Published in final edited form as:
Am J Physiol Regul Integr Comp Physiol. 2008 January ; 294(1): R66–R75.

Amiloride-sensitive NaCl taste responses are associated with genetic variation of ENaC α-subunit in mice

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Abstract

An epithelial Na^+ channel (ENaC) is expressed in taste cells and may be involved in the salt taste transduction. ENaC activity is blocked by amiloride, which in several mammalian species also inhibits taste responses to NaCl. In mice, lingual application of amiloride inhibits NaCl responses in the chorda tympani (CT) gustatory nerve much stronger in the C57BL/6 (B6) strain than in the 129P3/J (129) strain. We examined whether this strain difference is related to gene sequence variation or mRNA expression of three ENaC subunits (α, β, γ). Real-time RT-PCR and in situ hybridization detected no significant strain differences in expression of all three ENaC subunits in fungiform papillae. Sequences of the β- and γENaC subunit genes were also similar in the B6 and 129 strains, but αENaC gene had three single nucleotide polymorphisms (SNPs). One of these SNPs resulted in a substitution of arginine in the B6 strain to tryptophan in the 129 strain (R616W) in the αENaC protein. To examine association of this SNP with amiloride sensitivity of CT responses to NaCl, we produced F_2 hybrids between B6 and 129 strains. Amiloride inhibited CT responses to NaCl in F_2 hybrids with B6/129 and B6/B6 αEnaC R616W genotypes stronger than in F_2 hybrids with 129/129 genotype. This suggests that the R616W variation in the αENaC subunit affects amiloride sensitivity of the ENaC channel and provides evidence that ENaC is involved in amiloride-sensitive salt taste responses in mice.

Keywords

salt taste; amiloride sensitivity; epithelial sodium channel; single nucleotide polymorphisms; nerve recording

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Amiloride, an epithelial Na^+ channel (ENaC) blocker (2,29), alters Na^+ currents in rat taste cells (8,34) and decreases NaCl responses of the chorda tympani (CT) nerve in various species of mammals, such as rats (14), mice (43,44), gerbils (20), hamsters (15), rhesus monkeys (16), and chimpanzees (17). Behavioral experiments in rodents demonstrated that amiloride abolished behavioral discrimination between sodium and non-sodium salts (18,58,60), indicating that the amiloride-sensitive component of NaCl responses plays a major role in the perception of sodium salt taste. In mice and rats, there are prominent strain differences in the amiloride sensitivity of neural responses to NaCl (8,10,11,28,44,47,49,50). For example, in C57BL/6 (B6), amiloride suppressed the CT re sponses to NaCl to ~50% of control, whereas

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in 129P3/J (129) mice, the compound produced very small ~20% of control) or no significant inhibition of the NaCl response (11,47,50).

One type of amiloride-sensitive ENaC that is expressed in taste cells (31,32,34,56,57) may play a role in the salt taste transduction. ENaC consists of at least three subunits (α, β, and γ) encoded by three different genes, *Scnn1a*, *Scnn1b*, and *Scnn1g*, respectively (gene names: sodium channel, non-voltage-gated, type I, α, β, and γ). Each ENaC subunit possesses two hydrophobic membrane-spanning domains (M1 and M2) with intracellular NH_2 and COOH termini and a large extracellular loop containing two or three cysteine-rich domains (24) and shares 3~40% sequence identity in mammals (4,5,36). The α ENaC confers a low-amplitude, amiloride-sensitive sodium current, whereas β- and γ-subunits are required for the maximal channel activity. Several naturally occurring and induced mutations in ENaC subunits were shown affect ENaC activity and its sensitivity to amiloride (3,13,19,22,23,25–27,33,52). These findings raise the question of whether polymorphisms of genes encoding ENaC subunits may be involved in the differences in amiloride sensitivity among mouse strains.

In this study, therefore, we investigated relationships of the amiloride sensitivity with single nucleotide polymorphisms (SNPs) and mRNA expression levels in fungiform papillae in the anterior tongue of three subunits of ENaC (α, β, γ). To accomplish this, we used mice from two inbred strains, amiloride high-sensitive B6 and amiloride low-sensitive 129, and their F_2 hybrids.

MATERIALS AND METHODS

Animals

All experimental procedures were approved by the committee for Laboratory Animal Care and Use at Kyushu University (Fukuoka, Japan). Subjects were adult male and female C57BL/6NCrj mice [B6, 8–16 wk of age, ranging in weight from 20 to 32 g, obtained from Charles River (Tokyo, Japan)], 129P3/J mice [129, 8–16 wk of age, ranging in weight from 23 to 34 g, obtained from the Jackson Laboratory (Bar Harbor, ME)], and F_2 hybrid mice generated by intercrossing B6 and 129 strains [8–16 wk of age, ranging in weight from 23 to 35 g].

Sequencing analysis

To compare nucleotide sequences of three ENaC subunit genes of B6 and 129 mice, total RNA was extracted from the anterior tongues as described earlier (56). A cDNA was generated by reverse transcription [oligo$(dT)_{12–18}$ primer] with the superscript II (Invitrogen, San Diego, CA). The primers ($\alpha_{1\sim4}$ for α-subunit, $\beta_{1\sim3}$ for β-subunit, $\gamma_{1\sim4}$ for γ-subunit) used for DNA amplification are shown in Table 1. PCR was performed on PE9700 with the following conditions: 95°C for 5 min (1 cycle); 94°C for 30 s, 58°C for 30 s, and 72°C for 120 s (35 cycles); and 72°C for 5 min (1 cycle). The PCR solution contained 10 mM Tris · HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 μM each primer, 200 μM dNTP, and 0.05 U/μl of Ex HS Taq polymerase (Takara Bio, Otsu, Japan). The PCR products were sequenced by the dideoxy chain termination method; confirmation that these products were fragments of ENaC subunits was accomplished by BLAST search of GenBank.

Genotype determination for α616 ENaC polymorphism in F_2 hybrid mice

Genomic DNA was extracted from mouse tails of F_2 mice using the NaOH/Tris method. The αENaC region with sequence variation between B6 and 129 strains, C1877T (R616W), which was revealed in sequencing analysis, was amplified using primer α_5 shown in Table 1. The genotypes (B6/B6, B6/129, or 129/129) were determined by sequencing as described above.

Electrophysiological experiments: recordings of responses from the CT nerve

Electrophysiological recordings were obtained from the three groups of F_2 hybrid mice with different αENaC C1877T SNP genotypes [129/129 (*n* = 7), B6/129 (*n* = 14), and B6/B6 (*n* = 11)] and from B6 inbred mice (*n* = 10). The data for 129 parental strain (*n* = 15) were derived from our recent study (50). These mice were operated under pentobarbital anesthesia. The CT nerve was exposed for electrophysiological recording. The procedures of dissection and recording were the same as those used previously (43,45,48). Briefly, under pentobarbital anesthesia, the trachea of each animal was cannulated, and the mouse was then fixed in the supine position with a head holder to allow dissection of the CT nerve. The right CT nerve was exposed at its exit from the lingual nerve by removal of medial pterygoid muscle. The CT nerve was then dissected free from surrounding tissues and cut at the point of its entry to the bulla. For whole nerve recording, the entire nerve was placed on a silver wire electrode. An indifferent electrode was placed in nearby tissue. Neural responses resulting from chemical stimulations of the tongue were fed into an amplifier (K-1; Iyodenshikogaku, Nagoya, Japan), monitored on an oscilloscope and audiomonitor, recorded on a recorder (WS-641G; Nihon-kohden, Tokyo, Japan), and stored on magnetic tape for later analysis. Whole nerve responses were integrated by an integrator having a time constant of 1.0 s.

Chemical stimulations to the tongue

The anterior half of the tongue was enclosed in a flow chamber made of silicone rubber (42). Solutions were delivered into the chamber by gravity flow and flowed over the tongue for a controlled period. Solutions used as chemical stimuli were as follows: 0.03, 0.1, and 0.3 M NaCl with and without 100 μM amiloride; 0.03, 0.1, and 0.3 M KCl with and without 100 μM amiloride; and 0.1 M NH_4Cl (Nacalai Tesque, Kyoto, Japan). These chemicals were dissolved in distilled water and used at ~24°C. The order of chemical stimulations for whole nerve recordings was 0.1 M NH_4Cl, 0.0330.3 M NaCl, 0.03~0.3 M KCl, and 0.03~0.3 M NaCl with 100 μM amiloride and 0.03~0.3 M KCl with 100 μM amiloride. After the series of stimulations with amiloride, 0.03~0.3 M NaCl without amiloride was repeatedly applied to check the recovery after amiloride inhibition. In most cases of whole nerve recording after confirming the recovery (>85% of control levels of responses), these series of stimulations were repeated. During chemical stimulation of the tongue, the test solution flowed for 30 s at the same flow rate as the distilled water used for rinsing the tongue (~0.1 ml/s). The tongue was rinsed with distilled water for 1 min between successive stimulations during all the series (including stimuli mixed with and without amiloride). The stability of each preparation was monitored by the periodic application of 0.1 M NH_4Cl. A recording was considered to be stable when the NH_4Cl response magnitudes at the beginning and end of each stimulation series deviated by no more than 15%. Only responses from stable recordings were used in the data analysis.

Real-time RT-PCR

To determine whether the expression of three ENaC subunits differed between B6 and 129 strains, we measured relative mRNA abundances using quantitative real-time RT-PCR. For each mouse (*n* = 9 and 8 for B6 and 129, respectively), 50 taste buds were isolated from fungiform papillae, and 50 taste buds were isolated from circumvallate papilla. The isolated taste buds from each type of papillae of each mouse were pooled. As a positive control for ENaC expression, 5 mg of homogenized whole kidney were also collected from each mouse (*n* = 5 and *n* = 5 for B6 and 129, respectively). Total RNAs were isolated from each sample using the RNeasy Micro Kit (Quiagen, Valencia, CA). Genomic DNA was removed from RNA by treating it with DNase (provided in the RNeasy Micro Kit) for 30 min at 37°C. Based on RNA concentration, volumes of RNA extracts that contain the same amount of RNA were determined and reverse transcribed using oligo(dT) and Ready to GO you-prime First-strand Beads (GE healthcare, Piscataway, NJ). Real-time PCR was performed on an Applied

Biosystems StepOne Real-Time PCR System using specific primers and the QuantiTect SYBR Green PCR Kit (Quiagen). The specific PCR primers, α_6, β_4 (41), γ_5, and actin were used for amplifying α, β, γENaC subunits and β-actin, respectively (Table 1). To ensure that SYBR green was not incorporated into primer dimers or nonspecific amplicons during the real-time PCR runs, the PCR products were analyzed by gel electrophoresis in initial experiments. Single bands of the expected size were obtained in all instances. Furthermore, analysis of SYBR green dissociation curves after completion of 40 PCR cycles revealed the presence of single amplicons for each primer pair. To ensure that residual genomic DNA was not being amplified, control experiments were performed in which reverse transcriptase was omitted during cDNA synthesis. Expression of each gene was assessed in a separate PCR reaction, with each reaction mixture containing 1 μl of cDNA template, 0.12 μmol forward and reverse primers, 10 μl of 2× Master Mix, and DNase RNase-free water to make a total volume of 20 μl. Samples were amplified as following: 95°C for 15 min (1 cycle); 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s (40 cycles). Relative expression of mRNAs was determined by a simplified comparative threshold cycle (C_T) method (37) using β-actin as an endogenous standard. Briefly, C_T values were averaged from each duplicate, and differences between the mean C_T values of the ENaCs and β-actin were calculated as $\Delta C_{T\ ENaC} = C_{T\ ENaC} - C_{T\ \beta\text{-actin}}$ for normalization. Finally, ENaC mRNA amounts relative to β-actin were determined as $2^{\Delta C_{T\ ENaC}}$.

In situ hybridization

In situ hybridization experiments were performed as described previously (54,55,56). PCR fragments amplified by using α_7, β_5, and γ_6 (Table 1) for each ENaC subunit were purified and cloned into the pGEM T-Easy vector (Promega, Madison, WI) and confirmed by direct sequencing and digestion with appropriate restriction enzymes. Digoxigenin-labeled antisense RNA probes were generated by in vitro transcription using digoxigenin-RNA labeling mix and SP6 or T7 RNA polymerase (Roche, Indianapolis, IN). Frozen blocks of the dissected anterior parts of the tongue embedded in the optimum-cutting temperature compound (Sakura Finetechnical, Tokyo, Japan) were sectioned into 5- to 7-μm-thick slices, which were mounted on silane-coated glass slides. To examine cellular distribution patterns of three ENaC subunits, horizontal sections in the middle part of taste buds (~30 μm below the level of the microvilli on the apical tongue surface) were used. The cryosections were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, treated two times with 0.1% diethyl pyrocarbonate in PBS for 15 min, washed with 5× SSC for 15 min at room temperature, and then prehybridized in a hybridization buffer consisting of 50% formamide, 5× SSC, 5× Denhardt's solution, 500 μg/ml denatured salmon testis DNA, 250 μg/ml denatured baker's yeast tRNA, and 1 mM dithio-threitol for 1 h at room temperature. Hybridization was carried out for 18 h at 58°C in a hybridization buffer with added 200 ng/ml antisense riboprobe. After hybridization, sections were washed two times in 5× SSC for 5 min each and two times in 0.2× SSC for 30 min each at 65°C. Subsequently, the sections were immersed in TBS consisting of 50 mM Tris·HCl (pH 7.5) and 150 mM NaCl for 5 min at room temperature, put in the blocking solution containing 0.5% blocking reagent (Roche) in TBS for 30 min, and incubated with anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (AP; 1:400 dilution; Roche) in the blocking solution for 60 min at room temperature. After three washes of 5 min each in buffer consisting of 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20, sections were immersed in AP buffer consisting of 100 mM Tris·HCl (pH 9.5), 100 mM NaCl, and 50 mM $MgCl_2$ for 5 min. The signals were developed using nitroblue-tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate as chromogenic substrates. Next, the reaction was stopped by rinsing the slides in Tris-EDTA buffer after which they were mounted. The signal specificities of mRNA for each gene in the taste tissues were tested by using a sense probe as a negative control.

Data analysis

In the analysis of whole nerve responses, the magnitudes of the integrated responses at 5, 10, 15, 20, and 25 s after stimulus onset were measured and averaged. With the use of these average values, relative response magnitude for each test stimulus was calculated with the response magnitude to 0.1 M NH_4Cl taken as a unity (1.0). These relative response values were used for all statistical analyses. An amiloride-sensitive component of the response was calculated for each mouse as the difference between responses to a taste solution without and with amiloride. The amiloride-sensitive component was also expressed as a percentage relative to responses to a taste stimulus without amiloride. We chose NH_4Cl as the standard stimulus to be consistent with previous studies on amiloride sensitivity of salt responses (10,11,28,45, 47,50,60). The relative response values for salts and percentages of the amiloride-sensitive component of salt responses in the B6 and 129 strains were not changed substantially when a different stimulus, quinine, was used as the standard (Yasumatsu, Ohkuri, and Ninomiya, unpublished observation).

Separate ANOVAs were conducted for parental strains and F_2 hybrids, and for each index of responses to NaCl or KCl. Genotype (strain or αENaC genotype) was a between-group factor; taste solution concentration and amiloride were within-group factors. When the interaction term of the ANOVA was significant, post hoc Tukey's honest significant difference tests were performed to compare individual means. The expression levels for ENaC mRNA in quantitative RT-PCR analysis were compared using *t*-tests. A P value of <0.05 was considered statistically significant. Calculations were performed using the statistical software packages StatView (Abacus Concepts, Berkeley, CA) or STATISTICA (StatSoft, Tulsa, OK).

Amiloride-sensitive components of neural responses to NaCl were analyzed using the Lineweaver-Burke (double-reciprocal) plots (35). These plots were constructed based on reciprocals of nerve responses (R) and taste solution concentrations (C) using mean values for parental strains (B6 and 129) and F_2 hybrids (with three αENaC genotypes). Analyses of these plots allowed us to characterize properties of receptors (or channels) using K_d (dissociation constant) and R_{max} (maximum neural response) values. The K_d values represent binding affinity (or ion conductance), and R_{max} values represent the total number of functional receptors (or channels) in the cell membrane (46,51,60).

RESULTS

Sequencing analysis of ENaC subunits (α, β, γ) in B6 and 129 strains

cDNA sequences of the αENaC subunit in B6 mice were identical with those reported in GenBank (AF112185: obtained from the B6 strain). In the 129 strain, three SNPs (T737C, G862A, and C1877T) in the α-subunit were detected (Fig. 1). One of these SNPs, C1877T, resulted in an amino acid substitution of arginine to tryptophan at position 616, R616W, near the predicted second transmembrane domain, which spans a region between Ser^{592} and Leu^{612} (53). These SNPs in αENaC in 129 strain were not reported in either GenBank (http://www.ncbi.nlm.nih.gov/SNP) or MGI (http://www.informatics.jax.org/) SNP databases. No SNPs were found in cDNA sequences of β- and γ-subunits between B6 and 129 strains (data not shown).

CT nerve responses before and after amiloride treatment in B6, 129, and their F_2 hybrid mice with different αENaC genotypes

CT nerve responses to 0.03–0.3 M NaCl and KCl in all groups of mice were concentration dependent and were not affected by mouse genotype (Fig. 2 and Table 2; genotypes of F_2 mice were determined by sequencing as described in MATERIALS AND METHODS). Consistent with this, no significant response magnitude differences between B6 and 129 strains or between

F_2 mice with different αENaC genotypes were found in post hoc tests for either NaCl or KCl. Effects of genotype revealed themselves mostly as interactions with effects of amiloride on NaCl responses.

Amiloride significantly suppressed NaCl responses (Fig. 2, *top*) both in the parental strains and in the F_2 hybrids (Table 2). However, significant interactions between effects of amiloride and genotype demonstrate genetic differences in amiloride sensitivity of NaCl response. Suppression of NaCl response was stronger in B6 mice than in 129 mice (Fig. 2, 2 panels on *left*) and it was stronger in F_2 mice with B6/B6 and B6/129 αENaC genotypes compared with F_2 mice with the 129/129 αENaC genotype (Fig. 2, 3 panels on *right*). Correspondingly, the amiloride-sensitive component of NaCl response was larger in B6 mice compared with 129 mice and in F_2 mice with B6/B6 and B6/129 αENaC genotypes compared with F_2 mice with 129/129 αENaC genotype (Fig. 3*A*; significant effects of genotype, concentration, and their interaction, Table 2). Mean amiloride-sensitive component for each strain and F_2 genotype are also presented as a double-reciprocal plot (Fig. 3*B*). The R_{max} values derived from this plot were much higher in B6 inbred and B6/129 and B6/B6 F_2 mice (1.17, 0.885, and 1.103, respectively) compared with those of 129 inbred and 129/129 F_2 mice (0.653 and 0.601, respectively). K_d values were similar in all five groups (B6 = 0.217, 129 = 0.226, B6/B6 = 0.161, B6/129 = 0.216, 129/129 = 0.219).

When the amiloride-sensitive component was expressed relative to NaCl response without amiloride, the effects of concentration were either nonsignificant (in parental strains) or relatively weak (in F_2 hybrids), and there were no interactions between effects of genotype and concentration (Table 2). Therefore, we present the percentage of the amiloride-sensitive component averaged across concentrations (Fig. 4). The percentage of amiloride-sensitive component was larger in B6 mice compared with 129 mice and in F_2 mice with B6/B6 and B6/129 αENaC genotypes compared with F_2 mice with 129/129 αENaC genotype. Similar amiloride-sensitive components in F_2 mice with B6/B6 and B6/129 αENaC genotypes demonstrate dominance of the B6 allele over the 129 allele. A difference in amiloride sensitivity between the two parental strains (45% suppression in B6 and 22% suppression in 129) was larger than the difference between F_2 hybrids with different αENaC genotypes (37–38% in B6/B6 and B6/129 F_2 and 21% in 129/129 F_2), that is, B6 mice were more than two times more amiloride sensitive (i.e., had 105% larger amiloride-sensitive component) than 129 mice, but B6/B6 and B6/129 F_2 mice were only 76–81% more amiloride-sensitive than 129/129 F_2 mice. Consistent with this, B6 inbred mice had slightly but significantly larger amiloride-sensitive component compared with B6/B6 F_2 mice ($P = 0.04$, 1-tailed *t*-test).

Although the effect of amiloride on KCl responses was statistically significant (Table 2), the suppression of KCl responses by amiloride was very small (Fig. 2, *bottom*). Amiloride suppressed KCl responses on average (across KCl concentrations) by 13.2 ± 4.8% (B6), 7.2 ± 1.8% (129), or 3.7 ± 1.6% (F_2). There were no effects of genotype on any index of KCl responses and no interactions between effects of mouse genotype and amiloride (Table 2).

Quantitative real-time RT-PCR analysis of ENaC subunits (α, β, γ) in the tongue and kidney tissues of B6 and 129 strains

mRNA for all ENaC subunits (α, β, γ) were detected in taste buds from fungiform and circumvallate papillae and kidneys in both B6 and 129 mice. For each ENaC subunit, mRNA levels normalized relative to the housekeeping gene, β-actin, did not differ significantly between B6 and 129 strains (Fig. 5). In all three tissues, ENaC subunit mRNAs were expressed at different levels, with $\alpha > \beta \doteqdot \gamma$. In fungiform papillae and kidney, an excess of α over β or γ was about twofold, but in circumvallate papillae it was about fivefold.

In situ hybridization analysis of ENaC subunits (α, β, γ) in anterior tongue of B6 and 129 strains

To compare taste cell distribution patterns of each ENaC mRNA in taste buds of B6 and 129 parental strains, in situ hybridization was performed. Signals for all ENaC subunits (α, β, γ) were clearly detected in some fungiform taste cells of mice from both B6 and 129 strains (Fig. 6). This was not evident when a sense probe was used. In the nontaste epithelial tissue, all three subunits were detected at low levels. The distribution patterns of all ENaC subunits in a taste bud were similar in 129 and B6 mice.

DISCUSSION

In the present study, we first investigated polymorphisms of three ENaC subunits (α, β, and γ) between amiloride high-sensitive B6 and amiloride low-sensitive 129 mice. The sequencing analysis detected three SNPs between B6 and 129 strains in the α-subunit, and one of them, C1877T, resulted in an amino acid substitution, R616W (Fig. 1). No SNPs were found in β- and γ-subunits in this study. The R616W variant is located within an arginine-rich region of the mouse αENaC, immediately following the predicted second transmembrane (M2) domain spanning a region between Ser^{592} and Leu^{612} of αENaC (53), which may constitute a part of the inner mouth of the pore of ENaC (21). This arginine-rich region (RRFRSRYWSPGR; 613–624 for the mouse and 586–597 for human) is a sequence of positively charged amino acids identical among five mammalian αENaC subunits (human, rat, mouse, bovine, and guinea pig) and is also conserved in the human δENaC homolog (RRLRRAWFSWPR; see Ref. 59). This region is not present in any of the β- and γENaC subunits.

We then examined whether the αENaC R616W polymorphism is associated with amiloride inhibition of NaCl responses of the CT nerve. Amiloride inhibited nearly 50% of CT responses to NaCl in B6 mice and only ~20% of NaCl responses in 129 mice (Figs. 2 and 4), which confirms a previously reported difference in amiloride sensitivity between these two strains (11,47). Although these previous studies did not detect any significant suppression of CT response to NaCl by amiloride in 129 mice, in this study suppression of NaCl responses of the CT nerve by 100 μM amiloride in 129 mice was small but significant. The apparent discrepancy between these results may be because of the greater statistical power in this study, in which we tested 15 mice compared with 5 mice in the earlier studies (11,47).

An association of differences in amiloride sensitivity of CT responses to NaCl and αENaC sequence variants in the two inbred strains, B6 and 129, could be a true causal relationship, but it could also be the result of a fortuitous fixation of the two independent characteristics in these strains during inbreeding. That is why we analyzed association between amiloride sensitivity and αENaC sequence variants in F_2 hybrids of B6 and 129 strains. In the F_2 hybrid generation, phenotypes and genotypes that fortuitously coincide in inbred progenitors would not cosegregate. We have found that amiloride inhibition of NaCl responses in F_2 hybrids with the αENaC 129/129 genotype was smaller than in F_2 hybrids with B6/129 and B6/B6 genotypes. This significant association shows that the αENaC polymorphism, R616W, is likely to be causally related with differences in amiloride sensitivity between B6 and 129 strains. However, a difference in amiloride sensitivity between the two parental strains was larger than that between F_2 hybrids with different αENaC genotypes. This suggests that there are other genes in addition to αENaC that influence the amiloride sensitivity and contribute to difference between B6 and 129 strains. Overall, these data provide evidence that ENaC is involved in the amiloride-sensitive salt taste responses in mice.

Similar amiloride sensitivity of F_2 hybrids with B6/B6 and B6/129 αENaC genotypes demonstrates dominance of the B6 allele of αENaC over its 129 allele. F_2 mice with heterozygous αENaC genotype (i.e., B6/129) carry two different αENaC alleles, one on each

chromosome. As a result, each αENaC-expressing diploid cell of such mice is likely to produce two allelic forms of the αENaC protein. Our data suggest that the presence of the 129 allele of αENaC in the same cell with its B6 allele does not affect the cell function compared with that of B6 homozygotes (i.e., B6/B6) that express only the B6 allele. This allelic interaction frequently occurs between wild-type (dominant) and mutant (recessive) alleles because mutant alleles are often hypofunctional, and the presence of a hypo-functional allele along with a normal functioning allele in the same cell may have no impact on cell properties. Similarly, amiloride sensitivity of taste cells could be determined by the presence of a more sensitive B6 allelic variant of αENaC, whether or not a less-sensitive 129 allele is present in the same cell. This suggests that the B6 variant of αENaC is likely to be a normal, wild-type allele, and its 129 variant is probably a mutation that recently occurred in this strain.

The amino acid residue of the mouse R616W αENaC variant corresponds to an arginine residue R589 of its human ortholog. Effects of double-point mutations of the positively charged arginine residues R589 and R591 (both in the post-M2 region) to negatively charged glutamate (R589E and R591E) of αhENaC were examined in a single-channel patch-clamp experiment using human αβγhENaC heterologously expressed in *Xenopus* oocytes (21). Compared with a wild-type αβγhENaC, the mutant αR589E, R591E βγhENaC had an increased chord conductance. The diameter of the channel pore was larger in the mutant αR589E, R591E βγhENaC channel than in the wild-type channel, suggesting that the R589E and R591E double mutations may produce changes in surface charge and structure of the inner mouth of the ENaC pore. This mutation also resulted in reduction of ion selectivity between Na^+ vs. K^+. The amino acid position of human R589 (mouse R616) and R591 in αENaC is also known to be a part of R-X-R (arginine-X-arginine) motif, which is implicated as an endoplasmic reticulum retention motif (61). The double mutations of the arginine residues of this motif (R589E and R591E) increase macroscopic amiloride-sensitive current and surface expression of αENaC (21). Although there is no available data for single mutations of the arginine residue in the arginine-rich region (also R-X-R motif), the mutation, R616W (equivalent to R589 in human), found in the 129 strain may also lead to some changes in the amiloride-sensitive Na^+ current. However, unlike the results from the above-mentioned study, the mutation of R616W in mice should not increase amiloride-sensitive Na^+ current, or change ion selectivity of amiloride inhibition between Na^+ vs. K^+, because mice with the R616W mutation showed smaller but selective inhibition by amiloride of responses to NaCl, but not to KCl. The single mutation from positively charged arginine to neutral tryptophan (R616W) may not result in as great changes in the charge at the inner pore of ENaC as it was observed for the double mutations from positive arginine to negative glutamate in the above-mentioned study (21). The K_d represents binding affinity between enzyme and substrate in enzyme kinetics (35). Therefore, the K_d values from double-reciprocal plots of the amiloride-sensitive component in this study may characterize channel conductance. The K_d values were similar among the five groups (parents and F_2 hybrids), suggesting that channel conductance itself is not greatly influenced by the R616W mutation. We hypothesize that a possible conformational change resulting from the substitution of arginine with tryptophan, which has an aromatic ring, may produce a new steric hindrance around the inner pore of ENaC that may lead to a decrease in the amiloride-sensitive current and thus result in lower amiloride inhibition of NaCl responses in mice with the R616W mutation.

The magnitude of R_{max} represents the total number of functional channels. The R_{max} values of the amiloride-sensitive component calculated from double-reciprocal plots (35) were higher in B6, B6/129, and B6/B6 (0.89~1.17) than in 129 and 129/129 (0.60~0.65) mice (Fig. 3*B*). The density of amiloride-sensitive channels on the apical side of the taste cell membrane has also been implicated in the strain difference in amiloride sensitivity between BALB/c and C57BL mice (40). These results suggest that mice with different αENaC genotypes may differ in the total number of functional amiloride-sensitive ENaC heteromeric channels [with $\alpha_2\beta\gamma$-

subunit composition (9,30,39)] on apical membranes of taste cells, although transcript abundances are almost the same in the cytoplasm. Intracellular trafficking of heteromeric receptors or channels is likely to be a tightly controlled process requiring proper folding and assembly, with the endoplasmic reticulum serving as the primary checkpoint of these complex events (38). Therefore, we hypothesize that the R616W mutation in the R-X-R motif may result in insufficiency of endoplasmic reticulum retention of αENaC, which may result in its improper folding or assembly with other β- and γ-subunits and, consequently, a decrease in expression of functional ENaC in the cell membrane. However, further studies using heterologus expression of ENaC mutants would be needed to examine the effects of R616W on channel assembly and cell surface expression.

In the current study, ENaC mRNA expression patterns observed in in situ hybridization analysis were similar in fungiform papillae between B6 and 129 mice (Fig. 6). In real-time RT-PCR analysis, no significant differences in relative expression levels of ENaC subunit mRNAs in fungiform, circumvallate papillae, and kidney were observed between B6 and 129 strains. However, in all three tissues, ENaC subunit mRNAs were expressed at different levels with $\alpha > \beta \doteqdot \gamma$. In fungiform papillae and kidney, an excess of α over β or γ was about twofold, but in circumvallate papillae it was about fivefold (Fig. 5). It was suggested that ENaC in the collecting duct has the composition $\alpha_2\beta\gamma$ (9,30,39). Therefore, our data on the relative subunit mRNA abundance in the kidney and fungiform papillae, but not in circumvallate papillae, correspond to this stoichiometry. It is known that the αENaC subunit by itself confers a low-amplitude, amiloride-sensitive, sodium current, whereas the β- and γ-subunits are required for the maximal channel activity (4,5,36). In the taste system, the importance of the β- and γ-subunits was also suggested because all three subunits are abundantly present in taste cells in amiloride-sensitive fungiform papillae, whereas the amiloride-insensitive posterior tongue region poorly expresses the β- and γ-subunits compared with the α-subunit (31,34,56). These studies did not detect differences in expression levels among three ENaC subunits in anterior tongue in intensity of immunoreactivity in rat (34), semiquantitative RT-PCR in rat (31), and numbers of positive cells in a taste bud in in situ hybridization analyses in mice (56). All three subunits were expressed at similar levels (1:1:1), but in posterior tongue, $\alpha > \beta \doteqdot \gamma$. Consistent with these previous studies, we also found that the difference in expression levels among the three ENaC subunits was greater in circumvallate papillae than in fungiform papillae. These results suggest that ENaC in fungiform papillae may function as an amiloride-sensitive sodium channel, like it functions in the kidney. However, the universal amiloride insensitivity of glossopharangeal nerve NaCl responses may be the result of disproportional expression levels of different ENaC subunits in circumvallate papillae. The discrepancy between our data and results of the previous studies in anterior tongue (2:1:1 vs. 1:1:1) may be because of the differences in methods (e.g., different PCR primers used in real-time PCR, which have different annealing temperatures, differences in the size of PCR product, and amplified cDNA region).

Our data suggest that, although the R616W polymorphism plays an important role in strain differences in amiloride sensitivity, this is probably not the only factor involved. For example, in our preliminary experiment, we found some splicing variants of αENaC expressed in taste cells in 129 and BALB mice (Shigemura and Ninomiya, unpublished observation). In rats, a splicing variant with a deletion of 49 amino acids in the NH_2-terminal region of the α-subunit showed amiloride-sensitive currents reduced to <20% of the values obtained with the full-length ENaC (7). Therefore, it is possible that differences in expression levels of these variants may cause differences in the amiloride-sensitive current, although no comparison has been made yet. Furthermore, expression levels of β- and γ-subunits in taste tissues were enhanced by increasing blood aldosterone levels in rat (34), suggesting a hormonal influence on amiloride sensitivity. Vasopressin, a hormone known to be involved in osmotic regulation, has also been shown to increase amiloride-sensitive inward Na^+ current in hamster fungiform taste cells (12). Collectively, with respect to ENaCs, in addition to mutations of amino acid sequences of

the channel, their normal vs. splicing variants and hormonal influences are possible factors responsible for strain differences in amiloride sensitivity of NaCl taste responses. Genes controlling these alternative factors would, therefore, be targets for future genetic approaches to understanding salt taste transduction.

In summary, in this study, using B6 and 129 mice and their F_2 hybrids, we examined possible relationships of the amiloride sensitivity of CT responses to NaCl with SNPs and mRNA expression levels in fungiform papillae of three subunits of ENaC (α, β, γ). Of the three SNPs in the α-subunit detected between the B6 and 129 strains, one resulted in an amino acid substitution, R616W. Subsequent electrophysiological study using F_2 hybrids with 129/129, B6/129, B6/B6 genotypes of this SNP revealed that the 129/129 genotype was associated with smaller amiloride inhibition of NaCl responses compared with B6/B6 and B6/129 genotypes. Real-time RT-PCR and in situ hybridization analyses found no significant differences between B6 and 129 mice in the expression levels of ENaC subunits in fungiform papillae. These results suggest that ENaC is involved in the amiloride-sensitive salt taste responses in mice, and an amino acid change at R616W of αENaC subunit may produce smaller amiloride sensitivity in mice with the 129 genotype than in mice with the B6 genotype.

Perspectives and Significance

Taste receptors remained elusive until recent discoveries of T1R and T2R proteins involved in sweet, umami, and bitter taste and a demonstration that PKD1L3 and PKD2L1 proteins may be involved in sour taste reception. However, the molecular identity of a salty taste receptor remains controversial (1,6) despite numerous studies showing that, in rodents, amiloride affects sodium taste responses and suggesting that ENaC is involved in salty taste reception. The complexity of the salty taste may be due to the fact that animals have several types of salt taste transduction systems that evolved to help them to survive in their environments. Results of our study strongly support the role of ENaC in the amiloride-sensitive salt taste responses in mice and demonstrate that strain and perhaps species differences in salt taste sensitivity could be attributed to amino acid variants of ENaCs. These findings contribute to better understanding of molecular mechanisms of salty taste.

Acknowledgements

We thank Maria Theodorides, Natalia Bosak, and Yutaka Ishiwatari for assistance with F_2 DNA work.

GRANTS

This work was supported by Grants-in-Aid 18791370 (N. Shigemura), 18077004 (Y. Ninomiya), and 18109013 (Y. Ninomiya) for Scientific Research from the Japan Society for the promotion of science and by National Institutes of Health Grant R01 DC-00882 (G. K. Beauchamp and A. A. Bachmanov).

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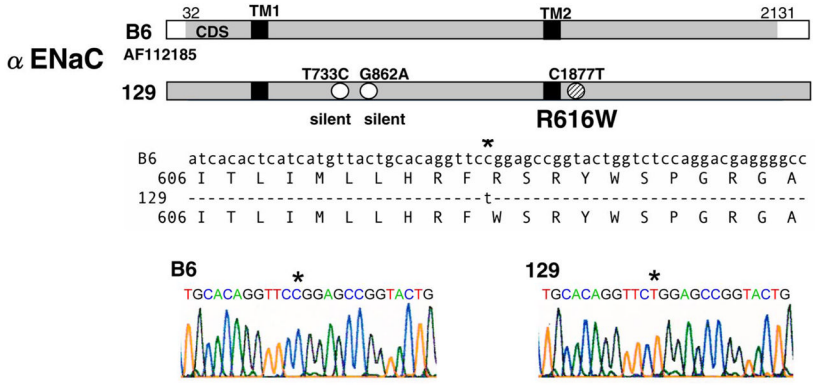

Fig. 1.

Identification of single nucleotide polymorphisms in α-subunit of the epithelial Na^+ channel (ENaC) from taste cells of B6 and 129 mice by sequencing and alignment of B6 and 129 αENaC nucleotide and protein sequences. Open circles, silent mutations without amino acid substitution; filled circle, a missense mutation with amino acid substitution in position 616 (arginine in B6 to tryptophan in 129 strain). *Nucleotide no. 1877 in AF112185. CDS, start of coding sequence; TM, predicted transmembrane domains.

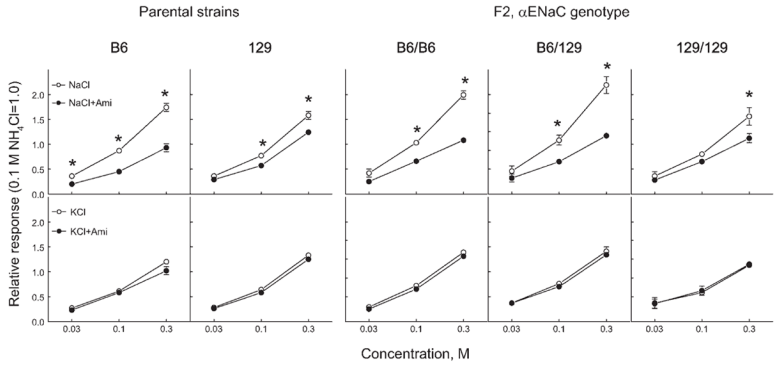

Fig. 2.

Relative chorda tympani nerve responses (0.1 M NH_4Cl = 1.0) to 0.03–0.3 M NaCl and 0.03–0.3 M KCl with and without 100 μM amiloride in the B6 and 129 strains and in their F_2 hybrids with different αENaC genotypes (R616W). Values indicated are means ± SE. *$P < 0.05$, Tukey's honest significant difference (HSD) post hoc tests.

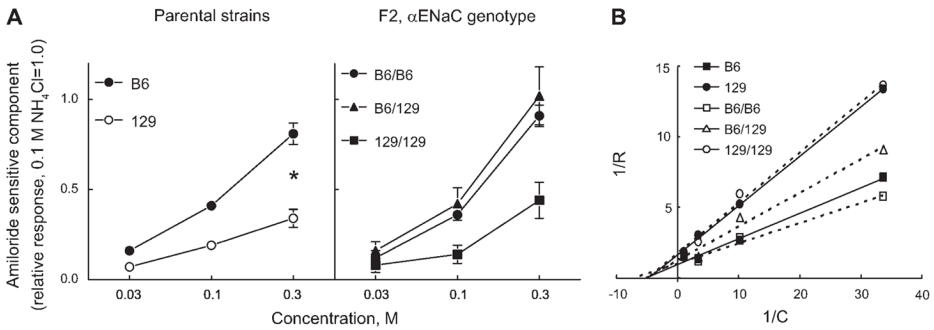

Fig. 3.

A: amiloride-sensitive component of chorda tympani nerve responses to 0.03–0.3 M NaCl (responses to NaCl without amiloride – responses to NaCl with 100 μM amiloride) in the B6 and 129 strains and in their F_2 hybrids with different αENaC genotypes (R616W). Values indicated are means ± SE. **P* = 0.05, Tukey's HSD post hoc tests. Although no significant differences between F_2 mice with different αENaC genotypes were found for individual NaCl concentrations, post hoc tests of data collapsed across concentrations have shown that 129/129 F_2 mice have a significantly lower amiloride-sensitive component than B6/129 F_2 mice (*P* = 0.015). *B*: double-reciprocal plots of the amiloride-sensitive component of chorda tympani nerve responses to 0.03–0.3 M NaCl in parental B6 and 129 strains and their F_2 hybrids with different αENaC genotypes (R616W). The reciprocal values were calculated using means for each genotype. C, concentration (M); R, relative response (0.1 M NH_4Cl = 1.0).

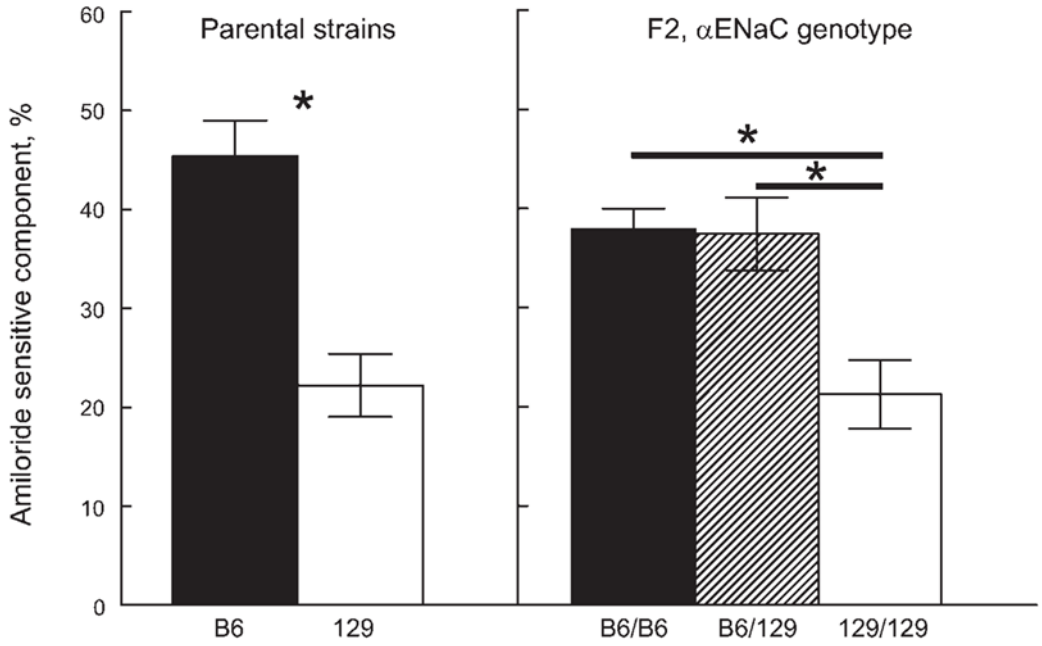

Fig. 4.

Amiloride-sensitive component of chorda tympani nerve responses to NaCl expressed as a percentage of responses to NaCl without amiloride in the B6 and 129 strains and in their F_2 hybrids with different αENaC genotypes (R616W). Values indicated are means ± SE. *P < 0.05, *t*-test (parental strains) or Tukey's HSD post hoc tests (F_2).

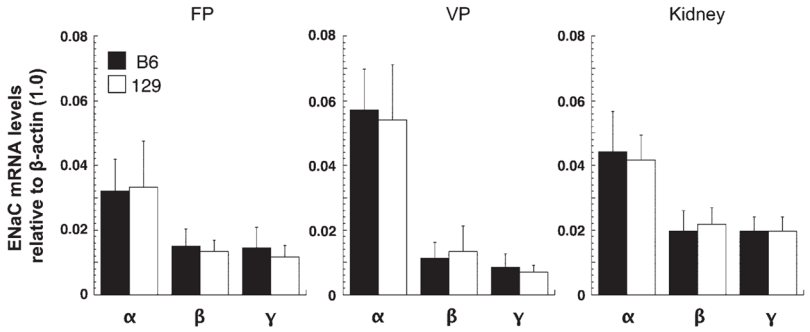

Fig. 5.

Real-time PCR analysis of ENaC subunits (α, β, γ) in taste buds isolated from fungiform (FP) and circumvallate (VP) papillae and kidneys of B6 and 129 mice. Values indicated are means ± SD. The expression of ENaC subunit mRNAs was normalized to that of a housekeeping gene, β-actin. All ENaC subunits were detected in fungiform, circumvallate papillae, and kidney in both B6 (n = 9, 9, and 5, respectively) and 129 (n = 8, 8, and 5, respectively) mice. No significant differences were observed between B6 and 129 strains in mRNA abundance of all ENaC subunits in three tissues (t-test, $P > 0.05$).

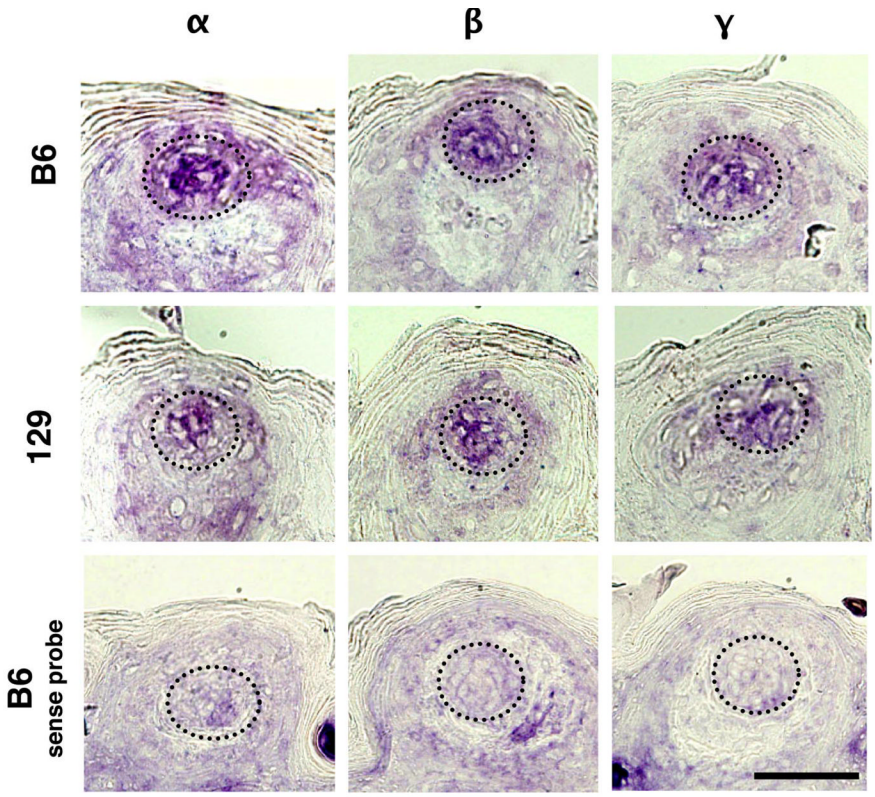

Fig. 6.

In situ hybridization analysis of ENaC subunits (α, β, γ) in the fungiform papillae on the anterior tongue in B6 and 129 mice (horizontal sections in the middle part of taste buds, ~30 μm below the level of the microvilli on the apical tongue surface). All subunits of ENaC were expressed in a subset of fungiform taste bud cells of both B6 and 129 mice. The expression patterns were similar in the B6 and 129 strains. The dotted lines indicate the outlines of sample taste buds. Bar = 50 μm.

Table 1

Nucleotide sequences for the primers used in sequencing, genotyping, and expression analyses

Genes	Accession No.	Name	Forward	Reverse	Region
aENaC	AF112185	α_1	CCCATTCTGCCTCCATA	AGCCTGGCGAGTGTAGGAAGA	9-622
		α_2	GTCACTGTGTGCACCCTTAATC	GGAAGACATCCAGAGATTGG	494-1072
		α_3	TACCTGTCGTTTCAACCAGGC	ACAGATGAGCCGAACCACAG	952-1800
		α_4	CGTCTGTGAAGTCCCAGGAT	GCTGTGGAAGATGTGCTGAA	1593-2194
		α_5	GTGGTTCGGCTCATCTGTG	GGTAGCATAGGCAGGTGGAG	1783-2056
		α_6	TGCTCCTGTCACTTCAGCAC	CCCCTTGCTTAGCCTGTTC	2163-2242
		α_7	CTAATGATGCTGGACCACACC	AAAGCGTCTGTTCCGTGATGC	26-581
βENaC	AF112186	β_1	CTCACACTGGAGCAGCTTCC	GATAGGCGTGAAGTTCCGATG	1-821
		β_2	GAAGCACCTGCAATGCCCAG	AGATGGCCAGTCAGCCATGG	571-1421
		β_3	CCACTACTTATACCCACTGCC	CGGCTCTTTGTACAAGGCCTA	1235-2046
		β_4	GGCTAGAGTCCTCTCCTGGTT	GCCAACTAGGGCAAGGATTCT	2213-2294
		β_5	GCCAGTGAAGAAGTACCTCC	CCTGGGTGGCACTGGTGAA	38-669
γENaC	AF112187	γ_1	TGTACACACGCCAGCCGTGA	TTCCGACCAGTGAAGAAGTC	1-620
		γ_2	AAGCAGGATCCATGCGGTCCA	GTCCTCTGTGCACTGGCTGTA	500-1212
		γ_3	GCTTGTCCATCAGCAGAATG	ATCCTTGGCTTTCTGCCACTG	1074-1800
		γ_4	GGCTTCTGAGAAATGGTTGC	GCAGGCTGTTAGACATGTATC	1509-2138
		γ_5	CTGCCAAATGGTACTCCTATCC	TGTAGGCTGGTGTTTGTTATGC	2665-2760
		γ_6	AAGAATCTGCCAGTTCGAGGC	TACCACTCCTGGATGGCATTG	106-776
β-Actin	X03765	Actin	GGTCAGAAGGACTCCTATGTGG	TGTCGTCCCAGTTGGTAACA	64-166

ENaC, epithelial Na^+ channel.

Table 2

ANOVA results for chorda tympani responses to NaCl and KCl in parental strains (B6 and 129) and F_2 mice with different αENaC genotypes

Effect	Parental Strains			F_2: αENaC genotype		
	DF	*F*	*P*	DF	*F*	*P*
NaCl, response magnitude						
Genotype	1, 23	0.6	0.4	2, 29	2.1	0.14
Amiloride	1, 23	257.8	<0.0001	1, 29	95.4	<0.0001
Concentration	2, 46	384.2	<0.0001	2, 58	403.7	<0.0001
Genotype × amiloride	1, 23	38.5	<0.0001	2, 29	4.4	0.02
Genotype × concentration	2, 46	0.3	0.7	4, 58	1.9	0.12
Amiloride × concentration	2, 46	119.3	<0.0001	2, 58	69.2	<0.0001
Genotype × amiloride × concentration	2, 46	21.5	<0.0001	4, 58	3.3	0.02
NaCl, amiloride-sensitive component						
Genotype	1, 23	38.5	<0.0001	2, 29	4.6	0.02
Concentration	2, 46	119.3	<0.0001	2, 58	73.3	<0.0001
Genotype × concentration	2, 46	21.5	<0.0001	4, 58	3.2	0.02
NaCl, %amiloride-sensitive component						
Genotype	1, 23	22.4	<0.0001	2, 29	7.7	0.002
Concentration	2, 46	1.8	0.2	2, 58	13.1	<0.0001
Genotype × concentration	2, 46	0.4	0.7	4, 58	0.6	0.6
KCl, response magnitude						
Genotype	1, 23	3.1	0.09	2, 29	1.5	0.2
Amiloride	1, 23	28.2	<0.0001	1, 29	6.9	0.01
Concentration	2, 46	332.4	<0.0001	2, 58	383.8	<0.0001
Genotype × amiloride	1, 23	1.3	0.3	2, 29	2.9	0.07
Genotype × concentration	2, 46	3.4	0.04	4, 58	2.8*	0.03
Amiloride × concentration	2, 46	7.0	0.002	2, 58	0.8	0.4
Genotype × amiloride × concentration	2, 46	2.6	0.08	4, 58	1.2	0.3
KCl, amiloride-sensitive component						
Genotype	1, 23	1.3	0.3	2, 29	3.0	0.07
Concentration	2, 46	7.0	0.002	2, 58	2.4	0.10
Genotype × concentration	2, 46	2.6	0.08	4, 58	1.8	0.14
KCl, %amiloride-sensitive component						
Genotype	1, 23	1.8	0.2	2, 29	3.3	0.052
Concentration	2, 46	1.3	0.3	2, 58	1.7	0.2
Genotype × concentration	2, 46	3.5	0.04	4, 58	1.4	0.3

Response magnitudes were analyzed using 3-way ANOVA assessing effects of genotype (a between-group factor), amiloride, and concentration (within-group factors) and their 2- and 3-way interactions. Amiloride-sensitive components were analyzed using 2-way ANOVA assessing effects of genotype (a between-group factor) and concentration (a within-group factor) and their 2-way interactions. DF, degree of freedom; *F*, *F*-values; *P*, *P* values. The data in this table could be presented for example as $F(1,23) = 0.6$, $P = 0.4$ (for NaCl response magnitude in the parental strains). Genotype is strain (for parental strain analyses) or αEnaC genotype (for F_2 analyses).