Neuroscience Letters 533 (2013) 104–108

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

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

Expression of equilibrative nucleoside transporter 1 in rat circumvallate papillae

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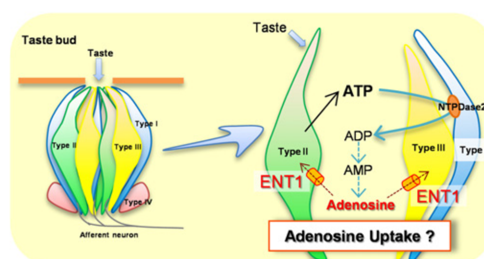
HIGHLIGHTS

- mRNAs for ENT1, but not ENT2, and CNT1, 2 or 3, are expressed in rat taste buds.
- ENT1 is expressed in type II and III taste cells.
- ENT1 contributes to extracellular adenosine clearance in taste buds.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 19 September 2012
Received in revised form 27 October 2012
Accepted 31 October 2012

Keywords:
Adenosine
Taste bud
Circumvallate papillae
ENT

ABSTRACT

In gustatory function, communication between four types taste buds cells plays crucial roles. ATP is one of the intercellular signaling molecules in taste buds, and the extracellular ATP fate is regulated by its cellular clearance, but there is little information on it. Therefore, we examined the expression profiles of nucleoside transporters (NTs) as a clearance system for ATP metabolite adenosine in rat circumvallate papillae (CP) by RT-PCR, real-time PCR and immunohistochemistry. Among NTs, mRNA for *Ent1* was expressed by the CP, and significantly was greater in the CP as compared with non-CP. ENT1 immunoreactivity was detected in PLC-β2-positive type II (71.0 ± 8.5%), chromogranin-A-positive type III (64.9 ± 7.4%), and SNAP25-positive type III (77.0 ± 10.4%) taste cells, but not in NTPDase2-positive type I ones. These results indicate that ENT1-expressing type II and III taste cells might comprise an adenosine clearance system in taste buds of the CP. ENT1 expression in taste cells is important for elucidation of complicated taste signaling.

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1. Introduction

ATP is an intracellular energy source and also plays a crucial role in maintenance of homeostasis as a cell-cell communication molecule [8]. ATP released into the intercellular space actives P2 receptors, and then cellular signals are transmitted to host cells [9]. In addition to intact ATP, adenosine, as its metabolite, also plays a role in cellular signaling *via* activation of P1 receptors [10]. In the central nervous system, ATP is extensively metabolized into adenosine by ecto-enzymes expressed by neuronal cells, and the generated adenosine is taken up into the cells *via* nucleoside transporters (NTs) [18–20]. Previously, we demonstrated that among NTs expressed by cerebral neurons and glial cells, equilibrative NT (ENT) 2 in astrocytes plays a major role in adenosine clearance in brain neuronal cells [19]. Thus, the ATP clearance system consisting of ATP metabolism and adenosine uptake is critical for maintenance of cellular homeostasis through modulation/termination of the ATP/adenosine signaling [1,3,11], and the revealing of cell-type specific expression profile of NT isoforms is important for a better understanding the ATP/adenosine signaling system in tissues.

In taste buds, ATP is an intercellular communication molecule, and plays a role in gustatory function [8]. ATP is reported to be released from type II receptor taste cells stimulated by taste compounds *via* pannexin1 hemichannels and exocytosis [14,15,21], and the signaling is transmitted to type III presynaptic taste cells and/or

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0304-3940/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neulet.2012.10.063

gustatory afferent nerve fibers *via* P2 receptors [6,24]. As for ATP metabolism, type I and III taste cells have been demonstrated to express ectonucleoside triphosphate diphosphohydrolase 2 (NTPDase2) and ecto-5′-nucleotidase (NT5E), respectively, and both types of cells also express prostatic acid phosphatase, and are considered to contribute to the modulation/termination of ATP signaling [2,4]. Recently, Dando et al. and Kataoka et al. reported that among P1 receptors, A2B receptors (A2BRs) are expressed by type II taste cells and modulate the sweet taste in the circumvallate papillae (CP), indicating that in addition to ATP, adenosine is also a taste-signaling molecule [4,16]. This also suggests that clearance of adenosine from the extracellular space in taste cells is important for regulation of taste signaling, but there is no information on the adenosine clearance system in taste buds.

Therefore, in this study, we examined the expression of NTs in taste cells and identified cell-type specific isoform(s) of NTs.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (200–300 g; Japan SLC, Hamamatsu, Japan) were used in this study. All experiments were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University, and were performed according to the Guidelines for Animal Experimentation of Kyoto Pharmaceutical University.

2.2. Dissection of circumvallate papilla taste epithelia

SD rats were perfused transcardially with saline under deep anesthesia (pentobarbital sodium, 25 mg/kg, i.p.). For reverse transcription (RT)-PCR, taste epithelia containing CP were picked up by injection of an enzyme mixture comprising 2.5 mg/mL dispase II, 1.0 mg/mL collagenase D, and 1.0 mg/mL trypsin inhibitor for 30 min at RT, and then the taste epithelia were treated with an RNAlater® solution (Life Technologies, Tokyo, Japan) at −20 °C.

2.3. RT-PCR analysis

Total RNA was extracted and reverse transcribed with a NucleoSpin® RNA XS kit (MACHEREY-NAGEL, Düren, Germany) and a PrimeScript™ RT reagent kit (Takara, Shiga, Japan) according to the manufacturers' instruction manuals, respectively. RT-PCR was performed using rTaq DNA polymerase (Takara, Shiga, Japan) or SYBR Premix Ex Taq (Takara). The primer sets used were as follows: rat *Ent1* (NM_031684): 5′-AACTGCTACTTCATCCCTGTGG-3′ (sense), 5′-GCAGGTAGTGGTGCTGCTTC-3′ (antisense); rat *Ent2* (NM_031738): 5′-TGTGTTGGTCTTCACAGTCAC-3′ (sense), 5′-AGCAGGAAGCAACAGATAGG-3′ (antisense); rat *Cnt1*: 5′-TGAACAAAGCAGAGCGGAAG-3′ (sense), 5′-AGAAGCCAAGACATGCAGTAGAGA-3′ (antisense); rat *Cnt2* (NM_031664): 5′-TGGGTACCACAGCTGCAGAGA-3′ (sense), 5′-TAGGGACGGATGAGCAGAGG-3′ (antisense); rat *Cnt3* (NM_080908): 5′-TCCCATTGAGTCCGTAGTTG-3′ (sense), 5′-TTCTGACTTGGTGACGTGTG-3′ (antisense); rat *Ntpdase2* (NM_172030): 5′-GGGTGACTGCCAACTACCTG-3′ (sense), 5′-GACTGTGAAGAGCAGCAGGAG-3′ (antisense); rat *Plcb2* (NM_053478): 5′-AAGGCATATCTGAGCCAAGG-3′ (sense), 5′-TTGCAAGGTGACAGGCACTG-3′ (antisense); rat *Snap25* (NM_030991): 5′-ATGCTGCAGCTGGTCGAAGA-3′ (sense), 5′-GAGTCAGCCTTCTCCATGAT-3′ (antisense); rat *Actb* (NM_031144): 5′-AGGTCATCACTATTGGCAACGA-3′ (sense), 5′-CACTTCATGATGGAATTGAATGTAGTT-3′ (antisense); and rat *Gapdh* (NM_017008): 5′-TCATTGACCTCAACTACATGGTC-3′ (sense), 5′-CGTTCAGCTCTGGGATGAC-3′ (antisense).

2.4. Immunohistochemical analyses

The animals were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) containing 0.2% picric acid under deep anesthesia (pentobarbital sodium, 25 mg/kg, i.p.). The tissues containing CP were sectioned at 40 μm thickness with a freezing microtome, and free-floating sections were immunoreacted with primary antibodies, rabbit anti-ENT1 (1:200; #11337-1-AP, Proteintech Group, IL), sheep anti-NTPDase2 (1:200, #AF5797, R&D Systems, MN), goat anti-PLC-β2 (1:200, #sc31759, Santa Cruz, CA), goat anti-chromogranin A (1:30, #sc1488, Santa Cruz, CA), or mouse anti-SNAP25 (1:500, #S5187, Sigma, MO) in Can Get Signal® B solution (TOYOBO, Osaka, Japan) for 3 days at 4 °C, followed by incubation for a day at 4 °C with Alexa Flour® 594-conjugated donkey anti-sheep, -goat or -mouse IgG antibodies (1:1000) and Alexa Flour® 488 conjugated donkey anti-rabbit IgG antibodies (1:1000; Life Technologies, Tokyo, Japan) in Can Get Signal® B solution. Negative controls were prepared by omitting the primary antibodies. For the preadsorption test, anti-ENT1 antibodies (1.8 μg/total vol. 300 μL) were adsorbed with ENT1 antigen peptide (8.1 μg/total vol. 300 μL; #ag1881, Proteintech Group, IL). The sections were mounted on glass slides and then enclosed using a Prolong® antifade kit (Life Technologies, Tokyo, Japan). Photomicrographs were obtained under a confocal laser microscope (LSM510META; CarlZeiss, Germany).

3. Results

By semi-quantitative RT-PCR analyses, the mRNAs for ENT1 and 2, and CNT3 were expressed by the rat CP (Fig. 1A), and mRNA levels for ENT1 corrected by those for GAPDH in the rat CP were approximately 1.2-fold greater than that in the non-CP. On the quantification analysis, the expression level of mRNA for ENT1 in the rat CP was significantly greater than that in non-CP, *i.e.*, the juxta-regions of CP (Fig. 1B). On immunohistochemistry, as shown in Fig. 1C, ENT1-immunoreactivity was detected in taste buds of the CP. On the other hand, the preadsorption and negative controls did not show any apparent fluorescent signals (Fig. 1C). These results suggest that ENT1 may be expressed in taste buds of the CP.

NTPDase2 is an ecto-ATPase expressed by type I taste cells in taste buds [2]. ENT1-immunoreactivity (green) was not detected in NTPDase2-positive taste cells (red), and the ENT1-positive taste cells were surrounded by these cells (Fig. 2). It is well-known that type II taste cells are surrounded by type I cells in taste buds, and that their typical morphology is spindle-shaped or pyriform and the majority of them are immunoreactive for PLC-β2 [22]. The ENT1-immunoreactivity was detected together with the PLC-β2-signals (arrowheads in Fig. 2). The double labeling ratio of the ENT1-immunoreactive taste cells as to the PLC-β2-positive taste cells was 71.0 ± 8.5% (mean ± SEM, N = 3). Type III taste cells are thin and spindle-shaped, and are immunoreactive for chromogranin-A and SNAP25 [7,24]. ENT1-immunoreactivity (green) was also found in the type III taste cells (red, Fig. 2). The double labeling ratios of the ENT1-immunoreactive taste cells as to chromogranin-A- and SNAP25-positive taste cells were 64.9 ± 7.4% (N = 4) and 77.0 ± 10.4% (N = 5), respectively. These results suggest that the ENT1-expression levels in type II and III taste cells are greater than that in type I ones.

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