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Dopamine β-Hydroxylase–like Immunoreactive Cells in the Frog Taste Disc

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Abstract

We immunohistochemically examined the existence of dopamine β-hydroxylase (DBH), a noradrenalin (NA)-synthesizing enzyme from dopamine, in the taste disc of frog, *Rana catesbeiana*. DBH-like immunoreactive cells were located in the intermediate layer in the taste disc; the cells showed an apical process reaching the surface of the disc and one or several basal processes. Cells with a thick apical process and those with a thin apical process were both immunoreactive: these cells corresponded to type II and III receptor cells of the frog taste disc. Immunoreactive granules were observed in the cytoplasm of those cells. In the frog taste disc, only type III cells are reported to have afferent synapses with the nerve via basal processes but those basal processes have not been reported in type II cells. In the present study, we found that type II–like cells possessed a long basal process extending toward the basal lamina. Mucous (type Ia) cells, wing (type Ib) cells, and glia-like sustentacular (type Ic) cells were all immunohistochemically unreactive. The present observations support the argument that NA (or adrenalin) may work as a chemical transmitter in the frog taste organ.

Key words: dopamine β-hydroxylase, frog, glossopharyngeal nerve, neurotransmitter, noradrenalin, taste cell

Introduction

Taste information in frogs is transmitted from taste cells to the glossopharyngeal nerve via synapses. Despite various pharmacological and histochemical studies, precise identification of synaptic transmitters from taste cells to afferent nerves has not yet been achieved in frogs or in other vertebrates (reviewed by Nagai et al. 1996; Yamamoto et al. 1998; Roper 2006). Although serotonin and adenosine triphosphate are proposed to be strong candidate transmitters in mammalian taste buds (reviewed by Roper 2006), these substances have not yet been precisely identified as transmitters.

On the basis of structural features, frog taste disc cells are divided into 6 types (types Ia, Ib, Ic, II, III, and IV) (Osculati and Sbarbati 1995; Li and Lindemann 2003). Efferent synapses from the nerve to the taste disc cells are found in type II, III, and IV cells, but afferent synapses are found only in type III cells (Osculati and Sbarbati 1995). Serotonin-like immunoreactivity was observed in type IV cells (Kuramoto 1988; Toyoshima 1994), but these cells (Merkel cell-like basal cells) do not possess apical processes reaching the free surface of the taste disc, hence they are not considered to be taste receptor cells.

Graziadei and DeHan (1971) observed by electron microscopy that basal processes of frog taste disc cells had synaptic contacts with afferent nerves and contained dense core vesicles, which suggests that those vesicles contain catecholamine. In frogs, noradrenalin (NA) was proposed to be a candidate neurotransmitter of taste cells from pharmacological experiments using NA, its blockers, and depleting agents (Morimoto and Sato 1982; Nagahama and Kurihara 1985). NA-like immunoreactivity was observed in taste bud cells of rats (Herness et al. 2002). Fungiform papillae in frogs were found to contain measurable amounts of NA by means of high-performance liquid chromatography (HPLC) (Zancanaro et al. 1995), but whether the substance was derived from taste cells was not proven. So far, the existence of NA-related substances has not been shown in frog taste disc cells.

In the present study, we observed immunohistochemically the existence of dopamine β-hydroxylase (DBH), an NA-synthesizing enzyme from dopamine, in taste disc cells of the frog, *Rana catesbeiana*.

Materials and methods

Materials

Twelve bullfrogs, *R. catesbeiana*, (body weight 250–400 g) were used. The experiments were performed in accordance

with the guideline for animal experiments at Matsumoto Dental University. Frogs were anesthetized with diethyl ether and pithed to destroy the brain and spinal cord.

Immunohistochemistry

Light microscopy

To locate DBH-like immunoreactive cells, the tongue was cut out with the lower jaw and fixed by intra-arterial perfusion with 4% paraformaldehyde and picric acid in 0.05 M Tris buffer (pH 7.6) containing 1.5% NaCl. Taste discs were screened and further fixed by immersing in the same fixative overnight at 4 °C. After fixation, they were rinsed in 0.05 M Tris buffer (pH 7.6) containing 1.5% NaCl (1.5 T) for 1 day or longer and cryoprotected in 1.5 T with 20% sucrose overnight at 4 °C. The specimens were embedded in Tissue Tek II OCT compound and frozen in isopentane cooled with dry ice. Frozen sections (20 μm) were obtained using a cryostat (Damon/IEC Division, Needham Heights, MA). The sections were freely floated in the solutions during the immunohistochemical procedure. First, the sections were treated with 0.3% (w/v) H_2O_2 in methanol for 30 min at room temperature to eliminate intrinsic peroxidase activity and rinsed in 1.5 T. Next, they were incubated with the primary antiserum, rabbit anti-DBH (provided by Dr I. Nagatsu, ex-professor of Fujita Health University), diluted 1:20 000 with 1% normal goat serum in 1.5 T for 24 h at 4 °C, and rinsed in 1.5 T for 30 min. Immunoreactivity of this antibody against DBH has been confirmed in the frog adrenal gland (Nagatsu et al. 1979). The sections were then treated with biotinylated goat anti-rabbit IgG (Vector Lab Inc., Burlingame, CA) for 30 min at room temperature and rinsed in 1.5 T for 30 min. They were then treated with Vectastain Elite ABC Reagent (Vector Lab Inc.) for 30 min and rinsed in 1.5 T for 30 min. 3,3′-Diaminobenzidine-tetrahydrochloride (DAB), 0.03% (w/v) in 0.05 M Tris buffer (pH 7.6) containing 0.006% (w/v) H_2O_2, was applied for about 5 min at room temperature to visualize the distribution of the antigen with the deposition of dark brown reaction products. The peroxidase reaction was stopped by transferring the sections to distilled water. Finally, the sections were mounted on glass slides, dehydrated with a graded ethanol series, cleared in xylene, and mounted in Bioleit (Oken Shoji, Tokyo, Japan). As a control, preparations were processed in the same manner except that normal rabbit serum (Vector Lab Inc.) was used instead of the primary antiserum. The preparations were observed under a microscope and photographed (BX-51, Olympus, Tokyo, Japan).

Electron microscopy

The same preparations as for light microscopy were fixed with 4% paraformaldehyde and 0.1–2% glutaraldehyde in 0.05 M Tris buffer (pH 7.6) containing 1.5% NaCl. They were sectioned and treated in the same manner as for light microscopy and further treated with 1% OsO_4 to obtain osmium black substances with DAB on the immunoreactive sites. After washing in buffer solution and dehydrating in a graded ethanol series, they were embedded in Quetol-812 (Nissin EM, Tokyo, Japan). Ultrathin sections were cut using a microtome (LKB, Browman, Sweden) and observed under an electron microscope (H-7600, Hitachi Ltd., Tokyo, Japan).

Results

A large number of DBH-like immunoreactive spots were observed in the horizontal section of the taste disc (Figure 1A1). In the vertical section of the taste disc, numerous DBH-like

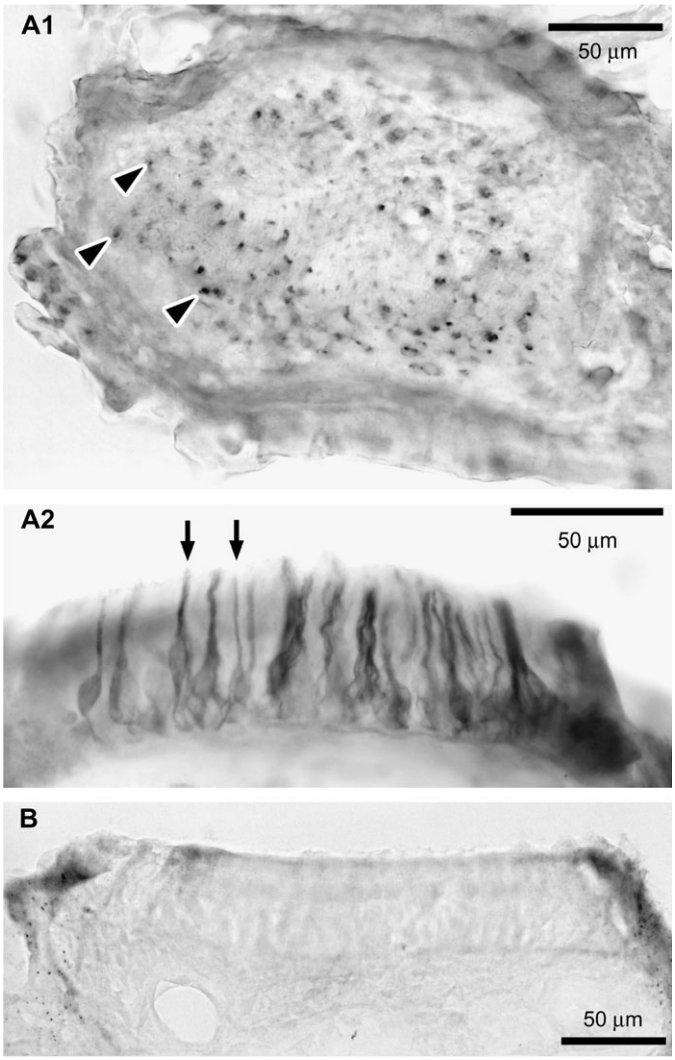

Figure 1 Photomicrographs of DBH-like immunoreactivity in the frog taste disc. **(A1)** A large number of DBH-like immunoreactive spots (arrowheads) were observed in the taste disc (horizontal section). **(A2)** Numerous DBH-like immunoreactive cells (arrows) were observed in the taste disc (vertical section). **(B)** No immunoreactivity was observed within the taste disc when normal rabbit serum was used instead of anti-DBH antiserum. Dark material around the taste disc is nonspecific DAB reaction products.

immunoreactive cells were observed (Figure 1A2). No immunoreactivity was observed within the taste disc when normal rabbit serum was used instead of anti-DBH antiserum (Figure 1B). Some dark material was observed around the taste disc, which was thought to be nonspecifically attached 3,3′-diaminobenzidine (DAB) reaction products.

DBH-like immunoreactive cells showed a rod-shaped apical process reaching the free surface of the disc (Figure 2). Cell bodies of immunoreactive cells were located in the intermediate layer of the taste disc (Figure 2B). Immunoreactive cells possessed either a single or several basal processes (Figure 2B).

Osculati and Sbarbati (1995) classified frog taste disc cells that possessed a rod-shaped process into 2 groups: type II cells with a thick apical process and type III cells with a thin apical process. In the present study, DBH-like immunoreactivity was observed in both types of apical processes (Figure 3).

Apical processes of type II cells tend to form clusters resembling a small bud in the taste disc (Osculati and Sbarbati 1995; Li and Lindemann 2003). Figure 4 shows immunoreactive cells forming groups, which seemed to be composed of either type II cells (Figure 4B) or a mixture of type II and III cells (Figure 4C), though it was difficult to distinguish the thickness of each apical process because they overlapped.

In the present study, type II–like cells, which showed a thick apical process, possessed a long basal process extending toward the basal lamina (Figure 4B–D). This basal process was observed regardless of whether the cells formed a group (Figure 4B,C) or not (Figure 4D).

Figure 5 shows immunoreactive apical processes in the horizontal section of the taste disc. Large immunoreactive spots are supposed to represent thick apical processes of type II cells and small spots represent thin apical processes of type III cells. Figure 5B shows a horizontal section sliced at a level closer to the free surface of the taste disc than in Figure 5A. Polygonal-shaped mucous (type Ia) cells were located in the superficial layer of the taste disc and were separated from each other by apical sheet-like prolongations of wing (type Ib) cells (in agreement with Osculati and Sbarbati 1995). Thick immunoreactive processes were observed at the junction of 3 or 4 type Ia cells, whereas thin immunoreactive processes were observed to be in close contact with type Ia and Ib cells. A similar cellular arrangement of type Ia, Ib, II, and III cells in the superficial layer, as in Figure 5B, has been

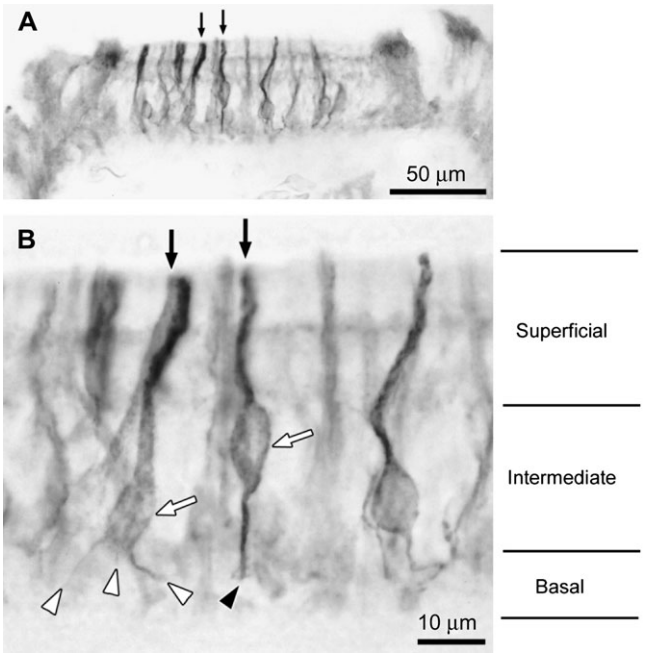

Figure 2 Photomicrographs of DBH-like immunoreactive cells in the frog taste disc (vertical section). **(B)** Magnification of **(A)**. The taste disc is divided into 3 layers (superficial, intermediate, and basal). Cell bodies of DBH-like immunoreactive cells were located in the intermediate layer of the taste disc (white arrows), and those cells showed an apical process reaching the free surface of the disc (black arrows). Immunoreactive cells possessed either a single basal process (black arrowhead) or several basal processes (white arrowheads).

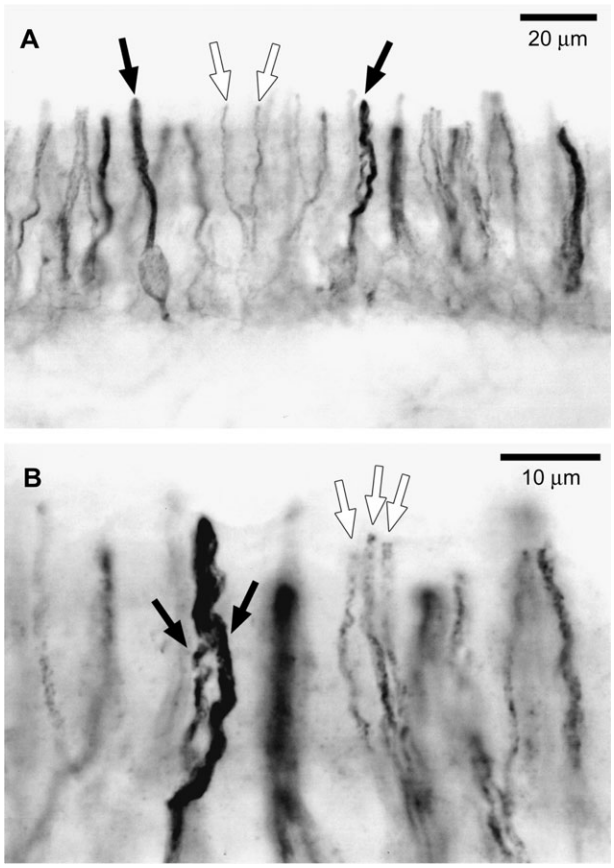

Figure 3 Photomicrographs of rod-shaped apical processes of DBH-like immunoreactive cells (vertical section). **(A)** Both thick (black arrows) and thin (white arrows) apical processes were immunoreactive: the former were supposed to belong to type II cells and the latter to type III cells in Osculati and Sbarbati's nomenclature (Osculati and Sbarbati 1995). Some of those apical processes are magnified in **(B)**.

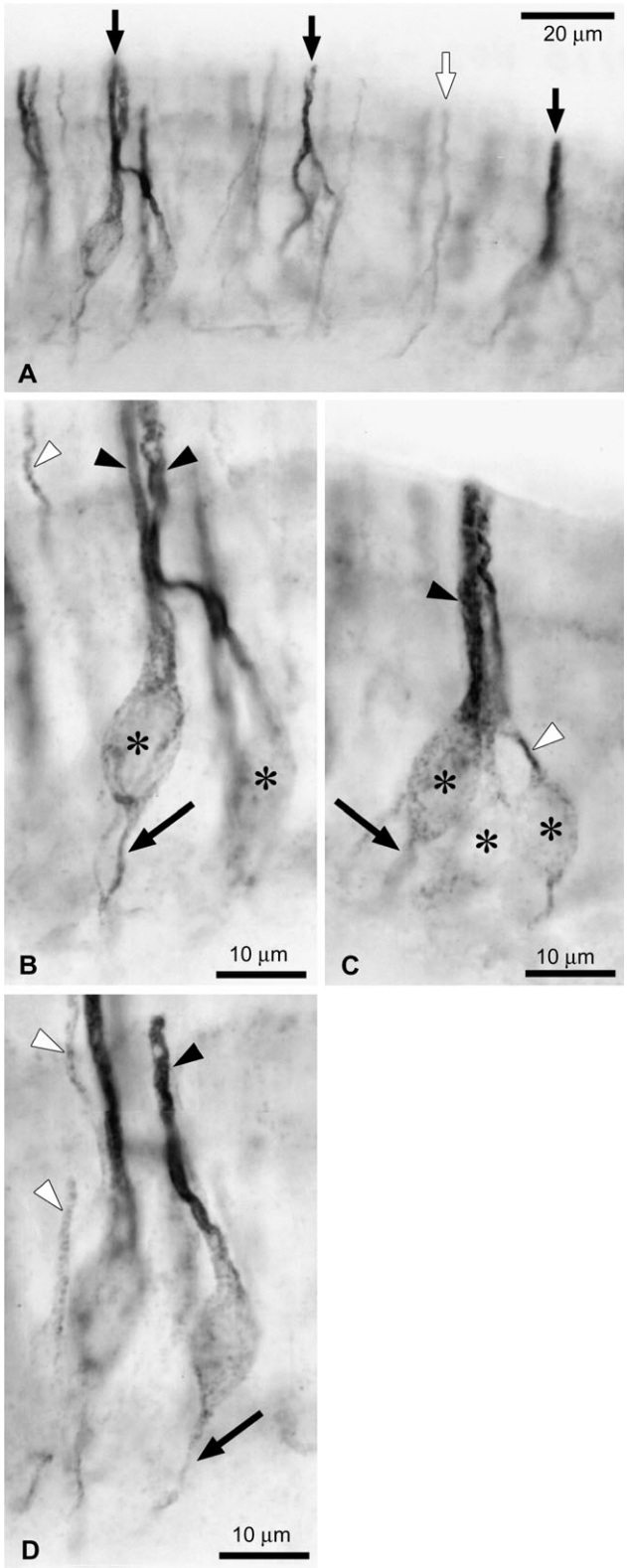

Figure 4 Photomicrographs of DBH-like immunoreactive cells forming a group (vertical section). **(A)** Some immunoreactive apical processes tended to form a group (black arrows). White arrow shows a type III–like cell with a thin apical process. **(B)** Cell group is shown by an arrow on the left side of (A). Thick apical processes (black arrowheads) of 2 immunoreactive cells (*) ran close together. A cell with a thick apical process possessed a long basal process (arrow). A basal process to the left of the arrowed process is probably from another cell at the back. White arrowhead shows a thin apical process. **(C)** Cell group shown by an arrow on the right side of (A). Apical processes of at least 3 immunoreactive cells (*) ran close together. A cell with a thick apical process (black arrowhead) possessed a basal process (arrow) extending near the basal lamina. A basal process to the left of the arrowed process is probably from another cell at the back. White arrowhead shows a thin apical process. **(D)** A solitary cell with a thick apical process (black arrowhead) possessed a basal process (arrow) extending near the basal lamina. White arrowheads show thin apical processes.

described by other researchers (Witt 1993; Osculati and Sbarbati 1995; Li and Lindemann 2003).

Figure 6 shows an electron micrograph of DBH-like immunoreactive apical processes of receptor-like cells. Mucous (type Ia) cells, wing (type Ib) cells, and glia-like (type Ic) cells showed no immunoreactivity. The immunoreactive apical process in Figure 6B was probably from a type II cell because it was about 2 μm thick and was enclosed by type Ic cells as described by Osculati and Sbarbati (1995).

DBH-like immunoreactivity was observed in the cytoplasm around the nucleus as well as in apical processes and basal processes (Figure 7A,B). Immunoreactivity was seen in granules of the cytoplasm (Figure 7C,D) and also in ground substances of the cytoplasm (Figure 7D). No immunoreactivity was observed in the nucleus (Figure 7C,D). Space between cells in the specimen was an artifact derived from the procedure of frozen sectioning and immunohistochemistry.

Table 1 shows the density and the ratio of immunoreactive apical processes of type II– and type III–like cells in a taste disc, which were calculated from 6 well-stained taste disc slices. The area of the discs ranged from 8352 μm^2 to 25 634 μm^2, (mean ± standard deviation [SD] 15 572 ± 7688 μm^2, $n = 6$). DBH-immunoreactive apical processes of type II– and type III–like cells were counted on each taste disc, and the densities of those processes were expressed as numbers in an area of 1000 μm^2. The mean densities of processes calculated are shown in the table.

Discussion

It was observed by electron microscopy that basal processes of presumed taste sensory cells had synaptic contacts with afferent nerves and contained dense core vesicles in the taste disc of frogs (Graziadei and DeHan 1971) and toads (Witt 1993). Osculati and Sbarbati (1995) observed that basal processes of type III cells contained dense core vesicles in the frog taste disc. These dense core vesicles are presumed to contain catecholamine. Zancanaro et al. (1995) showed by means of HPLC technique that fungiform papillae in frogs contained measurable amounts of NA. In frogs, some pharmacological experiments proposed NA as a candidate neurotransmitter of taste cells (Morimoto and Sato 1982; Nagahama and Kurihara 1985). Morimoto and Sato (1982) reported that intravascularly administered adrenalin, dopamine, and NA all increased afferent nerve impulses in

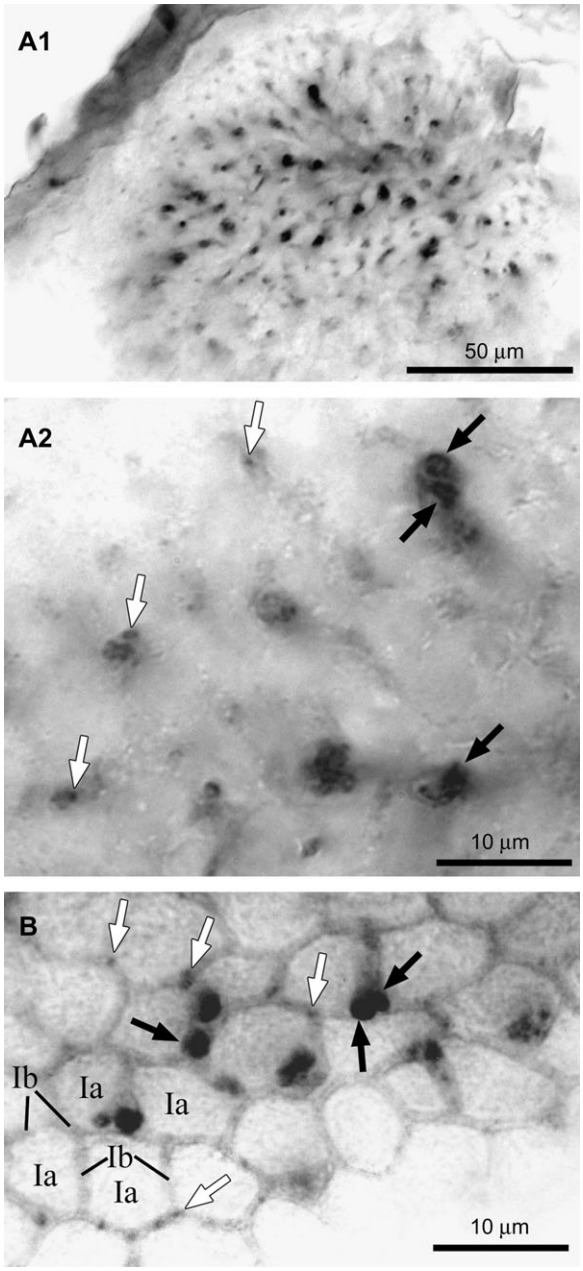

Figure 5 Photomicrographs of apical processes of DBH-like immunoreactive cells (horizontal section). **(A1)** A large number of immunoreactive spots were observed. **(A2)** Magnification of (A1). Large (black arrows) and small (white arrows) spots were observed. **(B)** The section was sliced at a level close to the taste disc surface. Thick (black arrows) and thin (white arrows) immunoreactive processes were clearly observed. Ia: mucous cells, Ib: wing cells.

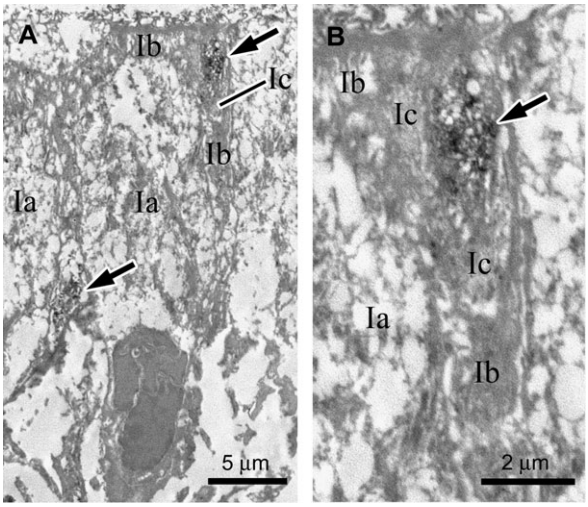

Figure 6 Electron micrographs of DBH-like immunoreactive cells in the frog taste disc (oblique section). **(A)** Apical processes of receptor-like cells (arrows) showed DBH-like immunoreactivity. Mucous (type Ia) cells, wing (type Ib) cells, and glia-like (type Ic) cells did not show immunoreactivity. **(B)** Magnification of the upper right area of (A).

the glossopharyngeal nerve in frogs, with the threshold concentrations of 10^{-3}, 10^{-7}, and 10^{-9} M, respectively. They also reported that intravascularly administered adrenalin and NA enhanced nerve impulses responding to taste stimuli but not to tactile stimuli and that those nerve impulses responding to taste stimuli decreased with intravascularly administered adrenergic blocker (phentolamine or phenoxybenzamine). These observations support that NA is the strongest candidate neurotransmitter among the 3 catecholamines. Although NA has not been shown in frog taste disc cells, NA-like immunoreactivity was observed in the taste bud cells of rats (Herness et al. 2002).

In the present study, DBH-like immunoreactivity was observed in taste disc cells. DBH catalyzes the conversion of dopamine to NA within catecholamine-secreting vesicles (chromaffin granules) of the adrenal medulla and dense-cored synaptic vesicles of the sympathetic nervous system. In adrenal chromaffin granules, DBH exists as both soluble and membrane-bound protein (reviewed by Stewart and Klinman 1988). Using the same anti-DBH serum as in the present study, Nagatsu et al. (1979) observed DBH immunoreactivity only inside chromaffin granules in chromaffin cells of the frog adrenal gland. In the present study, DBH-like immunoreactive products were mostly observed in granules in the cytoplasm of frog taste disc cells under an electron microscope. In some cells, however, reaction products were also observed in the ground substance of the cytoplasm. Redick et al. (1974) also reported a similar localization of DBH to the outer side of the vesicular membrane, but Nagatsu et al. (1979) mentioned that it may be an artifact based on damage to chromaffin granules. It is possible that the reactivity seen outside of granules in some cells in the present study is also an artifact caused by the histochemical procedure and that the result does not necessarily represent the correct localization of the enzyme inside cells.

Osculati and Sbarbati (1995) suggested 6 types of cells (type Ia, Ib, Ic, II, III, and IV) in the frog taste disc on the basis of structural features. Li and Lindemann (2003) confirmed the morphology of these types of cells using various fluorescent dyes. In this study, we follow the classification and nomenclature of cell types by Osculati and Sbarbati (1995).

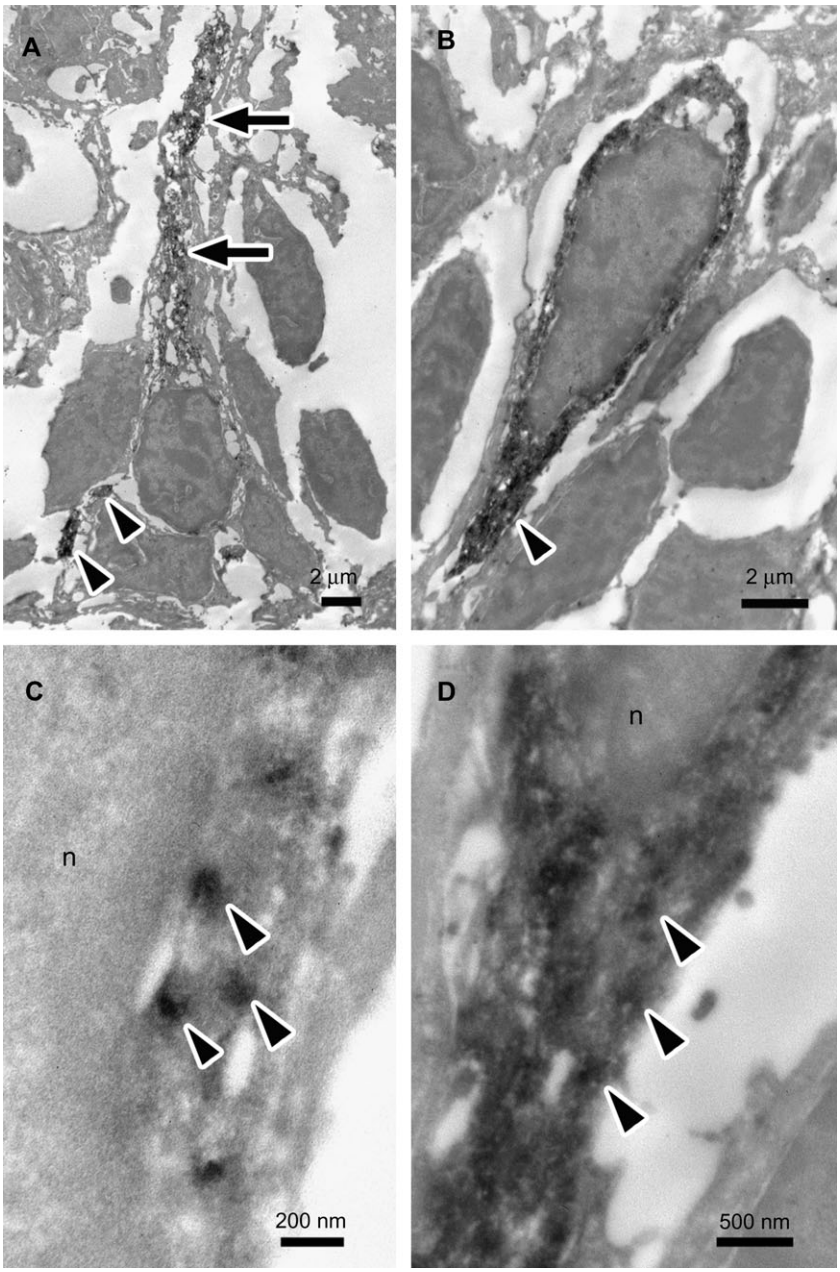

Figure 7 Electron micrographs of DBH-like immunoreactive cells in the taste disc (vertical section). **(A,B)** DBH-like immunoreactive cells possess an apical process (arrows) and one or several basal processes (arrowheads). **(C)** Immunoreaction products were observed in granules (arrowheads) of the cytoplasm. **(D)** Immunoreaction products were observed not only in granules (arrowheads) but also in ground substances of the cytoplasm. No immunoreactivity was observed in the nucleus (n).

A large number of DBH-like immunoreactive cells were observed in the taste disc. These cells all possessed a cell body located in the intermediate layer of the disc, thick or thin apical processes reaching the free surface of the disc, and a single or several basal processes; these are characteristics of type II and III cells.

Richter et al. (1988) observed about 760 apical processes of type III cells stained with a fluorescent dye in a taste disc, approximately 200 μm in diameter, of *Rana esculenta*. Düring and Andres (1976) described that branching of the apical process of taste cells is seldom seen, hence the number of apical processes is supposed to approximately represent the number of the cells. Osculati and Sbarbati (1995) observed about 600–800 type III cells in a taste disc under an electron microscope, in agreement with Richter et al. (1988). In the present study, DBH-immunoreactive apical processes of type II and III cells in a taste disc of 200 μm in diameter were estimated as 139 ± 23.4 and 353.3 ± 82.2

Table 1 Density and ratio of immunoreactive apical processes of type II– and type III–like cells in frog taste discs

	Mean ± SD (n = 6)
Density of II (number/1000 μm^2)	4.4 ± 0.7
Density of III (number/1000 μm^2)	11.3 ± 2.6
Ratio III/II	2.5 ± 0.3

(mean ± SD, n = 6), respectively, from the densities shown in Table 1. The number of apical processes of type III cells is about half of those shown by Richter et al. (1988). Presuming that the densities of those cells are similar regardless of the species difference, at least half of all type III cells are assumed to be DBH immunoreactive.

Li and Lindemann (2003) observed at least 100 and 300 apical processes of type II and III cells, respectively, in a taste disc of *R. esculenta* by different staining methods with fluorescent dyes from Richter et al. (1988). The numbers of processes of type II and III cells were similar to those in the present study.

Type I cells

Type I cells contain mucous (type Ia) cells, wing (type Ib) cells, and sustentacular, glia-like (type Ic) cells (Osculati and Sbarbati 1995). In the present study, none of these type I cells showed DBH immunoreactivity.

Type II cells

Osculati and Sbarbati (1995) reported that type II cells possessed a thick apical dendrite and made contact with glossopharyngeal nerve fibers at the soma level, although presynaptic vesicles were absent. Electrophysiologically, type II cells are reported to produce action potentials (Takeuchi et al. 2001; Suwabe and Kitada 2004) and respond to quinine (bitter taste) stimuli (Takeuchi et al. 2001). These observations suggest that type II cells may work in taste reception in frogs.

Herness and Sun (1999) reported that exogenous NA enhanced calcium-activated chloride currents via β-adrenergic receptors in rat taste bud cells. Herness et al. (2002) showed that NA application inhibited outward potassium currents and elevated intracellular calcium levels in rat taste bud cells. They also observed the expression of multiple α and β subtypes of adrenergic receptors in lingual epithelium using the reverse transcription–polymerase chain reaction technique and further observed NA-like immunoreactivity in taste bud cells. From these observations, the authors suggested adrenergic paracrine communication among taste bud cells.

Although the existence of adrenergic receptors has not been reported in frog taste disc cells, the present finding of immunoreactivity of the NA-synthesizing enzyme, DBH, in type II–like cells supports the idea that type II cells transmit information to afferent nerves or to other taste disc cells via NA.

Osculati and Sbarbati (1995) did not show the basal process of type II cells in their schema but described that some type II cells showed a short basal process. Düring and Andres (1976) did not observe basal processes in cells that possessed a thick apical process, which were presumably type II cells. In the present study, we found that type II–like cells, which showed a thick apical process, possessed a long basal process extending toward the basal lamia. Further study is needed to clarify whether this long basal process makes synapses with afferent nerves.

Type III cells

Osculati and Sbarbati (1995) reported that type III cells possessed a single (sometimes branching) thin apical dendrite and one or more basal foot processes that made a synapse-like neuroepithelial junction and considered them as receptor cells. In the present study, DBH-like immunoreactivity was observed in type III–like cells with a thin apical process and one or more basal processes. This supports the argument that NA works as a chemical transmitter from the cells to afferent nerves and/or to other cells in frog taste discs, as suggested in rat taste bud cells (Herness et al. 2002).

Nagahama and Kurihara (1985) showed that taste responses increased when L-dopa, dopamine, or NA was administered intravascularly in frogs to which NA-depleting agents had previously been administered. Because the immunoreactivity of tyrosine hydroxylase, which synthesizes L-dopa from L-tyrosine, was not observed in the frog taste disc (Kuramoto 1988), taste cells in frogs might take up L-dopa or dopamine for NA synthesis.

Apical processes of type II cells tend to form clusters resembling a small bud in the taste disc (Osculati and Sbarbati 1995; Li and Lindemann 2003). In the present study, apical processes of type II and III cells were found to run close together and those immunoreactive cells seemed to form groups in the taste disc. In mammals, Roper (2006) presented a hypothetical model that taste information is transmitted from type II cells, which possess taste receptors, to type III cells, which do not show taste receptors but make synapses to the afferent nerve. In frogs, such cell-to-cell communications among type II and III cells (either between IIs, between IIIs, or between II and III) have not been reported. Further study is needed to clarify whether those cells communicate with one another and whether the group formation of those cells has any roles in taste transduction.

Type IV cells

In the present study, type IV cells could not be identified and DBH immunoreactivity of those cells was not clarified.

In mammals, serotonin-like immunoreactivity is reported in taste cells (Kim and Roper 1995). In frogs, Zancanaro et al. (1995) showed that fungiform papillae contained

serotonin by means of HPLC, but serotonin-like immunoreactivity has been found only in Merkel cell-like basal (type IV) cells of the frog taste disc (Kuramoto 1988; Toyoshima 1994). As those cells do not possess apical processes reaching the free surface of the taste disc, they are not considered to be taste receptor cells receiving taste substances; however, Morimoto and Sato (1977) observed that afferent nerve impulses in the glossopharyngeal nerve increased with intravascularly administered serotonin, and Imendra et al. (2000, 2002) reported that bath-applied serotonin modulated the electrical properties of taste receptor cells in frogs. These findings suggest that serotonin in type IV cells plays certain roles in the taste transduction of frogs.

In summary, immunoreactivity of DBH, an NA-synthesizing enzyme, was observed in type II– and type III–like cells, both of which possess an apical process reaching the free surface of the taste disc and are candidates for taste receptor cells in frogs. The result supports that NA works as a transmitter of taste transduction in frog taste discs.

Zancanaro et al. (1995) showed by means of HPLC that there was 20 times the amount of adrenalin than NA in frog fungiform papillae. They also showed that the content of adrenalin in fungiform papilla was higher than that of the general tongue epithelium, but the content of NA was not significantly different. Because adrenalin is synthesized from NA, the possibility cannot be excluded that adrenalin instead of NA works as a chemical transmitter in the taste disc.

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