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Biochemical studies of taste sensation: Binding to taste tissue of ^{3}H-labeled monellin, a sweet-tasting protein*

(binding sites/taste receptors/sweeteners/taste specificity)

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ABSTRACT **Binding of ^{3}H-labeled methylated monellin to taste receptor tissue was demonstrated *in vitro*. Preparations of bovine and human circumvallate (taste) papillae bound more of the ligand than did lingual and nonlingual epithelial preparations devoid of taste buds. Binding to the taste preparations saturated at high ligand concentrations. Furthermore, sugars and other sweet-tasting molecules appeared to compete to some extent with this sweet-tasting protein for its binding sites. These binding measurements of the intensely sweet-tasting protein monellin to taste receptor preparations help to establish the binding interaction as an initial step in taste sensation.**

Relatively little is understood about the biochemical basis of sweet taste despite considerable knowledge of a wide variety of sweet-tasting chemical compounds (1–6). Most of the behavioral, physiological, and biochemical research on sweet taste has used sucrose or other sugars. The inherently weak binding (6–8) of sugars to taste receptors, with values of K_D in the range of 10^{-1} to 10^{-3} M (9, 10), presents a substantial difficulty in directly studying the biochemical basis of how a sugar binds to and triggers a taste receptor to respond. The interest in our laboratory (11–16) in the sweet-tasting protein monellin stemmed in part from the possibility that it could be used as a "probe" for sweet taste receptor sites (5). Monellin is 10^4–10^5 times more effective than sucrose in eliciting a sweet sensation, leading to the hypothesis (5) that the binding constant of monellin would be more favorable than those of sugars for biochemical investigation; this hypothesis is confirmed by the present study. Using ^{3}H-labeled methylated monellin ([^{3}H]-Me-monellin), we demonstrate that it binds to bovine and to human taste tissue preparations.

MATERIALS AND METHODS

Monellin was isolated from the fruit of *Dioscoreophyllum cumminsii* (11, 13) and methylated (16, 17) with [^{3}H]formaldehyde (100 Ci/mol, 1 Ci = 3.7×10^{10} becquerels; New England Nuclear). We recently showed (16) that partially methylated monellin retains its sweetness. To prepare the [^{3}H]-Me-monellin for the bovine experiments, we methylated it as described (16), dialyzed (or ultrafiltered) it, and then chromatographed it on carboxymethyl-cellulose. Three preparations showed specific radioactivities of 14, 7.9, and 4.0 Ci/mol; no differences in binding results could be attributed to the different preparations. The reaction conditions for preparing the [^{3}H]-Me-monellin were modified for the experiments with human tissue to increase the utilization of the radioactive precursor. The [^{3}H]formaldehyde was added to the monellin first, followed by two successive additions of $NaBH_4$, rather than the reverse order. For this preparation, 200 mg (5 mg/ml) of monellin reacted on ice with 32 μl of [^{3}H]formaldehyde (the original [^{3}H]formaldehyde was diluted with unlabeled formaldehyde to yield 2.7 M and a specific radioactivity of 9.5 Ci/mol). This was followed by reduction with 100 μl of 1.2 M $NaBH_4$ (in water) for 5 min and then with 400 μl for another 5 min. The preparation was washed at room temperature with 10 mM sodium phosphate buffer (pH 7.2) on an Amicon model 202 ultrafiltration apparatus (UM2 filter) to remove unreacted [^{3}H]formaldehyde (16). This yielded a preparation of specific radioactivity 2.2 Ci/mol.

Cow and steer tongues were obtained fresh from local slaughterhouses and human tissue specimens were provided at autopsy. Nonpathological tissue specimens were provided within 30 hr (median 16 hr) after death. The bovine tongues were stored at −15°C for 1–4 days, and the human specimens were stored at −65°C for up to a month. The binding activity seemed likely to survive low-temperature storage because we had earlier compared the binding results with fresh bovine tissue (carried out in two experiments immediately upon arrival in the laboratory) with those using previously frozen bovine tissue; the values for binding activities were not significantly different with the fresh or frozen-stored tissues.

To prepare the tissue fraction, we thawed the frozen tongues or human tissue samples and dissected the circumvallate papillae free, with the top surface (0.2–0.5 mm) having been removed with a scalpel. Small (5 × 5 mm) blocks of tongue epithelium provided control tissue devoid of taste buds (bovine and human), and pectoral skin (human) was also used as a control tissue where indicated. The epidermal sidewalls of the papillae and the upper layer from the control epithelial blocks were teased off with forceps (18, 19) and each of these two tissue samples was placed in a small volume (1 ml) of cold homogenization buffer (0.15 M NaCl/0.3 mM $CaCl_2$/0.01 M Tris·HCl, pH 7.4) (18). The homogenization and centrifugation procedures were carried out in the cold (0°–4°C). The two samples were minced vigorously with a fine scissors for 6 min and each was transferred to a 7-ml all-glass TenBroeck homogenizer (A. H. Thomas) with a tight-fitting pestle. After settling for 3 min, the sediment volumes were equalized, usually by removing some of the control material. The samples were homogenized manually (16 strokes) and allowed to settle for 5 min; the supernatant was then withdrawn. The homogenizing and settling was repeated with 3 ml of fresh homogenizing buffer. The combined supernatants were centrifuged in an International

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Abbreviation: [^{3}H]Me-monellin, [^{3}H]methylmonellin.

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Clinical centrifuge at approximately 50 × g for 10 min (bovine) or 20 min (human). The turbid supernatant (S1) was decanted, the pellet was homogenized again in 3 ml of homogenizing buffer, and the few remaining larger fragments were allowed to settle. The supernatant was combined with S1 and centrifuged in a Sorvall RC2-B at 7000 × g for 30 min. The clear supernatant was decanted and discarded, and the pellet was suspended in homogenizing buffer for the binding studies.

Binding was measured in a system based on that used for quantitating taste ligand binding to catfish taste receptors (20). In preliminary studies with [^{3}H]Me-monellin, unacceptably high levels of counts were retained by the Millipore (type HAWP) filters themselves, even after their treatment with bovine serum albumin in buffer (10 μg/ml–35 mg/ml). Several additional types of commercial filters were tested; the type BA85 filter (formerly type B6) from Schleicher & Schuell (pore size 0.45 μm), pretreated with bovine serum albumin (20 mg/ml in homogenizing buffer), performed satisfactorily and was adopted for our standard procedure. For the bovine experiments, the assay mixture contained 0.25 ml of the suspension, 25 μl of [^{3}H]Me-monellin (in 10 mM sodium phosphate buffer, pH 7.2), and 50 μl of the sugar (in water) where added as a competitor. The tissue preparation averaged 2 mg/ml in the assay mixture. After incubation on ice for 1 hr, the sample was filtered with suction, after which the filter was washed with a 10-ml portion of buffer. Radioactivity was measured in 10 ml of scintillation fluid (21) with a Packard Tri-Carb model 3375 liquid scintillation counter (efficiency, 37% for tritium). Protein was determined by the Lowry method (22), with human serum albumin as a standard. The binding assays with human preparations were similar except that 0.15–0.25 ml of tissue suspension in buffer was used, to which was added [^{3}H]Me-monellin in 10 mM sodium phosphate buffer (pH 7.2) to give a final volume of 0.40 ml. In all cases, blanks without the tissue fraction enabled correction for retention of radioactivity by the filters.

The competition experiments with human tissue were of two types. In one type, the sweetener (potential competitor) was present in the assay mixture throughout the binding assay period. In the other type, it was added to the filter chamber after the binding assay samples were filtered. A sweet compound was added (1 ml), allowed to remain in place for 10 min, and then filtered as usual. Comparable controls were included to which buffer was added. Sucrose (0.5 M), lactose (0.5 M), and sodium saccharin (1 mM) were tested with both approaches. In addition, each of the sweeteners neohesperidin dihydrochalcone (50 μM), calcium cyclamate (12.5 mM), L-aspartyl-L-phenylalanine methyl ester (Aspartame) (7.5 mM), and stevioside (1.5 mM) were tested in one experiment by use of only the former approach. Preparations were also tested with 10 mM ammoniated glycyrrhizin, but they became gelatinous, thereby trapping a large amount of fluid with a resultant high level of nonspecific radioactivity.

RESULTS AND DISCUSSION

Binding of [^{3}H]Me-Monellin to Taste Receptors. Binding of [^{3}H]Me-monellin (Table 1) was greater to the bovine circumvallate sidewall epithelium than to tongue epithelium devoid of taste buds. The binding of this sweet-tasting protein is therefore substantially greater to the preparation containing taste receptors than to that devoid of taste receptors. Addition of excess native monellin (75 μM) displaced the bound [^{3}H]-Me-monellin (6 μM) by an average of 54% ($n = 6$; $P < 0.02$). This finding demonstrated that native monellin competes for binding with the labeled derivative. The lower than expected extent of displacement cannot be readily explained, but it may be related to the question of nonspecific entrapment of label (see below).

Table 1. Binding of [^{3}H]Me-monellin to taste and nontaste tissue from bovine tongue

Additions*	Binding, pmol/mg† Circumvallate sidewall	Tongue epithelium
[^{3}H]Me-Monellin	196 ± 51	70 ± 28
[^{3}H]Me-Monellin + sucrose	124 ± 26	26 ± 7
[^{3}H]Me-Monellin + lactose	133 ± 21	59 ± 15

* [^{3}H]Me-monellin was at 6 μM and, where present in the assay mixture, sucrose or lactose was at 0.15 M.

† Data are from 11 experiments, in each of which duplicate samples were run. Values are mean ± SEM. A two-factor analysis of variance showed a difference between the circumvallate and epithelial values [F (1,20) = 9.336; $P < 0.01$] and a difference among the three addition conditions [F (2,40) = 4.300; $P < 0.025$]. Repeating the analysis of variance on the circumvallate and epithelial values separately showed that the significant differences among the addition conditions are in the sidewall values [F (2,20) = 3.758; $P < 0.05$] and not in the epithelial values [F (2,20) = 1.347; $P > 0.2$]. The Duncan multiple range test showed that the circumvallate values with sucrose and with lactose are each different from the control ($P < 0.05$).

In addition to the experiments shown in Table 1, preliminary studies were carried out with isolated bovine taste bud cells (18), but these failed to show significant levels of binding of [^{3}H]-Me-monellin. Control experiments suggested that the negative results with the cell suspensions were not due to the collagenase treatment, but rather that the exposure to Ficoll could be responsible. The collagenase treatment, which is an early step of the cell isolation procedure, was initially postulated to be responsible. Accordingly, control experiments were carried out in which bovine circumvallate sidewall and epithelial preparations were incubated in collagenase (see ref. 18) for 1 hr at 0° or 37°C. The tissue preparations were then sedimented by centrifugation and washed prior to the binding assay with [^{3}H]Me-monellin. No deleterious effect of the collagenase was observed. Because the final stage of the cell preparation is carried out in Ficoll, its effect was tested by adding it to the binding assay with [^{3}H]Me-monellin as a ligand. In a preliminary study, Ficoll (15 mg/ml) partially inhibited binding with the circumvallate sidewall preparation, but possibly not with the epithelium. Whether the Ficoll itself acts as a competitive inhibitor or an impurity present in it was responsible has not been determined.

The limited amount of monellin currently available does not allow direct taste tests with cows. It is clearly established, however, that monellin is intensely sweet tasting to humans (5, 11–16). Because of its effectiveness at low concentrations, monellin was postulated (5) to have a binding constant more favorable for biochemical study than those of sugars. The binding method was therefore applied to human taste tissue. Binding of [^{3}H]Me-monellin was measured over a range of concentration of the ligand (Fig. 1). Substantially greater binding occurred to the circumvallate preparation, which contained taste receptors, than to the control preparations devoid of taste receptors. Saturation of binding to the circumvallate occurred at the higher ligand concentrations, and the value of K_D appeared to be in the range of 10^{-5} M. Heating the circumvallate tissue preparation in a boiling water bath for 30 min resulted in loss of binding activity. A preliminary determination of the initial portion of the saturation curve (up to 40 μM) with the bovine preparation suggested that the maximal binding may be lower.

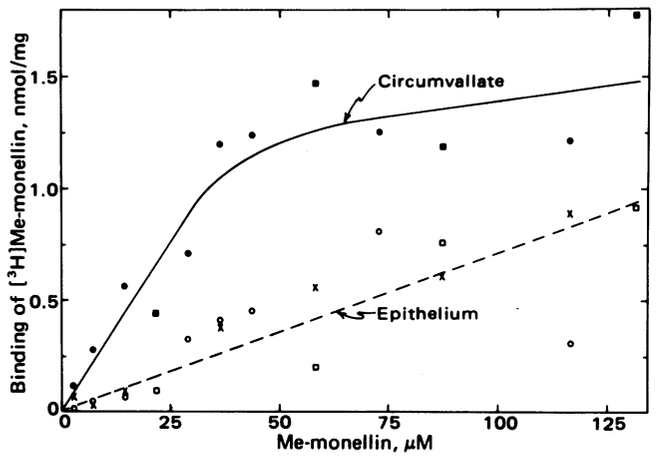

FIG. 1. Binding of [^{3}H]Me-monellin to preparations from human taste papillae, control tongue epithelium, and control pectoral skin. Data from five experiments are summarized. Single samples were run in each case, with each point shown representing either a single value (■, □) or the mean of two to five values (●, ○). For the pectoral skin (x), each point is a single sample. Data were grouped into four concentration ranges and subjected to a two-factor analysis of variance with repeated measures. This showed an effect of monellin concentration [F (3,8) = 9.330; $P < 0.01$] and that the amount bound to circumvallate is significantly greater than that bound to epithelium [F (1,8) = 48.555; $P < 0.001$].

Although significantly less binding occurs to the nonreceptor epithelial preparations than to the taste tissue preparations, nevertheless the absolute levels of ligand bound to the control preparations are substantial. Whether this measure indicates a nonspecific phenomenon or a lower number of actual binding sites cannot be definitively stated. It appears likely, however, that a substantial portion of the binding to the epithelial preparations represents nonspecific entrapment of label in the preparation retained on the filter disc. It must therefore be assumed that a portion measured as "bound" to the circumvallate could similarly be nonspecific entrapment. Recent studies suggest that a portion of the taste ligand L-alanine measured as "bound" in a preparation of catfish taste receptors may be contained within vesicles (23). This possibility appears less likely in the present case, in which the ligand is a protein. Definitive resolution of the meaning of nonspecific binding in the present case must await isolation of purified receptor molecules.

Competition of Sweet Compounds for [^{3}H]Me-Monellin Binding. Understanding the specificity of the receptor sites with which sweet compounds interact is of considerable theoretical and practical interest. The ability of other sweet-tasting compounds to act as competitors with [^{3}H]Me-monellin in the binding assay could provide information about the relative specificity of the monellin binding sites. Sucrose is preferred by cows and lactose less so in two-choice taste preference tests when each sugar is paired with water (24). Furthermore, sucrose, and to a lesser extent, lactose, bind preferentially to bovine taste receptor-containing preparations (9, 10) compared with control preparations devoid of taste receptors. Both sugars taste sweet to humans, with sucrose sweeter than lactose. In our binding competition studies, sucrose and lactose inhibited binding of [^{3}H]Me-monellin by 37% and 32%, respectively (Table 1); the decreases were statistically significant. No significant diminution of the lower level of binding to the epithelial preparations was caused by the sugars. The competition data therefore show some overlap in binding specificity, but further refinement of the system will be necessary in order to draw more extensive conclusions. The data suggest that monellin is either an agonist (i.e., a taste stimulus) or an antagonist to the action of these two sugars in the bovine. The first hypothesis (agonist) predicts monellin to be preferred by cows; the second hypothesis (antagonist) predicts that monellin would decrease the effectiveness of these sugars in mixtures of sugar plus monellin.

Binding of [^{3}H]Me-monellin to human taste tissue appeared to be decreased partially by several other sweet-tasting compounds. Two types of competition experiments were carried out (see *Materials and Methods*). In one type, the potential competitor was present in the binding assay mixture; in the other it was added to the filter chamber after the binding assay sample was filtered and allowed to remain in place in order to displace the bound [^{3}H]Me-monellin. A preliminary survey of the effects of several sweeteners was carried out. Sucrose (0.5 M), lactose (0.5 M), and sodium saccharin (1 mM) were tested with both approaches and found to displace by 20–40% the bound [^{3}H]Me-monellin (36 μM). In addition, neohesperidin dihydrochalcone (50 μM), calcium cyclamate (12.5 mM), and Aspartame (75 mM) were tested in one experiment with the former approach and found to displace [^{3}H]Me-monellin (73 μM) by 20–40%. Under the same conditions, stevioside (1.5 mM) showed no effect.

The competition studies, both with the bovine and human samples, were carried out to survey whether or not displacement occurs. The results of the competition studies, though at a relatively preliminary stage, when taken together suggest some degree of overlap in specificity among certain other sweet-tasting compounds and monellin. Although it is difficult to formulate a detailed picture of the binding interaction, monellin is postulated to interact with taste receptors in a multipoint attachment. This would mean that two or more regions of the protein would be involved directly in binding to regions of the receptor cell membrane. This would allow for regions of overlap between other sweeteners and the monellin binding sites without necessitating other sweeteners to interact at every locus of the monellin interaction. This postulate also need not imply that the monellin binding sites are the only receptor sites for all sweet-tasting stimuli. Hough and Edwardson (25) recently reported preparing an antibody to the sweet-tasting protein thaumatin. Several other sweet compounds, including monellin, Aspartame, sodium saccharin, and cyclamate, were able to react with the antibody. Electrophysiological recordings from the taste nerves of monkeys (26) showed that the response to sucrose was decreased when the tongue was first exposed to monellin. Binding measurements of ^{14}C-labeled sugars with bovine preparations indicated that competition occurs among the sugars (10).

Based on our results, we tend to consider a hypothesis of a "narrowly tuned" receptor site for monellin as less tenable than the alternatives. Considerable interest is being maintained in structure–taste activity correlations among sweet-tasting compounds (1–4, 6, 27–37). While sometimes of practical importance, they do not directly measure the receptor site interaction. The degree to which receptor sites are "broadly tuned" or "narrowly tuned" has not been known, nor is information available about the possible multiplicity of types of sites. Structure–activity correlations by taste evaluations cannot by themselves answer such questions, whereas direct binding studies could. Up to now, the lack of a suitable biochemical system to experimentally answer these questions has been a major impediment to a full understanding of sweet taste specificity. The experimental approach described in this paper provides an opportunity for more detailed definition of taste binding sites for sweet compounds.

Receptor Macromolecules for Sweet Stimuli. While competition studies in relatively crude systems are useful, highly purified, defined receptor molecules would enable more definitive studies of binding interactions and competition among ligands. *No convincing demonstration of the isolation of a taste receptor molecule has yet appeared.* The first biochemical study purporting to have isolated a sweet receptor protein was carried out over a decade ago (38) and received wide and often uncritical acceptance. Dastoli and Price reported the isolation (38) and characterization (39), from bovine tongues, of a "sweet-sensitive protein." Interactions with sweet compounds were measured by using changes in refractive index upon mixing a sweet compound with the protein fraction; they also reported that difference spectral changes in the ultraviolet region could equally well be used. Electrophysiological and behavioral studies by Sato and coworkers (40, 41) have included difference spectra studies in attempting to correlate their data in rats and monkeys with a biochemical parameter. Unfortunately, the resulting values of ΔA are very small. For example, maximal values of ΔA (with sucrose) were 0.01–0.05 when measured at A_{280} values of 0.4–1.0, respectively. Usually the differences measured were considerably smaller.

Recently, two groups independently examined in detail the Dastoli and Price (38) protein preparation and assay method. Nofre and Sabadie (42) found that the bovine tongue protein underwent changes in refractive index upon addition of sugars, but nonsweet compounds could equally well elicit the changes. Furthermore, bovine gamma globulin, used as a control protein, showed the same changes. Nofre and Sabadie (42) concluded that the "sweet-sensitive protein" isolated earlier (38) is *not* a receptor protein. Ostretsova *et al.* (43) repeated the experiments of Dastoli and Price (38) and also extended the studies by measuring binding with a radioactively labeled ligand, [^{14}C]-glucose, an approach that we and others have found (9, 10, 20, 23) to be a more reliable technique for studying binding to taste tissue. Ostretsova *et al.* (43), using difference spectra, were unable to substantiate the conclusions of Dastoli and Price (38) and of Hiji *et al.* (40). In particular, they showed that use of extracts of circumvallate or of fungiform papillae, which contain taste buds, resulted in spectra changes no different from those elicited by extracts of tongue epithelium devoid of taste buds. Yet it has been shown (9, 10) that sugars preferred by cows in behavioral taste tests (24) do bind to a greater extent to tissue homogenates and membranes containing taste receptors than to those derived from lingual tissue that is devoid of taste buds when ^{14}C-labeled sugars are used as ligands. Using equilibrium dialysis with [^{14}C]glucose as a ligand, Ostretsova *et al.* (43) presented evidence for binding activity, but the activity was in a sedimentable fraction rather than in the soluble phase from whence the Dastoli and Price protein derives. The observation of binding activity in a sedimentable fraction agrees with results from experiments with bovine (10) and catfish (20, 23) taste receptors. In addition to the failure of the other groups, in direct experimental tests, to substantiate the earlier claims, Koyama and Kurihara (44) showed that the "sweet-sensitive protein" is present in large amounts throughout the bovine tongue epithelium whether or not taste buds are present. Later reports show that unique proteins do exist in preparations derived from taste papillae and taste bud cells (45, 46), but their function has yet to be elucidated.

The accumulated evidence during the past decade therefore fails to support the original claim (38, 39) that a "sweet-sensitive protein," which is the receptor molecule for sweet compounds, was in fact isolated. The weak binding interactions of sugars with receptors underlies the earlier lack of a reliable assay method, which has hindered more definitive approaches. The present results directly demonstrate binding of radioactively labeled monellin. The binding assay could provide an *in vitro* assessment of the effectiveness of sweet-tasting stimuli.

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