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Efferent Fibers Innervate Gustatory and Mechanosensitive Afferent Fibers in Frog Fungiform Papillae

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Abstract

A possibility of efferent innervation of gustatory and mechanosensitive afferent fiber endings was studied in frog fungiform papillae with a suction electrode. The amplitude of antidromic impulses in a papillary afferent fiber induced by antidromically stimulating an afferent fiber of glossopharyngeal nerve (GPN) with low voltage pulses was inhibited for 40 s after the parasympathetic efferent fibers of GPN were stimulated orthodromically with high voltage pulses at 30 Hz for 10 s. This implies that electrical positivity of the outer surface of papillary afferent membrane was reduced by the efferent fiber-induced excitatory postsynaptic potential. The inhibition of afferent responses in the papillae was blocked by substance P receptor blocker, L-703,606, indicating that substance P is probably released from the efferent fiber terminals. Slow negative synaptic potential, which corresponded to a slow depolarizing synaptic potential, was extracellularly induced in papillary afferent terminals for 45 s by stimulating the parasympathetic efferent fibers of GPN with high voltage pulses at 30 Hz for 10 s. This synaptic potential was also blocked by L-703,606. These data indicate that papillary afferent fiber endings are innervated by parasympathetic efferent fibers.

Key words: efferent synapse, frog fungiform papilla, gustatory afferent fiber, parasympathetic efferent fiber, substance P

Introduction

Sensory cells in auditory, vestibular, and lateral line organs are innervated by afferent and efferent nerve fibers, and their afferent fibers are also innervated by efferent fibers (Smith and Sjöstrand 1961; Hama 1965; Hillman 1969; Nakajima and Wang 1974). Activity of the sensory cells and the afferent fibers are modulated by efferent fibers depending on changes in the physiological environment around the sensory organs (Fex 1962; Llinás and Precht 1969; Flock 1971; Furukawa 1981; Sewell and Starr 1991). Efferent innervation of taste receptor cells has been suggested histologically for a long time (Nomura et al. 1975; Jaeger and Hillman 1976; Yoshie et al. 1996; Reutter et al. 1997). In 2002, efferent synaptic potentials were first recorded from taste cells in frog fungiform papillae (Sato et al. 2002). Slow hyperpolarizing postsynaptic potentials corresponding to slow inhibitory postsynaptic potentials (IPSPs) are elicited in frog taste cells by stimulation of parasympathetic efferent nerve fibers (Sato et al. 2005). The receptor potentials in frog taste cells induced by gustatory stimuli are inhibited or enhanced by efferent stimulation (Sato et al. 2005, Sato, Nishishita, Mineda, et al. 2007, 2009). Slow depolarizing postsynaptic potentials are elicited in frog taste cells when lingual blood circulation declines (Sato et al. 2007a, 2007b).

There is a possibility that parasympathetic efferent fibers innervate the gustatory and mechanosensitive nerve fiber endings in the fungiform papillae in the frog. The purpose of the present experiment is to examine this possibility.

The taste disk as a frog taste organ is situated at the top of each fungiform papilla. Numerous fungiform papillae distribute on the whole dorsal surface of the tongue. The taste disk has 6 types of cells that are horizontally arranged to make 4 layers (Osculati and Sbarbati 1995; Li and Lindemann 2003). Single gustatory fibers in glossopharyngeal nerve (GPN) bifurcate several times near the fungiform papillae and innervate on average 6 fungiform papillae (Rapuzzi and Casella 1965; Hanamori et al. 1990). Chemical and electrical stimulation of one fungiform papilla induces both orthodromic neural impulses toward the central nervous system and antidromic neural impulses toward the other fungiform papillae (Sato et al. 1987). Because the diameter of taste

disks is as large as 100–300 μm in diameter, and the fungiform papillae have 5–10 myelinated afferent nerve fibers (Jaeger and Hillman 1976), frog taste organ is suitable for cell physiological and neurophysiological investigations.

Materials and methods

Preparation

All the experiments were carried out under a guidance for Animal Experimentation in Nagasaki University with approval of the Institutional Animal Care and Use Committee. Bullfrogs (*Rana catesbeiana*) weighing 370–620 g were used in spring and autumn. The animals were anesthetized by intraperitoneal injection of a 50% urethane in Ringer solution at a dose of 2 g/kg body weight. Both hypoglossal nerves were cut to remove spontaneous twitches of the tongue. Both GPNs were severed near the corpus of hyoid bone, separated from the connective tissues and immersed in paraffin oil. The tongue was pulled out and pinned on a cork plate. Before start of experiments atropine (a blocker of muscarinic acetylcholine [Ach] receptor) was intravenously injected at 1 mg/kg to completely block the large physicochemical junction potential generated between secreted saliva and lingual fluid when the GPN was strongly stimulated (Sato et al. 2000). All experiments were carried out during normal blood circulation for 4 h of the tongue (Sato et al. 2002, 2007b). The room temperature of laboratory was kept at 22–25 °C.

Intracellular recording of slow synaptic potential from taste cell (Method I)

Glass microelectrodes used were filled with a 3 M KCl and had a resistance of 30–60 MΩ. A microelectrode tip was slowly advanced into the taste disk of fungiform papilla to penetrate a taste cell of type III or type II cell located in deeper part of the disk (Figure 1A) (Osculati and Sbarbati 1995). The criteria for identifying the taste cell penetration was the same as in the previous work (Sato, Nishishita, Mineda, et al. 2007). Briefly, a signaling of taste cell penetration was an appearance of the resting membrane potential with 3 steps. An indifferent chlorided silver wire electrode was inserted into the forelimb muscles. Slow synaptic potentials recorded from taste cells were amplified with a microelectrode amplifier (Nihon Kohden MEZ 8101) and displayed on a pen recorder (Sato et al. 2002, 2004).

Autonomic efferent fibers in GPN were orthodromically stimulated with high voltage pulses at 30 Hz for 5 s to induce a slow hyperpolarizing synaptic potential (Sato et al. 2002, 2004). Stimulus duration was 0.1 ms in this and all other experiments. Threshold voltage for exciting unmyelinated autonomic efferent fibers in the fungiform papillae was evaluated by using a slow synaptic potential from a taste cell.

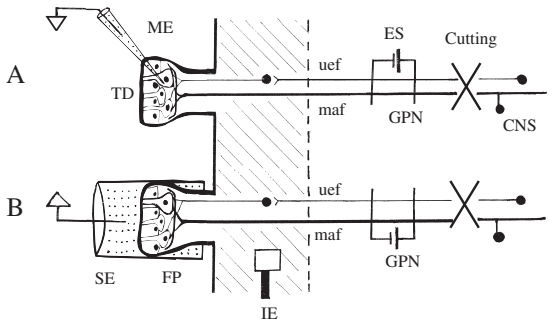

Figure 1 Schematic drawing of experimental methods. **(A)** Recording of slow hyperpolarizing synaptic potential from a taste cell (type III or type II cell) of taste disk of the fungiform papilla with a microelectrode while orthodromically stimulating high threshold–efferent fibers in GPN with 1–10 V pulses at 30 Hz for 5 s. (Method I). **(B)** Recording of antidromically conducting afferent spike potentials or extracellular slow synaptic potentials from the fungiform papilla with a suction electrode while stimulating afferent or efferent fibers of GPN with low (0.05–0.5) or high (1–10) V pulses at 30 Hz. (Method II or Method IV). ME, microelectrode; SE, suction electrode; IE, indifferent electrode; ES, electrical stimulator; FP, fungiform papilla; TD, taste disk (several type cells are shown); CNS, central nervous system; maf, myelinated afferent fiber; uef, unmyelinated efferent fiber.

Extracellular recording of antidromically conducting spike potential from papillary myelinated afferent fiber (Method II)

Antidromic stimulation of afferent fibers from papillae

Neural impulses resulting from antidromic stimulation of single afferent fibers from a papilla were recorded extracellularly with a suction electrode (Rapuzzi and Casella 1965; Sato et al. 1987) placed over a papilla and an indifferent silver wire electrode located on the dorsal surface of the tongue (Figure 1B). The suction electrode had a tip diameter of 180–280 μm and was filled with Frog Ringer solution. The suction electrode picked up electric activity at the node of Ranvier of afferent fiber inside the papilla and the indifferent electrode picked up the activity at the node underneath the papilla (Miyamoto et al. 1985). Recordings were made with a resistance–capacitance (R-C) coupled amplifier (Nihon Kohden AVB-10) with low and high signal filters of 50 Hz and 3 kHz. To establish the threshold for antidromic stimulation of afferent neurons, the intensity of single low voltage pulses applied to the GPN was gradually increased (0.05–0.5 V) while observing the amplitude and shape of action potentials with an oscilloscope.

A theory for estimation of synaptic activity at afferent fiber endings

As shown in Figure 1B, action potential of afferent axons elicited from stimulating electrodes lifting the cut GPN will travel antidromically to the papilla. Under resting conditions with a negative membrane potential, the outside of the axon will be positive relative to the cytosol along its length. When electrically stimulated, the segment of axon membrane

carrying action potential will have a reversal of the membrane potential with the outside negative to the inside. Thus, when the action potential is conducted antidromically, it will first pass over the indifferent electrode that will be negative relative to the suction electrode, resulting in recording a positive spike potential. When the action potential reaches the recording electrode in the papilla, it will record a negative potential to the indifferent electrode, resulting in an appearance of downward negative spike potential. If efferent fibers form synaptic connections with afferent endings in the papillae, they may produce excitatory postsynaptic potentials (EPSPs) in afferent fibers in which case the external membrane voltage will became less positive relative to the cytosol. Thus, the magnitude of the positive spike potential produced when an action potential is conducted antidromically over the indifferent electrode will be reduced, while the negative spike potential produced when the action potential reaches the suction electrode will be unaffected because the EPSPs do not spread in afferent fibers outside the papillae. Conversely, if the efferent fibers form synapses that produce IPSPs in the afferent fibers, the outside of afferent fibers in the papilla will become more positive relative to the cytosol. Therefore, the magnitude of the positive spike potential will be larger as the antidromic action potential carried by the afferent axon passes over the indifferent electrode.

Electrical integration of neural impulses recorded from papillary afferent fibers (Method III)

Papillary neural impulses were recorded according to Method II. In order to quantitatively analyze the time course of inhibition in the amplitude of papillary afferent impulses by parasympathetic efferent fibers, an integrator (Nihon Kohden, EI-601G) with a time constant of 1 s was used. Output of the integrator was proportional to the amplitude and frequency of pulses applied. When 0.5-ms pulses were applied to the input of integrator at various frequencies, the amplitude of integrated responses was proportionate to frequencies up to 1 kHz. When 0.5-ms pulses at 10 Hz were applied to the integrator at a range of 10 μV–10 mV, the amplitude of integrated responses was proportionate to the intensity of voltages given. The full sizes of positive and negative components of neural impulses were integrated. When antidromically conducting impulses of afferent fibers were evoked, the distal part of severed GPN was stimulated with 0.05–0.5 V at 30 Hz for ~50 s. On the other hand, when orthodromically conducting impulses of efferent fibers were evoked, efferent fibers of the GPN were stimulated with 1–10 V at 30 Hz for 10 s. In some experiments, efferent fibers were stimulated at a frequency of 1–100 Hz.

Extracellular recording of slow synaptic potential from taste disk and afferent terminal (Method IV)

Slow positive or slow negative efferent synaptic potentials were evoked in a taste disk or an afferent terminal of the papilla by orthodromically stimulating efferent fibers of GPN with high voltage pulses at 30 Hz for 10 s. The synaptic potentials were recorded from papillae sucked with a suction electrode filled with Ringer solution and amplified with a direct current (DC) amplifier (Nihon Kohden, MEZ 8101). An indifferent electrode was put in the lower mandible muscles in order to remove electrocardiograms. To remove the afferent fiber-induced neural spike potentials, high-frequency signals were filtered at 100 Hz. A slow positive synaptic potential was recorded from a taste disk of an intact papilla. On the other hand, after strongly breaking the bottom of the taste disk with a fine forceps, a slow negative synaptic potential was recorded from afferent terminals of a taste disk-broken papilla.

Chemical and mechanical stimulation

Whether a GPN-induced impulse in a papillary nerve fiber was gustatory or mechanosensitive was identified by chemically and mechanically stimulating the fungiform papillae around the sucked papilla. A small brush (length 5 mm, diameter 0.2 mm) and 5 taste stimuli of 0.5 M NaCl, 1 mM $CaCl_2$, 10 mM quinine-HCl (Q-HCl), 0.3 mM acetic acid, and 1 M sucrose were used. The brush was manually touched on the fungiform papillae. Chemical stimuli were flowed on the papillae at a rate of 0.5 mL/s through a syringe. The tongue surface was adapted to a Frog Ringer solution, which was composed of 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES (4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid) and adjusted to a pH of 7.2 by a Tris (tris(hydroxymethyl)aminomethane) solution.

Drugs

Tubocurarine chloride (a blocker of nicotinic ACh receptor), atropine sulfate, L-703,606 oxalate salt (a blocker of substance P receptor) (Sato et al. 2004, 2007a) and flufenamic acid (a blocker of nonselective cation channel; Hescheler and Schultz 1993) were used. All the drugs were purchased from Sigma-Aldrich Co.. Stock solutions of tubocurarine and flufenamic acid were prepared with ethanol. Stock solution of L-703,606 oxalate was prepared with methanol (Sato et al. 2004, 2005, 2007a, 2007b). These stock solutions were dissolved in Frog Ringer solution to make dilute drug solutions. The concentration finally prepared was 0.5 mg/mL for tubocurarine and 2.5 mg/mL for L-703,606 and flufenamic acid. Atropine was directly dissolved in Ringer solution and the concentration prepared was 0.5 mg/mL. All drugs were injected into precaval vein or postcaval vein.

Statistics

Data were expressed as means ± standard error of means. The level of significance was set at $P < 0.05$ with a Student's *t*-test.

Results

Difference in threshold of papillary afferent and efferent fibers

In the fungiform papillae, there are 2 types of nerve fibers: myelinated afferent and unmyelinated efferent fibers. We measured a threshold difference in the 2 types of papillary fibers to examine the synaptic interaction between them. Figure 2A,B show an example of a slow hyperpolarizing postsynaptic potential from a taste cell of the papilla (A) and a train of antidromically conducting impulses from 8 papillary afferent fibers (B) while measuring efferent and afferent thresholds of GPN with high and low voltage pulses. In these 2 recordings, the threshold was 5 V for an efferent fiber and a range of 0.06–0.5 V for 8 afferent fibers. Figure 2C,D shows histograms of thresholds of autonomic unmyelinated efferent fibers (C) and myelinated afferent fibers (D). The mean threshold of efferent and afferent fibers was 4.4 ± 0.4 V ($n = 32$) and 0.26 ± 0.11 V ($n = 49$). The mean threshold of the efferent fibers was 17 times larger than that of the afferent fibers.

Inhibition of papillary afferent fiber response by efferent fiber stimulation

Figure 3A shows the amplitude of spike potentials of a single papillary afferent fiber evoked by antidromically stimulating the GPN with a low voltage pulse (0.25 V) before and after high threshold–efferent fibers in the GPN were stimulated with high voltage pulses of 4.5 V at 30 Hz for 10 s (heavy bar). When high threshold–efferent fibers were strongly stimulated, low threshold–afferent fibers were simultaneously stimulated because of an impossible separation of each fiber type in GPN. Control total amplitude of the afferent spike potentials evoked before the efferent stimulation was inhibited by 20% at 5.1 s and 10% at 11.2 s after the end of the efferent stimulation. In the afferent spike potentials, the upper deflected positive spike component came from between a positive active suction electrode and a negative indifferent electrode, and the lower deflected negative spike component appeared between a negative active electrode and a positive indifferent electrode. The amplitude of positive spike potentials was inhibited by 28 ± 5% ($n = 6$) 5 s after 10-s efferent stimulation, whereas negative spikes barely changed (2 ± 2%, $n = 6$). Figure 3B illustrates a time course of inhibition of spike amplitude following 10-s efferent stimulation. The afferent inhibition lasted ~40 s after 10-s efferent stimulation. Spike potentials in an afferent fiber in Figure 3A were from a gustatory fiber because 0.5 M NaCl stimulation of the surrounding fungiform papillae induced the same large impulses as seen in the investing fiber in Figure 3A,C. No enhancement of papillary afferent fiber responses was observed by efferent stimulation of GPN at 30 Hz.

If spontaneous firing of taste cells and afferent fibers occurred orthodromically during recording from the papilla with a Ringer-filled suction electrode, the shapes of cell and afferent spike potentials would be monophasic (Avenet

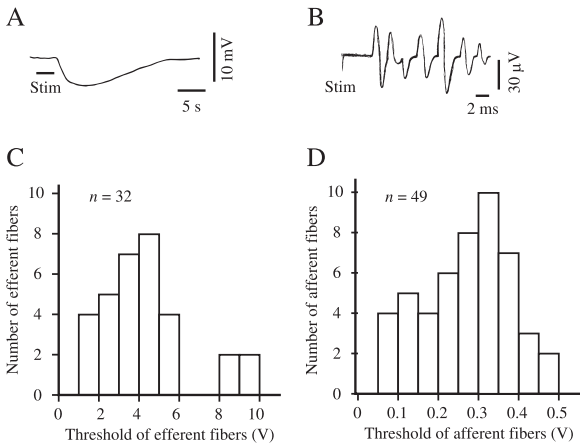

Figure 2 Difference in thresholds for exciting myelinated afferent and unmyelinated efferent fibers in fungiform papillae. **(A)** Slow hyperpolarizing synaptic potential recorded from a taste cell of a papilla by orthodromically stimulating high threshold–efferent fiber in GPN. A microelectrode was used. GPN efferent fibers were stimulated with pulses of 0.1 ms in duration and 5 V in intensity at 30 Hz for 5 s. (Method I). **(B)** Antidromically conducting spike potentials recorded from 8 afferent fibers in a papilla while antidromically stimulating low threshold–afferent fibers in GPN. A suction electrode was used. When this trace was taken, GPN afferent fibers were stimulated with 0.5 V. Stim means stimulation. (Method II). **(C)** and **(D)** Histograms of threshold voltages of papillary unmyelinated efferent fibers in GPN (C) and papillary myelinated afferent fibers in GPN (D).

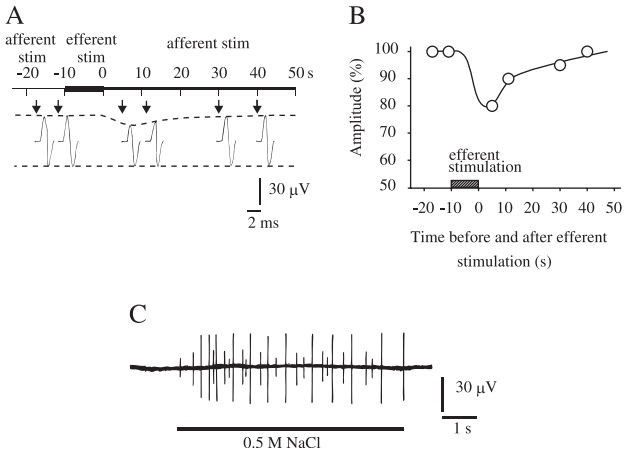

Figure 3 Inhibition of gustatory fiber spike potentials by efferent fiber stimulation. **(A)** Inhibition of amplitude of spike potentials antidromically conducting a papillary low threshold–afferent fiber elicited by antidromic afferent stimulation of GPN with 0.25 V immediately after 10-s stimulation of high threshold–efferent fibers in GPN with 4.5 V pulses at 30 Hz for 10 s (heavy bar). At arrows, the afferent fiber was stimulated with a single pulse (0.1 ms, 0.25 V). (Method II). **(B)** Time course of the amplitude of afferent spike potentials inhibited by efferent stimulation of GPN in data of Figure 3A. **(C)** Gustatory impulses conducted antidromically along 3 branched fibers during 0.5 M NaCl stimulation of the fungiform papillae around the sucked papilla used in the study of Figure 3A (Method II).

and Lindemann 1991) and negative-positive going biphasic (Miyamoto et al. 1985), respectively. These shapes of the orthodromic action potentials were quite different from GPN-induced antidromically conducting afferent spike potentials (Figure 3A).

In order to quantitatively analyze an inhibition process of afferent spikes, neural impulses of papillary fibers were integrated with an integrator of 1-s time constant. Figure 4A shows an integrated afferent fiber response induced by antidromic stimulation of a single low threshold–afferent fiber in GPN at 30 Hz. The amplitude of integrated responses remained constant during antidromic stimulation with low voltages. Immediately after high threshold–efferent fibers were stimulated at 30 Hz for 10 s (thick bar), an integrated afferent response elicited by antidromic stimulation with low voltage at 30 Hz was inhibited in amplitude for ~40 s (Figure 4B). This is clearly seen from a slow increase of integrated response compared with a rapid rise in control (Figure 4A). Initial 10-s strong stimulation (designated efferent stimulation) of small diameter-efferent fibers induced a large response of large diameter-afferent fibers. In integrated responses, a component of electrical stimulus artifacts was included (Figure 4C). This must be subtracted to obtain a real afferent response as shown in Figure 4D. The subtraction was carried out manually. Of 12 papillary afferent fibers examined, 7 fibers were gustatory and 5 fibers were mechanosensitive. To identify fiber types, the tongue surface around the suction electrode sucking a papilla was stimulated with taste stimuli and a small brush. Of 7 gustatory fibers, 6 fibers were identified by responsiveness to NaCl. The remaining one fiber did not respond to NaCl but responded to Q-HCl and acetic acid. When an investing fiber did not respond to 5 types of taste stimuli, a mechanical stimulus was applied to the tongue to identify the mechanosensitive fiber. The responsiveness of gustatory fibers to mechanical stimuli (Hanamori et al. 1990) was not analyzed. The amplitude of integrated responses in afferent fibers 5 s after 10-s efferent stimulation at 30 Hz was inhibited by 27 ± 6% in gustatory fibers (n = 7) (Figure 4D) and by 25 ± 7% in mechanosensitive fibers (n = 5). No difference existed between inhibition rates in the 2 types of fibers ($P > 0.05$). The magnitude of inhibition in afferent responses was dependent on the frequency of efferent stimulation (Figure 5).

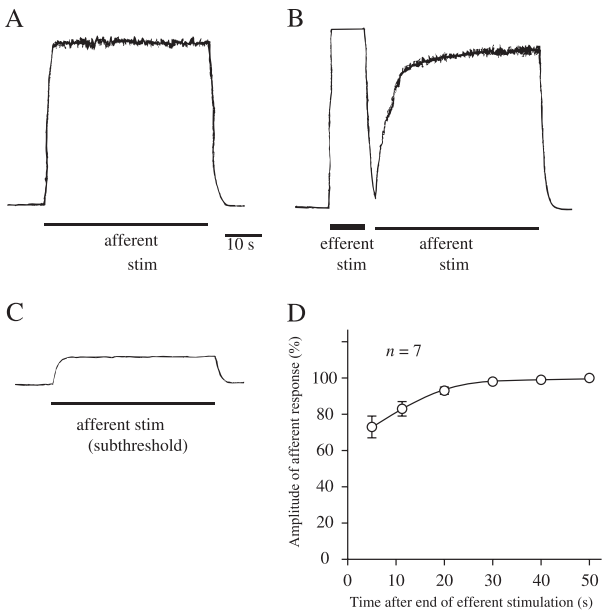

Figure 4 Inhibition of amplitude of integrated response of gustatory afferent spike potentials. **(A)** Control integrated response of antidromically conducting spike potentials in a low threshold–gustatory afferent fiber of the fungiform papilla induced by antidromic stimulation of GPN with 0.32 V pulses at 30 Hz. **(B)** Inhibition of an integrated response of antidromically conducting afferent spike potentials after orthodromic 30-Hz stimulation of high threshold–efferent fibers in GPN with 6.1 V pulses for 10 s (thick bar). Initial integrated response during 10-s strong efferent stimulation scaled out. **(C)** Integrated response of stimulus artifacts when afferent fiber of CPN was stimulated antidromically with just-subthreshold intensity at 30 Hz. **(D)** Mean time course of inhibition of integrated gustatory fiber responses induced by antidromic afferent fiber stimulation of GPN with low voltage pulses (0.2–0.4 V) at 30 Hz for 50 s after orthodromic 10-s stimulation of efferent fibers of CPN with 6–7 V pulses at 30 Hz. The amplitude of control afferent response without efferent stimulation was taken as 100%. Seven gustatory fibers were tested. (All data, Method III).

Effect of tubocurarine on efferent-induced inhibition of afferent response

In order to block nicotinic ACh receptors in autonomic ganglia, tubocurarine was used. Figure 6A shows a control inhibition of integrated spike potential response in a single papillary afferent fiber after the efferent fibers in GPN were stimulated at 30 Hz for 10 s. The afferent fiber responded to gustatory stimuli of 0.5 M NaCl and 0.3 mM acetic acid. Figure 6B illustrates an integrated response of the same afferent fiber after 10-s strong GPN stimulation (efferent stimulation) at 30 Hz in the tubocurarine-injected tongue (1 mg/kg). This record was taken 15 min after the drug

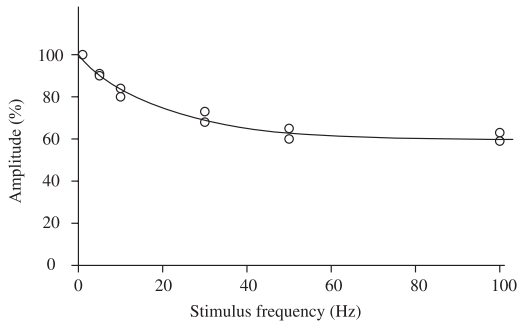

Figure 5 Effect of frequency of efferent fiber stimulation on inhibition of integrated papillary afferent responses. Efferent fibers of GPN were orthodromically stimulated with 6 V pulses at 1–100 Hz for 10 s and then an afferent fiber of GPN was antidromically stimulated with 0.23 V pulses at 30 Hz for 50 s. Neural impulses were recorded with a suction electrode. Ordinate indicates the amplitude of integrated afferent responses measured 5 s after end of efferent stimulation. (Method III).

injection. The inhibition of afferent response did not occur following the strong efferent stimulation. This implies that parasympathetic ganglia located near the fungiform papillae were blocked by tubocurarine and strong stimulation of parasympathetic preganglionic fibers included in the distal part of transected GPN (Figure 1) became ineffective in inhibiting the afferent activity. Figure 6C shows the percent of inhibition in the amplitudes of gustatory and mechanosensitive fiber responses induced by low-threshold afferent stimulation 5 s after end of 10-s efferent stimulation, before and after tubocurarine was injected at 1 mg/kg. The inhibition of afferent responses was 27% in both gustatory (5) and mechanosensitive (5) fibers before application of tubocurarine and was 0% in both fibers after application of the drug. No difference was found between both afferent fiber responses ($P > 0.05$). In these experiments, control and test data before and after the drug injection were obtained from different fungiform papillae of the same frogs. In a tubocurarine-treated tongue in which parasympathetic preganglionic fiber activity was blocked, the amplitude of action potentials of a single papillary afferent fiber induced by strong afferent stimulation (9.8 V) of GPN at 30 Hz was the same as that induced by weak stimulation (0.4 V) of GPN (Figure 6Da,b). When 7 afferent fibers included in a papilla of tubocurarine-treated tongue were all excited for ~60 s by strong stimulation of afferent fibers in GPN at 30 Hz, the amplitude of the integrated response never changed (Figure 6Dc). These data indicate that low threshold–afferent fibers in GPN were not inactivated when high threshold–parasympathetic efferent fibers in GPN were stimulated by high voltages (<10 V) of 0.1 ms in duration.

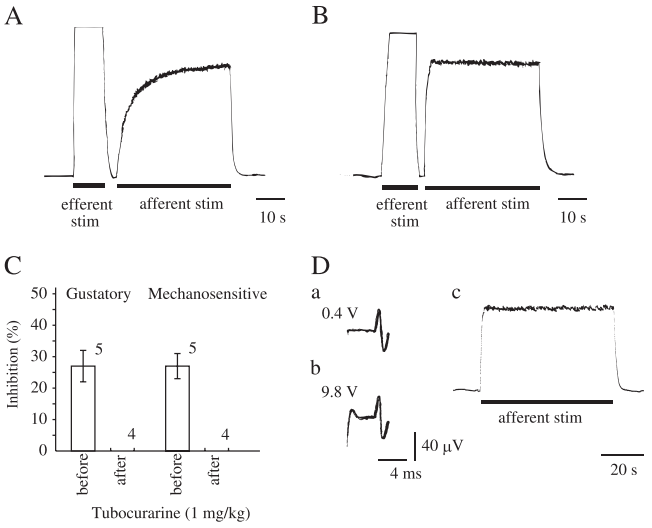

Figure 6 Effect of tubocurarine on efferent fiber stimulation inhibiting integrated afferent fiber response. **(A)** Control effect of 10-s efferent fiber stimulation of GPN on the following antidromically conducting afferent fiber response before injection of tubocurarine. Initial 10-s strong efferent stimulation was applied to GPN with 7.2 V at 30 Hz. The following afferent stimulation was done with 0.23 V at 30 Hz. **(B)** At 15 min after intravenous injection of tubocurarine at 1 mg/kg, the effect of 10-s strong efferent stimulation on antidromically conducting afferent fiber response. In (A) and (B), the integrated afferent responses were obtained from the same afferent fiber. Initial 10-s large response induced by strong efferent stimulation of GPN scaled out. **(C)** Percent of inhibition of gustatory and mechanosensitive fiber responses induced by antidromic GPN stimulation with 0.15–0.34 V at 30 Hz for 50 s 5 s after 10-s strong orthodromic efferent stimulation of GPN with 5–10 V at 30 Hz. Data were obtained before and after application of tubocurarine at 1 ng/kg. Numeral above each column is number of afferent fibers tested in this Figure and Figures 7 and 8. Data of control (before) and test (after drug) were obtained from different papillae of the same frog. Three frogs were used in obtaining data of (C). **(D) (a)** and **(b)** Action potentials of a single papillary afferent fiber induced by antidromic GPN afferent stimulation with 0.4 V (a) and 9.8 V (b) in tubocurarine-treated tongue. Five action potentials were superimposed in each trace. **(c)** Integrated response of papillary 7 afferent fibers evoked by antidromic GPN stimulation with 9.2 V at 30 Hz in tubocurarine-treated tongue. (Method III excepting (a) and (b) of D [Method II]).

Effects of L-703,606 and flufenamic acid on efferent-induced inhibition of afferent response

A substance P receptor blocker, L-703,606, was intravenously injected at a dose of 5 mg/kg. As shown in Figure 7A, an efferent fiber-induced inhibition of gustatory spike potentials in the fungiform papilla was gradually recovered during 15 min after the drug injection. In either gustatory or mechanosensitive fibers, the percent of inhibition of papillary afferent fibers 5 s after 10-s efferent stimulation was 26–27% before the drug injection (Figure 7B). There was no difference between both fibers ($P > 0.05$). After the drug injection, no inhibition of the amplitude of afferent spike potentials was induced by strong efferent stimulation at 30 Hz.

After a blocker of nonselective cation channels, flufenamic acid (Sato et al. 2004) was intravenously injected at 5 mg/kg,

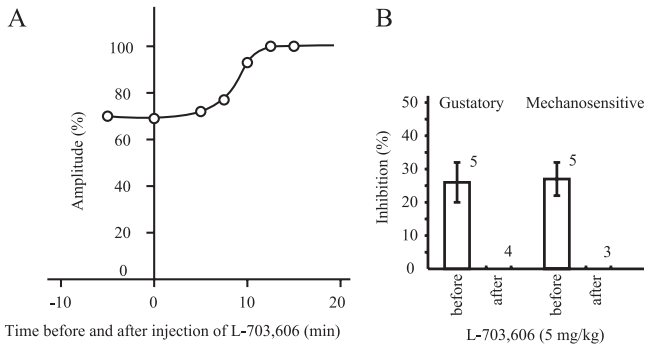

Figure 7 Effect of L-703,606 on efferent fiber stimulation inhibiting afferent fiber response in the fungiform papilla. **(A)** Recovery of antidromic papillary afferent response inhibited by orthodromic efferent fiber stimulation in GPN with 7 V at 30 Hz for 10 s after L-703,606 injection at 5 mg/kg. Ordinate denotes the amplitude of antidromically conducting papillary afferent fiber responses 5 s after the end of efferent stimulation. Afferent fiber of GPN was antidromically stimulated with 0.25 V at 30 Hz. At time 0, the drug was injected. **(B)** In gustatory and mechanosensitive fibers, percent of inhibition in the amplitude of antidromically conducting afferent responses 5 s after 10-s GPN efferent stimulation with 5–10 V at 30 Hz. Data of control (before) and test (after drug) were obtained from different papillae of the same frog. Four frogs were used. (Method III).

the amplitude of papillary afferent spike potentials inhibited by efferent stimulation at 30 Hz was gradually recovered during 15 min after the drug injection (Figure 8A). In either gustatory or mechanosensitive fibers, the percent of inhibition of papillary afferent responses induced by efferent stimulation at 30 Hz was 25–26% in the control and 0% after the drug injection (Figure 8B).

In Figures 6C, 7B, and 8B, of a total of 27 gustatory fibers examined 23 fibers were identified as gustatory ones by NaCl. Of the remaining 4 gustatory fibers which did not respond to NaCl, 2 fibers were identified by $CaCl_2$ and 2 fibers were identified by either acetic acid or Q-HCl. The responsiveness of the taste fibers to mechanical stimuli was not examined.

Recording of slow negative synaptic potential from papillary afferent ending

Figure 9A shows a typical slow positive synaptic potential recorded from an intact fungiform papilla with a suction electrode after efferent fibers of the GPN was orthodromically stimulated with high voltage pulses of 7 V at 30 Hz for 10 s. The mean time course of the synaptic potentials was as follows: a latency of 9.3 ± 0.8 s (n = 13), a rise time of 31.8 ± 3.5 s (n = 13), and a fall time of 89.5 ± 3.7 s (n = 13). The mean amplitude of slow positive synaptic potentials was 0.64 ± 0.06 mV (n = 29). This positive synaptic potential was mostly derived from an efferent synaptic potential recorded from whole taste cells of a taste disk in the papilla. Figure 9B illustrates a slow negative synaptic potential recorded from the whole afferent terminals of the papilla sucked with a suction electrode after the bottom of the taste disk was mechanically broken with a fine forceps. The efferent fibers of GPN was stimulated with 7 V at 30 Hz for 10 s. The time course of slow negative synaptic potentials was a latency of 5.9 ± 1.0 s (n = 16), a rise time of 9.9 ± 0.1 s (n = 16), and a fall time of 36.1 ± 2.3 s (n = 16). The mean amplitude of the slow negative synaptic potentials was 0.45 ± 0.05 mV (n = 24). This amplitude was a size of 70% of slow positive potential. It was possible to destruct some afferent fiber terminals when the bottom of the taste disk was mechanically broken.

Histograms of slow positive (C) and slow negative (D) synaptic potentials are shown in Figure 9C,D. These slow negative synaptic potentials from papillary afferent terminals may derive from intracellular slow depolarizing synaptic potentials generated in the afferent fiber terminals by the parasympathetic efferent stimulation of GPN. No slow positive synaptic potentials were recorded from papillary afferent terminals following efferent stimulation of GPN.

Figure 10A,B illustrate the relationships between stimulus intensity applied to GPN and slow negative potential (A) and between stimulus frequency applied to GPN and slow negative potentials (B). The slow negative synaptic potential recorded from afferent terminals of the papilla with a broken taste disk gradually increased with increasing stimulus intensity and frequency. Maximal slow negative synaptic potential was evoked by efferent stimulation of GPN at 30 Hz (A) and with 10 V (B). After a blocker of substance P receptors, L-703,606 was intravenously injected at a dose of 5 mg/kg, the amplitude of slow negative synaptic potentials evoked by

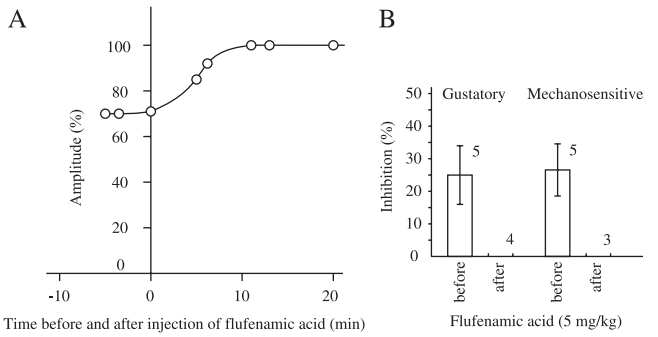

Figure 8 Effect of flufenamic acid on efferent fiber stimulation inhibiting afferent fiber response in the fungiform papilla. **(A)** Recovery of antidromically conducting afferent response inhibited by orthodromic 10-s efferent stimulation of GPN after flufenamic acid injection at 5 mg/kg. Ordinate denotes the amplitude of papillary afferent responses 5 s after end of efferent stimulation. Efferent fibers of GPN were initially stimulated with 7 V at 30 Hz for 10 s and then an afferent fiber was stimulated with 0.25 V at 30 Hz. At time 0, the drug was injected at 5 mg/kg. **(B)** In gustatory and mechanosensitive fibers, percent of afferent fiber inhibition 5 s after 10-s efferent stimulation of GPN. Efferent fibers were orthodromically stimulated with 5–10 V at 30 Hz for 10 s, and afferent fibers were antidromically stimulated with 0.25–0.35 V at 30 Hz for 50–65 s. Control and test data were obtained from different papillae of the same frog. Three frogs were used. (Method III).

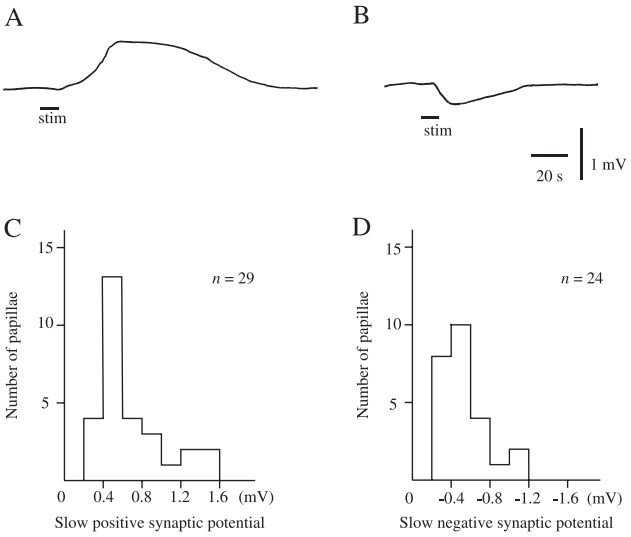

Figure 9 Slow synaptic potentials recorded from fungiform papillae. **(A)** Slow positive synaptic potential recorded from an intact papilla after orthodromic GPN efferent stimulation with 7 V at 30 Hz for 10 s. **(B)** Slow negative synaptic potential recorded from afferent fiber terminals in a papilla containing a broken taste disk. Situation of orthodromic GPN efferent stimulation was the same as (A). Synaptic potentials were recorded using suction electrodes. **(C)** and **(D)** Histograms of slow positive synaptic potentials (C) and of slow negative synaptic potentials (D). Ordinate denotes number of papillae tested. (Method IV).

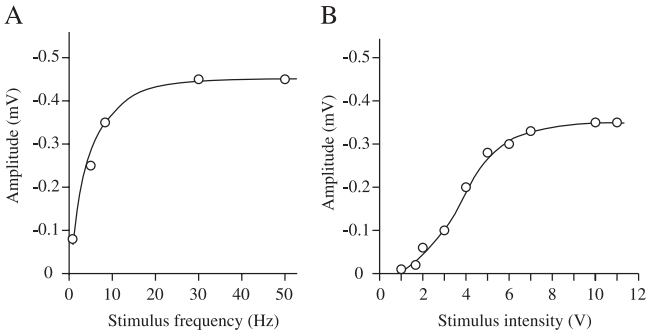

Figure 10 Effects of stimulus frequency and intensity applied to efferent fibers of GPN on slow negative synaptic potentials. **(A)** Relationship between stimulus frequency of GPN and amplitude of slow negative synaptic potential. Stimulus intensity and time were 7 V and 10 s. **(B)** Relationship between stimulus intensity of GPN and amplitude of slow negative synaptic potential. Stimulus frequency and time were 30 Hz and 10 s. Efferent fibers in GPN were orthodromically stimulated. In (A) and (B), each circle point is the mean of data from afferent terminals of 2 fungiform papillae with broken taste disks. (Method IV).

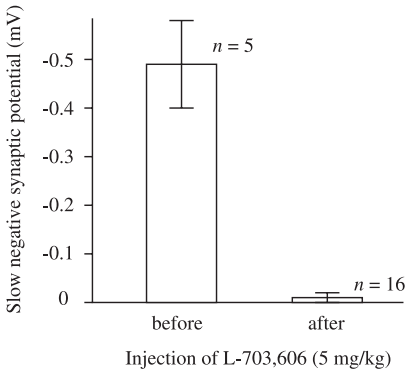

Figure 11 Effect of L-703,606 on slow negative synaptic potentials. Before application of the drug control, synaptic potentials were recorded from afferent nerve fiber terminals of 5 taste disk-broken fungiform papillae. After intravenous injection of the drug at 5 mg/kg, slow synaptic potentials were recorded from afferent terminals of 16 taste disk-broken papillae. Efferent fibers in GPN were orthodromically stimulated with 7 V at 30 Hz for 10 s. Control and test data were obtained from different papillae of the same frog. Two frogs were used. (Method IV).

GPN efferent stimulation was inhibited by 96% of the control (Figure 11), indicating that activation of the receptors at the afferent terminals is dependent on substance P from parasympathetic fibers.

Discussion

The possibility that frog taste cells are innervated by efferent fibers was clarified in 2002 by recording slow postsynaptic potentials from taste cells (Sato et al. 2002). When lingual circulation is normal, only slow hyperpolarizing postsynaptic potential regarded as slow IPSP is recorded by parasympathetic efferent stimulation at 30 Hz (Sato et al. 2005, 2007b). Synaptic substance released from the terminals of parasympathetic efferent fibers is supposed to be substance P because slow hyperpolarizing postsynaptic potentials are blocked by L-703,606 (Sato et al. 2004). It has been supposed that slow hyperpolarizing postsynaptic potential of taste cells is generated by inhibiting nonselective cation channels in the basal membrane of the cells (Sato et al. 2004). The amplitude and form of taste receptor potentials in frog taste cells are modulated in various degrees by parasympathetic efferent stimulation (Sato et al. 2005, Sato, Nishishita, Mineda, et al. 2007).

The present experiment suggests that efferent innervation exists in gustatory and mechanosensitive afferent fibers in fungiform papillae as in auditory and vestibular organs (Smith and Sjöstrand 1961; Hillman 1969). It was very difficult to intracellularly and extracellularly record synaptic potentials from the single endings of gustatory and mechanosensitive afferent fibers in the papillae. Therefore, at the first stage of the present experiment, we studied indirect evidence of existence of efferent synaptic activity on afferent fibers in the papillae. As shown in the recording method in Figure 1, if efferent activity did not exist in afferent fiber endings, afferent fiber spike potentials recorded with a suction electrode would not be modulated in amplitude. The decrease in spike potential size of gustatory and mechanosensitive fibers suggests an appearance of slow EPSPs by efferent fiber stimulation. If the spike potential size was increased by efferent stimulation, it is suggested that slow IPSPs would appear in afferent fiber endings. We could not find the increase of afferent spike potential. Therefore, we consider that slow EPSP alone is evoked in afferent axon endings of the papillae by parasympathetic efferent stimulation. After intravenous injection of tubocurarine, the efferent fiber stimulation did not inhibit the afferent spike size. This is due to blockage of parasympathetic ganglia located near the papillae (Sato et al. 2005). Therefore, stimulation of parasympathetic preganglionic fibers included in a stimulated GPN position does not induce impulses in the parasympathetic postganglionic fibers. Stimulation of the GPN induces directly impulses in sympathetic postganglionic fibers because sympathetic ganglia are located near the spinal cord (sympathetic nerve trunk) (Inoue et al. 1992). However, sympathetic response had no effect on the papillary afferent response. This implies that parasympathetic nerve fibers alone innervate the endings of papillary afferent fibers.

Intravenous injection of substance P receptor blocker, L-703,606 and nonselective cation channel blocker, flufenamic acid both inhibited the reduction of afferent impulse sizes induced by parasympathetic nerve stimulation. Therefore, as in parasympathetic efferent innervation on frog taste cells (Sato et al. 2004), it is suggested that substance P is released from the ending of parasympathetic postganglionic fibers and that cation channels are related to generation of slow synaptic potentials in afferent fiber of the papillae.

The afferent fibers in frog fungiform papillae are composed of gustatory (60%) and mechanosensitive (40%) fibers

(Hanamori et al. 1990). The time course of inhibition of papillary afferent responses induced by parasympathetic efferent stimulation did not differ between gustatory and mechanosensitive fibers. The blocking effects of L-703,606 and flufenamic acid on efferent-induced inhibition of afferent responses were the same in both types of afferent fibers (Figures 7 and 8). Therefore, the mechanisms generating slow EPSPs in afferent terminals by parasympathetic efferent activity might be the same in gustatory and mechanosensitive fibers.

The fungiform papillae of the bullfrog have one taste disk of 250 μm in diameter at the top. The myelinated afferent fibers run underneath the basement membrane of taste disk and lose the myelin sheath. The myelin sheath-lost axon frequently bifurcates and innervates several tens taste cells. The length of radially branched axons having a diameter of 0.5–2.5 μm is 20–120 μm (Graziadei and DeHan 1971; Düring and Andres 1976). There are 2 Ranvier nodes inside the fungiform papilla. The first node is located at the distance of 60 μm from the original point of myelin sheath (Miyamoto et al. 1985). When antidromically conducting afferent action potentials are recorded from the frog papilla with a suction electrode, the papillary action potentials are led off between the first node of Ranvier and the third node underneath the papilla (Miyamoto et al. 1985). The parasympathetic nerve-induced slow negative synaptic potential (corresponding to slow EPSP) might originally appear at some places of unmyelinated axons of 20–120 μm in length. The length constant (λ) of unmyelinated axons of 0.5–2.5 μm in diameter is calculated as 0.4–0.7 mm by using physiological values of the membrane resistance and internal resistance of dendrites of mammalian motoneurons (Rall 1959). This means that the distance where the original slow EPSP generated at a synaptic site is decreased to 1/e is 0.7 mm for 2.5-μm diameter axon and 0.4 mm for 0.5-μm dianeter axon. The maximal length from the end of longest unmyelinated axon to the first node of Ranvier in the papilla is 180 μm. Therefore, the original amplitude of slow EPSP generated at unmyelinated gustatory and mechanosensitive axon endings may maximally attenuate by 30% at the action potential-generating first node of myelinated afferent fiber in fungiform papilla. These data support that GPN-induced antidromically conducting afferent action potentials were inhibited in amplitude at the first node as shown in Figures 3–8. Gustatory and mechanical stimuli-induced synaptic potentials also may appear at the unmyelinated afferent axon endings. When both tastant (mechanical stimulus)-induced synaptic potential and parasympathetic nerve-induced slow synaptic potential appear simultaneously at unmyelinated axon endings, both types of synaptic potentials would be summated, resulting in an increase in the number of fired impulses in gustatory and mechanosensitive fibers. Taste nerve responses of cooled tongue in frogs greatly decrease (Yamashita 1964), so that an increment of gustatory and mechanosensitive neural responses by the activation of parasympathetic nerve may play an important role in enhancing the quality of life in poikilothermal frogs in low temperature seasons.

The time course (45 s) of slow external synaptic potentials recorded from whole afferent fiber terminals in a fungiform papilla using a suction electrode is similar to that of slow depolarizing synaptic potentials in frog taste cells elicited by stimulation of parasympathetic efferent fibers in blood circulation–declined frog (Sato et al. 2007a, 2007b). Probably, similar intracellular Ca^{2+}-activated nonselective cation channels might be involved in generation of slow negative synaptic potential in afferent endings of the papillae. It has been suggested that the prolonged time course of the slow synaptic potentials might be due to an involvement of metabotropic receptor, G-protein and several cell signal transducing proteins (Gwynne and Bornstein 2007, 2009; Sato et al. 2007b). In generation of 2 types of slow hyperpolarizing and slow depolarizing synaptic potentials in efferent synapses of frog taste cells, we have proposed that 2 second messengers, diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP_3) are mediated (Sato et al. 2007b). The slow hyperpolarizing synaptic potential might be elicited by closing one type of nonselective cation channels. The closing is due to phosphorylation of the channels by protein kinase C (PKC) through the DAG pathway. On the other hand, the slow depolarizing synaptic potential might be elicited by opening the intracellular Ca^{2+}-activated nonselective cation channels through IP_3 pathway (Sato et al. 2007b). The molecular mechanisms of slow type of synaptic potentials are still unknown in detail.

Electrical stimulation of frog parasympathetic nerve in GPN induces a slow IPSP in taste cells (Sato et al. 2002, 2004). The amplitude of 0.5 M NaCl-induced receptor potential during a slow IPSP is larger than that without the slow IPSP (Sato et al. 2005). This may result in the generation of larger 0.5 M NaCl response in frog taste nerve. The present study suggests that summation of tastant-induced EPSP and parasympathetic nerve-induced slow EPSP in frog gustatory fiber endings of the papillae may elicit larger gustatory neural responses. Presynaptic and postsynaptic action of parasympathetic efferent nerve in the frog taste organ in which taste cells make synapses with gustatory fibers is likely to be a mechanism of the enhancement of gustatory neural responses.

In conclusion, parasympathetic efferent fibers of frog GPN are suggested to innervate gustatory and mechanosensitive afferent fiber endings in the fungiform papillae.

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