

# Amiloride Inhibition on NaCl Responses of the Chorda Tympani Nerve in Two 129 Substrains of Mice, 129P3/J and 129X1/SvJ

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## Abstract

Amiloride, a sodium channel blocker, is known to suppress NaCl responses of the chorda tympani (CT) nerve in various mammalian species. In mice, the NaCl suppressing effect of amiloride is reported to differ among strains. In C57BL mice, amiloride inhibits NaCl responses to about 50% of control, whereas no such clear suppression was evident in prior studies with 129 mice. However, evidence from behavioral studies is not entirely consistent with this. Recently, it has been found that genetic backgrounds of 129 mice differ within substrains. 129X1/SvJ (formerly 129/SvJ) mice differ from the 129P3/J (formerly 129/J) strain by 25% of sequence length polymorphisms. Therefore, we examined possible substrain difference between 129P3/J and 129X1/SvJ mice in the amiloride sensitivity of electrophysiologically recorded NaCl responses. Amiloride significantly suppressed CT responses to NaCl without affecting responses to KCl both in 129P3/J and 129X1/SvJ mice. However, the magnitude of the amiloride inhibition was significantly larger (~50% of control in response to 0.01–1.0 M NaCl by 100  $\mu$ M amiloride) in 129X1/SvJ than in 129P3/J mice (~20% of control in response to 0.03–0.3 M NaCl by 100  $\mu$ M amiloride). Threshold amiloride concentration for suppression of responses to 0.3 M NaCl was 30  $\mu$ M in 129P3/J mice, which was higher than that in 129X1/SvJ mice (10  $\mu$ M). In 129X1/SvJ mice, the threshold amiloride concentration eliciting inhibition of NaCl responses and the magnitude of the inhibition were comparable with those in C57BL/6 mice. These results suggest that amiloride sensitivity of NaCl responses differs even among the 129 substrains, 129P3/J and 129 X1/SvJ, and the substrain difference of 129 mice in amiloride sensitivity is as large as that between two inbred strains (129P3/J and C57BL/6).

**Key words:** amiloride, chorda tympani nerve, mouse substrain difference, salt taste

## Introduction

Amiloride, a sodium channel blocker for various epithelial tissues, is known to suppress responses of the chorda tympani (CT) nerve to NaCl in various species of mammals, such as rats (Heck *et al.*, 1984; Brand *et al.*, 1985; Formaker and Hill, 1988; Ninomiya *et al.*, 1988; Elliott and Simon, 1990; Ye *et al.*, 1993), mice (Ninomiya *et al.*, 1989, 1991, 1996; Gannon and Contreras, 1995; Ninomiya, 1996, 1998; Yasumatsu *et al.*, 2003), hamsters (Herness, 1987; Hettinger and Frank, 1990), gerbils (Jakimovich, 1985), rhesus monkeys (Hellekant *et al.*, 1988), and chimpanzees (Hellekant and Ninomiya, 1991). In mice, the amiloride inhibition of NaCl responses of the CT nerve was shown to differ among strains (Ninomiya *et al.*, 1989, 1996; Gannon and Contreras, 1995). For example, amiloride suppressed the CT responses to NaCl to ~50% of control in C57BL/6 and C3H/He mice (Ninomiya *et al.*, 1989), whereas the compound did not significantly inhibit the NaCl responses in 129P3/J mice (Gannon and

Contreras, 1995; Ninomiya *et al.*, 1996). In BALB/c and DBA/2 mice, amiloride inhibits NaCl response of the CT nerve at temperature lower than ~24°C but only slightly at ~24°C or more (Ninomiya *et al.*, 1989, 1996, unpublished data). Since mouse strain differences have been successfully used in genetic approaches to reveal receptor mechanisms for sweet, umami, and bitter tastes (Lush *et al.*, 1995; Bachmanov *et al.*, 1997, 2001; Adler *et al.*, 2000; Matsunami *et al.*, 2000; Kitagawa *et al.*, 2001; Li *et al.*, 2001; Max *et al.*, 2001; Montmayeur *et al.*, 2001; Nelson *et al.*, 2001; Sainz *et al.*, 2001), differences in amiloride sensitivity among the mouse strains, if established, may also provide a genetic tool for understanding salt taste reception mechanisms.

Recent behavioral studies using several mouse strains are not entirely consistent with the electrophysiological studies reviewed above. That is, amiloride attenuated behavioral discrimination between sodium and nonsodium salts as

detected by short-term lick tests (5–6 s) in C57BL/6, BALB/c, DBA/2, and 129P3/J (formerly 129/J) mice, indicating that even 129P3/J mice may possess the amiloride sensitivity in the peripheral taste system (Eylam and Spector, 2003, 2005).

The 129 inbred strain, used in previous taste genetic studies, is known to have a number of substrains derived mainly from two major parent stocks, 129/J and 129/Sv (see Sanematsu *et al.*, 2005). About a decade ago, it was found that there is substantial genetic variation among substrains of the 129 inbred strain (Simpson *et al.*, 1997; Threadgill *et al.*, 1997). Some of this variation has apparently arisen as a result of genetic contamination and the rest appears to be due to residual heterozygosity and/or alleles introduced from other strains during various backcrossing programs. For example, of 86 simple sequence length polymorphisms (SSLPs) examined, 37 (~43%) were found to exhibit at least one polymorphism among the fifteen 129 substrains and ten 129-derived ES cell lines (Simpson *et al.*, 1997). Differences between substrains ranged from 0 to 19 of the 86 loci tested. To avoid further confusion, it has been proposed (Festing *et al.*, 1999) that nomenclatures for 129 substrains be revised as follows: 129X1/SvJ (formerly 129/SvJ) and 129P3/J (formerly 129/J).

The discrepancy between electrophysiological and behavioral effects of amiloride in mice from the 129 strain led us to reexamine electrophysiologically determined amiloride sensitivity in 129 mice. In the present study, we compared the amiloride inhibition of the CT responses to NaCl between two 129 substrains, 129P3/J (formerly 129/J) and 129X1/SvJ (formerly 129/SvJ). The results suggest that there is a substantial difference in the amiloride inhibition of the CT response to NaCl between these two 129 substrains.

## Materials and methods

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 mice of 129X1/SvJ (8–40 weeks of age,  $n = 9$  males and 6 females) and 129P3/J (8–40 weeks of age,  $n = 9$  males and 6 females) strains ranging in weight from 18 to 35 g, originally purchased from The Jackson Laboratory (Bar Harbor, ME). The gender and age differences did not affect the data obtained from the present study. Each mouse was anesthetized with intraperitoneal injection of sodium pentobarbital (50–60 mg/kg intraperitoneal, Somnopentyl; Schering-Plough Co., Kenilworth, NJ) and maintained at a surgical level of anesthesia with supplemental injections of sodium pentobarbital. The trachea was cannulated, and the mouse was then fixed in the supine position with a head holder to allow dissection of the CT nerve. The hypoglossal nerve was transected bilaterally to prevent tongue movements. 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 positioned nearby in the wound. Neural responses resulting from chemical stimulations of the tongue were fed into an amplifier (Iyodenshikogaku K-1, Nagoya, Japan) monitored on an oscilloscope and audiometer. Whole nerve responses were integrated with a time constant of 1.0 s and recorded in a computer for later analysis using PowerLab system (PowerLab/sp4; AD Instruments, Australia).

## Chemical stimulation

The anterior half of the mouse's tongue was enclosed in a flow chamber made of silicone rubber (Ninomiya and Funakoshi, 1981). Solutions were delivered into the chamber by gravity flow and flowed over the tongue for a controlled period. Solutions used were: 0.01–1.0 M NaCl with and without 100  $\mu$ M amiloride, 0.1 and 0.3 M NaCl with 10 or 30  $\mu$ M amiloride, 0.01–1 M KCl with and without 100  $\mu$ M amiloride, and 0.1 M NH<sub>4</sub>Cl (Wako Pure Chemicals Industries, Osaka, Japan). These chemicals were dissolved in distilled water and used at ~24°C. After the series of stimulations with amiloride, 0.01–1.0 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 during the interval of ~1 min between successive stimulations. The stability of the preparation was monitored by periodic application of 0.1 M NH<sub>4</sub>Cl. A recording was considered to be stable when magnitudes of NH<sub>4</sub>Cl response 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.

## Data analysis

In the analysis of whole nerve response to each stimulus, the magnitudes of the integrated response at 5, 10, 15, 20, and 25 s after stimulus onset were measured and averaged. In each animal, responses for three repeated trials of the same stimulus were averaged. Relative response magnitude (averaged) for each test stimulus was calculated when the response magnitude to 0.1 M NH<sub>4</sub>Cl was taken as a unity (1.0), and this was used for statistical analysis. Data were analyzed using a two-way repeated measures analysis of variance (ANOVA) with strain as a between-group factor and solution concentration as a within-group factor. Differences between individual means were assessed using Student's *t*-test as *post hoc*. A *P* value of <0.05 was considered statistically significant. All calculations were performed using the statistical software package StatView (Abacus Concepts, Inc., Berkeley, CA).

## Results

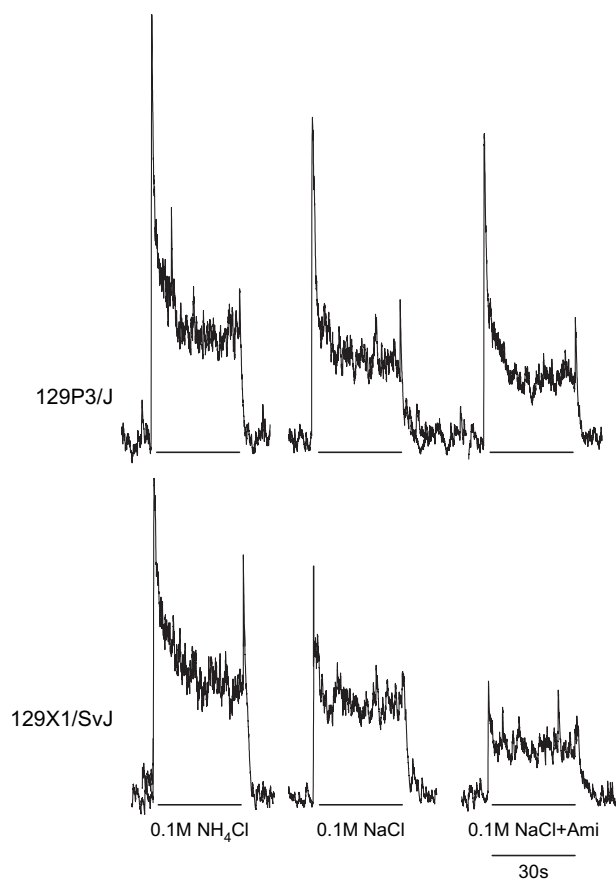
Figure 1 shows sample recordings of the integrated responses of the CT nerve of 129P3/J and 129X1/SvJ mice to 0.1 M NaCl with and without 100  $\mu$ M amiloride. The response to NaCl was clearly suppressed by amiloride in 129X1/SvJ mice, whereas only a slight inhibition by amiloride was found in 129P3/J mice. Figure 2 shows concentration–response relationships for NaCl and KCl with and without 100  $\mu$ M amiloride in the two strains. Although reduction of responses by amiloride appeared much smaller in 129P3/J than in 129X1/SvJ, in both strains, responses to NaCl with amiloride were significantly different from those without amiloride [in 129P3/J—effect of amiloride:  $F(1,28) = 9.79$ ,  $P < 0.01$  and amiloride-concentration interaction:  $F(4,92) = 2.33$ ,  $P > 0.05$ ; in 129X1/SvJ—effect of amiloride:  $F(1,28) = 53.7$ ,  $P < 0.001$  and amiloride-concentration interaction:  $F(4,91) = 12.2$ ,  $P < 0.001$ ]. This indicates that both 129 substrains possess amiloride sensitivity in the CT nerve. *Post hoc* Student's *t*-tests confirmed inhibition of responses to NaCl at all concentrations tested (0.01–1.0 M) in 129X1/SvJ ( $P < 0.01$  or 0.001), whereas significant inhibition was observed in

responses to NaCl at the concentrations from 0.03 to 0.3 M ( $P < 0.05$ –0.01) in 129P3/J mice. No inhibition was evident in responses to KCl in both strain [in 129P3/J—effect of amiloride:  $F(1,28) = 0.76$ ,  $P > 0.05$  and amiloride-concentration interaction:  $F(4,92) = 0.14$ ,  $P > 0.05$ ; in 129X1/SvJ—effect of amiloride:  $F(1,21) = 1.03$ ,  $P > 0.05$ ; amiloride-concentration interaction:  $F(4,76) = 0.21$ ,  $P > 0.05$ ]. The magnitude of responses to NaCl without amiloride in 129X1/SvJ mice was not significantly different from that in 129P3/J mice [effect of strain:  $F(1,28) = 3.84$ ,  $P > 0.05$ ; strain-concentration interaction:  $F(4,98) = 0.72$ ,  $P > 0.05$ ]. In contrast, the amiloride-sensitive response component (obtained by subtraction of the residual response after amiloride from the control response before amiloride) of the NaCl response was significantly larger in 129X1/SvJ mice than in 129P3/J mice [effect of strain:  $F(1,28) = 22.0$ ,  $P < 0.001$ ; strain-concentration interaction:  $F(4,85) = 10.1$ ,  $P < 0.001$ ].

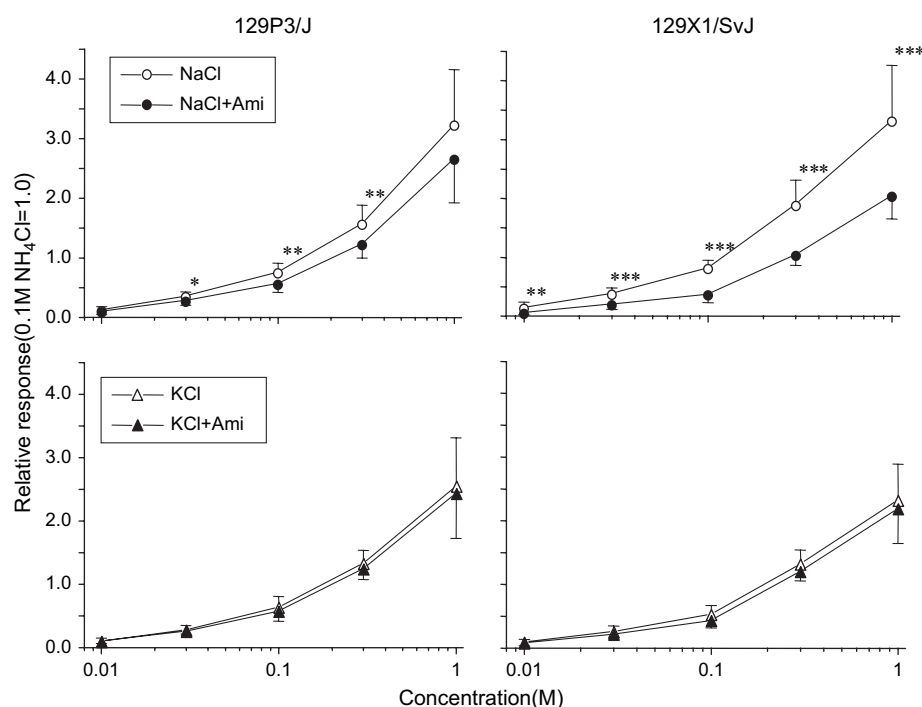
As shown in Figure 3, in response to 0.1 M NaCl, the threshold concentration of amiloride producing significant inhibition was 10  $\mu$ M in both strains ( $P < 0.01$  or 0.001), whereas in response to 0.3 M NaCl, the threshold was higher in 129P3/J mice (30  $\mu$ M) than in 129X1/SvJ mice (10  $\mu$ M). Figure 4 shows percent responses (relative to control expressed as 100%) to 0.1 and 0.3 M NaCl with 10, 30, or 100  $\mu$ M amiloride in the two strains. In 129P3/J mice, the magnitude of reduction of NaCl response by amiloride was  $\sim 20\%$  of control at 100  $\mu$ M ( $\sim 80\%$  control response after amiloride), which was significantly smaller than the magnitude for 129X1/SvJ mice ( $\sim 50\%$  of control) [ANOVA— $F(1,27) = 18.0$ ,  $P < 0.001$  for 0.1 M NaCl and  $F(1,28) = 32.6$ ,  $P < 0.001$ , for 0.3 M NaCl]. From these results, we concluded that there is a substantial difference in amiloride sensitivity between the two 129 substrains, 129P3/J and 129X1/SvJ.

## Discussion

In the present study, we examined the effect of amiloride on CT response to NaCl in two 129 substrains, 129P3/J and 129X1/SvJ. We found that amiloride (100  $\mu$ M) suppressed CT responses to NaCl in both strains, although the magnitude of suppression of NaCl response by amiloride was much smaller in 129P3/J than in 129X1/SvJ mice. Previous electrophysiological studies reported that 129P3/J mice (formerly 129/J) showed no significant suppression of CT responses to NaCl by amiloride (Gannon and Contreras, 1995; Ninomiya *et al.*, 1996). However, in the report by Ninomiya *et al.* (1996), a nonsignificant tendency for suppression was evident, but only five of 129/J mice were tested. Thus, this apparent discrepancy between our past electrophysiological results and those in the current paper may be due to the greater power in this study: we tested 15 mice here (data for 10 mice were added to that of the previous study) and five in the previous study, and with the modest decrease of  $\sim 20\%$  (the magnitude of suppression was similar



**Figure 1** Sample recordings of the integrated responses of the CT nerve of 129P3/J and 129X1/SvJ mice to 0.1 M  $\text{NH}_4\text{Cl}$  and 0.1 M NaCl with and without 100  $\mu$ M amiloride.



**Figure 2** Concentration–response relationships for NaCl and KCl with and without 100  $\mu$ M amiloride in 129P3/J and 129X1/SvJ mice. Values indicated are means  $\pm$  SDs. Numbers of subjects are 10–15 for both 129P3/J and 129X1/SvJ mice. Significant difference between NaCl with amiloride (filled circles) and without amiloride (open circles)—*t*-test: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

in the previous and the present study) we could not detect amiloride sensitivity in the earlier work.

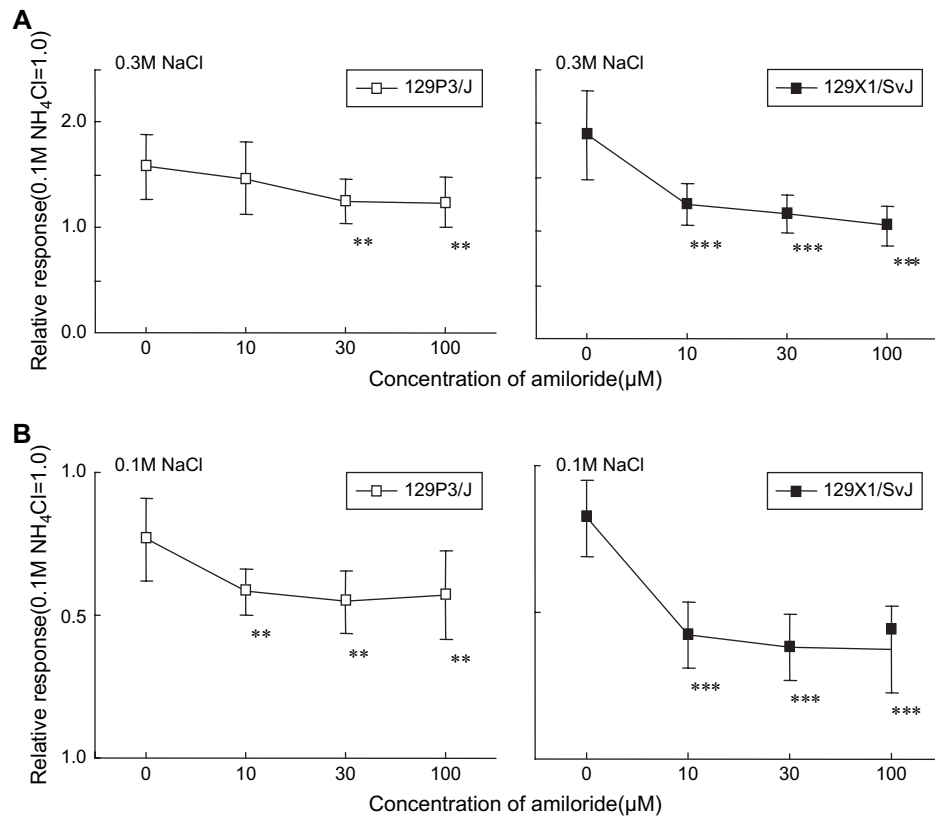
Recent behavioral studies (Eylam and Spector, 2003, 2005) also demonstrate that 129P3/J (formerly 129/J) mice possess amiloride sensitivity responsible for discrimination between NaCl and KCl. Therefore, with regard to the amiloride sensitivity, the results from this study are consistent with those of the behavioral study using the same strain of mice. Even a modest decrease of  $\sim 20\%$  inhibition, as determined by recording from the CT, appears to be sufficient to reduce behavioral discriminations.

The 129/J and 129P3/J mice are basically the same strain as they were originally derived from the same parental stock (129/J) in The Jackson Laboratory (only symbol has changed). Genetic variation among 129 substrains was documented in 1997 (Simpson *et al.*, 1997; Threadgill *et al.*, 1997). Thus, there are no available data for the genetic background of the 129/J mice used in electrophysiological studies conducted prior to 1997.

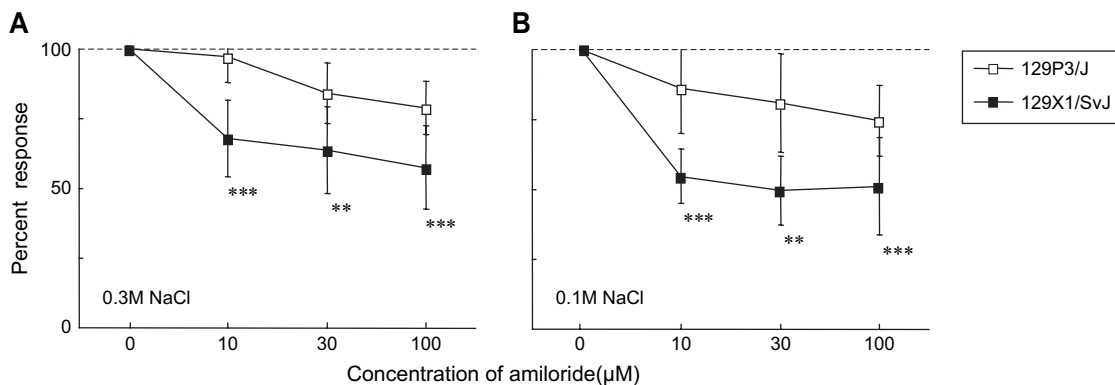
In the present study, we found clear substrain differences in amiloride inhibition of the CT responses to NaCl in 129 mice. The magnitude of inhibition of NaCl responses by amiloride was significantly larger in 129X1/SvJ ( $\sim 50\%$  of control for 0.01–1.0 M NaCl) than in 129P3/J mice ( $\sim 20\%$  of control for 0.03–0.3 M NaCl). Also, the threshold concentration of amiloride for inhibition of responses to 0.3 M NaCl was lower (10  $\mu$ M) in 129X1/SvJ than in

129P3/J mice (30  $\mu$ M). The magnitude of amiloride inhibition and the threshold concentration of amiloride in 129X1/SvJ mice are comparable with that in C57BL/6 and C3H/He mice (Ninomiya *et al.*, 1989, 1996, Bachmanov *et al.*, 1999; Yasumatsu *et al.*, 2003), suggesting almost no difference in amiloride sensitivity between 129X1/SvJ versus C57BL/6 or C3H/He strain.

The difference in amiloride sensitivity between two 129 substrains may be due to difference in genetic composition. Threadgill *et al.* (1997) demonstrated that the 129/SvJ (129X1/SvJ) strain differs in 52–54 out of 212 SSLPs ( $\sim 24\%$ ) from 129/J (129P3/J) and other 129 substrains, suggesting that about one-fourth of genomic sequences may be different between the two substrains. The 129X1/SvJ strain may thus be an inbred strain that is a mixture of the 129/Sv parent strain and an unknown strain (Threadgill *et al.*, 1997). The genes involved in high amiloride sensitivity in 129X1/SvJ may be derived from an unknown strain possessing high amiloride sensitivity, such as C57BL/6 and C3H/He mice (Ninomiya *et al.*, 1989). Taste receptor mechanisms for sweet and umami tastes have previously been revealed by examining neural and behavioral responses to particular taste compounds between 129 and C57BL/6 inbred mouse strains (Bachmanov *et al.*, 1997, 2000, 2001; Li *et al.*, 2001; Inoue *et al.*, 2004). The results from the present study, demonstrating substrain difference between 129P3/J and 129X1/SvJ in amiloride sensitivity, imply that



**Figure 3** Relative responses to 0.1 and 0.3 M NaCl with 10, 30, or 100  $\mu$ M amiloride and without amiloride (0) in 129P3/J and 129X1/SvJ mice. Values indicated are means  $\pm$  SDs. Numbers of subjects are **(A)** 10–15 for 129P3/J and 15 for 129X1/SvJ mice, and **(B)** 8–15 for 129P3/J and 8–14 for 129X1/SvJ mice. Significance of differences between the response to NaCl with and without amiloride was determined by ANOVA, followed by comparison of mean values (using *t*-tests) of control mice (without amiloride) and experimental mice (with amiloride at 10, 30, or 100  $\mu$ M); \*\**P* < 0.01, \*\*\**P* < 0.001.



**Figure 4** Percent response (relative to control without amiloride taken as 100%) of 129P3/J and 129X1/SvJ mice to 0.1 and 0.3 M NaCl with 10, 30, or 100  $\mu$ M amiloride and without amiloride (0). Values indicated are means  $\pm$  SDs. Numbers of subjects are **(A)** 10–15 for 129P3/J and 15 for 129X1/SvJ mice, and **(B)** 8–15 for 129P3/J and 8–14 for 129X1/SvJ mice. Significance of differences between the 129P3/J (open squares) and 129X1/SvJ (filled squares) strains were determined by ANOVA, followed by comparison of means (using *t*-tests) for each concentration; \*\**P* < 0.01, \*\*\**P* < 0.001.

further genetic study of these strains could aid in understanding the mechanisms of salt taste reception.

The amiloride-sensitive component of NaCl responses of the CT nerve is thought to reflect the action of a transduction

pathway that involves the relatively selective entry of  $\text{Na}^+$  through the epithelial sodium channels (ENaCs) in the apical membrane of a subset of taste receptor cells (Heck *et al.*, 1984; Brand *et al.*, 1985; DeSimone and Ferrell,

1985; Avenet and Lindemann, 1988; Formaker and Hill, 1988; Ninomiya and Funakoshi, 1988; Elliott and Simon, 1990; Ye *et al.*, 1993). There are many factors influencing the functional properties of ENaCs that may relate to substrain and strain differences in amiloride sensitivity. For example, ENaCs are known to consist of at least three subunits ( $\alpha$ ,  $\beta$ , and  $\gamma$ ), each of which possesses two transmembrane domains (Canessa *et al.*, 1993, 1994; Lingueglia *et al.*, 1993). The  $\alpha$ ENaC confers a low amplitude, amiloride-sensitive, sodium current, whereas  $\beta$  and  $\gamma$  subunits are required for the maximal channel activity. Apparent amiloride binding affinity was found to be attenuated by mutations at several specific sites of the subunits. For example, mutations at mouse  $\alpha$ Ser-583,  $\beta$ Gly-525, and  $\gamma$ Gly-542, which are located in a region preceding the second membrane-spanning domain, altered amiloride  $K_i$  (Schild *et al.*, 1997; Kellenberger *et al.*, 2003; Ji *et al.*, 2004; Kashlan *et al.*, 2005).

Moreover, it is known that amiloride clearly inhibits NaCl responses in the CT nerve, innervating the anterior tongue, whereas no such amiloride inhibition is evident in the glossopharyngeal nerve, innervating the posterior tongue (Formaker and Hill, 1991; Ninomiya *et al.*, 1991; Ninomiya, 1998; Kitada *et al.*, 1998). Recent studies suggested the possibility that these tongue regional differences in amiloride sensitivity may be related to differences in expression patterns of the three subunits because all three subunits are abundantly present in taste cells in amiloride-sensitive fungiform papillae, whereas expression levels of  $\beta$  and  $\gamma$  subunits are lower than those for  $\alpha$ ENaC in amiloride-insensitive circumvallate papilla in rodents (Kretz *et al.*, 1999; Lin *et al.*, 1999; Shigemura *et al.*, 2005). Furthermore, expression levels of  $\beta$  and  $\gamma$  subunits were enhanced by increasing blood aldosterone levels (Lin *et al.*, 1999), indicating 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  $\text{Na}^+$  current in hamster fungiform taste cells (Gilbertson *et al.*, 1993). Finally, the density of amiloride-sensitive channels on the apical side of the taste cell membrane has been implicated in the strain difference in amiloride sensitivity between BALB/c and C57BL mice (Miyamoto *et al.*, 1999). Collectively, with respect to ENaCs, mutations of amino acid sequences of the channel, the relative expression patterns of three subunits, the density of the channel on the apical side of the taste cell membrane and hormonal influences are all possible factors responsible for substrain and strain differences in amiloride sensitivity. Genes controlling these candidate factors would, therefore, be likely targets for genetic approaches to understanding salt taste transduction.

In summary, the present study examined amiloride sensitivity of the CT nerve responses to NaCl in two 129 substrains, 129P3/J and 129X1/SJ, and found prominent substrain differences in the amiloride sensitivity. Further study of these two 129 substrains could provide insights into the mechanisms of amiloride-sensitive salt taste reception in mice.

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