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Effect of the umami peptides on the ligand binding and function of rat mGlu4a receptor might implicate this receptor in the monosodium glutamate taste transduction

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1 The effect of several metabotropic ligands and di- or tripeptides were tested on the binding of [^{3}H]-L(+)-2-amino-4-phosphonobutyric acid ([^{3}H]-L-AP4) on rat mGlu$_4$ receptor. For selected compounds, the functional activity was determined on this receptor using the guanosine-5′[γ-^{35}S]-thiotriphosphate [γ-^{35}S]-GTP binding assay.

2 Using the scintillation proximity assay, [^{3}H]-L-AP4 saturation analysis gave binding parameters K_D and B_{max} values of 150 nM and 9.3 pmoles mg^{-1} protein, respectively. The specific binding was inhibited concentration-dependently by several mGlu receptor ligands, and their rank order of affinity was established.

3 Several peptides inhibited the [^{3}H]-L-AP4 binding with the following rank order of potency: glutamate-glutamate > glutamate-glutamate-leucine = aspartate - glutamate > > glutamate - glutamate-aspartate > lactoyl-glutamate > > aspartate-aspartate. Aspartate-phenylalanine-methyl ester (aspartame) was inactive up to 1 mM and guanosine-5′-monophosphate and inosine-5′-monophosphate were inactive up to 100 μM.

4 The [γ-^{35}S]-GTP binding functional assay was used to determine the agonist activities of the different compounds. For the rat mGlu$_4$ agonists, L-AP4 and L-glutamate, the correlation between their occupancy and activation of the receptor was close to one. The peptides, Glu-Glu, Asp-Glu and Glu-Glu-Asp failed to stimulate the [γ-^{35}S]-GTP binding at receptor occupancy greater than 80% and Glu-Glu-Leu appeared to be a weak partial agonist. These peptides did not elicit a clear dose-dependent umami perception. However, Glu-lac showed a good correlation between its potency to stimulate the [γ-^{35}S]-GTP binding and its affinity for displacement of [^{3}H]-L-AP4 binding. These data are in agreement with the peptide taste assessment in human subjects, which showed that the acid derivatives of glutamate had characteristics similar to umami.

Keywords: Umami peptide; metabotropic glutamate receptor; [^{3}H]-L-AP4; rat mGlu$_{4a}$ receptor; [γ-^{35}S]-GTP binding; radioligand binding; taste transduction

Abbreviations: Asp-Asp, aspartate-aspartate; Asp-Glu, aspartate-glutamate; CHPG (R,S)-2-chloro-5-hydroxyphenylglycine; EGLU, 2(S)-α-ethylglutamic acid, Glu-Glu, Glutamate-glutamate; Glu-Glu-Asp, glutamate-glutamate-aspartate; Glu-Glu-Leu, glutamate-glutamate-leucine; Glu-lac, lactoyl glutamic acid; GMP, guanosine 5′-monophosphate; GTPγS, guanosine-5′-O-(3-thiotriphosphate); IMP, inosine 5′-monophosphate; L-AP4, L(+)-2-amino-4-phosphonobutyric acid; L-CCGI, 2S,1′S,2′S-2-(2′-carboxycyclopropyl)glycine; L-SOP, L-serine-O-phosphate; MAP4, (S)-2-amino-2-methyl-4-phosphono butanoic acid; MCCG, (2S,3S,4S)-2-methyl-2-(carboxycyclopropyl)-glycine; MCPG, (+)-α-methylcarboxyphenylglycine; mGlu, metabotropic glutamate; MPPG, (RS)-α-methyl-4-phosphonophenyl glycine; MSG, monosodium glutamate; MSOP, (RS)-α-methylserine-O-phosphate; MSPG, (RS)-α-methyl-4-sulphonophenylglycine; MTPG, (RS)-α-methyl-4-tetrazolyl-phenylglycine; SPA, scintillation proximity assay; 1S,3R-ACPD, (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid

Introduction

To date, eight G-protein-coupled metabotropic glutamate (mGlu) receptors have been cloned (for review, see Conn & Pin, 1997). On the basis of their sequence similarities, signal transduction and agonist rank order of potencies, these receptors have been sub-divided into three groups: Group I, mGlu receptors, which couple to phosphoinositide (PI) hydrolysis, are activated by the weak group I selective agonist (S)-3,5-dihydroxyphenylglycine and the potent non selective agonist quisqualate (Aramori & Nakanishi, 1992; Ito *et al*., 1992). It comprises mGlu$_1$, which is preferentially blocked by (+)-2-methyl-4-carboxyphenylglycine (Salt & Turner, 1998); and mGlu$_5$, which is selectively activated by the weak agonist (R,S)-2-chloro-5-hydroxyphenylglycine (Doherty *et al*., 1997); Group II, mGlu receptors, which are negatively coupled to cyclic AMP production, are activated by the group II specific and potent agonist LY354740 (Schoepp *et al*., 1997) and blocked by the potent although not group II selective antagonist LY341495 (Kingston *et al*., 1998). It comprises mGlu$_2$ for which there is so far no subtype selective agonist or antagonist and mGlu$_3$ which is preferentially activated by N-acetyl-L-aspartyl-L-glutamic acid (Wroblewska *et al*., 1997). Finally, Group III, mGlu receptors, which are also negatively coupled to cyclic AMP production, are activated by the group III selective agonist L(+)-2-amino-4-phosphonobutyric acid (L-AP4). It is the largest group and comprises mGlu$_4$, mGlu$_6$,

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$mGlu_7$ and $mGlu_8$ for which there is so far no subtype selective agonist or antagonist (for review see Conn & Pin, 1997).

Additionally to its function as an excitatory amino acid, glutamate elicits in vertebrate a particular taste response termed 'umami' (Ikeda, 1909). Many investigators believe that the taste of glutamate is a separate quality and cannot be formed by any combination of the four basic tastes (sweet, sour, bitter and salty) (Yamaguchi, 1991, Yamamoto *et al*., 1991). An attempt to identify the receptor responsible for the taste of monosodium glutamate (MSG) has led to the cloning of an mGlu4-like receptor (Group III) from the rat taste buds (Chaudhari *et al*., 1996). More recently, the same group claimed that this $mGlu_4$-like receptor appeared to be a truncated form with a deletion of a large segment of the putative glutamate binding site in the N-terminal domain (Fedorov & Chaudhari, 1997), although apparently the full-length receptor was also present in the taste buds (Chaudhari, personal communication).

There is a large discrepancy in the literature concerning the ability of various di- and tripeptides to elicit umami taste in humans (van den Oord & van Wassenaar, 1997). Usually the taste assessments are performed by a panel of trained analysts who evaluate and describe the sensory properties of the particular substances. We attempted to relate the sensory data of the peptide taste assessment to the results obtained using *in vitro* $mGlu_4$ receptor assays. Since the conditioned taste aversion experiments (Chaudhari *et al*., 1996) revealed that to rats the perceived taste of L-AP4 was similar to that of MSG, we decided to investigate the effect of several peptides with alleged umami properties on the [^{3}H]-L-AP4 binding and to test their functional activity on the rat brain $mGlu_4$ receptor using the [γ-^{35}S]-GTP binding assay.

Methods

Materials

Aspartate-phenylalanine-methyl ester (aspartame), L-cystine, inosine 5′-monophosphate (IMP), guanosine 5′-monophosphate (GMP) and GTPγS were obtained from Sigma (Buchs, Switzerland). L-glutamate and quisqualate were from RBI (Zürich, Switzerland). L(+)-2-amino-4-phosphonobutyric acid (L-AP4), L-serine-O-phosphate (L-SOP), (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD), 2(S)-α-ethylglutamic acid (EGLU), 2S,1′S,2′S-2-(2′-carboxycyclopropyl)glycine (L-CCGI), (S)-2-amino-2-methyl-4-phosphono butanoic acid (MAP4), (2S,3S,4S)-2-methyl-2-(carboxycyclopropyl)glycine (MCCG), (+)-α-methylcarboxyphenylglycine (MCPG), (RS)-α-methyl-4-phosphonophenyl glycine (MPPG), (RS)-α-methylserine-O-phosphate (MSOP), (RS)-α-methyl-4-sulphonophenylglycine (MSPG), (RS)-α-methyl-4-tetrazolyl-phenylglycine (MTPG), and [^{3}H]-L-AP4 (specific activity 54 Ci mmol^{-1}) were purchased from Tocris Cookson (Bristol, U.K.). [γ-^{35}S]-GTP (specific activity 1000 Ci mmol^{-1}) was purchased from Amersham (Zürich, Switzerland).

Glutamate-glutamate (Glu-Glu), glutamate-glutamate-leucine (Glu-Glu-Leu), aspartate-glutamate (Asp-Glu), glutamate-glutamate-aspartate (Glu-Glu-Asp) and aspartate-aspartate (Asp-Asp) were obtained from Fluka (Switzerland) and Bachem (Switzerland). According to the suppliers data sheet the peptides were checked for their purity by micro analysis and thin layer chromatography and were at least 90–95% pure (remaining moisture). We additionally performed identity analysis by LC-mass spectrometry. All test samples contained L-amino acids only. The glutamic acid derivative lactoyl glutamic acid (Glu-lac), was kindly synthesized and provided by A. Goeke (Givaudan Roure, Dübendorf).

Cells

cDNA encoding the rat $mGlu_{4a}$ receptor was obtained from Prof S. Nakanishi (Kyoto, Japan). The cDNA was subcloned into the Semliki Forest Virus (SFV) expression vector pSFV2.gen. The recombinant Semliki Forest virus particles expressing the rat $mGluR_4$ receptor gene was generated as described previously (Lundstrom *et al*., 1994). Briefly, mRNAs were transcribed *in vitro* from linearized pSFV2.gen- $mGlu_4$, and pSFV-Helper2 (Berglund *et al*., 1993) plasmids in 40 mM HEPES/KOH, pH 7.4, 6 mM MgOAc, 10 mM spermidine/HCl in presence of 1 mM CAP, 10 mM DTT, 1 mM each ATP, UTP and CTP, 0.5 mM GTP, 50 U RNAse inhibitor and 60 U SP6 RNA polymerase at 37°C for 1 h. The transcripts were co-electroporated into baby hamster kidney (BHK-21) cells at 1500 V and 25 μF. The virus stocks were collected 24 h after electroporation, and activated by α-chymotrypsin digestion. Confluent BHK cells were infected with the recombinant virus particles and receptor expression was assayed by ^{35}S-methionine labelling 14–16 h post-infection. Labelled cell lysates were subjected to 10% SDS–PAGE and the protein expression analysed following autoradiography. Subsequently, the CHO cells grown in monolayer cultures were infected with the $mGlu_4$ virus particles. The infected cells were harvested 14–16 h post infection to prepare the membranes. The recombinant SFV-LacZ expressing β-galactosidase (negative control) was prepared similarly using linearized pSFV-LacZ vector.

Membrane preparation

Cells, cultured as above, were harvested and washed three times with cold PBS and frozen at −80°C. The pellet was resuspended in cold 20 mM HEPES-NaOH buffer containing 10 mM EDTA (pH 7.4), and homogenized with a polytron (Kinematica AG, Littau, Switzerland) for 10 s at 10,000 r.p.m. After centrifugation for 30 min at 4°C, the pellet was washed once with the 20 mM HEPES-NaOH buffer containing 0.1 mM EDTA (pH 7.4), and resuspended in a smaller volume of the same buffer. The membrane suspension was frozen at −80°C before use. Protein content was measured using the Pierce method (Socochim, Lausanne, Switzerland) using bovine serum albumin as standard.

[^{3}H]-L-AP4 binding

After thawing, the membranes were centrifuged and resuspended in cold binding buffer (mM): HEPES-NaOH 30, $MgCl_2$ 1.2, NaCl 110, $CaCl_2$ 2.5, cystine 100 μM, pH 8. The final concentration of membranes in the assay was 35 μg protein per 200 μl. Binding experiments were performed using the scintillation proximity assay from Amersham. Membranes, wheatgerm agglutinin SPA beads (RPNQ0001, Amersham) (2 mg per assay), compounds and [^{3}H]-L-AP4 in a total volume of 200 μl were placed in a 96-well picoplate (Canberra-Packard, Zürich, Switzerland), the plate was sealed and incubated for 1 h, shaking. The beads were then allowed to settle for 30 min and the radioactivity was counted in a Top-Count (Canberra-Packard, Zürich, Switzerland). The saturation isotherm was determined by incubating various [^{3}H]-L-AP4 concentrations (10–500 nM) at room temperature, for 1 h. For inhibition experiments, membranes were incubated

with 50 nM [^{3}H]-L-AP4 at room temperature, for 1 h, in presence of various concentrations of inhibitors. Non specific binding was measured in the presence of 100 μM L-SOP.

[γ-^{35}S]-GTP binding

After thawing, the membranes were washed once and resuspended in cold 20 mM HEPES-NaOH buffer containing 1 mM $MgCl_2$ and 100 mM NaCl (pH 7.4). Wheatgerm agglutinin SPA beads were resuspended in the same buffer (40 mg of beads ml^{-1}). The membranes and the beads were mixed (beads: 13 mg ml^{-1}, membranes: 400 μg protein ml^{-1}) and incubated with 5 μM GDP at room temperature for 1 h, under mild agitation. [γ-^{35}S]-GTP binding was performed in 96-well microplates in a total volume of 180 μl with 30 μg membrane protein and 0.3 nM [γ-^{35}S]-GTP. Non-specific binding was measured in the presence of 10 μM cold GTPγS. Plates were sealed and incubated at room temperature for 2 h, under agitation. The beads were allowed to settle and the plate counted in a Top-Count using quench correction.

Data analysis

The inhibition or activation curves were fitted with a four parameter logistic equation giving EC_{50} or IC_{50} values, and Hill coefficients using the iterative non linear curve fitting software Origin (Microcal Software Inc., Northampton, MA, U.S.A.). Saturation experiments were analysed with the same program using the rectangular hyperbolic equation derived from the equation of a bimolecular reaction and the law of mass action, $B = (B_{max}*[F])/(K_D + [F])$, where B is the amount of ligand bound at equilibrium, B_{max} is the maximum number of binding sites, [F] is the concentration of free ligand and K_D is the ligand dissociation constant. Each experiment was performed three times at least in triplicate.

Taste assessments

The panel consisted of ten persons (four females and six males, 28–55 years old). All were members of the Givaudan Roure Research, experienced in organoleptic testing of flavours and foods in model systems and routined to evaluate and describe taste. The test substances were offered to the panel at three concentrations (2, 5 and 10 mM) at pH 6.0 in 0.4% NaCl solution. All the substances, including monosodium glutamate, were presented blind, and the panellists were asked to compare them to the reference (0.4% NaCl solution) and describe the perceived flavour. In between the tastings sufficiently long pauses were taken to neutralise any taste effects in the mouth and pure water was offered for rinsing the mouth.

Results

[^{3}H]-L-AP4 binding

No specific binding was observed in the membranes prepared from the CHO cells infected with SFV-LacZ virus particles expressing β-galactosidase. In contrast, 55–60% specific [^{3}H]-L-AP4 binding was observed in the membrane preparations from the SFV-mGlu$_4$-infected CHO cells. Saturation analyses were performed at equilibrium using concentrations of [^{3}H]-L-AP4 from 10–500 nM (Figure 1). The analysis of the curve gave the following binding parameters, K_D value of 150$\pm$10 nM and maximum number of binding sites (B_{max}) of 9.35$\pm$0.36 pmoles mg^{-1} protein.

Following determination of the binding properties of [^{3}H]-L-AP4 on SFV-mGlu$_4$-infected cell membranes, the effects of a number of other compounds were examined on [^{3}H]-L-AP4 binding. Displacement curves for selected glutamate analogues are shown in Figure 2, and the results are summarized in Table 1. The specific binding was inhibited concentration-dependently by several mGlu receptor ligands with the following order of potency: L-AP4 > > L-SOP > L-CCG I = L-glutamate > D-AP4 > MSOP > MPPG > MAP4 > 1S,3R - ACPD > >

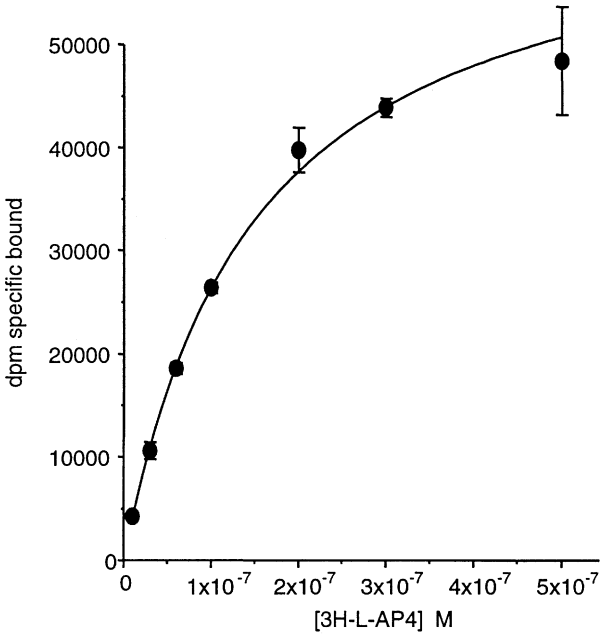

Figure 1 Saturation analysis of [^{3}H]-L-AP4 binding on rat mGlu$_4$ receptor-expressing cell membranes. Membranes were incubated at room temperature for 1 h with various concentrations of [^{3}H]-L-AP4. Values are specific [^{3}H]-L-AP4 bound in d.p.m. and are the mean$\pm$s.d. (bars) of three individual experiments, performed in quadruplicate.

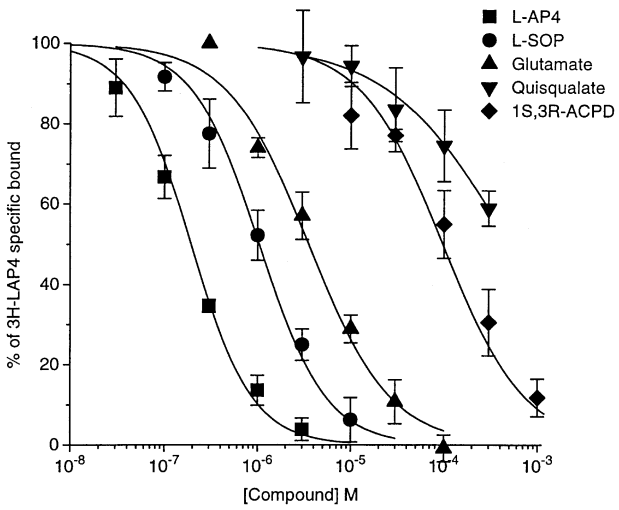

Figure 2 Concentration-dependent inhibition of [^{3}H]-L-AP4 binding to rat mGlu$_{4a}$ receptor-expressing cell membranes. Results are expressed as % of [^{3}H]-L-AP4 specific bound and are the mean$\pm$s.d. (bars) of three individual experiments, performed in triplicate.

quisqualate. MCPG, MSPG, MCCG, MTPG and EGLU all had IC_{50} values higher than 1 mM.

Several di- and tripeptides described in literature as having umami taste were assayed for their ability to inhibit [^{3}H]-L-AP4 binding to the rat $mGlu_{4a}$ membranes. The peptides which we tested were displacing [^{3}H]-L-AP4 (Figure 3) and their IC_{50} values ranged from 44 to over 3000 μM with the following rank order of potency: Glu-Glu > Glu-Glu-Leu = Asp-Glu > > Glu-Glu-Asp > Glu-lac > > Asp-Asp (Table 1). Aspartate-phenylalanine-methyl ester was inactive up to 1 mM and guanosine-5′-monophosphate and inosine-5′-monophosphate were inactive up to 100 μM (data not shown).

[γ-^{35}S]-GTP binding

In order to determine the ability of the 'umami' peptides to activate the $mGlu_4$ receptor, [γ-^{35}S]-GTP binding studies were performed using the SFV-$mGlu_4$-infected CHO cell membranes. L-AP4 and L-glutamate treatment of membranes produced a concentration-dependent increase in counts of radioactivity, with baseline and maximal L-AP4-mediated counts of 1105 and 2878 d.p.m. per 35 μg protein, respectively, giving an approximate signal to noise ratio of 2.6. Since the basal and agonist-mediated responses in [γ-^{35}S]-GTP binding assays varied occasionally between experiments depending on the membrane preparation, for comparative purposes the data were normalized as percentage of the maximal response obtained using 100 μM L-AP4. The agonist dose-response curves for L-AP4 and L-glutamate are shown in Figure 4. L-AP4 was more potent than L-glutamate (EC_{50} of 0.2 and 3.59 μM respectively, Table 2), although the latter was more efficacious than the former (Figure 5).

In contrast, most of the 'umami' peptides were very weak activators of the receptor with the following rank order of EC_{50}: Glu - Glu - Leu < < Glu - lac < < Glu-Glu = Asp-Asp = Asp-Glu = Glu-Glu-Asp (Figure 4, Table 2). For graphical clarity, the data for Glu-Glu, Asp-Asp and Glu-Glu-Asp were omitted.

For L-AP4, L-glutamate, Glu-lac, Asp-Glu and Glu-Glu-Leu a functional-occupancy relationship curve was made

Table 1 IC_{50} values and Hill coefficients (±s.d.) for the inhibition of [^{3}H]-L-AP4 binding on rat $mGlu_4$ receptor-expressing cell membranes

Compound	*IC_{50} (μM)*	*Hill*
L-AP4	0.174±0.015	1.14±0.14
L-SOP	1.04±0.15	1.05±0.13
L-CCG I	3.51±0.22	0.932±0.028
L-glutamate	3.52±0.36	0.988±0.081
D-AP4	7.93±0.57	0.896±0.039
MSOP	12.2±0.27	0.839±0.039
MPPG	27.2±1.7	0.906±0.035
MAP4	30±1.6	0.948±0.02
1S,3R-ACPD	117±17	0.89±0.097
Quisqualate	512±180	0.671±0.2
Glu-Glu	44±2.3	0.73±0.031
Glu-Glu-Leu	64±4	0.779±0.077
Asp-Glu	71±14	0.854±0.11
Glu-Glu-Asp	374±65	1.32±0.32
Glu-lac	814±150	0.842±0.15
Asp-Asp	3890±1900	0.818±0.17

Values were obtained from the fit of the mean data from three individual experiments performed in quadruplicate.

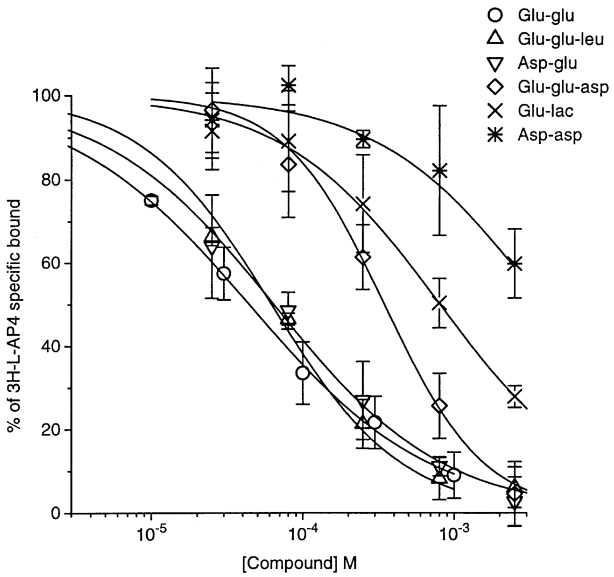

Figure 3 Concentration-dependent inhibition of [^{3}H]-L-AP4 binding to rat $mGlu_{4a}$ receptor-expressing cell membranes. Results are expressed as % of [^{3}H]-L-AP4 specific bound and are the mean±s.d. (bars) of three individual experiments, performed in triplicate.

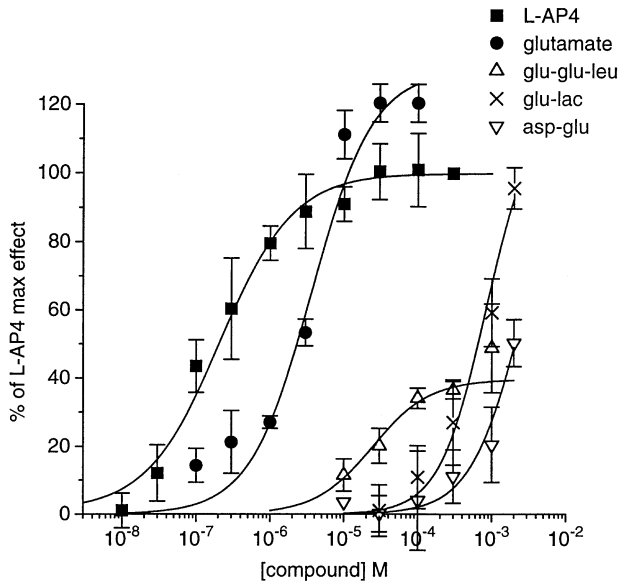

Figure 4 Concentration-dependent stimulation of [γ-^{35}S]-GTP binding on rat $mGlu_{4a}$ receptor-expressing cell membranes. Results are expressed as % of L-AP4 maximal effect and are the mean±s.d. (bars) of at least three individual experiments, performed in quadruplicate.

Table 2 EC_{50} E_{max} values and Hill coefficients (±s.d.) for the inhibition of [γ-^{35}S]-GTP binding on rat $mGlu_4$ receptor-expressing cell membranes

Compounds	*EC_{50} (μM)*	*Hill*	*E_{max}*
L-AP4	0.2±0.045	0.87±0.12	100
L-glutamate	3.59±0.59	1.05±0.11	130±6
Glu-Glu-Leu	24.3±100	1.12±0.53	39±5
Glu-lac	830±10	1.38±0.3	122±10
Glu-Glu	2690±1800	0.93±0.5	83±3.87
Asp-Glu	2700±700	1.22±0.5	120±21
Asp-Asp	2820±1400	1.4±0.8	190±14
Glu-Glu-Asp	2860±880	0.76±2.7	105±34

Values were obtained from the fit of the mean data from three individual experiments performed in quadruplicate.

(Figure 5). For L-AP4 and L-glutamate the occupancy and the functional response correlates in almost a one to one fashion. Interestingly, Glu-lac functional-occupancy curve was close to the curve of the two standards whereas the Glu-Glu-Leu curve indicates that this compound was able to occupy a large fraction of the receptor without inducing a proportional response. Asp-Glu appeared to be unable to activate the receptor at an occupancy level close to 80%.

To assess the specificity of the metabotropic agonist effects, a group III antagonist, MPPG was applied in this assay following the receptor activation by L-AP4, L-glutamate, and Asp-Asp. We found that 300 μM MPPG completely inhibited the [γ-^{35}S]-GTP binding in the $mGlu_4$ membranes stimulated with approximate EC_{95} concentrations of L-AP4 (10 μM), L-glutamate (100 μM) (data not shown). Interestingly, Asp-Asp potencies in both assays were rather similar (3890 and 2820 μM for [^{3}H]-L-AP4 and [γ-^{35}S]-GTP binding, respectively) which indicates that this compound should behave as an agonist on this receptor, however, 300 μM MPPG only partially reversed the effect of 4 mM of this compound (data not shown).

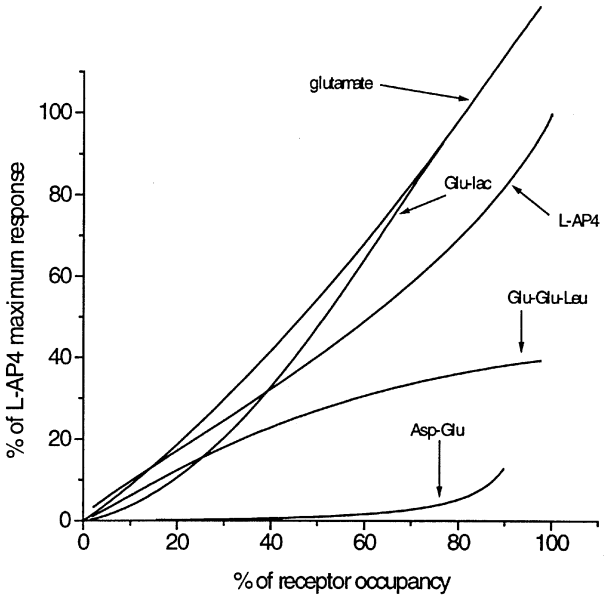

Figure 5 The relationship between % receptor occupancy and effect (as % of L-AP4 maximal effect) for L-AP4, L-glutamate, Glu-lac, Glu-Glu-Leu and Asp-Glu. % of occupancy was calculated by use of the law of mass action and K_i values derived from the binding experiments.

Sensory evaluation

The dipeptides selected for our studies were of the general formula Glu-X and Asp-X, where X is another amino acid or lactoyl group. Additionally two tripeptides Glu-Glu-Asp and Glu-Glu-Leu were tested as described in Methods. The panellists were emphasized to report the umami or any taste enhancing or modifying effect in the test samples compared to the reference (0.4% NaCl solution). The results emerging from the sensory evaluation of the peptide solutions at varying concentrations are summarized in Table 3 and Figure 6. Glu-Glu-Asp and Glu-Glu-Leu were not perceived as umami at the tested concentrations and pH and were excluded from the table. Both had a pronounced chemical, bitter taste, which could effectively mask any possible 'umami-like' properties of these compounds in the sensory evaluations.

Apart from MSG, of all the compounds with reported umami-like taste, only Glu-lac was recognised in a concentration-dependent manner (Figure 6). Glu-Glu, Asp-Glu, and Asp-Asp had a weak mouthfeel effect which could not be clearly defined and did not increase at higher concentrations of the compounds. It can be concluded that in all the Glu-X di- and tripeptides the strong umami effect of the parent L-glutamic acid has been lost, and Glu-lac was the only derivative still possessing the defined umami properties.

Discussion

The present study was undertaken to gain an insight into the molecular mechanisms of the taste transduction, and in particular to examine the implication of the metabotropic glutamate receptor $mGlu_4$ in the responses to the compounds with characteristic 'umami' taste. Umami is the taste quality attributed to monosodium glutamate and nucleotides. The

Table 3 Evaluated peptides and their experimentally determined taste properties

Compound	*Concentration* (mM)	*No. of persons (of ten) recognizing umami-like taste*	*Taste/flavour description*
Glu-lac	2	3	umami, mouth-watering
	5	5	umami, bouillon
	10	6	umami, bouillon, long-lasting, mouthfeel
MSG	2	7	umami, mouthfeel, MSG, bouillon
	5	8	umami, mouthfeel, MSG, mouth-watering, bouillon
	10	10	umami, mouthfeel, MSG, long-lasting, mouth-watering, bouillon
Asp-Asp	2	3	umami, mouthfeel, long-lasting
	5	2	umami, spicy
	10	3	mouthfeel, mouth-watering, long-lasting
Glu-Glu	2	4	mouthfeel, mouth-watering, long-lasting
	5	3	umami, mouthfeel
	10	4	umami, mouthfeel, long-lasting
Asp-Glu	2	2	mouthfeel, long-lasting
	5	1	long-lasting
	10	1	long-lasting

Summary of the sensory evaluation: (1) Glu-Glu-Asp and Glu-GLu-Leu have a strong chemical, bitter taste and could not be perceived as umami at any concentration; (2) All the panellists reporting the umami-like taste of Glu-Glu and Asp-Asp noted that it was weak and difficult to define; (3) Monosodium glutamate was clearly recognized by majority of the panel at 2 mM. Glu-lac evoked the strongest umami taste, although at a higher concentration than MSG.

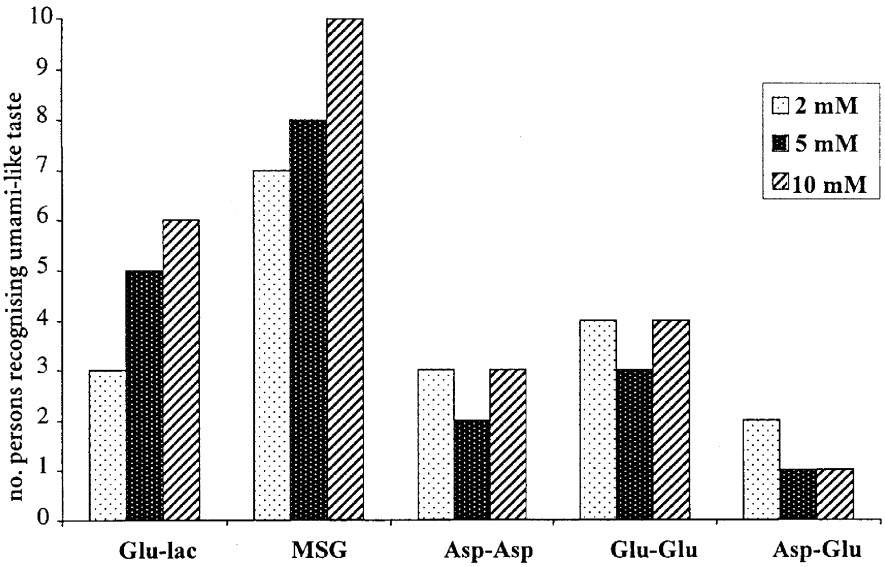

Figure 6 Number of persons recognizing the umami-like taste of the compounds, presented as 2, 5 and 10 mM solutions in 0.4% NaCl, pH 6.0. The tasting panel consisted of ten individuals.

behavioural and electrophysiological effects of umami were extensively studied for a number of years (Yamaguchi, 1991; Yamamoto *et al.*,1991), although the mechanisms involved in this and other taste pathways remained elusive.

However, it has been long suggested that certain chemical stimuli, such as saccharin, sugar and denatomium (a bitter compound) as well as amino acids, are transduced *via* G-protein-coupled receptors of the taste cells (Lindemann, 1995). Although the biochemical nature of such receptors is still poorly understood, a novel G protein α subunit (α-gustducin) has been identified in taste tissue and shown to be a close homologue of transducins, the rod and cone photoreceptor G proteins (McLaughlin *et al.*, 1992). Gustducin can be activated by certain bitter compounds in presence of taste tissue, and couples the receptor(s) to phosphodiesterase (Ruiz-Avila *et al.*, 1995; Hoon *et al.*, 1995; Ming *et al.*, 1998).

Recently, the putative bitter and sweet taste receptors, TR1 and TR2, were cloned from the taste receptor cells of the tongue (Hoon *et al.*, 1999). These are the novel members of the seven-transmembrane protein family, specifically expressed in subsets of taste receptor cells and localized to the taste pore of taste buds, the only region of the cells exposed to the taste stimuli. Interestingly, although the two cloned receptors show no significant homology with most of the known GPCRs, they are resembling the Ca^{2+} sensing receptor (30% identity), a family of putative pheromone receptors, and metabotropic glutamate receptors (both 20% identity). All these families show low, but striking similarity, and are distinguished from the other GPCRs by the presence of a very long N-terminal extracellular domain. The notion that TR1 encodes the receptor for the sweet and TR2 for the bitter taste transduction originates from the correlation between their topographic distribution and anatomical representation of sweet and bitter-transducing taste buds in fungiform and circumvallate papillae, respectively, which do not overlap with the umami taste transducing buds. However, so far it has not been possible to determine the exact ligand specificity of TR1 and TR2 due to the difficulties of expressing these novel genes in heterologous systems.

The idea that the excitatory brain receptors and umami taste receptors might be structurally similar originated from the fact that many known ligands of brain glutamate receptors (monosodium glutamate, L-AP4, ibotenic acid, tricholomic acid, etc.) taste umami and are considered flavour potentiators (Faurion, 1991). Later it was shown that although several brain mGlu receptors are expressed in the lingual epithelium, a single metabotropic receptor $mGlu_4$ is expressed selectively in the sensory cells (taste buds in foliate and vallate papillae) (Chaudhari *et al.*, 1996).

Two splice variants of the rat $mGlu_4$ have been isolated from the brain cDNA libraries (Tanabe *et al.*, 1992; Thomsen *et al.*, 1997). When $mGlu_{4a}$ was expressed in cell lines, the degree of inhibition of the forskolin-induced cyclic AMP formation by certain compounds was relatively moderate, never exceeding 60% of induced cyclic AMP levels (for 0.1 mM L-SOP) (Tanabe *et al.*, 1993; Eriksen & Thomsen, 1995). For the $mGlu_{4b}$ receptor, a splice variant of $mGlu_4$ where 64 amino acids at the C-terminus were replaced by 135 new amino acids, a minor inhibition of cyclic AMP formation (20%) was observed when expressed in insect cells by recombinant baculovirus (Thomsen *et al.*, 1997).

In this study, the [^{3}H]-L-AP4 radioligand binding to the Group III mGlu receptor was characterized using the scintillation proximity assay (SPA). Using this method, the saturation analysis gave binding parameters K_D and B_{max} values of 150 nM and 9.3 pmoles mg^{-1} protein, respectively (Figure 1) for membranes prepared from the CHO cells infected with the Semliki Forest virus expressing the rat $mGlu_{4a}$. This K_D value was significantly lower than the one described earlier for both splice variants a and b (K_D of 441 nM for $mGlu_{4a}$ in BHK cells and K_D of 480 and 369 nM for $mGlu_{4a}$ and $mGlu_{4b}$, respectively, in Sf9 cells (Eriksen & Thomsen, 1995; Thomsen *et al.*, 1997) and might reflect a better coupling of the receptor in this expression system used. However in all these previous publications, centrifugation was used to separate free from bound radioligand and it is well known that this method of separation gives binding data which have a lower degree of accuracy compared to data obtained using an homogeneous system. Moreover, Eriksen & Thomsen (1995) stressed that the [^{3}H]-L-AP4 saturation obtained had a Hill number lower than one indicating some degree of heterogeneity in the receptor affinity. Finally, the K_D value reported for the amino-terminal domain of mGlu4 recently published by Han &

Hampson (1999) was of 120 nM although they found a K_D value of 470 nM for the full length receptor.

The maximum number of binding site value was also higher than previously reported (B_{max} of 3.0 pmol mg^{-1} for $mGlu_{4a}$ in BHK cells and B_{max} of 4.2 and 0.8 pmol mg^{-1} for $mGlu_{4a}$ and $mGlu_{4b}$, respectively, in Sf9 cells. Eriksen & Thomsen, 1995; Thomsen *et al*., 1997), reflecting a higher efficiency of expression obtained using the Semliki Forest virus vector.

The radioligand [^{3}H]-L-AP4 is well suited for characterization of $mGlu_4$ receptors in this assay and was employed to establish the rank order of affinity of various compounds, including the di- and tripeptides (Figure 2 and Table 1). The rank order of potency found for reference metabotropic ligands in our model was similar to the one reported by Thomsen *et al*. (1997). The peptides assayed here were previously reported as having umami taste (Nakata *et al*., 1995; Kuramitsu *et al*. 1996; Frerot, 1997). Although all the peptides were displacing [^{3}H]-L-AP4, the IC_{50} values varied considerably, ranging from 44 for Glu-Glu, up to 814 μM for Glu-lac. Asp-Asp was the weakest binding inhibitor, with an IC_{50} value of 3890 μM (Table 1).

The [γ-^{35}S]-GTP binding assays can be utilized as an alternative to the laborious cyclic AMP assays, or coupling of the Group III receptors to the promiscuous or chimeric G-proteins (Gomeza *et al*., 1996). The [γ-^{35}S]-GTP binding method was previously assessed using the metabotropic receptor ligands in CHO cells expressing human $mGlu_2$ and $mGlu_4$ (Kowal *et al*., 1998a), and recently the same authors showed that inhibition of [^{3}H]-L-AP4 binding and activation of [γ-^{35}S]-GTP binding correlate for the human $mGlu_{4a}$ (Kowal *et al*., 1998b). The rank order of affinity for the compounds tested by these authors on the human receptor in the [^{3}H]-L-AP4 binding assay was similar to the one described here for the rat receptor. Similar to their data, for the rat $mGlu_{4a}$ we found a significant correlation between receptor occupancy and functional activities of L-AP4 and L-glutamate (Figure 5). These similarities are not surprising taking into account that the receptors have 96% amino acid identity (Flor *et al*., 1995; Wu *et al*., 1998).

Based on our results for the rat $mGlu_{4a}$ and the published data for the human receptor, it can be concluded that the combination of [^{3}H]-L-AP4 and [γ-^{35}S]-GTP binding assays provides a good tool for evaluating the novel compounds. The concentration response curves for the peptides (Figure 4) demonstrate that although Asp-Glu is more potent than Glu-lac in the binding experiments (IC_{50} of 71 and 814 μM, respectively), the former was unable to activate the receptor at concentration which almost fully occupied it (EC_{50} of 2700 and 830 μM, respectively, Table 2). Of all the umami peptides tested only Glu-lac and Asp-Asp induced a functional response almost proportional to their receptor occupancy. However, the maximum stimulation (E_{max}) reached with Asp-Asp was substantially higher than the E_{max} obtained with glutamate and the stimulatory properties of Asp-Asp were not antagonized significantly by the group III antagonist MPPG. This might indicate that, in this membrane preparation, this compound activates the [γ-^{35}S]-GTP binding not only *via* the $mGlu_4$ receptor but also by activating an endogenous G-protein coupled receptor or by a direct action on the G-protein. Noticeably, the sensory evaluation of the taste properties of the same peptides which was carried out simultaneously, and independently of these studies showed that only Glu-lac had significant umami-like effects (Figure 6).

Although both rat and human receptors have similar pharmacology, we isolated the human $mGlu_{4a}$ from the brain cDNA library and expressed it in CHO cells using SFV vectors in order to test it with the 'umami' peptides. Our preliminary data demonstrate the same rank order of potency for these peptides in the $mGlu_4$ receptors from both species. This observation validates the comparison of the *in vitro* results obtained using the rat receptor, and the sensory evaluation carried out by human subjects, provided that in both species the $mGlu_4$ is involved in the umami taste transduction.

For the peptides which did not activate the receptor efficiently, the panel members concluded that none of them (Glu-X dipeptides, or Glu-Glu-X tripeptides) had a pronounced umami taste. These observations are in agreement with the earlier sensory reports (van den Oord & van Wassenaar, 1997). Only the Glu-acid derivative had the taste characteristics similar to MSG, including flavour enhancing and mouthfeel effects (Figure 6). However, the umami taste of Glu-lac was considerably weaker than that of MSG at the same concentration. This might be explained by its lower affinity (IC_{50} of 814 and 3.5 μM for Glu-lac and MSG, respectively, Table 1).

In summary, this study is the first to characterize in the scintillation proximity assay the binding of the radioligand, [^{3}H]-L-AP4, to the rat mGlu4a expressed in CHO cells using SFV vectors. Here we were able to compare the binding results to the functional studies, and to demonstrate the corresponding potencies for the agonists L-AP4 and L-glutamate. In addition, we have applied both [^{3}H]-L-AP4 and [γ-^{35}S]-GTP binding assays to characterize a series of peptides with alleged umami taste. Several of them were identified as $mGlu_4$ agonists, albeit with varying potencies and more important very different efficacies. The independent taste evaluation of the same peptides demonstrated that, in agreement with its efficacy on the mGlu4 receptor, only Glu-lac tasted distinctly umami, similar to monosodium glutamate. The combination of the taste assessment and *in vitro* receptor binding and activation results, as well as the receptor localization in the taste buds (Chaudhari *et al*., 1996), suggest that a $mGlu_4$ receptor similar to the one in brain might be involved in transducing the umami taste stimulus.

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