A metabotropic glutamate receptor variant functions as a taste receptor

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Sensory transduction for many taste stimuli such as sugars, some bitter compounds and amino acids is thought to be mediated via G protein-coupled receptors (GPCRs), although no such receptors that respond to taste stimuli are yet identified. Monosodium L-glutamate (L-MSG), a natural component of many foods, is an important gustatory stimulus believed to signal dietary protein. We describe a GPCR cloned from rat taste buds and functionally expressed in CHO cells. The receptor couples negatively to a cAMP cascade and shows an unusual concentration–response relationship. The similarity of its properties to MSG taste suggests that this receptor is a taste receptor for glutamate.

Chemoreceptor cells in taste buds monitor the chemical environment in the oral cavity and generate signals that lead to taste perceptions. Taste transduction for simple salts involves altered permeation of the receptor cell membrane by ions such as Na^+, K^+ or H^+ (ref. 1). The resulting receptor currents in taste bud cells stimulate neurotransmitter release to excite sensory afferents, ultimately leading to perceptions such as 'salty' or 'sour'. Taste transduction for larger organic molecules such as sugars, amino acids or a heterogeneous collection of compounds that elicit perception of bitterness, is thought to include binding at specific receptors on the taste cell plasma membrane[1,2].

Some of the proteins that orchestrate this plethora of sensory transduction mechanisms have been identified using molecular biological methods. Taste receptor cells express G proteins, including α-gustducin[3], α-transducin[4], a number of additional Gα subunits[5,6], several Gβ subunits and a taste-specific Gγ[7]. Phosphodiesterases[4] and a cyclic nucleotide-gated channel[8] cloned from mammalian taste buds could potentially participate in sensory transduction pathways. Epithelial sodium channels demonstrated in taste buds presumably underlie 'salty' and 'sour' transduction[9–11]. Detectable receptor activity for 'bitter' stimuli is found in membrane preparations from taste tissue[12]. Although a number of candidate taste-GPCRs have been proposed[13–15], their functional significance in taste transduction has not been established[2]. This report describes the cloning and functional characterization of a taste receptor and its natural taste stimulus.

Sweet, sour, salty, bitter and (arguably) *umami* constitute basic taste qualities. *Umami* denotes the taste of the glutamate moiety in monosodium L-glutamate (L-MSG), a compound that occurs naturally in protein-rich and other foods. Taste transduction for glutamate is hypothesized to entail stimulation of neurotransmitter-like ionotropic and metabotropic glutamate receptors[16–18]. A number of ionotropic glutamate receptors are expressed in lingual tissue, although none seems preferentially localized to taste buds[17]. Metabotropic glutamate receptors (mGluR1–8) constitute a family of GPCRs that are found in many neuronal cells[19]. In taste receptor cells, molecular, physiological and behavioral evidence implicates a metabotropic receptor similar or identical to mGluR4 in taste transduction for L-glutamate[20]. Such evidence includes the findings that mGluR4 is expressed in rat taste buds[17,21] and that an mGluR4-selective ligand, L-AP4, mimics the taste of L-MSG in conditioned taste aversion in rats[17] and in human psychophysical measurements[22]. Further, both L-MSG and L-AP4 interact synergistically with nucleotide monophosphates to elicit *umami* taste responses[23,24]. Additionally, stimulating taste buds with glutamate decreases cellular cAMP (X. Zhou & N. Chaudhari, *Chem. Senses* **22**, 834, 1997) and alters membrane conductances[25], a signaling cascade also triggered by mGluR4. Collectively, these findings are consistent with the transduction of L-glutamate taste by an mGluR4-like receptor. Nevertheless, several lines of evidence indicate apparent discrepancies between *umami* taste and the properties of mGluR4. The concentrations of glutamate needed to elicit taste and to activate the neurotransmitter receptor mGluR4 differ markedly. The detection threshold for L-MSG in recordings from sensory afferents is 0.1–0.3 mM in juvenile and 1–3 mM in adult rodents[26,27], whereas mGluR4 requires glutamate in the micromolar range. Further, the ability of glutamate agonists to stimulate mGluR4 does not correlate fully with their *umami* taste[28]. Additionally, *umami* taste does not seem to be blocked by a known antagonist of mGluR4[23]. These observations suggest that the receptor(s) transducing *umami* taste may differ significantly from mGluR4, particularly in the glutamate-binding domain.

The glutamate-binding domain of the mGluR is contained within the large extracellular *N* terminus. Although detailed structural information is lacking, a model of the *N* terminus of mGluRs is based on the structure of a bacterial periplasmic leucine-isoleucine-valine binding protein (LIVBP)[29]. Experimental verification of this model includes mutation of contacting amino acids[29], expression of truncated extracellular domains that retain binding characteristics[30] and chimeric receptors with distinct agonist sensitivities[31]. Thus, the extracellular *N* terminus might be a plausible site for differences between neurotransmitter and taste receptors for glutamate. We found an unusual variant of mGluR4, taste-mGluR4, expressed in lingual epithelium.

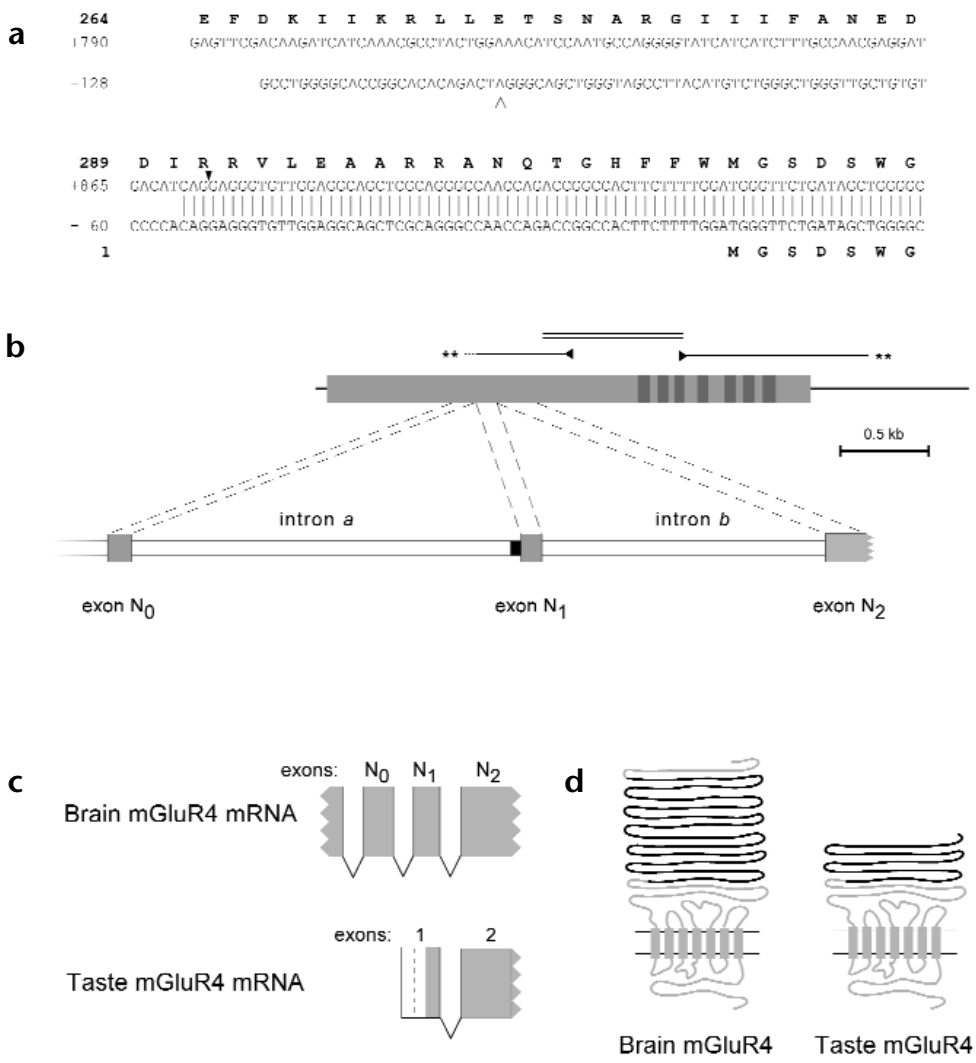

Fig. 1. The 5′ end of mGluR4 cDNA from taste papillae contains novel sequence derived from an intron. (**a**) The 5′ end of mGluR4 cDNA from taste papillae (lower lines) is aligned with the corresponding region of mGluR4 cDNA from brain (upper lines). Amino acid (bold) and translated nucleotides (regular) for each sequence are numbered. A stop codon (△), in frame with the long open reading frame, is found within the novel region of the cDNA from taste tissue. Sequence identity between the two cDNAs begins abruptly at the codon for R291. This position also corresponds to the location of intron *a* (▼). (**b**) Schematic showing the full-length cDNA for mGluR4 from brain, including 5′ and 3′ untranslated regions (line), translated region (shaded bar) and regions encoding seven putative transmembrane segments (gray stripes). The 800-bp segment (double line) expressed in rat taste buds[17] as well as the 3′ and 5′ RACE products (**) we characterized are shown above the brain-derived mGluR4 cDNA. The corresponding genomic organization shows two intervening sequences. The last 60 bp at the 3′ end of intron *a* (in black) are identical to the first 60 bp at the 5′ end of the taste-derived cDNA; the taste-derived cDNA includes part of intron *a* followed by exon N_1. (**c**) mGluR4 mRNA from brain includes several exons upstream of N_0, whereas mGluR4 mRNA derived from taste tissue begins with an extended exon N_1 spliced to exon N_2. In the taste-derived mRNA, the presence of the upstream stop codon prevents translation of the first 128 bp (white); translation presumably initiates at the next methionine codon (M309 in brain-derived cDNA). (**d**) Predicted transmembrane topology of brain- and taste-mGluR4 showing the truncated extracellular *N* terminal domain followed by seven putative transmembrane helices and cytoplasmic *C* terminus. The LIVBP-like glutamate-binding domain[29] and its truncated version are shown as heavy lines.

The corresponding protein is predicted to lack approximately half the *N* terminus, including a large portion of the LIVBP-like putative glutamate-binding domain. Taste-mGluR4 cDNA expressed in CHO cells conferred sensitivity to L-glutamate at concentrations ~100-fold higher than needed for brain-mGluR4, and its expression level was negatively coupled to cAMP concentration. These findings correspond well with the concentrations of glutamate needed to elicit *umami* taste and resolve the discrepancy between neurotransmitter receptors for glutamate and taste receptors for glutamate. Some of these results were presented in abstracts (A. Fedorov & Chaudhari, N., *Chem. Senses* **23**, 593, 1998; A. M. Landin *et al., Chem. Senses* **24**, 586, 1999).

Results

In-situ hybridization shows that mGluR4 is expressed in rat taste buds and cannot be detected in surrounding non-sensory epithelium[17,21]. However, it is unclear whether mGluR4 in taste buds is identical to that in the brain. Earlier RT-PCR and *in-situ* hybridization analyses focused on an ~800-bp core region conserved among mGluR1-8 (Double line in **Fig. 1b**). The *N* terminus of mGluRs comprises a large extracellular glutamate-binding domain[29]. The cytoplasmic *C* terminus of mGluRs participates in interactions with G proteins. Because *N* and *C* termini of mGluRs determine essential functional characteristics, we analyzed the corresponding sequences for mGluR4 expressed in taste tissue.

Full-length cDNA for mGluR4 from taste tissue

In the brain, mGluR4 mRNA is found in two forms, mGluR4a and mGluR4b, which differ at the 3′ end[32]. The longer mRNA includes an exon containing an in-frame stop codon, and thus generates a shorter protein product, mGluR4a. To analyze the *C* terminus of mGluR4 in taste cells, we used poly(A)RNA from taste (circumvallate and

foliate) papillae and gene-specific primers located in the previously characterized 800-bp core region. We performed 3′ RACE (rapid amplification of cDNA ends) reactions and cloned the longest specifically amplified bands. DNA-sequence analysis of representative clones yielded 100% identity with mGluR4a cDNA from the brain[33]. We also carried out RT-PCR with a primer pair straddling the alternatively spliced exon. The principal amplification product was confirmed by DNA sequence analysis to be mGluRa. A faint band corresponding in size to mGluR4b was detected only in occasional PCRs (not shown). Thus, we conclude that the *C*-terminal sequence in taste tissue was predominantly of the mGluR4a type.

Gene-specific reverse primers in the 800 bp core region were also used in 5′ RACE reactions. Previously cloned cDNAs for rat mGluR4a include a 5′ untranslated region (5′ UTR) of either 69 bp[33] or 854 bp[29]. Thus, we estimated that our 5′ RACE products should range between 1450 and 2235 bp. Using brain poly(A)RNA to validate the method, amplification products extending to ≥2000 bp were obtained as expected. In contrast, the 5′ RACE product obtained in parallel from taste tissue terminated abruptly at approximately 600 bp. Similarly truncated products from taste-derived mRNA resulted from 5′ RACE reactions with at least four different gene-specific reverse primers and two sources of reverse transcriptase.

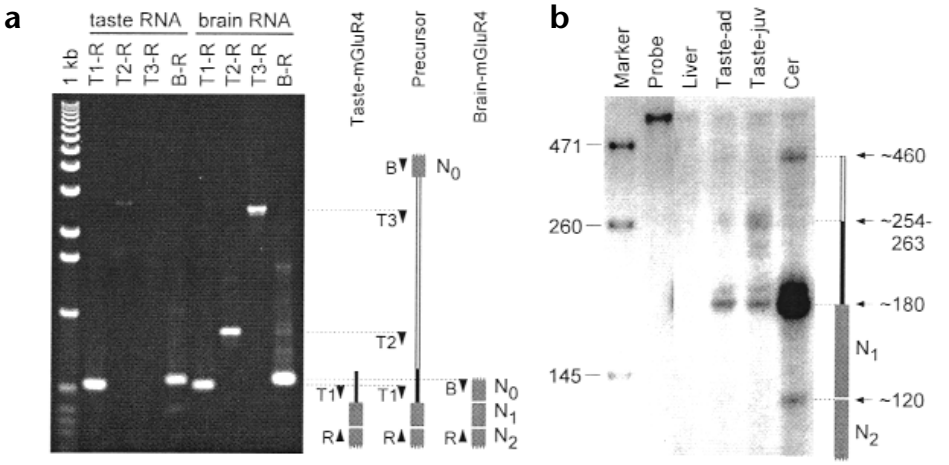

Fig. 2. The truncated mRNA in taste papillae is a mature mRNA. (**a**) RT-PCR with a reverse primer (R) in exon N_2 and forward primers B (within exon N_0) or T1, T2, T3 (within intron *a*) using poly(A)RNA from taste tissue or from brain. With taste RNA, PCR product is visible in lanes amplified with T1-R and B-R primer pairs but not with the T2-R or T3-R primer pairs, demonstrating that only a portion of intron *a* sequence is present in the RNA. With brain RNA used as template, PCR products are clearly seen with all three primer pairs, T1-R, T2-R and T3-R, implying that sequences from intron *a* are present in unspliced precursor for the known mGluR4 mRNA. A 1-kb ladder (BRL) is in the far left lane. Schematics on the right show RNA templates postulated to yield the PCR products obtained (taste-mGluR4, precursor of brain-mGluR4 and mature brain-mGluR4, respectively). (**b**) RNase protection assay demonstrates that taste tissue contains significant amounts of the truncated mGluR4 RNA. [^{32}P]-labeled probe was hybridized with poly(A)RNA from liver, cerebellum (0.05 μg, cer) and taste papillae (2–3 μg) from adult (ad) or juvenile (juv) rats. Lengths are indicated in nucleotides; RNA markers are on the left, and RNase-protected fragments are on the right. A schematic of the probe used (right) shows exons (gray) and the segment of intron *a* found in taste-derived mGluR4 cDNA (black).

A distinct mGluR4 cDNA found in taste tissue

Sequence analysis of the cloned RACE product indicated that the 5′ terminal 40–60 bp of the taste-derived cDNA clones were not similar to any region of brain mGluR4 cDNA (**Fig. 1a**). A stop codon was found near the 5′ end, in-frame with the long open reading frame, implying that the 5′ end is untranslated sequence. We found two potential start codons in frame, 102 bp and 189 bp downstream of the stop codon. Thus, the sequence of mGluR4 cDNA from taste tissue is unique for the first ~60 bp and then is identical for at least 2220 bp to mGluR4a cDNA from brain.

The point of divergence between unique and identical sequences for taste- and brain-derived cDNAs, located at amino acid R291 of mGluR4a, resembles a splice acceptor site. To test this, we analyzed a genomic fragment amplified from this region and determined that an intron (intron *a*, **Fig. 1b**) interrupts the codon for R291. The 3′ end of intron *a* is identical to the ~60-bp unique sequence at the 5′ end of the taste-derived mGluR4 cDNAs (**Fig. 1a** and **b**). One additional intron was also identified further downstream.

Taste-tissue mGluR4 is a mature mRNA

The presence of intron sequence in the taste-derived mGluR4 cDNA raised the possibility that it was amplified from a nuclear (precursor) RNA rather than a mature mRNA. Because introns are spliced out intact, a precursor RNA should include the complete sequence of intron *a*. This prediction was tested by RT-PCR (**Fig. 2a**). We used three forward primers (T1, T2 and T3) along intron *a*. The reverse primer (R) was selected from a separate downstream exon to preclude amplification from genomic DNA. From brain poly(A)RNA, approximately equal amounts of product were detected for all three reactions using forward primers within intron *a* (**Fig. 2a**). This result implies that intron *a* may be a late-spliced intron and that brain poly(A)RNA contains precursor RNAs that include the complete intron. In contrast, taste poly(A)RNA showed amplification product only from the farthest-downstream intronic primer, T1, and not from upstream primers T2 or T3. Thus, poly(A)RNA in taste tissue included only a short segment from the 3′ end of intron *a*, suggesting that the truncated mGluR4 cDNAs obtained in 5′ RACE were probably derived not from an unspliced precursor RNA, but from a mature mRNA. A forward primer (B) located in the next exon upstream (N_0), amplified from the previously known mGluR4 mature mRNA, served as a control. PCR product from mGluR4 mRNA lacking this intron was detected in poly(A)RNA from both brain and taste papillae. Precursor RNAs are typically found in tissue at considerably lower concentration than their respective mature mRNAs. In taste tissue, the low concentration of mature full-length mGluR4 mRNA[17] precludes detecting its precursor RNA (as RT-PCR products with T3 and T2 primers).

RNA secondary structure can cause premature termination of reverse transcripts and yield truncated products in 5′ RACE. This did not seem to be the case for the truncated taste-mGluR4 cDNA because brain poly(A)RNA did yield long 5′ RACE products, and because precursor RNAs in brain samples were readily

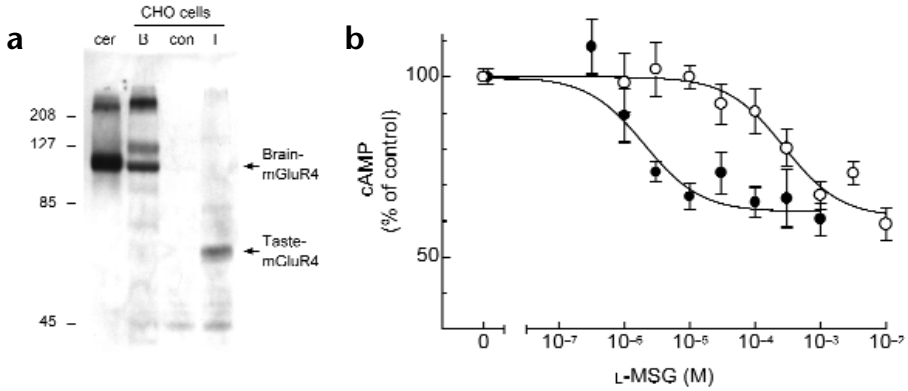

Fig. 3. Taste-mGluR4 is activated by glutamate at much higher concentrations than brain-mGluR4. (**a**) Immunoblot of CHO cells stably transfected with brain-mGluR4 (B) or taste-mGluR4 (T) shows a prominent immunoreactive band of predicted molecular weight (~102 kDa and ~68 kDa, respectively) when probed with an antibody against the shared *C* terminus of both forms. CHO cells transfected with non-recombinant vector (con) do not show either band. Extracts of cerebellum (cer) and brain-mGluR4 expressing CHO cells also show a higher band, presumably a dimerized receptor at ≥208 kDa. (**b**) CHO cells expressing brain-mGluR4 (●) and taste-mGluR4 (○) both respond to glutamate by decreasing cAMP levels. Cells were stimulated with L-glutamate in the presence of forskolin and IBMX (see Methods). Six independent experiments were carried out, each including triplicate wells of cells for each concentration. Three of the experiments were conducted on lines of clonal transformants and another three on non-clonal lines of stably expressing CHO cells. The values represent mean ± s.e.; the curves are a sigmoidal fit to the data. Control cells, transfected with the non-recombinant vector did not significantly alter cAMP levels when stimulated with L-glutamate (1 mM, 127 ± 16.0%; 10 mM, 132 ± 19.0%; n = 9). The absolute values of cAMP per well in forskolin-stimulated cells in the absence of glutamate were: 6.07 ± 0.60 pmole, 6.88 ± 0.78 pmole and 4.33 ± 0.42 pmole for cells transfected with brain-mGluR4, taste-mGluR4 and vector, respectively.

reverse transcribed and amplified through intron *a* (**Fig. 2a**). Thus, based on the 5′ RACE and RT-PCR analyses above, we tentatively concluded that taste tissue may contain two forms of mGluR4 mRNA—one similar to the known mGluR4a[33] and another with a truncated 5′ end.

Because RT-PCR can detect RNAs that are present in minor (nonphysiological) quantities in cells, we tested whether the truncated mGluR4 RNA found in taste tissue was present at significant concentration using RNase protection (**Fig. 2b**), an independent method not based on amplification. The probe for this assay consisted of the last ~400 nucleotides of intron *a*, followed by 178 nucleotides in two consecutive exons, N_1 and (part of) N_2. No bands were generated with RNA from liver, a control tissue that does not express mGluR4, demonstrating the specificity and RNase sensitivity of the probe. The known full-length form of mGluR4 mRNA (henceforth designated 'brain-mGluR4') should protect a band of 178 nucleotides, as it includes no sequence from intron *a*. Indeed, consistent with an identity as brain-mGluR4, a protected band of ~180 nucleotides was detected in poly(A)RNA from cerebellum and, at a lower concentration, from taste papillae. In addition, we detected two bands corresponding to precursor nuclear RNA when cerebellar poly(A)RNA was used in RNase protection. Because exons N_1 and N_2 are not contiguous in the genome, precursor RNA protected fragments ~460 nucleotides long (400 nucleotides of intron *a* plus exon N_1) and ~120 nucleotides (fragment of exon N_2). The absence of these precursor bands in taste samples indicated that genomic DNA (which would protect the same size bands as precursor RNA) was not a significant contaminant in the taste samples.

In addition to bands derived from the known mGluR4 mRNA and its precursor RNA, hybridization with poly(A)RNA from taste papillae yielded an additional band. This was a broad band, 254–263 nucleotides in length, as might be expected for an RNA that included the 178 nucleotides of exon (N_1 plus N_2) sequence and extended 76–85 nucleotides forward into intron *a*. This protected band was consistent with the mGluR4 cDNA cloned in the 5′ RACE experiments. The band was not detected in cerebellar RNA lanes. Thus, we designate this form of mGluR4 RNA, which includes a part of intron *a*, as 'taste-mGluR4'. We note that the ~178-bp band derived from brain-mGluR4 is sharp, as expected, whereas the broad taste-mGluR4-derived band indicates more heterogeneity in length. The 5′ ends of mRNAs frequently show such heterogeneity, spanning 3–15 nucleotides.

We observed the broad band corresponding to taste-mGluR4 mRNA in three separate protection experiments using different batches of poly(A)RNA from taste tissue of juvenile rats. Densitometric analysis from three experiments indicated that taste-mGluR4 mRNA is present at 70–120% of the concentration of brain-mGluR4 mRNA in taste tissue from juvenile rats. Interestingly, the taste-mGluR4 band was found at lower concentration when poly(A)RNA from adult rather than juvenile rats was used for protection (**Fig. 2b**).

Taste-mGluR4 is a functional mRNA

To determine whether taste-mGluR4 is a functional mRNA, we generated full-length clones for both forms of mGluR4 in pcDNA3.1 vector, transfected them into CHO cells and selected stable transfectants. When probed with an antibody against the *C* terminus of mGluR4a, immunoblots (**Fig. 3a**) of cerebellar extracts contained a strong band of the expected molecular weight, ~102 kDa, as previously reported[34]. A presumed dimer at ~210 kDa was also detected. Clones of cells stably transfected with brain-mGluR4 showed prominent bands of the same size as in cerebellum (**Fig. 3a**). In contrast, CHO cells transfected with the taste-mGluR4 construct consistently showed a prominent band of ~68 kDa, corresponding to the size predicted from the cDNA sequence. Neither the ~102-kDa nor ~68-kDa bands was present in parallel lanes containing lysates of CHO cells transfected with non-recombinant pcDNA vector. Thus, the truncated taste-mGluR4 mRNA characterized from taste tissue was indeed a functional mRNA that was translated into immunologically recognizable protein.

Taste-mGluR4 is negatively coupled to cAMP

In transfected CHO cells, activation of group III mGluRs leads to a suppression of forskolin-stimulated cAMP synthesis[35]. CHO cells stably expressing brain-mGluR4 responded predictably to L-glutamate (**Fig. 3b**). The EC_{50} for this response was 2 μM glutamate, consistent with earlier reports on mGluR4a (5 μM)[35]. Cells expressing taste-mGluR4 displayed no response to L-glutamate at concentrations of 30 μM or below. The EC_{50} for L-glutamate for taste-mGluR4

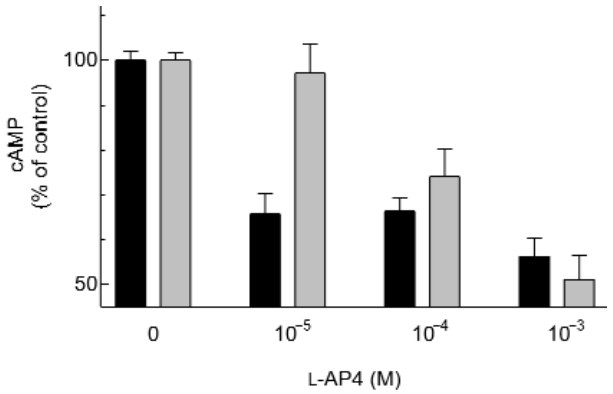

Fig. 4. Taste-mGluR4 is activated by L-AP4 at higher concentrations than is brain-mGluR4. CHO cells expressing brain-mGluR4 (solid bars) and taste-mGluR4 (shaded bars) were stimulated with the indicated concentrations of L-AP4. Six independent experiments were carried out (three each on clonal and non-clonal lines of stably transfected cells). For each concentration, triplicate wells of cells were used in each experiment. The values represent mean ± s.e.

was calculated to be 280 μM glutamate. We considered the possibility that a low cell-surface density of taste-mGluR4 might explain its low efficacy (high EC_{50}). Thus, we examined two separate lines of CHO cells expressing taste-mGluR4. Expression levels of taste-mGluR4 (as determined by immunoblot) were 100-fold and 2-fold lower, respectively, than in parallel lines expressing brain-mGluR4. Nevertheless, the EC_{50} values for the two lines expressing taste-mGluR4 were very similar, 300 and 250 μM, respectively. The consistently high EC_{50} for taste-mGluR4 suggests that low receptor density does not explain its low efficacy. Instead, the data suggest that taste-mGluR4 is approximately two orders of magnitude less sensitive to L-glutamate than is brain-mGluR4. Direct measurements of affinity will be necessary to confirm this interpretation.

Because the concentration of L-glutamate required for taste-mGluR4 was high, we considered that osmotic or other nonspecific effects might influence the cAMP response. Thus, we stimulated mGluR4-expressing cells with D-glutamate, an isomer that does not elicit *umami* taste[36]. Relative to controls, cells stimulated with 1 mM D-glutamate yielded cAMP levels of 126 ± 17% (brain-mGluR4) or 99 ± 3% (taste-mGluR4). Control CHO cells transfected with non-recombinant vector did not alter cAMP concentrations upon treatment with either L- or D-glutamate. Thus, receptor-independent mechanisms do not seem to decrease cAMP.

Taste-mGluR4 responds to L-AP4

In rat and human behavioral studies, L-AP4 mimics the taste of L-MSG[17,22]. Thus, we predicted that the taste receptor for L-MSG should also be stimulated by L-AP4. We directly tested whether taste-mGluR4 meets the criterion of a taste receptor by measuring its response to L-AP4 in a concentration range effective for taste. CHO cells expressing brain-mGluR4 responded to L-AP4 by suppressing forskolin-stimulated cAMP production. The response seemed to saturate at all concentrations of L-AP4 above 10 μM (**Fig. 4**), as expected from the published EC_{50} of 0.5–1.0 μM[35]. In cells expressing taste-mGluR4, 10 μM L-AP4 was ineffective, whereas 100 μM and 1 mM L-AP4 gave progressively larger responses. For rats in a behavioral assay, 100 μM L-AP4 is near the detection threshold[17]. Thus, taste-mGluR4 responds to L-AP4 over a concentration range similar to that observed for L-AP4 taste. The cAMP levels in control cells transfected with non-recombinant vector did not change upon treatment with L-AP4.

Discussion

MGluR4 is a metabotropic glutamate receptor originally characterized from the brain. *In situ* hybridization analyses have shown that this receptor is also expressed in taste buds[17,21]. The present report demonstrates that mGluR4 in taste tissue is expressed as a structurally and functionally distinct form that we have termed 'taste-mGluR4'. Taste-mGluR4 is a truncated version of the previously characterized brain receptor, and lacks ~50% of the receptor's extracellular *N* terminus. This truncation is particularly significant, because the *N* terminus of metabotropic glutamate receptors is believed to contain the glutamate-binding domain[29], and changes in this region are likely to influence the affinity of the receptor for ligands. Indeed, we report here that the truncated taste-mGluR4 is much less sensitive to L-glutamate and L-AP4 than the full-length brain form, implying a reduced affinity for these agonists. Importantly, the reduced sensitivity of taste-mGluR4 corresponds well with the concentrations of L-glutamate and L-AP4 needed to elicit a response in gustatory receptor cells *in situ*. The data are fully consistent with the interpretation that the novel taste-mGluR4 is a taste receptor for *umami* (the taste quality elicited by L-MSG).

The molecular identification and characterization of taste receptors has lagged behind research on other sensory receptors, notably, receptors for vision and olfaction. GPCRs are present in taste tissue[13,14]; receptors with sequences related to those of the mGluRs[15]. Although mRNA and/or protein for such candidate taste receptors has been demonstrated in lingual tissue, the lack of functional expression has hampered ligand identification and validation of their physiological significance. One of the challenges for studying the function of taste receptors is the high concentrations of stimuli needed. In the case of sugars, salts, and glutamate, the detection thresholds of gustatory sensory cells in nerve recordings or behavioral tests are in the range of a few hundred micromolar and higher. The low sensitivity of taste receptors, which may result from low affinity for ligands, has complicated binding assays and functional tests. By utilizing a high concentration of glutamate and a selective glutamate receptor ligand (L-AP4), we have been able to characterize the function of taste-mGluR4 in transfected cells.

The taste-mGluR4 cDNA in this report was cloned from posterior (circumvallate and foliate) taste papillae of juvenile rats. The threshold concentration for activating taste-mGluR4 (30 μM) matches well with the threshold (100 μM) reported for glutamate taste responses in the glossopharyngeal nerve of juvenile mice[26]. Interestingly, taste nerve thresholds in adult mice and rats, at 2–10 mM, are significantly higher[26,27]. In our studies, we found that the mRNA for taste-mGluR4 is expressed at lower concentration in adult than in juvenile rats[17] (**Fig. 2b**), which may explain the decreased sensitivity to MSG taste in adult rodents.

L-AP4 is a highly effective ligand at brain-mGluR4 (ref. 35). L-AP4 mimics the taste of glutamate in rats[17] and is an *umami* stimulus in humans[22]. Here we show that L-AP4 also stimulates taste-mGluR4, at concentrations effective as taste stimuli. MAP4, an antagonist of brain-mGluR4, fails to block taste nerve responses to glutamate and L-AP4 in chorda tympani nerve recordings[23]. Tests of MAP4 on cloned taste-mGluR4 may help to resolve this seeming paradox. Given the drastically altered *N* terminus of taste-mGluR4, it is impossible to predict its response to the various glutamate analogs known to activate or antagonize brain-mGluR4.

A distinctive feature of *umami* is the potentiation of glutamate responses by the nucleotide monophosphates of inosine and guanosine (IMP and GMP). This synergy is well documented in human psychophysical studies[37], animal behavioral experiments[24], gustatory nerve recordings[27] and patch-clamp studies[25]. Synergistic interactions are also found between L-AP4 and nucleotides[23,24], further underscoring the importance of taste-mGluR4 to *umami* transduction. The site of interaction between glutamate and nucleotides remains to be defined, but might involve synaptic convergence of separate gustatory sensory cells onto common afferent fibers, or converging signaling pathways from separate receptors within the same glutamate-sensing taste bud cell. It is also possible that both glutamate and nucleotides interact with a common receptor molecule. For instance, ligand-binding studies on bovine taste membranes suggested an allosteric model for nucleotide effects on glutamate taste[38]. The cloned taste-mGluR4 will allow direct tests of such models of receptor-taste stimulus interactions.

Our RT-PCR and RNase protection analyses indicate that both taste-mGluR4 and brain-mGluR4 are expressed in taste tissue (**Fig. 2a** and **b**). Glutamate is implicated as a neurotransmitter for taste bud afferent synapses[39], and brain-mGluR4 could serve as an autoreceptor at such synapses. We suggest that taste-mGluR4 is likely to function as an *umami* receptor, whereas brain-mGluR4 may serve as a neurotransmitter receptor at synapses in or near taste papillae.

Glutamate receptors other than mGluR4 may be expressed in gustatory sensory cells. For example, patch-clamp recordings, Co^{2+} uptake, and Ca^{2+} imaging on rat and mouse taste buds suggest that glutamate activates both ionotropic and metabotropic glutamate receptors[18,25,39,40]. However, all these experiments exposed both apical and basolateral membranes of taste receptor cells to glutamate. Given that *bona-fide* taste stimulation reaches only the apical membrane of taste receptor cells, it is critical to note that behavioral studies and nerve recordings (which stimulate only the apical membrane) indicate a minimal role for ionotropic-like glutamate receptors in taste transduction for L-MSG[17,22–24]. Thus, basolateral synapses on gustatory receptor cells may include glutamate receptors other than taste-mGluR4 (reviewed in ref. 20).

Transcripts of several mGluRs, including mGluR1 and mGluR5, are alternatively spliced[41,42]. For mGluR4, alternative splicing gives rise to receptors with either long or short *C* termini[32]. In spite of considerable structural diversity, most alternative-splicing variants of mGluRs show only subtle changes in their functional properties. For instance, mGluR1c and mGluR1a elicit distinct temporal patterns of Ca^{2+} release[42]. The present case of taste-mGluR4 demonstrates substantially different function of two receptors derived from the same gene. We do not presently know the molecular mechanism by which the truncated mRNA is produced in taste cells. It is possible that an alternative splice acceptor site is located within intron *a* and that our 5′ RACE reactions did not progress into additional exons upstream of the ~60 bp in intron *a*. Nevertheless, the presence of an in-frame stop codon within these ~60 nucleotides implies that any additional exons would constitute 5′ UTR of the mRNA and would not affect the sequence of the translated protein. An alternative possibility is that the origin for transcription of taste-mGluR4 mRNA is located within intron *a*. Multiple promoters yielding mRNAs with distinct 5′ exons occur in the mGluR5 gene[43].

Methods

Tissues and RNA. All tissues were from Harlan Sprague-Dawley rats. Circumvallate and foliate taste papillae were dissected from tongue, and rapidly frozen on dry ice. Poly(A)mRNA was extracted from tissues by direct binding to oligo dT-cellulose (FastTrack II kit, Invitrogen, Carlsbad, California). Unless stated otherwise, taste tissue samples were from juvenile (pre-weaning 16–20 day-old) rats.

5′ and 3′-RACE and RT-PCR. Initial 5′ RACE (rapid amplification of cDNA ends) reactions were carried out using superscript reverse transcriptase followed by terminal deoxynucleotidyl transferase (both from Gibco-BRL, Gaithersburg, Maryland)[44]. Subsequently, the Marathon RACE system (Clontech Laboratories, Palo Alto, California) was employed for generating and cloning RACE products. Poly(A)RNA, extracted from vallate and foliate papilla, was used to synthesize double strand cDNA, which was then ligated to an adapter-primer. Nested gene-specific primers were designed in the core region of rat mGluR4 cDNA sequence within the 800-bp region known to be expressed in taste buds[17]. RACE reactions in both directions were carried out using Klentaq DNA polymerase (Clontech) or Elongase (BRL) to ensure amplification of long products. Annealing steps were at the highest temperature permitted by respective primers. Amplification proceeded for 25–30 cycles to minimize nonspecific products. Amplification products were electrophoresed and blot hybridized to identify mGluR4-related bands, which were then cloned into pGEM-T vector (Promega) and sequenced on an ABI Sequencer Model 373A.

The following primers based on mGluR4a cDNA[33] (with identifying amino acid positions) were used in reverse transcriptase-polymerase chain reaction (RT-PCR):

B: 5′ (D266) CGACAAGATCATCAAACTGCCTAC 3′

R: 5′ (F455) GAAGTTGACGTTCCTGATGTACT 3′

To isolate a genomic fragment that contained 'intron *a*', genomic DNA was amplified with primers located in cDNA sequence: forward primer B (above) and a reverse primer at F307–N301. The following primers were based on intron sequence derived from genomic clones, and were used to map whether the entire intron *a* was represented in precursor nuclear RNAs (**Fig. 2a**):

T1: 5′ (48 bp upstream of R292) CAGCTGGGTAGCCTTACATGTCT 3′

T2: 5′ (400 bp upstream of R292) TCTGGAGTAGGATCAGGTGGATG 3′

T3: 5′ (2000 bp upstream of R292) AAAGGCTGCTATCTCGTGGACT 3′;.

RNase protection assay. The template for mGluR4 probe was constructed by ligating together a genomic fragment and a cDNA fragment at a shared *Afl*III site located 32 bp upstream of the junction between intron *a* and exon N_1 (**Figs. 1b** and **2b**). The genomic fragment contained the downstream 400 bp of intron *a* (extended for 368 bp upstream of the *Afl*III site). The 210 bp cDNA segment of the chimeric probe began at the *Afl*III site in intron *a* and included two sequential exons, N_1 and part of N_2. The resulting construct in pGEM-T vector was used to transcribe an antisense probe (**Fig. 2b**) with at least 238 nt complementary to taste-mGluR4 mRNA, as predicted from the sequence of the longest 5′ RACE clone (see Results). [^{32}P]-labeled antisense RNA was transcribed using T7 RNA polymerase. Hybridization and RNase digestion were performed using the High-Speed Hybridization RPA kit from Ambion (Austin, Texas). Protected fragments were analyzed on denaturing 6% polyacrylamide-urea gels.

Transfection. Full-length cDNAs for brain-mGluR4 and taste-mGluR4 (see Results) were reconstructed from cerebellar or taste poly(A)RNA respectively, by RT-PCR using Elongase (BRL) for high-fidelity amplification. The brain-mGluR4 insert included 7 nucleotides of 5′ untranslated region; the taste-mGluR4 insert included 100 nucleotides of presumed 5′ UTR upstream of the first in-frame start codon. Both cDNA inserts terminated at their common stop codon (following I912), yielding the mGluR4a version of the *C* terminus[33]. The inserts were cloned into the EcoRI site of pcDNA3.1 vector (Invitrogen).

CHO cells were transfected with brain-mGluR4 and taste-mGluR4 constructs and with non-recombinant pcDNA3.1 vector, all in parallel, using a cationic lipid, DMRIE-C (Gibco-BRL). Cells with stably integrated plasmid were selected and maintained in 300 μg per ml G418 starting 24 h after transfection. The medium consisted of Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1.7 mM proline and 100 units per ml penicillin-streptomycin[35]. Clones were lifted within two weeks and screened for expression by western blot analysis. A single clone for each form was propagated for functional assays. Stably transfected but non-clonal lines of cells from independent transfections with both forms of mGluR4 and non-recombinant vector were also maintained and used in functional assays.

Immunoblot analysis. Anti-peptide anti-serum directed against the *C*-terminal 18-amino-acid sequence of mGluR4a was raised in rabbits and antibody was purified by affinity chromatography (Zymed Laboratories, San Francisco, California). This epitope is shared between both brain-mGluR4 and taste-mGluR4 described here. Lysates of transfected CHO cells were electrophoresed and tested for expression of brain- and taste-mGluR4 by immunoblot analysis[45] on PVDF membrane. Detection was with alkaline phosphatase-conjugated secondary antibody and CSPD chemiluminescent substrate (both from Tropix, Bedford, Massachusetts). Bands on autoradiographs were densitometrically quantified as needed.

Functional assay. CHO cells, stably transfected with brain-mGluR4, taste-mGluR4 or non-recombinant pcDNA3.1, were maintained as sub-confluent cultures and refed every two days to minimize chronic stimulation of expressed receptors by glutamate released from dead cells. Cells were plated 20 h before assay in a 96-well microtiter plate at 2×10^4 cells per 200 µl well. Fresh medium was replaced for one hour immediately before assaying receptor function as described[35]. Briefly, cells were incubated in Dulbecco's phosphate buffered saline containing 1 mM IBMX for 20 min followed by stimulation for 10 min in 10 µM forskolin and 1 mM IBMX, with or without agonists. Stimulation buffer was rapidly removed, and cells were lysed in 200 µl 0.25% dodecyltrimethyl-ammonium bromide in 50 mM acetate buffer (Amersham, Piscataway, New Jersey). Released cAMP in 20% of each lysate was assayed directly using an Amersham EIA-based kit and plotted as mean ± s.e. of 3–6 experiments, each performed with triplicate wells of cells.

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