Binding of Umami Substance to Plasma Membrane Isolated from Bovine Circumvallate Papillae

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Fractionation of several kinds of membranes from the taste organ, circumvallate papillae, of bovine has been done to investigate localization of the umami substance binding site. Monosodium glutamate (MSG) and nucleotides are known as umami substances. The membrane fractions obtained from a series of centrifugation were examined for radioactive MSG binding activities by the ligand-binding methods, indicating a synergistic effect between MSG and nucleotides. They were also measured for activities of the marker enzymes. The membrane fraction that seemed to be rich in plasma membrane, on the basis of the marker enzyme activities, had specific MSG binding activity. These results suggest that umami substances bound to the plasma membrane of circumvallate papillae.

Taste sensation has a significant role in survival in nature. Diverse receptors and channels at the taste buds in the tongue and oral cavity can recognize the five basic tastes. Stimulus interactions with the receptors and channels in the taste cells are the initial processes in gustatory transduction. Sweet and umami tastes (sugar and amino acid) involve the mechanism of receptor-mediated second messengers that are cyclic nucleotides and inositol 1,4,5-trisphosphate (IP₃), as indicated by biochemical and patch-clamping methods. Evidence has been obtained for a IP₃ receptor-mediated release of Ca²⁺ from intracellular stores in response to certain bitter stimuli. Although many and various studies of the taste response have been reported, the molecular mechanism of the transduction in taste receptor cells is not clear because of low affinity of the receptors and their unstabilities and sparseness, which caused investigation to stall for long years.

Umami is a novel and unique basic taste. It is well known that a synergistic effect appears between MSG and 5'-nucleotides such as GMP and IMP. It has been proposed that the nucleotides are not a real umami substance but they act as an enhancer in the synergistic effect. Many studies of the umami sensation were done in mammals, for example, rat, mouse, hamster, bovine, and human. A bovine tongue is suitable to study taste sensation, because 91% of the taste buds concentrate in about 20 pieces of the circumvallate papillae. The circumvallate papillae have been the most effective material for the studies of MSG binding protein, since these papillae have a lot of taste buds. A membrane fraction from bovine circumvallate papillae was shown to have MSG binding activity, however, types of membrane involved in the MSG binding were not elucidated. In this study, bovine circumvallate papillae have been used for the characterization of synergism involving MSG and nucleotide. Purification of the plasma membrane from the taste bud has been done to investigate the location of the MSG binding site.

Materials and Methods

Materials. Bovine tongues were obtained fresh from a local slaughterhouse within 30 min after slaughtering and kept on ice during transportation. After they were rinsed several times with Ca²⁺ and Mg²⁺-free Hank's solution, the tongues were used for the experiments. 1,3,4(5)H]Glutamic acid (60 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (MO, U.S.A.). Sodium bicincominate and N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) was obtained from Dojindo Laboratories (Kumamoto, Japan); cytochrome c, aprotinin, and GMP disodium salt, from Sigma (MO, U.S.A.); clear-sol, from Nacalai Tesque. Other chemicals were reagent grade.

Tissue fractionation. Circumvallate papillae and epithelial tissue were obtained by a previously reported method with slight modifications. Briefly, two areas were dissected from the left and right posterior surfaces of fresh bovine tongue. The middle portion of each papilla was punched and the punched papillae were excised with a scalpel and put into a cold homogenization buffer (25 mm HEPES pH 7.4, 0.3 M sucrose, 3 mm EDTA and protease inhibitors 0.1 mm phenylmethylsulfonyl fluoride, 10 μM pepstatin A, 10 μM leupeptin, and 16 μM aprotinin). Epithelia were teased off around papillae into the same buffer. The papillae and epithelia were minced vigorously with fine scissors and homogenized with TenBroeck all glass homogenizer. Undisrupted tissues and nuclei were removed by centrifugation at 1000 x g for 10 min. The pellet (1000 x g pellet) was washed with the homogenization buffer and centrifuged. The supernatants were combined and centrifuged either at 5000 x g or 10,000 x g for 20 min to allow larger fragments to settle (5000 x g pellet and 10,000 x g pellet, respectively). Each supernatant was further centrifuged in a Hitachi 65T rotor in a Hitachi 70F Ultracentrifuge at 200,000 x g for 1 h. The resulting each pellet was suspended with the homogenization buffer without protease inhibitors (suspension buffer).

For further fractionation, the resulting pellet (200,000 x g pellet) from the supernatant of the 10,000 x g centrifugation was suspended in 49% (w/v) sucrose in 25 mm HEPES buffer (pH 7.4), transferred to an ultracentrifugation tube. The suspension was overlaid by discontinuous sucrose gradients composed of 2.5 ml each of 38, 32, 28, and 8% (w/v) sucrose in 25 mm HEPES buffer (pH 7.4). The gradient sample was centrifuged at 75,000 x g for 17 h in Hitachi RPS40T rotor. The sucrose gradients were fractionated into 0.4 ml fractions and monitored at 280 nm. Four peaks were collected into separate tubes and diluted with 25 mm HEPES buffer containing 3 mm EDTA to fill the tubes. Membranes were sedimented by centrifugation at 200,000 x g for 1 h and suspended in a small volume of the suspension buffer using a gentle hand homogenizer. These suspensions were stored at -20 °C for up to 1 week to measure enzyme activity. The activities of marker enzymes for each membrane fraction were studied to assess the purity. Each fraction was sonicated and the activities

Abbreviations: EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; HEPES, N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; IP₃, inositol 1,4,5-trisphosphate; LY, lysosome; MSG, monosodium glutamate; MT, mitochondria; PM, plasma membrane.
were assayed. Ouabain-sensitive (Na\(^+-\)K\(^+-\)Mg\(^2+\))-ATPase, 5'-nucleotidase, and alkaline phosphatase were marker enzymes for plasma membrane (PM), succinate dehydrogenase for mitochondria (MT), NADPH-cytochrome c reductase for endoplasmic reticulum (ER), and acid phosphatase for lysosome (LY). Ouabain-sensitive (Na\(^+-\)K\(^+-\)Mg\(^2+\))-ATPase was assayed as described by Wallach and Ullrey, modified by Lo. 5'-Nucleotidase was assayed by the method of Michell and Hawthorne. Acid phosphatase was measured as described. NADPH-cytochrome c reductase was measured spectrophotometrically. Succinate dehydrogenase was assayed by the method of Pennington.

**Binding assay.** The cells and the membrane prepared from circumvallate papillae and epithelium were assayed for MSG binding activity immediately after preparation. Twelve μl of 1-3.4μg/ml glutamic acid neutralized with 1.2μl of 0.1 n NaOH was used for a binding assay. Assay mixture for enzymes composed of 16 mm radioactive and cold MSG, 3 mm CaCl\(_2\), each buffer and/or 1.6 mm nucleotides were preincubated for 1 min, added the membrane fraction or cell (2 × 10\(^3\)) to final 60 μl, and then incubated various times following to be filtered with millipore GV (0.22 μm) membrane in multiscreen assay system. Filter was washed with 25 mm HEPES buffer with 3 mm CaCl\(_2\) three times, dried, and counted with Clear-sol scintillation fluid.

**Protein assay.** Protein was measured by the micro sodium biocinchoninate protein assay method using bovine serum albumin as a standard.

**Results and Discussion**

**Time-course and characteristic of MSG binding**

The binding of MSG to the circumvallate papilla and epithelium as a function of time is shown in Fig. 1. The binding of glutamate in the presence of GMP to the circumvallate papilla subfraction increased among incubation time and reached a plateau after 30 min. The binding of MSG in the absence of GMP occurred to lesser extent compared to that in the presence of GMP. There was an about 3.3-fold enhancement of MSG binding activity by addition of GMP at 30 min of incubation. The binding of MSG to epithelium subfraction increased linearly with the incubation time. There was no significant difference between the MSG bindings in the presence and absence of GMP (data not shown). Substantially greater binding occurred to the circumvallate preparation, which contained taste receptors, than to the epithelium, which was a control preparation devoid of taste receptors. The binding activity at 30°C was enhanced 4-fold compared to that at 4°C, that was similar to the result reported by Lum and Henkin. The synergistic effect of GMP on the MSG binding represents specific binding and suggests that putative MSG binding protein existed in the circumvallate papilla, but not in the epithelium. As the binding of MSG to the epithelium was still increasing after 30 min, the binding assay was done for 30 min at 30°C through this paper.

**MSG binding to subcellular fractions of tongue tissue**

It has been shown that MSG binding activity was recovered in the pellet from 7000 × g centrifugation. However, in our several preliminary experiments, the binding activity was observed in the supernatant from similar centrifugation. Accordingly, we examined MSG binding activity for subcellular fractions of the tongue tissues. As shown in Table 1, the binding activity was observed among the subcellular fractions and was changeable depending on the fractions. Much specific MSG binding was found in the 200,000 × g pellet of circumvallate papillae. The supernatant of the papilla also had significant activity of the specific binding. Considerable MSG binding activity was present in the 10,000 × g pellet, but was not attributed to the specific binding. The highest enhancement of MSG binding by GMP appeared in the 1000 × g pellet; that was due to the unbroken cells and contamination by large plasma membrane, which also had the specific binding, while efficiency of the binding was fairly low compared to that in other subcellular fractions. The subcellular fractions of epithelial tissues had lower levels of MSG binding activity and no significant specific binding, compared to those of the papilla. The specific binding of MSG to bovine circumvallate papillae has been reported by Torii and Cagan, where subcellular fraction of 7000 × g pellet was concerned with the binding. In this investigation, the binding activity was found substantially in the post-10,000 × g centrifugal supernatant such as the 200,000 × g pellet and supernatant (Table 1). When centrifugation at 5000 × g for 20 min followed by the 200,000 × g centrifugation was admitted into the subcellular fractionation, the resulting pellet had less enhancement of MSG binding by GMP (3.5-fold, data not shown) than the 200,000 × g pellet (5.6-fold, Table I). It is likely that homogenization circumvallate papillae with the TenBloee homogenizer might result in disintegration of the tissue and/or their cells to a greater extent in this experiment. This may account for the appearance of MSG binding activity in the Supernatant as well as in the 200,000 × g pellet.

The subcellular fractions of tongue tissues were examined for activities of the marker enzymes, to discover the characteristics of each fraction. The results are shown in Table 1.

![Fig. 1. Binding of L-3H glutamate to Preparations of Circumvallate Papillae and Epithelial Tissues from Bovine Tongue.](image)

The supernatant from 5000 × g centrifugation was submitted to the assay. Circumvallate papilla, epithelium.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Homogenate (μmol/mg protein)</th>
<th>1000 × g pellet</th>
<th>10,000 × g pellet</th>
<th>200,000 × g pellet</th>
<th>Supernatant (μmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circumvallate (+)</td>
<td>140.0</td>
<td>70.0</td>
<td>459.0</td>
<td>568.5</td>
<td>358.1</td>
</tr>
<tr>
<td>Circumvallate (-)</td>
<td>160.4</td>
<td>7.7</td>
<td>587.4</td>
<td>102.2</td>
<td>108.9</td>
</tr>
<tr>
<td>Epithelium (+)</td>
<td>82.3</td>
<td>43.8</td>
<td>37.9</td>
<td>146.0</td>
<td>48.9</td>
</tr>
<tr>
<td>Epithelium (-)</td>
<td>68.4</td>
<td>32.9</td>
<td>128.9</td>
<td>53.2</td>
<td>73.3</td>
</tr>
</tbody>
</table>

+ and −, in the presence and absence of GMP, respectively; 1000 × g, 10,000 × g and 200,000 × g pellets, precipitates from the 1000 × g, 10,000 × g and 200,000 × g centrifugations, respectively; supernatants were from the 200,000 × g centrifugation.
Table II. Activities of Marker Enzymes in Subcellular Fractions from Bovine Circumvallate Papillae

<table>
<thead>
<tr>
<th>Marker enzymes</th>
<th>Location</th>
<th>Enzyme activities (μmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Homogenate 1000 × g pellet 10,000 × g pellet 200,000 × g pellet Supernatant</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>MT</td>
<td>3.1 2.9 4.4 7.4 2.1</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>LY</td>
<td>7.1 1.0 5.6 14.3 3.9</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase</td>
<td>ER</td>
<td>0.020 0.013 0.022 0.084 0.028</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>PM</td>
<td>10.3 3.8 5.5 53.5 8.4</td>
</tr>
<tr>
<td>Ouabain-sensitive ATPase</td>
<td>PM</td>
<td>0.61 0.67 0.31 1.37 0.45</td>
</tr>
</tbody>
</table>

1000 × g, 10,000 × g and 200,000 × g pellets and supernatant are the same as in Table I.

Fig. 2. Fractionation of Membrane Components of Bovine Circumvallate Papillae by Discontinuous Sucrose Density Gradient Centrifugation.

The pelot from the 200,000 × g centrifugation was fractionated into membrane components.

in Table II. The enzyme activities appeared through the subcellular fractions where the activities were distributed in different proportions among the fractions. The marker enzyme for plasma membrane showed significant localization in the 200,000 × g pellet. The enzyme for endoplasmic reticulum was also concentrated in the 200,000 × g pellet compared with mitochondrial and lysosomal enzymes. Such localization of the marker enzymes was observed for the Supernatant in higher degree compared to the 10,000 × g pellet, but in less significantly compared to the 200,000 × g pellet. These results demonstrate that plasma membrane and endoplasmic reticulum were concentrated in the 200,000 × g pellet and the supernatant. Thus, the plasma membrane and/or endoplasmic reticulum seems to be responsible for the specific MSG binding. The distribution of marker enzymes in the subcellular fractions of epithelial tissues was similar to that of the circumvallate papillae (data not shown). This may imply a complete difference of the specific MSG binding function between those tissues.

MSG binding to membrane fractions of circumvallate papilla

To discover more about specificities of the MSG binding, the 200,000 × g pellet that had the most significant specific MSG binding was centrifuged on a discontinuous sucrose density gradient with a view to fractionating subcellular membrane components. It has been reported that plasma membranes from bovine circumvallate papillae were sedimented in the interface between 11 and 35% sucrose layers and between 8.5 and 33% sucrose layers. In our investigation, a discontinuous gradient consisting of 8, 28, 32, 38, and 49% sucrose concentrations was chosen for a satisfactory fractionation of the membrane components.

Table III. Binding of L-[3H]Glutamate to Membrane Fractions from Bovine Circumvallate Papillae

<table>
<thead>
<tr>
<th></th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-[3H]Glutamate bound (μmol/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plus GMP</td>
<td>18.0</td>
<td>3.5</td>
<td>1.7</td>
</tr>
<tr>
<td>none</td>
<td>4.2</td>
<td>0.8</td>
<td>1.7</td>
</tr>
</tbody>
</table>

II, III, and IV refer to the peak numbers II, III, and IV, respectively, from the discontinuous sucrose gradient centrifugation shown in Fig. 2.

Table IV. Activities of Marker Enzymes in Membrane Fractions from Bovine Circumvallate Papillae

<table>
<thead>
<tr>
<th>Marker enzymes</th>
<th>Location</th>
<th>Enzyme activities (μmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>II</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>MT</td>
<td>T</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>LY</td>
<td>37.3</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase</td>
<td>ER</td>
<td>0.12</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>PM</td>
<td>11.8</td>
</tr>
<tr>
<td>Ouabain-sensitive ATPase</td>
<td>PM</td>
<td>0.96</td>
</tr>
</tbody>
</table>

II, III, and IV are the same as in Table III. T refers to a trace.

The discontinuous sucrose gradient centrifugation of the pellet from 200,000 × g centrifugation gave four distinct peaks, I, II, III, and IV, as shown in Fig. 2. Peak I had higher absorbance at 260 nm than at 280 nm and was omitted in the following experiment. Peaks (membrane fractions) II, III, and IV were examined for the MSG binding activity and the results are presented in Table III. Membrane fraction II had the binding activity, which was synergistically increased (4.3-fold) by the addition of GMP. Membrane fraction III also had the specific MSG binding, while efficiency of the binding was considerably lower than membrane fraction II. Membrane fraction IV had no significant activity of MSG binding.

Activities of the marker enzymes in the membrane fractions described above were assayed and the results are shown in Table IV. Among the enzyme activities, those for plasma membrane were localized in membrane fraction II. The activities of the marker enzymes for endoplasmic reticulum and mitochondria were lower in membrane fraction II than in the fractions III and IV. The lysosomal enzyme activity was distributed evenly in the membrane.
fractions. These results indicate that plasma membranes were concentrated more in the membrane fraction II than in the other fractions. Thus, it may be concluded that plasma membrane of the circumvallate papillae participates in the specific MSG binding.

References