Expression of ATP-gated P2X₃ receptors in rat gustatory papillae and taste buds

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Summary. It has recently become evident that ATP and other extracellular nucleotides could play an important role in signal transductions. ATP mediates excitatory signaling by means of P2X receptors. P2X₃, one of its subtypes, a membrane ligand-gated ion channel, is strongly expressed in peripheral sensory neurons. The aim of the present study was to examine the distribution of nerve fibers expressing P2X₃ receptors in taste buds in the gustatory papillae and soft palate of rats by immunohistochemistry. We found that the fluorescence ATP marker quinacrine stained subsets of taste bud cells. Numerous nerve fibers innervating taste buds were intensely immunostained with the P2X₃ receptor antibody. These nerve fibers ascended among intragemmal cells and terminated just below the taste pores. In order to examine whether P2X₃ receptors are involved in signal modulation within taste buds, we used fluorescent double stainings to analyze the distribution of P2X₃ receptors and their relationship to α -gustducin immunopositive taste receptor cells. Many varicose nerve fibers expressing P2X₃ receptor-immunoreactivities were entangled with α -gustducin-immunopositive taste receptor cells and ended closely below the taste pores. In fungiform papillae, nerve fibers expressing both P2X₃ receptors and PGP 9.5 were

observed. In contrast, only PGP 9.5 immunoreactive nerve fibers were recognized in filiform papillae. These results suggest that P2X₃ receptors might be involved in taste transmission pathways within taste buds. ATP may act as a neurotransmitter, co-transmitter, or neuromodulator at P2X₃ receptors to generate activating gustatory nerve fibers.

Introduction

Taste buds, first recognized over a century ago, have remained a subject of continued interest. These organs are barrel-shaped clusters of specialized epithelial cells which include taste receptor cells. The taste bud in mammals is made up of approximately 60 to 80 spindle-shaped cells tapering apically to end in microvilli. Most of the taste buds concentrate in the gustatory papillae, i.e., circumvallate, foliate, and fungiform papillae of the tongue. Taste buds also appear on the soft palate, epiglottis, larynx, and pharynx. The filiform papillae, the most numerous papillae of the tongue, are not associated with taste buds.

From ultrastructural observations, it is now generally accepted that mammalian taste buds consist of four distinct cell types (for a review, see Murray, 1973). Type I cells are characterized by large, dense granules in their apical cytoplasm and are thought to have a supporting function. Type II cells have an electrolucent cytoplasm and a characteristic large circular nucleus without apparent synaptic vesicles. It has been shown that type II cells express all of the taste transduction cascade for sweet, bitter, and umami, including T1R and T2R taste receptor families (Hoon *et al.*, 1999), and the downstream taste transduction effectors such as α -gustducin, the GTP-binding α -subunit of a trimeric G-protein complex (McLaughlin *et al.*, 1992; Boughter *et al.*, 1997; Yang *et*

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al., 2000), phospholipase $C\beta 2$ ($PLC\beta 2$), and type III IP_3 receptors (IP_3R_3) (Clapp *et al.*, 2001, 2004; Miyoshi *et al.*, 2001). In this context, the type II cells are considered taste transducing cells for these taste qualities. Further, α -gustducin and $PLC\beta 2$ have often been used as marker proteins of type II taste cells. Type III cells make synaptic contact with dendrites; synaptic vesicles are clustered in these III cells at the synapses. Type III cells express the synaptic membrane proteins such as the synaptosomal-associated protein of 25kDa (SNAP-25, Yang *et al.*, 2000) and synaptotagmin 1 (Kohno *et al.*, 2005). It has been suggested that mammalian degenerin 1 (MDEG 1), candidate sour-taste receptors, are expressed in the type III cells (Ugawa *et al.*, 1998).

It has recently become evident that ATP and other extracellular nucleotides could play an important role in cell-cell communication. ATP mediates the excitation of sensory neurons through P2 receptors (purinoceptors). The P2 receptors are composed of two main families: the P2X ionotropic ligand-gated cation channel family, and P2Y metabotropic G-protein-coupled family (for reviews, see Burnstock, 1997; Ralevic and Burnstock, 1998). To date, seven ionotropic P2X receptor subtypes, P2X₁-P2X₇, have been cloned. Of these, the P2X₃ receptor is well known for its strong expression in sensory neurons (Vulchanova *et al.*, 1997; Llewellyn-Smith and Burnstock, 1998; Brouns *et al.*, 2000; Alavi *et al.*, 2001; also a review, see Burnstock, 2000).

Bo *et al.* (1999) was first report the appearance of abundant P2X₃ receptor-immunoreactive nerve fibers in rat circumvallate and fungiform papillae, and suggested that ATP might function as a neurotransmitter in taste transduction. Further, P2Y₁ receptors and their mRNA were reported to be expressed in a subset of taste bud cells (Kataoka, *et al.*, 2004). Rong *et al.* (2000), on the other hand, examined whole nerve recordings of the somatosensory trigeminal lingual nerve and gustatory chorda tympani nerve, and found that ATP preferentially activated the somatosensory afferent nerve but not the taste sensory nerve. They concluded that ATP and P2X receptors played a role in nociception, rather than taste sensation in the tongue. In order to investigate whether P2X₃ receptors are involved in taste signal modulation within the taste buds, we used fluorescent double immunolabelings to analyze the distribution of P2X₃ receptors and their relationship to α -gustducin-immunoreactive taste receptor cells.

The protein-gene product 9.5 (PGP 9.5) is a soluble protein isolated from human brain extracts and a major component of the neuronal cytoplasm (Jackson and Thompson, 1981; Gulbenkian *et al.*, 1987). Since it has been reported that PGP 9.5 is a general marker for

both the trigeminal branch of the somatosensory nerve (Fristad *et al.*, 1994) and the glossopharyngeal gustatory nerve (Wakisaka *et al.*, 1996), we further compare the distribution of PGP 9.5-immunoreactive nerves and P2X₃-immunoreactive nerves in the fungiform and filiform papillae by double immunolabeling.

Materials and Methods

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

Quinacrine accumulation in taste buds

Sprague-Dawley rats of both sexes, weighing about 200 g were used in the present study. Neonatal rats from 1 day to 14 days were also utilized in the present study. Rats were administered intraperitoneally with quinacrine (85 mg/kg, Sigma, St Louis, MO, USA), a fluorescent dye with an affinity for ATP and other adenine nucleotides, and sacrificed 48 h later. The circumvallate papillae were removed from rats that had been overdosed with an intraperitoneal injection of chloral hydrate. Tissues were then embedded in Tissue-Tek and frozen in a dry ice-isopentane mixture. Cryostat sections were cut at 8 μ m, mounted on Mas-coated glass slides (Matsunami, Japan), and instantly fixed on the slide using 4% phosphate-buffered paraformaldehyde. The sections were rinsed briefly in PBS and mounted in Vectashield (Vector Laboratory, USA). Tissue sections were examined in a fluorescence microscope equipped with an epi-illumination system. Quinacrine fluorescence was viewed and photographed immediately because of its rapid loss. No fluorescent cells were detected in the circumvallate taste buds of animals not treated with quinacrine.

Tissue preparation and sectioning

Rats were anesthetized with an intraperitoneal injection of chloral hydrate (350 mg/kg) and transcardially perfused with a fixative containing 4% paraformaldehyde and 0.5% picric acid in a 0.1M phosphate buffer for 15 min. The areas of the circumvallate, foliate, and fungiform papillae were removed and rinsed overnight in 30% sucrose in PBS at 4 °C. Tissues were then embedded in Tissue-Tek and snap-frozen in a dry ice-isopentane mixture. Sections were cut at 8 μ m in a cryostat, thaw-mounted onto Mas-coated glass slides (Matsunami), and air-dried for at least 30 min prior to processing for immunostaining.

Fig. 1. Quinacrine staining in taste bud cells of the adult rat circumvallate papilla. Positive signals for quinacrine are observed in a subset of taste bud cells (A). Expression of P2X₃ receptors (green) in the circumvallate papilla (B). Numerous nerve fibers expressing intense P2X₃-receptor immunoreactivity can be observed ascending parallel to the trench epithelium just beneath the taste buds and penetrating into taste buds. Double-labeled immunohistochemical images of P2X₃-receptors (green) and CK 18 (red), a marker of taste buds. P2X₃-immunoreactive nerves are seen to penetrate into the taste buds (C). Scale bars: 50 μ m

Single or double immunolabelling of P2X₃ receptors and taste bud cells

After a brief wash in PBS, the sections were pretreated with 10mM citrate buffer (pH 6.0) for 10 min in an autoclave at 121°C. The sections were placed in a humidified chamber and preincubated with a solution of 0.15% normal goat serum (Vector Laboratories) in PBS for 40 min. Sections were then processed for indirect single or double immunofluorescence staining by incubation the section overnight at 4 °C in a guinea pig anti-P2X₃ receptor antiserum (diluted 1:800, Neuromics, USA) or mixture of primary antibodies from different species for double labelling. These antibodies include rabbit polyclonal antibodies against α -gustducin (diluted 1:500, Santa Cruz biotechnology, USA), Protein gene product 9.5 (PGP 9.5) (diluted 1:1600, UltraClone, England), and a monoclonal antibody against cytokeratin 18 (CK18, diluted 1:100, Progen, Germany).

Following incubation with the primary antibodies, sections were rinsed in PBS three times for 5 min each and then treated at room temperature with a single secondary antibody or a mixture of secondary antibodies. P2X₃ was visualized using Alexa Fluor® 488 conjugated anti-guinea pig IgG secondary antibody (Molecular Probe, USA). Both PGP 9.5 and α -gustducin were visualized using Alexa Fluor® 546 anti-rabbit secondary antibody,

and Alexa Fluor® 546 anti-mouse secondary antibody was used for CK 18. The sections were then washed in PBS and coverslipped with Vectashield (Vector Laboratories). All preparations were examined with an epifluorescence microscope (Olympus, Japan) and analyzed by using filter combinations with a cooled CCD camera that enabled the separate visualization of multiple fluorophors. Images were overlaid with Adobe Photoshop (Adobe Systems, USA). P2X₃-immunoreactivity was seen as a green reaction product, α -gustducin, PGP 9.5, and CK 18 immunoreactivities as red.

Control experiments used the protocol as described above, but the primary antibodies were substituted with PBS. The substitution of primary antibodies resulted in a complete loss of specific staining.

Results

Quinacrine histochemistry and P2X₃ receptor immunoreactivity

When the presence of ATP in taste buds of the circumvallate papilla was examined using the quinacrine fluorescence technique, a certain subpopulation of taste bud cells accumulating fluorescent compound was observed (Fig.1A).

Fig. 2. Double-labeled immunohistochemical images of P2X₃ receptor-immunoreactive nerves (A) and CK18 (B) and their overlay (C), in the fungiform papilla. Many nerve fibers expressing P2X₃ receptors are clearly entangled with CK18-immunolabeled taste bud cells (C). Double-labeled immunohistochemical images of P2X₃ receptor-immunoreactive nerves (D) and PGP9.5-immunoreactive nerves (E), and their overlay (F), in the fungiform papilla and the filiform papilla. Only PGP9.5-immunoreactive nerves (arrowheads) appear in the filiform papilla (E, F). Double-labeled immunohistochemical images of P2X₃ receptor-immunoreactive nerves (G) and CK18-immunolabeled taste bud cells (H), and their overlay (I), in the soft plate of the rat. P2X₃ receptor-immunoreactive nerves exclusively innervate the taste bud.

Scale bars: 50 μ m

Fig. 3. Double labeled immunohistochemical images of P2X₃-immunoreactive nerves (green, **A**) and α -gustducin (red, **B**), and their overlay (**C**), in circumvallate taste buds. Numerous P2X₃ receptor-immunoreactive nerves are entangled with α -gustducin immunoreactive taste cells and end just below the taste pore. A horizontal section of circumvallate taste buds double labeled with antibodies against P2X₃ receptors (**D**) and α -gustducin (**E**), and their overlay (**F**). Note the close relationship between P2X₃ receptor-immunoreactive nerves and α -gustducin immunoreactive taste cells (**I**). Double-labeled immunohistochemical images of P2X₃ receptor-immunoreactive nerves (**G**) and α -gustducin (**H**), and their overlay (**I**), in rat circumvallate papilla at postnatal day 1. Many nerve fibers expressing P2X₃ receptors are clearly entangled with α -gustducin-immunoreactive taste cells. Scale bars: 50 μ m (**C**, **F**), 20 μ m (**I**)

In the adult rat circumvallate papilla, nerve fibers expressing intense P2X₃-immunoreactivity were observed ascending parallel to the trench epithelium in the lamina propria and formed a dense plexus just beneath the taste buds (Fig. 1B). After branching out, these nerve fibers entered the taste bud and ascended among the CK18-immunopositive intragemmal cells to terminate close below the taste pore (Fig. 1C). No fibers — or very few — expressing P2X₃ receptors were recognized in the epithelium outside the taste buds. In foliate papillae, nerve fibers expressing P2X₃ receptor-immunoreactivity also appeared in the lamina propria immediately below the taste buds. Just before entering the taste buds, these nerves divided into thinner branches with small varicosities, and terminated close below the taste pores (data not shown). In fungiform papillae, a single, centrally located taste bud was observed at the top of the papilla. A bundle of nerve fibers expressing P2X₃ receptor-immunoreactivities was seen ascending the core of the fungiform papilla toward the taste bud (Fig. 2A). These fibers entered the taste bud and ascended with small varicosities among CK18-immunoreactive taste bud cells, reaching to just below the taste pore (Fig. 2B,C). The number of intragemmal nerve fibers expressing P2X₃ receptors was greatest near the basal lamina.

Although both PGP 9.5 and P2X₃ receptor immunoreactive nerve fibers were present in fungiform papillae (Fig. 2D,E), no P2X₃ receptor-immunoreactive nerve fiber was recognized in the filiform papillae (Fig. 2F).

In the palate, P2X₃ receptor-immunoreactive nerves innervated only taste buds, and no nerve fibers expressing P2X₃ receptors were recognized in the epithelium outside the palatal taste buds (Fig. 2G, H, I).

Relationship between P2X₃ receptors and α -gustducin-immunoreactive taste receptor cells

To examine the relationship between P2X₃ receptor-immunoreactive nerves and α -gustducin-immunoreactive taste receptor cells, a double immunostaining method using two antibodies was applied. In adult taste buds, many varicose nerves showing P2X₃ receptor-immunoreactivities were distributed close to the α -gustducin-immunoreactive taste receptor cells (Fig. 3A, B, C). P2X₃ receptor-containing nerve fibers were entangled with taste receptor cells and ended in bulging processes just below the taste pore. Next, we also carried out double labeling in horizontal section of circumvallate taste buds. P2X₃ receptor-immunoreactive nerves were also observed to associate closely with α -gustducin-immunoreactive taste receptor cells in the taste buds (Fig. 3D, E, F).

In an attempt to understand better the relationship

between P2X₃ receptor-immunoreactive nerves and α -gustducin-immunoreactive taste receptor cells, a double immunolabeling method using the two antibodies was applied to the developing taste buds in the circumvallate papillae (Fig. 3G,H,I). At postnatal day 1, a limited number of α -gustducin-immunoreactive cells appeared in the trench wall epithelium of the circumvallate papilla (Fig. 3H). In the lamina propria, thick P2X₃ receptor-immunoreactive nerve elements were observed just below the trench wall epithelium. Nerve fibers arising from subepithelial thick bundles penetrated the basal lamina into the epithelium (Fig. 3G). Numerous nerve fibers expressing P2X₃ receptors were clearly entangled with α -gustducin-immunoreactive taste receptor cells (Fig. 3I). The number of α -gustducin-positive cells in a single taste bud increased markedly during the first 2 postnatal weeks and reached the adult value by postnatal day 14. At postnatal day 14, numerous nerve fibers expressing P2X₃-immunoreactivity were distributed close to the α -gustducin-positive taste receptor cells in the taste buds. P2X₃-expressing fibers in the taste buds were found to be entangled closely with α -gustducin-positive cells and terminated near the taste pore in the same fashion as in adult rats (data not shown).

Discussion

Burnstock (1972) first proposed a transmitter role for ATP in nervous systems. It is now well accepted that purines and pyrimidines are important mediators of a broad range of cell to cell communications in these systems (for reviews, see Gordon, 1986; Burnstock, 1997, 2000, 2006; North, 2002). It has been recognized that ATP is synthesized and stored within the cytoplasm and used as an important energy source for many intracellular reactions. Since Irvin and Irvin (1954) showed that quinacrine, antimalarial drug, had a strong affinity for ATP, quinacrine has been used for the possible intracellular storing site of ATP (for a review, see Bodin and Burnstock (2001)). It is now well accepted that ATP and other adenine nucleotides are stored in synaptic vesicles of the purinergic neurons and released by vesicular exocytosis as a neurotransmitter (Silinsky, 1975; Bodin and Burnstock, 2001).

In the present study, a close association between P2X₃ receptor-immunoreactive nerves and α -gustducin-expressing taste cells was observed. It is now known that α -gustducin (McLaughlin *et al.*, 1992; Boughter *et al.*, 1997; Yang *et al.*, 2000) is a marker protein of type II cells. In addition, type II cells also express all of the taste transduction cascade for sweet, bitter, and umami,

including the T1R and T2R taste receptor families (Hoon *et al.*, 1999) and the downstream taste transduction effectors (Miyoshi *et al.*, 2001; Clapp *et al.*, 2001, 2004). In this context, the type II cells can be considered as taste transducing cells for these taste qualities. In their recent study using P2X₂ and P2X₃ knockout mice, Finger *et al.* (2005) described how these mice showed greatly reduced behavioral responses to sweeteners, glutamate, and bitter substance. They concluded that ATP signaling via P2X₃ receptors is crucial for communication from the taste bud cells to gustatory nerves. From their results, it is reasonable to speculate that type II gustatory cells are potential sources for ATP. However, on the contrary to what has been expected, type II cells contain no synaptic vesicles. Further, because of its charge and molecular size, it has been believed that ATP cannot cross the plasma membranes by simple diffusion into the extracellular milieu (for a review see Gordon, 1986; Burnstock, 2006). If ATP is a neurotransmitter, then questions arise as to how type II cells release ATP toward the extracellular milieu where numerous P2X₃ receptor-expressing nerves exist.

According to a review article by Burnstock (2006), there is an ongoing debate about the precise ATP-transport mechanism(s) involved. Recently, however, there have been reports that exocytosis is not the only way by which ATP is released by non-neuronal cells (Ferguson *et al.*, 1997; also reviews, see; Burnstock, 1999, 2002, 2006; Cook and McCleskey, 2000; Bodin and Burnstock, 2001). In their study using the rabbit urinary bladder (Ferguson *et al.*, 1997), ATP was released from urinary bladder epithelial cells as the bladder filled and stretched. For ATP release from such non-neuronal cells, various transport mechanisms have been proposed, including ATP binding cassette (ABC) transporters, a ubiquitous family of transport ATPases, connexin or pannexin hemichannels, or possibly plasmalemmal voltage-dependent anion channels, as well as vesicular release (for a review, see Burnstock, 2006). According to a study by Schwiebert (1999), ABC proteins can transport ATP, but it may also be that these proteins regulate exocytosis. Bodin and Burnstock (2001) suggested the possibility that non-neuronal cells could release ATP both by exocytosis and through the activation of ABC proteins, and concluded that, whichever way, ATP is released from cells, and its extracellular importance in cell-cell communication can no longer be dismissed.

Although nothing conclusive can as yet be said about the releasing mechanisms of ATP in the taste buds, it is of interest to speculate that ATP may be released from non-vesicular type II taste cells into the extracellular milieu through the activation of ABC proteins in a similar

fashion as that suggested in non-neuronal cells. The full significance of ATP and its receptors, P2X₃, in taste buds is not yet fully understood, but ATP appears to be an important regulator of the taste bud function, which further supports the centrality of taste cells in the life history of the taste buds. Further studies into this problem remain an intriguing field for research.

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