Biochemical and Biophysical Research Communications 388 (2009) 1–5

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of the vesicular nucleotide transporter (VNUT) in taste cells

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ARTICLE INFO

Article history:
Received 14 July 2009
Available online 18 July 2009

Keywords:
ATP
Vesicular transporter
VNUT
Taste cell
Neurotransmitter

ABSTRACT

Taste cells are chemosensory epithelial cells that sense distinct taste qualities. It is the type II taste cell that express G-protein coupled receptors to sense either umami, sweet, or bitter compounds. Whereas several reports have suggested involvement of ATP in taste signal transduction, there is a paucity of molecular information about how ATP is stored and being released. The recent discovery of a novel vesicular nucleotide transporter (VNUT) led us to examine whether VNUT exist in the taste tissue where ATP is to be released for taste signal transmission. Here, we report that VNUT is selectively expressed in type II cell but not in type III taste cell. In addition, we show that during taste bud development VNUT expression is always accompanied by the expression of type II taste cell markers. Our results, together with previous studies, strongly suggest that VNUT plays a role in type II taste cell.

Introduction

Neurotransmitters such as glutamate, acetylcholine, GABA, and serotonin act to transduce signals through synapses from one neuron to another. These neurotransmitters are generally stored in a specialized vesicular compartment(s) where particular vesicular transporters play a role in molecular selection [1–3]. For the past decades, studies have suggested that ATP, besides energy currency, could be one of the neurotransmitters and it is now accepted that ATP indeed is a neurotransmitter [4,5]. ATP signaling was initially found in non-adrenergic, non-cholinergic nerves but soon became evident that ATP signaling is seen among many cell types including peripheral and central nervous systems [6]. This appears to be true in specialized sensory organs including taste tissues where purinergic receptors are abundant [7].

Taste cells are chemosensory epithelial cells that sense distinct taste quality such as umami, sweet, bitter, sour, and salty. These cells could be divided morphologically into dark, light, and intermediate cells, or type I, II, and III cells, respectively [8,9]. It is well studied and now widely accepted that the type II cells have receptors for sugars, amino acids, and bitter compounds [10]. One of the unsolved issues in the system is how type II taste cells transduce taste information to gustatory nerves despite that they do not form conventional synapses between taste cells and neurons. Taste signals are known to be transmitted from type II taste cells to the particular type of sensory nerves that bring information into the central nervous system [11–13]. Among several neurotransmitters ATP has been suggested to play a role in the taste signal transduction since P2X2 and P2X3 expression were found on the nerve fibers that innervate taste buds [14]. Finger et al. have recently reported using purinergic receptor knock out mice that ATP signaling is essential for taste transduction [15]. Furthermore, nucleoside triphosphate diphosphohydrolase-2 (NTPDase2), an ATP and ADP selective ecto-nucleotidase, has been found in type I taste tissue indicating that purinergic signals are tightly regulated [16]. These raised a question of how ATP is being stored and released in taste system. Recently, a novel vesicular nucleotide transporter (VNUT) has been identified and became a candidate molecule for storing ATP in taste cells. In the present study, we show VNUT is selectively expressed in particular type of taste cells that transmit taste signals to neurons.

Materials and methods

Isolation of taste buds. Animals were purchased from Charles River Laboratories (Kanagawa, Japan). Twenty 10-week-old C57BL/6 mice were sacrificed, tongues were excised and placed in phosphate buffered saline (PBS) solution minus calcium plus 2 mM EGTA. The excised tongues were injected sub-epithelially with a mixture of dispase II and collagenase A (both from Roche) and incubated for 20 min at 37 °C. The epithelia were peeled off from the rest of the tongue and CV region were isolated under dissecting microscope.

Cells. Human embryonic kidney (HEK) 293 cell line was purchased from ATTC (Manassas, VA, USA). Cells were maintained in Dulbecco's modified culture medium (Sigma) supplemented with 10% fetal calf serum and 2 mM glutamine (Invitrogen).

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doi:10.1016/j.bbrc.2009.07.069

RNA preparation and RT–PCR. The total RNAs were prepared by homogenization of tissues or cultured cells directly in guanidium thiocyanate/phenol monophasic solution using ISOGEN (Nippon Gene). Then total RNAs were used for first strand cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen). The RT–PCRs were carried out as previously described [17] for 30 cycles using following sets of primers: 5′-GTGGTATTTCAGTCAACATTCA GG-3′ and 5′-CACATCAGTAGTGCTGAGAGATCC-3′, which amplifies 841 bp of VNUT; 5′-TCATCCATAAGAATGGTTACAGC-3′ and 5′-CCC ACAGTCGTTTAATGATTTC-3′, which amplifies 231 bp of alpha-gustducin; 5′-TTCCTTGGTAGCTGGGAGTTGC-3′ and 5′-GGACGGCA GAGAATCACGTAGC-3′, which amplifies 627 bp of mT1R1; 5′-CTTC TGGAGACATTTCCAGACG-3′ and 5′-CTAGCTCTTCCTCATCGTGTAGC-3′, which amplifies 722 bp of mT1R2; and 5′-CTCTTCAACTACAGCA TCAGCAGC-3′ and 5′-GAAGGTCAGGTTGTACATGTTCTC-3′, which amplifies 459 bp of hT1R3; and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′, which amplifies 451 bp of G3PDH. PCR products were analyzed on a 1.5% agarose gel.

Immunohistochemistry. Antibodies against N-terminal region of mouse PLCbeta2 were generated in rat using synthetic peptide (PASPSKKPGGVAEGSLP). Antibodies against mouse T1R3 were generated in rabbit using mixture of four synthetic peptides (TEE-ATLNQRTQPNSILC, ARDLTLQFDAEGNVDMEY, QLQQSKMYWPGNQ VP, and CNQDQWSPEKSTA). These anti-serums were examined for their ability to detect both native and recombinant PLCbeta2 or T1R3 by immunohistochemistry and immunocytochemistry (Supplementary Figs. 1 and 2). Pre-immuned serum failed to detect specific signals from either examination. The other antibodies used were; rabbit anti-VNUT [18], mouse anti-IP3R3 (BD Biosciences), rabbit anti-PLCbeta2 (against C-terminus; Santa Cruz), mouse anti-NCAM (5B8, Developmental Studies Hybridoma Bank), mouse anti-Mash1 (BD Biosciences), mouse anti-V5 (Invitrogen). Immunohistochemical analysis was performed as follows. Tongues from adult mice or rats, or newborn mice at postnatal days 0, 3, and 6 (P0, P3, and P6) were fixed for 1 h at 4 °C in 4% paraformaldehyde in PBS, transferred to 30% sucrose in PBS, and incubated overnight at 4 °C. Frozen sections (12 μm) were rinsed in PBS and blocked in 0.3% Triton X-100 and 2% donkey serum in PBS for 1hr at room temperature. The primary antibodies were added to the sections in a humidified chamber and incubated overnight at 4 °C. Sections are washed in PBS, then the secondary antibodies (Alexa Fluor 488- or 555-conjugated donkey anti-mouse, rabbit, or rat; Invitrogen) were added (1:1000), incubation continued for 60 min, then rinsed in PBS. Zenon Alexa Fluor 488 IgG labeling kit (Invitrogen) was used to directly label PLCbeta2 antibodies.

Expression vector construction and transfection. V5-tagged fusion protein constructs were made by inserting full length of VNUT cDNA into pcDNA6/V5-His (Invitrogen). The plasmids were transfected to HEK 293 cells by Fugene 6 (Roche) for heterologous expression.

Results

Detection of VNUT expression by semi-quantitative RT–PCR in taste tissue

Since VNUT has recently identified we examined whether VNUT expression could be detected in taste tissues. Total RNAs isolated from either circumvallate papillae (CV) or surrounding non-sensory lingual tissue were subjected to RT–PCR analysis to examine selective expression of VNUT in taste tissues (Fig. 1). An elevated expression of VNUT in CV was seen along with T1R1, T1R2, and alpha-gustducin, but not in lingual epithelial tissue indicating that VNUT expression is either specific or selective to taste tissue. We failed to detect other known vesicular transporters such as vesicular glutamate transporters (VGLUT 1–3), vesicular inhibitory amino acid transporter (VIAAT), vesicular acetylcholine transporter (VAchT), and vesicular monoamine transporters (VMAT 1–2), examined by RT–PCR (data not shown).

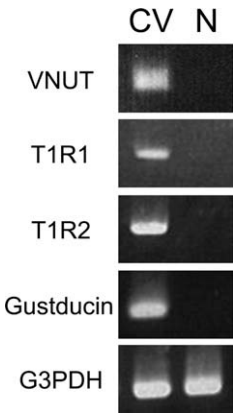

Fig. 1. VNUT transcripts are expressed in the taste tissue. RT–PCR analysis (30 cycles) was performed to examine expression of VNUT mRNAs in circumvallate papillae (CV) and non-sensory lingual tissue (N). Selective expression of VNUT expression in CV was observed together with T1R1, T1R2, and alpha-gustducin. Identity of each band was confirmed by nucleotide sequencing. G3PDH was used as a positive control for both tissues.

Selective expression of VNUT in type II taste cells

To determine which cell type of taste cells express VNUT protein, immunohistochemical analysis was performed using VNUT specific antibodies (Fig. 2). The antibody specificity has been shown previously [18]. In addition, we have further confirmed its specificity by pre-immune serum test, peptide adsorption test, and immunocytochemistry with VNUT overexpression system using HEK 293 cell line (Fig. 2F–H). A strong VNUT immunoreactivity was detected in the taste bud cells with round nucleus (Fig. 2A–E). By co-immunostaining CV sections with series of antibodies to type II or type III taste cell markers, such as PLCbeta2, IP3R3 for type II markers (Fig. 2A–C) or NCAM and Mash1 for type III markers (Fig. 2E and F), respectively, we found that VNUT immunopositive cells mostly co-localized with type II taste cell markers. On the contrary, VNUT immunoreactive cells were exclusive with type III taste cell markers. These results suggest that VNUT protein expression is restricted to type II cell.

VNUT and taste signaling elements are expressed similarly in the developing tongue

To examine whether VNUT protein expression is associated with taste bud development, we monitored the expression of type II taste cell markers along with VNUT expression. PLCbeta2 and T1R3 immunoreactive cells were weakly detected at the day of birth (P0) but was clearly detected at P3 in the mouse CV. The number of these cells increased gradually towards P6 when morphologically mature taste buds were observed (Fig. 3A–C). As had been expected, T1R3 immunopositive cells always expressed PLCbeta2 confirming that PLCbeta2 is a marker for Type II cell. Similarly, VNUT expression was always associated with PLCbeta2 expression, though its expression could be detected from P3 and thereafter along with maturation of taste buds (Fig. 3D–G). These indicate that VNUT may have functions related to type II cell.

Discussion

Although ample circumstantial evidence support ATP is compartmentalized with other neurotransmitters, there had been no

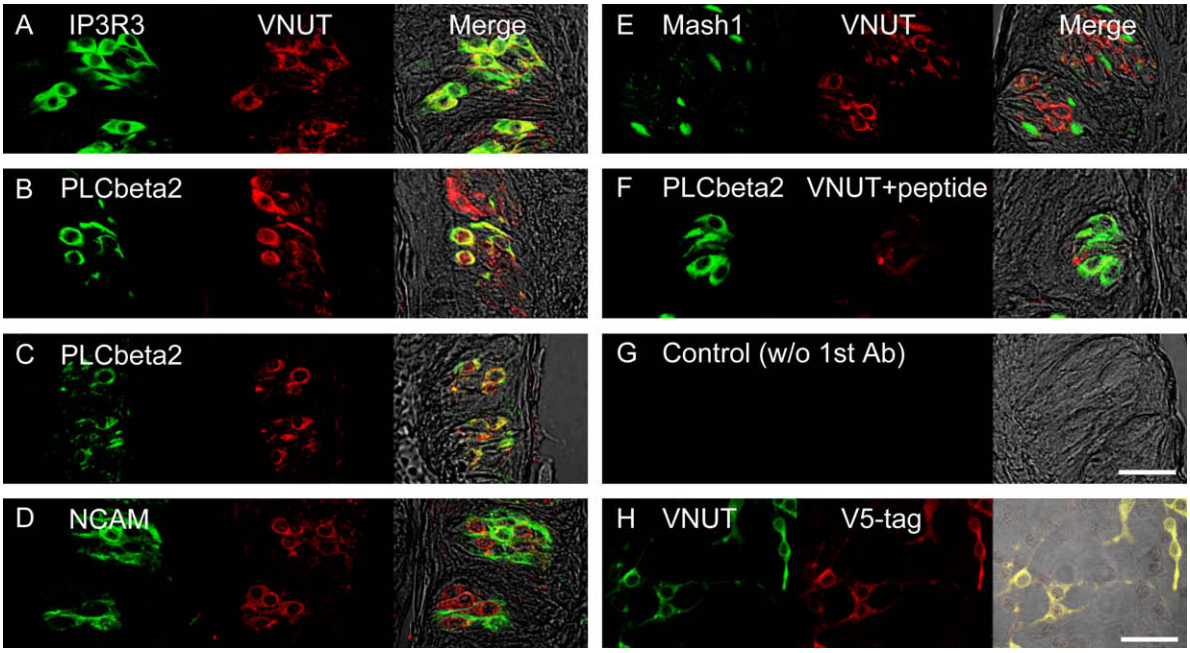

Fig. 2. VNUT is selectively expressed in type II taste cells of circumvallate papillae (CV). (A–C) VNUT expression pattern is similar to that of type II cell markers. IP3R3 (A, green) and PLCbeta2 (B and C, green) expression show significant overlap with VNUT (red). Right panels show the merged images of individual staining. On the contrary, type III taste markers such as NCAM (D, green) and Mash1 (E, green) were exclusive from VNUT (red) immunoreactive cells. (C) Anti-PLCbeta2 antibodies were directly labeled with 488 Alexa dye with Zenon reagents (see Materials and methods) and co-stained with anti-VNUT antibodies. (F–H) Controls. Antibodies against VNUT were neutralized by blocking peptides which contain the same epitope sequence to generate the antibodies (F). No signal was detected from the control section without primary antibodies (G). A recombinant VNUT protein (red) with V5 epitope tag (red) was produced in HEK cell line to confirm that the antibodies that are being used are specific to VNUT proteins (H). Bars, 25 μm (A–H).

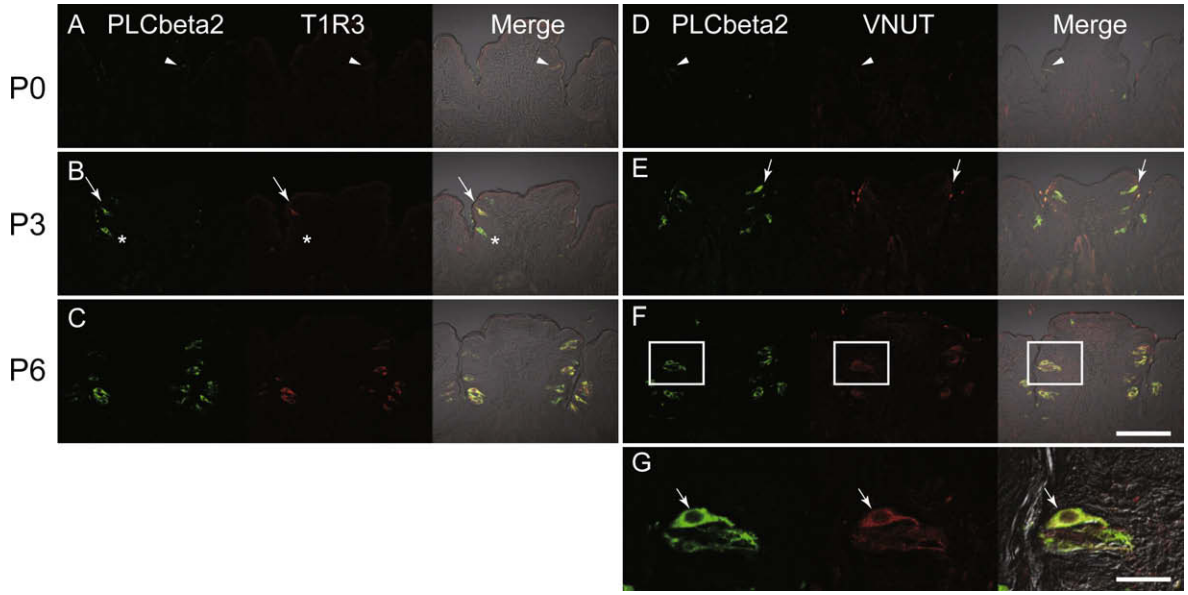

Fig. 3. VNUT expression is developmentally regulated and is correlated with the expression of type II taste cell markers. (A–G) Fluorescent micrographs of mouse CV sections from P0 (A,D), P3 (B,E), and P6 (C,F,G) stained with antibodies against PLCbeta2 (green), T1R3 (green), and VNUT (red). Right panels are the merged images. (A–C) PLCbeta2 and T1R3 immunoreactive cells were clearly detected from P3 and their numbers increased by P6. (D–F) Immunohistochemical analysis of PLCbeta2 and VNUT revealed that VNUT expression is first detected at P3 and is always associated with PLCbeta2 expression. (G) Magnified micrograph of the inset in (F). Note that most cells that are immunoreactive to PLCbeta2 are VNUT positive cells (see arrows for the double positive cell). Arrowheads in (A) and (D) (both at P0) show weak immunoreactive cells for PLCbeta2 and T1R3 but not for VNUT. Arrows in (B) (P3) show the cell that is both PLCbeta2 and T1R3 positive, while asterisks in (B) indicate the cell with only PLCbeta2 immunoreactivity. Bars, 100 μm (A–F), 25 μm (G).

report of vesicular transporters for ATP storage. This made us unable to identify the cell type and the tissue where ATP is stored and being released. A novel vesicular transporter, VNUT, that selectively transports nucleotides such as ATP and ADP, has enabled us to explore the link between ATP releasing cells and neurons or whatever cells that receive ATP [18].

We have found that VNUT is selectively expressed in type II taste cell. This report is the first report to show that VNUT is

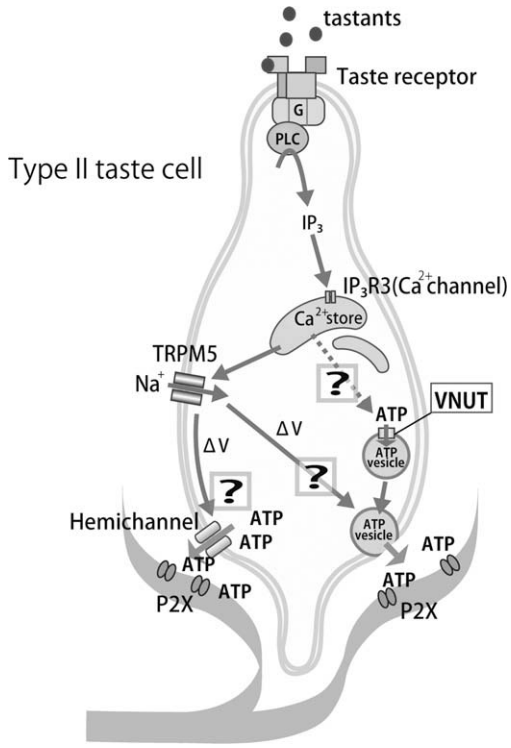

Fig. 4. Two possible models of ATP signaling pathways in taste system. VNUT is selectively expressed in type II taste cells where ATP is released. When type II taste cells are depolarized after taste stimuli, ATP secretion occurs by either through opening of hemichannels or/and exocytosis of vesicles. In either case, ATP signal is transmitted to gustatory afferent nerves that innervate taste buds. G, G-protein; PLC, phospholipase C; IP3, inositol triphosphate; IP3R3, IP3 receptor 3; ΔV, depolarization signal.

expressed in chemosensory epithelial cells. During mouse tongue development after birth, VNUT's temporal- and spatial-specific expression pattern was similar to those of PLCbeta2 and T1R3 analyzed by immunohistochemistry. Since PLCbeta2 and T1R3 are not only type II specific taste cell markers but also key molecules to transduce umami and sweet taste signaling [19,20], we speculate that VNUT also have a role in type II taste cell. The results we show here are partly consistent with the previous report that gene ablation of purinergic receptors affects type II taste cell mediated taste preferences: KO mice cannot discriminate sweet, bitter, and umami substances by behavioral test, however, on the other hand, electrophysiology data showed that gustatory nerves in the KO mice were devoid of sensing all taste stimuli [15]. Further studies should help to explain discrepancies between two experiments.

Initially, VNUT expression has been shown to be associated with chromaffin granules in adrenal gland and with secretory granules in PC12 cells [18]. Since ATP is often secreted with other neurotransmitters, we hypothesized that in type II taste cell ATP is compartmentalized in a vesicle that express VNUT. Although type II taste cell does not form conventional synapses [10], it has been indicated that presynaptic vesicular proteins are also expressed in type II taste cell, suggesting exocytotic pathways exist to transducer taste signals in type II taste cell as well as in type III taste cells [21,22]. Furthermore, syntaxin and SNAP-25, presynaptic membrane proteins are also found in type II and III taste cells [23,24]. These observations suggest that even though type II taste cells do not have conventional synapses they might have neurotransmitter vesicles similar to chemical synapses. In fact, a subset of taste cells, including type II taste cell, are known to secrete hormones (GLP-1 or CCK) and neurotransmitters [25,26]. Together, our present study implies the presence of secretory granules in type II taste cell that might be associated with VNUT expression and plays a role in exocytosis.

Whereas VNUT is a good candidate that function to store and release ATP through exocytosis from taste cells, there had been other reports suggesting that hemichannels, components of tight junction, such as pannexin 1 and connexins could mediate ATP signal transmission by releasing ATP from taste cells [27,28]. Pannexin 1 and connexins are both found in taste tissues [28], however, these molecules are expressed non-selectively in multiple tissues (data not shown). To determine which molecule is responsible for ATP release awaits further studies. Therefore, we cannot conclude at this point which molecule is more likely to be involved in a machinery for ATP release, either VNUT or hemichannel. We have developed schematic models of how ATP is released from type II taste cell after taste stimuli (Fig. 4). In this model, release of ATP is carried out by either opening of hemichannels (non-vesicular release) or/and exocytosis (vesicular release) after depolarization.

In conclusion, we have identified VNUT in type II taste cell, which are newly found vesicular transporter specialized to transport nucleotides such as ATP. Together with previous studies, our results suggest that there is a mechanism to store ATP in the cell and ATP is released from type II cells. Our observation provides a novel view of ATP release that is via exocytosis of vesicles containing ATP. Further experiments will be needed to elucidate the biological function of VNUT.

Acknowledgments

We thank Kenichiro Nakamura and Mariko Ozawa for excellent technical assistance. We also thank Drs. Yuzuru Eto and Rieko Tanegashima for critical comments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.07.069.

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