Chem. Senses 26: 965–969, 2001

Amiloride Blocks Salt Taste Transduction of the Glossopharyngeal Nerve in Metamorphosed Salamanders

Takatoshi Nagai, Daisuke Nii[1] and Hiro-aki Takeuchi[1]

Department of Physiology, Teikyo University School of Medicine, Tokyo 173-8605 and
[1]Department of Biology and Geoscience, Faculty of Science, Shizuoka University,
Shizuoka 422-8529, Japan

Correspondence to be sent to: Takatoshi Nagai, Department of Biology, Keio University School of Medicine, Hiyoshi, Yokohama 223-8521, Japan. e-mail: takatosh@hc.cc.keio.ac.jp

Abstract

Studies in the last two decades have shown that amiloride-sensitive Na^+ channels play a role in NaCl transduction in rat taste receptors. However, this role is not readily generalized for salt taste transduction in vertebrates, because functional expression of these channels varies across species and also in development in a species. Glossopharyngeal nerve responses to sodium and potassium salts were recorded in larval and metamorphosed salamanders and compared before and after the oral floor was exposed to amiloride, a blocker of Na^+ channels known to be responsible for epithelial ion transport. Pre-exposure to amiloride (100 μM) did not affect salt taste responses in both axolotls (*Ambystoma mexicanum*) and larval Ezo salamanders (*Hynobius retardatus*). In contrast, in metamorphosed Ezo salamanders the nerve responses to NaCl were significantly reduced by amiloride. In amphibians amiloride-sensitive components in salt taste transduction seem to develop during metamorphosis.

Introduction

Salt taste transduction is mediated primarily by entry of Na^+ ions into taste receptor cells, because amiloride, a blocker of epithelial Na^+ channels (ENaC) (Kleyman and Cragoe, 1988), reduces the intensity of NaCl taste perceived by humans (Schiffman *et al.*, 1983; Smith and Ossebaard, 1995) and the response of the chorda tympani nerve to sodium salts in rats (Heck *et al.*, 1984). Whole cell clamp studies show that amiloride-sensitive (blockable) sodium channels (ASSCs) are present in taste receptor cells (Avenet and Lindemann, 1988; Doolin and Gilbertson, 1996). Furthermore, antibodies directed against ENaC in rats successfully localized the channels in the taste cell membrane (Stewart *et al.*, 1995; Lin *et al.*, 1999). When the lingual epithelium is exposed to amiloride solution a reduction in taste nerve responses to sodium salts occurs in species other than rats, such as hamster (Hettinger and Frank, 1990) and monkeys (Hellekant *et al.*, 1988). However, transduction mediated by ASSCs is not readily generalized for salt taste transduction in vertebrates [for a review see Halpern (Halpern, 1998)], because the effect of amiloride is not specific to sodium salts in some species, such as dog (Nakamura and Kurihara, 1990), and amiloride does not reduce taste nerve responses to sodium salts in some strains of laboratory mice (Gannon and Contreras, 1995; Ninomiya *et al.*, 1996). Furthermore, involvement of ASSCs in salt taste transduction is less clear in non-mammalian vertebrates.

In amphibia salt solutions induce responses in the glossopharyngeal nerve, which innervates a majority of taste receptor cells in the lingual epithelium (Akaike *et al.*, 1976; Samanen and Bernard, 1981; Takeuchi *et al.*, 1994). However, salt responses in the nerve are not affected by amiloride in mudpuppies (*Necturus maculosus*) (McPheeters and Roper, 1985) and frogs (*Rana catesbeiana*) (Okada *et al.*, 1991; Kitada and Mitoh, 1998). In contrast to these studies, whole cell clamp studies of isolated amphibian taste cells show that ASSCs are present in tiger salamanders (*Ambystoma tigrinum*) (Sugimoto and Teeter, 1991) and frogs (*Rana esculenta/ridibunda*) (Avenet and Lindemann, 1988). Whether such opposing results derive from a difference in species or electrophysiological methods of measurement is not clear.

In the present study we determined the effects of amiloride on glossopharyngeal nerve responses in two species of amphibians, axolotls (*Ambystoma mexicanum*) and Ezo salamanders (*Hynobius retardatus*). In Ezo salamanders we examined not only larval animals but also metamorphosed adults, because susceptibility of taste nerve responses to amiloride may develop with growth, as has been reported in the mammalian taste system (Hill and Bour, 1985; Hill and

Mistretta, 1990). A preliminary account of this study has appeared in abstract form (Nii *et al*., 1998).

Materials and methods

Animals

Two species of salamanders, axolotls (*A. mexicanum*) and Ezo salamanders (*H. retardatus*), were used. Axolotl larvae of the wild and white strains were obtained from the Indiana University Axolotl Colony and raised until ~1 year of age (adult) in our laboratory (Shizuoka University). Neotenic axolotls do not metamorphose under natural conditions whereas Ezo salamanders do. Pre-metamorphic and post-metamorphic salamanders were selected from animals raised in our laboratory. Larval Ezo salamanders were younger than phase 0 and metamorphosed ones were older than phase 5. The phases are defined by our previous study on the metamorphosis of the taste buds in Ezo salamanders (Takeuchi *et al*., 1997).

Electrophysiological recordings

Neural activity of the glossopharyngeal nerve in response to chemical stimuli was recorded in axolotls and larval and metamorphosed Ezo salamanders. Methods of neural recordings and stimulation are described in detail in our physiological study on the glossopharyngeal nerve in axolotls (Takeuchi *et al*., 1994). The same methods were applied to obtain recordings from Ezo salamanders. Briefly, in anesthetized animals the peripheral end of the glossopharyngeal nerve was exposed in the caudal end of the external mandibular levator and hooked onto bipolar platinum wire electrodes. The overall activity of the nerve was differentially amplified and stored on a digital magnetic tape recorder (PC204AX; Sony, Tokyo, Japan), together with an electrical signal and voice cues marking the onset of stimulation. Taste stimuli (for 10–15 s) and distilled water rinses (for 100 s) were alternately presented to the rostral part of the oral floor through a peristaltic pump with a flow rate of 6 ml/min. In the mudpuppy (Samanen and Bernard, 1981; McPheeters and Roper, 1985) and the axolotl (Takeuchi *et al*., 1994) gustatory response in the glossopharyngeal nerve is known to vary over time during chemical stimulation. Therefore, the number of stimulations in a stimulus series was set to a minimum: stimulation by the 0.5 M KCl standard was followed by between two and four concentrations of NaCl and then the 0.5 M KCl standard again. When the response to the second 0.5 M KCl was reduced by >30%, data were discarded from the subsequent quantitative analysis. The effect of amiloride on the response to NaCl and KCl solutions was evaluated after the oral floor had been exposed for 10 min to 100 μM or 1 mM amiloride (Sigma, St Louis, MO) dissolved in distilled water. After exposure to amiloride, the post-stimulation rinse was with amiloride solution. The chemical stimuli were distilled water solutions of reagent grade NaCl and KCl. Stored neural activities were played back and subjected to subsequent data analyses. The activities obtained from axolotls and Ezo salamanders were fed to an integrator (time constant 0.3 s). Some of those from metamorphosed Ezo salamanders were fed to a spike counter (ET-612J; Nihon Koden, Tokyo, Japan), because in some animals the whole nerve recordings consisted of relatively few units (see Figure 4) and thus adequate neural integration could not be carried out for these recordings. What caused such a difference in neural recordings is not known, but morphological changes in the oral floor that accompany the change in innervation during metamorphosis are possibly involved (Takeuchi *et al*., 1997). Action potentials from the nerve and integrated nerve responses were recorded on a thermal array recorder (RTA-1200M; Nihon Koden). The neural responses were quantified by measuring the area under the output from the integrator with a computer-assisted digitizer or from the digital output of the spike counter. The spontaneous ongoing activity just before stimulation was subtracted from the activity induced for 10 s after the onset of stimulation. All responses were normalized by comparing each response with the response obtained to the standard solution (0.5 M KCl) recorded prior to the test solutions and are shown as relative magnitudes of responses.

Results

Effect of amiloride on salt taste responses in axolotls

In axolotls amiloride did not reduce the glossopharyngeal nerve responses to NaCl solutions (Figure 1). The response

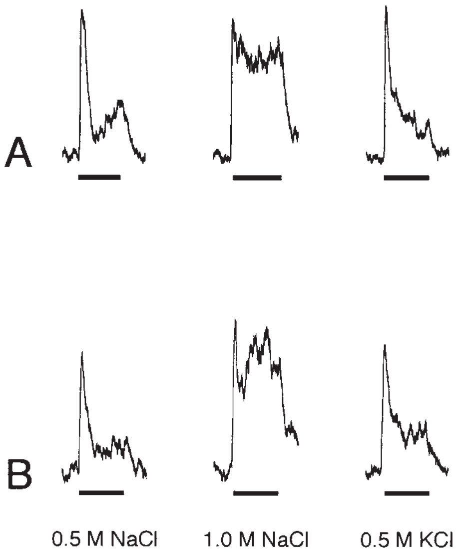

Figure 1 Example of integrated glossopharyngeal nerve responses of axolotl to 0.5 and 1.0 M NaCl and 0.5 M KCl in the control **(A)** and after the oral floor was exposed to 100 μM amiloride **(B)**. Each trace shows integrated neural responses for 25 s, including 5 s before stimulation. Marking for stimulus duration is shown below each trace of the response. The magnitude of the ordinate is an arbitrary unit, but a single unit applies to all integrated responses.

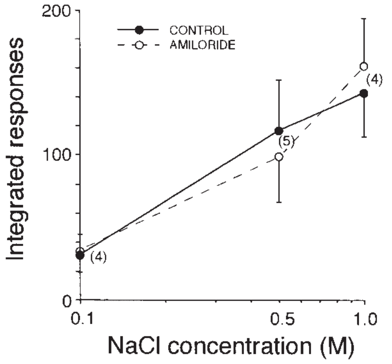

Figure 2 Intensity–response curves for NaCl in the control (●) and after exposure of the oral floor to 100 μM amiloride (○) in axolotls. The responses were normalized to the standard (0.5 M KCl) response obtained in the control. Data points are means ± SEM of the normalized responses for three concentrations of NaCl. The number of measurements is shown in parentheses.

to 0.5 M KCl was also not affected by amiloride and thus was adopted as the standard to normalize the responses to NaCl. The intensity–response curves of NaCl solutions did not show any significant reduction after the oral floor was exposed to 100 μM amiloride (0.1 M NaCl, $P = 0.3459$; 0.3 M NaCl, $P = 0.1880$; 1.0 M NaCl, $P = 0.2612$; 0.5 M KCl, $P = 0.3750$; not significant at $P > 0.05$ by two-tailed *t*-test; Figure 2). Amiloride at 100 μM is thought to be sufficient to suppress NaCl taste responses of the chorda tympani nerve in rats (Brand *et al*., 1985), but the effective concentration may differ between species. When a much higher concentration of amiloride (1 mM) was used a significant reduction in response was induced at 0.5 M NaCl (reduced by 40%, $P = 0.0193$, significant at $P < 0.05$ by two-tailed *t*-test), but not at lower and higher concentrations (0.1 M NaCl, reduced by 23%, $P = 0.0621$; 1.0 M NaCl, reduced by 15%, $P = 0.5297$; not significant at $P > 0.05$ by two-tailed *t*-test). A lack of reduction in the responses at lower concentrations of NaCl may show that amiloride at such a high concentration did not actually block ASSCs, but exerted some other effects on the oral floor. In axolotls the ASSCs involved in NaCl taste transduction are few and of very low affinity, if any, for amiloride.

Salt taste responses in larval and metamorphosed Ezo salamanders

A similar lack of sensitivity to amiloride was seen in larval Ezo salamanders (neural data not shown). Amiloride did not significantly reduce the neural responses to 0.5 and 1.0 M NaCl and 0.5 M KCl (0.5 M NaCl, $P = 0.1225$; 1.0 M NaCl, $P = 0.1129$; 0.5 M KCl, $P = 0.6339$; not significant at $P > 0.05$ by two-tailed *t*-test; Figure 3).

In contrast to axolotls and larval Ezo salamanders, amiloride reduced the nerve responses to NaCl in metamorphosed Ezo salamanders, leaving those to KCl unaffected (Figure 4). Intensity–response curves for NaCl were shifted to the right, showing a reduction in NaCl responses at all concentrations tested (Figure 3). The reductions in the responses to NaCl were statistically very significant (0.3 M NaCl, $P = 0.0071$; 0.5 M NaCl, $P = 0.0018$; 1.0 M NaCl, $P = 0.0058$; significant at $P < 0.01$ by two-tailed *t*-test). The responses at 0.1 M NaCl were not analyzed statistically due to the small sample size ($n = 3$).

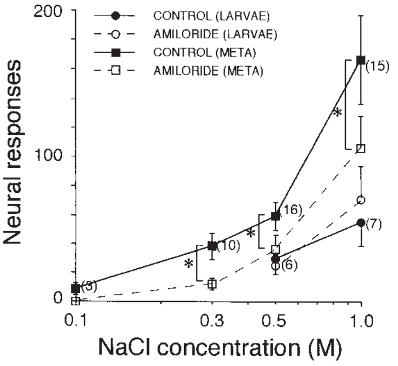

Figure 3 Intensity–response curves for NaCl in larval (●, ○) and metamorphosed (■, □) Ezo salamanders. For each animal the curves for NaCl in the control (filled symbols) and after exposure of the oral floor to 100 μM amiloride (open symbols) are shown. The responses were normalized to the standard (0.5 M KCl) response obtained in the control. Data points are means ± SEM of the normalized responses for two (larvae) and four (metamorphosed salamanders) concentrations of NaCl. Asterisk shows statistically significant difference in the mean values. The number of measurements is shown in parentheses.

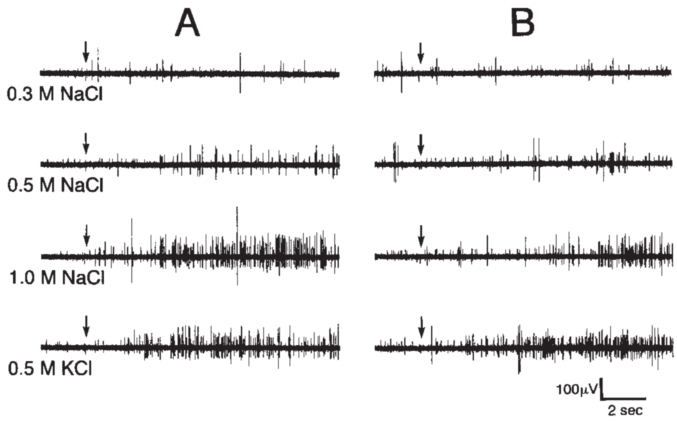

Figure 4 Example of multiunit activity of the glossopharyngeal nerve response of metamorphosed Ezo salamander to 0.3–1.0 M NaCl and 0.5 M KCl in the control **(A)** and after the oral floor was exposed to 100 μM amiloride **(B)**. Each trace shows multiunit recording of neural responses for 13 s, including 2 s before stimulation. Arrows show the onset of stimulation. Note that the neural spike activities to NaCl were reduced, leaving those to 0.5 M KCl relatively unaffected. Scale bars 2 s (horizontal) and 100 μV (vertical).

Discussion

We previously studied the morphology of taste organs on the tongue in two species of salamanders, axolotl and Ezo salamander, and found that salamanders undergo morphological changes of the taste organs during metamorphosis (Takeuchi *et al.*, 1997). The axolotls used in the present study were larvae and had a taste bud morphology similar to that of larval Ezo salamanders (i.e. barrel-shaped taste buds). In both axolotls and larval Ezo salamanders neural responses to NaCl recorded from the glossopharyngeal nerve were not reduced by pre-exposure of the lingual epithelium to 100 μM amiloride (Figures 1–3), a specific blocker of ENaC. In contrast, in metamorphosed Ezo salamanders these responses were reduced by pre-exposure to amiloride (Figures 3 and 4). Therefore, larval salamanders lack amiloride-sensitive components in glossopharyngeal nerve responses to NaCl, but it is likely that the nerve acquires such components during metamorphosis.

In mammals neural responses to NaCl increase during development of the gustatory system (Hill and Mistretta, 1990). The increase in NaCl response is attributed to ASSCs, added to taste cell membranes during development (Hill and Bour, 1985). The present study on salamanders suggests that addition of ASSCs during development also occurs in non-mammalian species. In mudpuppies recordings from the glossopharyngeal nerve failed to show that these channels are involved in transduction of NaCl (McPheeters and Roper, 1985). However, this does not necessarily mean innate absence of the channels in the mudpuppy taste cell. We speculate that mudpuppies would develop functional ASSCs in the taste cell if they metamorphosed, although neotenic mudpuppies do not metamorphose in their natural environment. In support of this speculation our preliminary experiment using axolotls artificially induced to metamorphose by thyroxin administration showed a reduction in NaCl responses by amiloride in the glossopharyngeal nerve (a single experiment; unpublished observation). Although axolotls artificially induced to metamorphose are a good model for studying the development of ASSCs in amphibians, axolotls administered thyroxin do not survive well so that a few metamorphosed animals were amenable only to morphological examination (Takeuchi *et al.* 1997). As an alternative we have chosen naturally metamorphosed Ezo salamanders to study the issue by a physiological approach. Ezo salamanders acquire amiloride-sensitive components in the glossopharyngeal nerve responses to NaCl during development. However, such acquisition may be attributable to adaptation from an aquatic to a terrestrial environment rather than to developmental change *per se*.

Absence of amiloride-sensitive components in the glossopharyngeal nerve responses to NaCl is also shown by frogs (Okada *et al.*, 1991; Kitada and Mitoh, 1998). However, whole cell clamp recordings of isolated taste cells in frogs (Avenet and Lindemann, 1988) show the presence of ASSCs in the taste cell membrane. The same channels are also present in larval tiger salamanders (Sugimoto and Teeter, 1991). However, these single cell studies do not necessarily argue against the glossopharyngeal nerve recordings, because the studies did not clarify whether ASSCs are distributed in the apical membrane of taste cells or in the basal membrane. If the channels are distributed in the basal membrane of taste cells amiloride, which is impermeable through tight junctions (Briggman *et al.*, 1983; Ye *et al.*, 1993), would be unable to prevent the flow of Na^+ ions into taste cells through the basal membrane, while Na^+ ions would be able to pass into the intercellular space facing the basal cell membrane by means of the paracellular pathway (Ye *et al.*, 1991). Therefore, absence of suppression by amiloride in the glossopharyngeal nerve responses simply suggests absence of ASSCs in the apical taste cell membrane, leaving the possibility that these channels are present in the basal membrane. In fact, a recent immunohistochemical study shows that ASSCs are present in both the apical and basal portions of taste cells in rats (Lin *et al.*, 1999). Furthermore, ASSCs are expressed in a very early stage of development (post-natal day 2) in rat taste buds (Kossel *et al.*, 1997). Therefore, these channels may be present in the basal portion of the taste cell in larval salamanders to mediate the amiloride-insensitive part of salt taste transduction. On the other hand, metamorphosed Ezo salamanders may have developed ASSCs in the apical portion of the taste cell, as expression of ASSCs in the apical region of rat taste buds can be induced by aldosterone (Lin *et al.*, 1999) and aldosterone participates in regulating ASSCs in frog skin, where the channels play a role in active Na^+ transport. Takada *et al.* showed that ASSCs develop when the skin of tadpoles is cultured with aldosterone (Takada *et al.*, 1995). Furthermore, development of these channels is suppressed by an as yet unknown mechanism(s) in tadpole skin *in vivo*, but suppression seems to be removed by an increase in endogenous thyroid hormone during development (Takada *et al.*, 1999). Similar hormonal regulation may operate in the development of ASSCs in the lingual epithelium of salamanders, including taste buds. Metamorphosed salamanders develop taste buds with a morphology not seen in larval salamanders (Takeuchi *et al.*, 1997). These 'adult type' taste buds, particularly with respect to the apical region, may be the site at which the ASSCs are expressed. Immunohistological demonstration of these channels in amphibian taste buds will clarify the point.

Acknowledgements

We thank Indiana University Axolotl Colony for the continuous supply of axolotls. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan (T.N.) and by a grant from the Saneyoshi Scholarship Foundation (H.-A.T.).

References

Akaike, N., Noma, A. and **Sato, M.** (1976) *Electrical responses of frog taste cells to chemical stimuli.* J. Physiol., 254, 87–107.

Avenet, P. and **Lindemann, B.** (1988) *Amiloride-blockable sodium currents in isolated taste receptor cells.* J. Membr. Biol., 105, 245–255.

Brand, J.G., Teeter, J.H. and **Silver, W.L.** (1985) *Inhibition by amiloride of chorda tympani responses evoked by monovalent salts.* Brain Res., 334, 207–214.

Briggman, J.V., Graves, J.S., Spicer, S.S. and **Cragoe, E.J. Jr** (1983) *The intracellular localization of amiloride in frog skin.* Histochem. J., 15, 239–255.

Doolin, R.E. and **Gilbertson, T.A.** (1996) *Distribution and characterization of functional amiloride-sensitive sodium channels in rat tongue.* J. Gen. Physiol., 107, 545–554.

Gannon, K.S. and **Contreras, R.J.** (1995) *Sodium intake linked to amiloride-sensitive gustatory transduction in C57BL/6J and 129/J mice.* Physiol. Behav., 57, 231–239.

Halpern, B.P. (1998) *Amiloride and vertebrate gustatory responses to NaCl.* Neurosci. Biobehav. Rev., 23, 5–47.

Heck, G.L., Mierson, S. and **DeSimone, J.A.** (1984) *Salt taste transduction occurs through an amiloride-sensitive sodium transport pathway.* Science, 223, 403–405.

Hellekant, G., DuBois, G.E., Roberts, T.W. and **van de Wel, H.** (1988) *On the gustatory effect of amiloride in the monkey (*Macaca mulatta*).* Chem. Senses, 13, 89–93.

Hettinger, T.P. and **Frank, M.E.** (1990) *Specificity of amiloride inhibition of hamster taste responses.* Brain Res., 513, 24–34.

Hill, D.L. and **Bour, T.C.** (1985) *Addition of functional amiloride-sensitive components to the receptor membrane: a possible mechanism for altered taste responses during development.* Dev. Brain Res., 20, 310–313.

Hill, D.L. and **Mistretta, C.M.** (1990) *Developmental neurobiology of salt taste sensation.* Trends. Neurosci., 13, 188–195.

Kitada, Y. and **Mitoh, Y.** (1998) *Amiloride does not affect the taste responses of the frog glossopharyngeal nerve and submandibular branch of the facial nerve to NaCl.* Chem Senses, 23, 222.

Kleyman, T.R. and **Cragoe, E.J. Jr** (1988) *Amiloride and its analogs as tools in the study of ion transport.* J. Membr. Biol., 105, 1–21.

Kossel, A.H., McPheeters, M., Lin, W. and **Kinnamon, S.C.** (1997) *Development of membrane properties in taste cells of fungiform papillae: functional evidence for early presence of amiloride–sensitive sodium channels.* J. Neurosci., 17, 9634–9641.

Lin, W., Finger, T.E., Rossier, B.C. and **Kinnamon, S.C.** (1999) *Epithelial Na^+ channel subunits in rat taste cells: localization and regulation by aldosterone.* J. Comp. Neurol., 405, 406–420.

McPheeters, M. and **Roper, S.D.** (1985) *Amiloride does not block taste transduction in the mudpuppy,* Necturus maculosus*.* Chem. Senses, 10, 341–352.

Nakamura, M. and **Kurihara, K.** (1990) *Non-specific inhibition by amiloride of canine chorda tympani nerve responses to various salts: do Na^+-specific channels exist in canine taste receptor membranes?* Brain Res., 524, 42–48.

Nii, D., Takeuchi, H.-A. and **Nagai, T.** (1998) *Amiloride-sensitive sodium channels in taste organ of metamorphosing urodeles.* Zool. Sci., 15 (suppl.), 102.

Ninomiya, Y., Fukami, Y., Yamazaki, K. and **Beauchamp, G.** (1996) *Amiloride inhibition of chorda tympani responses to NaCl and its temperature dependency in mice.* Brain Res., 708, 153–158.

Okada, Y., Miyamoto, T. and **Sato, T.** (1991) *Vasopression increases frog gustatory neural responses elicited by NaCl and HCl.* Comp. Biochem. Physiol., 100A, 693–696.

Samanen, D.W. and **Bernard, R.A.** (1981) *Response properties of the glossopharyngeal taste system of the mud puppy (*Necturus maculosus*).* J. Comp. Physiol., 143, 143–150.

Schiffman, S.S., Lockhead, E. and **Maes, F.W.** (1983) *Amiloride reduces the taste intensity of Na^+ and Li^+ salts and sweeteners.* Proc. Natl Acad. Sci. USA, 80, 6136–6140.

Smith, D. V. and **Ossebaard, C. A.** (1995) *Amiloride suppression of the taste intensity of sodium chloride: evidence from direct magnitude scaling.* Physiol. Behav., 57, 773–777.

Stewart, R.E., Lasiter, P.S., Benos, D.J. and **Hill, D.L.** (1995) *Immunohistochemical correlates of peripheral gustatory sensitivity to sodium and amiloride.* Acta Anat., 153, 310–319.

Sugimoto, K. and **Teeter, J.H.** (1991) *Stimulus-induced currents in isolated taste receptor cells of the larval tiger salamander.* Chem. Senses, 16, 109–122.

Takada, M., Yai, H. and **Takayama-Arita, K.** (1995) *Corticoid-induced differentiation of amiloride-blockable active Na^+ transport across larval bullfrog skin* in vitro*.* Am. J. Physiol., 268, C218–C226.

Takada, M., Shiibashi, M. and **Kasai, M.** (1999) *Possible role of aldosterone and T_3 in development of amiloride-blockable SCC across frog skin* in vivo*.* Am. J. Physiol., 277, R1305–R1312.

Takeuchi, H.-A., Masuda, T. and **Nagai, T.** (1994) *Electrophysiological and behavioral studies of taste discrimination in the axolotl (*Ambystoma mexicanum*).* Physiol. Behav., 56, 121–127.

Takeuchi, H.-A., Ido, S., Kaigawa, Y.-I. and **Nagai, T.** (1997) *Taste disks are induced in the lingual epithelium of salamanders during metamorphosis.* Chem. Senses, 22, 535–545.

Ye, Q., Heck, G.L. and **DeSimone, J.A.** (1991) *The anion paradox in sodium taste reception: resolution by voltage-clamp studies.* Science 254, 724–726.

Ye, Q., Heck, G.L. and **DeSimone, J.A.** (1993) *Voltage dependence of the rat chorda tympani response to Na^+ salts: implications for the functional organization of taste receptor cells.* J. Neurophysiol., 70, 167–178.

Accepted May 1, 2001