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Immunohistochemical Localization of Carbonic Anhydrase Isozyme II in the Gustatory Epithelium of the Adult Rat

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Abstract

The distribution of carbonic anhydrase isozyme II (CA II)-like immunoreactivity (-LI) in the gustatory epithelium was examined in the adult rat. In the circumvallate and foliate papillae, CA II-LI was observed in the cytoplasm of the spindle-shaped taste bud cells, with weak immunoreaction in the surface of the gustatory epithelium. No neuronal elements displayed CA II-LI in these papillae. There was no apparent difference in the distribution pattern between the anterior and posterior portions of the foliate papillae. In immunoelectron microscopy, immunoreaction products for CA II were diffusely distributed in the entire cytoplasm of the taste bud cells having dense round granules at the periphery of the cells. No taste bud cells displaying CA II-LI were detected in the fungiform papillae, but a few thick nerve fibers displayed CA II-LI. In the taste buds of the palatal epithelium, neither taste bud cells nor neuronal elements exhibited CA II-LI. The present results indicate that CA II was localized in the type I cells designated as supporting cells in the taste buds located in the posterior lingual papillae of the adult animal.

Introduction

Carbonic anhydrase (CA) is an enzyme that promotes the hydration of CO_2 to HCO_3^- and H^+. To date, six isozymes have been purified from mammals, and at least three mammalian genes encoding new isozymes for CA have been characterized. Among these CA isozymes, the highly catalytic cytoplasmic CA isozyme II (CA II) is thought to play an important role in the regulation of ion transportation and intracellular pH (Dodgson, 1991). Histochemical and immunohistochemical studies have revealed that this enzyme occurs in ductular, tubular or surface epithelia high in ion transport such as in the kidney, salivary gland, gastrointestinal tract and oral epithelium (Spicer *et al.*, 1979, 1990; Brown *et al.*, 1983; Asari *et al.*, 1989; Ogawa *et al.*, 1992). CA has been also found in rat type I skeletal fibers (Jeffery *et al.*, 1986), in large-sized primary afferent neurons (Kazimierczak *et al.*, 1986; Aldskogius *et al.*, 1988; Carr and Nagy, 1993; Ichikawa *et al.*, 1994) and in myelinated nerve fibers in the peripheral nervous system (Cammer and Tansey, 1987).

The taste buds are specialized sensory receptors for detecting chemical substances, and are mostly present in the lingual and palatal epithelia. In the lingual epithelium, the taste buds are located in three lingual papillae: circumvallate, foliate and fungiform papillae. The taste buds in the palatal epithelium are mostly localized in three regions: in the nasopalatine ducts (incisive papilla), at the border between hard and soft palates in a structure referred to as the 'Geschmacksstreifen', and along the surface of the soft palate (Miller and Spangler, 1982). The taste buds are also present in extra-oral regions such as the pharynx and larynx (Travers and Nicklas, 1990). The taste buds have been reported to contain various bioactive substances (summarized in table 3 of Welton *et al.*, 1992). It is reported that acetazolamide, a CA inhibitor, causes altered taste perception (Miller and Miller, 1990), suggesting the involvement of CA in the perception of taste. The presence of CA in the gustatory epithelium has been reported by various researchers: Brown *et al.* (1984) have reported the presence of CA in the taste buds of the rat circumvallate papilla, but they focused on the presence of CA in the circumvallate papilla by Hansson's histochemical method which cannot discriminate the isozymes of CA, and failed to reveal the distribution of CA in other lingual papillae. Suzuki *et al.* (1996) have shown CA activity in the type I cells (supporting cells) of mouse circumvallate papilla as revealed by enzyme histochemistry. Böttger *et al.* (1996) have demonstrated the localization of CA isozymes in the lingual papillae by immunohistochemistry, but they did not show the ultra-

structural localization of these isozymes. A recent study has revealed the presence of gene encoding CA in the rat taste-bud-enriched lingual epithelium (Hoon and Ryba, 1997). The present study was therefore designed to examine the immunocytochemical localization of CA II in the gustatory epithelium in the adult rat using a specific antibody against CA II. Moreover, since CA has been reported to be localized in the myelinated axons (Cammer and Tansey, 1987; Aldskogius *et al.*, 1988), its distribution was compared with that of protein gene product 9.5 (PGP 9.5), a general neuronal marker (Jackson and Thompson, 1981; Thompson *et al.*, 1983; Gulbenkian *et al.*, 1987), in the gustatory epithelium.

Materials and methods

All experiments were reviewed and approved by Osaka University Faculty of Dentistry Animal Use and Care Committee prior to the experiments.

Animals and tissue preparation

A total of 10 male Sprague–Dawley rats, weighing 200–250 g, were used in the present study. Animals (n = 5) were deeply anesthetized and perfused transcardically with 0.02 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The tongue and palatal epithelium were dissected out and post-fixed in the same fixative at 4°C for 2–3 days, then cryoprotected in 20% sucrose/PBS at 4°C overnight. Frontal sections (tongue and incisive papilla) or longitudinal sections (border between hard and soft palates) were cut at a thickness of 12–14 μm with a cryostat, thaw-mounted onto poly-L-lysine subbed glass slides and air-dried for at least 60 min for light microscopy. Two animals were sacrificed, and circumvallate papillae were quickly removed, fixed in Carnoy's solution for 2 h, embedded in paraffin and cut at a thickness of 5 μm.

For immunoelectron microscopy, animals (n = 3) were fixed by a transcardic perfusion with a mixture of 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, and immersed in 20% sucrose/PBS. Circumvallate papillae were cut at a thickness of 60–80 μm with a Microslicer (Dosaka EM, Kyoto, Japan), collected in 0.02 M PBS and treated as free-floating sections.

Immunohistochemistry

Paraffin-embedded sections were de-paraffinized with xylene and rehydrated through a descending series of ethanol. The sections were rinsed in PBS several times, then treated with methanol containing 0.3% H_2O_2 for 30 min to block endogenous peroxidase activity. Following treatment with PBS containing 1% bovine serum albumin (Sigma, St Louis, MO) for 30 min, they were incubated with polyclonal sheep anti-human CA II (1:4000; The Binding Sites, Cambridge, UK) for 16–18 h each at room temperature. Sections were incubated with biotinylated donkey anti-sheep IgG (1:300; The Binding Sites) and subsequently with ABC complex (Vector, Burlingame, CA) for 90 min each at room temperature. Horseradish peroxidase (HRP) was visualized with incubation with 0.05 M Tris–HCl buffer (pH 7.5) containing 0.04% 3,3′-diaminobenzidine (DAB) and 0.03% H_2O_2. Immunoreactions were intensified with nickel ammonium sulfate (NAS; 0.08–0.1%). Sections were counterstained with methyl green, dehydrated through an ascending series of ethanol and coverslipped with Permount (Fisher Scientific, NJ).

For immunoelectron microscopy, circumvallate papillae were immunostained by the ABC method as mentioned above, except that NAS intensification was omitted. These sections were further fixed with 1% OsO_4 reduced with 1.5% potassium ferrocyanide for 30 min at room temperature. Sections were dehydrated through an ascending series of ethanol and embedded in Epon 812 resin. Ultrathin sections were prepared with a diamond knife and observed with a Hitachi transmission electron microscope (H-7000, Hitachi Co., Tokyo) at an accelerating voltage of 75 kV after slight staining with uranyl acetate and lead citrate.

To examine the correlation between CA II and PGP 9.5, sections were first incubated with polyclonal sheep anti-human CA II antibody (1:2000) and then with polyclonal rabbit anti-PGP 9.5 antibody (1:1000; Ultraclone, Cambridge, UK) for 16–18 h each at room temperature. These sections were incubated with biotinylated donkey anti-sheep IgG (1:300) and subsequently with fluorescein isothiocyanate (FITC)-conjugated streptavidin (1:50; Amersham, Bucks, UK) for 90 min each at room temperature. Lissamine rhodamine (LRSC)-conjugated anti-rabbit IgG (1:500; Jackson ImmunoResearch, West Grove, PA) was applied to label PGP 9.5 for 90 min at room temperature. All incubations were carried out in a moist chamber. These sections were rinsed in PBS, coverslipped with PermaFluor (Immunon, Pittsburgh, PA), examined with an Olympus fluorescent microscope equipped with an appropriate excitation filter (B filter for FITC and G filter for LRSC) and photographed with Kodak Tri-X pan film (ASA 400).

The specificity of the primary antibody to human CA II was examined by an absorption test. The diluted primary antibody was preabsorbed with CA II purified from red blood cells of male Sprague–Dawley rats by affinity chromatography as described elsewhere (Ogawa *et al.*, 1992). These sections incubated with the preabsorbed primary antibody did not show any immunoreactions. The characterization and origin of the PGP 9.5 antiserum have been reported in the literature (Gulbenkian *et al.*, 1987). Thus, we considered that the immunoreactions observed in the present study were specific for CA II and PGP 9.5.

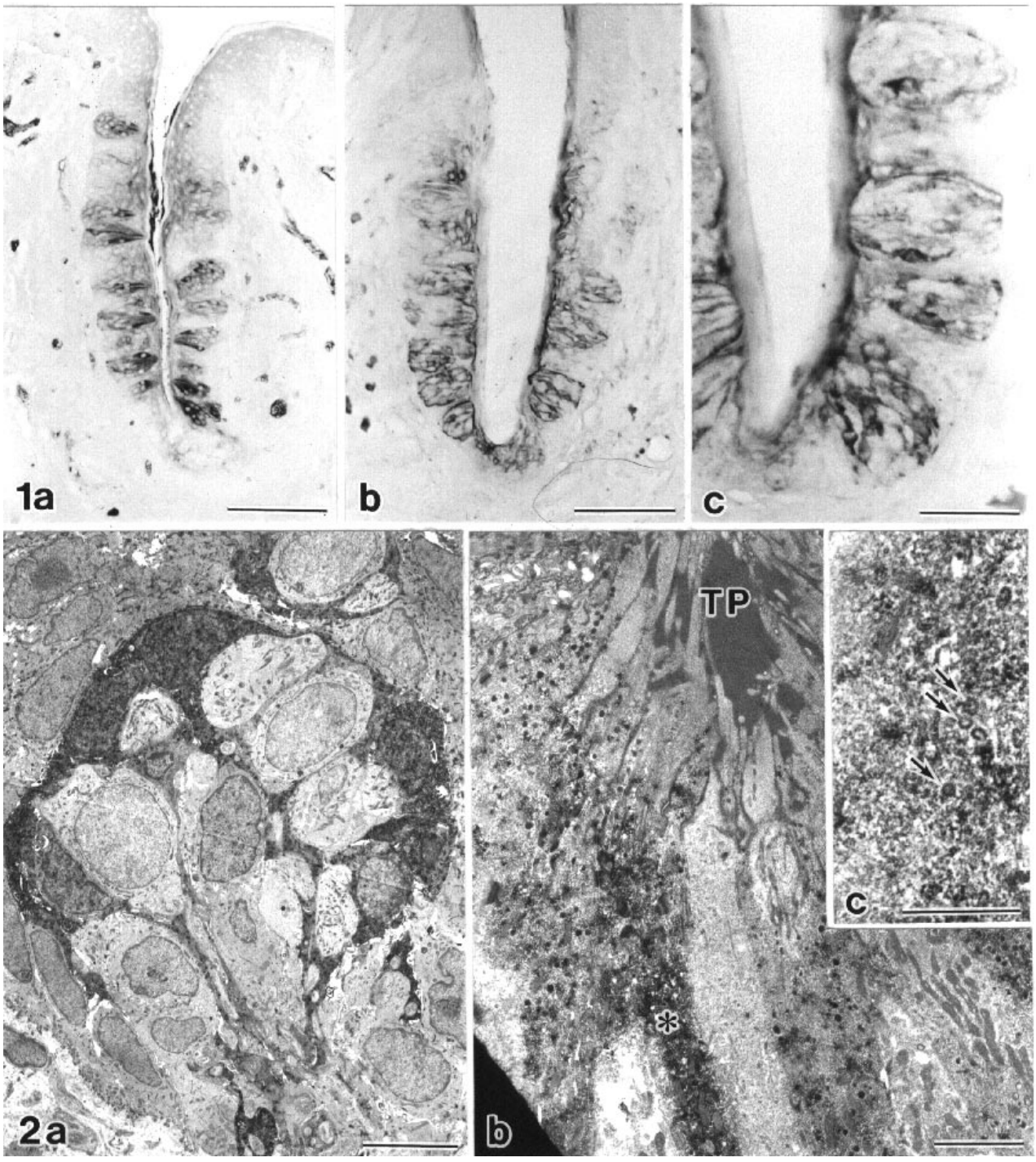

Figure 1 CA II-LI in the circumvallate papilla fixed with Carnoy's solution **(a)** and fixed in 4% paraformaldehyde **(b, c)**. (a) CA II-LI is observed in the cytoplasm of spindle-shaped cells located in the lower half of the trench wall epithelium of circumvallate papilla. (b) Strong immunoreactions for CA II are observed in the membrane of the taste bud cells, and weak reactions are detected in the cytoplasm. (c) A magnified view of CA II-LI in the taste bud cells. Moderate to weak immunoreactions for CA II are seen in the cytoplasm of the spindle-shaped cells. Note the strong shrinkage of the tissues in Carnoy's solution fixed sections (compare a and b; photographs were taken at the same magnification). Bars: a and b, 100 μm; c, 50 μm.

Figure 2 Immunoelectron micrographs of CA II-IR cells in the circumvallate papilla. **(a)** Immunoreaction products are localized in the cytoplasm of dark cells. **(b)** Numerous dense round granules gather at the periphery of the CA-IR cell (asterisk). Not all dark cells display CA II-LI. **(c)** Higher magnification view of the immunoreactive cell. Note the many granules (arrows) in its cytoplasm. TP, taste pore. Bars: a, 5 μm; b, 2 μm; c, 1 μm.

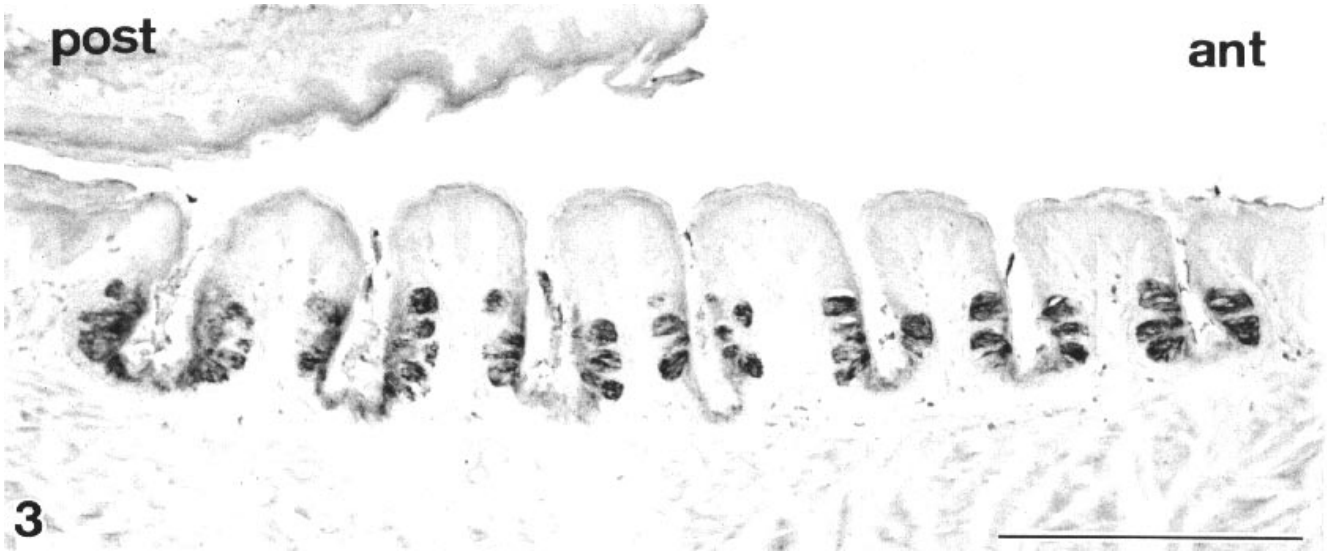

Figure 3 A photomicrograph of CA II-LI in the foliate papillae. CA II-IR cells are observed in the lower half of every trench. No apparent differences are observed in the distribution pattern of CA II-IR cells between anterior (ant) and posterior (post) portions of the foliate papillae. Bar: 500 μm.

Results

A subpopulation of spindle-shaped taste bud cells in the circumvallate papilla displayed CA II-LI (Figure 1a–c). In the paraffin sections fixed with Carnoy's solution, CA II-LI was detected throughout the cytoplasm of the spindle-shaped taste bud cells at the lower half of the trench wall (Figure 1a). In the frozen sections fixed with 4% paraformaldehyde, the spindle-shaped taste bud cells showed strong CA II-LI at the membrane of the taste buds cell, but moderate to weak reaction in the cytoplasm (Figure 1b,c). Most taste buds contained several CA II-immunoreactive (-IR) cells (Figure 1a–c). In addition to taste bud cells, weak immunoreaction for CA II was occasionally observed in the surface of the trench wall epithelium (Figure 1a–c). No neuronal elements showed CA II-LI in the circumvallate papilla (Figure 1a–c). Under the electron microscope, immunoreaction products for CA II were distributed diffusely throughout the entire cytoplasm of taste bud cells (Figure 2a). These cells had many dense round granules at the periphery of the cytoplasm, and not all cells with round granules showed CA II-LI (Figure 2b,c).

The distribution and localization of CA II-LI in the foliate papillae were comparable to those in the circumvallate papilla; CA II-LI was recognized in the spindle-shaped taste bud cells. No apparent differences in the distribution and numbers of CA II-IR cells existed between the anterior and posterior trenches of foliate papillae (Figure 3).

The taste buds in the fungiform papillae lacked apparent CA II-IR cells, but a few thick nerve fibers which were immunoreactive for PGP 9.5-LI displayed CA II-LI (Figure 4a,b).

In the incisive papilla, neither taste bud cells nor neuronal elements exhibited CA II-LI, but their epithelium exhibited weak CA II-LI (Figure 5). The taste buds at the border of the soft and hard palate did not have CA II-IR cells (Figure 6a,b). As with the border between the hard and soft palates, no taste bud cells or nerve fibers displayed CA II-LI in the taste buds in the epithelium of the posterior soft palate (data not shown).

Discussion

The present study demonstrated the distribution of CA II-LI in the gustatory epithelium of the rat. The distribution of CA II in the circumvallate papilla was in line with the previous histochemical study of CA in the circumvallate papilla (Brown *et al.*, 1984). However, Brown *et al.*'s histochemical technique showed the presence of CA activity in the lingual glands, but our immunohistochemistry with an antibody against CA II failed to demonstrate the occurrence of CA II-LI in the lingual gland. The difference of staining pattern may be due to the different methodology (Hansson's histochemical method for CA versus immunohistochemistry for CA II).

For detection of CA by histochemistry and immunohistochemistry, Carnoy's solution has often been used as a favorite fixative. In the present study, we compared the staining pattern between the sections fixed with Carnoy's solution and those fixed with 4% paraformaldehyde, and found much stronger cytoplasmic immunoreactions for CA II in the Carnoy's solution fixed sections than the 4% paraformaldehyde-fixed frozen sections. Carnoy's solution cannot be processed for electron microscopy and shrinks the specimens during tissue preparation owing to the ethanol content. Thus, in the present study, we used a 4% para-

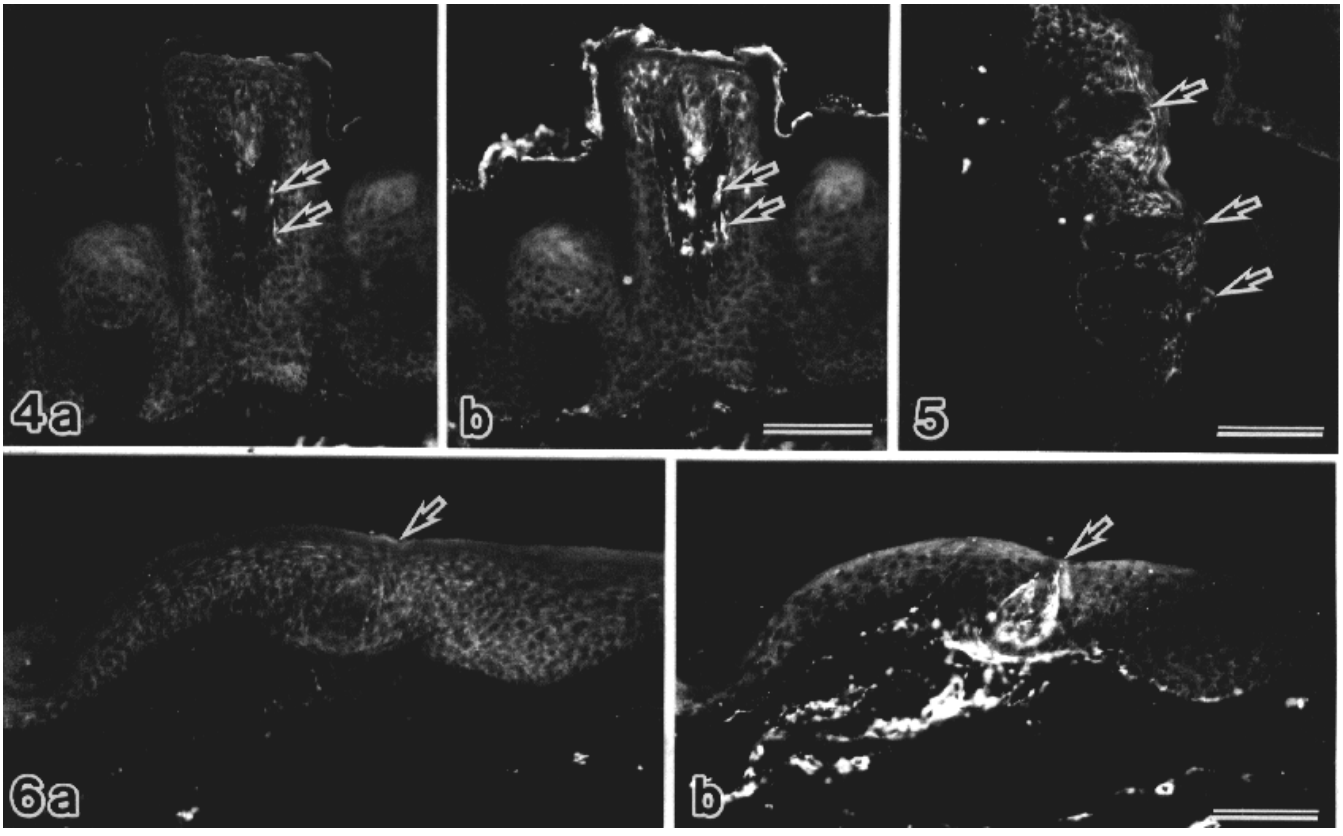

Figure 4 A paired photomicrograph of CA II-LI **(a)** and PGP 9.5-LI **(b)** in the fungiform papilla. No apparent CA II-IR cells are detected, but thick nerve fibers exhibit CA II-LI (arrows in a and b), which are also positive for PGP 9.5-LI (b). Bar: 50 μm.

Figure 5 A photomicrograph of CA II-LI in the incisive papillae. Neither taste bud cells nor neuronal elements show CA II-LI. The epithelium shows slight CA II-LI. Arrows indicate the taste buds. Bar: 100 μm.

Figure 6 A paired photomicrograph of CA II-LI **(a)** and PGP 9.5 **(b)** in the taste buds at the border between soft and hard palates. Neither taste bud cells nor neuronal elements display CA II-LI. Arrows indicate the taste buds. Bar: 100 μm.

formaldehyde solution as a fixative for light and electron microscopical observations.

It is generally accepted that the taste buds are composed of four types of cells: dark, light, intermediate and basal cells. The dark, light and intermediate cells correspond to type I, II and III cells respectively. The present light microscopic observation showed that CA II-IR cells were spindle-shaped, implicating that they are not basal cells. A previous ultrastructural study reported that type I cells are characterized by the presence of dense round granules at the periphery of the cells in the rat taste buds of the circumvallate papilla (Takeda and Hoshino, 1975). The present immunoelectron microscopy revealed that CA II-IR cells contained rich dense round granules at the apical portion of the cells, indicating that CA II was localized in the type I cells. This observation is consistent with a previous report that CA activity is present in the type I cells as demonstrated by enzyme histochemistry (Suzuki *et al.*, 1996).

It is of interest that CA II-IR cells were observed in the taste buds located in the posterior lingual papillae, i.e. circumvallate and foliate papillae, but not in other taste buds in the fungiform papillae, nasopalatine duct or palate epithelia. There are three major differences in the characteristics of lingual papillae between the anterior and posterior portions of the tongue. Firstly, the taste buds in the posterior lingual papillae (circumvallate and foliate papillae) receive a gustatory nerve supply from the lingual–tonsilar branch of the glossopharyngeal nerve, while those in the fungiform papillae receive a gustatory innervation from the chorda tympani. Therefore, different gustatory innervations may be account for the different distribution of CA II-LI in the taste buds. However, a few taste buds in the anterior portion of the foliate papillae are also innervated by the chorda tympani, and the distribution and numbers of CA II-IR cells in the anterior portion of the foliate papillae did not seem to differ significantly from those in the posterior portion of the foliate papillae. Thus, it seems better to conclude that no particular correlation was found between

the distribution pattern of CA II-IR taste bud cells and the innervation pattern of the gustatory nerve. Secondly, the taste buds in the posterior lingual papillae have been reported to be more sensitive to bitter stimulation than those in the anterior lingual papillae (Frank, 1991). A recent *in vitro* study indicated that the intracellular free calcium concentration increased following the application of bitter substances in single taste bud cells of the guinea pig (Orola *et al.*, 1992). As CA II serves in the regulation of intracellular pH, it is likely that CA II in the posterior lingual papillae may be associated with the regulation of intracellular pH in responses to specific kinds of taste stimuli, presumably bitter stimuli, rather than the direct involvement of the transmission of gustatory information. This idea is supported by the present immnoelectron microscopical analysis showing that CA II-LI was localized in the type I cells (supporting cells, or dark cells) but not in the type III cells (gustatory receptor cells), and that neuronal elements rarely displayed CA II-LI in the taste buds. Similar observations have been reported in the central nervous system showing that CA II is present in the oligodendrocytes and, to some extent, also in astrocytes—supporting cells in the nervous system—and rarely in the neurons (Ghandour *et al.*, 1980; Cammer and Tansey, 1988; Agnati *et al.*, 1995). Thirdly, the relation between the taste buds and the salivary gland should be considered. The duct of von Ebner's glands opens to the base of the trench of circumvallate and foliate papillae, and the epithelium at the base of the trench wall transiently changes to the epithelium of the duct, suggesting that these two epithelia share similar cytochemical characteristics. This speculation is supported by previous histochemical results showing strong CA activity in the ductal epithelium and secretory cells of von Ebner's gland as well as the gustatory epithelium of the circumvallate papillae (Brown *et al.*, 1984; Suzuki *et al.*, 1996; the present study). If this speculation is likely, it is reasonable that the taste buds in the palatal epithelium lacked CA II-LI since the duct of the palatal gland opens directly to the soft palate epithelium, not to the taste buds in the palatal epithelium. Further analysis is required to examine the expression of CA II in the developing von Ebner's glands and gustatory epithelium, and this study is now under progress in our laboratory.

In the fungiform papillae, thick nerve fibers exhibiting CA II-LI were present in the lamina propria, which is in agreement with the results of a previous study (Böttger *et al.*, 1996). The anterior portion of the tongue receives gustatory innervation from the chorda tympani, whose cell bodies are localized in the geniculate ganglion, and somatosensory innervation from the lingual nerve derived from the trigeminal ganglion. It is difficult to determine the exact origin of CA II-IR nerve fibers observed in the fungiform papillae since neurons in both the geniculate and trigeminal ganglia have CA activity (Aldskogius *et al.*, 1988; Ichikawa *et al.*, 1994). Further analysis using a retrograde tracing technique may clarify the exact origin of these nerve fibers.

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