Functional expression of the extracellular-Ca\(^{2+}\)-sensing receptor in mouse taste cells

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Summary

Three types of morphologically and functionally distinct taste cells operate in the mammalian taste bud. We demonstrate here the expression of two G-protein-coupled receptors from the family C, CASR and GPRC6A, in the taste tissue and identify transcripts for both receptors in type I cells, no transcripts in type II cells and only CASR transcripts in type III cells, by using the SMART-PCR RNA amplification method at the level of individual taste cells. Type I taste cells responded to calcimimetic NPS R-568, a stereoselective CASR probe, with Ca\(^{2+}\) transients, whereas type I and type II cells were not specifically responsive. Consistent with these findings, certain amino acids stimulated PLC-dependent Ca\(^{2+}\) signaling in type III cells, but not in type I and type II cells, showing the following order of efficacies: Phe~Glu>Arg. Thus, CASR is coupled to Ca\(^{2+}\) mobilization solely in type III cells. CASR was cloned from the circumvallate papilla into a pIREs2-EGFP plasmid and heterologously expressed in HEK-293 cells. The transfection with CASR enabled HEK-293 cells to generate Ca\(^{2+}\) transients in response to the amino acids, of which, Phe was most potent. This observation and some circumstantial evidence lead to the conclusion that type III cells can serve a novel chemosensory function by expressing the polymodal receptor CASR. A role for CASR and GPRC6A in physiology of taste cells of the type I remains to be unveiled.

Key words: Ca\(^{2+}\)-sensing receptor, Taste transduction, Identified taste cells, Exponential RNA amplification

Introduction

Existing evidence implicates two unrelated families of G-protein-coupled receptors (GPCRs) specifically expressed in taste cells, T1R and T2R, in mediating sweet, bitter and umami transduction. Three closely related GPCRs from the T1R family occur as at least two dimeric receptors. The heterodimer of T1R2 and T1R3 functions as a promiscuous sweet receptor, whereas T1R1 and T1R3 form a broadly tuned L-amino acid sensor. The T2R family includes nearly 30 GPCRs that distinguish bitter ligands (reviewed by Chandrashekar et al., 2006; Sugita 2006; Roper, 2007).

Previously, it was widely accepted that taste transduction could involve multiple signaling pathways, even within the particular taste modality (e.g. Margolskee, 2002). However, studies performed within the last few years have identified phospholipase Cβ2 (PLCβ2), as a principal GPCR effector, and TRPM5, as a pivotal transduction channel, to convey intracellular signals downstream of the T1R and T2R receptors. PLCβ2 and TRPM5 are largely co-expressed in taste bud cells (Perez et al., 2002; Perez et al., 2003; Zhang et al., 2003; Romanov et al., 2007), including separate populations of T1R- and T2R-positive cells (Zhang et al., 2003; Perez et al., 2003). The presence of PLCβ2 and TRPM5 in taste cells is vital for normal neuronal and behavior responses to sweet and bitter compounds and to amino acids (Zhang et al., 2003; Dotson et al., 2005; Mueller et al., 2005; Damak et al., 2006; de Araujo et al., 2008). Thus, although recognition of diverse sarcolemmal receptors relies on a variety of distinct GPCRs, transduction in non-overlapping subpopulations of sweet-, bitter- and umami-responsive cells apparently involves basically the same signaling pathway (discussed by Chandrashekar et al., 2006; Sugita 2006; Roper, 2007; Abe, 2008).

To validate the T1R1+T1R3 and T1R2+T1R3 dimers as the principal umami and sweet receptors in vivo, Zhao et al. (Zhao et al., 2003) generated Tlr1-, Tlr2- and Tlr3-knockout (KO) mice. They reported that the selective elimination of the particular receptor specifically abolished detection and perception of stimuli of the sweet and umami modalities but did not affect bitter, salty and sour responses. Somewhat different results were obtained in complementary studies of behavioral and neuronal responses of T1R3-KO mice, which reportedly were only slightly less capable than wild-type (WT) counterparts in discriminating monosodium glutamate from sucrose (Damak et al., 2003; Delay et al., 2006). These findings imply that apart from the T1R1+T1R3 and T1R2+T1R3 dimers, additional taste receptors are probably involved in detecting sweet compounds and amino acids (Damak et al., 2003; Delay et al., 2006).

The T1R1, T1R2 and T1R3 receptors belong to the subfamily C of mammalian GPCRs, which also includes metabotropic receptors for glutamate and GABA, the extracellular-Ca\(^{2+}\)-sensing receptor (CASR), and several orphan receptors, particularly, GPRC6A (Chandrashekar et al., 2006; Gloriam et al., 2007). The last has been deorphanized and shown to serve as a promiscuous receptor for L-amino acids (Wellendorph et al., 2005; Christiansen et al., 2007) and as one more extracellular-Ca\(^{2+}\)-sensing receptor (Pi et al., 2005). The multimodal receptor CASR is stimulated by a variety of different compounds, including extracellular Ca\(^{2+}\) and amino acids (Brown and MacLeod, 2001; Hofer and Brown, 2003). Given that calcium solutions taste bitter (Tordoff, 1996) and that the existence of unknown umami receptor(s) is quite possible (Chaudhari et al., 2009), it is not unlikely that the promiscuous receptors, GPRC6A and CASR, are employed by taste cells to detect...
Results
Evidence for expression of the CASR and GPRC6A receptors in taste cells

In a typical experiment \((n=5)\), RNA was sequentially isolated from a single circumvallate (CV) and foliate papilla and two pieces of the lingual epithelium with and without fungiform papillae. Using RT-PCR and gene-specific primers, we detected CASR transcripts in mRNA from the taste papillae but failed to amplify an appropriate product from preparations of the control non-taste epithelial tissue (Fig. 1A, lanes 2-5). These results imply that CASR is expressed mostly, if not exclusively, in taste cells.

A similar RT-PCR analysis of the taste tissue versus the non-taste tissue was carried out with GPRC6A-specific primers \((n=6)\), and a related product was amplified from all tissue preparations used (Fig. 1B, lanes 2-5). Since taste cells could not be isolated without adherent epithelial cells, both cells types could contribute to the RT-PCR signals. To ascertain the source of the GPRC6A transcripts in taste tissue preparations and to profile CASR and GPRC6A expression in a population of taste cells, we employed the SMART (switching mechanism at the 5’ end of RNA templates)-PCR-based RNA amplification technique at the single-cell level.

Single-cell profiling of RNA transcripts

A heterogeneous population of mammalian taste bud cells includes morphologically and functionally different taste cells classified into three subgroups, type I to III. Consequently, we performed the exponential SMART-PCR RNA amplification with identified taste cells only. Apart from CASR and GPRC6A, we analyzed transcripts for certain markers, as a positive control, which were chosen from a number of proteins reported to be basically expressed in non-overlapping subpopulations of taste cells. Of them, the glutamate-aspartate transporter, nucleoside triphosphate diphosphohydrolase 2 (NTPDase2), and epithelial amiloride-sensitive Na+ channel are believed to be specific for type I cells (Lawton et al., 2000; Bartel et al., 2006; Vandenbeuch et al., 2008). Virtually all cells of type II express phospholipase Cβ2 (PLCβ2), the InsP3 receptor type 3 (IP3R3), and the channel subunit TRP5 (Clapp et al., 2001; Perez et al., 2002), although evidence exists that IP3R3 also operates in a subpopulation of type III cells (Clapp et al., 2004; DeFazio et al., 2006). In the CV papilla, the taste-specific G-protein gustducin and GPCR T1R3 are most probably expressed in separate subpopulations of type II cells (Kim et al., 2003; Shindo et al., 2008).

The expression profiles of the neural cell adhesion molecule (NCAM), synaptosome-associated protein of 25 kDa (SNAP-25), and the channel subunit PKD2L1 are apparently restricted to type III cells (Yee et al., 2001; Clapp et al., 2006; DeFazio et al., 2006; Kataoka et al., 2008). Voltage-gated (VG) Ca2+ channels operate exclusively in type III cells (Romanov and Kolesnikov, 2006; Clapp et al., 2006), which particularly express two ω1-subunits of L-type Ca2+ channels, Cac.1.2 and Cac.1.3 (Roberts et al., 2009; Romanov et al., 2009).

For single-cell profiling of RNA transcripts, taste cells were isolated from CV papillae (see Materials and Methods). Each individual taste cell was patch-clamped, identified by its VG integral currents (Romanov and Kolesnikov, 2006; Romanov et al., 2007) (Fig. 3), and carefully rinsed to minimize possible contaminations with RNA from lysed cells. Next, the cell was sucked into the recording pipette (supplementary material Fig. S1), and the cellular material was expelled into a tube for the SMART-PCR cDNA synthesis and amplification (Hillmann et al., 2009) (see Materials and Methods) followed by PCR with gene-specific primers to detect certain transcripts. In addition to CASR and GPRC6A, each attested cell was analyzed for the expression of NTPDase2, SNAP-25, PKD2L1, TRPM5, gustducin and T1R3.

Using these combined techniques, we examined 27 identified taste cells, and CASR transcripts were detected in type I \((n=9)\) and type III \((n=9)\) but not in type II cells (Table 1, supplementary material Fig. S2). GPRC6A transcripts were seen exclusively in type I cells \((n=9)\) but were undetectable in taste cells of the other types (Table 1, supplementary material Fig. S2). It therefore appears that type I cells express both CASR and GPRC6A, type II cells express none of them, whereas type III cell express solely CASR, at least in the mouse CV papilla. Despite the limited number of taste cells being analyzed, our methodology (and therefore our findings) was validated by the following facts: (1) excepting SNAP-25, transcripts for a protein relevant to the particular cell type (e.g. NTPDase2 for the type I; gustducin, T1R3 and TRPM5 for the type II; PKD2L1 for the type III) were not detected in taste cells of the other two types (Fig. 2, Table 1); (2)
Based on the RT-PCR data (Fig. 1) and single-cell profiling of CASR and GPRC6A transcripts (Fig. 2), we searched for taste cells specifically responsive to CASR and/or GPRC6A agonists. In our experience, individual taste cells examined with the patch-clamp technique were much less responsive to a variety of GPCR agonists compared with cells assayed non-invasively with Ca\(^{2+}\) imaging (not shown). Perhaps, mechanical disturbance accompanying patch-clamping somewhat desensitized taste cells. Although these observations favored Ca\(^{2+}\) imaging, a priori, it was unclear whether this method would allow for unequivocal identification of taste cells. Meanwhile, the electrophysiological approach could ‘fingerprint’ taste cells that exhibited subtype-specific VG currents (Romanov and Kolesnikov, 2006; Romanov et al., 2007). We therefore tried to correlate electrophysiological characteristics of taste cells with their responsiveness, in terms of Ca\(^{2+}\) signaling, to certain chemical stimuli.

VG Ca\(^{2+}\) channels are present in type III cells and not functional in taste cells of the other types (Clapp et al., 2006; DeFazio et al., 2006; Romanov and Kolesnikov, 2006). Consistently, depolarization elicits well-resolved Ca\(^{2+}\) transients in type III cells, whereas in the type I and type II cells, VG Ca\(^{2+}\) entry is small or negligible (Romanov et al., 2008). Thus, type III taste cells could be distinguished non-invasively by specific Ca\(^{2+}\) responses to bath K\(^{+}\). Type I cells are markedly responsive to bath ATP owing to expression of P2Y receptors coupled to Ca\(^{2+}\) mobilization (Baryshnikov et al., 2003). Thus, ATP-dependent and KCl-dependent Ca\(^{2+}\) transients are hallmarks of type I and type III taste cells, respectively. Consequently, we monitored intracellular Ca\(^{2+}\) in a population of dissociated taste bud cells loaded with Fluo-4, stimulated them with 50 mM KCl and/or 10 \(\mu\)M ATP, and then identified responsive and non-responsive cells electrophysiologically (Fig. 3).

Overall 71 individual taste cells were assayed sequentially with Ca\(^{2+}\) imaging and the patch-clamp technique. Of 34 cells classified as type III, 29 cells (85%) showed marked Ca\(^{2+}\) responses (\(\Delta F/\Delta F_0 > 1\)) to 50 mM KCl with \(\Delta F/\Delta F_0 = 1.55 \pm 0.19\) on average (Fig. 4B,C), whereas five cells (15%) responded to the stimulation poorly (\(\Delta F/\Delta F_0 < 0.5\) or negligibly. Among 21 cells of the type I, only two cells were responsive to 50 mM KCl, but only weakly (\(\Delta F/\Delta F_0 < 0.4\); Fig. 4A, middle trace; Fig. 4B,C). Similarly, we found only two out of 26 cells of type II, in which Ca\(^{2+}\) detectably deviated from a resting level in response to 50 mM KCl (Fig. 4A, bottom trace; Fig. 4B,C). Therefore, consistent with the previous studies (DeFazio et al., 2006; Roberts et al., 2009), only robust type III taste cells are capable of generating marked (\(\Delta F/\Delta F_0 > 1\)) Ca\(^{2+}\) transients in response to 50 mM KCl (Fig. 4A).

Among type I 49 cells, 40 cells (82%) generated high (\(\Delta F/\Delta F_0 > 1\)) Ca\(^{2+}\) responses to 10 \(\mu\)M ATP (~EC\(_{50}\) for type I cells) with \(\Delta F/\Delta F_0 = 1.18 \pm 0.15\) on average (Fig. 4D-F). Nine cells of type I and most of cells of the other types responded to ATP weakly or negligibly (Fig. 4E,F). Overall, 36 type II cells and 35 type III cells were examined, and only four cells and three cells (13% and 11%, respectively), generated detectable Ca\(^{2+}\) transients in response to 10 \(\mu\)M ATP (Fig. 4D-F). We found only two cells out of these seven ATP-responsive cells, one type II and another type III, that generated relatively high ATP responses with \(\Delta F/\Delta F_0 = 0.3\) (Fig. 4D, middle and bottom traces). The other five cells showed much smaller deviations of Fluo-4 fluorescence from a basal level in response to 10 \(\mu\)M ATP. Thus, only type I taste cells can markedly respond to 10 \(\mu\)M ATP (\(\Delta F/\Delta F_0 > 1\)) and with the high probability of about 80% (Fig. 4E,F).

Because they can detect sweet, bitter or umami compounds, type II taste cells could be identified as responsive to a mixture of different tastants. However, in our experiments, the fraction of
were not responsive to the jump of bath Ca\(^{2+}\) from 1 mM to up to 568 as an indication that CASR was functional there.

In line with the data of single cell profiling of RNA transcripts (Fig. 4), where the abundance of type II cells was close to 70%.

Responses of taste cells to bath Ca\(^{2+}\) and NPS R-568

In line with the data of single cell profiling of RNA transcripts (Fig. 2, Table 1), we expected that taste cells of type I and type III might respond to CASR/GPRC6A agonists. Reportedly, a variety of diverse compounds stimulates CASR in expression systems (McLarnon and Riccardi, 2002; Breitwieser et al., 2004). Among them, phenylalkylamine derivatives, such as NPS R-467 and R-568, are known to modulate CASR stereoselectively by enhancing its sensitivity to external Ca\(^{2+}\) (Nemeth, 1996; Hofer and Brown, 2003). These compounds can therefore be used as a CASR-specific pharmacological probe. In a number of experiments, we searched for taste bud cells specifically responsive to calcimimetic NPS R-568 (5 mM) and neomycin (500 μM; n=11), another CASR agonist, elicited well resolved Ca\(^{2+}\) transients with 1 mM Ca\(^{2+}\) in the bath (Fig. 5A). The above mentioned effects of NPS R-568 and neomycin indicated conclusively that CASR was functional in type III taste cells. Note that HEK-293 cells transfected with CASR cDNA have been reported to show much higher sensitivity to bath Ca\(^{2+}\), generating saturated responses at nearly 10 mM Ca\(^{2+}\) (e.g. Zhang et al., 2002) (Fig. 9), the concentration that is close to the threshold of about 6 mM obtained for cells of the type III most sensitive to bath Ca\(^{2+}\) (Fig. 5A,B). Perhaps in type III cells, efficacy of coupling of Ca\(^{2+}\)-bound CASR to Ca\(^{2+}\) mobilization is low, compared with HEK-293 cells.

Table 1. Transcripts detected in individual taste cells

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The relatively low, moderate and high levels of signals are indicated by +, ++ and ++++, respectively.

Responsive cells was too small (<10%) to rely on taste responses as a label of type II cells. We therefore searched for those cells in a population that were weakly or negligibly responsive (ΔF/F₀<0.3) to both 50 mM KCl and 10 μM ATP (Fig. 4), where the abundance of type II cells was close to 70%.
enhanced intracellular Ca\(^{2+}\) signals elicited by external Ca\(^{2+}\) nor triggered Ca\(^{2+}\) transients by themselves (Fig. 5C).

Taste cells of the type II, which were identified as not responsive to either KCl or ATP, were also not found to be responsive to the CASR agonists and bath Ca\(^{2+}\) (Fig. 5D). These facts point out that in type II cells: (1) activity of Ca\(^{2+}\) entry channels is insignificant, in agreement with our previous observations (Romanov et al., 2008); (2) any extracellular-Ca\(^{2+}\)-sensing receptors are not functional, thereby supporting the data of the single cell SMART-PCR analysis (Fig. 2, Table 1).

**Responses of taste cells to amino acids**

Reportedly, a number of L-amino acids stimulate recombinant CASR in the presence of external Ca\(^{2+}\), including tryptophane and phenylalanine (aromatic), which were found to be the most potent (Wellendorph and Bräuner-Osborne, 2009). Evidence indicates that GPRC6A is a promiscuous amino acid receptor most sensitive to basic L-amino acids (arginine, ornithine) and allosterically potentiated by Ca\(^{2+}\) (Pi et al., 2005; Wellendorph and Bräuner-Osborne, 2009). We studied responsiveness of taste cells to phenylalanine (Phe) and arginine (Arg), which were chosen as relatively specific for CASR and GPRC6A, respectively, and to glutamate (Glu) that was taken as an umami compound.

While in an expression system, a recombinant receptor is usually dominant, a variety of different receptors and signaling pathways mediate responsiveness of natural cells. This factor compromises a functional assay of receptor-mediated intracellular signaling in situ. In particular, taste cells express several amino acid-sensing receptors, including the broadly tuned T1R1-T1R3 heterodimer and several glutamate receptors (Chaudhari et al., 2009; Yasumatsu et al., 2009). As a means to distinguish cellular responses mediated by CASR and/or GPRC6A, which are activated by amino acids and external Ca\(^{2+}\) with synergism, taste cells were sequentially stimulated by 3.5 mM Ca\(^{2+}\) or 1 mM Phe (Arg, Glu) and by 3.5 mM Ca\(^{2+}\) + 1 mM Phe (Arg, Glu), and synergistic responses to the agonists applied at these moderate concentrations were considered as CASR or GPRC6A-mediated.

**Type III taste cells**

Overall 93 cells of type III were assayed, and 75 cells (81%) generated Phe-dependent Ca\(^{2+}\) transients in response to the step-like change of bath Ca\(^{2+}\) from 1 mM to 3.5 mM. When 3.5 mM Ca\(^{2+}\) or 1 mM Phe was applied singly, cells responded weakly or negligibly, but together, these compounds produced a well-resolved Ca\(^{2+}\) response in the cell cytoplasm (Fig. 6A,B). The addition of 8 mM U73122, an inhibitor of phospholipase C (PLC), completely and irreversibly abolished Ca\(^{2+}\) transients (n=4; Fig. 6A), although responsive cells typically generated multiple responses to 3.5 mM Ca\(^{2+}\) + 1 mM Phe (n=70; Fig. 7A). These observations implicated CASR in mediating PLC-dependent Ca\(^{2+}\) signaling stimulated by Phe in type III cells.

The relative contribution of Ca\(^{2+}\) release and Ca\(^{2+}\) entry, the principal mechanisms of agonist-dependent Ca\(^{2+}\) signaling, could be assessed by comparing cellular responses in control cells and in
the absence of extracellular Ca\(^{2+}\). Because this approach was inapplicable in our case, we relied on Mn\(^{2+}\) ions passing through diverse Ca\(^{2+}\)-permeable channels that mediate store- and receptor-operated Ca\(^{2+}\) entry. Activity of these channels can be evaluated by a decline in Fluo-4 fluorescence induced by bath Mn\(^{2+}\). Not causing the true quench, these ions can inhibit Ca\(^{2+}\)-dependent fluorescence of Fluo-4 by competing with Ca\(^{2+}\) for binding sites of the dye and stimulating its fluorescence ineffectively (Molecular Probes Handbook; www.probes.com).

Fig. 6B shows the results of a representative experiment (n=4) performed with this approach. A cell chosen by its high response to 50 mM KCl was stimulated by 3.5 mM Ca\(^{2+}\) + 1 mM Phe, and the characteristic Ca\(^{2+}\) signal was observed. With 3.5 mM Ca\(^{2+}\) in the bath, the addition of 2 mM Mn\(^{2+}\) caused a decline in Fluo-4 fluorescence due to high enough activity of Ca\(^{2+}\)-entry channels (Fig. 6B). The application of 1 mM Phe elicited nearly double the Ca\(^{2+}\) transient, which inactivated rapidly (Fig. 6B). These phenomena indicate that 1 mM Phe stimulated both Ca\(^{2+}\) release and Ca\(^{2+}\) entry.

Indeed, if Ca\(^{2+}\) release contributed negligibly, the Phe responses should have been mediated largely by receptor-operated Ca\(^{2+}\) channels, and 1 mM Phe + 2 mM Mn\(^{2+}\) should have enhanced the decline of Fluo-4 fluorescence elicited by 2 mM Mn\(^{2+}\) in a resting cell (Fig. 6B, right panel) rather than stimulated an increased response (Fig. 6B, middle and right panels). By contrast, if Ca\(^{2+}\) release was mainly responsible for the initial Ca\(^{2+}\) rise stimulated (Fig. 6B). The application of 1 mM Phe elicited nearly double the Ca\(^{2+}\) transient, which inactivated rapidly (Fig. 6B). These phenomena indicate that 1 mM Phe stimulated both Ca\(^{2+}\) release and Ca\(^{2+}\) entry.

Fig. 5. Representative responses of taste cells of type III (A,B), type I (C) and type II (D) to bath Ca\(^{2+}\) and calcimimetic NPS R-568. Cells were loaded with Fluo-4. In A-D, the changes in bath Ca\(^{2+}\) are indicated by the continuous thin lines and the application of NPS R-568 is marked by the thick straight lines, above the fluorescence traces. In A and B, two different type III cells were assayed.

Fig. 6. PLC-dependent Ca\(^{2+}\) release and Ca\(^{2+}\) entry mediate responses of type III cells to Ca\(^{2+}\) and Phe. (A,B) Fragments of representative recordings of Fluo-4 fluorescence and its deviations in response to the different compounds applied as indicated. The application of the particular compound is shown by the thick straight lines above the fluorescence traces. The changes in bath Ca\(^{2+}\) are indicated by the thin lines. In A and B, two different cells were assayed.

Fig. 7. Representative responses of type III cells to bath Ca\(^{2+}\) and amino acids. (A) Fragments of recordings of Fluo-4 fluorescence from three different cells. (B) Averaged responses to 1 mM Phe, Arg and Glu at different concentrations of bath Ca\(^{2+}\). All responses were normalized to one elicited by 1 mM Phe + 3.5 mM Ca\(^{2+}\). The data are presented as mean ± s.d. In B, n indicates the number of cells examined under the particular recording conditions.
by 1 mM Phe, an enhancement of cellular responsiveness would have been expected in the presence of Mn2+, an effective CASR agonist (e.g. Rodriguez et al., 2005). Compared with control, 3.5 mM Ca2+ + 2 mM Mn2+ + 1 mM Phe should stimulate a larger fraction of CASRs, and therefore, a larger cellular response should be generated.

The lack of rapid response inactivation in control (Fig. 6B, middle and right panels) indicated that Mn2+ entered the cell cytoplasm and 'quenched' Fluo-4 fluorescence. To effectively compete with Phe-elevated Ca2+ for Fluo-4, Mn2+ should also be present in the cell cytoplasm at a higher concentration. The fact that in the presence of 2 mM Mn2+, Fluo-4 emission declined at rest and after a Phe-elicited Ca2+ peak with similar rates (Fig. 6B, right panel) suggests that 1 mM Phe also stimulated Mn2+ influx, by Mn2+-permeable Ca2+-entry channels.

Type III cells sensitive to Phe also responded to arginine (Arg) and glutamate (Glu; Fig. 7A). With 1 mM Ca2+ in the bath, 1 mM Phe elicited small cellular responses, and most of the cells responded poorly to Arg and Glu (both at 1 mM; Fig. 7A). At 2.5 or 3.5 mM Ca2+, cells were markedly sensitized, and some of them generated comparable responses to all three amino acids (Fig. 7A). U73122 inhibited responses to Glu and Arg (not shown), as was the case with Phe (Fig. 6A), implicating a common PLC-dependent transduction pathway. As summarized in Fig. 7B, efficacy of the particular amino acid depended on extracellular Ca2+. With 3.5 mM Ca2+ in the bath, averaged cellular responses yielded the sequence: Phe-Glu>Arg (Fig. 7B).

**Type I and type II taste cells**

Similar experiments were performed with taste cells of the two other types, although the effects of 1 mM Glu were not studied. In type I cells, 100 μM Glu might mobilize Ca2+ and stimulate inward currents (data not shown), suggesting the presence of neuron-like glutamate receptors like those found previously in non-specified taste cells (Chaudhari et al., 1996; Hayashi et al., 1996; Lin and Kinnamon, 1999). The sensitivity of type II cells to Glu was not examined as they functionally express T1R1-T1R3 heterodimeric receptors that are more sensitive to Arg than to Glu (Wellendorph and Bräuner-Osborne, 2009).

Type I cells were identified by Ca2+ transients on 10 μM ATP and by a drop in intracellular Ca2+ elicited by 50 mM KCl (Fig. 8A, left and middle panel). The addition of 3.5 mM Ca2+ to the bath slowly elevated cytosolic Ca2+, but Phe and Arg (1-10 mM) never enhanced such Ca2+ signals (n=9; Fig. 8A, right panel) and there was no stimulation of Ca2+ signaling with 1 mM Ca2+ in the bath (not shown). These results were similar to those obtained in the experiments with NPS R-568 and neomycin (Fig. 5C).

Type II taste cells were not responsive to either KCl or ATP. Each cell was stimulated by 3.5 mM Ca2+ in the absence and in the presence of the particular amino acid (or in the opposite order). Overall, 19 cells were assayed, and none of them responded to Phe (Fig. 8B) or Arg (not shown), both being applied at 1-10 mM.

Thus, by examining responsiveness of taste cells to NPS R-568, neomycin and Phe, potent CASR agonists, and to the effective GPRC6A agonist Arg, we obtained no functional evidence for coupling of CASR or GPRC6A to Ca2+ mobilization in type I and type II taste cells (Fig. 5C,D and Fig. 8). Although such a result could be expected for type II cells, because they are unlikely to express CASR and GPRC6A (Table 1, supplementary material Fig. S2), the lack of response of type I cells to the amino acids (Fig. 8A), neomycin and NPS R-568 (Fig. 5C) was rather surprising.

**Fig. 8. Responses of type I and type II cells to bath Ca2+, KCl, ATP and amino acids. (A,B) Fragments of representative recordings of Fluo-4 fluorescence from a type I cell (A) and from type II cell (B).**

**Studies of CASR in a heterologous system**

The sensitivity of CASR to L-amino acids has been intensely studied in heterologous systems, and their potencies were characterized by EC50 values obtained largely at 1 mM bath Ca2+ (see Wellendorph et al., 2009). Reduced to the CASR agonists used here, the rank of potencies is Phe>Glu>Arg, with more than tenfold higher efficacy for Phe than Glu and Arg (Wellendorph et al., 2009). In our experiments, the averaged response to 1 mM Phe did not exceed those to 1 mM Arg and 1 mM Glu by more than three times (Fig. 7B); in fact, these amino acids were similarly effective in certain cases (Fig. 7A, cell no. 1). The following factors might underlie this inconsistency: (1) in expression systems, CASR was presumably overexpressed, whereas responses of type III cells were generated at a physiologically relevant surface density of this receptor; (2) transduction of CASR agonists in type III cells is probably mediated by G proteins and/or downstream signaling pathways distinct from those in CASR-transfected cells; (3) the data presented here and in the literature were obtained under somewhat different recording conditions.

The above mentioned problem provided the rationale to compare CASR-mediated Ca2+ signaling in type III cells with that in an expression system. CASR in the taste tissue was cloned into a pIRES2-EGFP plasmid, heterologously expressed in HEK-293 cells, and cellular responses to bath Ca2+, amino acids and NPS R-568 were studied. Importantly, control non-transfected HEK-293 cells were negligibly responsive to a battery of these stimuli (supplementary material Fig. S3), indicating non-essential functional activity of endogenous Ca2+-entry channels and Ca2+-sensing receptors. The heterologous expression of CASR induced a dramatic change in responsiveness of HEK-293 cells to bath Ca2+ and rendered them capable of generating specific responses, oscillatory in many cases, which were markedly enhanced by 1 mM Phe, Glu and Arg (Fig. 9A). Basically, the potentiation of Ca2+ responses of CASR-transfected HEK-293 cells by these amino acids was reminiscent of the effects of Phe, Glu and Arg on type III taste cells (Fig. 7).

In a number of experiments, CAR- and Phe-positive HEK-293 cells were stimulated by NPS R-568 (Fig. 9B). With 1 mM Ca2+ in the bath, this compound effectively elicited Ca2+ transients in the cytoplasm of transfected HEK-293 cells but not in control non-transfected cells (supplementary material Fig. S3). As was the case with taste cells
Normalized magnitude of Ca\(^{2+}\) response versus NPS R-568 concentration. The circles, thick line) and in the presence of 0.1 \([\text{Ca}^{2+}]_{1/2}\),

and in the presence of 0.1

0.1 \([\text{Ca}^{2+}]_{1/2}\),

where 

\[\frac{R}{R_0} = \frac{[\text{Ca}^{2+}]^n}{[\text{Ca}^{2+}]_{1/2}^n + [\text{Ca}^{2+}]^n}\]  (1)

In all cases cells were loaded with Fura-2. (Fig. 5A), NPS R-568 markedly enhanced Ca\(^{2+}\) sensitivity of CASR-positive HEK-293 cells, which remained sensitized for a long time after removal of this CASR agonist (Fig. 9B; first and fourth responses).

Cellular responses to bath Ca\(^{2+}\) recorded in control conditions and in the presence of 0.1 \(\mu\text{M}\) NPS R-568, 1 \(\mu\text{M}\) Phe or 1 \(\mu\text{M}\) Arg (Fig. 9A,B) were averaged over all experiments and converted into dose-response curves (Fig. 9C) that were fitted with the Hill-like equation:

It should be noted that the monotonic slowly inactivating responses of type III cells (Fig. 5A and Fig. 7A) and oscillatory responses of CASR-positive HEK-293 cells (Fig. 9A,B) to the amino acids and NPS R-568 clearly indicate that they were generated by involving different signaling pathways. This makes the direct comparison of CASR-mediated responsiveness of taste cells and transfected HEK-293 cells impossible. Nevertheless, there is a common feature, namely, the same sequence, Phe-Glu-Arg, which characterizes sensitivity of these cells to the particular amino acid. This fact, the strong Ca\(^{2+}\)-amino acid synergism (Fig. 7), and the highest potency of Phe (Fig. 7B), favor CASR as the predominant receptor subtype endowing type III taste cells with the ability to detect amino acids. Indirectly, this inference is supported by the following.

Existing evidence indicates that T1R1 and T1R3 do not operate in type III cells (Clapp et al., 2006; DeFazio et al., 2006) (Table 1), and therefore cannot mediate their responses to amino acids. One more sensor of micromolar Glu, the truncated mGlu4 receptor, is coupled to adenylyl cyclase (Chaudhari et al., 2000) and presumably expressed in a subpopulation of type II cells (Chaudhari et al., 2009). The neuronal-like receptors basically operate at micromolar concentrations of Glu, whereas external Ca\(^{2+}\) is not obligatory for their activation (Hermans and Challiss, 2001). In our experiments, 100 \(\mu\text{M}\) Glu elicited small (\(\Delta F/F_0 \sim 0.1\)) deviations of intracellular Ca\(^{2+}\) from a resting level only in two type III cells out of 17 tested, of which, 14 cells were well responsive to 1 \(\mu\text{M}\) Glu but in the presence of 3.5 \(\mu\text{M}\) Ca\(^{2+}\) (not shown). The ionotropic receptors can also be excluded, given that 100 \(\mu\text{M}\) to 1 \(\mu\text{M}\) Glu never elicited current transients in resting type III cells (n=21) that could be associated with activation of Glu-gated channels (not shown).

Discussion

In the present work, we demonstrated that mouse taste cells express two more GPCRs from the subfamily C, CASR and GPRC6A, profiled their expression in a diverse population of CV taste bud cells at the single-cell level, and studied responses of identified taste cells to calcimimetic NPS R-468 and certain L-amino acids known to stimulate CASR and GPRC6A with different potencies. In addition, CASR was cloned from the taste tissue and heterologously expressed in HEK-293 cells, wherein CASR-mediated Ca\(^{2+}\) signaling was studied. Using the SMART-PCR cDNA amplification technique, transcripts for CASR were detected in individual type I taste cells, where it is largely coexpressed with GPRC6A, and in type III taste cells in which GPRC6A transcripts were undetectable. It would appear that type II taste cells do not express either CASR or GPRC6A (Fig. 2, Table 1).

By assaying identified taste cells with Ca\(^{2+}\) imaging (Figs 5, 6, 7), we revealed that: (1) only type III cells were specifically responsive to bath Ca\(^{2+}\). NPS R-468 and amino acids (Phe, Glu and Arg), which mobilized intracellular Ca\(^{2+}\) in a PLC-dependent manner; (2) type II cells were insensitive to these CASR agonists; (3) type I cells, unresponsive to the amino acids and NPS R-468, responded to external Ca\(^{2+}\) but non-specifically, that is, because of

et al., 2007; Lee et al., 2007), NPS R-5681 and the tested amino acids potentiated cellular responses at intermediate concentrations of extracellular Ca\(^{2+}\) by shifting the dose-response curve to lower concentrations. The dependence of cellular responses on NPS R-568 concentration was obtained at 1 mM bath Ca\(^{2+}\), and one was well fitted using Equation 1 with \([\text{R-568}]_{1/2}=0.11 \mu\text{M}\) and \(n=1.25\) (Fig. 9D, solid curve).

One more sensor of micromolar Glu, the truncated mGlu4 receptor, is coupled to adenylyl cyclase (Chaudhari et al., 2000) and presumably expressed in a subpopulation of type II cells (Chaudhari et al., 2009). The neuronal-like receptors basically operate at micromolar concentrations of Glu, whereas external Ca\(^{2+}\) is not obligatory for their activation (Hermans and Challiss, 2001). In our experiments, 100 \(\mu\text{M}\) Glu elicited small (\(\Delta F/F_0 \sim 0.1\)) deviations of intracellular Ca\(^{2+}\) from a resting level only in two type III cells out of 17 tested, of which, 14 cells were well responsive to 1 \(\mu\text{M}\) Glu but in the presence of 3.5 \(\mu\text{M}\) Ca\(^{2+}\) (not shown). The ionotropic receptors can also be excluded, given that 100 \(\mu\text{M}\) to 1 \(\mu\text{M}\) Glu never elicited current transients in resting type III cells (n=21) that could be associated with activation of Glu-gated channels (not shown).

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an increase in Ca\(^{2+}\) influx via Ca\(^{2+}\)-entry channels active at rest. In addition, the amino acids were found to stimulate Ca\(^{2+}\) signaling in type III cells and in CASR-positive HEK-293 cells with a similar order of potencies: Phe–Glu–Arg (Figs 7, 9). This fact, and presumably negligible contribution of other amino acido-sensing receptors, indicates that CASR is predominantly responsible for the sensitivity of type III cells to the amino acids.

In light of these findings, it was surprising that type I cells, which express both CASR and GPRC6A, were not specifically responsive, in terms of Ca\(^{2+}\) signaling, to Phe, Glu, Arg and calcimimetic NPS R-468. Note, however, that apart from PLC, reportedly the major downstream effector of CASR, this receptor can also stimulate cAMP signaling (Hofer and Brown, 2003; Geibel et al., 2006) and the E-cadherin–PI3K pathway (Tu et al., 2008), and modulate inwardly rectifying K\(^{+}\) channels (Huang et al., 2007). With such a diversity of CASR-mediated intracellular signaling, it is not unlikely that instead of Ca\(^{2+}\) mobilization in type I cells, CASR is coupled to some other signaling pathways, perhaps, with cAMP as a second messenger (see Hofer and Brown, 2003; Hofer et al., 2004; Breitwieser et al., 2004). Consistent, at least in the case with Glu, that this amino acid has been documented to modulate cAMP production in the taste tissue (Abaffy et al., 2003; Trubey et al., 2006), although a cellular source of Glu-dependent cAMP signals and the underlying mechanisms remain to be identified.

In fact, CASR is a multimodal sensor that can recognize a variety of amazingly diverse compounds either as agonists or as allosteric modulators. The list of such substances includes ions (H\(^{+}\), Ca\(^{2+}\), Mg\(^{2+}\), Sr\(^{2+}\), Pb\(^{2+}\), Gd\(^{3+}\), Yt\(^{3+}\)), polyamines (spermine, spermidine), inwardly rectifying K\(^{+}\) channels (Huang et al., 2007). With such a cAMP signaling (Hofer and Brown, 2003; Geipel et al., 2006) and the dimeric structure of the functional receptor. This feature enables CASR to detect small Ca\(^{2+}\) deviations at physiologically relevant concentrations (Hofer and Brown, 2003; Breitwieser et al., 2004) (Fig. 8). In parathyroid cells, CASR is believed to serve as a homeostatic Ca\(^{2+}\) detector, which adjusts secretion of the parathyroid hormone, following small changes in blood Ca\(^{2+}\) (Brown and MacLeod, 2001). Its physiological functions in other cell types are less well understood. A cellular function of GPRC6A is even more elusive, although the recent studies with GPRC6A-null mice suggest that “the overall function of GPRC6A may be to coordinate the anabolic responses of multiple tissues through the sensing of extracellular amino acids, osteocalcin and divalent cations” (Pi et al., 2008).

The presence of CASR and GPRC6A in type I cells (Table 1) and in type III cells poses a question about their roles in taste bud physiology. Since GPRC6A is also expressed in the lingual epithelium, given the existence of a group ofafferent gustatory nerve fibers preferably responsive to calcium and magnesium chlorides (Ninomiya et al., 1982). Although mechanisms of calcium and magnesium taste are basically obscure, recent evidence implicates the T1R3 receptor (Tordoff et al., 2008a). Moreover, it has been speculated that T1R3 may produce a functional Ca\(^{2+}\)-sensing receptor by dimerizing with CaSR (Tordoff et al., 2008b; Tordoff et al., 2008b). If so, populations of CaSR-positive cells and T1R3-expressing cells should overlap in the taste bud. The single-cell profiles of receptor transcripts obtained by us (Table 1) suggest that CASR is not expressed in type II cells, apparently the only taste bud cells expressing T1R3 (DeFazio et al., 2006), thereby arguing against the existence of a T1R3-CASR dimer. Gabriel and coauthors (Gabriel et al., 2009) found immunohistochemical evidence that CASR is expressed in a small subgroup of PLC\(\beta\)-positive cells. Since PLC\(\beta\) is basically expressed in type II cells (DeFazio et al., 2006), they suggested that CASR could be expressed in certain type II cells. Given that a limited number of cellular preparations were analyzed with the double immunostaining (Gabriel et al., 2009) as well as using single-cell SMART-PCR RNA amplification (present work), additional experiments are required to address this inconsistency.

### Materials and Methods

#### Taste cell isolation

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (www.nap.edu/books/0309053773/html/index.html). Taste cells were isolated from mouse (NMRI, 6-8-week-old) circumvallate (CV) papilla as described previously (Baryshnikov et al., 2003). A tongue was injected between the epithelium and muscle layers with 0.7 mg/ml collagenase B, 1 mg/ml dispase II, 0.2 mg/ml elastase (all from Roche Diagnostics), and 0.5 mg/ml trypsin inhibitor (Sigma-Aldrich) dissolved in a solution (mM): 140 NaCl, 20 KCl, 0.3 MgCl\(_2\), 0.7 CaCl\(_2\), 10 HEPES-NaOH (pH 7.4). The tongue was incubated in an oxygenated Ca\(^{2+}\)-free solution (mM): 120 NaCl, 20 KCl, 1 MgCl\(_2\), 0.5 EGTA, 0.5 EDTA, 10 HEPES-NaOH (pH 7.4) for 30-35 min. The epithelium was then peeled off from the underlying muscle, pinned serosal side up in a dish covered with Sylgard resin, and incubated in the Ca\(^{2+}\)-free solution for 10-30 min. The isolated epithelium was kept at room temperature in a solution (mM): 130 NaCl, 10 NaHCO\(_3\), 5 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 10 HEPES-NaOH (pH 7.4), 5 glucose, 2 Na-pyruvate). Taste cells were removed from the CV papilla by gentle suction with a firepolished pipette with an opening of 70-90 \(\mu\)m and then expelled into an electrophysiological chamber.

#### Electrophysiology

Ion currents were recorded, filtered at 2 kHz, and analyzed using an Axopatch 200A amplifier, a DigiData 1322A interface, and the pClamp8 software (all from Axon Instruments). Series resistance, which was generally in the range 7-15 M\(\Omega\), was routinely compensated for by up to 70%. The recording chamber (~150 \(\mu\)l) and the perfusion system have been described previously (Kolesnikov and Margolskee, 1998). External solutions were delivered at the rate of about 0.1 ml/second. Voltage gated currents were elicited by 100-msec step polarizations from the holding potential of ~70 mV. Generally, the perforated patch approach was used with recording pipettes containing (mM): 140 CsCl, 1 MgCl\(_2\), 0.5 EGTA, 10 HEPES-CsOH (pH 7.2), 400 mg/ml amphotericin B. The basic bath solution contained (mM): 140 NaCl, 5 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 10 HEPES-NaOH (pH 7.4), 5 glucose. When needed, it was modified in that 140 mM NaCl was substituted for 95 mM NaCl+45 mM KCl, or in that the concentration of CaCl\(_2\) was lowered from 1 mM to a higher value.

### Imaging and photometry

For Ca\(^{2+}\) imaging, taste cells were loaded with 4 \(\mu\)M Fluo-4/AM for 15 minutes in the presence of Pluronic (0.002%) at room temperature. Dye-loaded cells were plated onto a coverslip coated with Cell-Tak (BD Biosciences) inside an attached ellipsoidal papilla. The isolated papilla was kept at room temperature in a solution containing (mM): 140 CsCl, 1 MgCl\(_2\), 0.5 EGTA, 10 HEPES-CsOH (pH 7.2), 400 mg/ml amphotericin B. The basic bath solution contained (mM): 140 NaCl, 5 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 10 HEPES-NaOH (pH 7.4), 5 glucose. When needed, it was modified in that 140 mM NaCl was substituted for 95 mM NaCl+45 mM KCl, or in that the concentration of CaCl\(_2\) was lowered from 1 mM to a higher value.

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and recorded at 510–20 nm with a microscope ratio photometry system (RM-D model, Photon Technology International). Transfected 108k microsponge and a Plan-Neofluar ×40, NA=0.75 objective (Zeiss). A corrected experiment 340/380 nm ratio was converted into a Ca²⁺ concentration using the Grynkiewicz equation (Grynkiewicz et al., 1985) at the apparent dissociation constant for Ca²⁺-fura-2 binding 232–25°C.

**RT-PCR**

Total RNA was extracted from the taste and non-taste tissues using the RNeasy mini kit (Qiagen) with on-column DNase digestion. Isolated RNA was reverse-transcribed with an oligo(dT) primer and Superscript RT (Invitrogen) according to manufacturer’s protocol and was used as a template for PCR with gene-specific primers to analyze the presence of transcripts for CASR and GPRC6A.

**SMART cDNA synthesis and amplification technique**

First-strand cDNA was synthesized directly from cell lysate harvested via patch pipettes (supplementary material Fig. S1) and expelled into a PCR tube containing 3.3 pmol modified oligo(dT) primer (5'-AACGAATGATCTAGCGAGATTAC(T)30VN-3') and 5 pmol template-switching primer (5'-AACGAATGATCTAGCGAGATTACG (CCAGATGATCCGGGGG-3') in water. As negative control, the bath solution with no cells was sucked into a pipette and expelled into a PCR tube. The sample (2.5 μl) was incubated at 70°C for 2 minutes, cooled to 50°C, and then a 2.5 μl RT mixture was added into each tube. The reaction mixture was incubated at 50°C for 30 minutes, and then the addition of the RNA solution (for incorporation of template-switching primer), it was additionally incubated at 50°C for 1 h 30 minutes. For cDNA amplification, 67.5 μl PCR mixture (5 μl 10 mM dNTP mix, 1 μl 10 mM DTT, 0.5 μl 10 mM dNTPs, 10 IU RNaseOUT inhibitor (Invitrogen), 50 IU Superscript III RT) with a 20-cycle PCR amplification was performed according to the schedule from the kit user manual. After completion, the samples were purified using a PCR purification kit (Qiagen) and eluted with 50 μl EB buffer.

**Gene-expression profiles of individual taste cells were analyzed by PCR on SMART-amplicon cDNAs with gene-specific primers, which were designed to amplify cDNA but not genomic DNA. Primers for CASR were 2238-2259 sense and 2470-2491 antisense oligonucleotides (GenBank NM_013803.2), yielding a 254 bp product; primers for GPRC6A were 1478-1499 sense and 1722-1743 antisense oligonucleotides (GenBank NM_009849.1), yielding a 217 bp product; primers for gustducin were 812-832 sense and 1015-1039 antisense oligonucleotides (GenBank NM_00108143.1), yielding a 228 bp product; primers for T1R3 were 1091-1113 sense and 1634-1656 antisense oligonucleotides (GenBank NM_031872.2), yielding a 566 bp product; primers for TRPMs were 2360-2379 sense and 2790-2813 antisense oligonucleotides (GenBank NM_020771.1), yielding a 454 bp product; primers for Pkd2li were 1082-1105 sense and 1669-1690 antisense oligonucleotides (GenBank NM_181422.2), yielding a 690 bp product. All of the primers were synthesized by Evrogen (Moscow, Russia). PCR products were analyzed on a 1.2% agarose gel electrophoresis using GeneRuler 1 kb DNA ladder (Fermantas) to assess amplicon sizes.**

**CASR cloning**

Murine CASR was cloned from the circumvalate papilla by RT-PCR with the primers 5'-GTACTCGAGGCAAGACCATAACATCT-3' and 5'-ATGCGTTCCTAATCTCCCTCATTAG-3' and subcloned into XhoI and BamHI sites of pIRE2-EGFP plasmid (Clontech). Accelryme Taq cDNA polymerase High Fidelity (Invitrogen) was used for amplification. A 3320-bp amplified fragment was validated by sequencing. The right insertion was determined by restriction map.

**Cell culture and transient transfection**

The HEK-293 cell line was routinely cultured in the Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) containing 10% (vol/vol) bovine fetal serum (Hy Clone), glutamine (1%) and the antibiotic gentamicin (100 μg/ml) (Invitrogen). Cells were grown in 12-well culture plates in a humidified atmosphere (5% CO₂, 95% O₂) at 37°C. Before the day of transfection, cells were plated in 12-well culture plates at the density of 0.75–1×10⁶ cells in 1 ml DMEM per well. The plasmid cDNAs were transiently transfected into HEK-293 cells by replacing the growth medium in each well with the transfection mixture, containing 1.6 μg of DNA and 4 μl of Lipofectamine™ (Invitrogen) in 1 ml of serum-free DMEM. After 6 hours incubation, the transfection mixture was replaced with the normal culture medium. Cells were assayed 48–96 h after transfection.

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Supplementary material available online at http://jcs.biologists.org/cgi/content/full/123/6/972/DC1

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