ROLE OF NITRIC OXIDE AND DISTRIBUTION OF NITRIC OXIDE SYNTHASE IN THE GUSTATORY SYSTEM

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Abstract

미각계에서 산화질소의 역할과 산화질소 합성효소의 분포

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말초 미각계 및 중추 미각계에서 산화질소의 역할과 그것의 합성효소의 존재는 아직 규명되지 않고 있다. 본 연구는 말초미각계인 혀와 미각구심성신경 그리고 중추미각계인 뇌간고속핵에서 산화질소 합성효소의 분포 및 면역조직화학 방법과 고삭신경의 extracellular recording 뇌간고속핵 절편 whole cell patch 방법으로 조사하였다. 신경성 산화질소 합성효소는 혀의 전방에 위치한 심상유두와 유곽유두에 약하게 존재하였으며 미뢰주위와 결체조직에 존재하는 신경섬유 및 혀의 상피층에 풍부하게 존재하였다. 혀에 소금물을 가하여 증가된 고삭신경의 복합전위는 산화질소 유리제인 SNP에 의해 증가되었으며 내인성 산화질소 합성효소 억제제인 L-NAME와 soluble guanylate cyclase 억제제인 ODQ에 의해 억제되었다. 문측 연수에 존재한 문측 고속핵과 진전핵에서 nNOS가 풍부하게 존재하였다. 문측 고속핵의 신경들은 안정막전위가 -48±52mV였고 활동전위의 크기는 74±11mV였다. SNP에 의해 뇌간 고속핵 신경들이 탈분극되었으며 current clamp하였을 때 활동전압의 빈도가 증가하였다. 또한 SNP에 의한 문측 고속핵의 탈분극과 활동전압 빈도증가는 L-NAME와 ODQ에 의해 감소되었다.

이상의 실험결과는 산화질소 합성효소가 혀와 뇌간고속핵에 존재하며 여기서 유리된 내인성 산화질소가 말초성 및 중추성 미각기전에 관여하리라 사료된다.

Ⅰ. Introduction

Gustatory system is divided into peripheral gustatory system (PGS) and central gustatory system (CGS), in which PGS consists of taste bud mainly embeded in lingual papillae, and afferent taste nerves project to CGS. Taste buds are specialized neuroepithelial structures containing dark (type Ⅰ, Ⅳ) and light (type Ⅱ, Ⅲ) cells[1]. Taste buds in the rat are grouped into several populations: in fungiform papillae on the anterior portion of the tongue, in vallate and foliate papillae on the posterior tongue, in the epithelium of the nasoincisor ducts and "Geschmacks-streifen" of the palate, and on the laryngeal surface of the epiglottis[2-3]. They differ in their gustatory sensitivities, as shown by electrophysiological studies of the peripheral taste fibers.

In rats, chorda tympani nerve fibers innervating the fungiform

papillae are most sensitive to salts and acids[5]. The greater superficial petrosal nerve, which innervates taste buds on the palate, is most responsive to sweet-tasting stimuli[6,7]; and glossopharyngeal nerve fibers innervating the foliate and vallate taste buds respond well to acids and bitter-tasting stimuli[8]. However, the mechanism of taste receptor in response to various taste stimuli and synaptic relay between taste buds and afferent nerves are not well known.

CGS consists of nucleus tractus solitarius, thalamus, and primary taste sensory cortex. The rostral extent of the nucleus of the solitary tract (rNTS) is the site of termination of afferent gustatory fibers[9]. Morphological analysis of the gustatory zone of the rat rNTS shows that it contains at least three neuron types[10]. Fusiform neurons are characterized by an elongated soma and two large primary dendrites; multipolar neurons have a stellate-shaped soma and three to five primary dendrites; ovoid neurons have small soma and two or three primary dendrites. The small ovoid neurons connect locally in the medulla, whereas the multipolar and fusiform neurons project rostrally to the parabrachial nucleus[11]. Multipolar neurons are reportedly the predominant rostral projection neuron in the rat[12]. The transmitters used by afferent taste nerves to relay gustatory information from taste buds to second order neurons in the rNTS has been known to

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include GABA, substance P and glutamate.

Nitric oxide (NO) was first identified as a factor released by vascular endothelial cells, relaxing their underlying smooth muscle[13]. Subsequently, NO was reported to mediate the N-metyl-D-aspartate (NMDA)-induced increase in cGMP in the central nervous system (CNS)[14]. NO has been implicated in a wide range of physiological roles as a neurotransmitter mediating non-adrenergic, non-cholinergic transmission, synaptic plasticity and long-term potentiation related to the learning and memory[15]. NO is also known to be involved in neurocytotoxicity, hyperalgia, and in neurodegenerative diseases such as Alzheimer's disease[16-18].

NO is synthesized from the guanidino nitrogen of L-arginine by NO synthases (NOS) through a process that consumes five electrons and results in the formation of L-citrulline[13]. Three isoforms of NOS have been identified: neuronal NOS (nNOS, bNOS, type Ⅰ), inducible NOS (iNOS, type Ⅱ), and endothelial NOS (eNOS, type Ⅲ)[19-21]. As a gas, NO is able to freely cross cell membranes, giving rise to the notion that it acts as a retrograde messenger at synapses from which it is released and possibly at neighboring synapses as well. One of the best-documented targets of NO is an intracellular soluble guanylate cyclase, the enzyme that synthesizes cGMP[22]. cGMP can bind to at least three distinct classes of proteins. First, cGMP can directly gate channels. Cyclic nucleotide-gated channels have been found in both the PNS and CNS[23,24]. Second, cGMP can activate cGMP-dependent protein kinase (PKG)[25]. Finally, two subtypes of phosphodiesterases (PDE) are known to be regulated by cGMP: type Ⅱ PDE is stimulated by cGMP, whereas type Ⅲ PDE is inactivated[26]. However, the detailed action mechanisms of NO have not been elucidated in many tissues. Besides, existence of NOS in PGS and CGS and role of NO in taste signaling of the PGS have not been studied.

The present study was aimed to investigate the existence of NOS in the tongue and NTS and the role of NO in taste signaling in the PGS. The existence of nNOS was examined by immunohistochemistry and the activity of chorda tympani was assessed by extracellular recording.

Ⅱ. Materials and Methods

1. Extracellular recording of the chorda tympani

Rats (Sprague-Dawley) of either sex, weighing 200~250g, were used. They were anesthetized with sodium pentobarbital (I.P., 50mg/kg), and were secured in a head holder. The left chorda tympani nerve was exposed through a mandibular approach, cut near its entrance into the tympanic bullar, desheathed and placed on a platium wire electrode connected to the AC preamplifier (DAM 80, WPI. U.S.A.), with an indifferent electrode in nearby tissue. A mixture of vaseline and mineral oil (1:1) was filled around the nerve to prevent dehydration. Intergrated responses from the whole nerve were recorded on a curvilinear pen recorder (Grass physiograph) with the time constant set at 0.5 sec, monitored on an oscilloscope and an audiomonitor.

Chemicals used for taste stimuli were a series of concentrations ranging from 0.025 to 0.75M KCl, NaCl, NH$_4$Cl, and sucrose each dissolved in distilled water. Ten ml of each solution were slowly applied to the anterior tongue with a glass syringe. The tongue was rinsed with distilled water after 20 sec. At least one minute was allowed to elapse before application of another stimulus. The stability of the preparation was monitored with 0.1M NaCl as the control before and after applying a concentration series of each chemical.

The response magnitude at 10 sec after application of stimulus provided a "steady-state" (tonic) response (Fig. 1), in which a tractile component that might occur in the early portion of the response (phasic response) was reasonably avoided[27]. Therefore, a response was defined as an increase above baseline of the integrated neural activity and measured scale (mm) of pen recorder deflection at 10 sec following the stimulus onset.

To describe changes of the response to different taste stimuli as a function of concentration in each animal, a ratio of every response relative to 0.1M NaCl response was calculated. Therefore, a response magnitude equal to that elicited by stimulating the tongue with 0.1M NaCl had a ratio of 1.0.

At each concentration group, differences in the neural response of the taste stimuli were compared using Student t-test.

2. NOS immunohistochemistry

The rats were anesthetized with pentobarbital sodium (I.P., 50mg/kg) and perfused through the abdominal aorta with a solution of 4% paraformaldehyde in PBS (NaCl: 137mM, KCl: 2.7mM, Na2HPO4: 4.3mM, and KH$_2$PO$_4$: 1.4mM). Tongue and brain stem were removed and placed in the fixative solution for 2 h at 4℃ and then rinsed in PBS. They were dehydrated, paraffin-embedded and cut into 5μm sections. Each section was stained with H-E for the morphological characterization. Immunohistochemical staining was carried out using Vectastain Elite ABC Kit (Vector Lab., Burlingame, CA, U.S.A.). Purified monoclonal mouse anti-neuronal NOS (Transduction Lab., Lexington, KY, U.S.A.) were used as primary antibodies. The sections were deparaffinized with xylene and rinsed with PBS. Endogenous peroxidase was blocked by incubation in 3% H$_2$O$_2$ in water for 5 min. They were then incubated in 10% horse serum for 10 min to block non-specific reactions, and reacted in primary antibody diluted to 1:400 with 0.3% bovine serum albumin in PBS overnight. The tissues were washed with PBS and incubated in biotinylated anti-mouse IgG secondary anti-

body for 10 min. The sections were incubated in streptavidin/peroxidase complex for 5 min and developed with 3-amino-9-ethylcarbazole for 6 min. The sections were counterstained with Mayer's hematoxylin and mounted with canada balsam for photography in light microscope.

Ⅲ. Results

1. Distribution of nNOS in the tongue

Fungiform papillae were found on the anterior dorsal surface of the tongue (Fig. 1). Their superior and lateral sides were covered with squamous epithelium of which base was connected with the
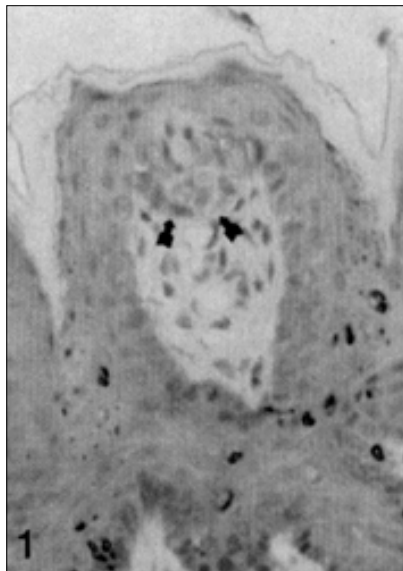

Fig. 1. Immunostaining with antiserum to neuronal nitric oxide synthase (nNOS) in the fungiform papillae. nNOS is demonstrated in the taste bud (arrows) and epithelial layer arounds the taste bud. (Mayer's hematoxylin stain, ×400)
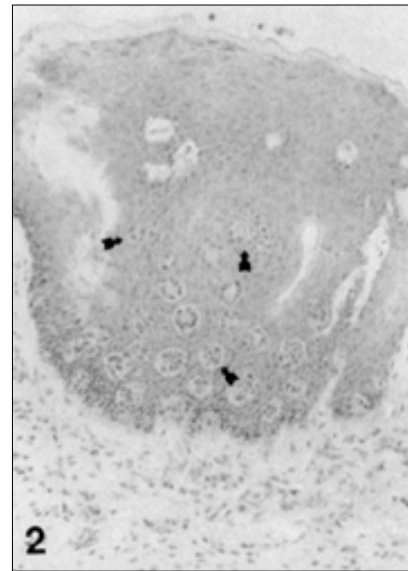

Fig. 2. Demonstration of nNOS in the circumvallate papillae. nNOS immunoreactivity was detected in the taste bud receptor cells (arrows) and nerve fibers adjacent to the taste buds. (Mayer's hematoxylin stain, ×150)
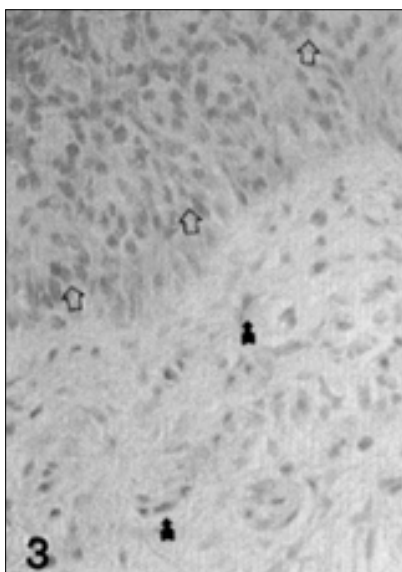

Fig. 3. Higher magnification of the taste buds and innervating nerves of the circumvallate papillae. Circumvallate taste buds may contain nNOS-positive cell (open arrows). Most nerves (block arrows) around the taste buds show the nNOS-positive immunoreactivity. (Mayer's hematoxylin stain, ×400)
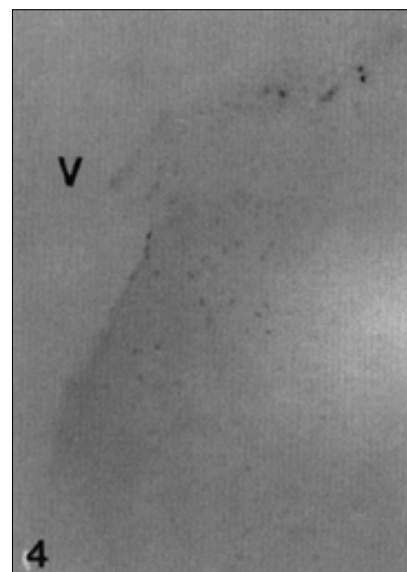

Fig. 4. Immunostaining with antiserum to nNOS in the nucleus tractus solitarios (NTS). nNOS is present along solitary tract from rostral to caudal rNTS. (Mayer's hematoxylin stain, ×200)

connective tissue. The taste buds having goblet shapes were always embedded on the superior side of the fungiform papillae, surrounded by the epithelium (Fig. 1). Various nerve fibers and blood vessels ran in the connective tissue and around the taste bud. The single circumvallate papilla having many taste buds was located at the junction of the oral and pharyngeal parts of the tongue (Fig. 2). However, foliate papillae were not observed in the tongue. The structures around circumvallate taste buds were similar to those of the fungiform taste buds, except the existence of minor salivary glands adjacent to the circumvallate taste buds. nNOS immunoreactivity was strongly observed in nerve fibers around taste buds, connective tissues, and lingual epithelial layer embedding taste buds. The taste receptor cell of the fungiform and circumvallate taste buds also showed nNOS-positive immunoreactivity (Figs. 1, 2, 3). Connective tissues and minor salivary glands did not show any immunoreactivity to nNOS

2. Localization of nNOS in the brain stem

The nucleus tractus solitarius (NTS) was cylindrically-shaped, and located close to the 4th ventricle in the dorsomedial medulla extending from the level of the gracile and cuneate nuclei in the caudal medulla to the facial motor nucleus rostrally. nNOS-immunoreactivity was strongly observed along the solitary tract extending from rostral to caudal medullae (Fig. 4).

3. Neural responses of the chorda tympani to salt and sugar stimuli

Integrated responses of the chorda tympani multifibers in one animal to a concentration series of various salts and sucrose are shown in Fig. 5. All responses were consisted of a sustained tonic phase with rapid onset upon application of the stimulus and rapid return to
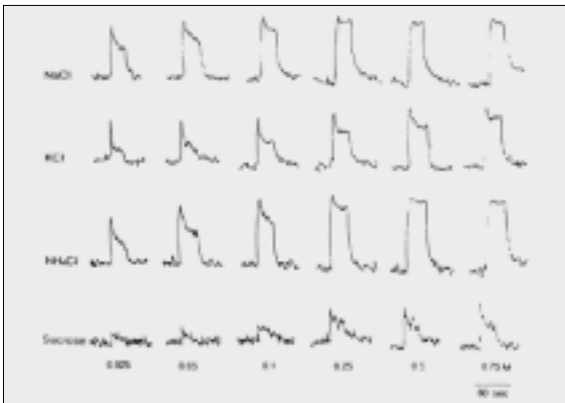

Fig. 5. Integrated neural responses recorded from the multifiber of chorda tympani nerve to varied concentrations of salt and sugar (sucrose) stimuli. Ten ml of each chemical was applied to the anterior tongue for 20 sec, and distilled water was continuously flowed to rinse the applied chemical.
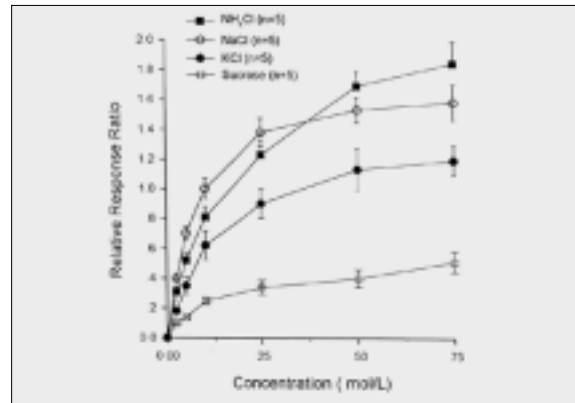

Fig. 6. Concentration-responses to NaCl, KCl, NH4Cl, and sucrose in the chorda tympani nerve, relative to 0.1M NaCl. A response magnitude equal to that elicited by 0.1M NaCl yields a ratio of 1.0.
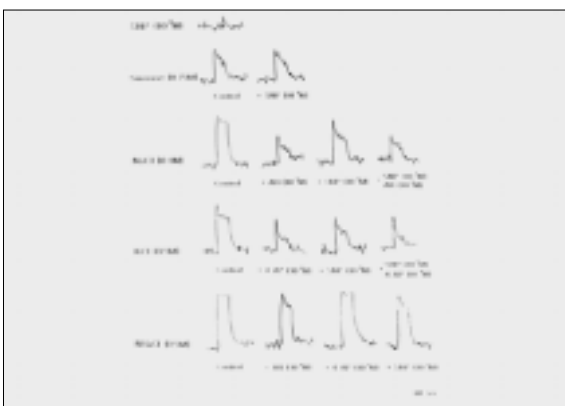

Fig. 7. Effects of sodium nitroprusside, amiloride, and 4-aminopyridine on the integrated neural responses to various salts and sucrose. Sodium nitroprusside (10^{-4}M), amiloride (10^{-4}M), and 4-aminopyridine (10^{-4}M) solutions were continuously flowed starting 5 min before application of the control solution to the tongue. SNP; sodium nitroprusside, AR; amiloride, 4-AP; 4-aminopyridine
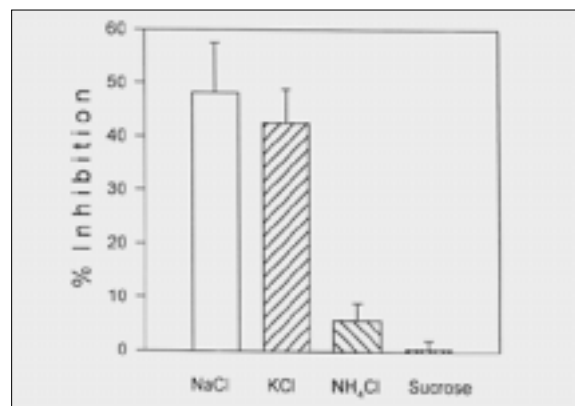

Fig. 8. Percent inhibiton of the chorda tympani responses to NaCl (0.1M), KCl (0.5M), NH4Cl (0.5M), and sucrose (0.75M) by SNP (10^{-4}M). The numbers of experiments are 5 each.
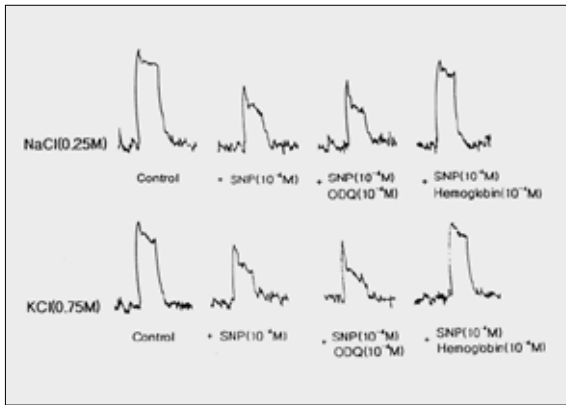
Fig. 9. Effects of ODQ or hemoglobin on the integrated chorda response to NaCl or KCl. SNP (10^{-4}M) solution was continuously flowed from 5 min prior to the application of NaCl or KCl solution to the tongue, and ODQ (10^{-4}M) or hemoglobin (10^{-4}M) was simultaneously added with NaCl or KCl solution.
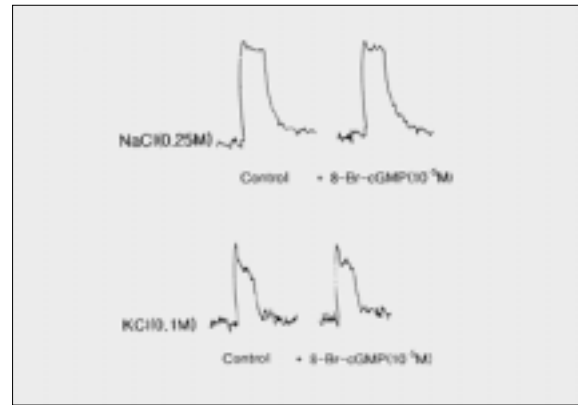

Fig. 10. Effects of 8-Br-cGMP on the NaCl- or KCl-elicited chorda response. 8-Br-cGMP (10^{-5}M) solution was continuously flowed from 5 min prior to the application of NaCl or KCl solution to the tongue.
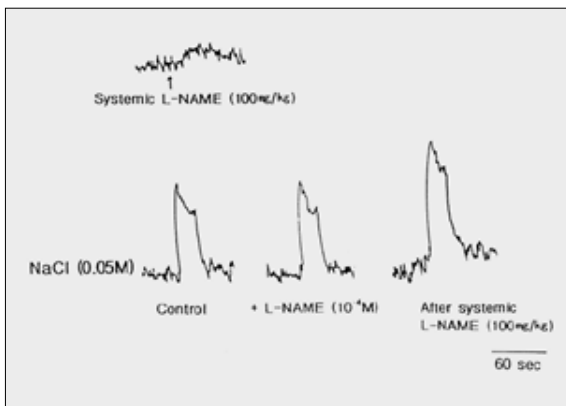

Fig. 11. Effects of L-NAME on the integrated chorda response to NaCl. NaCl solution (0.05M) was applied to the tongue at 5 min after the systemic administration of L-NAME (100㎎/㎏) or the local application of L-NAME (10^{-4}M) to the tongue.

baseline levels upon rinsing with continuous overflow of distilled water. Stimulation with various salt and sugar (sucrose) stimuli to the tongue elicited robust responses in a dose-dependent manner (Figs. 5, 6). The average response ratios for each chemical, relative to 0.1 M NaCl response, are shown in Fig. 6. The degree of magnitudes in response to the various taste stimuli ordered NaCl > NH₄Cl > KCl > sucrose (Figs. 5, 6).

4. Inhibitory effects of sodium nitroprusside on the chorda responses to various salt and sugar stimuli

Both phasic and tonic responses to NaCl (0.1M) were inhibited by amiloride (10^{-4}M), a Na⁺ channel blocker. Sodium nitroprusside (SNP, 10^{-4}M), a NO donor, also markedly inhibited the NaCl-elicited response, whereas it did not affect the inhibitory effect of amiloride on the NaCl-elicited response (Figs. 7, 8). 4-Aminopyridine (4-AP,

10^{-4}M), a potassium blocker, remarkably suppressed the KCl (0.5M)-elicited chorda response.

SNP (10^{-4}M) markedly depressed the KCl-elicited response, whereas it did not affect the effect of 4-AP on the KCl-elicited response (Figs. 7, 8).

The chorda response to NH₄Cl (0.5M) was not affected by 4-AP, but was slighlty decreased by amiloride (10^{-4}M) or SNP (10^{-4}M) (Figs. 7, 8). The response to sucrose was not affected by SNP (Figs. 7, 8).

The degree of inhibition on salt-elicited responses by SNP was in the order of NaCl > KCl > NH₄Cl (Fig. 8).

5. Effects of ODQ, hemoglobin, 8-Br-cGMP, and L-NAME on the inhibitory effects of SNP

Effects of soluble guanylate cyclase inhibitor on the inhibitory effects of SNP to the salts-elicited neural responses were examined to investigate whether NO may function through activating soluble guanylate cyclase or not.

The blocking effect of SNP on the NaCl- or KCl-elicited response was not attenuated by ODQ (10^{-4}M), a soluble guanylate cyclase inhibitor, whereas it was attenuated by hemoglobin, a NOS scavenger (Fig. 9).

In additon, the chorda tympani response to NaCl (0.25M) or KCl (0.1M) was not affected by 8-Br-cGMP (10^{-5}M), a cell permeable and non-degraded cGMP (Fig. 10).

Systemic administration of L-NAME (100㎎/㎏), a NOS inhibitor, slightly increased the resting discharge of the chorda tympani nerve. NaCl-elicited chorda response was not affected by local application of L-NAME (10^{-4}M) to the tongue, whereas it was enhanced by systemic administration of L-NAME (100㎎/㎏) (Fig. 11).

Ⅳ. Discussion

Signal transduction in the PGS can be devided into two consecutive mechanisms: the taste receptor potential within taste bud cells resulting from direct contact with any chemicals applied to the taste pore, and the synaptic relay between taste bud cells and afferent taste nerves[28]. The generating mechanism of the receptor potential in the taste bud with basic taste senses (salty, sour, sweet, bitter) have not been elucidated. In the present study, the integrated chorda response to salt stumuli was greater than that to sugar, suggesting that the taste buds innervated by the chorda tympani nerve is more sensitive to salt stimuli than the sweet. In addition, amiloride, a sodium channel blocker, markedly inhibited the NaCl-elicited chorda response, and 4-AP, a potassium channel blocker, greatly depressed the KCl-elicited chorda response. The NH4Cl-elicited response was slighlty decreased by amiloride, but was not affected by 4-AP. These findings are in agreement with those previous investigators have reported[29-31], demonstrating that the receptor potential evoked by NaCl is mediated through an activation of the amiloride-sensitive Na^+ channel and that evoked by KCl is generated through an activation of the 4-AP-sensitive K^+ channel. However, the receptor potential evoked by NH_4Cl is only in part mediated through the amiloride-sensitive Na^+ channel activation and is in majority through other unknown pathways.

The synaptic transmission in the taste pathway between taste buds and afferent nerve fibers has not been clarified. Existence of various cell types within the taste bud has been shown by morphological analysis, revealing that there are synaptic interactions among the cells present within the taste bud. By using light and electron microscopic immunocytochemistry, various neurochemicals including serotonin, GABA, calcitonin gene-related peptide, substance P, vasoactive intestinal peptide and galanin were proposed as the candidates for neurotransmitter or neuromodulator of taste signaling in taste buds[32].

Recently, NO has been also implicated in a wide range of physiological roles as a neurotransmitter or neuromodulator in the nervous system[33]. Although NOS is present in the organs and tissues innervated by the autonomic nervous system, the role of NO system in taste signaling within taste buds has not been examined. Hu et al. reported that NADPH- d-positive nerve fibers are present within and outside the taste buds in the dog fungiform papillae using NADPH diaphorase histochemistry, a simple method to demonstrate NOS. However, they failed to find NOS in the taste receptor cells within taste buds. In the present study, nNOS was present in receptor cells within the taste buds and lingual epithelium as well as various nerve fibers around taste buds. These results suggest that NO plays a role in synaptic transmission between taste buds and afferent taste nerve fibers.

Effects of NO on neuronal activity are controversial: NO has an excitatory or an inhibitory influence in the nervous system. In vagal motoneurons, locus caeruleus and NTS, NO donors increase the neural firing rate, and NOS inhibitors reduce the excitatory effect of NDMA[34,35]. On the contrary, in the carotid body, rostral or caudal ventrolateral medulla and spinal cord, NO decreases the neural activity[36-38]. In the present study, SNP, a NO donor, inhibited the NaCl- or KCl-elicited chorda response, whereas it did not affect the inhibitory effect of amiloride or 4-AP on the NaCl- or KCl-elicited neural response. Hemoglobin, a NO scavenger, attenuated the inhibitory effect of SNP on NaCl- or KCl-elicited chorda response. However, SNP did not affect the chorda response to sucrose, but slightly decreased the NH_4Cl-elicited chorda response. These results suggest that NO has an inhibitory effect on the chorda response to salt stimuli through modulating ion channels, particulary amiloride-sensitive Na^+ channel and 4-AP-sensitive K^+ channel in the apical membrane of the taste bud receptor cells.

NO has been indeed known to modulate various ion channels in the other organ or tissues such as cardiovascular tissues, gastrointestinal tract, nervous system[39-41]. It inhibits sodium influx in the renal collecting duct directly by blocking amiloride-sensitive $Na+$ channel[42] and blocks calcium influx induced by glutamate in neurons[43]. In hippocampal slices, NO directly depresses the activity of voltage- and Ca^{2+}-dependent K^+ channels[44], whereas in the intestine and cardiac ventricular cell, it increases Cl^- channel activity[45,46]. In the present study, ODQ, an inhibitor of soluble guanylate cyclase, did not affect the inhibitory effect of SNP on NaCl- or KCl-elicited chorda response. Although it is generally known that NO acts through binding the heme of intracellular soluble guanylate cyclase, the enzyme that synthesized cGMP[22], NaCl- or KCl-elicited chorda response was not affected by 8-Br-cGMP, an undegradable cell permeable cGMP. It is suggested that NO has inhibitory effects on the chorda responses to various salt stimuli through modulating the amiloride-sensitive Na^+ channel or 4-AP-sensitive K^+ channel, but not through cGMP pathway.

However, it cannot be excluded that NO is involved in synaptic transmission between taste bud and afferent taste nerves, modulating the chorda response because NO has been recently reported to be involved in neurotransmitter release and uptake[47-49]. Releases of both acetylcholine and dopamine from nerve growth factor-treated PC-12 cells is blocked by NOS inhibitors, which is reversed by excess of L-arginine[50]. Furthermore, NO has been implicated in hippocampal long-term potentiation (LTP) and the NO-induced enhancement of neurotransmitter release facilitates the neurotransmission that accounts for LTP[51]. In the present experiment, systemic administration of L-NAME, a NOS inhibitor, increased the resting chorda nerve activity and NaCl-elicited chorda response, while local application of L-NAME to the tongue did not affect the chorda response to NaCl. These results support the possibility that NO is synthesized within the

taste bud but not in the epithelium near the taste pore, being involved in synaptic transmission between taste buds and afferent nerves. However, the exact mechanisms by which NO regulates the synaptic transmission have to be further defined.

In summary, NO plays a role in the salt taste signaling mechanisms in part through modulating the amiloride-sensitive Na^+ channel and 4-AP-sensitive K^+ channel in apical membrane of the taste buds. It is also suggested that NO has a role in regulating the synaptic transmission between taste buds and afferent nerves.

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