

Development of Rat Chorda Tympani Sodium Responses: Evidence for Age-Dependent Changes in Global Amiloride-Sensitive Na⁺ Channel Kinetics

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Hendricks, Susan J., Robert E. Stewart, Gerard L. Heck, John A. DeSimone, and David L. Hill. Development of rat chorda tympani sodium responses: evidence for age-dependent changes in global amiloride-sensitive Na⁺ channel kinetics. *J Neurophysiol* 84: 1531–1544, 2000. In rat, chorda tympani nerve taste responses to Na⁺ salts increase between roughly 10 and 45 days of age to reach stable, mature magnitudes. Previous evidence from in vitro preparations and from taste nerve responses using Na⁺ channel blockers suggests that the physiological basis for this developmental increase in gustatory Na⁺ sensitivity is the progressive addition of functional, Na⁺ transduction elements (i.e., amiloride-sensitive Na⁺ channels) to the apical membranes of fungiform papilla taste receptor cells. To avoid potential confounding effects of pharmacological interventions and to permit quantification of aggregate Na⁺ channel behavior using a kinetic model, we obtained chorda tympani nerve responses to NaCl and sodium gluconate (NaGlu) during receptive field voltage clamp in rats aged from 12–14 to 60 days and older (60+ days). Significant, age-dependent increases in chorda tympani responses to these stimuli occurred as expected. Importantly, apical Na⁺ channel density, estimated from an apical Na⁺ channel kinetic model, increased monotonically with age. The maximum rate of Na⁺ response increase occurred between postnatal days 12–14 and 29–31. In addition, estimated Na⁺ channel affinity increased between 12–14 and 19–23 days of age, i.e., on a time course distinct from that of the maximum rate of Na⁺ response increase. Finally, estimates of the fraction of clamp voltage dropped across taste receptor apical membranes decreased between 19–23 and 29–31 days of age for NaCl but remained stable for NaGlu. The stimulus dependence of this change is consistent with a developmental increase in taste bud tight junctional Cl⁻ ion permeability that lags behind the developmental increase in apical Na⁺ channel density. A significant, indirect anion influence on apical Na⁺ channel properties was present at all ages tested. This influence was evident in the higher apparent apical Na⁺ channel affinities obtained for NaCl relative to NaGlu. This stimulus-dependent modulation of apical Na⁺ channel apparent affinity relies on differences in the transepithelial potentials between NaCl and NaGlu. These originate from differences in paracellular anion permeability but act also on the driving force for Na⁺ through apical Na⁺ channels. Detection of such an influence on taste depends fundamentally on the preservation of taste bud polarity and on a direct measure of sensory function, such as the response of primary afferents.

INTRODUCTION

Numerous behavioral, neurophysiological, and electrophysiological studies indicate that the amiloride-sensitive Na⁺ channel is a transduction pathway for taste system Na⁺ stimuli in numerous mammals (Brand et al. 1985; DeSimone et al. 1981; Gilbertson et al. 1993; Heck et al. 1984; Hill et al. 1990; Schiffman et al. 1983; reviewed in Stewart et al. 1997a) including some primates (Hellekant et al. 1997a,b). In mammals where this Na⁺ sensing system is functional, passive movement of stimulus Na⁺ ions across the taste cell apical membrane causes membrane depolarization and, consequently, release of neurotransmitter onto primary taste afferents (reviewed in Roper 1992). Notably in several species, including rat and hamster, neural responses to NaCl are not completely suppressed by amiloride (Brand et al. 1985; DeSimone and Ferrell 1985; Heck et al. 1984; Hettinger and Frank 1990). The unsuppressed part of the neural response to NaCl has been termed the amiloride-insensitive component (Formaker and Hill 1988; Ye et al. 1993a).

The amiloride-insensitive component of the neural taste response to NaCl is highly dependent on the stimulus anion (Formaker and Hill 1988) and is thought to result from activation of transduction sites below taste cell tight junctions (Ye et al. 1991, 1993a). Specifically, when Na⁺ salts with small, highly permeant anions, such as Cl⁻, are applied to the lingual surface, electroneutral diffusion of Na⁺ and the stimulus anion through taste cell tight junctions is facilitated. In contrast, when Na⁺ salts with larger, tight junction-impermeant anions are applied to the tongue, electroneutral diffusion of Na⁺ and the stimulus anion is severely limited. Consequently, neural responses to Na⁺ salts with large, organic anions, such as acetate or gluconate, are transduced entirely by the apical pathway and completely inhibited by amiloride (Formaker and Hill 1988; Ye et al. 1993a). Therefore the composite neural response to NaCl comprises distinct apical, voltage-sensitive and basolateral, voltage-insensitive components.

Interestingly, significant developmental alterations in gustatory Na⁺ responses have been documented in several mamma-

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lian species (Ferrell et al. 1981; Hill 1988; Hill and Almlı 1980; Mistretta and Bradley 1983; Ninomiya et al. 1991). In rats, impressive age-dependent increases in the magnitude of whole chorda tympani nerve responses to Na^+ (and Li^+) stimuli occur (Ferrell et al. 1981; Hill and Almlı 1980). Specifically, when expressed relative to an NH_4Cl reference response, NaCl and LiCl response magnitudes increased progressively between ~ 10 and 45 days postnatal. By ~ 45 days of age, NaCl and LiCl response magnitudes reached stable, adult levels. Analyses of single chorda tympani fiber responses during development have established clearly that the developmental increase in rat whole chorda tympani sensitivity to NaCl and LiCl is due specifically to increases in single chorda tympani fiber responses to these salts (Hill et al. 1982). In contrast, responses to acids, ammonium chloride and other monochloride salts are robust in early postnatal rats and remain so during development. The fact that Na^+ exhibits a unique developmental time course among taste stimuli suggests that underlying physiological changes originate at the level of the receptor cells responsible for transduction of the Na^+ taste signal. More recent work has examined the potential mechanisms that underlie developmental changes in neural sensitivity to Na^+ stimuli.

Changes in amiloride-sensitive Na^+ channel function have been proposed to account for the developmental increase in gustatory system Na^+ sensitivity. For example, Hill and Bour (1985) showed that the sensitivity of whole chorda tympani NaCl responses to the suppressive effects of amiloride increased in parallel with developmental increases in sensitivity to NaCl and LiCl . In particular, chorda tympani responses to NaCl and to LiCl in 12- to 13-day-old rats were unaffected by $100 \mu\text{M}$ amiloride. However, in rats aged 29–31 and 90–110 days, chorda tympani responses were potently suppressed by amiloride. Furthermore the degree of response inhibition caused by amiloride was proportional to overall taste system sensitivity to these stimuli at these age points. An implication of these results is that the apical Na^+ transduction pathway develops postnatally, while the basolateral Na^+ transduction pathway appears to be in place and functional around the time of birth. These findings were later replicated by Sollars and Bernstein (1994). Hill and Bour (1985) concluded that the concomitant, progressive increases in gustatory sensitivity to NaCl (and LiCl) and to amiloride were due to an orderly increase in the functional expression of amiloride-sensitive Na^+ channels in taste cell apical membranes. With this notion in mind, Stewart et al. (1995) hypothesized that the developmental increase in gustatory Na^+ sensitivity was due to a progressive addition of newly synthesized, functional amiloride-sensitive Na^+ channels. They tested this hypothesis by correlating the presence of Na^+ channel-like antigen in fungiform papilla taste buds with the developmental increase in gustatory Na^+ and amiloride sensitivity.

Surprisingly, although the rat chorda tympani does not exhibit significant amiloride sensitivity before 7–10 days postnatal, antigenic determinants of amiloride-sensitive Na^+ channel are observed in taste cells of fungiform papilla taste buds as early as the day after birth (Stewart et al. 1995). However, limited microscopic resolution in that study precluded definitive localization of Na^+ -channel-like immunoreactivity to the apical membrane. Likewise, Kossel and co-workers (1997) used whole cell recordings to identify amiloride-blockable

currents in isolated, single taste cells of fungiform papilla taste buds from rats as young as 2 days postnatal. They determined that the percentage of neonatal rat taste cells that exhibit amiloride-blockable currents is stable throughout development; the normalized density of amiloride-sensitive channels in taste cells is stable throughout development; and the amiloride-inhibition constant of taste cell Na^+ channels is stable throughout development. The authors suggest that redistribution of fully functional channels from the basolateral to apical domain and/or maturation (i.e., progressive opening) of taste pores could account for the developmental increase in neural Na^+ and amiloride sensitivity in rats. On the other hand, progressive pore opening would be expected to influence the apparent development of neural responses to all stimuli and not only those to Na^+ salts. Nonetheless these electrophysiological and histochemical observations suggest that the Na^+ transduction apparatus is functionally mature well before 7–10 days of age and therefore diverge considerably from earlier neurophysiological data.

In an attempt to clarify the nature of this apparent discrepancy, we have used in the present study *in vivo* lingual receptive field voltage clamping with simultaneous whole chorda tympani recordings to resolve age-related changes in taste cell apical Na^+ channel function. This approach offers several advantages over the use of either isolated, *in vitro* preparations or conventional neurophysiological recordings. First, the topological arrangement of taste buds and, therefore segregation of apical and basolateral transduction pathways, remains intact. Second, aggregate apical membrane Na^+ channel properties can be studied quantitatively without the use of amiloride. This is especially important because it has been suggested that restricted access of amiloride to taste cell apical membranes caused by unopened taste pores may contribute to the apparent lack of amiloride sensitivity in young rats (Kossel et al. 1997). Finally, the combination of epithelial voltage clamp with neural recording provides a measure of how taste cells “report” exclusively taste-related membrane events. We hypothesized that the developmental increase in gustatory Na^+ sensitivity in rats is due to the progressive addition of functional amiloride-sensitive Na^+ channels to taste cell apical membranes. The results we obtained are consistent with such an age-related increase in the apparent number of functional, amiloride-sensitive Na^+ channels in taste cell apical membranes. In addition, the results provide entirely new evidence that the apparent affinity of the aggregate apical Na^+ transduction pathway also increases during postnatal development. Finally, our results indicate that these age-related changes in apical Na^+ channel function are temporally distinct. Therefore the onset of mature gustatory Na^+ sensing in rat appears to rely on developmental sequelae that are more complicated than a simple, monotonic increase in the number of functional Na^+ transduction sites. Portions of these findings have been presented previously in preliminary form (Hendricks et al. 1998; Stewart et al. 1997b).

METHODS

Animals

Sprague-Dawley rats at 12–14 days of age, 19–23 days of age, 29–31 days of age, or 60 days of age and older (hereafter termed 60+ day old) were used in these studies. Rats were bred and raised in our colony with the exception of several rats in the 60+ day old group,

which were obtained directly from the supplier. These adult animals, and all breeding stock, were obtained from Harlan Sprague-Dawley. Rats were provided standard laboratory chow (Purina) and water ad libitum and maintained on a 12 h:12 h light:dark photoperiod (lights on at 0700). Breeding harems (3 females and 1 male) were established, and the male remained in the cage for 14 days. After 14 days, the male was removed, and the females were segregated into individual maternity cages. Maternity cages were checked for births each day at 0800 and 1800 h. The day of birth (postnatal day 0) was recorded as the date when litters were first observed. If necessary, on the day after birth, litters were culled to 10 pups (5 female, 5 male, when possible); litters with fewer than 8 pups were not used for these studies. Generally, a single pup from each litter was sampled for each age range noted in the preceding text. However, each age range includes two data sets obtained from litter mates. Remaining offspring were weaned to the preceding diet at 21 days of age and then transferred to sex-segregated cages no later than 45 days of age.

Chorda tympani recording

Rats at the ages noted in the preceding text were deeply anesthetized by intraperitoneal injection with pentobarbital (50–65 mg/kg). Supplemental injections (20–30 mg/kg) were given as needed to maintain surgical anesthesia. Body temperature was maintained with a circulating water heating pad (K-MOD 100, Allegiance Healthcare, McGaw Park, IL). Bilateral hypoglossotomy to prevent tongue movement was performed after blunt dissection of surrounding tissue, and the trachea was cannulated to facilitate free breathing. Following placement of the animal into a nontraumatic head holder, the left or right chorda tympani nerve was exposed via an epimandibular approach. The nerve was freed of surrounding connective tissue, cut near its entrance into the tympani bulla, desheathed, and placed onto a 28-gauge platinum wire electrode. A reference electrode was placed in nearby tissue. The wound was filled with a mixture of petroleum jelly and mineral oil to prevent drying of the nerve. Neural activity was fed to a fixed gain isolation amplifier (BioAmp 100, Axon Instruments, Foster City, CA) and then to a Grass P511 preamplifier (AstroMed, West Warwick, RI). The amplified signal was monitored with an oscilloscope and audio loudspeaker. Amplified neural activity was RMS-rectified and integrated with a time constant of 1–2 s. The integrated output was recorded on one channel of a Linseis L6514 4-channel rectilinear chart recorder (Linseis, Princeton Junction, NJ).

Lingual receptive field voltage clamp

Simultaneous lingual epithelial voltage clamping and chorda tympani neural recordings have been described in detail (Stewart et al. 1996; Ye et al. 1993a). Briefly, a portion of the anterior dorsal lingual epithelium was enclosed within a vacuum-applied cast acrylic stimulation chamber. For rats aged 29–31 and 60+ days of age, the chamber enclosed a 28-mm² patch of dorsal tongue, while the chamber used for 12–14 and 19–23 day old rats enclosed a 12.5-mm² patch. The outer vacuum groove in the chamber creates a mechanically stable and electrically tight seal between the chamber and lingual epithelium. Stimulus and rinse solutions (see following text) were injected (4 ml; 1 ml/s) into the chamber via tubing fitted to a dedicated port (dead space: ~0.5 ml). The chamber was fitted with separate Ag-AgCl electrodes for measurement of current and potential, while reference electrodes were placed noninvasively on the ventral lingual epithelium. The current-passing electrode in the chamber served as virtual ground, ensuring that only current passing through the stimulated patch was collected. Command currents and potentials were delivered by a voltage-current clamp amplifier (VCC600; Physiologic Instruments, San Diego, CA). Transepithelial potential and current responses were recorded on two channels of a Linseis L6514 4-channel rectilinear chart recorder.

Stimulus solutions and stimulus application

All solutions used were made from reagent grade chemicals (Sigma, St. Louis, MO; or Fisher, Pittsburgh, PA) dissolved in glass-distilled H₂O. The rinse solution contained 15 mM KHCO₃ (pH 8.7). A Na⁺-depleted Krebs-Henseleit solution (DKH) that contained (in mM) 6 KCl, 2 CaCl₂, 1.2 MgSO₄, 1.3 NaH₂PO₄, 25 NaHCO₃, and 5.6 glucose (pH 7.5) was applied after each stimulus series to help maintain a stable transepithelial potential. Transepithelial potential in the presence of 150 mM NaCl rarely fluctuated more than ±5 mV during the course of an experiment. In addition, those preparations that exhibited absolute transepithelial potentials of greater than ±20 mV in the presence of 150 mM NaCl were excluded from analysis. Stimuli were concentration series (50, 100, 200, and 500 mM) of NaCl and Na⁺ gluconate (NaGlu). Sodium gluconate was selected as a stimulus in addition to NaCl primarily because the lower shunt permeability of gluconate relative to chloride significantly reduces the voltage-independent (i.e., basolateral) Na⁺ response component (Ye et al. 1991, 1993a). Therefore the chorda tympani response to NaGlu should derive primarily from voltage-dependent, apical transduction of Na⁺.

Stimuli were applied under zero-current clamp (equivalent to open circuit conditions), and steady-state potentials were recorded from the front panel display of the voltage-current clamp amplifier. Next, chorda tympani responses to the stimuli were obtained under voltage clamp at +60 and –60 mV relative to the zero-current clamp potential recorded for each stimulus. In this way, neural responses to concentration series were obtained under three membrane potential conditions: effective open circuit, and +60 and –60 mV voltage clamp. Each stimulus series was bracketed by the application of a 500 mM NH₄Cl reference stimulus under zero-current clamp, and intervening data were retained for analysis only when the magnitude of the NH₄Cl reference responses before and after the stimulus series varied by <10%. Steady-state response magnitudes, measured as the height of each integrated chorda tympani response 30 s after application of the stimulus, were expressed relative to the mean NH₄Cl response magnitude bracketing a stimulus series. Ammonium chloride was selected as a reference stimulus for several reasons. First, expression of integrated neural data relative to NH₄Cl provides a valid and reliable measure for comparisons of multiunit taste responses among subjects and between treatment groups (Beidler 1953). Importantly, because the responsiveness of the rat taste system to NH₄Cl does not change during postnatal development (Hill et al. 1982), it also provides an appropriate, unchanging reference response for comparisons of Na⁺ responses among age groups.

The protocol for stimulation of the lingual epithelium was generally as follows. The reference NH₄Cl stimulus was applied and allowed to remain in the chamber for 40–50 s. The solution was then rinsed from the chamber by repeated application of the KHCO₃ rinse solution for ≥60 s. Then, the stimuli of interest (NaCl or NaGlu) were applied as a concentration series with each concentration allowed to remain in the chamber for 40–50 s before being rinsed from the chamber repeatedly with KHCO₃, as noted in the preceding text. About 60 s after the final stimulus in a concentration series was rinsed from the chamber, the reference NH₄Cl solution was reapplied then rinsed. At this point, DKH was injected into the chamber, allowed to remain on the tongue for 60 s, and then rinsed from the chamber with repeated applications of the KHCO₃ rinse solution. Last, another reference stimulus was applied and rinsed, and the next concentration series was begun.

Statistical analyses

Data are expressed as means ± SE unless otherwise noted. Age-dependent differences in zero-current clamp chorda tympani response magnitudes at each stimulus concentration were determined using ANOVA with Student-Newman-Keuls (SNK) post hoc comparisons where appropriate.

TABLE 1. *Chorda tympani* relative response magnitudes obtained under zero-current clamp (CC_0)

Stimulus/ Age, d	Relative Response Magnitude, mM			
	50	100 ^a	200 ^a	500 ^a
NaCl				
12–14	0.11 ± 0.03	0.14 ± 0.03 ^{b-d}	0.18 ± 0.04 ^{b-d}	0.23 ± 0.04 ^{b-d}
19–23	0.14 ± 0.01	0.33 ± 0.04 ^{b,c}	0.46 ± 0.04 ^{b,c}	0.53 ± 0.09 ^{b,c}
29–31	0.20 ± 0.03	0.48 ± 0.02 ^{d,e}	0.81 ± 0.05 ^{d,e}	0.94 ± 0.06 ^{d,e}
60+	0.21 ± 0.04	0.54 ± 0.98 ^{d,e}	0.98 ± 0.09 ^{d,e}	1.07 ± 0.11 ^{d,e}
NaGlu				
12–14	0.05 ± 0.01	0.07 ± 0.01	0.10 ± 0.01 ^b	0.13 ± 0.01 ^{b-d}
19–23	0.07 ± 0.03	0.14 ± 0.03	0.28 ± 0.05	0.45 ± 0.09 ^{b,c,e}
29–31	0.11 ± 0.03	0.18 ± 0.08	0.36 ± 0.12	0.70 ± 0.06 ^{d,e}
60+	0.14 ± 0.03	0.32 ± 0.08	0.48 ± 0.09 ^e	0.91 ± 0.07 ^{d,e}

Values are means ± SE (NaGlu: $n = 4-9$ /age; NaCl: $n = 5-10$ /age). ^a Significant main effect of age at concentration (ANOVA, $P < 0.05$). ^b Student's Newman-Keuls, $P < 0.05$ vs. 60+. ^c Student's Newman-Keuls, $P < 0.05$ vs. 29–31. ^d Student's Newman-Keuls, $P < 0.05$ vs. 19–23. ^e Student's Newman-Keuls, $P < 0.05$ vs. 12–14.

RESULTS

Development of chorda tympani responses to NaCl

Relative to the NH_4Cl reference response, integrated chorda tympani responses to NaCl and to NaGlu under zero-current clamp increased in a progressive manner between 12–14 days of age and adulthood. Table 1 provides summary NaCl and NaGlu response magnitude data. Because we were primarily interested in determining whether the present results were consistent with previously published data regarding Na^+ response development, we examined age-related differences in response magnitude only under the zero-current clamp condition, which is equivalent to conventional chorda tympani recording. Significant age-related differences in NaCl response

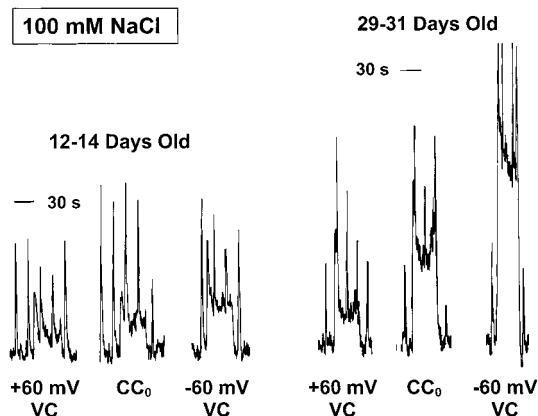


FIG. 1. Integrated chorda tympani responses to 100 mM NaCl under 0-current clamp (CC_0) and +60 and -60 mV voltage clamp (VC). The responses on the left are from a 12- to 14-day-old rat, while those on the right are from a 29- to 31-day-old rat. Two features of the responses are especially notable. Specifically, the CC_0 magnitude of the responses to 100 mM NaCl are clearly smaller in the 12- to 14-day-old rat as compared with those of the 29- to 31-day-old. In contrast, the influence of transepithelial voltage perturbation on 100 mM NaCl response magnitude appear considerably greater in the 29- to 31-day-old, especially under -60 mV VC, where the chorda tympani response is nearly doubled. These observations are consistent with increasing postnatal sensitivity to gustatory NaCl stimuli caused by an increase in the density of conducting apical Na^+ channels. Superimposed spikes are due to injected current (voltage clamp) and voltage (current clamp) pulses applied at 20-s intervals to monitor transmembrane conductance.

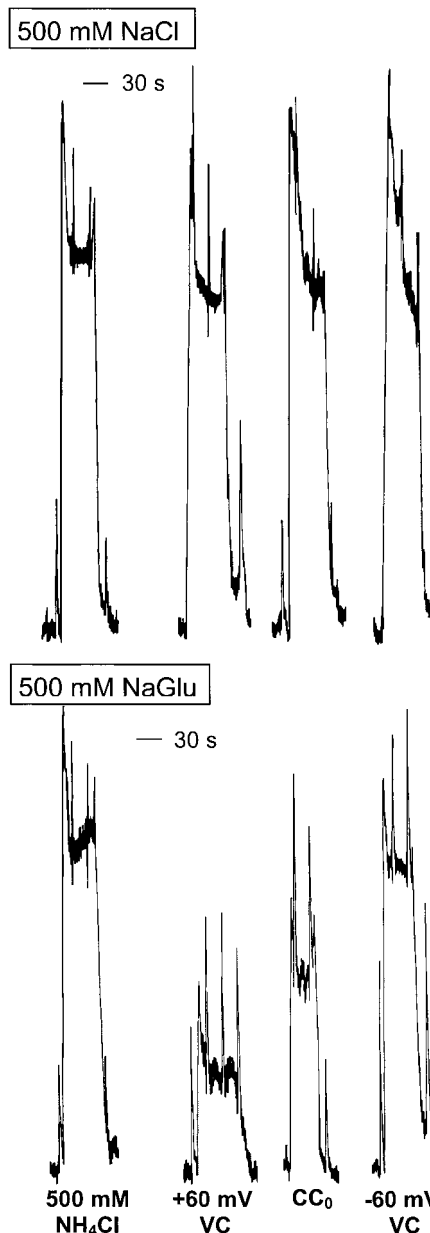


FIG. 2. Anion-dependent reduction of chorda tympani response voltage sensitivity. These integrated chorda tympani responses to the 500 mM NH_4Cl reference stimulus under 0-current clamp and to 500 mM NaCl (top) and 500 mM NaGlu (bottom) under 0-current clamp (CC_0), and ± 60 mV voltage clamp are from a single 60+-day-old rat. Note the striking, obvious divergence in the impact of transepithelial voltage perturbation on chorda tympani responses to these 2 Na^+ salts. Specifically, lingual epithelial voltage clamp has almost no effect on responses to 500 mM NaCl, whereas responses to 500 mM NaGlu are potentially modulated by transepithelial voltage perturbation. Superimposed spikes are caused by current and voltage pulses delivered at 20-s intervals to monitor transmembrane conductance.

magnitude under zero-current clamp were detected for NaCl concentrations 50 mM (ANOVA, $P < 0.05$) (Table 1). Student-Newman-Keuls posthoc comparisons indicated a significant, progressive, age-dependent increase in mean response magnitudes to 100, 200, and 500 mM NaCl $\leq 29-31$ days of age (SNK, $P < 0.05$) (Table 1). At 29–31 days of age, mean chorda tympani response magnitudes to these stimuli did not differ significantly from 60+-day-old rat response magnitudes.

Results obtained for NaGlu were in general very similar to those obtained for NaCl. However, significant, age-related differences in zero-current clamp response magnitudes to NaGlu were detected only at 200 and 500 mM (ANOVA, $P < 0.05$). Post hoc comparisons revealed a significant age-related difference in response magnitudes to 200 mM NaGlu between 12–14 and 60+ day old rats (SNK, $P < 0.05$), while significant age-related differences in chorda tympani response magnitudes to 500 mM NaGlu were detected among all groups, except 29- to 31- and 60+-day-old rats. The results for zero-current clamp responses to both Na^+ salts are consistent with previously published data regarding the development of chorda tympani NaCl responses in rats (Ferrell et al. 1981; Hill and Almlil 1980).

In conjunction with the developmental increase in Na^+ response magnitude, the sensitivity of chorda tympani NaCl responses to applied lingual epithelial voltage perturbations increased with age. For example, Fig. 1 depicts integrated chorda tympani responses to 100 mM NaCl under zero-current

clamp and +60 and -60 mV voltage clamp obtained from a 12- to 14- and a 29- to 31-day-old rat. It is clear that the effect of voltage clamp on responses to this NaCl concentration is considerably greater in the 29- to 31-day-old rat. In general, and consistent with previously published results (Ye et al. 1993a,b), chorda tympani responses to NaCl at all concentrations were elevated under submucosal negative voltage clamp, which increases the electrochemical driving force for Na^+ into the taste receptor cell, and suppressed under submucosal positive voltage clamp, which decreases the driving force for Na^+ into the taste receptor cell. However, at 250 and 500 mM NaCl, the influence of voltage clamp appeared blunted in 29- to 31- and 60+-day-old rats (Fig. 2). Chorda tympani responses to all concentrations of NaGlu obtained under voltage clamp also exhibited marked sensitivity to applied voltage perturbations, and the magnitude of this sensitivity appeared to increase with age. In contrast to the NaCl case, the effect of voltage clamp on NaGlu chorda tympani response magnitudes remained robust at higher concentrations

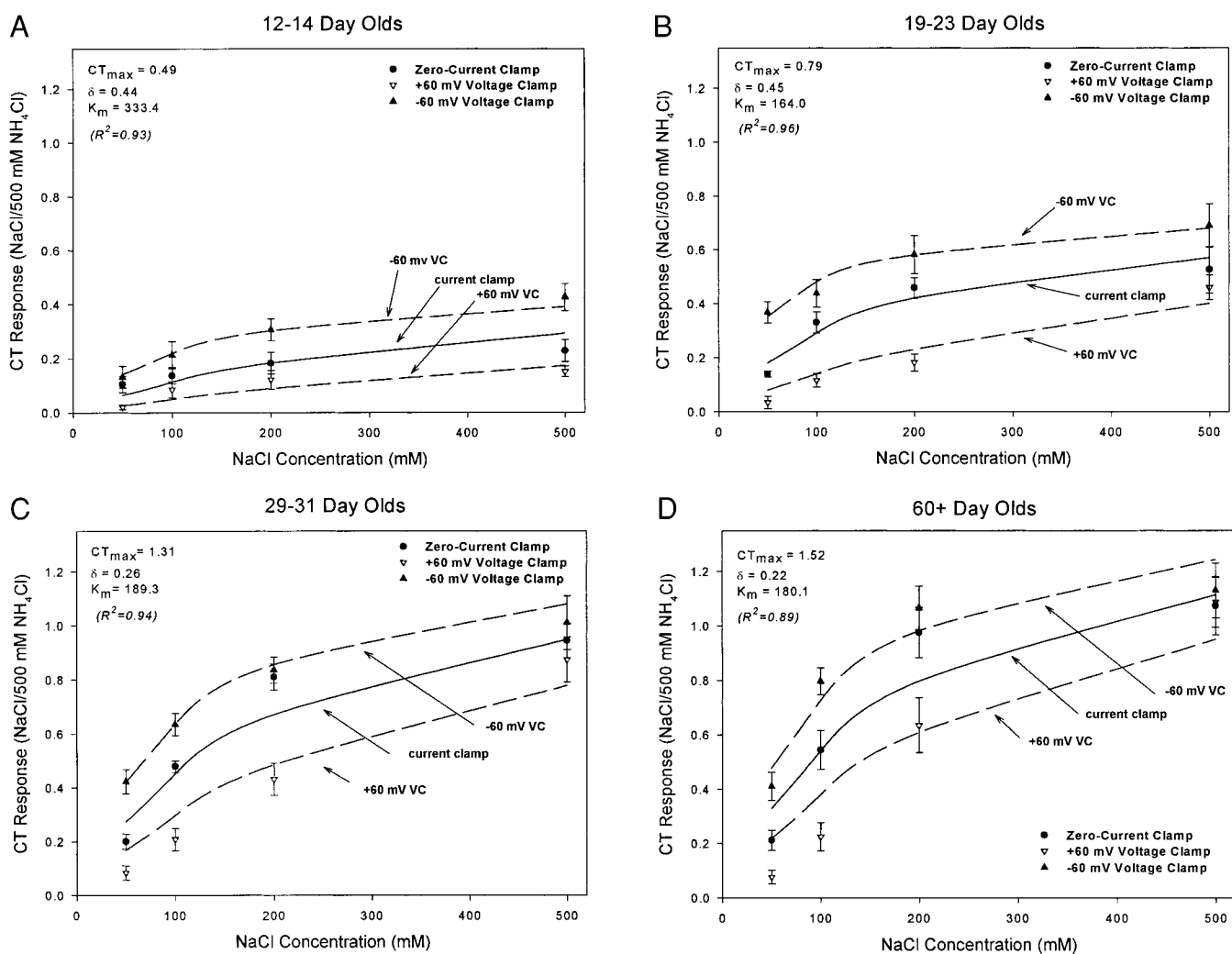


FIG. 3. Response-concentration plots of mean NaCl relative response data under the transepithelial potential conditions (symbols) for 12- to 14 (A)-, 19- to 23 (B)-, 29- to 31 (C)-, and 60+ (D)-day-old rats. Also plotted are theoretical lines of best-fit derived by fitting the data for each age group with the apical channel model (Eq. 1). Best-fit parameters are listed in each panel; the coefficient of determination R^2 , is provided as an indicator of the strength of the correspondence between the model and the data. An age-dependent increase in the magnitudes of NaCl responses is supported by the increasing predicted values of CT_{max} . Notably, the effect of transepithelial voltage clamp on chorda tympani responses to NaCl concentrations 100 mM becomes much diminished in 29- to 31- and 60+-day-old age groups (C and D). Data presented are means \pm SE ($n = 5-10$ per age group).

and at all ages studied (Fig. 2). The effects of lingual epithelial voltage clamp on chorda tympani response-concentration functions for NaCl and NaGlu are shown in Figs. 3 and 4, respectively.

Figures 3 and 4 clearly show that Na^+ salt chorda tympani responses are subject to modulation by imposed transepithelial voltage perturbations even at the earliest age-point studied (12–14 days of age). By considering ion channel flow kinetics (Mintz et al. 1986; Ye et al. 1993a) and by assuming that depolarizing Na^+ ion influx into taste cells is the rate-limiting event in the evoked neural response to Na^+ , we obtain the apical channel model equation, which is a modified form of the Beidler taste equation (Beidler 1954)

$$R = \frac{CT_{\max} c_e}{K_m + c_e} \quad (1)$$

where, K_m is the concentration of the NaCl that yields a half-maximal chorda tympani response, and CT_{\max} is the maximum value of R , which is the neural response obtained at a given Na^+ electrochemical concentration, c_e . It is in this last term that the major difference between the apical channel model (Eq. 1) and the Beidler taste equation is found. Although the apical channel model equation closely resembles the Beidler taste equation, it takes into account the assumption that depolarizing Na^+ flux through apical channels into taste receptor cells, which will depend on electrical potential as well as ion concentration, is the rate-limiting step in the generation of the neural taste response to Na^+ stimuli. That is, Na^+ flux through apical channels depends on electrochemical concentration, which is written as

$$c_e = c e^{-\delta\phi} \quad (2)$$

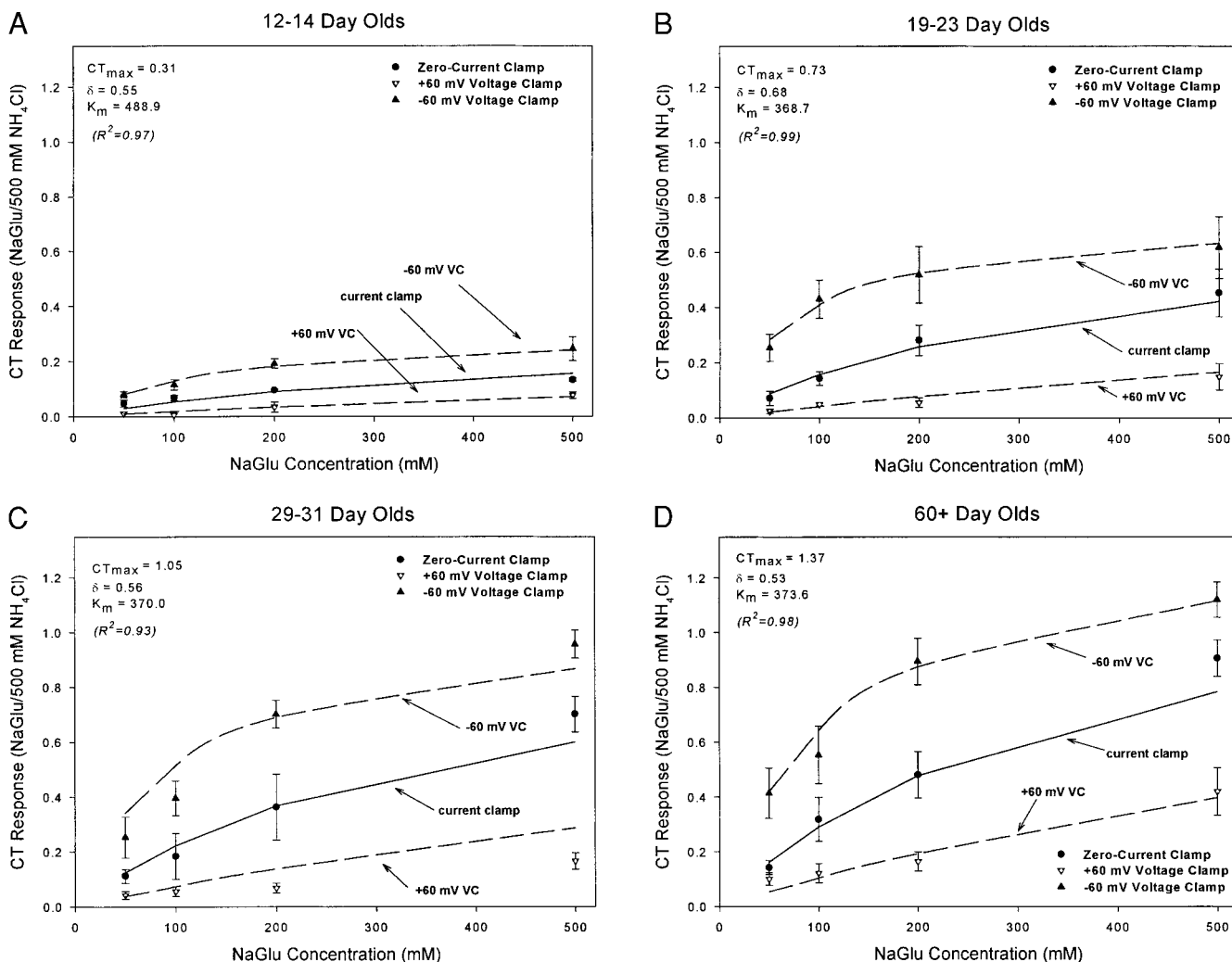


FIG. 4. Response-concentration plots of mean NaGlu relative response data under three transepithelial potential conditions (symbols) for 12- to 14 (A)-, 19- to 23 (B)-, 29- to 31 (C)-, and 60+ (D)-day-old rats. Also plotted are theoretical lines of best-fit derived by fitting the data for each age group with the apical channel model (Eq. 1). Best-fit parameters are listed in each panel; the coefficient of determination, R^2 , is provided as an indicator of the correspondence between the apical channel model and the data. Notably, the effect of transepithelial voltage clamp on chorda tympani responses to NaGlu clearly becomes more pronounced with advancing postnatal age as evidenced by the separation between response means for the 3 voltage conditions within each concentration. This observation, in conjunction with the age-related increase in predicted values of CT_{\max} , support the view that apical Na^+ channel density increased as a function of postnatal age. Interestingly, unlike chorda tympani responses to NaCl, there is no evidence of age- or stimulus concentration-related loss of NaGlu response voltage sensitivity. Data presented are means \pm SE ($n = 4-9$ per age group).

where, c is the stimulus Na^+ concentration and ϕ is $F\Delta V/RT$, where ΔV is the applied clamp voltage ($\text{J} \cdot \text{C}^{-1}$), at 35°C (i.e., 308°K), the approximate tongue surface temperature. In this instance, $F = 9.65 \times 10^4 \text{ C} \cdot \text{mol}^{-1}$ and $R = 8.314 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. Finally, δ is the fraction of ϕ that is "sensed" by Na^+ at taste cell apical membranes (Palmer 1984; Ye et al. 1993a). For each age group, the chorda tympani NaCl and NaGlu response data were fit with this equation.

Figures 3 and 4 depict for each age group the best-fit parameters obtained with the apical channel model for NaCl and NaGlu response data, respectively, as well as plots of the theoretical functions that follow from it. As expected for both stimuli, the channel model equation predicted an age-related, monotonic increase in CT_{max} , which is highly consistent with the postulated developmental increase in apical Na^+ channel number. Surprisingly, predicted values of K_m decreased in an age-related manner. This unexpected finding is also entirely consistent with the well-reported age-related increase in rat gustatory Na^+ sensitivity. An interesting feature of these changes is that the leftward shift in K_m along the concentration axis (i.e., an increase in channel affinity for Na^+) occurs between 12–14 and 19–23 days of age and is superimposed on a rapid increase in CT_{max} that occurs primarily between 12–14 and 29–31 days of age. Finally, and much to our surprise, a shift in the predicted value of δ occurred between 19–23 and 29–31 days of age when NaCl was the stimulus. In contrast, when NaGlu was the stimulus, this parameter appeared to remain stable across the developmental period studied. Using the parameters obtained in the preceding text, the chorda tympani response data shown in Figs. 3 and 4, were replotted as a function of NaCl electrochemical concentration, c_e . That is, the NaCl and NaGlu response data were replotted after

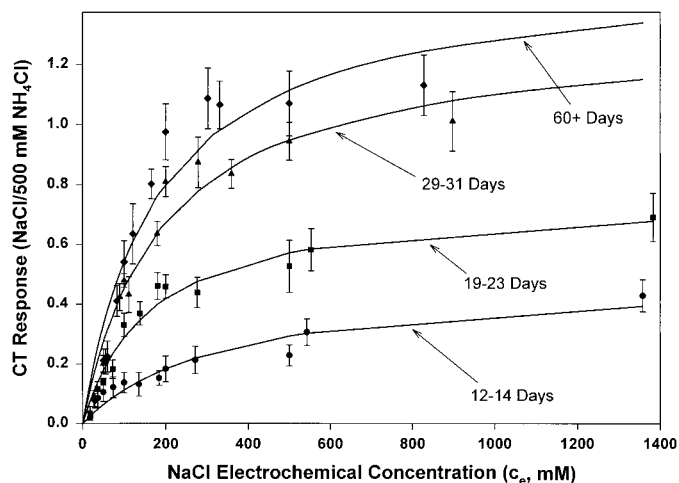


FIG. 5. Response-electrochemical concentration plots of mean NaCl relative response data (symbols) for all age groups. NaCl chemical concentrations were transformed to electrochemical concentration with Eq. 2 using best-fit parameters shown in Fig. 2 for each age group, and the data were replotted. The resulting theoretical lines indicate that the 3 curves from Fig. 2, corresponding to the 3 potential conditions, collapse onto a single curve for each age group. Therefore even at the youngest age point tested, the appropriate stimulus dimension for Na^+ stimuli is electrochemical concentration. Furthermore the age dependent increases in chorda tympani NaCl response magnitude and aggregate Na^+ channel affinity (increasing slope, i.e., decrease in K_m), are clearly evident from the theoretical lines. Data presented are means \pm SE (●: 12- to 14-day olds; ■: 19- to 23-day olds; ▲: 29- to 31-day olds; ◆: 60+- day olds).

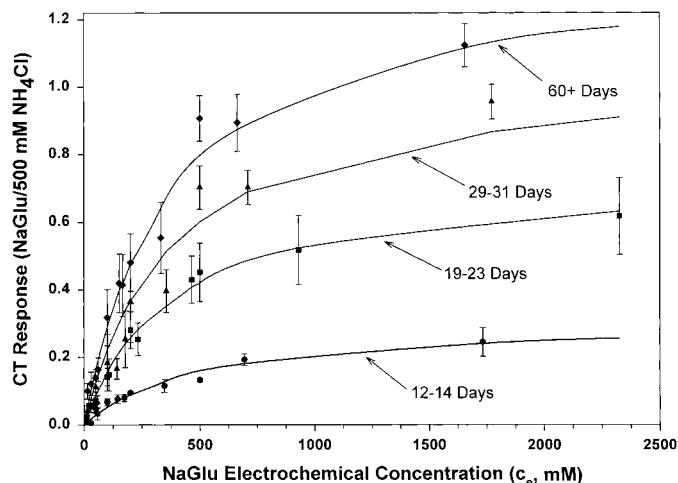


FIG. 6. Response-electrochemical concentration plots of mean NaGlu relative response data (symbols) for all age groups. NaGlu chemical concentrations were transformed to electrochemical concentration with Eq. 2 using best-fit parameters shown in Fig. 3 for each age group, and the data were replotted. The resulting theoretical lines obtained from Eq. 1 indicate that the 3 curves from Fig. 3, corresponding to the 3 potential conditions, collapse onto a single curve for each age group. Therefore even at the youngest age point tested, the appropriate stimulus dimension for Na^+ stimuli is electrochemical concentration. Furthermore the age dependent increases in chorda tympani NaCl response magnitude and aggregate Na^+ channel affinity (i.e., decrease in K_m), are clearly evident from the theoretical lines. Data presented are means \pm SE (●: 12- to 14-day olds; ■: 19- to 23-day olds; ▲: 29- to 31-day olds; ◆: 60+- day olds).

transforming the independent variables of clamp voltage and stimulus concentration into a single variable, c_e (Eq. 2).

Figures 5 and 6 depict the resulting response-electrochemical concentration functions for NaCl and NaGlu, respectively. It can be seen clearly that across developmental age the three response-concentration functions (which correspond to the 3 clamp voltage conditions) shown in each panel of Figs. 3 and 4 collapse onto a single curve for each age group. This transformation of the data reveals that even at the earliest age points studied the actual stimulus intensity for Na^+ is c_e , i.e., the driving force for the ion through a passive channel. Moreover, for both NaCl and NaGlu, the collapsed response-concentration functions for all four age groups manifest the age-dependent increase in CT_{max} and the age-dependent decrease in K_m , which is evidenced by the increasing initial slope of the theoretical functions. Although the channel model accounts for most of the variance in the data (minimum $R^2 \geq 0.89$), a notably larger deviation is observed for the 60+-day olds.

Such a deviation could arise from a secondary voltage-independent transduction process and could lead to errors in estimating CT_{max} . In an attempt to eliminate this as a source of error, the data were replotted as the voltage sensitivity index (VSI, Eq. 3). Because the VSI is defined as a difference in chorda tympani responses at two voltages, contributions from voltage-independent processes cancel out. The remaining, voltage-sensitive part of the chorda tympani response to NaCl (or NaGlu) should then arise exclusively from transduction by amiloride-sensitive Na^+ channels (Ye et al. 1993a). Consequently, the VSI can be used to provide a more accurate estimate of the relative densities of apical Na^+ channels among experimental groups (Ye et al. 1993b). In the present case the VSI is

$$VSI = R(c, -60) - R(c, +60) \quad (3)$$

where $R(c, -60)$ is the mean chorda tympani response magnitude of a stimulus at concentration, c , and a clamp voltage of -60 mV, and $R(c, +60)$ is the mean chorda tympani response magnitude of a stimulus at concentration, c , and a clamp voltage

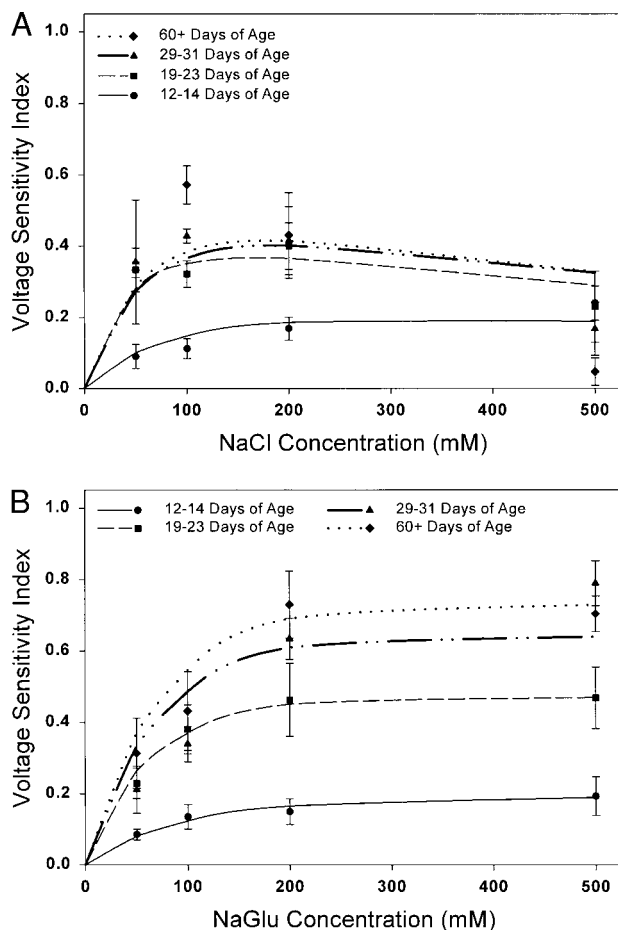


FIG. 7. A: plots of voltage sensitivity index (VSI) vs. NaCl concentration. Voltage sensitivity indices (symbols) were calculated for NaCl responses from each age group, according to Eq. 3, and plotted against NaCl concentration. The lines are theoretical functions obtained by substituting Eq. 1 into Eq. 3; they provide an estimate of the maximum NaCl response component that derives from transduction of Na^+ stimulus ions via apical Na^+ channels, "apical R_{\max} " (Table 2). Because of the loss of response voltage sensitivity at NaCl concentrations of ≥ 200 mM in the 29- to 31- and 60+-day old age groups, the correspondence between the apical channel model and the data diminishes considerably vs. the younger age groups (12- to 14-day olds, $R^2 = 0.72$; 19- to 23-day olds, $R^2 = 0.57$; 29- to 31-day olds, $R^2 = 0.27$; 60+-day olds, $R^2 = 0.22$). Despite this loss of predictive accuracy, the predicted relative apical channel densities obtained from the VSI model closely parallel those predicted by the apical channel model equation. B: plots of VSI vs. NaGlu concentration. Response data for NaGlu were transformed and plotted as for NaCl data in A. The resulting estimates of the maximum NaGlu response component that derives from transduction of Na^+ stimulus ions via apical Na^+ channels, apical R_{\max} are provided Table 2. A clear, age-related increase in the apical R_{\max} , an analog of apical Na^+ channel density, is apparent in the progressively increasing VSI, and is borne out by predicted R_{\max} values (Table 2). In striking contrast to NaCl, the correspondence between the apical channel model and the data are excellent across ages and concentrations for NaGlu (12- to 14-day olds: $R^2 = 0.95$; 19- to 23-day olds: $R^2 = 0.95$; 29- to 31-day olds: $R^2 = 0.84$; 60+-day olds: $R^2 = 0.88$). An important factor in the agreement between data and theory in this case is the preservation of response voltage sensitivity in the presence of the larger, tight junction-impermeant gluconate anion. Data presented are means \pm SE (NaCl: $n = 4-9$; NaGlu: $n = 3-9$).

TABLE 2. Apical R_{\max} values predicted by fit of apical channel model to VSI data

Stimulus	Predicted Apical R_{\max}			
	12-14 days	19-23 days	29-31 days	60+ days
NaCl	0.42 (25)	0.79 (47)	1.41 (84)	1.68
NaGlu	0.34 (25)	0.74 (54)	1.16 (84)	1.38

Values in parentheses represent the relative percentage of "mature" apical Na^+ channel density (i.e., apical R_{\max} at 60+ days of age) obtained by dividing predicted apical R_{\max} values for each age group and stimulus by the apical R_{\max} value predicted for each stimulus at 60+ days of age times 100. VSI, voltage sensitivity index.

of $+60$ mV. The theoretical VSIs for NaCl and NaGlu responses were determined by substitution of Eq. 1 into Eq. 3 with the values of K_m and δ obtained above. An "apical R_{\max} " (renamed from CT_{\max} to emphasize that it is the estimated maximum response component that arises exclusively from voltage-sensitive transduction processes) was obtained for each age group. The resulting theoretical curves for NaCl and NaGlu are shown in Fig. 7, A and B, respectively, while predicted apical R_{\max} values are provided in Table 2. For NaCl, when apical R_{\max} values for each age group are expressed as a ratio of the apical R_{\max} obtained for 60+-day-old rats, it can be inferred that fungiform papilla taste cell apical membranes in 12- to 14-day-old rats have $\sim 25\%$ the mature density of functional amiloride-sensitive Na^+ channels ($0.42/1.68$), those of 19- to 23-day-old rats have $\sim 47\%$ the mature density of functional channels ($0.79/1.68$), while those of 29- to 31-day-old rats have $\sim 84\%$ the mature density of apical Na^+ channels ($1.41/1.68$). Apical R_{\max} estimates obtained for NaGlu are in excellent agreement with those obtained for NaCl (Table 2).

DISCUSSION

In the present study, simultaneous *in vivo* lingual epithelial voltage clamping and chorda tympani recording were used to examine the increase in taste system Na^+ sensitivity that occurs during postnatal development in rat. The primary finding from these experiments is that the age-related increase in the chorda tympani Na^+ response results from the development of a transduction mechanism that is driven by stimulus electrochemical concentration. This observation implicates a set of apical membrane ion channels as the stimulus transducer and, furthermore, indicates that the postnatal increase in chorda tympani Na^+ responses is due primarily to an increase in transducer (i.e., ion channel) relative density. These results confirm previous reports, which, based on amiloride pharmacology, concluded that a progressive increase in the number of functional amiloride-sensitive Na^+ channels was probably the underlying mechanism for the developmental increase in taste system Na^+ sensitivity in rat (Hill and Bour 1985; Sollars and Bernstein 1994). Our current results also extend those findings by revealing that in addition to the age-dependent increase in the relative density of taste receptor cell functional Na^+ channels, the apparent Na^+ binding affinity of the amiloride-sensitive Na^+ channel (corresponding to a decrease in K_m) increases as a function of postnatal age. Together, these two age-related changes in the intrinsic functional properties of the Na^+ sensing apparatus contribute to the postnatal maturation of anterior tongue taste receptor Na^+ sensitivity in rat. An especially

interesting feature of these age-dependent changes in Na⁺ channel properties is that they are temporally distinct.

Results from our kinetic analyses of chorda tympani responses to NaCl and NaGlu during the postnatal period indicated differences in the timing of changes in apparent density and affinity of apical, amiloride-sensitive Na⁺ channels. Specifically, the age-related decrease in K_m values for both Na⁺ salts was observed to occur between 12–14 and 19–23 days of age. Estimated K_m for NaCl decreased by ~169 mM over this period and K_m for NaGlu by ~120 mM. Thereafter, K_m values for both salts remained stable into adulthood (Fig. 8A). In contrast, for both Na⁺ salts, CT_{max} values increased rapidly between 12–14 and 29–31 days of age, at which point the rate of growth in CT_{max} slowed considerably as mature, unchanging CT_{max} values were approached. The age-related trends in these parameters are considered in greater detail in the following sections.

Development of chorda tympani responses to NaCl and NaGlu: parallel trends

As described in RESULTS, the quantitative trends in chorda tympani response development are in general similar for these two Na⁺ salts, and this similarity was fully expected at the outset of these studies. However, inspection of the data presented in RESULTS clearly indicates that there are important quantitative differences in the parameters that describe the development of the Na⁺ sensing apparatus with respect to these two salts. Consideration of these differences provides important insight regarding the development not only of apical, amiloride-sensitive Na⁺ channel function but also the development of taste bud polar epithelial topology.

By describing our data with a channel model equation, we were able to follow, operationally, the development of the apical, voltage-dependent Na⁺ sensing apparatus by obtaining changing values in the system parameters CT_{max} , K_m , and δ . The predictive utility of this model requires that changes in Na⁺ response magnitude be the consequence of changes in apical (i.e., amiloride-sensitive) Na⁺ channel function. For NaGlu, across all age points examined, it is clear that CT response magnitude is a single-valued function of the electrochemical Na⁺ concentration (e.g., Fig. 6). Moreover, values of R_{max} (the estimate of relative channel density eliminating possible voltage-independent processes) for NaGlu predicted from consideration of the VSI revealed that the relative density of apical channels increases rapidly from ~25% of the mature density at 12–14 days of age to ~84% the mature density at 29–31 days of age. Because of the accelerating increase observed between these age points, we attempted to describe the age-dependent trend in whole system CT_{max} values (predicted from Eq. 1) using a bounded-growth model

$$CT_{max} = \frac{a}{[1 + e^{-(t-t_h)/b}]} \quad (4)$$

where CT_{max} is the maximum chorda tympani response value predicted by the channel model equation and t is the mean age in days for each age group studied, while b is the time in days required for the initial response magnitude at $t = 0$ days to increase by a factor of e , and t_h is the time at which the response magnitude has reached half the mature, unchanging, steady-state response magnitude, a . Values of CT_{max} obtained

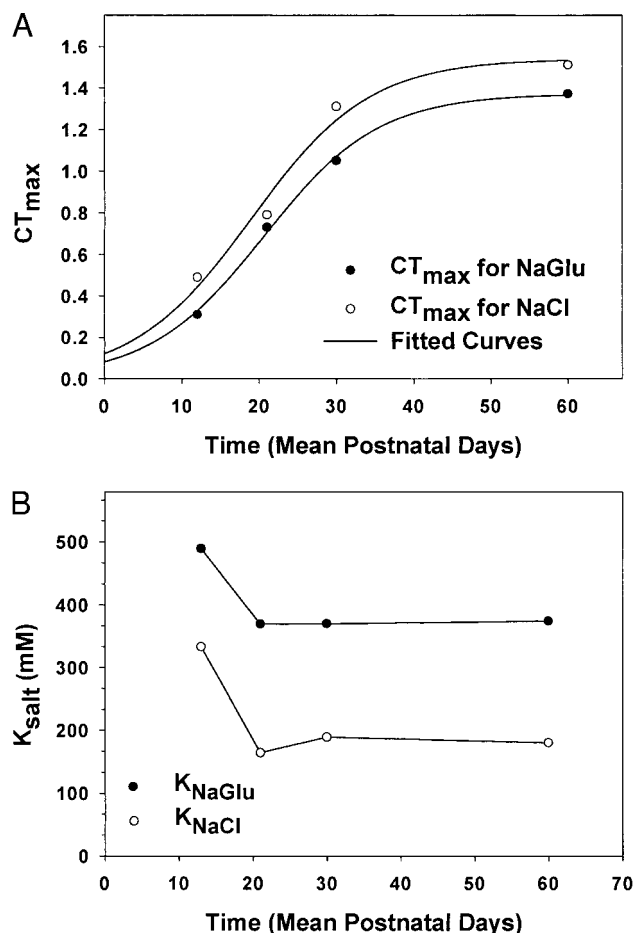


FIG. 8. Age-related trends in system CT_{max} and K_m . A: maximum chorda tympani response values (i.e., CT_{max}) for NaCl and NaGlu predicted by Eq. 1 (symbols) were fit with a bounded-growth function (Eq. 4). The resulting theoretical curve provides an exceptional description of the age-related increase in CT_{max} values observed for both NaCl ($R^2 = 0.98$) and NaGlu ($R^2 = 0.99$), which is exponential between 0 days of age and around the time of weaning (i.e., 21 days of age). The parallel nature of the theoretical functions reflects the presence of the additional basolateral, voltage-insensitive component of NaCl responses. Importantly, extrapolation of the theoretical line to time 0 (i.e., 0 days of age) permits estimation of the relative density of apical Na⁺ channels present at the time of birth. These estimates were 6 and 8% of the mature channel density for NaGlu and NaCl, respectively. B: the apparent affinity of apical Na⁺ channels as inferred from K_m values, declines between 12–14 and 19–23 days of age. Interestingly, K_m values estimated for NaCl were invariably lower than were those for NaGlu. This difference is probably due to anion-dependent differences in transmembrane potential attending application of these salts, which acts to modulate the driving force for Na⁺ entry through apical Na⁺ channels. That is, the native channel Michaelis constant, K , is anion dependent. Therefore in the intact system, it is more instructive to express specific values for each Na species studied (e.g., K_{NaCl} and K_{NaGlu}). This apparent anion-related modulation of an intrinsic channel property is the unique consequence of constraints imposed by taste bud polarity on the function of the in situ system.

for NaGlu were fit with this model, and Fig. 8A shows the data plotted with the resulting predicted line. The best fit of the model to the data was obtained when $a = 1.37$, $b = 7.4$ days, and $t_h = 20.6$ days; the model accounts for nearly all of the variance in the data ($R^2 = 0.99$). The predicted value for maximum system response to NaGlu is exactly equal to the CT_{max} value obtained from the channel model equation for 60+-day-old rats, which is consistent with the operational assumption that 60 postnatal days is sufficient time to achieve

asymptotic values ($t = \infty$). The time to half-maximal system response, t_h , corresponds closely to the time of weaning. When predicted values of CT_{\max} derived from this model are extrapolated to zero days of age (i.e., $t = 0$), a value of 0.08 is obtained, which suggests that, at birth, fungiform papilla taste receptor cell apical membranes contain a relative Na^+ channel density that is $\sim 6\%$ that of mature rats (0.08/1.37). By ~ 30 days of age, the developmental increase in CT_{\max} has slowed considerably, and thereafter gradually approaches the unchanging steady-state maximum (i.e., mature adult) value. This model is fully consistent with the hypothesis that the postnatal increase in taste system Na^+ sensitivity is due to an increase in the number of functional, apical amiloride-sensitive Na^+ channels. It furthermore suggests that activation of inactive channels is accelerated by the presence of some channels already activated. Not surprisingly, when CT_{\max} data for NaCl were fit with the bounded-growth model, a similarly accurate description of the developmental trend in gustatory Na^+ sensitivity was obtained.

Figure 8A also shows the theoretical bounded growth function for the development of NaCl response CT_{\max} . The best fit of the model to the data were obtained when $a = 1.54$, $b = 7.7$ days, and $t_h = 19.0$ days ($R^2 = 0.98$). This predicted maximum system NaCl response is nearly identical to the value of CT_{\max} for 60+-day-old rats obtained from the channel model equation. Moreover, the values of b and t_h are comparable to those obtained for NaGlu, and extrapolation of CT_{\max} values to zero days of age, yields a relative channel density of $\sim 8\%$ the adult density. This zero-day value is of particular interest with respect to the findings of Ye et al. (1993b), who, using the apical channel model, determined that fungiform taste receptors of adult rats raised under developmental dietary Na^+ restriction express a relative density of apical Na^+ channels that is $1/10$ th the density in the taste receptor cells of control animals reared on a Na^+ replete diet. However, the effect of developmental Na^+ restriction appears to be unique to channel density, as no diet-related difference in apparent K_m was reported by Ye et al. (1993b). Those results, in conjunction with the current findings, underscore the notion that channel density and affinity are subject to independent regulation, and, further, raise the intriguing possibility that developmental Na^+ restriction causes truncated development of the gustatory Na^+ sensing apparatus. That is, with respect to Na^+ sensing, fungiform taste receptor cells in developmentally Na^+ -restricted rats attain a state of maturity functionally equivalent to that of a newborn. It should be emphasized again, however, that these data relate only to the apical Na^+ sensing machinery.

With respect to the entire Na^+ sensing apparatus (i.e., apical and basolateral pathways), an important distinction between the bounded growth functions for NaGlu and NaCl relates to the parallel nature of CT_{\max} values across development. That is, system CT_{\max} values predicted by the channel model equation and by the bounded growth model, as well as the apical R_{\max} values predicted by the VSI function, are larger for NaCl than for NaGlu at all age points. This reliable difference in the magnitudes of NaCl and NaGlu responses was expected and corresponds to the presence of the amiloride-insensitive component of the chorda tympani response to NaCl. It is interesting to note that despite the presence of this amiloride-insensitive response component in NaCl responses from the earliest age points studied, the fundamental hypothesis that the postnatal

increase in gustatory Na^+ sensitivity is due to an orderly increase in the density of functional, apical (and, therefore voltage-sensitive) Na^+ channels is nonetheless validated by the apical channel model. In addition to its contribution to stimulus-dependent differences in measures of maximum response magnitude, the anion-dependent properties of the paracellular shunt in the intact taste bud contribute to differences between NaCl and NaGlu with respect to other Na^+ channel kinetic parameters.

In addition to parallel, age-dependent trends in chorda tympani CT_{\max} for NaCl and NaGlu, parallel, age-related changes in the apparent affinity (K_m) of Na^+ for the apical Na^+ channel were seen to occur (Fig. 8B). Specifically, K_m for both salts decreased from relatively high values at 12–14 days of age to lower values at 19–23 days of age, and then remained stable across succeeding age points. The similar trends in K_m for both salts clearly suggest that the affinity of the apical channel for Na^+ ions increases developmentally and, thus, contributes to the overall increase in gustatory Na^+ sensitivity that is seen during the postnatal period. We speculate that the change in channel K_m is most probably due to developmental alteration in intrinsic channel properties. It is possible, however, that changes in the “affinity” of processes intermediate to Na^+ binding/influx and the generation of the neural response (e.g., taste cell neurotransmitter release) are responsible for the apparent shift in channel affinity. Consistent with such a view, Hill et al. (1982) observed a developmental decrease in the latency of chemically-stimulated neural responses in single chorda tympani fibers. On the other hand, Hill and colleagues (1982) found no difference in single chorda tympani fiber response latency in rats aged 10–14 versus those aged 24–35 days of age. In the present study, the apparent shift in K_m occurs between 12–14 and 19–23 days of age, long before Hill et al.’s latency shift, which occurred between 24–35 days of age and adulthood. Regardless of the source of this apparent shift, the large differences in predicted K_m values observed for the two salts raise an important question: if K_m describes the affinity of Na^+ for a Na^+ specific channel, why then are the predicted values of K_m for the two salts so divergent? That is, why, in the presence of NaCl, is the apparent affinity of Na^+ for Na^+ channels so much greater than when NaGlu is the stimulus?

From the present experiments, and from previous work (Stewart et al. 1996), it is clear that the lingual transepithelial potential (TEP) that attends application of NaGlu is considerably more submucosa positive compared with the potential that attends application of an equimolar concentration of NaCl. This stimulus-dependent difference in TEP results from the lower shunt permeability of the gluconate anion relative to Cl^- . Given the polarized epithelial topology of the taste bud, it is predicted that the lower TEP evoked by NaCl results in a greater depolarization of the basolateral taste receptor cell membrane and in a relatively larger hyperpolarization across the apical taste receptor cell membrane compared with NaGlu (cf. Lindemann 1996; Ye et al. 1994). Importantly, this hyperpolarization at the apical membrane is “seen” by Na^+ ions as an increased intracellular electronegative potential and thus a relative increase in the driving force for Na^+ ions into the taste cell across the apical amiloride-sensitive conductance. In contrast, when gluconate is the stimulus anion, the more submucosa positive potential creates a relative depolarization of the

apical membrane and thus a relative decrease in driving force for Na^+ ions into the taste cell. In our model system, the net effect of these stimulus- and, hence, TEP-dependent differences in the driving force for apical Na^+ flux appears to be manifested as stimulus-dependent shifts in K_m . That is, the aggregate K values predicted by our model depend on anion- and, hence, stimulus-related differences in TEP.

The idea that stimulus-dependent TEP can potentially influence apparent K_m values for the intact system has been demonstrated previously (Stewart et al. 1996). Such stimulus-related modulation of the channel native Michaelis constant, here called K , is the unique consequence of applying the apical channel model to the intact taste system and illustrates how the polar topology of the taste bud imposes unavoidable constraints on the function of membrane effectors, such as ion channels. Moreover stimulus-dependent modulation of an apparent intrinsic channel property such as K emphasizes the importance of studying function under conditions that maintain the essential polarity of the biological system. Given these observations and the likelihood that Na^+ salts modulate their own apparent Na^+ channel affinity, it is perhaps most appropriate to assign individual Na^+ salt stimuli specific K values (e.g., K_{NaCl} and K_{NaGlu}) when such parameters are determined in the intact system. For the present study, it is critical to bear in mind that the consistent, stimulus-dependent difference between K_{NaCl} and K_{NaGlu} is superimposed on a large developmental change in the Na^+ channel "native" K . In addition to this clear and important influence of the stimulus anion on apparent system K_m , the stimulus anion also contributes to differences between NaCl and NaGlu with respect to other system properties.

As noted in RESULTS, age-dependent changes in the fraction of the clamp voltage, δ , that is sensed by Na^+ ions as they traverse the apical Na^+ channels were observed. However, in contrast to parallel trends in other system parameters, age-dependent changes in δ were observed for NaCl only. Specifically, and unexpectedly, estimated δ for NaCl declined from comparable values at 12–14 and 19–23 days of age (i.e., 0.44 and 0.45, respectively) to considerably lower values at 29–30 and 60+ days of age (i.e., 0.25 and 0.22, respectively). By comparison, estimated δ values for NaGlu were largely stable throughout the time period studied. For all age groups, δ values for NaCl are smaller than are those for NaGlu . The consistent difference in δ for the two stimuli is a consequence of the higher shunt permeability of Cl^- relative to that of gluconate. Moreover, the decline in δ for NaCl in the two oldest age groups implies that taste bud shunt properties change considerably as a function of postnatal age. Specifically, the decrease in δ for NaCl between 19–23 and 29–31 days of age suggests that tight junctional permeability to mobile anions, such as Cl^- , actually *increases* with advancing age. That is, taste bud tight junctions do not become tighter with advancing postnatal age, they become leakier. This decrease in tight junction resistance appears to be unique to taste buds, as measurements of relative transepithelial resistance and conductance did not vary as a function of postnatal age (data not shown). The dissociation between changing δ for NaCl and stable δ for NaGlu is not inconsistent and probably reflects the fact that even a considerable increase in the shunt permeability for smaller ions has no evident impact on permeability to large ions with low tight junctional mobility (e.g., gluconate). This remarkable develop-

mental change in the topological properties of the taste bud has direct functional correlates in the present findings.

The age-related change in δ for NaCl may explain in large part the failure of the theoretical VSI function to fit accurately the data for NaCl responses in 29- to 31- and 60+-day-old rats. While there was excellent agreement between the observed and predicted VSIs for NaCl for 12- to 14- and 19- to 23-day-old rats, by 29–31 days of age and continuing into adulthood, the predicted VSI underestimated the observed VSI at concentrations of NaCl below 200 mM and overestimated the observed VSI at the highest NaCl concentration (500 mM) (Fig. 7A). The degradation in the predictive accuracy of the model probably derives from the loss of voltage sensitivity in chorda tympani responses to higher concentrations of NaCl in 29- to 31- and 60+-day-old rats. A striking example of this can be seen in Fig. 2. Not surprisingly, the timing of the loss of chorda tympani response voltage sensitivity at higher NaCl concentration coincides with the timing of the decrement in δ observed for NaCl .

The critical parameter in the VSI of the chorda tympani response to NaCl is δ . That is, as δ approaches unity, the observed effect of voltage perturbations on chorda tympani responses becomes greater. In contrast to the case of NaGlu , where δ is stable and relatively large, δ appears to decline as a function of increasing NaCl concentration, especially in the older age groups. It is possible that in the presence of high NaCl concentrations, the paracellular diffusion potential of the stimulus ions is increased, and, under voltage clamp conditions, this increase in paracellular electroneutral diffusion potential for Na^+ and Cl^- provides a "sink" for current injected to maintain command voltage. Essentially, the paracellular shunt becomes a lower resistance pathway for ion movement (i.e., current) than is the apical, amiloride-sensitive Na^+ channel, and so a relatively larger fraction of the clamp voltage is dropped across the paracellular shunt as NaCl concentration increases. The consequences of this age-dependent increase in the paracellular shunt permeability (observed as a decline in δ) are two. One probable consequence, which is observed, is that dissipation of clamp voltage across the paracellular shunt diminishes the measurable voltage sensitivity of chorda tympani response to NaCl at high concentrations but only in older rats that have developed the lower paracellular shunt resistance. Another likely consequence is that as the rat matures, the relatively increased electroneutral diffusion of Na^+ and the stimulus co-anion through the tight junctions increases the magnitude of the voltage-independent and amiloride-insensitive response component, particularly at high stimulus concentrations. Such a change in shunt properties could also contribute to Na^+ response development, although available evidence suggests that the magnitude of the amiloride-insensitive component of chorda tympani NaCl responses is stable during development in rats (Hill and Bour 1985; Sollars and Bernstein 1994). This important developmental change in taste bud topological properties, which exerts influences on several fundamental parameters that describe system function, could only be appreciated through examination of the intact, functioning taste system.

It is important to note that the apical channel model assumes that δ is a constant. However, the present data clearly indicate that the value of δ necessarily depends on the potential for paracellular electroneutral diffusion of Na^+ and Cl^- , which is

determined jointly by NaCl concentration and the resistance of the shunt pathway (i.e., tight junctions) (Ye et al. 1993a). It should also be emphasized here that for Na⁺ salt stimuli that contain large, shunt-impermeant anions, such as gluconate, modulation of δ would not be observed. That is, modulation of δ is essentially anion dependent.

Potential mechanisms for developmental increases in apical Na⁺ channel number and affinity

Our current findings indicate that temporally distinct, age-dependent changes in the density of apical, amiloride-sensitive Na⁺ channels and in the apparent affinity of Na⁺ for those channels together provide the basis for postnatal development of mature gustatory Na⁺ sensitivity of the rat chorda tympani nerve. Our results confirm previous hypotheses (e.g., Hill and Bour 1985; Sollars and Bernstein 1994) regarding the postnatal increase in chorda tympani responses to Na⁺. The cellular basis for the increase in the density of functional channels remains to be elucidated, but several mechanisms could be involved. For example, a simple increase per taste bud in the number of taste cells that possess a fixed number of apical Na⁺ channels could contribute to an apparent increase in the number of apical amiloride-sensitive Na⁺ channels. There is, in fact, ample evidence that fungiform papilla taste bud size increases postnatally (Krimm and Hill 1998) and, furthermore, that this size increase may be due proliferation of taste cells as opposed to hypertrophy of individual cells (Kossel et al. 1997). On the other hand, recent developmental studies of taste cell proliferation revealed lower rates of cell addition to taste buds in rats aged zero and 29–31 days compared with those aged 60+ days (Hendricks and Hill 2000). Another obvious mechanism for an increase in the density of apical Na⁺ channels would be a progressive increase in the de novo synthesis and insertion into the apical membrane of functional Na⁺ channels. However, previous immunohistochemical results provided qualitative evidence that the fungiform papilla taste cells of 1-day-old rats contained significant amounts of apical Na⁺ channel-like immunoreactive material (Stewart et al. 1995). That result raises the possibility that from early in development, rat fungiform papilla taste receptor cells synthesize amiloride-sensitive Na⁺ channels, and these extant channels gradually become functional during the postnatal period. For example, it is possible that channels are synthesized and immediately inserted into taste cell apical membranes but that they remain inactive. Subsequent, developmentally regulated activation processes (e.g., covalent modification) (cf. Garty and Palmer 1997; Shimkets et al. 1998) might then act to increase the relative density of functional channels. Alternatively, it is possible that a redistribution of Na⁺ channels between basolateral and apical membrane compartments occurs during the early postnatal period. This potential mechanism was favored by Kossel and colleagues (1997) and by Stewart et al. (1995), who suggested that such membrane reallocation might be promoted by maturation of, and subsequent association of apical membrane channels with, taste cell cytoskeletal elements. Interestingly, cytoskeletal elements interact with and modulate the function of apical Na⁺ channels (Ismailov et al. 1997; Smith and Benos 1996; Smith et al. 1991). This intriguing possibility has so far evaded elucidation in the taste system, but recent advances in the molecular biology of apical Na⁺ chan-

nels in other tissues should facilitate its evaluation (Benos et al. 1997; Garty and Palmer 1997).

Strides in the understanding of the apical Na⁺ channel may also provide insights into the mechanism for the considerable increase in the affinity of Na⁺ for the apical channel that was observed to occur between 12–14 and 19–23 days of age. In the past several years, molecular biological techniques have allowed the cloning of distinct genes that encode the primary amino acid sequences for three homologous subunit proteins that in complementation comprise an amiloride-sensitive, Na⁺-selective conductance with biophysical properties similar to those of purified, native amiloride-sensitive Na⁺ channels characterized from several source tissues (Benos et al. 1997; Canessa et al. 1994; Garty and Palmer 1997). Specifically termed the epithelial Na⁺ channel, or ENaC, the heterotrimeric channel consists of alpha, beta, and gamma subunit proteins, all of which have been detected immunohistochemically in rat fungiform papilla taste cells (Kretz et al. 1999; Lin et al. 1999). When expressed alone, the alpha subunit comprises an amiloride-sensitive conductance, characterized by low maximum Na⁺ current and low amiloride binding affinity. However, co-expression of all three subunits results in a highly Na⁺-selective, amiloride-blockable conductance with large maximum Na⁺ current. It is notable that subunit complementation patterns can significantly influence, for example, the amiloride binding affinity and ion selectivity of the ENaC, in addition to the maximum Na⁺ current that can be sustained by the channel complex (Benos et al. 1997). Clearly, an age-related alteration in subunit complementation pattern could contribute to the developmental increase in gustatory Na⁺ sensitivity. One striking, though fully speculative, possibility with respect to the current results is that the age-related change in apparent channel affinity is the result of “immature” subunit complementation patterns. However, Kossel et al. (1997) present evidence that the inhibition constant of amiloride for blockade of whole cell inward currents in neonatal taste cells is comparable to that obtained for adult taste cells. Given other results that suggest alterations in subunit complementation can strongly influence ENaC amiloride-binding affinity (Benos et al. 1997), this result tends to argue against an altered subunit complementation pattern in young rat taste receptor cells. On the other hand, it is possible that in taste receptor cells apical Na⁺ channel affinities for Na⁺ ions and for amiloride are subject to different and distinct regulatory effectors (cf. Garty and Palmer 1997). Clearly, an important area for future investigation includes resolving, in the intact, developing taste system, whether the interaction of amiloride with the apical Na⁺ channel changes significantly as a function of postnatal age. Results from such experiments, in combination with more molecular studies that directly assess taste receptor cell ENaC subunit expression and complementation patterns during postnatal development, should provide important evidence regarding the cellular mechanisms that underlie the developmental increase in apical Na⁺ channel affinity.

APPENDIX

As shown in the RESULTS, the chorda tympani response to Na⁺ is a saturating function of the Na⁺ electrochemical concentration (cf. Eq. 1), i.e., the driving force for the flux of Na⁺ through a conductive pathway. This suggests that the form of Eq. 1 is the result of a kinetic

process involving the influx into taste receptor cells of Na^+ ions across apical membrane ion channels. This can be demonstrated using the Na^+ channel model proposed by Mintz et al. (1986) in their computational simulation of sodium transport across tight epithelia. It is assumed that the apical channels have pores connecting the mucosal solution phase with the cell interior and that each pore contains a single Na^+ ion binding site. In addition, each Na^+ ion entering the channel from either side must overcome a potential energy barrier to reach the binding site. While the binding site may be located at any point within the pore, we assume here that it is located at the pore exit into the cell interior. In this way, a Na^+ ion entering the channel from the stimulating solution will encounter the entire electrical potential gradient across the apical membrane. The binding association reaction between a Na^+ ion originating from the mucosal side (m) and the pore site has rate constant f_m ; the similar association reaction involving a Na^+ ion originating from the cellular side (c) of the channel has rate constant f_c . The binding dissociation reaction releasing Na^+ into the mucosal solution has rate constant k_m and that releasing Na^+ into the cellular medium has rate constant k_c . Because Na^+ ions entering channels from the mucosal side encounter the additional potential energy of the apical membrane potential, f_m and k_m can be written respectively as: $f_{m0} \exp[-\theta/2]$ and $k_{m0} \exp[\theta/2]$, where θ is the dimensionless apical membrane potential relative to the mucosal solution, and f_{m0} and k_{m0} are rate constants exclusive of electrical potential effects. The values of the pairs f_{m0} and k_{m0} and f_c and k_c may differ in magnitude depending on the size of the energy barriers on each side of the pore region, but the ratios, k_{m0}/f_{m0} and k_c/f_c are equal, defining the thermodynamic dissociation constant, K_e , between Na^+ ions and their channel binding sites.

Let N be the total number of channels per unit area of apical membrane, p_o , the fraction of pore binding sites that are unbound, and p_1 , the fraction of sites with a bound Na^+ ion. Conservation of total binding sites requires: $p_o + p_1 = 1$. In the steady state, the net Na^+ flux, J_{Na} is

$$J_{\text{Na}} = N(f_m c_m p_o - k_m p_1) = N(k_c p_1 - f_c c_c p_o) \quad (\text{A1})$$

Equation A1 in conjunction with the total binding site conservation relation noted above permits determination of p_o and p_1 as functions of the rate constants, the Na^+ concentration in the mucosal solution, c_m , the Na^+ concentration in the cell, c_c , and the apical membrane potential. Accordingly we obtain the following equation for J_{Na}

$$J_{\text{Na}} = \frac{Nk_c(c_m e^{-\theta/2} - c_c e^{\theta/2})}{c_m e^{-\theta/2} + k(c_c + K_e) + K_e e^{\theta/2}} \quad (\text{A2})$$

where $k = k_c/k_{m0}$. Because the cell electrical potential is electronegative with respect to the mucosal solution and the cell Na^+ concentration is kept at a low value by the Na-K pump, $c_c \exp[\theta/2]$ can be neglected compared with $c_m \exp[-\theta/2]$. We assume that the rate constant for dissociation of Na^+ into the cell is ≥ 10 times greater than the rate constant for dissociation into the mucosal solution, i.e., $k \geq 10$. K_e can be expected to be of the same order as c_c (i.e., from 10 to 30 mM), so $K_e \exp[\theta/2]$ can also be expected to be small compared with $k(c_c + K_e)$. The sodium flux can be, therefore approximated without loss of accuracy by

$$J_{\text{Na}} = \frac{Nk_c c_m}{c_m + k(c_c + K_e)e^{\theta/2}} \quad (\text{A3})$$

Under voltage-clamp conditions, equation A3 becomes

$$J_{\text{Na}} = \frac{Nk_c c_m e^{-\delta\phi}}{c_m e^{-\delta\phi} + k(c_c + K_e)e^{\theta/2}} \quad (\text{A4})$$

If the CT response is assumed to be proportional to J_{Na} , then comparing Eq. A4 with Eq. 1 in RESULTS, we can conclude that CT_{max} is proportional to the density of channels, N , and the rate constant for

dissociation of Na^+ from the channel to the cell interior, k_c . In terms of this model, we also note that K_m in Eq. 1 is

$$K_m = k(c_c + K_e)e^{\theta/2} \quad (\text{A5})$$

This suggests the likely source of the anion dependence that we observed in K_m , i.e., the reason why K_{NaCl} is much smaller than K_{NaGlu} . We note that k , c_c , and K_e should not change between NaCl and NaGlu. However, the potential across the apical membrane will be affected by the anion present because gluconate has a much lower paracellular shunt permeability than chloride. NaGlu, with its greater transepithelial potential relative to NaCl, should therefore also have a significantly more positive apical membrane potential because changes in the transepithelial potential will produce changes in potential across both the apical and basolateral taste receptor cell membranes. Substituting NaGlu for NaCl therefore effectively reduces the Na^+ influx accounting for the poorer CT stimulation at a given Na^+ concentration and, likewise, the apparently higher K_m . If we take the K_m values for NaGlu and NaCl at 60+ days (373.6 and 180.1 mM, respectively), Eq. A5 suggests that the apical membrane potential for NaGlu is ~ 38 mV more positive than for NaCl ($2(26) \ln [373.6/180.1]$). Reasonable values of parameters that would approximately account for these K_m values are: $k = 25$, $K_e = 10$ mM, and $c_c = 10$ mM for both salts, and $\theta \approx -2$, (-52 mV) for NaCl, and -0.6 (-15.6 mV) for NaGlu. These give 183.9 mM for NaCl and 370.4 mM for NaGlu and an apical potential difference between the salts of 36.4 mV. Thus although the Na taste transducer is a Na^+ channel, the anion present in the Na stimulus strongly modulates the density of depolarizing Na^+ current into the receptor cells.

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