IP3 receptor type 3 and PLCβ2 are co-expressed with taste receptors T1R and T2R in rat taste bud cells

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Abstract
The Ca2+ signaling cascade has been reported to be activated by many tastants in vertebrate taste systems. Recently we have shown that Gi2 and phospholipase Cβ2 (PLCβ2) are co-expressed in a subset of taste bud cells and are possibly involved in Ca2+ triggering of taste signaling in rats. We report here that, as a component downstream of PLCβ2, the type 3 isoform of the inositol 1,4,5-trisphosphate (IP3) receptor (IP3R3) is specifically expressed in the same cells as PLCβ2 in rat taste buds. We also show that cells expressing rT2R9, a probable cycloheximide receptor, are included among PLCβ2- and IP3R3-positive cells, as in the case of rT1R2, a different type of taste receptor. Our findings indicate that PLCβ2 and IP3R3 co-localize together with Gi2 as downstream components of two different types of taste receptors, T1R and T2R, in taste bud cells.

Introduction
In the taste buds of vertebrates the Ca2+ signaling cascade of PLC followed by inositol 1,4,5-trisphosphate (IP3)-dependent Ca2+ release from intracellular stores has been reported to be activated by various tastants, including many bitter tastants (Akabas et al., 1988; Hwang et al., 1990; Spielman et al., 1994; Ogura et al., 1997), some non-sugar sweeteners (Bernhardt et al., 1996) and several amino acids (Bryant and Leftheris, 1991). In our most recent report (Asano-Miyoshi et al., 2000) we showed that a subset of taste bud cells contain a taste transduction pathway comprising rT1R2 as a G protein-coupled receptor, G12 as a G protein and PLCβ2, although the ligand of rT1R2 is unknown.

Recently another type of G protein-coupled receptor, T2R, was isolated and shown to be specifically expressed in a subset of taste bud cells (Alder et al., 2000; Matsunami et al., 2000) independent of T1R2-expressing cells. It was shown that cells expressing T2Rs are gustducin-positive cells (Alder et al., 2000) and that one type of T2R, mouse mT2R5, responds to a bitter tastant, cycloheximide, and induces an increase in intracellular Ca2+ concentration in a heterologous expression system with G15 (Chandrashekar et al., 2000). However, the native signaling pathway downstream of these receptors is not yet known.

On the other hand, the inositol 1,4,5-trisphosphate receptor (IP3R) is a ligand-gated ion channel that releases Ca2+ from internal stores and directly causes an increase in intracellular Ca2+ concentration as one of the components downstream of PLC. Three subtypes of IP3R, IP3R1–IP3R3, have been identified and each shows a distinct tissue distribution (Newton et al., 1994), but it not known which types of IP3R are expressed in taste tissues.

In this study we have identified a type of IP3 receptor, IP3R3, a probable component downstream of PLCβ2, in rat taste bud cells. In addition, we examined the correlation between the expression of calcium signaling components such as PLCβ2 and IP3R3 and that of two different types of taste receptors in taste bud cells.

Materials and methods
RNA preparation and reverse transcription–PCR (RT–PCR)
The poly(A)+ RNA of the circumvallate and foliate papillae containing many taste buds was isolated as described previously (Asano-Miyoshi et al., 1998). The cDNA fragments encoding rat IP3Rs were amplified using the poly(A)+ RNA and degenerate primers:

5’-GA(AG)TA(CT)TG(CT)CA(AG)GG(GATC)CC-(ATC)TG(CT)CA(TC)GA(AG)AA(CT)CA-3’
and

5’-AA(AG)(CT)A(AG)(AG)TA(AG)TGC-CACAT(AG)TT(AG)TG(CT)TC-3’

corresponding to the amino acids sequences EYCQGP-CHENQ and EHNMWHYLCF, respectively, conserved among the three types of rat IP₃Rs. Each cDNA fragment was subcloned into the pBluescript SK(–) vector, sequenced and subjected to further analyses.

**In situ hybridization**

RNA probes labeled with digoxigenin-UTP were synthesized with T3 or T7 RNA polymerase from the cDNA fragments encoding rat IP₃Rs obtained above, the full-length cDNA of rT1R2 (Hoon et al., 1999), a gift from Dr C.S. Zuker, and a partial cDNA corresponding to the open reading frame of rT2R9 (Alder et al., 2000), which was obtained by PCR using a rat genomic DNA as template (GenBank accession no. AF227146).

The circumvallate papillae of the tongue of a 5-week-old rat (Wistar) were excised and 4-µm-thick frozen sections were prepared to be used for in situ hybridization as described previously (Asano-Miyoshi et al., 2000; Yasuoka et al., 1999). In the double labeling analysis a signal amplification method using two tyramide fluorogenic substrates was adopted according to the method described previously (Asano-Miyoshi et al., 2000). Fluorescent images were obtained under a fluorescence microscope (Olympus BX60) using a MicroMax cooled CCD camera system (Nippon Roper) and analyzed with MetaMorph imaging software (Universal Imaging Corp.).

**Immunohistochemistry**

The circumvallate papillae of the tongue of a 5-week-old rat (Wistar) were excised, and 10 µm thick frozen sections were prepared in the same way as for in situ hybridization. The sections were post-fixed in 4% paraformaldehyde for 10 min. After washing three times in phosphate-buffered saline (PBS), the sections were blocked with 3% normal goat serum and 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Then, the sections were incubated with a mixture of the primary antibodies, a monoclonal IP₃R3 antibody (Transduction Laboratories) and either a polyclonal PLCβ2 antibody (Q-15) (Santa Cruz Biotechnology) or a polyclonal Gust1 antibody (I-20) (Santa Cruz Biotechnology), each diluted 1:200 in 1% normal goat serum and 1% BSA at 4°C overnight. After washing three times...
times in PBS, the sections were incubated in the secondary antibodies. Texas Red-labeled donkey anti-mouse IgG and FITC-labeled donkey anti-rabbit IgG (Jackson Immuno Research), each diluted 1:100 in 1% normal goat serum and 1% BSA at room temperature for 1 h. After washing three times in PBS, the sections were mounted with Gel/Mount (Biomeda). Fluorescent images were obtained as described above.

Results

We first tried to identify IP3R isoforms expressed in rat taste tissues by RT–PCR using the degenerate primers corresponding to the amino acid sequences conserved among the three types of rat IP3Rs (see Materials and methods). As a result, two of types of IP3R cDNA, IP3R1 and IP3R3 (GenBank accession nos A36579 and A46719), were obtained. Next, in situ hybridization experiments were performed with these IP3R subtypes. The results clearly indicated that IP3R3 is highly expressed in a subset of cells in all taste buds with a frequency similar to that of PLCβ2 (Rosseler et al., 1998; Asano-Miyoshi et al., 2000) (Figure 1). No significant signals were observed when IP3R1 was used as probe (data not shown).

Next, we examined the correlation between expression of PLCβ2 and IP3R3 by in situ hybridization (Figure 2). The expression profiles of PLCβ2 and IP3R3 were compared in longitudinal (Figure 2a) and transverse (Figure 2b) serial sections of taste buds of the circumvallate papillae, where each cell would be divided into two or more adjacent sections. In this analysis when a certain cell expressed two genes, probes for the two genes should both give positive signals in the same cell divided between two serial sections (Asano-Miyoshi et al., 2000). As a result, it was revealed that PLCβ2 and IP3R3 are expressed in the same cells. We also carried out double labeling in situ hybridization experiments using digoxigenin- and FITC-labeled probes, which gave a clearer indication of the correlation (Figure 2c). The results of overlaid images of a single section were essentially the same as that obtained with the serial sections.

Figure 2 Co-expression of PLCβ2 and IP3R3. In situ hybridization experiments on 4 µm thick longitudinal (a) and transverse (b) serial sections of rat circumvallate papillae with antisense RNA probes for PLCβ2 and IP3R3 were performed. The serial sections in each panel are aligned along the arrow below the panel. The red arrowheads show cells positive to both probes. (c) The result of double label fluorescent in situ hybridization with PLCβ2 (green) and IP3R3 (red). Cells positive for both probes show yellowish signals as a result of overlap of the green (FITC) and red (Cyanine 3) signals. Bars represent 10 µm.
Figure 3  Correlation between the expression of taste receptors and calcium signaling components. In situ hybridization experiments on 4 µm thick transverse serial sections of rat circumvallate papillae with antisense RNA probes for the taste receptors rT1R2 (a) or rT2R9 (b), PLCβ2 and IP3R3 were performed. The serial sections in each panel are aligned along the arrow below the panel. The red arrowheads show the cells positive for both probes. (c–f) The results of double label fluorescent in situ hybridization with (c) PLCβ2 (green) versus rT1R2 (red), (d) PLCβ2 (green) versus rT2R9 (red), (e) IP3R3 (red) versus rT1R2 (green) and (f) IP3R3 (red) versus rT2R9 (green). Cells positive for both probes show yellowish signals as a result of overlap of the green (FITC) and red (Cyanine 3) signals. Bars represent 10 µm.
It is thus revealed that a certain subtype of IP3 receptor, IP3R3, is expressed in the same cells that express PLCβ2.

To gain further insight into the molecular process of the taste transduction pathway, we next examined the correlations between these cells and cells expressing two types of taste receptors, including a recently identified T2R, rT2R9, a rat homolog of the mouse cycloheximide receptor (mT2R5) (Alder et al., 2000), in taste buds of rat circumvallate papillae (Figure 3).

In order to examine the relationship between expression of taste receptors and calcium signaling components, we performed in situ hybridization experiments using 4 µm thick transverse serial sections of rat circumvallate papillae in the same way as described for Figure 2b. As shown in Figure 3a,b, all rT1R2- and rT2R9-positive cells expressed PLCβ2 and IP3R3. Next, we also carried out double labeling in situ hybridization experiments, which again revealed that all of the receptor-positive signals were in PLCβ2- (Figure 3c,d) and IP3R3-expressing (Figure 3e,f) cells. Consequently, the results suggest that both types of taste receptor can transduce the taste signal to activation of PLCβ2, leading to activation of IP3R3, and induce IP3-dependent Ca2+ release from intracellular stores.

Finally, we examined the existence of PLCβ2 and IP3R3 proteins by double labeling immunofluorescence staining (Figure 4a–d). The results show the same correlation as those of the in situ hybridization experiments (Figure 2), i.e., cells expressing IP3R3 protein and those expressing PLCβ2 protein were identical. On the other hand, gustducin-positive cells were also IP3R3 positive (Figure 4e–h).

**Discussion**

Although many reports have described the physiological features of Ca2+ signaling cascades in vertebrate taste systems (Akabas, 1993; Lindemann, 1996), there is little information concerning the molecular components. Our in situ hybridization results show that in taste buds a certain subtype of IP3R, IP3R3, is highly expressed in the same subset of cells that express PLCβ2 as one of two downstream signaling molecules generated by PLCβ2, IP3 and diacylglycerol. In addition, double labeling immunostaining
showed that IP$_{3}$R3 protein and PLCβ2 protein co-existed in the same cells, which included gustducin protein-containing cells, although further investigations are needed to determine their functional linkage in terms of subcellular localization, because it might be altered during experimental procedures, including fixation and permeabilization. These observations strongly suggest that these components mediate Ca$^{2+}$ responses induced by various taste stimuli. It is thus confirmed that Ca$^{2+}$ release from internal stores triggered by IP$_{3}$ has an important function in taste transduction.

IP$_{3}$R3 is a subtype that is expressed predominantly in gastrointestinal tissues, such as in the apical region of villous enterocytes in the jejunum and pancreatic acinar cells, as well as kidney, lung, testis and brain (Blondel et al., 1993). Its activation is considered to result in a single transient, but global, increase in the concentration of cytosolic Ca$^{2+}$ (Hagar et al., 1998), as observed in isolated taste bud cells (Akabas et al., 1988; Spielman et al., 1994; Ogura et al., 1997). Thus, IP$_{3}$R3 is better suited to signal initiation in taste cells than other types of IP$_{3}$Rs, whose properties are ideal for Ca$^{2+}$ oscillation.

The finding that both rT1R2- and rT2R9-positive cells express PLCβ2 and IP$_{3}$R3 (Figure 3) suggests that taste stimuli received by these receptors probably induce the calcium response mediated by PLCβ2 and IP$_{3}$R3. In our most recent report (Asano-Miyoshi et al., 2000) we found that PLCβ2 and G$_{12}$ are co-expressed in the same subset of cells and that gustducin-positive cells are also included in this subset. It is thus possible that the βγ subunits of G$_{12}$ are the general upstream components activating taste cell PLCβ2 and that those of gustducin might also act in a limited subpopulation of PLCβ2-positive cells. Since no correlation has been observed between expression of rT1R2 and gustducin (Hoon et al., 1999; Asano-Miyoshi et al., 2000), rT1R2 appears to transduce the taste signal of its unknown ligand through a pathway comprising G$_{12}$βγ PLCβ2 and IP$_{3}$R3. On the other hand, T2R-positive cells have been reported to be included among gustducin-positive cells and the βγ subunits (G$_{12}$βγ) of gustducin have been shown to mediate IP$_{3}$ responses to a bitter tastant, denatonium (Huang et al., 1999). Therefore, cycloheximide, a ligand of rT2R9, probably causes the Ca$^{2+}$ response in taste bud cells through a pathway comprising rT2R9, gustducin (G$_{12}$βγ) and/or G$_{12}$βγ PLCβ2 and IP$_{3}$R3.

In summary, a significant subset of taste bud cells have a Ca$^{2+}$ signaling pathway involving PLCβ2 and IP$_{3}$R3 and these cells are taste cells showing IP$_{3}$ responses to several of the tastants reported in previous physiological studies. In a subpopulation of Ca$^{2+}$-signaling PLCβ2- and IP$_{3}$R3-positive taste cells both types of taste receptors, T1R and T2R, might receive some specific taste stimulus, such as cycloheximide for rT2R9, and might transduce the signals through the Ca$^{2+}$ signaling pathway. Identification of downstream components following Ca$^{2+}$ release are needed, together with further physiological studies using native Ca$^{2+}$ signaling components, taste receptors and their specific ligands.

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References


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