RESEARCH COMMUNICATION

Some taste substances are direct activators of G-proteins

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Amphiphilic substances may stimulate cellular events through direct activation of G-proteins. The present experiments indicate that several amphiphilic sweeteners and the bitter tastant, quinine, activate transducin and G_i/G_o-proteins. Concentrations of taste substances required to activate G-proteins *in vitro* correlated with those used to elicit taste. These data support the hypothesis that amphiphilic taste substances may elicit taste through direct activation of G-proteins.

INTRODUCTION

G-proteins transfer and amplify signals between specific receptors and effectors through the exchange of GDP for GTP [1,2]. G-protein activation is terminated by the hydrolysis of GTP to GDP and P_i through the intrinsic GTPase activity of the α-subunits [3,4]. Recent studies have shown that a variety of cationic–amphiphilic neuropeptides and venom peptides (e.g. substance P, bradykinin and mastoparan) and non-peptide substances (e.g. compound 48/80 and alkylamines) activate G-proteins directly [5–7]. The amphiphilic properties of such compounds allow them to penetrate deeply into the plasma membrane, as predicted by Schwyzer's theory of insertion of amphiphilic peptides into membranes [8]. As the interaction of receptor molecules with G-proteins appears to be mediated by the third cytoplasmic loop of receptor proteins [5–7], it is assumed that amphiphilic peptides mimic receptor activities by interaction with G-proteins in a similar manner as receptors do. Hence, by their direct activation of G-proteins, amphiphilic substances may mimic certain cellular effects of receptor agonist. The physiological significance of such a signal-transduction pathway is not known at the present time.

Taste sensation is initiated by an interaction of taste stimuli with the exposed apical surface of the taste receptor cells, leading to membrane depolarization and synaptic transmission to second-order neurons [9]. In contrast with ionic stimuli (salt and sour), the transduction of sweet and bitter tastes has been proposed to involve putative specific membrane receptors [10]. Recent studies using PCR in rat lingual epithelia or bovine taste tissue [11,12] have identified novel G-protein-coupled receptors. However, it is not yet known whether these receptors are involved in taste transduction and, to date, no taste receptor has been isolated. Furthermore, multiple transduction mechanisms may be operative for both sweet and bitter tastes [13–16]. Hydrophobic interactions of bitter stimuli with lipid bilayers of gustatory tissue has led to the hypothesis that specific receptors are not needed for bitter sensation [17]. Indeed, bitter stimuli can depolarize N-18 mouse neuroblastoma cells, unrelated to taste [18]. Recently [16] it has been hypothesized that direct activation of G-proteins by amphiphilic and potentially bitter neuropeptides (e.g. bradykinin is bitter) is one of the diverse signal-transduction pathways for bitter sensation.

The involvement of G-proteins and intracellular signal molecules in mediation of sweet and bitter taste transduction has been shown [13,14,19,20]. Moreover, gustducin, a novel G-protein closely related to transducin (the major G-protein of the retina), has been identified and cloned from rat gustatory tissue [21]. Interestingly, transducin is also present in gustatory tissue [22]. These findings call for common routes of G-proteins in the senses of vision and taste.

Intriguingly, it has been shown that intravenous and intralingual administration of some non-sugar sweeteners may elicit taste [23,24] and taste nerve responses [25,26] independently of any interaction with putative receptors at the apical surface of the tongue. Since non-sugar sweeteners and bitter substances are amphiphilic, and as their hydrophobic characteristics are important for their taste potency [27–29], we undertook this study to test the hypothesis that such compounds are direct G-protein activators. With the lack of availability of gustducin in amounts needed for this study, we studied the effects of some known sweet and bitter taste substances on the GTPase activity of transducin and on a purified fraction containing G_i/G_o-proteins which belong to the same G-protein superfamily [1,2]. Mastoparan was used as a positive control for G-protein activation.

MATERIALS AND METHODS

Materials

Azolectin, mastoparan and the taste substances, sodium saccharin, sodium cyclamate, aspartame, neohesperidin dihydrochalcone (NHD), naringin, quinine chloride (hydrochloride) and sucrose, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Monellin was purchased from BioResources International (Somerset, NJ, U.S.A.). Single-chain monellin was kindly provided by Kirin Brewery Co. (Kanagawa, Japan). The guanidine SC-45647 sweetener was kindly given by The Nutra-Sweet Company (Mt. Prospect, IL, U.S.A.). Guanosine 5'-[γ-[^{35}S]thio]triphosphate ([^{35}S]GTP[S]) (1195/mmol) was purchased from du Pont New England Nuclear (Bad Homburg, Germany). Materials used for the GTPase assay were as described elsewhere [30].

Purification of G-proteins

G_i/G_o-proteins were purified from bovine brain membranes by a three-step column-chromatography procedure [31]. In brief,

Abbreviations used: NHD, neohesperidin dihydrochalcone; GTP[S], guanosine 5'-[γ-thio]triphosphate; EC_{50}, concentration giving half-maximal stimulation; DMSO, dimethyl sulphoxide.

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cholate extracts were subjected to chromatography on a DEAE-Sepharose Fast Flow column (Pharmacia, Freiburg, Germany), AcA 34 gel filtration (Serva, Heidelberg, Germany) and on a heptylamine–Sepharose column. Fractions were analysed for GTP[S] binding and immunoreactivity employing specific antibodies [32]. Heterotrimeric G-proteins were identified by SDS/PAGE and silver staining. Purity was greater than 90%. The purified mixture contained mostly G_o1, some G_o2, G_i1, G_i2, and traces of G_i3.

Heterotrimeric transducin was prepared essentially as described previously [33]. Heterotrimeric transducin was identified by SDS/PAGE followed by Coomassie Blue and immunostaining, and quantified by GTP[S] binding. Purity was greater than 90%. Both the transducin and the G_i/G_o-protein preparations were free of ATPase or low-affinity GTPase activities.

Reconstitution of G_i/G_o-proteins into phospholipid vesicles

Azolectin/cholate mixtures, 1 and 10% (w/v) respectively, in the buffer A, and G_i/G_o-proteins (28 pmol) were loaded on to a 10 ml gel-filtration AcA 34 (25 cm × 8.5 mm) column prepared with degassed buffer A containing 100 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA and 20 mM Hepes/NaOH, pH 8.0, 4 °C, according to [34] with slight modifications. Liposomes containing G_i/G_o-proteins were eluted (200 μl fractions) from the column with the above buffer and were quantified by GTP[S] binding. Pooled fractions were then used for the GTPase assays.

GTPase assay

GTP hydrolysis was determined essentially as described by Wenzel-Seifert and Seifert [30]. In agreement with a model suggested by Wieland et al. [35], GDP was included in reaction mixtures, unless otherwise specified, to enhance the relative stimulatory effects of taste substances on GTPase activity. For solubilized G_i/G_o-proteins, reaction mixtures (50 μl) contained 44 nM of G-proteins, 1 μM [γ-^{32}P]GTP (0.1 μCi/tube), 1 mM adenosine 5′-[β,γ-imido]triphosphate, 3 μM GDP, 0.1 mM EGTA, 0.5 μM $MgCl_2$, 1 mM dithiothreitol and 0.2% (w/v) BSA in 65 mM triethanolamine/HCl, pH 7.0. Reaction mixtures contained taste substances and mastoparan at various concentrations. NHD and naringin (200 mM) were solubilized first in 20% (v/v) dimethyl sulphoxide (DMSO) with a final concentration of 4% (v/v) DMSO (this concentration of DMSO did not affect basal GTPase activity). Tubes were incubated for 15 min at 25 °C. In the experiments with transducin, the release of P_i under the above conditions was very low. Thus the reaction mixture was modified to contain 270 nM of transducin, 25 nM [γ-^{32}P]GTP (0.15 μCi/tube) and 1 μM GDP. Tubes were incubated for 5 min at 25 °C. For G_i/G_o-protein-reconstitution experiments, the reaction mixture (100 μl) was modified and contained 5 nM G_i/G_o-proteins, 50 nM [γ-^{32}P]GTP (0.1 μCi/tube), 0.1 mM ATP, 5 mM creatine phosphate and 40 μg of creatine kinase without GDP.

Data analyses

Results of each experiment were analysed separately by analysis of variance, and Student's t test was used for the determination of statistical differences (at least at $P < 0.05$) between basal and stimulated G-protein activities. Experiments with each taste substance were performed at least twice, and three or four replicates were used for each treatment. Correlation was calculated between the minimal tastant concentration that stimulated significantly ($P < 0.05$) the GTPase activity of G_i/G_o-proteins (y-axis) versus the sweetener concentration (x-axis) needed to produce a sweet intensity level equal to that produced by 0.29 M sucrose (commonly used as a reference) [36–38]. The level of quinine bitter intensity was based on its mid-range bitter intensity [39].

RESULTS AND DISCUSSION

The results of the present study indicate that some sweet and bitter amphiphilic taste substances are effective G-protein activators *in vitro* (Figure 1). Taste substances activated the GTPase of either solubilized transducin or a solubilized mixture of purified G_i/G_o-proteins in a concentration-dependent manner. The bitter taste substance, quinine chloride, and the non-sugar sweeteners, NHD and sodium saccharin, were the most effective stimuli, showing stimulations of GTPase activity up to 2–3.5-fold. By comparison, mastoparan (400 μM) stimulated transducin and G_i/G_o-proteins 1.6- and 2.7-fold respectively (results not shown). These results suggest that some taste substances may be similarly effective and even more effective activators of G-proteins than mastoparan.

Lower concentrations of taste stimuli were usually required to stimulate GTPase of transducin than for the activation of G_i/G_o-proteins. Activation of transducin by taste substances showed saturation (e.g., sodium saccharin) or biphasic concentration–response curves (e.g. quinine chloride) (see Figure 1). In most experiments, analysis of variance resulted in high significance of the stimulatory effects of the taste substances ($P < 0.01$). In the experiments with transducin, EC_{50} (concn. giving half-maximal stimulation) values of 5 mM for sodium saccharin, 1.3 mM for NHD, 4 mM for sodium cyclamate and 4 mM for quinine chloride were observed. The sweet protein, monellin (used up to 250 μM), did not stimulate transducin, and the stimulation by the dipeptide, aspartame, was significant ($P < 0.05$) only when a concentration of 8 mM was used. With respect to the G_i/G_o-proteins, a saturated concentration–response curve was observed only for aspartame (EC_{50} = 7 mM). All of the six taste stimuli

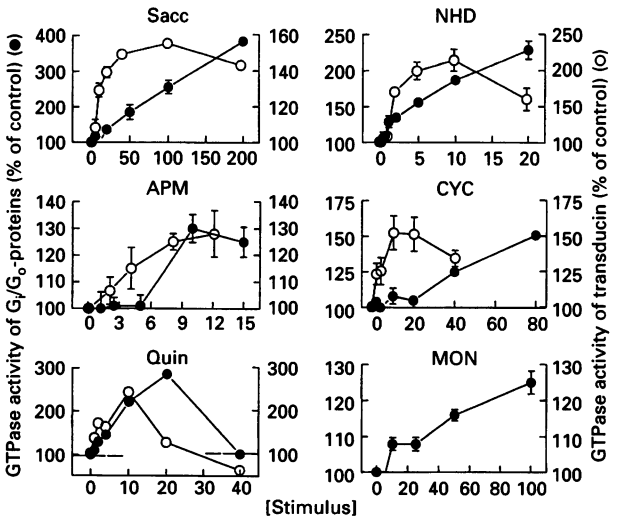

Figure 1 Stimulation of GTPase of G_i/G_o-proteins (●) and transducin (○) by taste substances

Results for the sweeteners, sodium saccharin (Sacc), NHD, aspartame (APM), sodium cyclamate (CYC), monellin (MON) and the bitter tastant, quinine chloride (Quin), are presented. Values are the means ± S.E.M. for three or four replicates of a representative experiment. When S.E.M. bars are missing, they were too small to be shown. Basal turnover numbers of the G_i/G_o-proteins and transducin were 0.07–0.08 min^{-1} and 0.00009–0.00011 min^{-1} respectively. Concentrations of stimulus are in mM, except for MON, where it is μM.

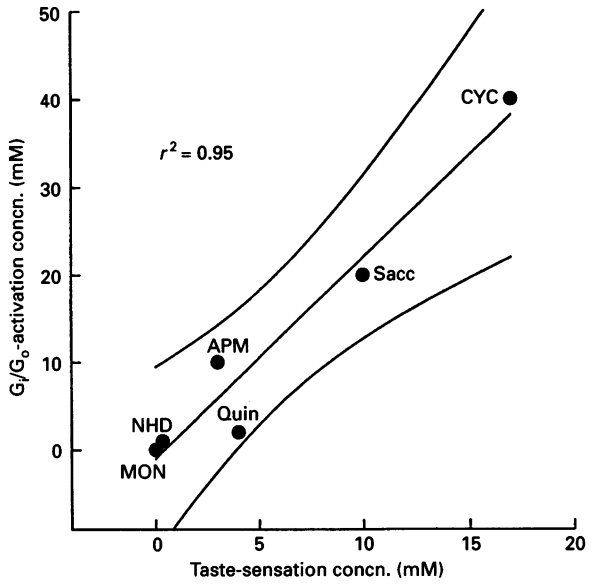

Figure 2 Correlation between concentrations of taste substances which stimulated GTPase of G_i/G_o-proteins and those used to elicit taste in humans

Correlation was calculated between the minimal tastant concentration that stimulated significantly ($P < 0.05$) the GTPase activity (y-axis) versus the sweetener concentration (x-axis) needed to produce a sweet intensity level equal to that produced by 0.29 M sucrose (commonly used as a reference) [36–38]. The level of quinine bitter intensity was based on its mid-range bitter intensity [39]. Abbreviations are as for Figure 1.

shown in Figure 1 significantly stimulated (at least at $P < 0.05$) the GTPase activity of G_i/G_o-proteins. As for native monellin, synthetic single-chain monellin (25 μM) also stimulated ($P < 0.05$) the GTPase of G_i/G_o-proteins by 25%.

We tested some other sweet proteins as possible activators of G-proteins. However, with the exception of purified monellin and single-chain monellin, other proteins were heavily contaminated by nucleotidase activity (results not shown) and, therefore, could not be studied. Stimulation by sodium saccharin or sodium cyclamate of GTPase of either transducin or G_i/G_o-proteins was observed independently of sodium, and the biphasic effects of quinine chloride on GTPase were not due to a change in pH or due to the presence of chloride (results not shown).

Initial experiments employing purified G_i/G_o-proteins reconstituted into phospholipid vesicles indicated that sodium saccharin (20 mM), sodium cyclamate (50 mM), NHD (10 mM), aspartame (9 mM), and quinine chloride (10 mM), i.e., at relative mid-range concentrations, stimulated (at least at $P < 0.05$) GTPase by 23, 32, 18, 13 and 82% respectively. Mastoparan (40 μM) stimulated GTPase by 83% under the same conditions.

Could the above receptor-independent activation of G-proteins be related to taste sensation? On the basis of the notion that some neuropeptides which are direct activators of G-proteins and which are potentially bitter stimuli (e.g. bradykinin tastes bitter), Spielman et al. [16] have recently hypothesized that direct G-protein activation is one pathway by which bitter taste is transduced. The present experiments, though not providing direct evidence, are the first to provide data which support the hypothesis of such a transduction pathway and extend the hypothesis to some non-sugar sweeteners. This pathway is likely to co-exist with taste sensations initiated by putative taste receptors located at the apical plasma membrane. Interestingly, the concentrations of taste substances which increased the GTPase activity of G_i/G_o proteins and transducin correlated closely (Figure 2) with those needed to elicit taste sensation in humans [e.g. 26,36–39]. The slopes of the concentration–response curves for saccharin, NHD, quinine and cyclamate activating transducin were steepest at the low millimolar range (Figure 1). Unspecific effects (e.g. changes in the microenvironment of G-proteins due to the substances tested) may be ruled out, since naringin, which is the precursor of NHD (reduction of one oxygen in the γ-ring of naringin gives NHD; see [37]), did not stimulate, but rather slightly inhibited, the GTPase of transducin and G_i/G_o-proteins when applied at the same concentrations (results not shown). In addition, the SC-45647 guanidine sweetener [40] did not increase the GTPase activity of transducin and the G_i/G_o proteins. Sucrose (0.5 M) inhibited (40%) GTPase of G_i/G_o-proteins. The mechanism for such a response is unclear.

There was a clear higher sensitivity of transducin, which is present in gustatory tissue [22], towards stimulation by taste substances, compared with the G_i/G_o-proteins (Figure 1). The results obtained from the reconstitution experiments suggest that some taste substances, when applied at concentrations needed to elicit taste, activate G-proteins associated with lipid bilayers.

With respect to possible physiological relevance of the present experiments, it should be noted that, in order for a taste stimulus to interact with the α-subunit of G-proteins directly under physiological conditions, it must penetrate the plasma membrane [5,6]. The concentrations of some taste stimuli required to elicit taste sensation are, for nutritional reasons, quite high [e.g., 14,38]. However, taste sensation of bitter stimuli (most are toxic constituents which produce aversive behavioural signals) and non-sugar sweeteners (most are synthetic) is in the low-millimolar range [26,36–39]. These substances are amphiphilic and, as such, they bear hydrophobic and hydrophilic domains. It is known that the hydrophobicity of amphiphilic drugs (e.g., chlorpromazine, imipramine, propranolol) allows these molecules to permeate through, and to accumulate, in the plasma membrane [41–43]. Some amphiphilic drugs get access to the surface of the intracellular membranes to which they become absorbed. Intracellularly, these substances are not evenly distributed, but may display affinities to cellular components [42]. Such effects occur at the millimolar concentration range, e.g. at concentrations similar to the ones needed to elicit taste by the above taste substances.

Non-sugar sweeteners are, as bitter stimuli, chemically diverse; e.g., these are aromatic compounds, sulphamates, dipeptides, guanidines and even proteins. These substances are amphiphilic, and according to the AH–B–γ sweet triangle theory of sweetness [27–29], it is the presence of a strong γ-hydrophobic binding site in these sweeteners that is responsible for their high sweet potency and that differentiates them from sugars, which contain mainly the hydrophilic (AH–B) sites. By analogy to many amphiphilic drugs [41–44], low-molecular-mass non-sugar sweeteners would be expected to possess the capacity to permeate through membranes. Even the well-known sweet taste protein, monellin, possesses hydrophobic and hydrophilic sites which may be important for its taste [45]. Interestingly, amphiphilic peptides may also cross membranes through a process referred to as 'electrophoretic transfer' [46]. Of note is the recent suggestion that receptors may interact with G-proteins through hydrophobic domains which appear to be more important for the activation of G-proteins than basic moieties [47]. Thus, although comprising diverse chemical structures, non-sugar sweeteners (as well as some bitter tastants) share amphiphilic characteristics. The rapid entry of saccharin into the bloodstream from the stomach and the gut, the rapid excretion (with active transport in the kidney) in the urine [48,49], and the fast absorption of quinine and its rapid distribution in tissues, including the fetus [50], may at least in part, be due to their amphiphilic characteristics.

The hypothesis that non-sugar sweeteners activate G-proteins directly under physiological conditions is consistent with the temporal characteristics of these compounds. Compared with sugars, almost all non-sugar sweeteners possess inferior sweet quality such as slow taste onset and lingering aftertaste (sweet persistence) [51,52]. The delays in onset and extinction of sensation may result from a process of stimulus penetration through the plasma membrane. Furthermore, some of the above taste compounds elicit taste [23,24] and taste nerve responses [25,26] following intravenous or intralingual administration, independently of stimulation of putative receptors at the apical surface of the tongue. Fishberg et al. [23] used intravenous injection of sodium saccharin to measure the circulation time in humans, i.e., subjects describe a sweet taste passing rapidly from the base to the tip of the tongue. These observations were usually interpreted as stimulation of putative taste receptors that may occur at the basolateral membrane of taste cells, in addition to those at the apical surface. Moreover, we found that, in addition to the stimulation of gustatory membranes, sodium saccharin increased significantly adenylate cyclase activity in membranes derived from tongue muscle, femur muscle and from liver [53]. These effects were concentration- and GTP-dependent and were also interpreted on the basis that receptors for saccharin may occur in other tissues. However, one may equally propose that the lack of tissue specificity for saccharin is due to saccharin penetrating the plasma membrane and interacting directly with G-proteins.

In summary, the present study shows that some taste substances are direct G-protein activators. Such a transduction pathway may especially be relevant to non-sugar sweeteners and bitter substances which are amphiphilic. Future studies will have to address the questions of whether the stimulatory effects by taste substances reported herein can also be seen with expressed gustducin and in native taste-cell membranes and which effector systems are activated.

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