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Distribution of neuropeptide-containing nerve fibers in the salamander taste organs

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Summary. Salamander taste organs were recognized as oval cell clusters within the dorsal surface of the tongue. A moderate number of SP, CGRP, VIP, NPY, and GAL immunoreactive nerve fibers terminated in the cell clusters, and some of them penetrated into the basal half of the cell clusters. Around the glands, VIP, NPY, and GAL fibers were numerous, and SP and CGRP fibers were less numerous. Immunoreactivity of SOM and FMRF was not detected either in the nerve fibers associated with the cell clusters or those around the glands. These findings suggest that the chemosensory mechanisms of the salamander gustatory organs are under the control of peptidergic innervation. In addition, the present study indicates that the caudate taste organs are structurally primitive but functionally mature.

Key words: Taste organ, Neuropeptides, Immunohistochemistry, Salamander (Caudate)

Introduction

There is a distinct difference in the histological appearance in amphibian taste organs between anurans and caudates. In adult anurans, they are seen as epithelial discs on the free tops of the fungiform papillae (DeHan and Graziadei, 1971; Graziadei and DeHan, 1971). The epithelial discs, which correspond to the mammalian taste buds, are composed of three cell types, taste/sensory, supporting, and basal cells, and are surrounded by ciliated columnar cells. They are richly innervated (Rapuzzi and Casella, 1965), and the afferent nerve endings establish synaptic contact with the taste cells (Uga and Hama, 1967; Graziadei and DeHan, 1971; Stensaas, 1971). On the other hand, the taste organs in adult caudates are barrel-shaped intraepithelial cell complexes (Toyoshima and Shimamura, 1987; Toyoshima et al., 1987). These cell complexes are composed of the same three types of cells as the epithelial discs in anurans.

In the lingual papillae of adult anurans, several kinds of neuropeptide-containing nerve fibers are distributed, and some of them are associated with the epithelial discs (Hirata and Kanaseki, 1987; Kuramoto, 1988; Kusakabe et al., 1996a). More recently, Kusakabe et al. (1996b) demonstrated the ontogeny of the peptidergic fibers in the developing bullfrog tongue through metamorphosis and further development. According to this report, the neuropeptide-containing nerve fibers first appeared in association with cell clusters in the epithelial layer of the immature tongue at the metamorphic stage; stage XX of Taylor and Kollros (1946). Histologically, the taste organs in adult caudates greatly resemble the cell clusters in developing anuran tongues. Phylogenically the caudates are placed in a lower position than the anurans. Information as to whether peptide-containing fibers exist in association with the caudate taste organs would aid in understanding the significance of gustatory mechanisms from a phylogenical viewpoint. As far as we are aware, there is no information on peptidergic innervation in the gustatory organs of caudates. Therefore, in the present study, the occurrence and distribution of seven neuropeptides, i.e., substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY), galanin (GAL), somatostatin (SOM), and FMRFamide (FMRF) were examined in the caudate taste organs.

Materials and methods

Seven adult Tokyo salamanders, *Hynobius nebulosus* tokioensis, were used in this study. After anesthesia by immersion in a 1% aqueous solution of tricaine-methanosulfate (MS-222) for a few minutes, the thoracic cavity was opened to expose the heart. Through a thin nylon tube inserted into the ventricle, the animal was briefly perfused with heparinized (1 IU/ml) 0.1M

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phosphate buffer saline (PBS), pH 7.4, and then with freshly prepared Zamboni's fixative solution (4% paraformaldehyde, 0.2% picric acid in 0.1M PBS, pH 7.4) for 10 minutes. The tongues were transversely cut into small blocks, and immersed in the same fixative for an additional 8 hours at 4 °C. After a brief washing with PBS, the specimens were transferred to 30% sucrose in PBS, and kept there overnight at 4°C. The specimens were then sectioned serially at 15 μm on a cryostat, and mounted on poly-L-lysine-coated slides. Some sections were stained with hematoxylin and eosin for general histology.

The sections were processed for immunohistochemistry according to the peroxidase-antiperoxidase (PAP) method. Prior to PAP treatment, sections were dipped in a fresh 0.3% solution of hydrogen peroxide in methanol for 30 min at room temperature to inhibit endogenous peroxidase activity. After washing in several changes of 0.3% Triton-X in 0.1M PBS (PBST), the sections were treated for 1 h with a protein blocking agent (Immunon, USA) at room temperature to block nonspecific protein binding sites. Then they were incubated at 4 °C overnight with the primary antisera against the following peptides: SP (Cambridge, UK), CGRP (Cambridge, UK), VIP (Incstar, USA), NPY (Incstar, USA), GAL (Cambridge, UK), SOM (Incstar, USA), and FMRF (Incstar, USA). The antisera were diluted with 0.2% bovine serum albumin, 1% normal goat serum, and 0.2% sodium azide in PBST. After rinsing in several changes of PBST, the sections were transferred for 2 h to anti-rabbit IgG (Cappel, USA) at room temperature. Next the sections were rinsed with several changes of PBS, transferred for 2 h to rabbit PAP complex (Jacaon, USA) and rinsed in several changes of PBS. The peroxidase activity was demonstrated with 3,3'-diaminobenzidine. This immunostaining procedure has been detailed in a previous report (Kusakabe et al., 1991).

Primary antisera were preincubated with 50 μM of the respective peptide. The absorbed antiserum was used for incubation of the section followed by incubation with the secondary antiserum to test the specificity of the primary antiserum.

Results

In sections stained with hematoxylin and eosin, the dorsal surface of the salamander tongue was covered with stratified epithelium composed of surface squamous cells and inner cuboidal or columnar cells. Typical fungiform and filiform papillae, such as are seen in the anuran tongue, were not found. The taste organs were recognized as oval cell clusters within the epithelium, and the taste/sensory and supporting cells extended throughout almost the entire thickness of the epithelium (Fig. 1). Many glands were located in the lamina propria (Fig. 1).

Immunoreactivity of SP (Fig. 2a), CGRP (Fig. 2b), VIP (Fig. 2c,d), NPY (Fig. 2e), and GAL (Fig. 2f) was recognized in the nerve fibers ascending from the base to the cell clusters, and in those distributed around glands (Fig. 2g,h) and blood vessels in the lamina propria of the tongue. Within the epithelium, there were no immunoreactive fibers except for those associated with the cell clusters. The immunoreactive fibers appeared as thin processes with many varicosities. There were some differences in the distribution and abundance of peptide-containing fibers. A moderate number of SP (Fig. 2a), CGRP (Fig. 2b), VIP (Fig. 2c,d), NPY (Fig. 2e), and GAL (Fig. 2f) immunoreactive fibers terminated in the cell clusters. In transverse sections through the middle of the cell clusters, some fibers penetrated into the basal half of the cell clusters, and some VIP and GAL fibers extended to the apical half of the cell clusters (Fig. 2c,e). Around the glands, VIP (Fig. 2g), NPY (Fig. 2h), and GAL fibers were numerous, and SP and CGRP fibers were less numerous. VIP, NPY, and GAL fibers were moderately abundant around blood vessels. Immuno-

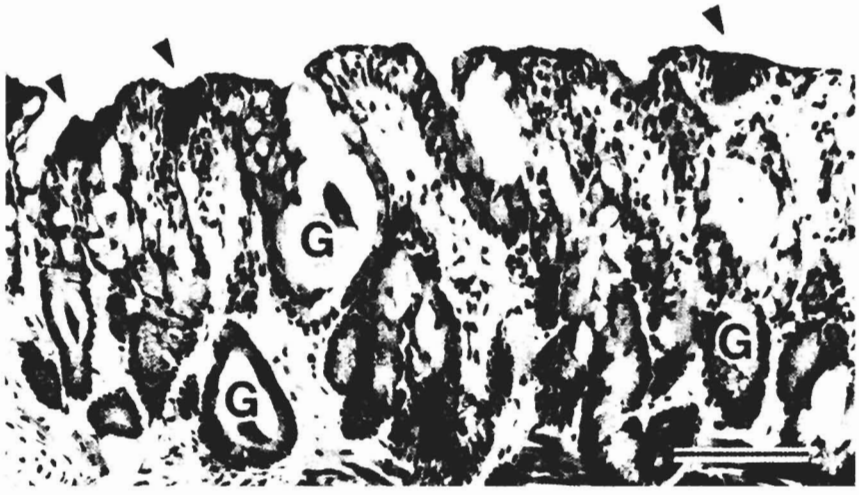

Fig. 1. Hematoxylin-eosin-stained section of salamander tongue. Three arrowheads indicate the cell clusters located within the epithelium. G: glands. Scale bar: 200 μm.

reactivity of SOM and FMRF was not detected either in the nerve fibers running from the base to the cell clusters, or in those around the glands and blood vessels. The occurrence of immunoreactivity for the seven neuropeptides is summarized in Table 1. No immunoreactivity for the five neuropeptides was detected in the sections incubated with preabsorbed antisera.

Table 1. Distribution and relative abundance of immunoreactive nerve fibers in the salamander tongue.

	SP	CGRP	VIP	NPY	GAL	SOM	FMRF
Epithelium							
in stratified epithelium	-	-	-	-	-	-	-
within cell cluster	++	++	++	++	++	-	-
Glands	+	+	+++	+++	+++	-	-
Blood vessels	+	+	++	++	+	-	-

Grading of frequency of immunoreactive fibers: -, absent; +, few; ++, moderate number; +++, many.

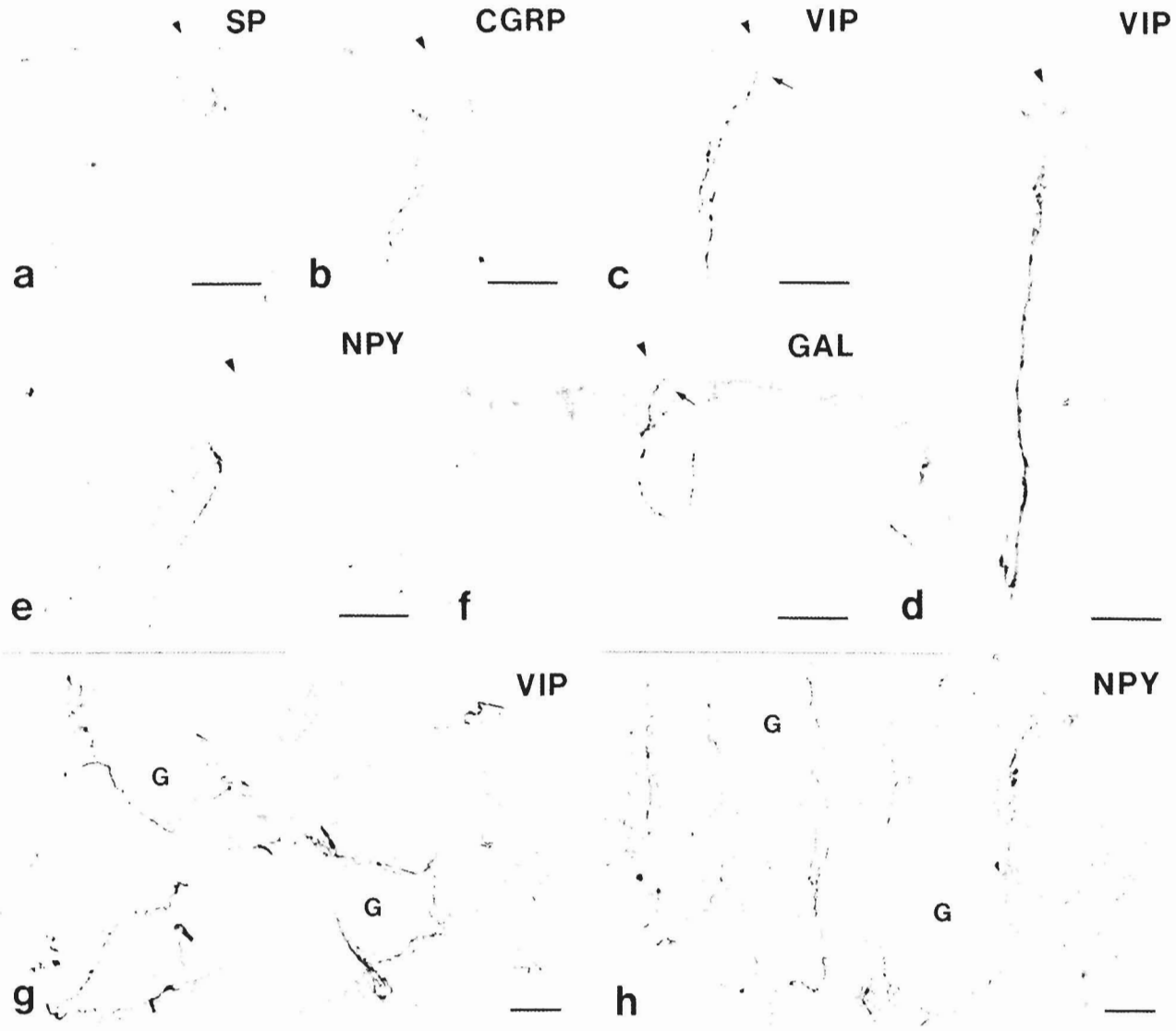

Fig. 2. SP (**a**), CGRP (**b**), VIP (**c, d**), NPY (**e**), and GAL (**f**) immunoreactive nerve fibers associated with the cell clusters (arrowheads), and VIP (**g**) and NPY (**h**) fibers around the glands (G). Arrows in figure 2c and 2f indicate the VIP and GAL fibers penetrating into the apical half of the cell clusters. Scale bars: 50 μm.

Discussion

In spite of the distribution of a number of neuropeptide-containing nerve fibers in the lingual papillae of a variety of vertebrates (Lundberg et al., 1979; Nagy et al., 1982; Baecker et al., 1983; Finger, 1986; Terengi et al., 1986; Luts et al., 1990; Montavon et al., 1991), the functional role of regulatory neuropeptides in the gustatory mechanisms remains to be elucidated. Concerning the participation in the anuran taste reception, we recently surmised that the chemoreceptor function of the bullfrog gustatory organ may be under the control of complicated peptidergic innervation (Kusakabe et al., 1996a), because Finger et al. (1986) suggested possible modulation of the taste bud sensitivity by the perigemmal SP-containing fibers in the rat lingual papillae. In the fine structure of the salamander taste organs, the light cells, which are considered to be gustatory transducer cells, have numerous dense-cored vesicles of 140-180 nm in diameter and synaptic vesicles of 60-80 nm in diameter in the basal cytoplasm, and they tend to accumulate in the cytoplasm facing the junction with nerve fibers (Toyoshima et al., 1987). In the present study, SP, CGRP, VIP, NPY, and GAL immunoreactive fibers terminated at the base of the cell clusters, and some of them penetrated into the apical half of the cell clusters. Considering together with the ultrastructural characteristics of the light cells, the peptidergic fibers associated with the salamander taste organs may be involved in the modulation of taste transmission through the synaptic contacts. To clarify this, it is necessary to perform immunohistochemistry at the electron microscopic level.

Although there was no physiological study on the caudate taste organs, tactile and sensory maturation of the anuran larvae tongue have been studied electrophysiologically (Honda et al., 1992). According to this report, the afferent discharges generated in the glossopharyngeal nerve were first recorded in response to both tactile and chemical stimuli of the tongue rudiments at the metamorphic stage; stage XX of Taylor and Kollros (1946). As mentioned above, the histological appearance of salamander taste organs closely resembles the cell clusters in developing bullfrog tongues at stage XX. More recently, the ontogeny of regulatory neuropeptides in the bullfrog taste organs has also been studied immunohistochemically (Kusakabe et al., 1996b). According to this study, only CGRP immunoreactive fibers appeared in association with the oval cell clusters (immature taste organs) at stage XX, although as the stages progress, SP, VIP, and GAL fibers appear within the primitive epithelial discs. In the cell clusters of the salamanders, however, in addition to CGRP fibers, SP, VIP, NPY, and GAL fibers were also observed. The caudates are classified as phylogenetically lower than the anurans. That is, the oval cell clusters in caudates should be more primitive than the epithelial discs in anurans. Nevertheless the neuropeptide-containing nerve fibers which are detected in the anuran taste organs in adults could be found. This indicates that the cell clusters in caudates are structurally primitive but functionally mature.

In conclusion, the taste mechanisms in both anurans and caudates are under the control of peptidergic innervation, although there is a definite structural difference in these two taxa.

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References

Baecker B., Yanaihara N. and Forssmann W.G. (1983). VIP innervation of the tongue in vertebrates. Anat. Embryol. 167, 173-189.

DeHan R.S. and Graziadei P.P.C. (1971). Functional anatomy of frog's taste organs. Experientia 27, 823-826.

Finger T.E. (1986). Peptide immunohistochemistry demonstrates multiple classes of perigemmal nerve fibers in the circumvallate papilla of the rat. Chem. Senses 11, 135-144.

Graziadei P.P.C. and DeHan R.S. (1971). The ultrastructure of frog's taste organs. Acta Anat. 80, 563-603.

Hirata K. and Kanaseki T. (1987). Substance P-like immunoreactive fibers in the frog taste organs. Experientia 43, 386-389.

Honda E., Hirakawa T., Nakamura S. and Nakahara S. (1992). Tactile and taste sensory maturation of the frog larvae tongue. Brain Res. 595, 74-78.

Kuramoto H. (1988). An immunohistochemical study of cellular and nervous elements in the taste organ of the bullfrog, *Rana catesbeiana*. Arch. Histol. Cytol. 51, 205-221.

Kusakabe T., Anglade P. and Tsuji S. (1991). Localization of substance P, CGRP, VIP, neuropeptide Y, and somatostatin immunoreactive nerve fibers in the carotid labyrinths of some amphibian species. Histochemistry 96, 255-260.

Kusakabe T., Kawakami T., Ono M., Sawada H. and Takenaka T. (1996a). Distribution of peptidergic fibers in bullfrog lingual papillae demonstrated by a combination of double immunofluorescence labelling and a multiple dye filter. Histochem. J. 28, 189-197.

Kusakabe T., Matsuda H., Kawakami T., Syoui M., Kurihara K., Takenaka T. and Sawada H. (1996b). Ontogeny of regulatory neuropeptides in the bullfrog taste organ. Dev. Brain Res. 96, 285-289.

Lundberg J.M., Hökfelt T., Anggard A., Pernow B. and Emson P. (1979). Immunohistochemical evidence for substance P immunoreactive nerve fibers in the taste buds of the cat. Acta Physiol. Scand. 107, 389-391.

Luts A., Montavon P., Lindstrad K. and Sundler F. (1990). Peptide-containing nerve fibers in the circumvallate papillae. Regul. Peptides 27, 209-226.

Montavon P., Lindstrad K., Luts A. and Sundler F. (1991). Peptide-containing nerve fibers in the fungiform papillae of pigs and rats. Regul. Peptides 32, 141-150.

Nagy J.I., Goedert M., Hunt A. and Bond A. (1982). The nature of substance P-containing nerve fibers in taste papillae of the rat tongue. Neuroscience 7, 3137-3151.

Rapuzzi G. and Casella C. (1965). Innervation of the fungiform papillae in the frog tongue. J. Neurophysiol. 28, 154-165.

Stensaas L.J. (1971). The fine structure of the fungiform papillae and epithelium of the tongue of South American toad, *Calyptocephalella gayi*. Am. J. Anat. 131, 443-462.

Taylor A.C. and Kollros J.J. (1946). Stages in the normal development of *Rana pipiens* larvae. Anat. Rec. 9, 7-25.

Terenghi G., Polak M., Rodrigo J., Mulderry P.K. and Bloom S. (1986). Calcitonin gene-related peptide-immunoreactive nerves in the tongue, epiglotis and pharynx of the rat: occurrence, distribution and origin. Brain Res. 365, 1-14.

Toyoshima K. and Shimamura A. (1987). Monoamine-containing basal cells in the taste buds of the newt *Tritrus pyrrhogaster*. Arch. Oral. Biol. 32, 619-621.

Toyoshima K., Miyamoto K. and Shimamura A. (1987). Fine structure of taste buds in the tongue, palatal mucosa and gill arch of the axolotl, *Ambystoma mexicanum*. Okajima Folia Anat. Jpn. 64, 99-110.

Uga S. and Hama K. (1967). Electron microscopic study on the synaptic region of the taste organ of carps and frogs. J. Electron Microsc. 16, 269-276.

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