, when presented simultaneously with NaCl solutions cannot inhibit salt

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responses completely have further supported the presence of amiloride-insensitive salt transduction mechanisms in mammalian species (Hettinger and Frank, 1990; Gilbertson and Gilbertson, 1994). Taken together, the evidence suggests that ASSCs may be absent in mammalian TRCs innervated by nerve IX. However, the relative contribution of these amiloride-insensitive pathways to NaCl detection remains unclear. In rat, pretreatment of the tongue with amiloride prevents the subsequent formation of LiCl-induced conditioned taste aversions to 100 mM NaCl but not 500 mM NaCl (Hill et al., 1990), suggesting that higher NaCl concentrations are required to stimulate amiloride-insensitive pathways.

The recent cloning of the ASSC from rat colon (Lingueglia et al., 1994; Canessa et al., 1994) led to experiments that questioned the conclusion that ASSCs are absent from glossopharyngeal-innervated TRCs. Using in situ hybridization and immunohistochemistry, ASSC mRNA and protein were localized in circumvallate TRCs and the surrounding epithelium (Li et al., 1994; Li and Snyder, 1994; Simon et al., 1993). The disagreement between immunohistochemical detection of ASSCs and the insensitivity of NaCl responses to amiloride was also shown in developing rat fungiform TRCs. While ASSCs may be detected immunohistochemically as early as postnatal day 1 (Stewart et al., 1993), amiloride-sensitive NaCl responses cannot be detected in chorda tympani nerve (VII) nerve recordings until 12–13 d postnatally (Hill and Bour, 1985). A preliminary report suggested that these immunohistochemically identified ASSCs are functional as early as postnatal day 2 (McPheeters et al., 1994). Though the reason for amiloride insensitivity in the afferent nerve recordings remains unclear, the recent finding that a class of basolaterally restricted ASSCs with a significantly higher K_i for amiloride (~50 μM; Mierson et al., 1994) may be present in rat TRCs suggests that early in development ASSCs may be restricted to the basolateral membrane.

The present study attempts to resolve the controversy between the apparent existence of ASSCs in the circumvallate papilla and the insensitivity of NaCl responses to amiloride in glossopharyngeal nerve recordings. Using a combination of whole-cell and nystatin-perforated patch–clamp recording configurations on TRCs maintained in taste buds isolated from fungiform, foliate, and circumvallate papillae, we examined the proportion of TRCs containing *functional* ASSCs. The results show that ASSCs are present in roughly two thirds of the fungiform TRCs, one third of the foliate TRCs, and that circumvallate TRCs apparently lack functional ASSCs. In agreement with earlier reports, all ASSCs in the present study exhibit K_is for amiloride in the submicromolar range (Avenet and Lindemann, 1988; Gilbertson et al., 1993). Using amiloride concentrations of up to 1 mM, we found no evidence to support the presence of a separate class of ASSCs with a higher K_i. The present results suggest that the lack of amiloride sensitivity of salt responses of the glossopharyngeal nerve is due to the absence of functional ASSCs in the TRCs innervated by nerve IX and that the detection of ASSC mRNA and protein may reflect a nonfunctional pool or a pool of channels that lacks sensitivity to amiloride. Part of these results have appeared in abstract form (Doolin and Gilbertson, 1995).

MATERIALS AND METHODS

Taste Bud Isolation

The procedure to isolate all three types of TRCs was similar to that described by Béhé et al. (1990) and Gilbertson et al. (1993). In brief, all three taste cell types were obtained from 2- to 5-month-old male Sprague–Dawley rats. Tongues were removed from rats and immediately immersed in cold Tyrode's solution. To isolate the fungiform TRCs, 3 ml of Tyrode's containing 15 mg of dispase (grade II; Boehringer Mannheim Corp., Indianapolis, IN), 3 mg of trypsin inhibitor (type I-S; Sigma Chemical Co., St. Louis, MO), 1.5 mg of collagenase A (Boehringer Mannheim Corp.), and 3 μM amiloride was injected beneath the epithelia containing these TRCs. The epithelium containing foliate and circumvallate TRCs was isolated by injecting 2 ml of the Tyrode's/dispase solution beneath the epithelium from the cut end of the tongue. Amiloride was included in the enzyme cocktail to protect the amiloride sensitive sodium channels from enzymatic degradation (Garty and Edelman, 1983; Gilbertson et al., 1993). The tongue was then placed in oxygenated Ca^{2+}–Mg^{2+}-free Tyrode's and incubated for 35 min. After incubation, the section of lingual epithelium containing the TRCs was peeled off and pinned in a Sylgard-lined petri dish with the serosal side up. The sections of epithelium containing fungiform, foliate, and circumvallate papillae, isolated in this manner, are visibly different (Fig. 1). Mild suction through a fire-polished pipette (~200 μm diameter) was used to isolate individual taste buds and to plate them on a Cell-Tak-coated slide (Collaborative Biomedical Products, Bedford, MA). Once isolated, however, no morphological differences were observed between the three TRC types (Fig. 1). Taste buds isolated in this manner retained this morphology for several hours with only minor rounding of the taste bud occurring at their apical pole.

Solutions

Normal extracellular solution (Tyrode's) contained (in mM): NaCl, 140; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; HEPES, 10; glucose, 10; and Na^+ pyruvate, 10. The pH was adjusted to 7.40 with NaOH. In Ca^{2+}–Mg^{2+}-free Tyrode's, 2 mM BAPTA (Molecular Probes Inc., Eugene, OR) replaced $CaCl_2$ and $MgCl_2$. Other components were identical to Tyrode's. The only alteration to Tyrode's to make a Na^+-free solution was to replace NaCl with equimolar *N*-methyl-D-glucamine (NMDG), a large impermeant cation.

Amiloride solutions (.001 μM to 1 mM) were made up in normal Tyrode's. For concentrations above 100 μM, amiloride was dissolved into dimethyl sulfoxide (DMSO) at a concentration of

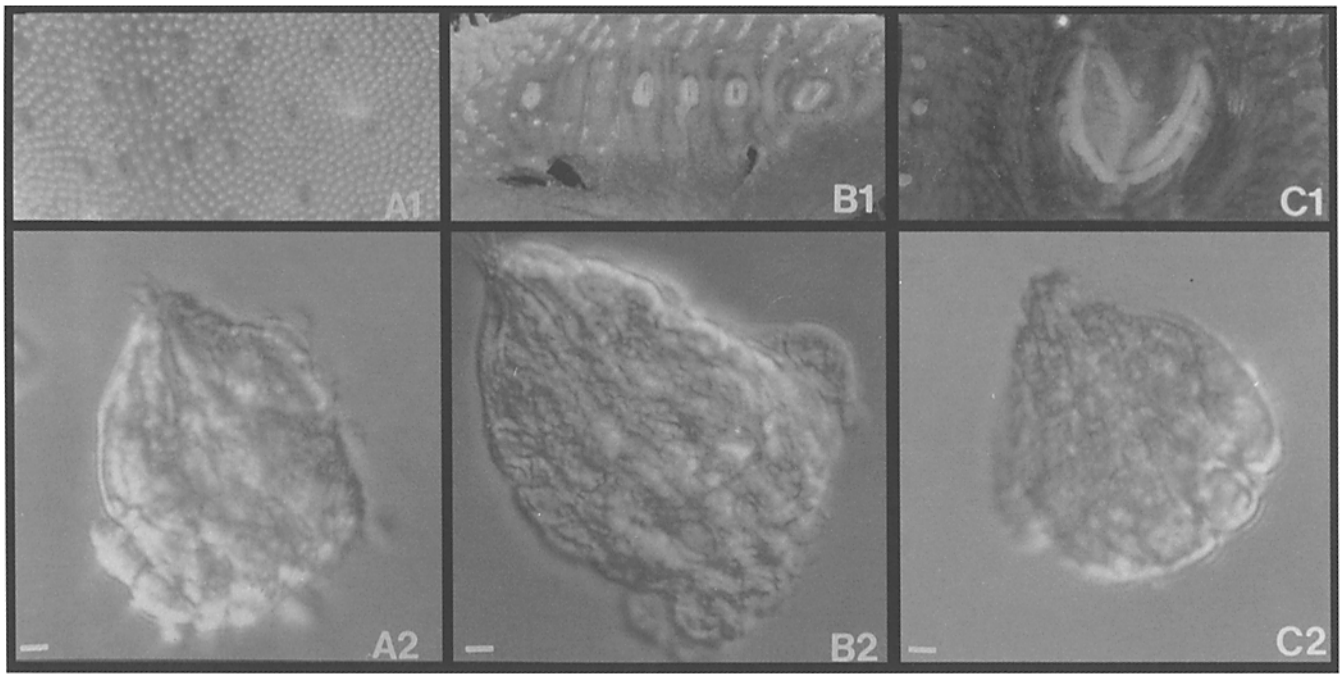

FIGURE 1. Isolated lingual epithelium and taste buds from the fungiform (*A1, A2*), foliate (*B1, B2*), and circumvallate papillae (*C1, C2*). Scale bar, 5 μm.

1 M and diluted to its final concentration. The final DMSO concentration in these solutions never exceeded 0.1%, and at this concentration, DMSO had no effect on TRCs (personal observation).

Two types of intracellular (pipette) solution were used in the present work. For whole-cell recordings, intracellular solution consisted of the following (in mM): KCl, 140; $CaCl_2$, 1; $MgCl_2$, 2; HEPES, 10; EGTA, 11; and ATP, 3. The pH was adjusted to 7.20 with KOH. For perforated patch recordings, intracellular solution was prepared by first dissolving nystatin in DMSO to a final concentration of 50 mg/ml. Fresh preparation of this stock solution for each new experiment is required for formation of successful perforated patches. The nystatin–DMSO solution was diluted to 250 μg/ml in a solution containing the following (in mM): KCl, 55; K_2SO_4, 75; $MgCl_2$, 8; and HEPES, 10 (pH 7.20 with Tris–OH). To this solution, pluronic (Molecular Probes, Inc.) was added to a final concentration of 0.2% (w/v), and this solution was sonicated. Pipette tips were filled with nystatin-free solution prior to being backfilled with nystatin solution. All chemicals were obtained from Sigma Chemical Co. unless otherwise indicated.

Electrophysiological Recording

The two patch–clamp configurations used to record steady-state currents were either whole-cell (Hamill et al., 1981) or nystatin perforated (Horn and Marty, 1988; Korn et al., 1991). The pipettes were made from microhematocrit tubes (Scientific Products, Inc., McGaw Park, IL) on a Flaming/Brown micropipette puller (model P-97; Sutter Instrument Co., Novato, CA) to a resistance of 4 and 8 MΩ when filled with intracellular solution. Typical seal resistances on TRC membranes ranged from 1 to 20 GΩ.

Whole-cell membrane currents for both voltage-gated channels and ASSCs were recorded with an Axopatch patch–clamp amplifier (model 200A, Axon Instruments, Inc., Foster City, CA) and were filtered at 2 kHz through a lowpass Bessel filter. pCLAMP 6.0.2 software (Axon Instruments, Inc.) was used for both stimulation and recording of membrane currents. Steady-state TRC responses to Na^+-free saline and salines containing amiloride (0.1 μM to 1 mM) were recorded using AxoTape software (version 2.0.2, Axon Instruments, Inc.) sampling at a rate of 10 kHz. The signal was displayed on a strip chart recorder (Gould RS-3200) filtered at 15 Hz. A stimulator (model S-900; Dagan Corp., Minneapolis, MN) was used to induce brief 15 mV hyperpolarizing pulses into the TRCs (held at −70 mV) every 3 s to monitor input resistance.

RESULTS

Both giga seal whole-cell and nystatin-perforated patch recording configurations were used in the present study. The use of nystatin-perforated patches provided an increase in recording duration and stability. In agreement with a previous study by Gilbertson et al. (1993), no other differences were observed between conventional whole-cell patches and perforated patch–clamp configurations. For this reason, results from both techniques were pooled. In all experiments, care was taken to record from TRCs in all regions of the isolated taste bud (i.e., cells on the inner and outer portions) and from multiple taste buds in a single preparation. In the present work and in our previous studies on hamster TRCs (Gilbertson et al., 1993), we have found no significant differences in the physiology of TRCs as a function of location within the taste bud.

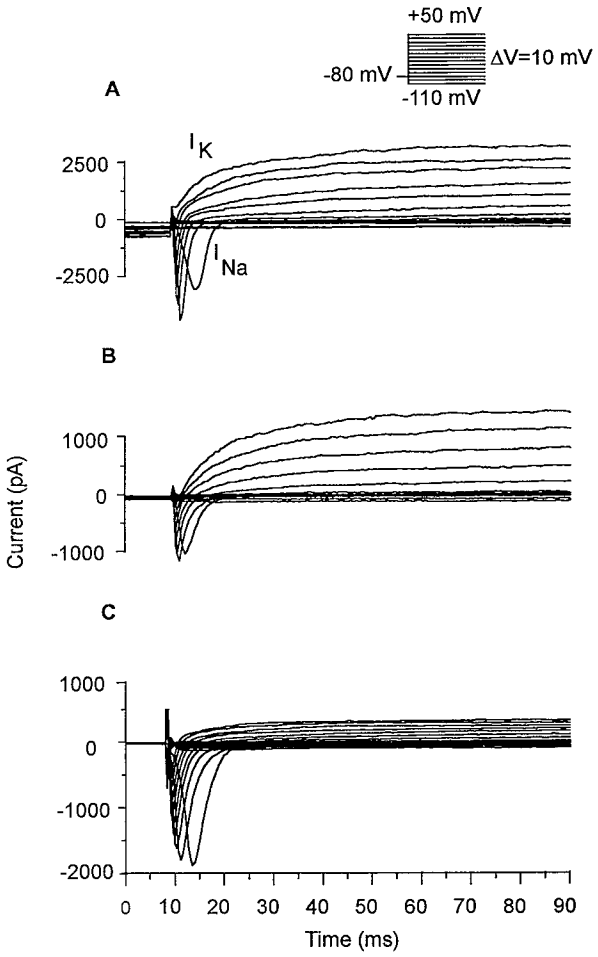

FIGURE 2. Whole-cell voltage-activated currents in isolated taste receptor cells. Fungiform (*A*), foliate (*B*), and circumvallate (*C*) TRCs exhibit voltage-activated Na^+ and K^+ currents upon depolarizations from −80 mV. The variability shown above represents the normal range of currents and does not reflect a consistent difference among TRC types. All three types of TRCs are capable of generating action potentials (data not shown).

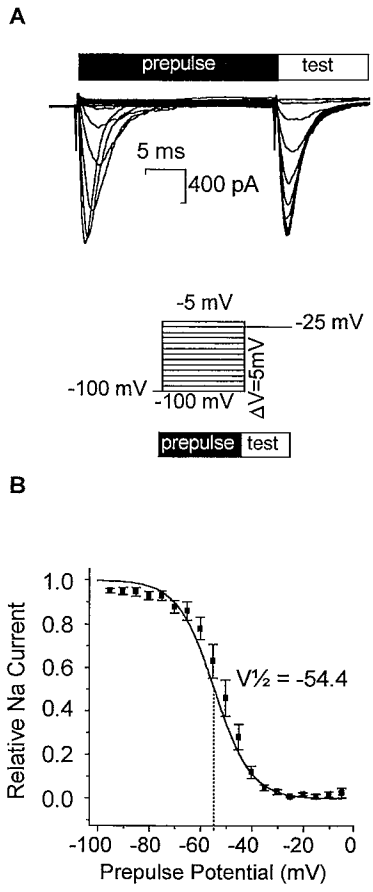

FIGURE 3. (*A*) Two-pulse protocol used to assess Na^+ channel inactivation. (*B*) Mean inactivation curves from fungiform TRCs (n = 5). Foliate (−52.0 mV; n = 5) and circumvallate (−57.3 mV; n = 5) TRCs had similar half-inactivation potentials (data not shown). Data points were fit by the relation $I = 1/(1 + \exp(V_p - V_{1/2})/k)$, as described in the text. Half-inactivation potentials ($V_{1/2}$) were not significantly different among taste bud types. Graph of mean ± SEM.

Voltage-activated Whole-cell Currents

As a precursor to our investigation on ASSC distribution in fungiform, foliate, and circumvallate TRCs, we recorded whole-cell voltage-activated currents from >200 cells. The goals of this first set of experiments were twofold. First, finding voltage-activated K^+ and/or Na^+ currents allowed the unequivocal identification of the cell as a TRC and not an epithelial cell (Kinnamon and Roper, 1988; Akabas et al., 1990; Gilbertson et al., 1993). Second, because there is comparatively little information on rat circumvallate TRCs and none on rat foliate TRCs, we intended to compare macroscopic whole-cell currents among TRC types.

In each of the three classes of TRCs, ~50% of cells exhibit voltage-activated, tetrodotoxin (TTX)-sensitive transient Na^+ currents and sustained outward K^+ currents similar to those reported in TRCs from rat and other species (Fig. 2) (for review see Gilbertson, 1993). Roughly one third of the cells had only voltage-activated K^+ currents, which may be indicative of developing TRCs (McPheeters et al., 1994). The remaining 15% of cells displayed no voltage-activated conductances, despite having the elongate morphology expected of TRCs. At the whole-cell level, no differences were observed among TRC types with respect to current densities. Observed variations reflect the normal variability among the classes of TRCs (Fig. 2).

Because action potential frequency in TRCs apparently encodes stimulus intensity (Avenet and Lindemann, 1991; Gilbertson et al., 1992; Cummings et al., 1993), we investigated the voltage-activated Na^+ channel kinetics as a possible distinguishing characteristic among TRC classes, which may contribute to underlying differences in salt responsiveness. The practical reason for our initial focus on Na^+ channel kinetics was that these experiments were complementary to our main goal of determining the ASSC distribution and required no additional preparation. Using a two-pulse protocol (Fig. 3 *A*), the kinetics of Na^+ channel inactivation were assessed by plotting the peak Na^+ current elicited during the test period as a function of the prepulse potential (V_p; Fig. 3 *B*). The resulting points

were fit to the following relation to determine the half-inactivation voltage ($V_{1/2}$) by the Marquardt–Levenberg algorithm (ORIGIN, Microcal Software, Northampton, MA) weighted to the inverse of the variance:

$$I = 1/(1 + \exp[V_p - V_{1/2}]/k) \quad (1)$$

where I is the relative Na^+ current, k is the slope coefficient, and V_p and $V_{1/2}$ are the prepulse and half-inactivation potentials, respectively. Half-inactivation potentials for the fungiform, foliate, and circumvallate TRCs were calculated to be −54.4, −52.0, and −57.3 mV, respectively, and were not significantly different from one another.

Amiloride-sensitive Na^+ Currents

Because of the discrepancy between reports showing the presence of ASSC protein and mRNA (Simon et al., 1993; Li et al., 1994) and those showing an apparent lack of amiloride sensitivity in glossopharyngeal nerve NaCl responses (Formaker and Hill, 1991), we attempted to determine the distribution of functional ASSCs in the three main classes of TRCs. To be included in this analysis, cells had to meet criteria that were previously established for mammalian TRCs (Gilbertson et al., 1993). In brief, TRCs had to display both voltage-activated Na^+ and K^+ currents (see above), have an elongate morphology, and provide a stable (i.e., drift-free) recording for >5 min. The requirement for both Na^+ and K^+ channel activity helped ensure that we were not including developing cells in our analysis (Kinnamon and Roper, 1988), which may not express the ASSC. To ensure, however, that we were not artificially selecting a distinct subpopulation of TRCs by limiting our analysis to only those cells that had both Na^+ and K^+ channels, in separate experiments we investigated the amiloride sensitivity of cells that contained only K^+ currents from each of the three classes.

Initially, TRCs that had both voltage-activated Na^+ and K^+ channels were tested for evidence of electrogenic Na^+ transport by monitoring steady-state currents at −70 mV during replacement of extracellular Na^+ with the large impermeant cation NMDG. In >90% of the TRCs of each type (Table I), Na^+ replacement caused a large reduction in inward current, concomitant with an increase in cellular input resistance. These effects are consistent with a decrease in inward Na^+ movement. A similar effect is seen when the subset of TRCs that possess ASSCs are superfused with Tyrode's containing amiloride (0.001–1000 μM; Fig. 4, *A* and *B*). As expected, there was a direct correlation between the magnitude of current reduction and the degree of change in the input resistance for both Na^+ removal and for amiloride inhibition (when effective). This relationship was similar for all TRC types and suggests that both the amiloride-sensitive and the amiloride-insensitive Na^+ pathways have similar permeability properties.

27 of 43 (62.8%) mature fungiform TRCs (i.e., those with both Na^+ and K^+ currents as in Fig. 2) were amiloride sensitive, a figure similar to the proportion of fungiform TRCs that were amiloride sensitive in hamster (Gilbertson et al., 1993). The remaining 16 cells responded to Na^+ replacement as described above, but did not show a conductance decrease to amiloride at any concentration (0.001–1000 μM). These responses may be reflective of the amiloride-insensitive Na^+ transport described in other preparations (McPheeters and Roper, 1985; Tonosaki and Funakoshi, 1989). On the other hand, although >90% of circumvallate TRCs responded to Na^+ replacement in the same manner as fungiform TRCs, no circumvallate TRC responded to amiloride with a conductance decrease (Figs. 4 *C* and 5 *C*; Table I), and there was no apparent change in input resistance (data not shown). Responses of foliate TRCs to amiloride lie intermediate between the other two classes. Approximately 36% of the foliate TRCs tested that responded to Na^+ replacement also responded to amiloride (n = 22 cells). When KCl was used to replace NaCl in the extracellular solution, a decrease in resting current occurred. At all potentials, amiloride had no effect on the resting current in high K^+ solutions in TRCs (data not shown). This re-

TABLE I

Distribution of ASSCs in Rat TRCs

Taste bud type	Voltage-activated currents	Responded to Na^+ removal	Percent Na^+ sensitive	Responded to amiloride	Percent amiloride sensitive
Fungiform	Na^+ and K^+	56 of 61	91.8	27 of 43	52.8
	K^+ only	8 of 8	100.0	6 of 8	75.0
Foliate	Na^+ and K^+	23 of 24	95.6	8 of 22	35.4
	K^+ only	5 of 6	83.3	2 of 5	40.0
Circumvallate	Na^+ and K^+	27 of 30	90.0	0 of 26	0.0
	K^+ only	6 of 8	75.0	0 of 5	0.0

Na^+ sensitivity was defined as those cells showing a decrease in conductance during perfusion of Na^+-free saline. Amiloride-sensitive cells showed a similar decrease in current and increase in resistance during bath perfusion on amiloride (0.001–1,000 μM) in normal saline. TRCs with only voltage-activated K^+ currents had a similar proportion of ASSCs as those with both voltage-activated Na^+ and K^+ currents (mature TRCs).

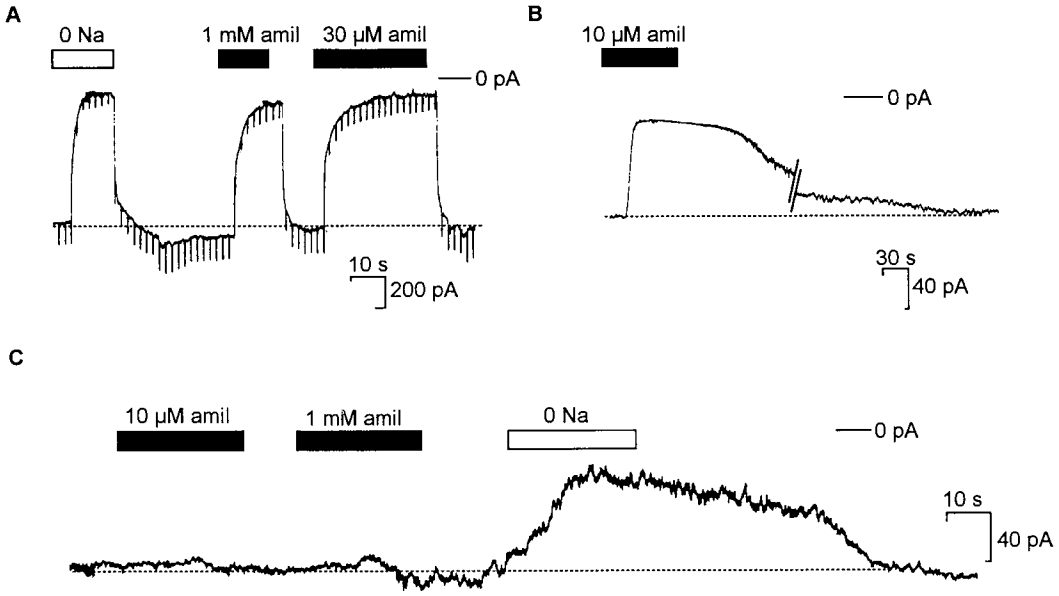

FIGURE 4. Effect of amiloride (*amil*) on steady-state currents in isolated rat fungiform (*A*), foliate (*B*), and circumvallate (*C*) TRCs. Cells were held at −70 mV, and either Na^+-free saline (*0 Na^+*; NMDG-Cl substituted for NaCl) or amiloride in normal saline was bath applied. An upward deflection (i.e., reduction in current) is consistent with a decrease in inward Na^+ movement. Brief downward deflections in fungiform trace are responses to brief 15 mV hyperpolarizations used to monitor input resistance. Amiloride had no effect on steady-state currents in circumvallate TRCs.

sult is consistent with the ASSCs in hamster fungiform taste tissue, which are highly Na^+ selective (over K^+) (Gilbertson et al., 1992).

Similar experiments to determine amiloride sensitivity were performed on TRCs that exhibited only voltage-activated K^+ currents from each of the three classes of taste buds. These cells, which may reflect developing, or immature, TRCs showed a similar distribution of amiloride sensitivities across the different bud types (Table I). Most responded to Na^+ replacement with a decrease in holding current and conductance decrease. Greater than half of the fungiform TRCs were amiloride sensitive; none of the circumvallate TRCs responded to amiloride; and the foliate TRCs fell in between the two. On the basis of amiloride sensitivity, there was no significant difference between TRCs that had K^+ currents only and those with both Na^+ and K^+ currents.

In the vast majority of TRCs, Na^+ removal caused a larger decrease in steady-state current than did maximal doses of amiloride. This is consistent with amiloride-sensitive Na^+ transport being a subset of the total Na^+ transport. In both fungiform and foliate TRCs that had ASSCs, the ratio of amiloride-sensitive Na^+ current to the total Na^+ current overlapped and was highly variable. In both fungiform and foliate TRCs, the proportion of amiloride-sensitive Na^+ current to the total Na^+ current ranged from as little as 20% to greater than 95% of the total Na^+ current. This high degree of variability was indicative of individual TRC differences, since this range was found within both single taste buds and individual experiments. Furthermore, there was no significant difference in the time course of the current response during Na^+ removal or amiloride application. The rates of current change seen in Fig. 4 represent individual cell variability (as above) and solely reflect, we believe, differences in perfusion rates. Our efforts to record from a variety of TRCs within a given taste bud create differences in the rate at which the perfusate is applied to individual TRCs.

To determine whether there exist multiple classes of ASSCs differing in their sensitivity to amiloride, as has been suggested in a recent preliminary report (Mierson et al., 1994), we determined the K_is for ASSCs in both fungiform and foliate TRCs. Increasing extracellular concentrations of amiloride caused subsequent decreases from the resting current of −70 mV in fungiform and foliate TRCs (Fig. 5, *A* and *B*). Maximal inhibition of the current occurs at amiloride concentrations between 10 and 20 μM. The apparent K_is for fungiform and foliate TRCs are in the submicromolar range (0.01–0.1 μM), similar to values reported for frog (Avenet and Lindemann, 1988) and hamster (Gilbertson et al., 1993) TRCs. There was no evidence of a separate class of amiloride-sensitive Na^+ currents that were inhibited at concentrations >30 μM. On the basis of these results, there appears to be a single functional and pharmacological class of ASSCs found in mammalian TRCs.

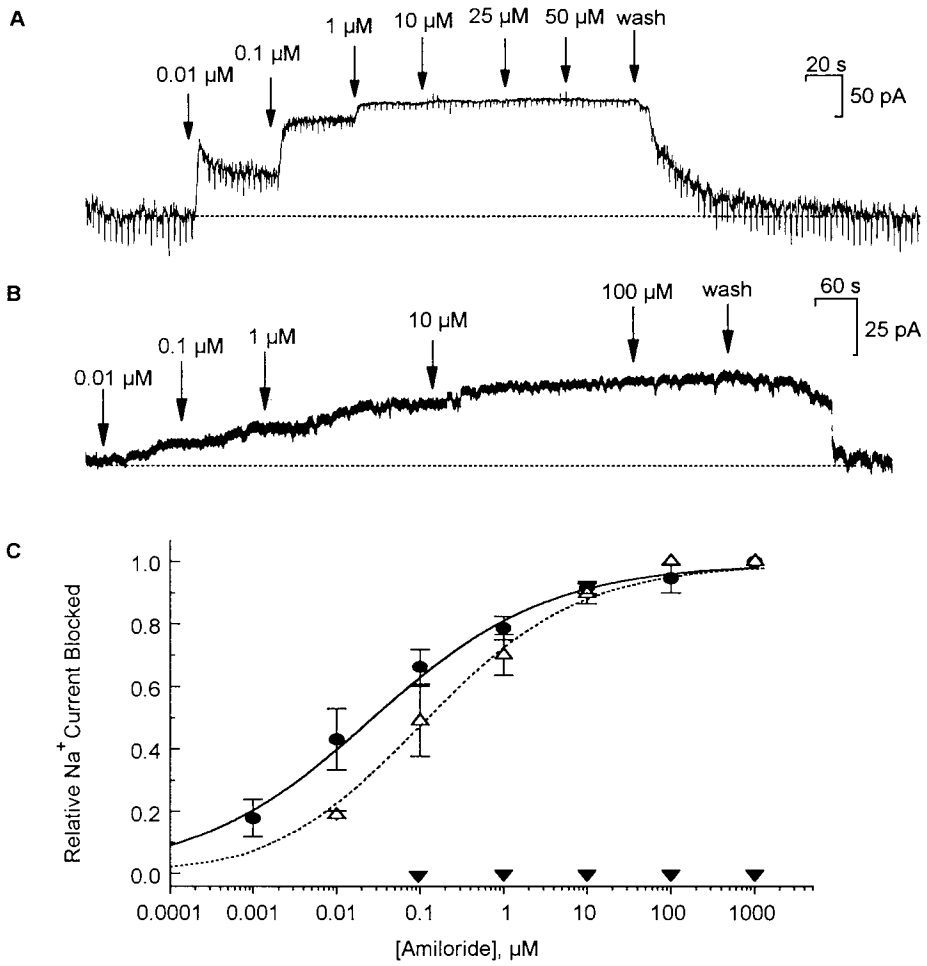

FIGURE 5. Dose–response of amiloride-inhibition of Na^+ current in a fungiform (*A*) and a foliate (*B*) TRC. Concentrations above ~20 μM amiloride maximally blocked ASSC activity. (*C*) Dose–response curve for inhibition of ASSCs. Amiloride inhibition constants (K_i) for inhibition of ASSCs in fungiform (—●—; n = 27) and foliate TRCs (···△···; n = 8) are in the submicromolar range, while amiloride has no effect upon Na^+ currents in circumvallate TRCs (▼; n = 26). Graph of mean ± SEM. Curves are best fits using standard dose–response relationship.

DISCUSSION

In the present study, we attempted to resolve conflicting reports on the distribution of functional ASSCs in mammalian TRCs. On one hand, responses of the glossopharyngeal nerve to NaCl are insensitive to mucosal amiloride (Formaker and Hill, 1991). However, recently, both ASSC mRNA (Li et al., 1994) and protein (Simon et al., 1993) have been localized to the circumvallate papillae. Using patch–clamp recording configurations on TRCs isolated from rat fungiform, foliate, and circumvallate papillae, we have shown that whereas functional amiloride-sensitive Na^+ channel activity is present in a significant proportion of fungiform and foliate TRCs, functional amiloride-sensitive sodium channel activity is absent from circumvallate TRCs. These results concur with the afferent nerve recordings and support the view that the labeling studies may be detecting a channel that is either nonfunctional or, alternatively, is a functional Na^+ channel that is not amiloride sensitive.

Voltage-activated Channels

Our preliminary investigations into the voltage-activated conductances present in fungiform, foliate, and circumvallate TRCs served two purposes. First, it allowed the identification of a cell as a mature TRC and not a developing cell or a non-taste epithelial cell, which do not express either voltage-dependent Na^+ or K^+ currents (Kinnamon and Roper, 1988; Akabas et al., 1990; Gilbertson et al., 1993). Second, because these experiments are the first in which TRCs from each of the three taste bud types were recorded in the same study, it allowed a direct comparison of the macroscopic voltage-activated conductances among cell types.

Our results show that there are no gross differences between whole-cell currents recorded in the three TRC types (Fig. 2). In physiological saline (Tyrode's), each of the TRC types displayed voltage-activated Na^+ currents and K^+ currents similar to those described in other mammalian preparations (see Gilbertson, 1993, for review). These currents were similar in terms of both current densities and activation/inactivation kinetics, and no consistent significant differences were noted. Similarly, those cells that displayed only voltage-activated K^+ appeared to form a homogenous group across taste bud classes. It was beyond the scope of these experiments to isolate pharmacologically each potential conductance and compare it among TRC

types; therefore, this conclusion must be considered preliminary.

Because TRCs produce action potentials (Roper, 1983; Kashiwayanagi et al., 1983) and the frequency of action potentials appears related to stimulus intensity in fungiform TRCs (Avenet and Lindemann, 1991; Gilbertson et al., 1992; Cummings et al., 1993), we investigated the ability of TRCs to generate action potentials by determining the inactivation properties of TTX-sensitive Na^+ channels from fungiform, foliate, and circumvallate TRCs. These experiments revealed that half-inactivation potentials for Na^+ currents were comparable for the three TRC types, each being near −55 mV. This information, when coupled with the fact that resting potentials for these cells are approximately −65 to −70 mV (T. Gilbertson, personal observation), indicates that the majority of Na^+ channels in all classes of TRCs are able to be activated upon depolarization. Thus, it does not appear that the relative insensitivity of the circumvallate TRCs to NaCl is due to an inherent inability to generate active responses during NaCl stimulation, but instead may be attributable to the underlying transduction mechanism(s) for NaCl itself.

Amiloride-sensitive Na^+ Channels

The chief finding from the present set of experiments is that functional ASSCs are present in a subset of fungiform and foliate TRCs but are apparently absent from TRCs isolated from the circumvallate papilla. Furthermore, the mature fungiform TRCs (those with both Na^+ and K^+ channels) contain a significantly higher proportion of amiloride-sensitive cells than do taste cells located within the foliate papillae (63% vs. 37%; Table I). Of the roughly one third of TRCs that expressed only voltage-activated K^+ currents, all had a similar distribution of functional ASSCs across taste bud types. These TRCs, which may reflect immature or developing TRCs, nonetheless apparently already have ASSCs, consistent with a preliminary report (McPheeters et al., 1994).

The distribution of amiloride sensitivities across taste bud types, coupled with afferent nerve recordings from rat (Formaker and Hill, 1991; Sollars and Bernstein, 1994), suggests that those TRCs that are innervated by nerve VII (chorda tympani), such as taste cells within fungiform and anterior foliate, may possess functional ASSCs, whereas those that receive glossopharyngeal innervation (e.g., taste cells within posterior foliate and circumvallate) do not. Our finding of 63% amiloride-sensitive fungiform TRCs roughly correlated with a study that found the chorda tympani nerve in hamster to be comprised of 55% NaCl-best fibers (Frank et al., 1988). This correlation suggests that ASSCs may be present predominately in NaCl-best fibers. Whereas the conclusion concerning correlation between innervation by nerve VII and amiloride sensitivity is intriguing, it is limited by the fact that in the present study, the relative distribution (i.e., anterior vs. posterior) of those foliate TRCs that were amiloride-sensitive was not determined.

Although the lack of functional ASSCs in circumvallate TRCs agrees with previously reported afferent nerve recordings, it is in direct contrast to recent work showing that circumvallate taste tissue has ASSC mRNA (Li et al., 1994) and apparently expresses ASSC protein (Simon et al., 1993). There are at least two possible explanations to account for this apparent discrepancy. One, ASSCs are present in circumvallate TRCs, but they are restricted to the basolateral membrane. This would explain the insensitivity of NaCl responses to mucosal amiloride recorded in the glossopharyngeal nerve. Two, ASSCs are present in the circumvallate papillae, but are nonfunctional. There may be complete nonfunctionality; that is, the expressed channel protein exhibits no sodium permeability owing to either errors in posttranslational modification or membrane targeting and incorporation. Alternatively, this nonfunctionality may extend only to the ability of amiloride to inhibit sodium flux through the channel.

Our data argue against the first alternative of there being a restriction of ASSCs to the basolateral membrane. Since the TRCs are isolated and amiloride is applied via bath perfusion, there is access to the entire cell. Therefore, unlike the glossopharyngeal nerve recordings in vivo, the present experiments do not restrict amiloride applications to the apical membrane of TRCs. Whereas it is possible that the enzymatic procedures used to isolate TRCs degrade ASSCs, care was taken to prevent this by using amiloride and trypsin inhibitors in all of the dissociating solutions. This step proved to be an effective method to prevent degradation of ASSCs (Garty and Edelman, 1983; Gilbertson et al., 1993). Clearly, we were able to record ASSC activity from both the fungiform and foliate TRCs. While the possibility cannot be ruled out that there is another class of ASSC that is selectively degraded by our dissociation treatment or is lost at some subsequent step, preliminary work measuring epithelial transport across the lingual epithelium, where dissociation conditions are very mild, supports our present findings. Recording from epithelia containing either fungiform or circumvallate TRCs has shown that Na^+ transport across the fungiform-containing epithelia is significantly amiloride sensitive, whereas across the epithelia containing the circumvallate papilla, it is not (Zhang et al., 1996).

Our results therefore suggest that ASSCs are functionally absent from circumvallate TRCs. The recent cloning and expression of ASSCs have shown that there are three subunits (α, β, and γ) which make up the

ASSC (Canessa et al., 1994). Full ASSC function requires that each of the three homologous subunits be present to form the heterotrimeric ASSC. When ASSC subunits are expressed alone or as dimers ($\alpha\beta$, $\alpha\gamma$, $\beta\gamma$) in oocytes, there is little amiloride-sensitive current (Canessa et al., 1994). What is not clear from these experiments is whether or not the homomers or dimers form channels that are permeable to sodium ions (see below). However, a recent study has shown that the α subunit when expressed alone in Xenopus oocytes is capable of generating Na^+ currents (Li et al., 1995). Both the in situ hybridization and immunocytochemistry studies targeting the ASSC in circumvallate tissue used probes against the α subunit of the ASSC (Li and Snyder, 1994; Simon et al., 1993). It is plausible that this subunit is found in the circumvallate TRCs, but not in conjunction with the β and γ subunits required to allow full expression of amiloride-sensitive sodium currents. To elucidate this point will require repeating the in situ hybridization and/or immunocytochemistry using other subunit specific probes of the ASSC.

Amiloride-insensitive Na^+ Currents

Though there were significant differences between the fungiform, foliate, and circumvallate in terms of percentage of cells showing ASSC activity, there was no such differentiation on the basis of responses to complete Na^+ replacement. Greater than 90% of all cells of each type showed a decrease in resting conductance upon replacement of extracellular NaCl with NMDG-Cl (Table I). This result suggests that there is a constitutive Na^+ influx at rest in physiological saline. In two thirds of fungiform TRCs and in a minority of foliate TRCs, at least a proportion of this influx is via ASSCs. However, in all TRC types (and completely in circumvallate TRCs), it is clear that there is a significant component of the Na^+ influx that is not through ASSCs. This Na^+ influx has been said to be via the amiloride-insensitive sodium pathway, the specifics of which have not been elucidated. Whatever the mechanism, it is clearly an important contributor to NaCl transduction in rat TRCs, particularly for those taste cells within the circumvallate papillae, and is the predominant mechanism in other species, including mudpuppy (McPheeters and Roper, 1985) and mouse (Tonosaki and Funakoshi, 1989).

One suggestion to account for the mechanism of the amiloride-insensitive NaCl response is by ASSCs situated basolaterally. Recently, a basolateral ASSC has been suggested to be present in rat, which has a significantly higher amiloride inhibition constant (Mierson et al., 1994). In this mechanism, Na^+ ions would permeate the tight junctions between TRCs, termed the paracellular pathway, and then enter the TRC via open basolateral ASSCs (DeSimone et al., 1984; Simon et al., 1993). Sodium transport across the tight junctions is anion dependent, which is believed to account for the different tastes among different Na^+ salts (Ye et al., 1991). While the paracellular pathway contributes to salt transduction, Na^+ influx through basolaterally restricted ASSCs cannot account for the amiloride-insensitive response. In the present experiments using isolated TRCs, amiloride, even at concentrations of 1 mM, could not completely inhibit sodium influx. Furthermore, we found no evidence of an ASSC in rat TRCs having an inhibition constant higher than that reported here. There clearly seems to be an additional pathway for Na^+ influx independent of ASSCs, which is important for both apical and basolateral routes of Na^+ entry.

It is possible, given the extensive labeling of ASSC mRNA and protein seen in the circumvallate papillae, that amiloride-insensitive Na^+ transport is itself through an alternatively spliced form of the ASSC or through a unique combination of the α, β, and γ subunits. This unique protein may possess the ability to form a sodium channel, but one that lacks sensitivity to amiloride. Alternatively spliced forms of ASSCs, including only those containing α subunits, have been shown to express Na^+ currents when expressed in oocytes (Li et al., 1995). Whether such a channel is present in TRCs remains speculation at this point; such a channel would be consistent with the recent molecular cloning studies that suggest unequal distribution of ASSC subunits in the circumvallate papillae (Li and Snyder, 1994), earlier afferent nerve recordings showing nerve IX insensitivity to amiloride (Formaker and Hill, 1991), and the present study on isolated TRCs.

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