Immunohistochemical identification of cells expressing steroidogenic enzymes cytochrome P450scc and P450 aromatase in taste buds of rat circumvallate papillae

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Summary. The present study demonstrated for the first time the localizations and patterns of expression of key enzymes for steroidogenesis, cytochrome P450 side-chain-cleavage (P450scc), and P450 aromatase in the taste buds of rat circumvallate papillae, using immunoblot analyses and immunohistochemistry. Immunoblot analyses showed that proteins with a molecular weight close to that of rat adrenal cytochrome P450scc and a molecular weight close to that of rat ovary cytochrome P450 aromatase were present in the rat circumvallate papillae. In immunohistochemistry, antibodies against cytochrome P450scc and P450 aromatase yielded the labelings of a subset of taste bud cells. In the double immunolabeling of P450scc and α-gustducin or phospholipase C β2 (PLC/β2), which were considered as markers of a majority of type II cells, P450scc was co-expressed in a subset of α-gustducin or PLC/β2, but did not co-express neural adhesion molecule (NCAM), a marker of major type III cells. Further double immunolabeled studies showed that P450 aromatase was co-expressed in a subset of α-gustducin or PLC/β2, but did not co-express PGP9.5, a marker of a majority of type III cells. The selective localization of cytochrome P450scc and P450 aromatase strongly suggests that estrogen biosynthesis from cholesterol might occur in a subset of type II cells of the rat taste buds. Although the full significance of estrogen in the taste bud function is not yet understood, estrogen appears to be an important regulator of taste transduction, as is the case with ATP (Finger et al., 2005), which further supports the centrality of taste cells in the life of taste buds.

Introduction

The demonstration using electron microscopy by Murray and Murray (1967) of synaptic contacts between nerves and certain light cells in taste buds of rabbit foliate papillae fostered the belief that this new cell type functions as a taste transducer. They first called cells having synaptic vesicles “type III cells”, and designated the dark cells as “type I cells” and the light cells without apparent synaptic vesicles, “type II cells”. The type I cell, appearing dark in its cytoplasm under both the light and electron microscopes, contains dense secretory granules in the apical cytoplasm that are apparently the precursors of dense substances in the taste pore, and is thought to play supportive functions. The type II cell, appearing light in its cytoplasm, has short microvilli with prominent microfilamentous cores extending deep into cytoplasm as rootlets. Although the type II cell abuts on a nerve fiber and is provided with an extensive subsurface cistern,
this cell type is devoid of synaptic vesicles (Murray et al., 1969; Fujimoto and Murray, 1970; Murray, 1973; Toyoshima and Tandler, 1987; Yoshiie et al., 1990; Seta and Toyoshima, 1995). In addition, the type II cell is characterized by an abundance of well-developed smooth endoplasmic reticulum (Takeda and Hoshino, 1975; Toyoshima and Tandler, 1987; Yoshiie et al., 1990). The presence of manifold forms of smooth endoplasmic reticulum in the type II cell suggests that these cells may have a variety of secretory functions (Toyoshima and Tandler, 1987). The type III cell, intermediate in cytoplasmic opacity, contains synaptic vesicles and forms typical synaptic contacts with afferent nerves. In addition to these cell types, a few basal cells, namely the type IV cell, are always present. This type of cell is considered to be differentiated into the above three cell types.

Recently, T1R or T2R families of taste receptors for sweet, umami, and bitter have been cloned (Hoon et al., 1999; Adler et al., 2000; Nelson et al., 2001). Further, the occurrence of all of the downstream molecules for the taste transduction effectors including a-gustducin, a taste related G protein (McLaughlin et al., 1992; Boughter et al., 1997; Yang et al., 2000a), phospholipase C β2 (PLCβ2: Clapp et al., 2004), and type III IP3 receptor (IP3R: Clapp et al., 2001; Miyoshi et al., 2001), have been reported. However, contrary to what has been expected, these taste receptors and taste transduction components are considered to be expressed in the type II cell.

The term neurosteroids (Baulieu, 1981) has been used to refer to neuroactive steroids produced not only in the brain, but also in the peripheral nervous system. In contrast to the circulating steroid hormones, many neurosteroids induce non-genomic effects by means of putative cell surface receptors in an auto / paracrine configuration (Collins and Webb, 1999; Russel et al., 2000; Shibuya et al., 2003). Since the steroid hormones are lipid soluble, they are able to diffuse easily through the cell membrane by non-vesicular diacrine secretion or transmembrane effusion. The term neurosteroid does not designate a particular class of steroids, but only refers to their site of synthesis, the nervous system (Schumacher and Baulieu, 1995).

Steroidogenesis begins with the conversion of cholesterol to pregnenolone by the cholesterol side-chain cleavage enzyme, cytochrome P450sc (CYP11A), which is a key enzyme catalyzing the conversion of cholesterol to pregnenolone, the first and rate-limiting enzyme step in the biosynthesis of all classes of steroid hormones (for reviews, see Baulieu, 1997, 1998; Kawato et al., 2003; Payne and Hales, 2004). It is known that the cholesterol substrate for cytochrome P450sc is transported into mitochondria by specific cholesterol-transporting proteins, the StAR in gonadal tissues, such as the testis, adrenal, and ovary (Stocco, 2000). Cytochrome P450 aromatase (CYP19) is also known as a key enzyme catalyzing the conversion of testosterone to estrogens, the final step of the estrogen synthesis. Thus, this enzyme is occasionally called estrogen syntase.

Recently, we have found for the first time that a subset of type II taste cells in the taste buds of the rat circumvallate papillae expresses steroidogenic enzymes P450sc and P450 aromatase and suggested that steroid hormone biosynthesis may occur within the taste buds. The expression of these enzymes in mammalian as well as non-mammalian taste buds has not been previously demonstrated to our knowledge. This study is undertaken with the hope of furthering our understanding of the taste transduction mechanisms.

Materials and Methods

The methods used in this study were approved by the Institutional Animal Care and Use Committee at Kyushu Dental College.

Immunoblot analyses

Adult Sprague-Dawley rats of both sexes were killed by an overdose of an intraperitoneal injection of sodium pentobarbital. The mitochondrial fractions were prepared as described below. The circumvallate papillae and adrenal glands were removed and homogenized immediately with 5 volumes of an ice-cold homogenization buffer of 0.32 M sucrose and 10 mM HEPES (pH 7.5) in the presence of protease inhibitor cocktails (Roche, Germany) and centrifuged at 600 × g for 10 min at 4°C. The supernatant was centrifuged at 9,000 × g for 20 min at 4°C to sediment the mitochondrial fraction. The pellets were resuspended in the homogenization buffer, and the mitochondrial fractions were again sedimented under same conditions. The washed mitochondrial fractions were resuspended in a SDS-sample buffer. The microsomal fractions were prepared as described below. The circumvallate papillae and ovaries were removed and homogenized immediately with 5 volumes of an ice-cold homogenization buffer in the presence of protease inhibitor cocktails (Roche) and then centrifuged at 1,000 × g for 10 min at 4°C to remove cellular debris. The supernatant was centrifuged at 105,000 × g for 60 min at 4°C, and resultant pellets were resuspended in a SDS-sample buffer.

Equal amounts of 20 μg of the samples were then subjected to 10% (w/v) SDS-PAGE, and the separated proteins were transferred electrophoretically to a
polyvinylidene difluoride (PVDF) filter (Millipore, MA, USA) according to the method by Laemmli (1970). Non-specific binding sites on the PVDF filter were blocked by incubation with 5% skim-milk for 3 h. The filter was then incubated with the rabbit anti-rat cytochrome P450 side-chain cleavage (P450scc) antibody (1:1,000; Chemicon International, Temecula, USA) or the rabbit anti-P450 aromatase (1:600; Abd Serotec, Oxford, UK) for 12 h at room temperature. After washing with 0.01 M phosphate-buffered saline (PBS) containing 0.03% (v/v) Triton X-100, the immunoreactive bands were made visible with Western Blot Chemiluminescence Reagent Plus (NEN™ Life Science Products, Inc., MA, USA) according to the manufacturer’s standard protocol. Negative controls were set up to replace the primary antibody with PBS. As positive control samples, tissues from rat adrenal glands and ovaries were used.

Immunohistochemistry

Adult SD rats of both sexes were intraperitoneally anesthetized deeply with chloral hydrate (350 mg/kg) and perfused transcardially with a fixative containing of 4% paraformaldehyde and 0.5% picric acid in a 0.1M phosphate buffer (pH 7.3). The circumvallate papillae were dissected out and rinsed overnight in a phosphate buffer containing 30% sucrose. Tissues were then embedded in Tissue-Tek and snap-frozen in a dry ice-isopentan mixture. Sections were cut at 5 µm in a cryostat, thaw-mounted onto Mas-coated superfrost slides (Matsunami, Japan), and stored in airtight boxes at ~80°C. After a brief wash in PBS, the sections were blocked for 2 h in 5% goat serum in PBS. Immunohisto stainings were performed using the indirect immunofluorescence method with antibodies to cytochrome P450scc (1:100) and cytochrome P450 aromatase (1:100) as the primary antibodies and Alexa Fluor 488 conjugated goat anti-rabbit IgG or anti-mouse IgG (1:1,000, Molecular Probes, Engene, OR, USA) as the secondary antibody overnight at 4°C in a humidified chamber. Slides were rinsed with PBS and coverslipped with Vectashield (Vector Laboratories, USA). The specificity of P450scc and P450 aromatase against adult rats were determined by substitutions of buffer for the primary antibodies.

For double immunostaining, the sections were incubated with anti-P450 aromatase (1:100) and anti-a-gustducin (1:1,000, polyclonal rabbit antibody; Santa Cruz Biotechnology, CA, USA), anti-PLCβ2 (1:1,000, polyclonal rabbit antibody; Santa Cruz Biotechnology), or anti-NCAM (1:500, polyclonal rabbit antibody; Chemicon International) antibodies as the primary antibodies and Alexa Fluor 488 conjugated goat anti-mouse IgG and Alexa Fluor 546 conjugated goat anti-rabbit IgG (1:1,000, Molecular Probes) as the secondary antibody overnight at 4°C in a humidified chamber. The negative control was performed by 2 combinations of antibodies: 1) anti-P450 aromatase and Alexa Fluor 546 conjugated anti-rabbit IgG; and 2) anti-a-gustducin, anti-PLCβ2, anti-NCAM rabbit polyclonal antibodies, and Alexa Fluor 488 conjugated anti-mouse IgG. No specific signal was observed with either antibody combinations.

For double immunohistochemical stainings using primary antibodies from same host species, the following procedures were used: the sections were incubated with the anti-P450scc antibody (1:100) as the primary antibody and with Alexa Fluor 488 conjugated goat anti-rabbit IgG (1:1,000) as the secondary antibody. For labeling the second primary antibodies, the manufacturer’s instructions for Zenon™ Alexa Fluor 546 Rabbit IgG Labeling Kit (Molecular Probes) was used. After rinsing with PBS, sections were incubated with a prelabeled anti-a-gustducin rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-PLCβ2 rabbit polyclonal antibody
Fig. 2. Double-labelled immunohistochemical images of P450sc and taste cell markers in rat circumvallate taste buds. 
A–C: Immunofluorescence of P450sc (green, A), α-gustducin (red, B), and their overlay (C) in a longitudinal section. About 40–50% of α-gustducin-immunoreactive cells co-expressed P450sc. D–F: Immunofluorescence of P450sc (green, D), PLCβ2 (red, E), and their overlay (F) in a longitudinal section. About 40–50% of PLCβ2-immunoreactive cells co-expressed P450sc. G–I: Immunofluorescence of P450sc (green, G), NCAM (red, H), and their overlay (I) in a longitudinal section. All cells positive for P450sc did not co-expressed NCAM. Scale bars = 30 μm.
Fig. 3. Double-labelled immunohistochemical images of P450 aromatase and taste cell markers in rat circumvallate taste buds. A–C: Immunofluorescence of P450 aromatase (green, A), α-gustducin (red, B), and their overlay (C) in a longitudinal section. Less than 10% of the α-gustducin immunoreactive cells co-expressed P450 aromatase.

D–F: Immunofluorescence of P450 aromatase (green, D), PLC β2 (red, E), and their overlay (F) in a longitudinal section. Less than 10% of the PLC β2 immunoreactive cells co-expressed P450 aromatase.

G–I: Immunofluorescence of P450 aromatase (green, G), PGP9.5 (red, H), and their overlay (I) in a longitudinal section. All cells positive for P450 aromatase did not co-express PGP9.5. Scale bars = 30 μm.
(Santa Cruz Biotechnology), or anti-NCAM rabbit polyclonal antibody (Chemicon International). Slides were rinsed with PBS and coverslipped with Vectashield (Vector Laboratories). Two kinds of negative controls were performed: 1) omission of the first primary antibody, and 2) replacement of the pre-labeled secondary antibodies by a neutralized labeled Fab fragment.

All images were obtained by changing the filter cube without altering the portion of the section and focus with CCD cameras (Olympus, Tokyo; Keyence, Osaka). Digital images were contrast and color adjusted, and plates were created using Adobe Photoshop (Adobe systems, USA) for Macintosh.

Results

Immunoblot analysis

The presence of cytochrome P450scc and P450 aromatase proteins was verified by immunoblot analyses (Fig. 1). In mitochondrial fractions of the circumvallate papilla, a single protein band was observed for P450scc. The electrophoretic mobility of the P450scc band indicated a molecular mass of about 48 kDa. This molecular mass was approximately equal to that in the adrenal glands. In the microsomal fraction of the circumvallate papilla, on the other hand, a single protein band was observed for P450 aromatase although the reaction product was low. The electrophoretic mobility of the P450 aromatase band indicated a molecular mass of about 58 kDa, which was almost identical to that in the ovary.

Immunohistochemistry

In cryosections of the circumvallate papillae, the antibody against cytochrome P450scc gave a labeling of a subset of taste bud cells — about 15 to 20 % — of all taste buds examined (Fig. 2A). The antibody against cytochrome P450 aromatase gave labeling of a subset of taste bud cells, about 5 to 10 %, of all taste buds examined (Fig. 3A). In contrast, no positive signals for either antibody was detected in the intragemmal and subgemmal nerve fibers. The immunoreactive taste bud cells for P450scc and P450 aromatase were spindle shaped with round nuclei and extended the entire thickness of the taste bud from the basal lamina to the taste pore. The difference between the number and density of the P450scc protein and P450 aromatase by immunohistochemistry was not significant between genders.

In double immunolabeling of P450scc and α-gustducin, which is considered a major marker protein of type II cells, approximately 40 to 50 % of the α-gustducin immunoreactive cells showed an immunoreactivity for P450scc (Fig. 2A–C). In double immunolabeling of P450scc and PLCβ2, which is also considered a major marker of a majority of type II cells and a small subset of type III cells (Clapp et al., 2001), about 40 to 50 % of the PLCβ2 immunoreactive cells showed an immunoreactivity for P450scc (Fig. 2D–F). In double immunolabeling of P450scc and NCAM, which is considered as a marker of type III cells (Takeda et al., 1992), P450scc expressing taste bud cells did not co-express NCAM (Fig. 2G–I).

In double immunolabeling of P450 aromatase and α-gustducin, less than 10 % of the α-gustducin immunoreactive cells showed an immunoreactivity for P450 aromatase (Fig. 3A–C). In double immunolabeling of P450 aromatase and PLCβ2, less than 10 % of the PLCβ2 immunoreactive cells showed an immunoreactivity for P450 aromatase (Fig. 3D–F). In double immunolabeling of P450 aromatase and PGP 9.5, which is considered as a marker of a majority of type III cells (Yee et al., 2001), P450 aromatase expressing taste bud cells did not co-express PGP 9.5 (Fig. 3G–I).

Discussion

The present study demonstrates for the first time the expression of key enzymes for steroid synthesis, — P450 side-chain-cleavage cytochrome P450scc (CYP11A) and P450 aromatase (CYP19) — in the taste buds of the rat circumvallate papillae by immunoblot analyses and immunohistochemistry. The immunoblot analyse of mitochondrial and microsomal fractions in the rat circumvallate papillae showed that about 48 kDa (P450scc) and about 58 kDa (P450 aromatase) were present. It has been established that steroidogenesis begins with the conversion of cholesterol to pregnenolone by the cytochrome P450scc, the enzyme catalyzing the first step of steroid biosynthesis of all classes of steroid hormones including cortisol and the sex steroid hormones (reviews, see Schumacher and Baulieu, 1995; Plassart-Schiess and Baulieu, 2001; Kawato et al., 2003; Payne and Hales, 2004). In their study using immunoelectron microscopy, Ishimura et al. (1985) showed that cytochrome P450scc was exclusively localized on the matrix side of the inner membranes of the mitochondria including the tubulovesicular crista in the bovine adrenal cortical cells.

In the present study, we have showed that the antibody against cytochrome P450scc gave an immunolabeling of a
subset of taste bud cells among the taste buds examined. This strongly suggests that a certain type of taste bud cells might be able to catalyze the conversion of cholesterol to pregnenolone. We further found that the antibody against cytochrome P450 aromatase (CYP19) gave an immunolabeling of a small subset of taste bud cells of all those examined. It is known that cytochrome P450 aromatase is also a key enzyme catalyzing conversion of the C19 androgens, androstenedione and testosterone, to the C18 estrogens, estrone and estradiol, respectively (Harada et al., 1993; Payne and Hales, 2004; Harada and Honda, 2005). Thus, the expression of cytochrome P450 aromatase in a small subset of taste bud cells strongly suggests that certain taste bud cells might be able to synthesize estrogens from its precursors.

In double immunolabeling of cytochrome P450sc and a-gustducin or PLCβ2, all P450sc expressing cells co-expressed both a-gustducin and PLCβ2. However, not all a-gustducin- and PLCβ2-immunoreactive cells were p450sc immunoreactive. It has been becoming increasingly evident that both a-gustducin and PLCβ2 are the main immunohistochemical markers of a majority of type II cells (Yang et al., 2000a; Clapp et al., 2001, 2004). Our results thus suggest that the initial steroid hormone biosynthesis from cholesterol by P450sc might occur in a subset of type II cells in rat taste buds. Further, in a double immunolabeling of cytochrome P450 aromatase and a-gustducin or PLCβ2, all cytochrome P450 aromatase expressing cells co-expressed both a-gustducin and PLCβ2. However, not all a-gustducin- and PLCβ2-immunoreactive cells were p450sc immunoreactive. Our present results thus further suggest that the biosynthesis of estrogens from testosterone by P450 aromatase might occur in a subset of type II cells.

It has recently become evident that non-gonadal tissues, such as neurons and glial cells in the brain and the cerebellum, are able to synthesize steroid hormone (Zwain and Yen, 1999; Sakamoto et al., 2003; also see reviews by Schmacher and Baulieu, 1995; Baulieu, 1997, 1998; Baulieu et al., 2001; Plassart-Schiess and Baulieu, 2001; Kawato et al., 2003). Moreover, the use of a real-time reversed transcription polymerase chain reaction (RT-RT-PCR) showed that steroidogenic enzyme mRNA, which would not be detectable using Northern analysis, is widely expressed not only in recognized gonads and adrenal cortex, but also in many non-gonadal and non adrenal human fetal tissues such as the aorta, brain, liver, kidney, heart, lung, pancreas, prostate, stomach, and thymus (Pezzi et al., 2003). The conversion of androgens to estrone also has been shown to occur in peripheral tissues, including the skin, muscle, fat, and bone. This conversion is catalyzed by the aromatase enzyme complex (for reviews, see Sasano and Harada, 1998; Payne and Hales, 2004).

Also of interest is the fact that some sensory organs, such as the retina of the golden hamster (Jaliffa et al., 2005) and carotid body of the rat (Joseph et al., 2006), express steroidogenic enzymes. In their study on the steroidogenic enzyme immunostaining of the rat carotid body, Joseph et al. (2006) noted that, although P450sc staining was strongly expressed in both late fetal and adult rats, a slight staining for P450 aromatase was expressed only during the late fetal period and was not detected in adult rats. Since estrogens are known to play important regulatory functions relating to specific neural growth factors such as neurotrophins and their receptors during neural development (Miranda et al., 1994), Joseph et al. (2006) suggested that estrogens might be involved in the development of a neural network in the late fetal stage of the rat carotid body. In the retina, on the other hand, Jaliffa et al. (2005) suggested that the steroid hormone might regulate GABAergic pathways both pre- and post-synaptically as local modulators in the retinal circadian activity of the hamster.

To date, however, the exact function of steroid hormones in the chemosensory organs remains unknown. In consulting standard textbooks, steroid hormones are said to be synthesized in gonads and adrenal cortex and reach their target organs at a distance via the general blood circulation (Fawcett, 1986). It is now widely accepted that steroid hormones are also synthesized and present in non-gonadal tissues including central and peripheral neurons, and have in fact been called neurosteroids (Baulieu, 1981). In their review article, Kawato et al. (2003) proposed that brain neurosteroids are fourth generation neuropeptides which are synthesized within the nerve cells and act as neurotransmitters through transmitter receptors. According to them, first generation neuropeptides are neurotransmitters such as glutamate, GABA, and acetylcholine, second generation neuropeptides are catecholamins such as dopamine and serotonin, and third generation neuropeptides are neuropeptides. It is well known that first to third generation neuropeptides are stored in the synaptic vesicles and released by exocytosis into synaptic clefts. On the other hand, neurosteroids (steroid hormones) are lipid soluble and are able to diffuse through the cell membrane by non-vesicular diacrine diffusion as paracrine messengers (Kawato et al., 2003). It is now becoming increasingly evident that, in contrast to the classical genomic effects of steroid hormones, many steroid hormones (neurosteroids) are able to induce non-genomic effects by means of putative cell surface receptors (Collins and Webb, 1999; Russel et al., 2000;
It has been known that the initial step in the conversion of cholesterol to the first steroid hormone, i.e., pregnenolone, is a cleavage of its side chain by cytochrome P450scc in the mitochondria. Several subsequent steroidogenic pathways, including the final step in the conversion of testosterone to estrogens by cytochrome P450 aromatase, are involved in the smooth endoplasmic reticulum (Payne and Hales, 2004). However, little is known about how the intermediate products in steroidogenesis are moved back and forth between mitochondria and the smooth endoplasmic reticulum to accomplish the successive biosynthetic steps (Fawcett, 1986).

Although the type II cells do not show any features of an afferent chemical synapse such as the membrane-thickening and accumulation of synaptic vesicles nor express the presynaptic proteins (Yang et al., 2000b; Kohno et al., 2005), it has been shown that this type of cell expresses T1Rs and T2Rs taste receptors of sweet, umami, and bitter, and taste transduction component molecules, and has been regarded as a taste transducer cell. Recently, Ueda et al. (2006) showed, however, that soluble N-ethylmaleimide-sensitive fusion protein attachment receptor (SNARE) proteins, i.e., synaptosomal-associated proteins, were present not only in type III cells but also in type II cells of the rat taste bud.

In the present study, we found that the number of P450 aromatase immunoreactive cells was relatively few as compared with P450scc immunoreactive cells. Though nothing conclusive can as yet be said, it seems likely that the intermediate products of steroidogenesis from cholesterol to estrogens, such as pregnenolone, progesterone, testosterone, etc., also might be involved in the taste bud function, as is the case with neurons (Baulieu, 1997; Kawato et al., 2003). Although the significance of the estrogens in the taste bud functions is yet to be assessed, it would be of interest to suppose that steroid hormones synthesized and stored in a subset of type II taste cells may be released by non-vesicular transmembrane effusion and may be involved in a role of signal transduction processes of taste as neuromessengers or neuromodulators, as is the case with brain neurosteroids.

Based on our present findings and given the current concept on brain neurosteroid functions, we are tempting to emphasize the new hypothesis that estrogens in certain type II taste cells may be involved in an important function for taste perception. A schematic model of the hypothetical possibilities of the intracellular events is depicted in Figure 4. Although the full significance of estrogens is not yet fully understood, estrogen appears to be an important regulator of the taste bud function, as is the case with ATP (Finger et al., 2005), which further
supports the centrality of taste cells in the life history of taste buds.

References


