Existence of subtypes of gustducin-immunoreactive cells in the vallate taste bud of guinea pigs*

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Summary. Vallate taste buds in the guinea-pig tongue were immunohistochemically investigated with regard to the colocalization of gustducin with calbindin-D28K (=spot 35 protein) and type III inositol triphosphate receptor (IP₃R-3) in order to characterize gustducin-immunoreactive cells. Individual taste bud cells ranged from totally immunopositive to totally immunonegative for these three molecules. Among the immunoreactive cells, gustducin-immunoreactive cells were divided into two cell populations: one immunopositive and the other immunonegative for calbindin-D28K. Applying our previous data to the present results, the former cells should belong to Type III cells designated by electron microscopy. This finding provides new evidence regarding the taste bud types of cells expressing gustducin in the guinea pig.

Introduction

The taste bud is a chemoreceptive sensory organ comprising specialized epithelial cells and neurites. Some nerve terminals make synaptic contacts with a discrete

type of taste-bud cell, *i. e.*, Type III cell (Murray *et al.*, 1969; Murray, 1986). From an ultrastructural perspective, therefore, Type III cells have been postulated to participate in taste reception (Murray *et al.*, 1969; Paran *et al.*, 1975; Takeda and Hoshino, 1975; Takeda, 1976; Idé and Munger, 1980; Yoshie *et al.*, 1990; Kanazawa, 1993).

Our previous immunocytochemical studies on guinea-pig vallate taste buds revealed that Type III cells exclusively displayed immunoreactivity for spot 35 protein (Yoshie *et al.*, 1991). This protein was originally isolated from bovine and rat cerebella (Yoshida and Takahashi, 1980; Yamakuni *et al.*, 1984, 1985) and was immunohistochemically localized in Purkinje cells (Yamakuni *et al.*, 1985). Spot 35 protein is synonymous with calbindin-D28K (Takagi *et al.*, 1986; see also Wasserman and Taylor, 1966).

For the last decade, meanwhile, gustation receptors and signal transduction molecules have been progressively elucidated as follows: *e.g.*, metabotropic (=G-protein-coupled) and ionotropic receptors including metabotropic glutamate receptors (mGluRs) and epithelial sodium channels (ENaCs), gustducin, phospholipase C, inositol triphosphate (IP₃), and phosphodiesterase (for reviews, see Kinnamon and Margolskee, 1996; Lindemann, 1996; Herness and Gilbertson, 1999; Gilbertson *et al.*, 2000). Among these molecules corresponding to reception-transduction systems, gustducin is a G-protein alpha subunit primarily cloned in murine taste buds (McLaughlin *et al.*, 1992). This subunit was then reported to be expressed in taste buds of several other mammalian species including humans (Takami *et al.*, 1994; Wong *et al.*, 1996; Boughter *et al.*, 1997; Cho *et al.*, 1998; Ming *et al.*, 1998; Tabata *et al.*, 2003).

In guinea pigs, however, no such data have been available. Consequently, we applied immunohistochemistry for gustducin to guinea pig-vallate taste buds and further attempted to specify the cell type(s) of gustducin-immunoreactive cells.

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Materials and Methods

Male and female guinea pigs weighing 250–350 g were used in this study. Animal treatment followed the instructions of the Institutional Animal Care and Use Committee at our university.

Tissue preparation

The animals were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and perfused through the arterial trunk with phosphate-buffered saline (pH 7.3) and successively with 4% paraformaldehyde in a 0.07 M phosphate buffer (pH 7.3). Circumvallate papillae were removed from the tongues and further immersed in the paraformaldehyde fixative for 2 to 12 h at 4°C.

The fixed specimens were rinsed overnight in the same phosphate buffer containing 30% sucrose at 4°C and rapidly frozen in isopentane precooled to –35°C. Sections 7 µm in thickness were made on a cryostat, mounted on APS-coated glass slides, and immediately processed for the following immunohistochemical procedures.

Antibodies and antigens

This study employed four kinds of primary antibodies. Mouse monoclonal antibodies against IP₃R-3 (type III IP₃ receptor) and calbindin-D28K were purchased from BD Biosciences (USA) and Sigma (USA), respectively. The rabbit polyclonal antibody against gustducin was from Santa Cruz Biotechnology (USA). The rabbit polyclonal antibody against spot 35 protein was generously donated by the Department of Neuropharmacology, Brain Research Institute, Niigata University, Niigata (see Yamakuni *et al.*, 1984). As secondary antibodies, fluorescent dye-conjugated IgGs (Alexa Fluor 555-labeled anti-mouse and Alexa Fluor 488-labeled anti-rabbit IgGs) were obtained from Molecular Probes (USA).

All corresponding antigens, except the spot 35 protein, were purchased from the primary antibody sources.

Immunohistochemical protocol

Immunohistochemistry was performed using the indirect fluorescence method.

For single immunomarking, individual specimen sections were incubated overnight at 4°C with one of the primary antibodies against IP₃R-3 (dilutions: 1:200 to 1:1600), calbindin-D28K (1:200 to 1:1600), spot 35 protein (1:500 to 1:2000), and gustducin (1:200 to

1:1600). After incubation, these sections were exposed to Alexa Fluor 555-labeled anti-mouse IgG (1:200), or Alexa Fluor 488-labeled anti-rabbit IgG (1:200) for 2 h at room temperature.

For double immunomarking, each section was incubated overnight at 4°C with mixtures of the anti-calbindin-D28K antibody (1:800) and anti-spot 35 protein antibody (1:1000), anti-calbindin-D28K antibody (1:800) and anti-gustducin antibody (1:400), or anti-gustducin antibody (1:400) and anti-IP₃R-3 antibody (1:400) followed by incubation with a mixture of Alexa Fluor 555-labeled anti-mouse IgG (1:100) and Alexa Fluor 488-labeled anti-rabbit IgG (1:100) for 2 h at room temperature.

The stained sections were observed and photographed under a confocal laser scanning microscope (LSM510, Zeiss, Germany) or fluorescence microscope equipped with a digital imaging system (Axioplan/AxioCam, Zeiss, Germany).

Specificity controls of the immunoreaction

All immunoreaction specificity, except for the spot 35 protein, was checked using the following three negative controls: omission of the primary antibodies, incubation of tissue sections with normal host animal sera in place of the primary antibodies, and incubation of sections with antibodies which had been preincubated at 4°C for 24 h with the corresponding antigens at 10–100 µg/ml. The immunospecificity for spot 35 protein has already been checked elsewhere (Iwanaga *et al.*, 1985; Yoshie *et al.*, 1988).

Results

All antibodies used in this study exhibited intense immunoreactivity of the taste bud cells. The immunohistochemical reactions after the three control procedures mentioned above were all negative, which consequently confirmed the specificities of all immunohistochemical findings (data not shown).

Confirming our previous findings (Yoshie *et al.*, 1988, 1991), immunoreactivity for spot 35 protein was also recognized in vallate taste bud cells (Fig. 1a). Double immunostaining for spot 35 protein and calbindin-D28K was detected in completely identical taste bud cells (Fig. 1a–c); therefore, both antibodies should recognize the same cells.

According to the immunostainability, the taste bud cells were divided into four classes: all cells immunopositive to those totally immunonegative for IP₃R-3, gustducin, and calbindin-D28K. Immunohistochemical reactivities

Fig. 1. Immunohistochemistry for spot 35 protein (**a**), calbindin-D28K (**b**), and overlay of **a** and **b** (**c**). Note that both immunoreactivities of the taste buds completely overlap. $\times 600$

Fig. 2. Immunohistochemistry for IP₃R-3 (**a**), gustducin (**b**), and an overlay of **a** and **b** (**c**). A large parts of IP₃R-3 immunoreactive cells are concomitantly immunoreactive for gustducin. $\times 1,200$

Fig. 3. Immunohistochemistry for calbindin-D28K (**a**), gustducin (**b**), and an overlay of **a** and **b** (**c**). Some parts of gustducin immunoreactive cells are also immunoreactive for calbindin-D28K. $\times 1,200$

of the individual taste bud cells to these substances were as follows. A subset — approximately 40% — of the cells was immunoreactive for IP₃R-3 (Fig. 2a). These IP₃R-3-immunoreactive cells were partly immunopositive for gustducin (Fig. 2a–c). Furthermore, some of the gustducin-immunoreactive cells exhibited immunoreactivity to calbindin-D28K (Fig. 3a–c). Conversely, all calbindin-D28K-immunopositive cells were immunostained for both gustducin and IP₃R-3, and all gustducin-immunopositive cells were immunostained for IP₃R-3. These immunohistochemical data are summarized in Table 1.

Discussion

The present study revealed that guinea pig vallate taste buds included gustducin-immunoreactive cells, some of which were concomitantly immunoreactive for calbindin-D28K, *i.e.*, spot 35 protein. This protein is a calcium-binding soluble protein isolated from cerebellar Purkinje cells (Yamakuni *et al.*, 1984; Hozumi *et al.*, 1986). Our previous electron-microscopic immunocytochemistry demonstrated that spot 35 protein immunoreactivity was localized exclusively in Type III cells of vallate taste buds in guinea pigs (Yoshie *et al.*, 1991).

Under a transmission electron microscope, the intrinsic constituents of mammalian taste buds are usually classified into four types of cells: basal or Type IV, Type I, Type II, and Type III cells (Murray *et al.*, 1969; Takeda and Hoshino, 1975; Takeda, 1976; Idé and Munger, 1980; Yoshie *et al.*, 1990; Kanazawa, 1993; for review, see Reutter and Witt, 1993). The basal cell resides at the baso-lateral region of the taste bud without reaching the taste pit apically, whereas three other types of cells are spindle-shaped extending apically to the taste pit. Among these types of cells, Type III cells make an afferent chemical synapse with the intragemmal nerve fiber and,

therefore, have been proposed to be primarily responsible for gustatory sensation (Murray *et al.*, 1969; Takeda and Hoshino, 1975; Takeda, 1976; Idé and Munger, 1980; Yoshie *et al.*, 1990; Kanazawa, 1993).

In the present study on guinea-pig taste buds, gustducin-immunoreactive cells were classified into two types: immunopositive and immunonegative to calbindin-D28K. Our previous papers regarding guinea pig taste buds demonstrated that only Type III cells were immunostained for spot 35 protein, (*i.e.* calbindin-D28K) (Yoshie *et al.*, 1990, 1991). Thus, the gustducin-immunoreactive cells with immunoreaction to calbindin-D28K should be Type III cells at least.

Concerning the cell type(s) expressing gustducin, various studies dealing with mammalian taste buds have been performed (Wong *et al.*, 1996; Boughter *et al.*, 1997; Yang *et al.*, 2000; Tabata *et al.*, 2003). These papers all described gustducin immunoreactivity as being confined to Type II cells, which have had an unclear function since the cell type was ultrastructurally characterized (Murray *et al.*, 1969). Although Type II cells make direct contact with the swellings of intragemmal nerve terminals, no chemical synaptic structures have been seen between Type II cells and the nerve terminal (Murray *et al.*, 1969; Takeda and Hoshino, 1975; Takeda, 1976; Idé and Munger, 1980; Yoshie *et al.*, 1990; Kanazawa, 1993).

On the other hand, a recent novel transmission mechanism — including the transmitter — has been proposed between Type II cells and the nerve terminal (Huang *et al.*, 2007; Romanov *et al.*, 2007). According to these authors, ATP as a transmitter passes through the hemichannel of pannexin penetrating the plasma membrane of Type II cells and binds to its ionotropic receptors, P2Xs, located on the gustatory nerve endings (Romanov *et al.*, 2007; see also Kataoka *et al.*, 2004, 2006).

In conclusion, the present study revealed that some taste bud cells showed immunoreactivity to both

Table 1. Immunoreactivities of taste bud cells in the guinea-pig vallate papilla

Antibodies against:	Calbindin-D28K	Gustducin	IP ₃ R-3
Cell ₀	—	—	—
Cell ₁	—	—	+
Cell ₂	—	+	+
Cell ₃	+	+	+

+: reactive, —: nonreactive

calbindin-D28K and gustducin in the guinea-pig tongue. This strongly suggests that some Type III cells express gustducin. To our knowledge, this is the first report concerning cell-type immunoreactivity to gustducin other than Type II cells. To confirm and determine the exact types of gustducin-immunopositive cells, an immunocytochemical examination at the electron microscopic level is currently underway.

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