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Chronic hypoxia alters calbindin D-28k immunoreactivity in lingual and laryngeal taste buds in the rat

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Summary. The distribution and abundance of the calcium binding protein, calbindin D-28k (CB) immunoreactivity in the taste buds of the circumvallate papillae and larynx were compared between normoxic and chronically hypoxic rats (10% O_2 for 8 weeks). In the normoxic rats, CB immunoreactivity was observed in some cells and fibers of the intragemmal region of the taste buds in the circumvallate papillae. In contrast, in the subgemmal region of the laryngeal taste buds, fibers but not cells were immunoreactive for CB. In chronically hypoxic rats, CB immunoreactive cells and fibers in the taste buds were decreased in the circumvallate papillae. In the laryngeal taste buds, the density of the subgemmal CB immunoreactive fibers in chronically hypoxic rats was greater than in normoxic rats. It is considered that function of the laryngeal taste buds is different from that of the lingual taste buds, so that laryngeal taste buds may be involved in chemosensation other than taste. The altered density of CB immunoreactive cells and fibers in the lingual and laryngeal taste buds is a predominant feature of hypoxic adaptation, and chronic hypoxic exposure might change the chemical sensitivity of the circumvallate papillae and larynx through the regulation of intracellular Ca^{2+}.

Key words: Chronic hypoxia, Calcium binding protein, Tongue, Larynx, Adaptation

Introduction

Most taste buds are found on the tongue but a substantial number are also distributed in the oral cavity, pharynx and larynx (Miller, 1977). The lingual taste buds are specialized chemosensory receptors responding to various chemical substances. Taste buds in the lingual epithelium of rats are present in three types of lingual papillae, i.e., the circumvallate, foliate, and fungiform papillae, and they are in an optimal location for modulating feeding and drinking because of routine contact with ingested substances. Taste buds in the rat pharynx and larynx account for 10% of all taste buds in the body (Travers and Nicklas, 1990). Since taste buds in the larynx are located at the entrance of the lower respiratory tract, they probably play a role in respiratory modulation or airway protection rather than in food selection (Stedman et al., 1980; Bradley, 2000; Nishijima and Atoji, 2004).

Calcium binding proteins are considered to play important roles in the storage and transport of intracellular Ca^{2+} (Baimbridge and Miller, 1982). These proteins can be divided into two groups, i.e., a "buffer" type and a "trigger" type (Andressen et al., 1993). Calbindin D-28k (CB), which was originally isolated from chick digestive tracts (Taylor, 1974), is a buffer type calcium binding protein (Andressen et al., 1993). Recently, the occurrence of CB immunoreactive nerve fibers and cells has been reported in the rat lingual taste buds (circumvallate; Johnson et al., 1992; Yamagishi et al., 1993; Miyawaki et al., 1996; foliate and fungiform; Miyawaki et al., 1996). Miyawaki et al. (1998) speculated that the CB fibers might participate in gustatory transmission, because the CB immunoreactive terminals made synaptic contact with the taste cells. As far as we are aware, however, the occurrence and distribution of CB immunoreactivity in the rat laryngeal taste buds have not been reported except for two papers (Yamamoto et al., 2000; Nishijima and Atoji, 2004), and the physiological role of CB fibers in the larynx remains unclear.

Hypoxic adaptation of CB immunoreactive fibers has been demonstrated in the rat arterial chemoreceptor organ, carotid body (Kusakabe et al., 2000a). According

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to this, relative abundance of CB fibers in the chronically hypoxic carotid bodies was less than in the normoxic carotid bodies. In the present study, the distribution and relative abundance of CB immunoreactive nerve fibers and cells in the taste buds located in the circumvallate papilla, which may be representative of lingual papillae, and the larynx were compared between normoxic and chronically hypoxic rats, and a possible role of this protein in adaptive changes to a low O_2 environment is discussed.

Materials and methods

Chronic hypoxic exposure

Eight-week-old Wistar rats were placed in an air-tight acrylic chamber (50x50x60 cm) with two holes. One hole, located at the top of the chamber walls, was connected to a multi-flowmeter (MODEL-1203, KOFLOC, Japan) to deliver a hypoxic gas mixture (10% O_2 in N_2: total 20 L/min) into the chamber. The flow of air and N_2 was regulated by the multi-flowmeter, and the O_2 and CO_2 levels within the box were monitored with a gas analyzer (Respina 1H26, NEC San-ei, Japan). The second hole was located at the bottom wall of the chamber and was used to exhaust the gas mixture. The temperature within the chamber was maintained at 25°C. This hypoxic condition was confirmed to be hypocapnic to the rats in a previous study (Hirakawa et al., 1997). Eight rats were exposed chronically in this chamber for three months with food and water available *ad libitum*. Eight control rats were housed for three months in the same chamber ventilated by air at the same flow rate. The chamber was opened for 10 min every 3 days for husbandry.

All experiments with animals were performed in accordance with the "Principles of Laboratory Animal Care" (NIH publ. no. 86-23, revised 1985) and with the "Guiding Principles for the Care and Use of Animals in the Fields of Physiological Sciences" published by the Physiological Society of Japan.

Tissue preparation

The animals were intraperitoneally anesthetized with sodium pentobarbital (0.05 mg/g). Anesthetized animals were perfused through a thin nylon tube inserted into the left ventricle of the heart with 0.1M heparinized phosphate-buffered saline (PBS), followed by Zamboni's fixative solution (0.2% picric acid and 4% paraformaldehyde in 0.1M PBS at pH 7.4) at a constant flow rate.

The circumvallate papillae of the tongue and the laryngeal segments were dissected, and postfixed in the same fixative solution for an additional 8 h at 4°C. After a brief washing in PBS, the specimens were transferred to 30% sucrose in PBS at 4°C for 24 h. Then they were cut serially at 20 µm on a cryostat, and mounted on poly-L-lysine-coated slides.

Immunohistochemistry

The sections were processed for immunohistochemistry by the peroxidase-antiperoxidase (PAP) method. Prior to PAP treatment, sections were dipped in a fresh 0.3% solution of hydrogen peroxide in methanol for 30 min to inhibit endogenous peroxidase activity. After washing in 0.3% Triton X-100 in 0.1M PBS (PBST), sections were treated for 1h with a protein blocking agent (Immunon, USA) at room temperature to block nonspecific protein binding sites. Then they were incubated at 4°C for 24h with rabbit polyclonal antiserum against calbindin D-28k (1:1000; Chemicon, USA). After rinsing in 3 changes of PBST, sections were transferred for 3h to anti-rabbit IgG (Organo Technica, USA) diluted 1:100 with PBST at room temperature. Then they were rinsed in 3 changes of PBS, and were transferred for 1.5 h to rabbit PAP complex (Jackson Immuno Research, USA) diluted 1:100 with PBS at room temperature. After rinsing in 3 changes of PBS, preparations were transferred to 0.05M Tris-HCl buffer at pH 7.8 for 10 min, and the peroxidase activity was demonstrated with 3,3'-diaminobenzidine and H_2O_2. The immunostaining procedure has been detailed in a previous report (Kusakabe et al., 1991). For immunohistochemical controls, non-immune rabbit serum and PBS were used instead of the primary antibody. No specific reaction was observed on the control sections.

Evaluation of sections

Two observers, blinded to experimental information, evaluated the staining scores independently. Staining intensity was assigned using a semiquantitative immunohistochemical scoring system as follows: very few (±), few (+), moderate number (++) and abundant (+++).

Results

Circumvallate papillae

In the normoxic rats, abundant CB immunoreactive fibers were observed in the lamina propria of the circumvallate papillae (Fig. 1A) and some of these fibers penetrated into the intragemmal region of the taste buds (Fig. 1B). In addition to CB immunoreactive fibers, one to three spindle-shaped cells in a taste bud at each section were immunoreactive for CB, and the immunoreactivity was observed in the cytoplasm (Fig. 1B). Some taste buds had no CB immunoreactive cells. In the chronically hypoxic taste buds in the circumvallate papillae, CB immunoreactive cells and fibers in the intragemmal region were less numerous than in the normoxic ones (Fig. 1D). In addition, in the chronically hypoxic rats, the number of CB immunoreactive fibers in the lamina propria was remarkably decreased (Fig. 1C). There was no

significantly definite change in the abundance of CB immunoreactive fibers in the subgemmal region of the taste buds between the normoxic and chronically hypoxic circumvallate papillae. The relative abundance of CB immunoreactive cells and fibers in the circumvallate papillae of both normoxic and chronically hypoxic rats is summarized in (Table 1).

Larynx

There were no obvious morphological differences between normoxic and chronically hypoxic laryngeal taste buds in the sections stained with hematoxylin and eosin as previously reported (Kusakabe et al., 2000b). In the laryngeal taste buds of the normoxic rats, immunoreactivity for CB was observed in a small number of nerve fibers located in the deep part of the lamina propria, and these positive fibers extended to the subgemmal region. Some of them penetrated into the taste buds, but no CB immunoreactive cells were observed (Fig. 2A). In the chronically hypoxic laryngeal taste buds, the relative abundance of CB fibers in the

Table 1. Distribution and relative abundance of calbindin D-28k cells and fibers in normoxic and hypoxic taste buds and lamina propria in the circumvallate papillae and the larynx.

	Normoxia	Hypoxia
Circumvallate papillae		
Taste cells	++	+
Intragemmal nerve fibers	++	+
Subgemmal nerve fibers	±	±
Lamina propria	+++	+
Larynx		
Taste cells	–	–
Intragemmal nerve fibers	±	±
Subgemmal nerve fibers	+	++
Lamina propria	+	+

Grading of frequency of immunoreactive cells and nerve fibers: ±, very few; +, few; ++, moderate number; +++, abundant.

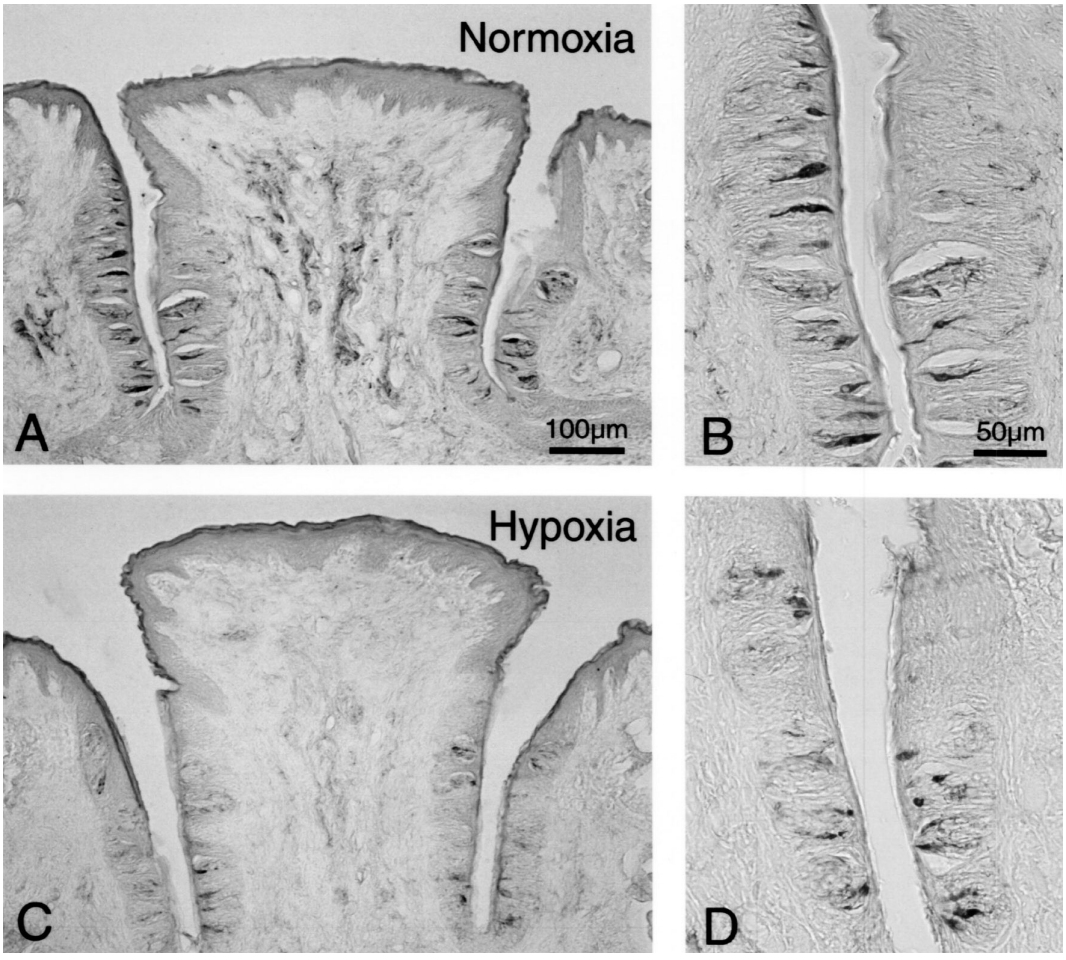

Fig. 1. Calbindin D-28k immnunoreactive fibers are observed in the lamina propria of the circumvallate papillae of the normoxic **(A)** and chronically hypoxic **(C)** rats. The number of calbindin D-28k immnunoreactive fibers in the lamina propria of the chronically hypoxic circumvallate papillae is remarkably decreased. Under high magnification, calbindin D-28k immnunoreactive cells and fibers in the taste buds in the chronically hypoxic circumvallate papillae **(D)** were less numerous than in the normoxic ones **(B)**.

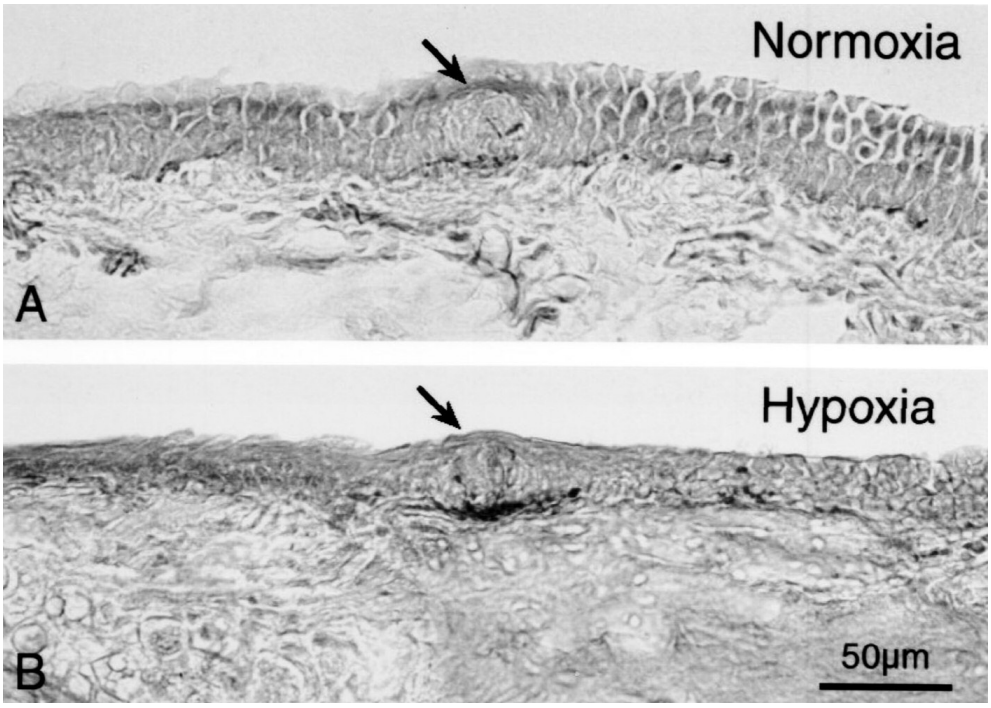

Fig. 2. Calbindin D-28k immnunoreactive fibers are observed in the laryngeal taste buds (arrow) of the normoxic **(A)** and chronically hypoxic **(B)** rats. Note the remarkable increase in the density of calbindin D-28k fibers in the subgemmal region of the chronically hypoxic laryngeal taste buds.

subgemmal region was greater than in the normoxic taste buds (Fig. 2B), but there was no distinct difference in the abundance of CB immunoreactive cells and fibers in the intragemmal region or the lamina propria between normoxic and chronically hypoxic rats. The relative abundance of CB immunoreactive fibers in the larynx of both normoxic and chronically hypoxic rats is also summarized in (Table 1).

In the circumvallate papillae and laynx, there was no difference of immunoreactivity for PGP 9.5, that is a general marker of the nervous system, between normoxic and chronically hypoxic rats (data not shown).

Discussion

The present study showed that there was a different staining pattern for CB between the lingual and laryngeal taste buds of normoxic rats. In the circumvallate papillae, CB immunoreactivity was observed in abundant nerve fibers in the lamina propria and in a moderate number of fibers in the intragemmal regions of the taste buds. Some cells were also positive for CB. On the basis of morphological features, CB immunoreactive cells in the taste buds of circumvallate papillae are thought to be taste cells receiving stimuli and transmiting information to afferent fibers (Miyawaki et al., 1998). Miyawaki et al. (1998) also speculated that CB immunoreactive nerve fibers might participate in the transmission of gustatory information because they establish synaptic contact with taste cells.

In the laryngeal taste buds of normoxic rats, immunoreactivity for CB was observed in nerve fibers at the intragemmal, subgemmal region and the lamina propria, but no CB positive taste cells were found. The laryngeal taste buds were observed mainly on the laryngeal surface of the epiglottis and on the aryepiglottic folds, but not on the vocal cords. The taste buds in the larynx probably play a role in respiratory modulation or airway protection rather than food selection, because they are located at the entrance of the lower respiratory tract (Stedman et al., 1980; Bradley, 2000; Nishijima and Atoji, 2004). Storey and Johnson (1975) have found that stimulation of the laryngeal mucosa with relatively innocuous fluids such as water or saturated sodium bicarbonate can produce a significant central apnea or disruption of respiration. The difference in the immunoreactivity for CB between the lingual and the laryngeal taste buds of normoxic rats may indicate a difference between the function of the lingual and the laryngeal taste buds.

In the chronically hypoxic rats, CB immunoreactive cells and nerve fibers in the intragemmal region and lamina propria of the circumvallate papillae were less numerous than those of the normoxic rats. Miyawaki et al. (1996, 1998) showed that CB immunoreactive cells were mainly localized in the taste buds of the posterior lingual papillae, i.e., the circumvallate and foliate papillae, and speculated that the presence of CB in cells of the lingual taste buds may be associated with responses to specific kinds of taste stimuli. Anorexia and hypophagia are common complaints of high altitude mountain climbers. High altitude hypoxia alters the

feeding behavior and may affect the pleasantness and intensity of taste. Singh et al. (1997) demonstrated with taste solution tests that rats preferred sweet solutions over other taste solutions, i.e., quinine sulfate, citric acid, and sodium chloride, during long-term exposure to simulated conditions of high altitude equivalent to 7,620 m. This finding suggests that taste sensory cues are important for palatability and are used as meaningful cues in the search for food acceptability, especially during nutritional stress caused by high altitude hypophagia. The changes in taste preference during hypoxia may be due to changes in taste sensitivity at the taste receptor level (Sharma et al., 1972, 1975, 1977) or the manifestation of modulations in higher levels along the neural axis regulating food intake (Koob and Annau, 1973; Sudakov et al., 1995). The present decreased density of CB immunoreactivity of taste cells and fibers in the taste buds and lamina propria of the circumvallate papilla in chronically hypoxic rats may suggest that taste sensitivity at the peripheral level is changed in a low O_2 environment, although it has not yet been clear whether CB is involved in the central modulation of food intake.

It has been generally accepted that CB takes part in calcium buffering, calcium transport, and regulation of enzymatic activity (Berridge et al., 1988; Baimbridge et al., 1992). In addition, CB might regulate intracellular calcium concentrations in laryngeal sensory corpuscles and may modulate signal transduction from taste cells to nerve fibers (Yamamoto et al., 2000). Therefore, the increased density of the subgemmal CB immunoreactive fibers in the laryngeal taste buds in chronically hypoxic rats might increase the sensitivity to stimuli other than taste.

In conclusion, the altered density of CB immunoreactive cells and fibers in the circumvallate papillae and larynx is a predominant feature of hypoxic adaptation. Chronic hypoxic exposure might change the chemical sensitivity in the circumvallate papillae and larynx through the regulation of intracellular Ca^{2+}.

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