An Immunohistochemical Study of Cellular and Nervous Elements in the Taste Organ of the Bullfrog, *Rana catesbeiana*

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Summary. The cellular and nervous elements of the bullfrog taste organ were examined by immunohistochemical methods using various antibodies.

The immunoreactivity for spot 35 protein, a soluble protein isolated from bovine cerebellum, was found in numerous taste cells located at the middle or slightly lower levels within the gustatory cell layer. The immunoreactive cells possessed cytoplasmic processes rising upward the free surface and also issued branched processes to the base of the epithelium. The immunoreaction for spot 35 protein was found diffusely throughout the cytoplasm from the apical to the basal parts of the taste cells.

NSE-immunoreactive taste cells were located at the upper or middle levels within the gustatory cell layer in the taste organ. The fact that the cells were smaller in number and size than spot 35 protein-reactive cells and further differed in localization distinguished the NSE-taste cells from the spot 35 protein cells.

Serotonin-like immunoreactivity was detectable in the basal cells localized at the base of the taste epithelium. The immunoreactive cells were arranged in a circle at the periphery of the taste organ, each extending a slender process toward the center. The terminal portion of this process spread leaf-like; numerous fine projections protruded from its margin. The serotonin-immunoreactive cells appear to coincide with the monoamine-containing basal cells, which have been previously reported.

Substance P-, calcitonin gene-related peptide (CGRP)-, vasoactive intestinal polypeptide (VIP)-, peptide HI (PHI)- or gastrin releasing peptide (GRP)-immunoreactive nerve fibers with varicosities were demonstrated within the taste organ. Some substance P-fibers ran along the bottom of the taste organ epithelium. A few thinner substance P-fibers ascended among the epithelial cells of the organ and terminated closely below the free surface. CGRP-fibers were found to correspond to substance P-fibers from their evidencing a double immunostaining. VIP- and PHI-fibers formed a meshwork in the basal area of the taste epithelium. Abundant substance P- and/or CGRP-fibers formed a meshwork among the ciliated cells located at the periphery of the taste organ. However, PHI- and GRP-fibers were detected less than substance P- and/or CGRP-fibers, though VIP-fibers were rarely present in the same region.

Neurofilament protein- or tyrosine hydroxylase-like immunoreactivities were found in thick nerve fibers in the taste organ, whereas no immunoreactivities were present in cellular elements within the taste organ.

The relationship between cellular and nervous elements in the taste organ was examined by double immunostainings. The substance P-fibers were closely related with both spot 35 protein- and serotonin-immunoreactive cells. In addition, VIP-fibers were found in connection with serotonin-immunoreactive cells.

From the present study, it is concluded that the bullfrog taste organ might be a chemo-mechanoreceptive sensory organ.

The taste organ of frogs is a disc-like structure composed of a thickened epithelium and underlying connective tissue rich in nerves and blood vessels. It is located at the top of the fungiform papillae, which are much smaller in number than the filiform papillae, another type of papillae on the bullfrog tongue surface. This organ has been long a favored material for electrophysiological studies of the taste transduction mechanisms (RAPUZZI and CASELLA, 1965; KASHIWAYANAGI et al., 1981; SATO et al., 1987). Morphological studies by light and electron microscopy have characterized the population of cells present in the taste organ (GRAZIADIEI and DEHAN, 1971; DURING and ANDRES, 1976; TOYOSHIMA et al., 1984). Especially, the ultrastructural features of frog taste cells
have been well described by DeHan and Graziaedi (1971, 1973) and Graziaedi and DeHan (1971). However, there seems to be no report demonstrating the taste cells of this organ by immunohistochemistry using a specific marker.

Spot 35 protein is a soluble protein first isolated from bovine cerebellum by Yoshida and Takahashi (1980). It was first demonstrated to be a Purkinje cell-specific substance (Yamakuni et al., 1984), but later immunohistochemical studies revealed that the immunoreactivity for this protein is also localized in other neuronal and paraneuronal cells, including small neurons in the amygdala, horizontal cells in the retina, intramural neurons in the gut, presumable gustatory cells in the taste bud, hair cells in the organ of Corti and a part of the olfactory cells; also immunopositive were various types of endocrine paraneurons including cells of the anterior pituitary, carotid body, adrenal medulla and pancreatic islet (Iwanaga et al., 1985; Hozumi et al., 1986; review: Fujita et al., 1988). Some other neuron-specific proteins have also been shown to be shared by paraneurons, though not in such a wide spectrum as in the case of spot 35 protein; these include neurofilament protein (NFP) and neuron-specific enolase (NSE) (Schmechel et al., 1978; Fujita et al., 1983; review: Fujita et al., 1988). Studies along this line have concentrated on mammalian cells, and very few data are available concerning the cells in lower vertebrates.

Zaccone (1986) has shown NSE-like immunoreactivity in Merkel cells in conger-eel epidermis. It is, therefore, possible that the neuron-specific proteins may be also shared by sensory paraneurons in lower vertebrates. In this study, we applied the antibodies to spot 35 protein, NFP and NSE to the taste organ of the bullfrog.

The taste organ of frogs is characterized by horizontally and radially arranged cells at the bottom of the epithelium. In the taste organ of the bullfrog, Hirata and Nada (1975) have shown by fluorescence histochemistry that the basal cells emit fluorescence for a monoamine, suggesting that the monoamine may be serotonin. However, immunohistochemistry seems to be more specific and sensitive for detecting than fluorescence histochemistry. In the present study, we examine the morphological features, immunohistochemical reactivities and innervation of the basal cells, giving special attention to the use of anti-serotonin antibody.

A close relationship between the gustatory cells and substance P-immunoreactive nerve fibers has been indicated in mammals, and it has been proposed that substance P might be involved in taste sensation (Lundberg et al., 1979; Yamasaki et al., 1984). Similarly, substance P-fibers have been reported to occur in the taste organ of the bullfrog and discussions on their function have followed (Hirata and Kanaseki, 1987). As to the possible occurrence of nerve fibers containing other peptides, little information is available on the frog taste organ. Using antisera for a variety of neuropeptides, we were able to identify a remarkable variety of peptide-containing fibers in the taste organ of bullfrogs.

The present study is the first detailed report on the cellular and nervous elements composing the bullfrog taste organ, as studied by immunohistochemical methods.

MATERIALS AND METHODS

Tissue preparation

Bullfrogs (Rana catesbeiana) weighing 160-400 g were used in the present study. Under anesthesia with 1% MS-222, the animals were first perfused through the heart with 0.64% saline solution, followed by perfusion of 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.3). The tongues were dissected out and immersed in the same fixative for 2-4 h. The tissues were then rinsed in 0.1M phosphate buffer for 1 h and placed in 0.1M phosphate buffer containing 30% sucrose for 2-4 days. They were frozen in liquid nitrogen, cut at 15-20 μm or serially at 10 μm in a cryostat and mounted on glass slides coated with poly-L-lysine.

Immunohistochemical procedures

Cryostat sections of the tongue were immersed in 0.3% Triton X-100 in phosphate buffered saline (PBS) for 1 h. They were soaked in 0.3% H2O2 in methanol for 10 min to block any intrinsic peroxidase activity. After sufficient rinsing with distilled water and PBS, they were incubated with normal swine serum diluted at 1:20 for 30 min, followed by incubation for 12-18 h with the first antibodies shown in Table 1. The sites of antigen-antibody reaction were detected by the peroxidase antiperoxidase (PAP) method according to Sternberger (1979). All incubations in this procedure were performed in a moisture chamber at room temperature.

A double immunofluorescence method was applied to examine the relationship in distribution between the two different antigens. After treatment with 0.3% Triton X-100 in PBS for 1 h, the cryostat sections were incubated with normal goat serum (1:20) for 30 min. They were then incubated with a mixture of
primary antibodies raised in a rabbit and others in a
rat for 3 h, followed by incubation with a mixture of
FITC-labeled goat anti-rabbit IgG (1:20; Biomedical
Technologies., Cambridge, USA) and rhodamine-
labeled goat anti-rat IgG (1:20; Cappel Laboratories,
West Chester, USA) for 1 h. This procedure was
carried out under the same conditions as the PAP
method. The immunostained sections were mounted
in a buffered glycerin, and viewed using a Leitz fluo-
rescence microscope equipped with filter sets L2.1
and N2.1 for FITC and rhodamine fluorescence, respec-
tively.

Controls
The specificity of each immunostaining was checked
as follows: Cryostat sections were incubated with
normal rabbit serum or with antibodies pretreated
with corresponding synthetic antigens (20-50μg/ml
of the diluted antibodies) for 24 h 4°C, under the same
conditions as described above.

RESULTS
In this study, the epithelium of the taste organ of
Rana catesbeiana was divided into two layers: the
apical one third and the basal two thirds. The former
was occupied by supporting cells, which contained
granules, and apical processes of taste cells. The
latter comprised numerous taste cells with both
apical and basal cytoplasmic processes. This layer
also contained basal cells with single slender process-
estended immediately above the basal lamina.
This cellular construction of the taste organ epithe-
ilum essentially agrees with the description by TOYO-
SHIMA et al. (1984).

**Spot 35 protein-like immunoreactivity**

When the tongue of the bullfrog was immunostained
with anti-spot 35 protein serum, an intensely positive
immunoreactivity was found in numerous cells within
the layer in the taste organ epithelium containing
gustatory cells. Immunonegative cells were intermin-
ged as often as immunopositive ones. The immuno-
reactive cells possessed an oval nuclear portion at the
middle or slightly lower levels in the layer and
extended one or more slender cytoplasmic processes
upward to the free surface (Fig. 1a). Single or bundled
processes ascended among the supporting cells and
reached the free surface (Fig. 1b). The immuno-
reactive cells also issued branched processes to the
base of the epithelium (Fig. 1a). The immunoreactive
material was distributed diffusely throughout the cyto-
plasm from the apical to the basal parts, but the nuclei
were free of the immunoreactivity.

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**Table 1. Details of primary antisera used for immunohistochemistry**

<table>
<thead>
<tr>
<th>Antisera</th>
<th>Code</th>
<th>Host</th>
<th>Dilution (PAP)</th>
<th>Dilution (Double-staining)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGRP</td>
<td>RPN1842</td>
<td>Rabbit</td>
<td>1:2,000</td>
<td>1:200</td>
<td>Amersham International plc, UK</td>
</tr>
<tr>
<td>GRP</td>
<td>R-6902</td>
<td>Rabbit</td>
<td>1:3,000</td>
<td></td>
<td>Dr. N. YANAIHARA</td>
</tr>
<tr>
<td>NFP (150K)</td>
<td></td>
<td>Rabbit</td>
<td>1:1,000</td>
<td></td>
<td>Dr. Y. TAKAHASHI</td>
</tr>
<tr>
<td>NSE</td>
<td></td>
<td>Rabbit</td>
<td>1:400</td>
<td></td>
<td>Wako Pure Chemical Industries, Ltd, Japan</td>
</tr>
<tr>
<td>PHI</td>
<td>R-8201</td>
<td>Rabbit</td>
<td>1:3,000</td>
<td></td>
<td>Dr. N. YANAIHARA</td>
</tr>
<tr>
<td>VIP</td>
<td>R-502</td>
<td>Rabbit</td>
<td>1:2,000</td>
<td>1:200</td>
<td>Dr. N. YANAIHARA</td>
</tr>
<tr>
<td>Serotonin</td>
<td></td>
<td>Rabbit</td>
<td>1:6,000</td>
<td></td>
<td>Dr. J. NISHITSUTSUJI-UWO</td>
</tr>
</tbody>
</table>
| Serotonin      | YC5/45   | Rat (mono-
clonal) | ---           | 1:400                       | Sera-Lab, UK                               |
| Spot 35 protein|          | Rabbit  | 1:100          | 1:100                       | Dr. Y. TAKAHASHI                           |
| Substance P    | RPN1572  | Rabbit  | 1:2,000        | 1:200                       | Amersham International plc, UK              |
| Substance P    | NC1/34HL | Rat (mono-
clonal) | ---           | 1:100                       | Sera-Lab, UK                               |
| Tryptophan     |          | Rabbit  | 1:1,600        |                             | Dr. A. ICHIYAMA                             |
| hydroxylase    |          |         |                |                             |                                              |
| Tyrosine       |          | Rabbit  | 1:1,000        |                             | Dr. F. IKUTA                                 |
| hydroxylase    |          |         |                |                             |                                              |
NFP-like immunoreactivity

NFP-like immunoreactivity was found in thick fibers of nerve bundles ascending within the fungiform papillae. The immunoreactive fibers, without varicosities, branched and formed a plexus under the taste epithelium. A few of them entered the epithelium and horizontally, just above the basal lamina, whereas no or very few immunoreactive fibers extended into the gustatory and supporting cell layers (Fig. 2a). No immunoreactivity was present in the cellular elements of the taste organ.

NSE-like immunoreactivity

The immunoreactivity for NSE was found in a small...
number of cells within the gustatory cell layer of the bullfrog taste organ. The immunoreactive cell extended one or more slender cytoplasmic processes to the free surface, ascending among the supporting cells, with other processes reaching downwards to the basal lamina. Their cell bodies were smaller in size than those of spot 35 protein-immunoreactive cells, and most were located at the upper or middle levels in the layer. Also, the basal cells showed a positive immunoreactivity for NSE (Fig. 2b). However, no immunoreactivity was present in nervous elements within the taste organ.

**Serotonin-like immunoreactivity**

Immunoreactivity for serotonin was detected in the
basal cells (Fig. 3a). In vertically cut taste organs, the immunoreactive cells showed up in variable transverse or longitudinal profiles according to the section site, and integration of these images indicated that the cells extended in a radial fashion on the basal lamina of the taste epithelium. Their round cell bodies were circularly arranged at the periphery of the taste epithelium. Each cell extended a single process along the basal lamina to the center of the epithelium (Fig. 3a, b); the distal portion of the process swelled in an attenuated spread (Fig. 3b), which was provided with numerous fine projections along its margin (Fig. 3c). Immunoreaction products for serotonin appeared granular in the cytoplasm, including the peripheral and central portions, the nuclei being free of the immunoreaction. No immunoreactivity was found in other cellular and nervous elements of the taste organ.

Immunoreactivity for tryptophan hydroxylase was found in the basally located cells of the taste organ (Fig. 3d). These cells exactly corresponded to the serotonin-containing basal cells with respect to their characteristic distribution and cell shape.

Peptide-containing nerve fibers

The present immunohistochemical observations demonstrated substance P-, CGRP-, VIP-, PHI- and GRP-immunoreactive nerve fibers in the bullfrog taste organ.

Substance P-fibers, with characteristic varicosities, were observed among nerve bundles ascending the

![Fig. 3. Micrographs of the basal cells immunoreactive for serotonin (a, b, c) and tryptophan hydroxylase (d) in the taste organ of a bullfrog. a. The serotonin-immunoreactive basal cells are shown in a longitudinal section located at the base of the taste epithelium. ×240. b. In a transverse (horizontal) section, the serotonin-immunoreactive basal cells reveal the round cell bodies arranged circularly at the periphery of the taste epithelium. Each cell extends a tail-like process to the center of the organ. The distended tail end is not focused in this micrograph. ×240. c. Processes of the serotonin-immunoreactive basal cells with numerous projections (arrows) protrude from the margin. Note the granular appearance of the immunoreaction. ×310. d. Tryptophan hydroxylase-like immunoreactivity demonstrated in the cell bodies (arrows) and processes (arrowheads) of the basal cells. ×1,380](image-url)
Fig. 4. Nerves supplying the bullfrog taste organ immunostained with anti-substance P (a), anti-CGRP (b), anti-VIP (c), anti-PHI (d), anti-GRP (e) and anti-tyrosine hydroxylase (f) antibodies. 

a. Substance P fibers form a dense plexus in the lamina propria under the epithelium. A few thinner substance P fibers ascend among the epithelial cells of the taste organ (arrowheads). Many substance P fibers enter the ciliated epithelium, forming a meshwork with varicosities (arrows).

b. CGRP fibers are numerous in the lamina propria. Fibers with varicosities also occur in a ciliated epithelium, most of them reaching only to the supranuclear level (arrows).

c. Varicose VIP fibers forming a meshwork in the basal area of the taste epithelium.

d. PHI fibers (arrows) run along a blood vessel. A few PHI fibers with varicosities (arrowhead) are present among the ciliated cells.

e. GRP fibers detected in the lamina propria.

f. Tyrosine hydroxylase-like immunoreactivity in thick fibers supplying a fungiform papilla. a-f: ×320
lamina propria in the fungiform papilla. These fibers formed a dense plexus in the propria under the epithelium, some fibers running along the bottom of the taste organ epithelium. Just before entering the epithelium, the substance P-fibers divided into thinner branches with smaller varicosities. A few thinner substance P-fibers ascended among the epithelial cells of the organ and terminated closely below the free surface (Fig. 4a).

Furthermore, a number of thin fibers containing substance P-like immunoreactivity entered the ciliated epithelium located at the periphery of the organ, forming, among the ciliated cells, a meshwork with smaller varicosities (Fig. 4a). Most of the fibers extended only to the level of the supranuclear region of the ciliated cells. Substance P-fibers were also distributed onto the walls of blood vessels supplying the fungiform papilla.

Varicose nerve fibers containing CGRP-like immunoreactivity were also found in the taste organ (Fig. 4b). The distribution pattern of the CGRP fibers to the taste organ cells and to blood vessels was similar to that of substance P-fibers, judging from the observation of sections.

Nerve fibers containing VIP-like immunoreactivity were recognized in the taste organ as commonly as substance P- and CGRP-fibers. The VIP-fibers, which were varicose in profile, ascended within the lamina propria together with nerve bundles and formed a dense plexus in the subepithelial region. After there branching out, they entered the taste organ epithelium and formed a meshwork in its basal area (Fig. 4c). No or very few VIP-fibers extended from basal

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**Fig. 5.** a and b. The same section of the taste organ double-immunostained with the antibodies to substance P (rhodamine labeling) (a) and to CGRP (FITC labeling) (b). Both immunoreactivities are seen in the same nerve fibers in the taste organ. ×480
area toward the free surface. VIP-fibers were scarcely found among the ciliated cells. There were some VIP-immunoreactive nerve fibers around blood vessels in the lamina propria.

Nerve fibers containing PHI-like immunoreactivity were often seen within the taste organ (Fig. 4d). On comparing serial sections, their distribution pattern closely resembled that of VIP-fibers. There were also some PHI-fibers among the ciliated cells (Fig. 4d). In addition, PHI-fibers were sometimes found along blood vessels within the papillae.

Nerve fibers with GRP-like immunoreactivity were also found in the taste organ. They were the least common of the five kinds of peptide-containing nerve fibers. Several GRP-fibers occurred in the lamina propria under the taste organ epithelium (Fig. 4e). Their branches rarely invaded the epithelium, although they did occasionally enter the ciliated epithelium; most of them did not exceed the supranuclear level of the ciliated cells. A few GRP-fibers were seen along the walls of blood vessels supplying the papilla.

The surface of the bullfrog tongue was covered not only with fungiform papillae, but also with numerous filiform papillae. Substance P-, CGRP-, VIP- and PHI-fibers wound upwards within the lamina propria in the latter papillae. There were abundant mucus glands opening on the tongue surface. The four kinds of peptide-containing fibers occurred also in the lamina propria around the glands. Especially, VIP- and PHI-fibers frequently approached the gland cells. GRP-fibers, on the other hand, innervated only a small number of filiform papillae. A few GRP-fibers were found in the lamina propria around the mucus glands.

**Tyrosine hydroxylase-like immunoreactivity**

Immunoreactivity for tyrosine hydroxylase was rec-
Fig. 7. The same sections (a and b; c and d) in the taste organ double-immunostained with the antibodies against serotonin (rhodamine labeling) (a, c), substance P (FITC labeling) (b) and VIP (FITC labeling) (d). a and b. The processes of the serotonin-containing basal cells (arrowheads) are in conjunction with substance P-positive nerve terminals (small arrows). A substance P-fiber (large arrow) runs immediately beneath the process of the serotonin-cell. c and d. VIP-fibers (arrows) from a dense meshwork in the basal region of the taste organ and are entangled in the processes of the serotonin-cells (arrowheads). × 450
Immunohistochemistry in the Taste Organ of Bullfrog

recognized in thick fibers within nerve bundles supplying most of the fungiform papillae. The immunoreactive fibers ascended the papillae to end under the epithelium. Branches of them could be seen neither in the lamina propria nor in the taste organs. No immunoreactivity was seen in any cellular elements within the taste organ (Fig. 4f).

**Relationship between substance P- and CGRP-fibers**

A double immunofluorescence staining method was carried out to examine the relationship in distribution between substance P- and CGRP-fibers. When the two antibodies were incubated on cryostat sections, substance P- and CGRP-like immunoreactivities were observed in the same nerve fibers within the taste organ (Fig. 5a, b).

**Relationship between spot 35 protein-immunoreactive cells and substance P-fibers**

To examine the relation between spot 35 protein-immunoreactive cells and substance P-fibers, a double immunostaining method using the two antibodies was applied. Substance P-fibers entering the taste epithelium gave dot-like varicosities on some of the spot 35 protein-immunoreactive cell bodies (Fig. 6a, b). In addition, a few substance P-fibers were entangled in the processes of spot 35 protein-immunoreactive cells (Fig. 6c, d).

**Relationship between serotonin-immunoreactive cells and peptide-containing nerve fibers**

The relation between serotonin-immunoreactive cells and substance P- or VIP-fibers was also investigated the double immunostaining method. In the taste organ, thinner substance P- fibers were distributed close to the serotonin-immunoreactive cell bodies. Certain substance P-fibers were found to run immediately beneath the processes of the serotonin-immunoreactive cells (Fig. 7a, b). VIP-fibers formed a dense meshwork in the basal region of the taste organ and were connected with the processes of the serotonin-immunoreactive cells (Fig. 7c, d).

**Cellular and nervous elements with a variety of immunoreactivities in the bullfrog taste organ are diagrammatically summarized in Figure 8.**

**Control of specificity**

No cellular or nervous elements with a positive immunoreaction were found in tongue sections which were incubated with normal rabbit serum or antibodies pretreated with corresponding antigens.

**DISCUSSION**

**Spot 35 protein-immunoreactive cells**

In the present study, spot 35 protein-like immunoreactivity was restricted to certain cells located in the gustatory cell layer of the bullfrog taste organ. GRAZIADEI and DEHAN (1971) presented a scheme of the taste organ, based on their ultrastructural investigations in *Rana pipiens*. The distribution patterns of the cell bodies and cytoplasmic processes of the gustatory cells which they illustrated closely resemble those of the spot 35 protein-immunoreactive cells we observed in both transverse and longitudinal sections. This therefore quite reasonably suggests that the spot 35 protein-immunoreactive cells correspond to the taste cells.

Cells negative for spot 35 protein were found in the gustatory cell layer of *Rana catesbeiana*. In *Rana esculenta* and *temporaria*, the gustatory cell layer has been claimed to contain non-gustatory elements including pre-taste (immature type) cells and supporting cells (DURING and ANDRES, 1976). Although a classification of cell types located in the gustatory cell layer has not been established for *Rana catesbeiana*, it seems reasonable to presume that the immunonegative cells may belong to the non-gustatory category of cells as mentioned above.

From electrophysiological studies in frog taste buds, it has been assumed that the taste cells are depolarized by various chemical stimuli applied to the tongue, and the subsequent response is synaptically transmitted to afferent gustatory nerves (KURIHARA et al., 1981; SATO et al., 1987). The present finding by the double immunostaining, that substance P-fibers are closely related to spot 35 protein-positive taste cells in the taste organ, strongly the above mentioned view that taste sensation is transmitted by substance P-containing nerves.

The experiment in the bullfrog by NAGAHAMA et al. (1982) indicated that Ca²⁺ is necessary for nerve fiber responses to stimuli on the tongue surface. They concluded that the responses are induced by Ca²⁺ influx into taste cells which causes the release of a chemical transmitter. It has been demonstrated in a variety of paraneurons that Ca²⁺ influx is essential for excitation caused by adequate stimuli (HAGIWARA...
A relationship between Ca\(^{2+}\) dynamics and spot 35 protein is postulated to occur in the taste cells, since the protein is a kind of Ca\(^{2+}\)-binding proteins (YAMAKUNI et al., 1985). It thus seems possible that the occurrence of spot 35 protein in the taste cells may somehow be involved in the Ca\(^{2+}\)-dependent release mechanisms of transmitters in the taste cells. Spot 35 protein-like immunoreactivity has also been demonstrated in a part of the gustatory cells in the taste buds of the guinea pig (IWANAGA et al., 1985). This therefore suggests that the gustatory cells of vertebrates from anurans to mammals may need a Ca\(^{2+}\)-binding protein such as spot 35 protein to achieve the action related to the Ca\(^{2+}\) dynamics.

The transmitter substances in the taste cells of the frog taste buds have been investigated by several authors. In their histochemical studies in *Rana pipiens*, DeHAN and GRAZIADEI (1971, 1973) revealed that noradrenaline is contained within the secretory granules, the presence of which had been ultra-structurally suggested in the cytoplasm of the taste cells (UGA, 1966; GRAZIADEI and DeHAN, 1971), proposing that noradrenaline is a candidate for transmission from taste cells to sensory nerve terminals. This view was supported by physiological experiments using bullfrogs, by MORIMOTO and SATO (1975, 1982). Evidence of the occurrence of noradrenaline and other catecholamines could not be offered by the present observation using antibody to tyrosine hydroxylase, the first catecholamine synthesizing enzyme. The immunoreactivity was found in thick nerve fibers supplying the fungiform papillae, but not in the taste organ. Whether or not noradrenaline or other catecholamines are produced in the taste cells of frogs remains a point of dispute. We could not exclude the possibility that the amount of catecholamines in the taste cells may be too small to be detected by the present method. On the other hand, it is possible that the messengers of frog taste cells are noradrenaline or other catecholamines, but other categories of substances.
Serotonin-immunoreactive cells

The serotonin-immunoreactive cells demonstrated in the present study exactly correspond, in their characteristic disposition, to the basal cells previously described by TOYOSHIMA et al. (1984) in Rana catesbeiana. The same cells have been reported in earlier works by HIRATA and NADA (1975, 1977) and NADA and HIRATA (1975a), who demonstrated by fluorescence histochemistry the presence of serotonin-fluorescent cells radially disposed at the base of the bullfrog taste organ, although the authors erroneously called them "gustatory cells". By this, we were able to confirm the occurrence of serotonin in the basal cells of the bullfrog taste organ by immunohistochemical methods, which are more sensitive and specific for the detection of the antigen than fluorescence-histochemical and histochemical methods used by the above mentioned authors.

The taste buds of the bullfrog are in contrast with those of the mouse in regard to their immunoreaction for serotonin. Serotonin-like immunoreactivity occurs in the basal cells in untreated bullfrogs, whereas it is detected in a part of the gustatory cells of taste buds in mice pretreated with 5-hydroxytryptophan, the precursor of serotonin, it is not detectable in the untreated mouse (UCHIDA, 1985). In addition, immunoreactivity for tryptophan hydroxylase, a key enzyme for indolamine synthesis, can be detected in bullfrog basal cells, but not in mouse taste buds (Kuramoto unpublished data). These differences of immunoreactivities support the view that serotonin is synthesized from its initial precursor, tryptophan in the basal cells of the bullfrog, and that this synthesis does not seem to occur in the taste cells of the mouse.

The present immunohistochemistry showed that the serotonin-containing basal cells lay their cell bodies at the periphery of the taste epithelium and extend to the center single processes ending in a leaf-like spread, the whole extent of the cell process lying on the basal lamina of the epithelium. This unique disposition and shape of the basal cells seem purposeful for the reception of tactile stimuli which are vertically given to the free surface of the taste epithelium, as suggested by TOYOSHIMA et al. (1984).

The occurrence of numerous fine projections on the margin of the process spread of the serotonin-containing basal cells deserves special attention. These projection probably correspond to the "digital cytoplasmic processes" of the bullfrog basal cells indicated by TOYOSHIMA et al. (1984), using electron microscopy. This supports the hypothesis that basal cells of bullfrogs represent a type of Merkel cells, since the presence of such digital projections is a criterion for identifying Merkel cells (review: FUJITA et al., 1988). In Rana esculenta and temporaria, DURING and ANDRES (1976) stressed the occurrence of Merkel cells at the base of the epithelium, demonstrating such ultrastructural features as finger-like process and dense cored vesicles. These cells correspond to our basal cells. Similarly, the basal cells with serotonin-fluorescence in newt taste buds have been indicated to be Merkel cells, possessing rod-like microvilli under the electron microscope (TOYOSHIMA and SHIMAMURA, 1987). It is hypothesized that the microprojections in mammalian Merkel cells are detectors of intraepidermal distortion (review: FUJITA et al., 1988). One could reasonably conclude that the radially disposed projection and finger-like microprojections of the serotonin-containing basal cells of frogs may serve to detect delicate mechanical deformations within the taste epithelium caused by pressure and tactile stimuli to the epithelial surface.

Ultrastructural investigations have shown that the basal cells of the bullfrog taste organ contain numerous cored vesicles in their cytoplasm, suggesting that the vesicles are the storage sites of monoamine, serotonin (HIRATA and NADA, 1975; NADA and HIRATA, 1975b; TOYOSHIMA et al., 1984). Our finding by the PAP method that granular immunoreaction for serotonin occurs in the cytoplasm of the basal cells may strongly support this suggestion. In mice treated with 5-hydroxytryptophan, it has been more clearly demonstrated by a protein A-collodial gold method that serotonin-like immunoreactivity is localized in the cored vesicles in the taste bud cells (UCHIDA, 1985).

In the present study, double immunostaining with the antibodies to substance P and serotonin showed the close apposition of a part of substance P-fibers to the serotonin-containing basal cells. This finding is compatible with the ultrastructural observations by NADA and HIRATA (1975b) and DURING and ANDRES (1976) that afferent nerve fibers are in close contact, over a basal lamina, with the basal cells. A similar relationship between serotonin-containing cells and afferent nerves has also been found in duodenal mucosa: FUJITA and KOYABASHI (1978) demonstrated bundles of nerve fibers in the lamina propria in juxtaposition to the base of enterochromaffin (EC) cells, suggesting that serotonin released from the EC cells may act as a transmitter on certain afferent elements in the nerves. The role of serotonin as a transmitter in the central nervous system of vertebrates and
invertebrates has also been suggested (Page, 1968). This leads to the hypothesis that serotonin released from the basal cells by mechanical stimuli acts as a transmitter upon afferent nerves containing, at least partly, substance P, which consequently conduct the tactile sensation from the taste organ to the central nervous system. In addition, serotonin contained in the basal cells may possibly act as a local hormone. Since serotonin has been exhibited to increase the excretion of mucus in the stomach (Lewis, 1958), it is possible that serotonin released from the basal cells may be dispersed by a paracrine fashion and either by diffusion or through local circulation, and may stimulate the secretion of the numerous mucus glands in the vicinity.

**NSE- and spot 35 protein-immunoreactive cells**

Immunoreactivity for NSE was shown in the present study in certain cells within the gustatory cell layer in the bullfrog taste organ. Immunoreactivity for spot 35 protein, on the other hand, appeared to be localized in a different population of cells. Careful comparison between the NSE- and the spot 35 protein-immunoreactive cells indicated that the former were smaller in number and size than the latter and, moreover, differed in localization. Therefore, the NSE-cells are distinguishable from the spot 35 protein-cells.

On the other hand, NSE-like immunoreactivity has been found in presumable gustatory cells in the dog taste buds (Fujita et al., 1983). The taste buds of the guinea pig revealed two kinds of gustatory cells with NSE-like immunoreactivity: a small cell type with intense NSE-immunoreaction and without spot 35 protein-immunoreactivity, and a large cell type with a weak immunoreaction for NSE and with an intense immunoreactivity for spot 35 protein (personal communication by Dr. S. Yoshie). This may imply the presence of subpopulations of gustatory cells in the taste buds. It thus seems that there may also be subpopulations of taste cells in the bullfrog taste organ: one positive for NSE and the other positive for spot 35 protein.

Previous immunohistochemical studies mainly in mammals have indicated that NSE and spot 35 protein are contained in a variety of sensory and endocrine paraneurons, both being localized either overlapping in the same cells or separated in different cells (Fujita et al., 1983; review: Fujita et al., 1988).

**NFP-immunoreactive nerve fibers**

In the present study, NFP-like immunoreactivity was found only in the nerve fibers supplying the taste organ. The immunoreactive fibers were rather thick and lacking in varicosities, and ran closely above the basal lamina within the taste epithelium. The NFP-fibers fail to accord in distribution and morphological characteristics with any of the peptide- and tyrosine hydroxylase-containing nerve fibers.

In mammals, numerous NFP-immunoreactive fibers have been detected in sensory or presumptive sensory nerves such as those in the skin (Iwanaga et al., 1982; Dalsgaard et al., 1984), in the iris (Seiger et al., 1984), and in the dental pulp (Maeda et al., 1987). These findings may support the view that the NFP-fibers observed in the bullfrog taste organ are also of a sensory nature. The taste organ receives, on the other hand, numerous fibers immunoreactive for substance P and/or CGRP, which generally are contained in the sensory fibers. Since the NFP-fibers definitely differ in localization and morphological features from the substance P- and/or CGRP-fibers, it is possible that the respective fibers may conduct different kinds of sensations. It is postulated that substance P- and/or CGRP-fibers in the bullfrog taste organ may transmit gustatory and tactile sensations. NFP-fibers, if they are sensory in nature as presumed above, remain undefined in regard to the amount of adequate stimuli.

**Peptide-containing nerve fibers in the taste organ**

The characteristic localization of ciliated cells at the margin of the disc of the bullfrog taste organ may presumably serve for the effective perception of taste stimuli. We recognized numerous substance P-fibers forming a meshwork among the ciliated cells. This finding is in accordance with that by Hirata and Kanaseki (1987), who suggested that the substance P-fibers regulate the ciliated cells, on the basis of their immunohistochemical result that the terminals of the substance P-fibers contain numerous synaptic vesicles and make contact with the ciliated cells. Although this idea can not be excluded, we consider yet another possibility that the substance P-fibers act as sensory nerves to perceive mechanical or chemical stimuli from the ciliated cells at the margin of the taste organ. Furthermore, in mammals, substance P-fibers have been found to be close to and within ciliated epithelia in the respiratory tract and nasal cavity, and experiments in mechanical denervation and capsaicin-treatment have shown that the substance P-fibers are of sensory origin (Lundblad et al., 1983; Lundberg et al., 1984b). These findings seem to support the possibility proposed above.
The double immunostaining method showed that CGRP- and substance P-like immunoreactivities were colocalized in a subpopulation of gustatory nerve fibers in the bullfrog taste organ. In rats CGRP-like as well as substance P-like immunoreactivities have been localized in sensory neurons of the trigeminal and spinal ganglia (ROSENFELD et al., 1983; GIBSON et al., 1984). The taste buds of mammals are supplied with numerous CGRP-fibers, which have been shown to be sensory in nature (TERENGHI et al., 1986). In addition, CGRP- and substance P-like immunoreactivities have been demonstrated to be localized in the same fibers within the taste buds of the rat (LEE et al., 1985). Thus, CGRP, in addition to substance P, is presumed to be involved in gustatory or tactile conduction.

The finding that VIP-, PHI- and GRP-fibers occur in the taste organ of the bullfrog is significant for the comprehension of the roles, besides the sensory one, of nerve fibers innervating the taste organ. Of especial interest are VIP- and PHI-fibers forming meshworks in the basal area of the taste organ. Investigation with double immunostaining for VIP and serotonin indicated a close relationship between VIP-fibers and serotonin-containing basal cells, suggesting an efferent action of the VIP-fibers to the basal cells. VIP has been reported to regulate the secretory activities of some paraneurons: it stimulates the release of glucagon from the pancreatic islet (AHRÉN and LINQUIST, 1982) and secretion of melatonin from pinealocytes through the elevation of cAMP (KANeko and KAKU, 1983). It seems reasonable to propose that the VIP-fibers may regulate the synthesis and secretion of serotonin in the basal cells.

PHI and VIP have been demonstrated by immunohistochemistry to coexist in mammalian neurons (YANAIHARA et al., 1983; LUNDBERG et al., 1984a), which supports the view that PHI represents a part of the molecule of prepro-VIP in mammals (ITO et al., 1983). In the present study, the distribution of PHI-fibers closely resembled that of VIP-fibers in the basal region of the taste organ, from observations of serial sections. However, it was impossible to decide by this observation alone whether or not PHI- and VIP-like immunoreactivities are localized in the same fibers. To examine the relation in distribution between PHI- and VIP-immunoreactive fibers in the bullfrog taste organ, a more effective method such as a double immunostaining method using two primary antibodies raised by different species will be necessary.

There is some evidence that the occurrence of PHI and VIP in the nervous system is different between mammals and lower animals. Our previous studies have that PHI-positive neurons occur much more extensively than VIP-positive ones in the central nervous system of the cockroach and Aplysia, and that numerous PHI-positive fibers, but no VIP-fibers, are present in the bullfrog adrenal gland (KURAMOTO et al., 1985; YUI et al., 1985; KURAMOTO, 1987). Such a discrepancy was seen also in the present study: among the ciliated cells of the bullfrog taste organ were some PHI-fibers, but no or very few VIP-fibers could be recognized. Therefore, it is suggested that in lower animals, PHI may be involved in more important regulatory functions than VIP, although the significance of the function of the PHI-fibers among the ciliated cells is unclear.

GRP-fibers, as well as the other four kinds of peptide-containing fibers, were found along blood vessels supplying the fungiform papillae. These fibers presumably act to regulate local circulation in the blood vessels.

The distribution of peptide-containing nerve fibers in the taste organ gives us the impression that they may be involved in the maintenance of the morphology and the regulation of the function of cellular elements of the taste organ. Nevertheless, a glosso-pharyngeal denervation experiment by TOYOSHIMA et al. (1984) has suggested that the morphological integrity of the frog taste organ does not necessarily require the supply of nerve fibers. Further studies will be necessary to clarify the possible effects upon the frog taste organ of nerve fibers containing such different kinds of peptides as demonstrated in the present study.

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