Biochemical and Biophysical Research Communications 406 (2011) 146–151

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Functional bitter taste receptors are expressed in brain cells

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ARTICLE INFO

Article history:
Received 18 January 2011
Available online 12 February 2011

Keywords:
Bitter taste receptors
T2R4
RT-PCR
Immunohistochemistry
Neurons
Glial cells
G-protein mediated calcium signaling

ABSTRACT

Humans are capable of sensing five basic tastes which are sweet, sour, salt, umami and bitter. Of these, bitter taste perception provides protection against ingestion of potentially toxic substances. Bitter taste is sensed by bitter taste receptors (T2Rs) that belong to the G-protein coupled receptors (GPCRs) superfamily. Humans have 25 T2Rs that are expressed in the oral cavity, gastrointestinal (GI) neuroendocrine cells and airway cells. Electrophysiological studies of the brain neurons show that the neurons are able to respond to different tastants. However, the presence of bitter taste receptors in brain cells has not been elucidated. In this report using RT-PCR, and immunohistochemistry analysis we show that T2Rs are expressed in multiple regions of the rat brain. RT-PCR analysis revealed the presence of T2R4, T2R107 and T2R38 transcripts in the brain stem, cerebellum, cortex and nucleus accumbens. The bitter receptor T2R4 was selected for further analysis at the transcript level by quantitative real time PCR and at the protein level by immunohistochemistry. To elucidate if the T2R4 expressed in these cells is functional, assays involving G-protein mediated calcium signaling were carried out. The functional assays showed an increase in intracellular calcium levels after the application of exogenous ligands for T2R4, denatonium benzoate and quinine to these cultured cells, suggesting that endogenous T2R4 expressed in these cells is functional. We discuss our results in terms of the physiological relevance of bitter receptor expression in the brain.

1. Introduction

The mammalian taste sensation provides valuable information about the nature and quality of food. Taste transduction predominantly involves the interaction of molecules (i.e. tastants) with taste receptor-expressing cells that reside in the taste buds located on the papillae of the tongue. Taste buds relay information to the brain on the nutrient content of food. Bitter taste which is sensed by bitter taste receptors (referred to as T2Rs), is one of the five basic taste modalities. Bitter sensing serves as a central warning signal against the ingestion of potentially harmful substances. Bitter compounds have diverse chemical structures and include natural alkaloids such as, quinine, caffeine, nicotine and morphine. Even in rodents, bitter sensing is well developed, as demonstrated by the ability of mouse or rat to respond to a wide variety of bitter molecules known to humans [1–3]. In fact, rodents express 29 bitter receptors compared to only 25 bitter receptors present in humans [2,3]. Previous studies have shown that in addition to taste cells in the oral cavity, T2Rs are also expressed in gastrointestinal (GI) neuroendocrine cells of the large intestine [3,4], chemosensory cells of nasal epithelium [5] and human airway cells [6,7]. However, the presence of T2Rs in other non-oral tissues has not been elucidated.

The expression "food for thought" is no longer just a metaphor, advances in nutritional and neuroscience research show that specific nutrients alter molecular pathways in the brain through changes in the G-protein coupled receptor (GPCR), ion channel and other receptor mediated signaling cascades. Electrophysiological studies of the brain neurons show that neurons are able to respond to different tastants. Electrophysiological studies in rats show that neurons in the nucleus accumbens (NAc) were activated in response to both sucrose (sweet tastant) and quinine (bitter tastant) [8]. However different sets of NAc neurons responded to the quinine than to sucrose, pointing to the possibility of different circuitry for processing aversive and rewarding stimuli. However, the presence of bitter taste receptors in brain cells has not been elucidated. In addition, the taste center(s) of the brain that respond to different tastes are not yet understood.

In this report, using reverse-transcriptase (RT)-PCR analysis we show the presence of transcripts corresponding to T2R4, T2R107 (also referred to as T2R10) and T2R38 in the brainstem, cerebellum, cortex and nucleus accumbens of rat brain. By immunohistochemistry (IHC) and double-labeling IHC experiments we

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doi:10.1016/j.bbrc.2011.02.016

demonstrate localization of T2R4 in the neuronal cells of the rat brain stem. Furthermore, functional studies on cultured cells of the rat brain show an increase in intracellular calcium levels after the application of denatonium benzoate and quinine (exogenous bitter ligands for T2R4), suggesting that T2R4 expressed in these cells is functional. We discuss various hypotheses regarding the physiological relevance of the expression of T2Rs in the brain.

2. Materials and methods

2.1. Materials

Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA, USA). The polyclonal antibody rabbit anti-rat T2R4 (based on old nomenclature was referred to as ratT2R16) was from Novus Biologicals (Littleton, CO, USA), the goat-anti mouse NeuN and goat anti-mouse GFAP antibodies are from Millipore (Billerica, MA, USA).

Table 1
Oligonucleotide primer sequences for the rat bitter taste receptor genes.

Type	Gene size (bp)	Primer sequence (5′-3′)	Predicted amplicon size (bp)
T2R4	915	F-CTGCTCAATGTGACCTTTGC R-CCAAAAGCTGCTCCTGTTTC	87
T2R107 or T2R10	927	F-ACATGCCTCTATCCCTGTGG R-GCACGTTAAGTGCTGCAGTG	96
T2R38	996	F-AGCCATCCTCACTCTCTGGA R-AATCTTGGCGCAGTAGAGGA	85
GAPDH	1307	F-ATGACTCTACCCACGGCAAG R-GATCTCGCTCCTGGAAGATG	105

2.2. Animals

Rat experimental procedures were approved by the University of Manitoba Protocol Management and Review committee (No. 09-003). Experiments were performed with adult male or female Sprague–Dawley rats (Winnipeg, MB; 110–150 g body weight).

2.3. RNA isolation

Total RNA from cells and tissues were isolated using RNeasy mini kit (Qiagen) according to manufacturer's protocol. Isolated RNA was treated with DNase I analyzed on an agarose gel by running the appropriate reverse transcriptase (RT) negative controls to ensure that no contaminating genomic DNA and then used for cDNA synthesis with SSIII RT (superscript III reverse transcriptase), dNTPs, Oligo dT primer and first strand buffer (Invitrogen).

2.4. Reverse transcriptase (RT)-PCR

Partial length T2R coding sequences were amplified from rat cDNA using oligonucleotide primers designed for T2R4, T2R10, T2R38 and GAPDH. The sequences of primers used and the predicted amplicon sizes are listed in Table 1. PCR was performed

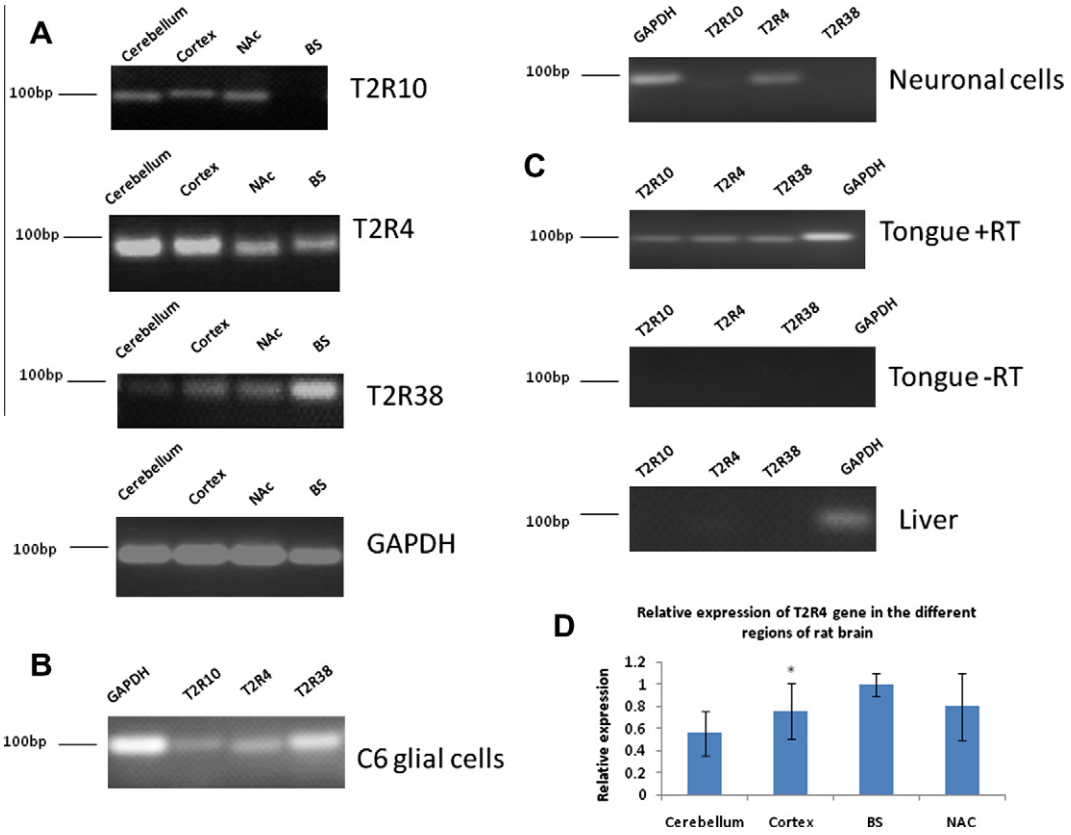

Fig. 1. Expression of bitter taste receptors (T2Rs) in different regions of the rat brain tissues, analyzed by reverse transcriptase (RT)-PCR and real time PCR. (A) 2% Agarose gel electrophoresis analysis of the RT-PCR products. GAPDH was used as an internal control for the PCR reactions. The molecular size markers (NEB) in base pairs and the bitter taste receptors (T2Rs) are indicated next to the gels. (B) Expression of T2Rs in C6 glial cells and neuronal cells, analyzed by RT-PCR. (C) Expression of T2Rs in rat tongue (positive control) and liver (negative control) along with the reverse transcriptase omitted samples (-RT) are shown. (D) Relative expression level of T2R4 in different regions of rat brain were determined by real-time PCR. Data presented are from experiments done in duplicate for three independent times. Values are plotted as mean ± SEM. Relative T2R4 expression levels were computed using $2^{-\Delta\Delta CT}$ method. Melt curve analysis confirmed the presence of a single PCR product in each reaction. Statistically significant values are indicated by asterisk.

in a total volume of 20 μl containing 1 μl reverse transcribed cDNA, 400 nM of each primer in hot start buffer and 0.4 U hot start Taq DNA polymerase (NEB) and 50 μM dNTPs. An initial denaturation step of 94 °C for 10 min was followed by 50 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s for T2R4 and T2R10 and 60 °C for 30 s for T2R38 and extension at 72 °C for 30 s and finished with a final extension at 72 °C for 2 min on a thermocycler (MJ mini cycler, Bio rad). Rat liver tissue (negative control) and tongue tissue (positive control) were also amplified for each primer pair to confirm the expression of T2Rs. The housekeeping gene GAPDH was used as a control in the PCR reactions. All PCR products were separated on 2% agarose gels and stained with ethidium bromide. Gel images were photographed under UV light.

2.5. Real-time PCR

For analysis by real-time PCR, reverse transcription was carried out as described above. Reaction mixtures, with a final volume of 25 μl consisted of 1 μl reverse transcribed cDNA, 0.4 μM primers, 1X SYBR Green containing dNTPs mix and Taq polymerase. The reaction conditions were similar to those used for RT-PCR, except for an initial denaturation step of 15 min at 95 °C. This was followed by melt curve analysis from 73 °C to 95 °C at every 1 °C increase in temperature for about 1 s for 23 cycles. Melt curve analysis confirmed the presence of a single PCR product in each reaction.

2.6. Primary neuronal cell culture

Cortical tissues from rat pups were dissociated by trituration and subsequently cultured in neurobasal medium supplemented with B27 on Poly-L-lysine coated 12-well plates and as described earlier [9]. For culturing primary neuronal cells, brain from embryonic day 19 or 20 white Dawley rats were placed in HBSS free of calcium and magnesium. Cortices were dissected out and placed in HBSS. Cells were dissociated with the help of sterilized glass pipettes for 5–10 times and transferred to a 15 ml sterile tube. Whole cell suspension was transferred to a 75 cm^2 tissue culture flask and placed in a CO_2 incubator for 1 h. Non-adherent cells were recovered and plated at 1.5–4.0 $\times$ 10^5 cells per well in 12 well plates, in neurobasal media (Invitrogen). Cells were allowed to grow for 10–14 days before use and 50% media was changed every 4 days to get a pure population of neuronal cells.

2.7. Histological procedures and Immunohistochemistry

Rats were anaesthetized with ketamine and xylazine and cardio-perfused with PBS followed by 4% paraformaldehyde (PFA) in PBS. Brains were removed and post fixed in 4% PFA overnight and cryoprotected in 30% sucrose in PBS for 1 day at 4 °C. Twenty five micrometer coronal sections were cut using a sliding microtome (Leica, Germany). The free floating sections were pre-treated with PBS containing 0.3% H_2O_2 for 1 h at room temperature to eliminate endogenous peroxidase activity, washed three times with 0.1 M TBS and blocked for 1hr at room temperature using blocking buffer. Sections were incubated overnight with a 1:100 dilution of rabbit anti-T2R4 antibody in 5% normal goat serum in TBS containing 0.25% Triton X-100. The sections were incubated for 1 h with the secondary antibody goat anti-rabbit HRP (1:500, Bio-Rad) followed by solution containing 0.02% 3, 3'-diaminobenzidine-4HCl (DAB), and 0.001% H_2O_2, and the reaction was stopped with a PBS wash. The control sections were treated with only secondary antibodies. To check the specificity of the antibody the tongue section was stained with the T2R4 antibody (Fig. 2C).

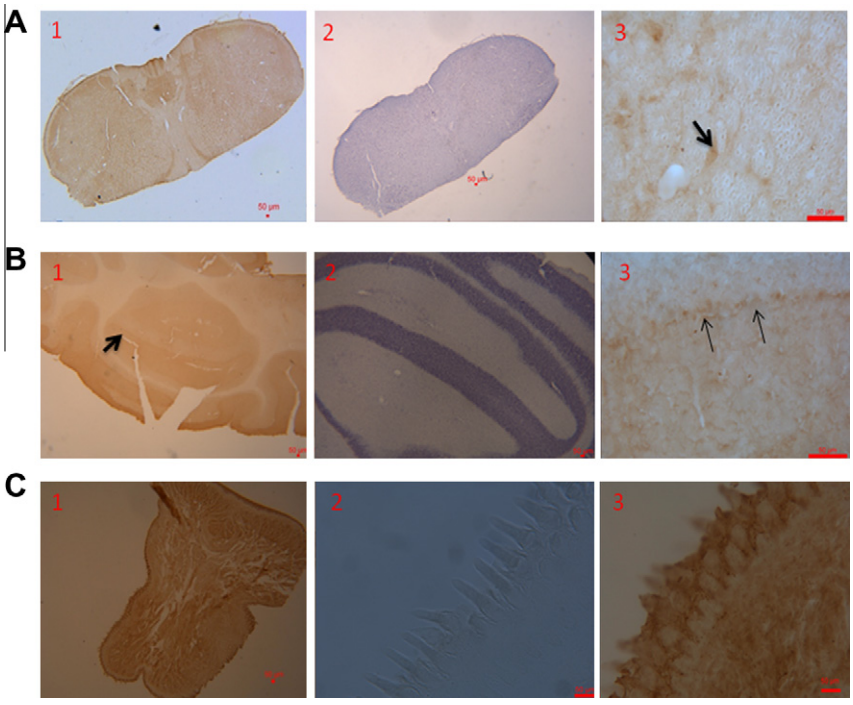

Fig. 2. T2R4 expression in the brain stem (BS) and cerebellar regions of rat brain. (A) BS stained with the T2R4 antibody and DAB brown as chromagen (1 and 3). The neurons of the brain stem stained positive for the receptor shown by arrows (3). (B) Cerebellar section stained with the anti-T2R4 antibody and DAB brown as chromagen (1 and 3). The neurons (purkinje) of the cerebellum stained positive for the receptor and are shown by arrows. In both A and B, the arrow shows stained neurons (3) at higher magnification of 40×. Control section without the T2R4 antibody but with the 2°Ab, goat anti-rabbit HRP and chromagen DAB brown and counter stained with hematoxylin solution (2). (C) Rat tongue stained with anti T2R4 antibody and DAB brown as chromagen (1 and 3). To check for the specificity of the anti-T2R4 antibody used in this study, rat tongue was used as a control. Calibration bar = 50 μm.

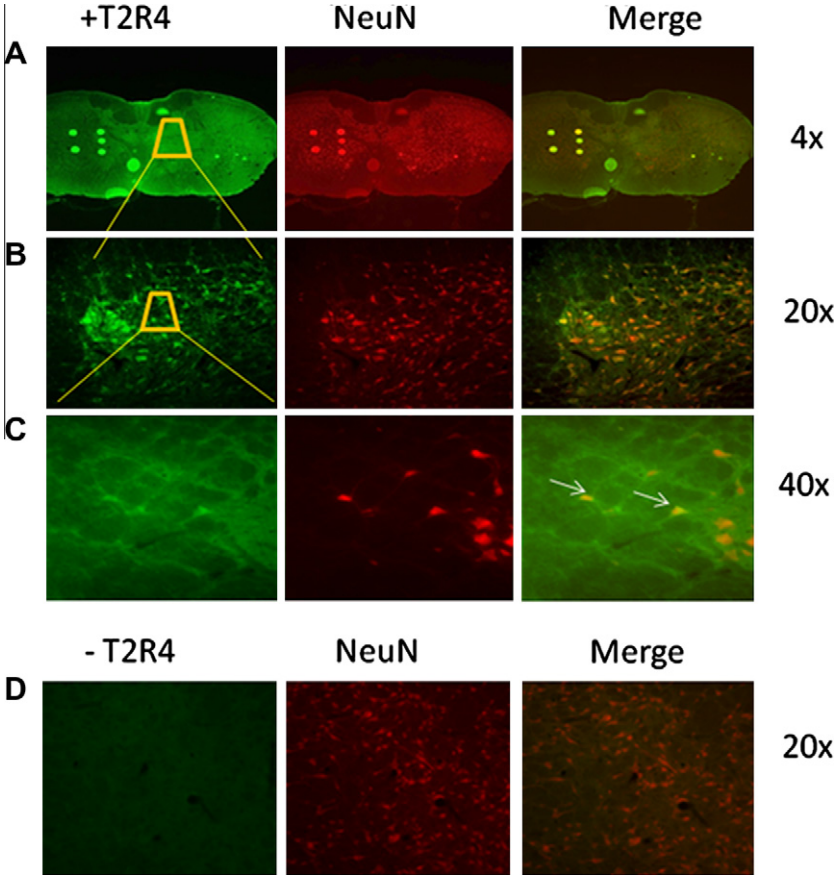

Fig. 3. Co-expression of T2R4 and nuclear neuronal marker, NeuN in the rat brainstem as assessed by double labeling immunohistochemistry. Fluorescence images of the rat brainstem labeled for T2R4, NeuN and merged images were shown at 4× (A), 20× (B) and 40× (C) magnifications along with section magnified shown in a box. The localization of T2R4 in neuronal cells is shown by arrows in the merged image, suggesting that T2R4 is predominantly expressed in neurons of brainstem. (D) Brainstem section incubated with normal rabbit antisera (control) and NeuN antibody, merged image shows no labeling with normal rabbit antisera.

For double labeling, brain sections were incubated overnight with both T2R4 antibody (1:300) and mouse anti-NeuN (1:1000) and then incubated with Alexa 488 donkey anti-rabbit IgG for 1 h, followed by Alexa 594 donkey anti-mouse IgG for 1 h, and finally mounted on slides using Gold anti-fade reagent (Invitrogen). Fluorescence images were obtained using an Olympus BX61 microscope.

2.8. Functional assays

Functional characterization of T2R4 was carried out using Fluo-4 NW (Invitrogen) and as described earlier [10]. The C6 glial cells and cultured primary neuronal cells stimulated with assay buffer were used as controls.

3. Results

3.1. Expression of T2R transcripts in brain tissues, C6 glial cells and primary neuronal cells

To determine the expression of T2R transcripts in brain tissues, three T2Rs (T2R4, T2R10 and T2R38) were selected for analysis by RT-PCR. The RNA was isolated from different brain regions including, the brain stem, cortex, cerebellum and nucleus accumbens (NAc), the cDNA synthesized and RT-PCR performed as described in methods. The transcripts for all the three T2Rs were detected in the regions analyzed, except for T2R10 which was not detected in the brain stem (Fig. 1A). This can be due to the low copy number or expression of T2R10 gene in the rat brainstem. Further, we examined the expression of T2Rs in invitro cultured C6 glial cells and primary neuronal cells. As shown in Fig. 1B, C6 glial cells express the transcripts for all the three T2Rs analyzed, while the primary neuronal cells express only T2R4. In addition, all three T2Rs were detected only in the tongue tissue (positive control) but are not found in the liver tissue (negative control) samples (Fig. 1C).

3.2. Localization of bitter taste receptor T2R4 in the rat brain

Among the three T2Rs examined by RT-PCR, T2R4 was expressed predominantly in the brain regions analyzed. In addition, only T2R4 transcripts were present in the primary cultured rat neurons, as such the bitter receptor T2R4 was selected for further analysis at the transcript level by quantitative real time PCR, and at the protein level by immunohistochemistry (IHC). Among the brain regions analyzed by real time PCR, the relative abundance of T2R4 mRNA was higher in the brain stem compared to the cerebellum, cortex and nucleus accumbens (Fig. 1D). Next, to visualize the localization of T2R4 receptor in the rat brain, we performed IHC analysis using a polyclonal T2R4 antibody. Our IHC analysis showed strong expression of T2R4 in the neurons of brain stem especially in the nucleus of solitary tract (NTS), and in the molecular layer of cerebellum (Fig. 2A and B). The IHC analysis is consistent with our real time PCR data.

To identify the specific cell type in the brain regions that express the T2R4, double labeling IHC was carried out using neuronal marker NeuN, and glial cell marker, GFAP. Double labeling IHC

experiments demonstrated localization of T2R4 in the neuronal cells of the rat brain stem. As shown in Fig. 3, the brain stem labeled with anti-T2R4 and anti-NeuN showed strong expression of T2R4 in the neuronal cells. However, we did not observe any co-localization of T2R4 with the glial cell marker (data not shown), indicating that T2R4 might be predominantly expressed in the neuronal cell population of the brain regions analyzed.

3.3. Functional assay of the expressed T2R4

Having demonstrated that invitro cultured primary neuronal and C6 glial cells express T2R4 (Fig. 1B), our next step was to determine whether the receptor expressed was functional. Previously it was shown, that addition of synthetic bitter compound denatonium benzoate (DB), or the natural alkaloid quinine to T2R4 expressing cells causes an increase in intracellular calcium through a heterotrimeric G-protein dependent pathway [3,11,12]. Except for neuronal cells stimulated with quinine, robust transient changes in intracellular calcium levels were observed for neuronal cells stimulated with DB and for glial cells stimulated with DB and quinine. In our assays, the addition of either DB (in millimolar doses) or quinine (micromolar doses) induced a dose dependant increase in intracellular calcium in both primary neuronal cells and C6 glial cells. EC_{50} values of 23.50 ± 4.0 mM and 22.97 ± 4.0 mM were observed for glial and neuronal cells stimulated with DB, and EC_{50} values of 1.6 ± 0.5 mM and 1.0 ± 0.3 mM for glial and neuronal cells stimulated with the quinine, respectively. Though these values are high, we could not find any experimentally derived literature values for the EC_{50} of quinine and DB for T2R4.

4. Discussion

There are multiple lines of evidence to support the hypothèses that chemosensory function of taste receptors is influenced by their localization in the human body. Humans have 25 T2Rs that are expressed in the oral cavity, GI neuroendocrine cells and airway epithelial and smooth muscle cells. In the oral cavity T2Rs are responsible for the detection of toxins in food prior to it being swallowed, in the GI tract they are hypothesized to play a role in food intake, and in limiting toxin absorption [13,14].

4.1. Expression of bitter taste receptors in the gastrointestinal tract

Elucidation of the taste signaling mechanisms in the GI tract at the molecular level came with the discovery that the T2Rs and taste receptor specific G protein, α-gustducin are expressed in peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) producing L cells of the intestine [15,16]. Furthermore it was shown that T2R9 affects glucose and insulin homeostasis in the gut [17] through the modulation of the incretin hormone GLP-1. The incretin hormone GLP-1 lowers the blood glucose level and controls food intake [18,19]. It has been shown that GLP-1 is secreted in a taste receptor-dependent manner by gut enteroendocrine cells in response to stimulation with natural and artificial stimuli [20].

Denatonium benzoate is a very bitter compound and an exogenous ligand for T2R4 [3]. Previous studies reported that denatonium benzoate inhibited feeding and delays gastric emptying in rats [21]. The physiological mechanism of action of DB is through its activation of T2Rs causing an increase in intracellular calcium and subsequent release of the peptide chloecystokinin (CCK) from the enteroendocrine cell line STC-1 which results in reduced food intake [22]. Results from our in vitro cell culture experiments demonstrated that primary neuronal cells cultured from the rat brain express T2R4. Functional assays involving G-protein mediated calcium signaling showed an increase in intracellular calcium after the application of DB, suggesting that T2R4 expressed in these cells is functional (Fig. 4). However, whether the DB induced calcium signaling in neuronal and glial cells results in any CCK or other peptide release is speculative, and remains to be determined.

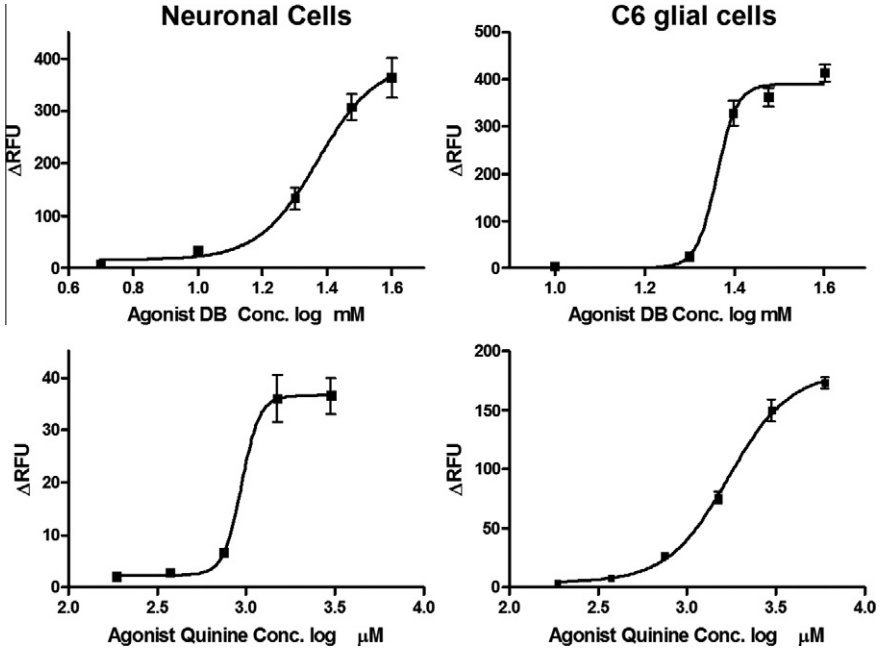

Fig. 4. Concentration-dependent changes in intracellular calcium [Ca^{2+}] induced by exogenous bitter ligands for T2R4 in primary neuronal and C6 glial cells. Dose–response curves of neuronal and C6 glial cells expressing endogenous T2R4 stimulated with increasing concentrations of the bitter compounds denatonium benzoate (mM) and quinine (μM). Data were collected from at least two independent experiments carried out in triplicate. EC_{50} values were calculated using Graph Pad Prism software, after subtracting the responses of cells stimulated with buffer alone.

4.2. Expression of taste receptors in cells of the central nervous system

The hypothesis that taste signaling mechanisms have physiological roles in the central nervous system has been proposed and tested. Previously, it has been shown that the putative mammalian sour taste receptor or PKD2L1 is expressed in the tongue and also in the neurons of the spinal cord [23]. Early electrophysiological studies described the presence of glucosensing neurons in brain regions known to influence glucose homeostasis, including the hypothalamus and the brain stem [24,25]. Recently it was shown that taste sensing GPCRs, T1Rs are expressed in the brain [26]. In addition, it was shown that the taste receptor specific G protein, gustducin, was expressed in brain cells [26]. In this manuscript, we report the presence of another taste sensing group of GPCRs, the bitter taste receptors in the brain cells. With this, we now know that brain has a full molecular component of the GPCR dependent taste signaling "cascade".

Quinine is a natural alkaloid similar to caffeine and is an important secondary metabolite. In our functional assay, quinine activates T2R4 with an EC_{50} of 1.0 ± 0.3 mM. This value is similar to the EC_{50} of 1.1 ± 0.5 mM obtained for the activation of T2R4 by quinine using a heterologous expression system (Pydi and Chelikani, unpublished results). A previous study reported that quinine is involved in the blockage of the neuronal gap junctions [27]. In addition, quinine induced hypoglycemia in healthy individuals by lowering the plasma glucose and increasing the plasma insulin level [28]. Studies have shown that quinine has the ability to cross the blood brain barrier and can be detected in the brain [29,30], however, whether it is present in physiological concentrations needed to activate T2Rs remains to be elucidated.

Our hypothesis is that the activation of T2Rs in the brain by the exogenous ligands results in an increase in intracellular calcium that might cause secretion of regulatory peptides (such as CCK) involved in regulation of food intake and other important physiological processes. However, the endogenous ligands for T2R4 in the brain are unknown. In addition, the presence of a ten-fold higher number of T2Rs (25 T2Rs in human and 29 in rodents) compared to only three sweet and umami receptors, brings up the question of whether T2Rs are developmentally regulated, an area that remains to be investigated.

Acknowledgments

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant and a New Investigator Salary Award from Heart and Stroke Foundation of Canada (HSFC) to PC. We thank members of the Department of Oral biology, Drs. J. E. Scott, Gilbert Kirouac, Sa Li, Jasbir Upadhyaya for providing useful suggestions and help with different techniques.

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