

Effects of Amiloride on Gustatory Neural Responses to Salts in the Frog

Yasuyuki Kitada, Kazuhisa Okuda-Akabane and Yoshihiro Mitoh¹

Department of Oral Physiology, School of Dentistry, Iwate Medical University, Iwate 020-8505 and ¹Department of Oral Physiology, Okayama University Dental School, Okayama 700-8525, Japan

Correspondence to be sent to: Yasuyuki Kitada, Department of Oral Physiology, School of Dentistry, Iwate Medical University, Iwate 020-8505, Japan. e-mail: ykita@iwate-med.ac.jp

Abstract

In frogs, the glossopharyngeal nerve (GL) innervates taste receptors on almost the entire tongue. The mandibular branch (MBF) and palatine branch (PN) of the facial nerve innervate taste receptors on a very small area at the base of the tongue and on the palate, respectively. In the present study, effects of amiloride, an epithelial sodium channel blocker, on the tonic responses of the GL, MBF and PN in frogs to NaCl, LiCl, KCl and CaCl₂ were investigated. In three nerves, amiloride at 0.5 mM, a relatively high concentration, did not affect the responses to 0.15 (concentration just above threshold)–0.5 M NaCl, 0.5 M LiCl and 0.3 M KCl, whereas it almost completely inhibited the response to 1.0 mM CaCl₂. Amiloride may exert an inhibitory action on the response to CaCl₂ by a competitive antagonism between Ca²⁺ and a monovalent cation of amiloride, because the response to Ca²⁺ is competitively inhibited by other cations such as Na⁺ and Mg²⁺. The lack of inhibitory effect of amiloride on the responses in the GL, MBF and PN to NaCl suggests that amiloride-sensitive sodium channels in the apical membrane of taste receptor cells are not involved in sodium taste transduction in frogs.

Introduction

In mammals, taste receptors on the anterior two-thirds and the posterior one-third of the tongue are innervated by the chorda tympani nerve (CT) and the glossopharyngeal nerve (GL), respectively. Taste receptors on the soft palate and in the naso-incisor ducts are innervated by the greater superficial petrosal nerve (GSP). The CT and the GSP are branches of the facial nerve. It has been demonstrated that amiloride, an epithelial sodium channel blocker, partially reduces the neural responses of the rat CT (Lindemann, 1996; Stewart *et al.*, 1997) and GSP (Harada *et al.*, 1997; Sollars and Hill, 1998) to NaCl, but it does not affect the NaCl response of the GL in rats (Formaker and Hill, 1991; Kitada *et al.*, 1998) and mice (Ninomiya *et al.*, 1991). These findings suggest that sodium taste processing involves both amiloride-sensitive and amiloride-insensitive transduction pathways. In frogs, unlike mammals, taste receptors on almost the entire tongue are innervated by the GL. When compared with those in the mammalian gustatory nerves, studies of amiloride-sensitive and amiloride-insensitive transduction pathways in the frog gustatory nerves are relatively few. Some investigators have reported that amiloride partially inhibited the responses of the frog GL to salts (Yoshii *et al.*, 1986; Herness, 1987). However, in their studies a single concentration (0.1 mM) of amiloride was tested, but the affinity of amiloride for inhibition was not quantitatively studied. Other investigators have reported

opposing results—that amiloride had no effect on the response to NaCl (Okada *et al.*, 1991). In their experiments only a single concentration was chosen for stimulation. No systematic studies have been done so far. Therefore, the effect of amiloride on neural responses of the frog to salts remains unclear.

In the present study, we investigated the effects of amiloride on the responses of the frog GL to a variety of salts (NaCl, LiCl, KCl and CaCl₂). In frogs, the mandibular branch of the facial nerve (MBF) innervates taste receptors on a very small area at the base of the tongue (Gaupp, 1899; Nomura and Suzuki, 1989) and the palatine nerve (PN), a branch of the facial nerve, innervates taste receptors on the palate (Gaupp, 1899; Pumphrey, 1935). Since in the rodent facial nerve NaCl responses are inhibited by amiloride (Lindemann, 1996; Stewart *et al.*, 1997), we also investigated the effects of amiloride on the responses of the MBF and of the PN to NaCl, LiCl, KCl and CaCl₂ in frogs.

Materials and methods

Neurophysiological recording

Bullfrogs (*Rana catesbeiana*), weighing 200–400 g, were anesthetized with urethane (3 g/kg body wt). Each animal was put in the supine position and the tongue was pulled out from the mouth and fixed on the plate of an experimental

chamber with pins. The hypoglossal nerve was transected bilaterally to prevent tongue movements. The GL or the MBF on one side was dissected free from the surrounding connective tissues and cut centrally. For recording the neural activities of the PN, the mandibles were removed from animals and the PN on one side was exposed from the ventral side by slitting the mucous membrane of the palate. The PN was cut near its entrance to the skull.

The nerve was placed on a silver recording electrode. Multifiber neural activity was differentially recorded in reference to a stainless-steel needle electrode placed in nearby tissue. The activities were displayed on an oscilloscope and passed through an integrator with a time constant of 0.5 s. The integrated neural activity was then displayed on a rectilinear pen recorder for analyses of response magnitudes.

Stimulation

The experiments were performed at 20–25°C. Since the frog GL response is sensitive to water and the water response is inhibited by low concentrations of NaCl (Zotterman, 1949), 0.05 M NaCl solution was used as an adapting solution. Taste receptors on the tongue were adapted with 0.05 M NaCl for at least 5 min before each stimulation. Stimulating solutions of 0.1–0.5 M NaCl, 0.1–0.5 M LiCl, 0.1–0.3 M KCl and 0.1–1.0 mM CaCl₂ were prepared with distilled water. Stimulating solutions were applied to the surface of the tongue at a flow rate of 20 ml/min for 30 s. To study the effect of amiloride, the adapting solution (0.05 M NaCl) containing 0.05–0.5 mM amiloride hydrochloride (Sigma) was flowed over the tongue for 1 min prior to each test and then the stimulating solution containing 0.05–0.5 mM amiloride was applied to the tongue. Stimulation of the palate was similar to that of the tongue.

Data analysis

The height of the pen recorder deflection at 25 s after stimulus application was used as the measure of response. In frogs, the phasic portion of the response is variable and depends on the adapting condition of taste receptors on the tongue as well as on concentration of salt stimuli, while the tonic response is practically independent of the adapting condition (Kashiwagura *et al.*, 1976). The response magnitudes were normalized relative to the magnitude of the response to 0.5 M NaCl, 0.5 M LiCl, 0.3 M KCl and 1.0 mM CaCl₂ for NaCl, LiCl, KCl and CaCl₂ stimulations, respectively.

Results

The GL responses

One GL innervates taste receptors on most of the tongue of the ipsilateral side (Figure 1A). The concentration of NaCl at threshold was 0.1–0.15 M when taste receptors on the tongue were adapted to 0.05 M NaCl. Amiloride at 0.1 mM did not affect the response of the GL to 0.15–0.5 M NaCl

(data not shown). Thus, 0.5 mM, a relatively high concentration, of amiloride was used in the following experiments for NaCl stimulations. Figure 1B shows the integrated responses of the GL to NaCl stimulation with and without 0.5 mM amiloride. The adapting solution containing 0.5 mM amiloride elicited a phasic response, indicating that amiloride itself is stimulatory in the GL responses. The responses to NaCl over the concentration range 0.15–0.5 M were unaffected by 0.5 mM amiloride. Figure 1C shows concentration–response curves for NaCl with and without 0.5 mM amiloride. Amiloride at 0.5 mM had no effect on the responses to NaCl stimulations at all concentrations tested.

The effects of amiloride on the responses of the GL to LiCl, KCl and CaCl₂ were examined. The concentration of LiCl or KCl at threshold was ~0.1 M. CaCl₂ is a very effective stimulus for eliciting impulses from the frog GL (Zotterman, 1949; Kusano and Sato, 1957; Nomura and Sakada, 1965; Kitada, 1978; Kitada and Shimada, 1980). The concentration of CaCl₂ at threshold was reported to be <0.01 mM and the maximum response was obtained at 1 mM CaCl₂ (Kitada, 1978). In the present study, 0.5 M LiCl, 0.3 M KCl and 1 mM CaCl₂ were employed as stimulating solutions. The reasons for choosing these concentrations are that the stable responses to the salts were obtained in these concentrations. Figure 2A shows the integrated responses to three salts. The responses to 0.5 M LiCl and 0.3 M KCl were not affected by the presence of 0.5 mM amiloride, but the response to 1.0 mM CaCl₂ was almost completely inhibited by 0.5 mM amiloride. Effects of amiloride on salt responses are summarized in 4–6 preparations (Figure 2B). No reduction in magnitudes of responses to 0.5 M LiCl and 0.3 M KCl was seen with 0.5 mM amiloride, whereas amiloride at 0.5 mM strongly inhibited the response to 1.0 mM CaCl₂.

Figure 3 shows the amiloride inhibition curves of CaCl₂ responses at two concentrations. Reducing the concentration of CaCl₂ from 1.0 to 0.1 mM shifted the curve to a low concentration range of amiloride, suggesting that the amiloride inhibition is due to competitive antagonism between Ca²⁺ ions and amiloride.

The MBF and PN responses

Figure 4A illustrates the small innervation area of the frog MBF. Application of 0.5 M NaCl solution to the innervation area of the tongue elicited impulses from the MBF. The concentration of NaCl at threshold was 0.1–0.15 M. Figure 4B,C shows integrated responses to NaCl and the concentration–response curves for NaCl with and without 0.5 mM amiloride, respectively. It is evident that amiloride did not affect the responses to NaCl stimulation. Effects of 0.5 mM amiloride on the responses to 0.5 M LiCl, 0.3 M KCl and 1.0 mM CaCl₂ in the MBF were similar to those in the GL (Figure 5). Amiloride at 0.5 mM did not affect the responses

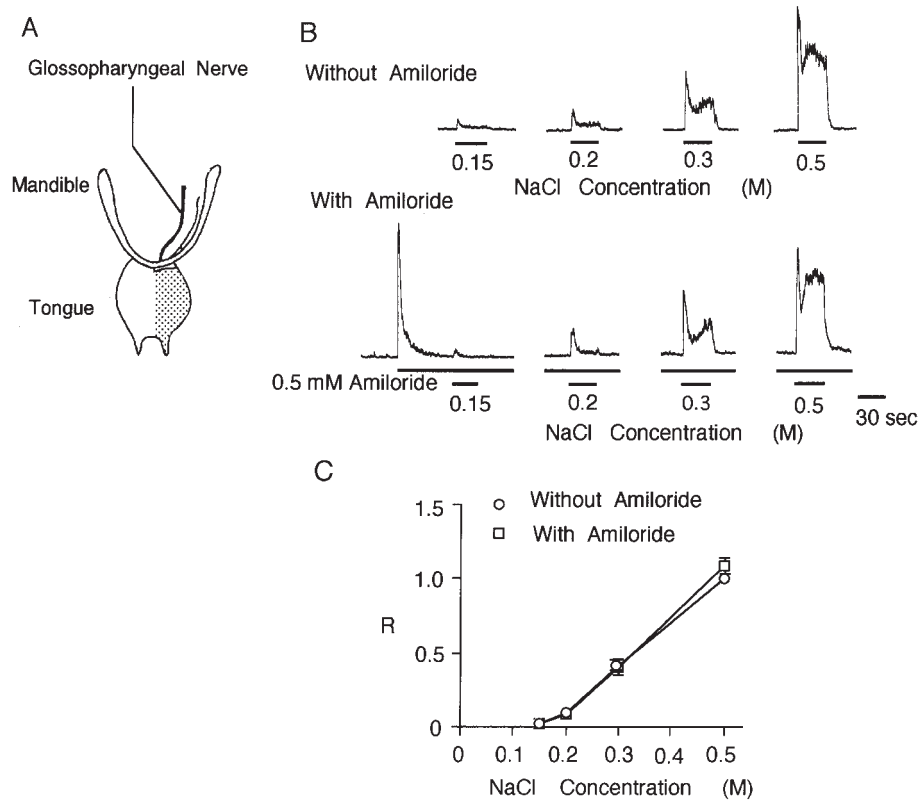


Figure 1 Effects of amiloride on the responses of the glossopharyngeal nerve (GL) to NaCl. **(A)** Innervation area of one GL (dotted area). **(B)** Integrated responses to 0.15–0.5 M NaCl with and without 0.5 mM amiloride. A solution of 0.05 M NaCl was used as the adapting solution before amiloride treatment. In amiloride treatment, adapting solution containing 0.5 mM amiloride was applied to the tongue for 1 min prior to each test and then stimulating solution containing 0.5 mM amiloride was applied. Note that the adapting solution containing 0.5 mM amiloride elicited a phasic response. **(C)** Average response ratios of the tonic responses to 0.15–0.5 M NaCl with and without 0.5 mM amiloride. The magnitude of the neural response to 0.5 M NaCl without amiloride is taken as unity on the ordinate. The relative magnitude of the response (R) is plotted against the concentration of NaCl. Points and bars represent mean \pm SEM, $n = 6$.

to 0.5 M LiCl and 0.3 M KCl, but it eliminated the responses to 1.0 mM CaCl₂ (Figure 5).

The frog PNs are responsive to salts (Pumphrey, 1935). As shown in Figure 6A, one PN innervates the taste receptors on the palate of the ipsilateral side. The concentration of NaCl at threshold was 0.1–0.15 M. Integrated responses of the PN to NaCl at 0.15, 0.2, 0.3 and 0.5 M are shown in Figure 6B. Effects of amiloride on the responses to NaCl in the PN were similar to those in the GL and MBF. As shown in Figure 6B,C, amiloride at 0.5 mM did not affect the responses to NaCl stimulations at all concentrations tested. Effects of 0.5 mM amiloride on the responses to 0.5 M LiCl and 0.3 M KCl were also examined. No reduction in the responses to 0.5 M LiCl and 0.3 M KCl were seen with 0.5 mM amiloride (Figure 7). The responses to 1.0 mM CaCl₂ were eliminated by 0.5 mM amiloride (Figure 7).

Discussion

In frogs, amiloride-sensitive sodium channels (ASSCs) were found in isolated taste cells by patch clamp recording (Avenet and Lindemann, 1988). The presence of ASSCs in taste cells suggests that the influx of Na⁺ ions through

ASSCs directly depolarizes the taste cells, eventually leading to the release of neurotransmitters onto taste afferent nerve terminals. Therefore, the frog GL response has been thought to be sensitive to amiloride. In the present study, we used 0.5 mM amiloride that is sufficient to block the influx of Na⁺ ions through ASSCs (Lindemann, 1996; Miyamoto *et al.*, 2000). We investigated the effects of amiloride on the responses to NaCl not only in the frog GL, but also in the MBF and PN (branches of the facial nerve). Our results show that amiloride does not affect the responses to NaCl in these nerves. The findings suggest that ASSCs in apical membrane of taste cells are not involved in salt taste transduction in frogs and that an amiloride-insensitive sodium pathway is the main contributor to salt taste transduction.

The distribution of ASSCs in taste buds isolated from the oral cavity has been investigated in rats (Doolin and Gilbertson, 1996; Gilbertson and Fontenot, 1998), hamsters (Gilbertson and Fontenot, 1998) and mice (Miyamoto *et al.*, 1999) by patch clamp recording. In rats, ASSCs were found in roughly two-thirds of fungiform taste receptor cells, in one-third of foliate taste receptor cells and in one-third of palate taste receptor cells (Doolin and Gilbertson, 1996;

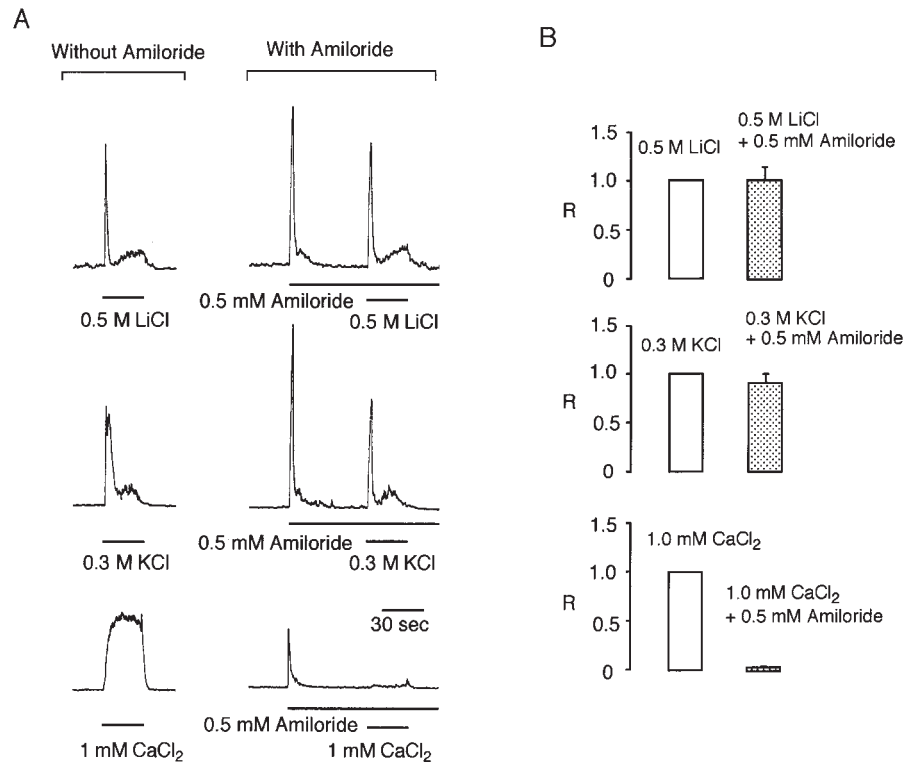


Figure 2 Effects of amiloride on the responses of the glossopharyngeal nerve to LiCl, KCl and CaCl₂. **(A)** Integrated responses to 0.5 M LiCl, 0.3 M KCl and 1.0 mM CaCl₂ with and without 0.5 mM amiloride. **(B)** Average response ratios of the tonic responses to 0.5 M LiCl ($n = 4$), 0.3 M KCl ($n = 6$) and 1.0 mM CaCl₂ ($n = 4$) with 0.5 mM amiloride. The response magnitudes were normalized relative to the magnitude of the response to 0.5 M LiCl, 0.3 M KCl and 1.0 mM CaCl₂ for LiCl, KCl and CaCl₂ stimulations, respectively.

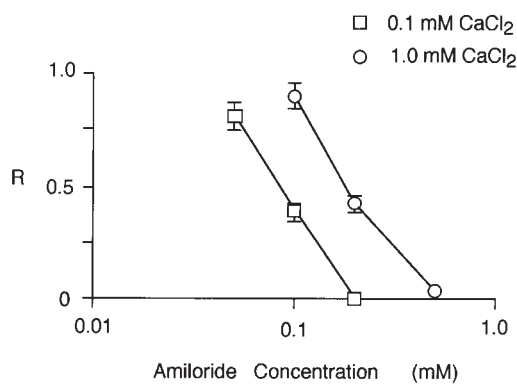


Figure 3 Inhibition by amiloride of the responses to 0.1 and 1.0 mM CaCl₂ in the glossopharyngeal nerve. A solution of 0.05 M NaCl was used as the adapting solution before amiloride treatment. In amiloride treatment, adapting solution with amiloride of various concentrations (0.05–0.5 mM) was applied to the tongue for 1 min prior to each test and then stimulating solution containing the same concentration of amiloride as that in the adapting solution was applied to the tongue. The magnitude of the tonic response to 1.0 mM CaCl₂ without amiloride treatment is taken as unity on the ordinate. The relative magnitude of the response (R) is plotted against the concentration of amiloride. Points and bars represent mean \pm SEM, $n = 4$.

Gilbertson and Fontenot, 1998). Fungiform and palate taste receptor cells and a part of foliate taste receptor cells are innervated by the facial nerve. On the other hand, no ASSCs

were found in the circumvallate taste receptor cells which are innervated by the GL (Doolin and Gilbertson, 1996). The pattern of the distribution of ASSCs in the oral cavity of rats suggests that the occurrence of ASSCs in the rat taste cells is linked with innervation by the facial nerve. In contrast, in frog taste cells ASSCs seem not to be linked with innervation by the facial nerve. That is, ASSCs were found in >50% of taste cells innervated by the GL (Avenet and Lindemann, 1988). Interestingly, in hamsters ASSCs were found in 68.4% of circumvallate taste receptor cells (Gilbertson and Fontenot, 1998). Hence, the distribution of ASSCs may not simply be linked with innervation by branches of the facial nerve.

For the amiloride-insensitive sodium pathway, Ye *et al.* (Ye *et al.*, 1993) proposed a model in which a flow of Na⁺ ions through tight junctions between taste cells stimulates the submucosal membrane of taste cells. The present results showed that the frog GL lacks amiloride sensitivity. Miyamoto *et al.* (Miyamoto *et al.*, 1989) reported that amiloride had no effect on the receptor potentials of taste cells induced by lingual application of NaCl solution in intracellular recordings in anesthetized frogs, suggesting that ASSCs are not present in the apical membrane of frog taste cells, but leaving a possibility that ASSCs exist in the vasolateral membrane of the cells. If this is the case, para-

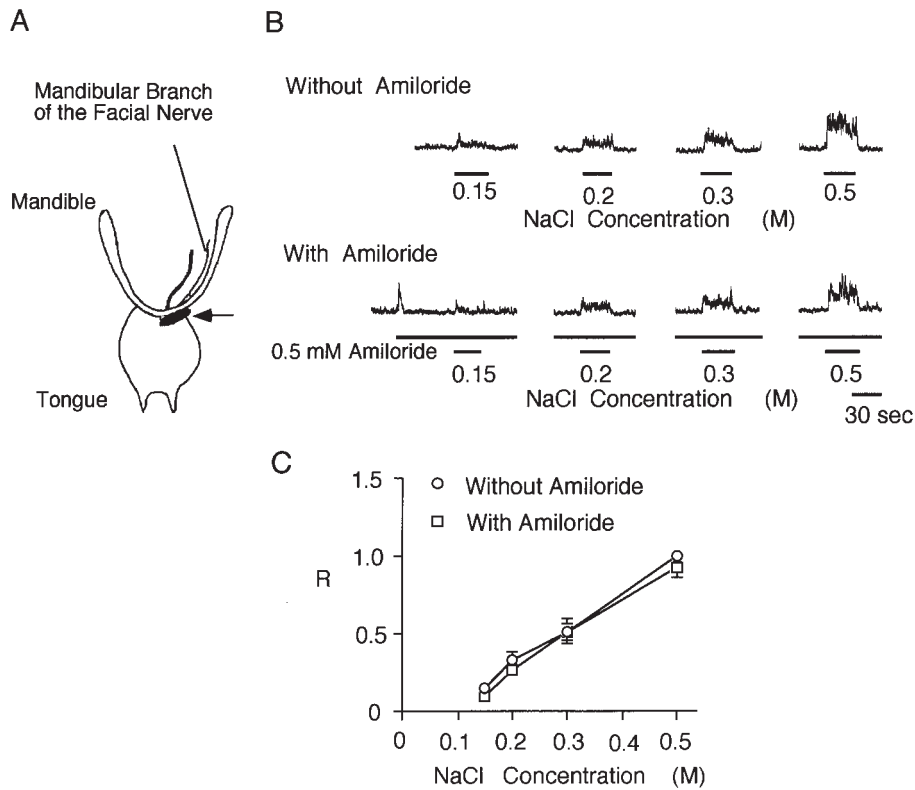


Figure 4 Effects of amiloride on the responses of the mandibular branch (MBF) of the facial nerve to NaCl. **(A)** The arrow represents small innervation area of one MBF (black area). **(B)** Integrated responses to 0.15–0.5 M NaCl with and without 0.5 mM amiloride. Note that the adapting solution containing 0.5 mM amiloride elicited a phasic response. **(C)** Average response ratios of the tonic responses to 0.15–0.5 M NaCl with and without 0.5 mM amiloride. The relative magnitude of the response (R) is plotted against the concentration of NaCl. The magnitude of the neural response to 0.5 M NaCl without amiloride is taken as unity on the ordinate. Points and bars represent mean \pm SEM, $n = 4$.

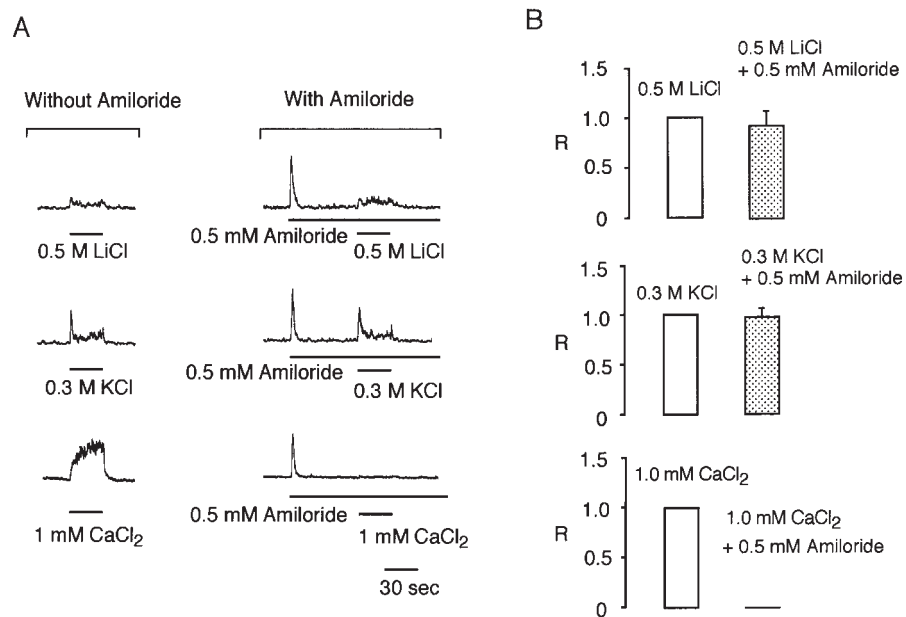


Figure 5 Effects of amiloride on the responses of the mandibular branch of the facial nerve to LiCl, KCl and CaCl₂. **(A)** Integrated responses to 0.5 M LiCl, 0.3 M KCl and 1.0 mM CaCl₂ with and without 0.5 mM amiloride. **(B)** Average response ratios of the tonic responses to 0.5 M LiCl ($n = 4$), 0.3 M KCl ($n = 4$) and 1.0 mM CaCl₂ ($n = 4$) with 0.5 mM amiloride. The response magnitudes were normalized relative to the magnitude of the response to 0.5 M LiCl, 0.3 M KCl and 1.0 mM CaCl₂ for LiCl, KCl and CaCl₂ stimulations, respectively.

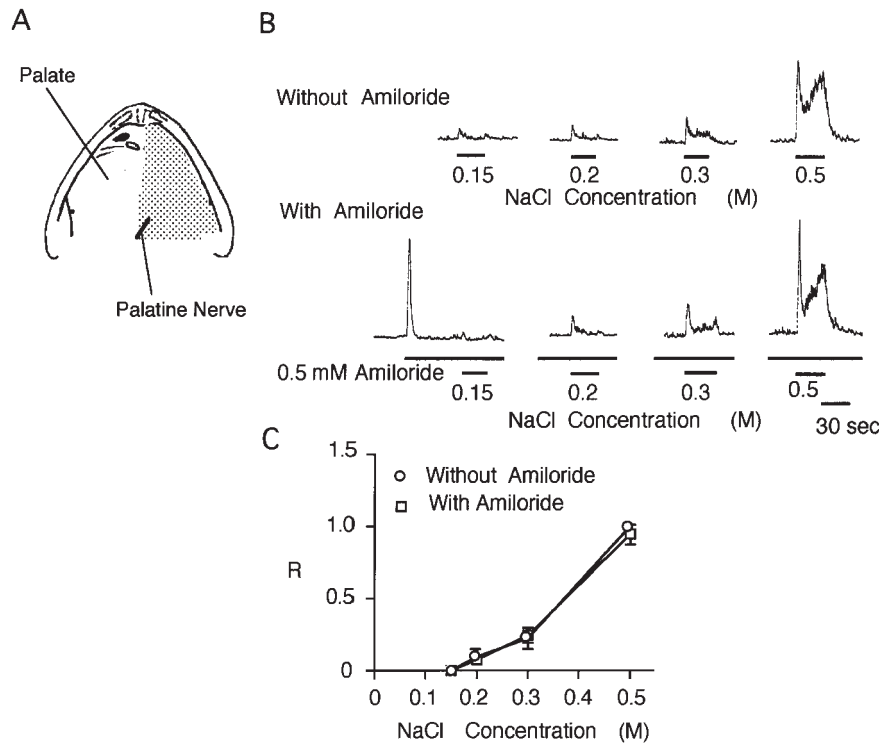


Figure 6 Effects of amiloride on the responses of the palatine branch (PN) of the facial nerve to NaCl. **(A)** Innervation area of one PN (dotted area) **(B)** Integrated responses to 0.15–0.5 M NaCl with and without 0.5 mM amiloride. Note that the adapting solution containing 0.5 mM amiloride elicited a phasic response. **(C)** Average response ratios of the tonic responses to 0.15–0.5 M NaCl with and without 0.5 mM amiloride. The magnitude of the neural response to 0.5 M NaCl without amiloride is taken as unity on the ordinate. The relative magnitude of the response (*R*) is plotted against the concentration of NaCl. Points and bars represent mean ± SEM, *n* = 4.

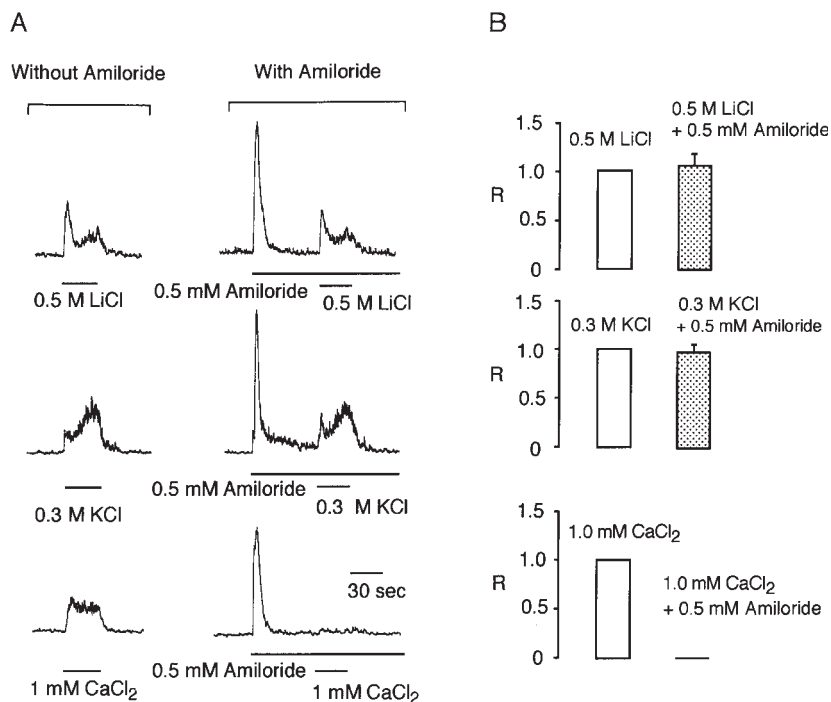


Figure 7 Effects of amiloride on the responses of the palatine branch of the facial nerve to LiCl, KCl and CaCl₂. **(A)** Integrated responses to 0.5 M LiCl, 0.3 M KCl and 1.0 mM CaCl₂ with and without 0.5 mM amiloride. **(B)** Average response ratios of the tonic responses of the PN to 0.5 M LiCl (*n* = 4), 0.3 M KCl (*n* = 4) and 1.0 mM CaCl₂ (*n* = 4) with 0.5 mM amiloride. The response magnitudes were normalized relative to the magnitude of the response to 0.5 M LiCl, 0.3 M KCl and 1.0 mM CaCl₂ for LiCl, KCl and CaCl₂ stimulations, respectively.

cellular transport of Na⁺ ions through tight junctions may contribute to sodium taste transduction in frogs. However, a large molecule of amiloride impermeable to the tight junctions cannot block the inflow of sodium ions through the submucosal ASSCs in taste cells. Therefore, this transduction pathway in the frog taste cells is amiloride-insensitive. There may be another amiloride-insensitive pathway. That is, Doolin and Gilbertson (Doolin and Gilbertson, 1996) reported that Na⁺ influx independent of ASSCs is important for apical and/or basolateral routes of Na⁺ entry into taste cells of the rat circumvallate taste buds. Thus, we cannot rule out the possibility that amiloride-insensitive Na⁺ currents in frog taste cells are responsible for the amiloride-insensitive component of neural responses to NaCl.

In the rat CT, responses to LiCl are partially inhibited by amiloride, as well as responses to NaCl (Brand *et al.*, 1985). Although responses of the rat CT to KCl are not affected by amiloride (Brand *et al.*, 1985), ASSCs in frog taste cells are somewhat permeable to K⁺ ions (Avenet and Lindemann, 1988). Therefore, it is important to examine effects of the responses to LiCl and KCl in the frog GL, MBF and PN. Our results showed that amiloride does not affect the responses of the three gustatory nerves to LiCl and KCl. The results suggest that ASSCs in the apical membrane of taste cells are not responsible for the responses to LiCl and KCl in the frog gustatory nerves.

Among the salts tested to date, Ca salts act as the most effective stimulus in the frog GL response (Zotterman, 1949; Kusano and Sato, 1957; Nomura and Sakada, 1965; Kitada, 1978). In the present study, amiloride at 0.5 mM almost completely inhibited the response to 1.0 mM CaCl₂. It has been reported that amiloride blocks the low threshold (T) Ca²⁺ channel in mouse neuroblastoma and chick dorsal root ganglion neurons (Tang *et al.*, 1988). However, the effect of amiloride on the response to 1.0 mM CaCl₂ in the frog gustatory nerve requires much higher concentrations (amiloride concentration of half-maximal inhibition ≈ 0.2 mM) than the blockade of the low threshold Ca²⁺ channel (amiloride concentration of half-maximal inhibition = 0.032 mM) (Tang *et al.*, 1988). There is no evidence for the presence of Ca²⁺ channel in frog taste cells so far (Avenet and Lindemann, 1987; Miyamoto *et al.*, 1991). It appears that the Ca²⁺ channel, as with that found in mouse neuroblastoma and chick dorsal root ganglion neurons, is not responsible for the response of the frog gustatory nerve to CaCl₂.

Kitada (Kitada, 1984, 1986) has found that low concentrations of pronase E inhibit the response to CaCl₂, but do not inhibit the response to NaCl and suggests that the calcium-receptor sites responsible for the response to Ca²⁺ may be a protein that is distinct from the sodium-receptor sites responsible for the response to Na⁺. Kitada (Kitada, 1978) found that all cations tested (Na⁺, K⁺, NH₄⁺, choline⁺, Mg²⁺, Ni²⁺, Co²⁺, Mn²⁺, La³⁺) inhibited the

response to CaCl₂. The inhibition of the response to Ca²⁺ by other cations is due to competition between Ca²⁺ and other cations for the same calcium-receptor sites (Kitada and Shimada, 1980). Therefore, the affinity of calcium-receptor sites for cations is not chemically specific, but charge-specific. Amiloride exists primarily as a monovalent cation with a positive charge in solution (Benos, 1982). Since the semilogarithmic concentration-inhibition curve of amiloride was shifted to the left as CaCl₂ was decreased (Figure 3), it is probable that amiloride inhibited the response to CaCl₂ in a competitive manner. Relatively high concentrations of amiloride (>0.1 mM) were required to inhibit the response to 1.0 mM CaCl₂ (Figure 3). It appears that amiloride does not act as a pharmacological agent, but simply acts as a cation. Complete inhibition of the response to CaCl₂ by amiloride suggests that calcium-receptor sites responsible for the response to CaCl₂ may reside in the apical membrane of frog taste cells.

In the mudpuppy, it has been reported that amiloride failed to inhibit the responses to NaCl in the GL (McPheeters and Roper, 1985). Since the present results showed that the responses of the frog gustatory nerves (GL, MBF and PN) to NaCl were not affected by amiloride, it is likely that ASSCs in the apical membrane are not associated with salt taste transduction in amphibians. Since the responses to NaCl in the mammalian facial nerve are sensitive to amiloride, the salt taste transduction mechanism in the frog facial nerve is different from that in the mammalian facial nerve.

Acknowledgements

This work was supported by grants-in-aid for High Performance Biomedical Materials Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Accepted August 17, 2001