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An *In Vitro* Assay Useful to Determine the Potency of Several Bitter Compounds

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Abstract

Gustducin and transducin are guanine nucleotide binding regulatory proteins (G proteins) expressed in taste receptor cells and implicated in transducing taste cell responses to certain compounds that humans consider bitter or sweet. These G proteins can be activated *in vitro* by taste receptor-containing membranes plus any of several bitter compounds. This activation can be monitored using limited trypsin digestion, sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting. Scanning of the autoradiograms enables one to quantitate the level of activation (defined as an activation index), obtain dose–response profiles and estimate the potency of the tastant. This assay may provide a useful substitute for, or adjunct to, the time-consuming human psychophysical analysis and costly animal studies typically used in taste sensory analysis. It may be used to identify and determine the concentration–response function of many bitter components of oral pharmaceuticals and food ingredients. A potential limitation of the assay is that only about half of all bitter compounds tested demonstrated *in vitro* activity, perhaps due to the presence of multiple transduction pathways. Nevertheless, the rapid throughput and microsample handling capability of this assay make it an ideal method to screen for high-potency bitterness inhibitors.

Introduction

Sensory analysis is an important tool in the development and evaluation of foods and beverages. Taste sensory analysis typically consists of the evaluation of three characteristics: taste quality (an important contributor to flavor), intensity (potency) and the perceived intensity measured over time (temporal profile). Psychophysical evaluation by a human taste panel is the standard method of measuring these characteristics. Although panelists are trained to make identifications and ratings of taste standards and test samples, their evaluation of taste quality, flavor, intensity and the temporal profile is complicated by several variables, including mixture suppression, salivary mixing, time resolution and panelist interpretation.

Animal behavioral tests such as two-bottle preference and conditioned taste aversion are useful to generate information relevant to the taste quality and concentration–response function of various tastants (Akaike *et al.*, 1965). Electrophysiological recording from the chorda tympani and glossopharyngeal nerves of animals provides a means to directly measure the temporal profiles of taste stimuli (Hanamori *et al.*, 1988; Hellekant *et al.*, 1991); recording from single taste nerve fibers is useful for gathering information relevant to the taste quality of the samples (Hellekant *et al.*, 1997). Disadvantages of human sensory analysis and animal testing include their time-consuming nature, requirement of major capital investments, extensive training of personnel, and difficulties in data analysis and interpretation. Moreover, psychophysical, electrophysiological or behavioral experiments are not amenable to screening very large numbers of samples rapidly or economically. For these reasons we sought to develop an *in vitro* assay that could substitute for, or be used as an adjunct to, human psychophysical analysis and animal studies in the assessment of taste quality.

Gustducin is a taste-specific G protein (McLaughlin *et al.*, 1992) that is biochemically indistinguishable from transducin, the G protein that mediates retinal phototransduction. Gustducin and rod transducin are closely related

and selectively expressed in taste receptor cells (Ruiz-Avila *et al.*, 1995). The targeted disruption of the α-gustducin gene caused a marked reduction in responsiveness of α-gustducin null mice to compounds considered by humans to be bitter (quinine and denatonium) or sweet (sucrose and SC 45647) (Wong *et al.*, 1996a). The responsiveness of α-gustducin null mice to these bitter and sweet compounds was restored by transgenic expression in taste receptor cells of the wild type α-gustducin cDNA (Wong *et al.*, 1996b). Gustducin and/or transducin are present in the taste receptor cells of several vertebrate species, including fish, amphibians, rodents, cattle and humans (D. Ming and R.F. Margolskee, unpublished data), suggesting that these G proteins play a fundamental role in vertebrate taste signal transduction.

We have demonstrated that gustducin and transducin can be activated in the presence of bovine taste receptor cell membranes by several bitter tastants—about half of all bitter compounds tested (Ming *et al.*, 1998). By measuring the activation of gustducin and/or transducin in the presence of taste membranes, it is possible to identify certain bitter tastants, determine their molecular mode of action, quantitatively determine their potency profiles and screen chemical libraries for potential agents that block their bitterness. Subsequent evaluation of candidate bitterness antagonists ('bitter blockers') by a trained human panel would be used to validate this method of flavor modifier compound discovery.

Materials and methods

Materials

Domestic cow (*Bos primigenius*) tongues were collected fresh from a local slaughterhouse and transported on ice to the laboratory. Bovine circumvallate and fungiform papillae were hand dissected, frozen in liquid nitrogen and stored at –80°C until use. All bitter tastants and buffer chemicals were of the highest purity available and purchased from either Sigma (St Louis, MO) or Boehringer Mannheim (Indianapolis, IN), unless otherwise noted. Rhodopsin was purified in the light as 6 M urea washed bovine rod outer segments using published procedures (Mazzoni *et al.*, 1991). Bovine transducin heterotrimer and βγ subunits were purified by standard procedures (Fung *et al.*, 1981). Rat α-gustducin was prepared by using an *in vitro* transcription/translation system from Promega (Madison, WI), following the manufacturer's suggested procedures in the presence of limiting [^{35}S]methionine from Amersham (Arlington Heights, IL). The rabbit polyclonal anti-transducin antibody was a kind gift from Drs Mel Simon and John Watson.

Taste cell membrane preparation

The collected taste tissues were homogenized with a Polytron™ homogenizer (three cycles of 20 s each at 25 000 r.p.m.) in a buffer containing 10 mM Tris, pH 7.5, 10% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10 μg/μl pepstatin A, 10 μg/μl leupeptin, 10 μg/μl aprotinin and 100 μM 4-(2-aminoethyl)-benzene-sulfonyl fluoride hydrochloride. After particulate removal by centrifugation at 1500 *g* for 10 min, the enriched taste cell membranes were collected by a two-step centrifugation at 7000 *g* for 10 min for the supernatant, followed by 45 000 *g* for 60 min for the pellet. The pelleted membranes were rinsed twice, resuspended in homogenization buffer lacking protease inhibitors and further homogenized by 20 passages through a 25-gauge needle. Aliquots were either flash frozen or stored on ice until use. Note that bovine circumvallate and fungiform papillae were processed separately. The protein concentration of the membrane preparations was measured by the Peterson modification of the micro-Lowry method (Peterson, 1977). The flow chart for this procedure is diagrammed in Figure 1.

Gustducin and transducin activation

Activation of *in vitro* translated recombinant α-gustducin and purified native transducin was based on the procedure of Neer *et al.* (Neer *et al.*, 1994). *In vitro* translated α-gustducin (10 μl of the transcription/translation mix) was incubated for 15 min at room temperature with βγ subunits from bovine retina [10 μl at 1 mg/ml in 10 mM Tris (pH 8.0), 10 mM CHAPS]. The gustducin mixtures were diluted 1:10 in incubation buffer [25 mM Tris (pH 7.5), 2 mM $MgCl_2$, 5 mM DTT, 100 mM NaCl, 100 μM GDP, 0.5 μM GTPγS] containing 0.25–1 mg/ml membrane protein equivalent of the membrane preparation. To assay activation of transducin, the reaction mix was modified to contain 5 mM $MgCl_2$, 150 mM NaCl and 3 mM DTT. Transducin was added to this mix to a final concentration of 0.4 μM. Aliquots of 20 μl were withdrawn from the premix solution, tastant or buffer was added from 20× stocks, and the final reaction mixtures were incubated for 1–3 h on ice (gustducin) or for 1 h at 30°C (transducin).

Trypsin assay and quantitation

l-1-Tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin (1:25 w/w trypsin to total protein) was added to gustducin/transducin which had been activated by incubation with taste membranes plus tastants. Trypsin digestions were performed at room temperature (15 min) and stopped by the addition of soybean trypsin inhibitor (6:1 mol/mol inhibitor to trypsin). The reaction mixtures were diluted 1:3 in 2× Laemmli buffer (Laemmli, 1970), then separated by SDS–PAGE using a 4–20% gel and a Tris–glycine buffer. For the transducin assays, the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes by an electro-blotter. The PVDF membrane with transferred proteins was blocked by the addition of 5% BLOTTO skim milk powder for 30 min, reacted with anti-transducin antibodies and HRP-labeled goat anti-rabbit secondary antibody, developed with the AmpLight enhanced chemi-

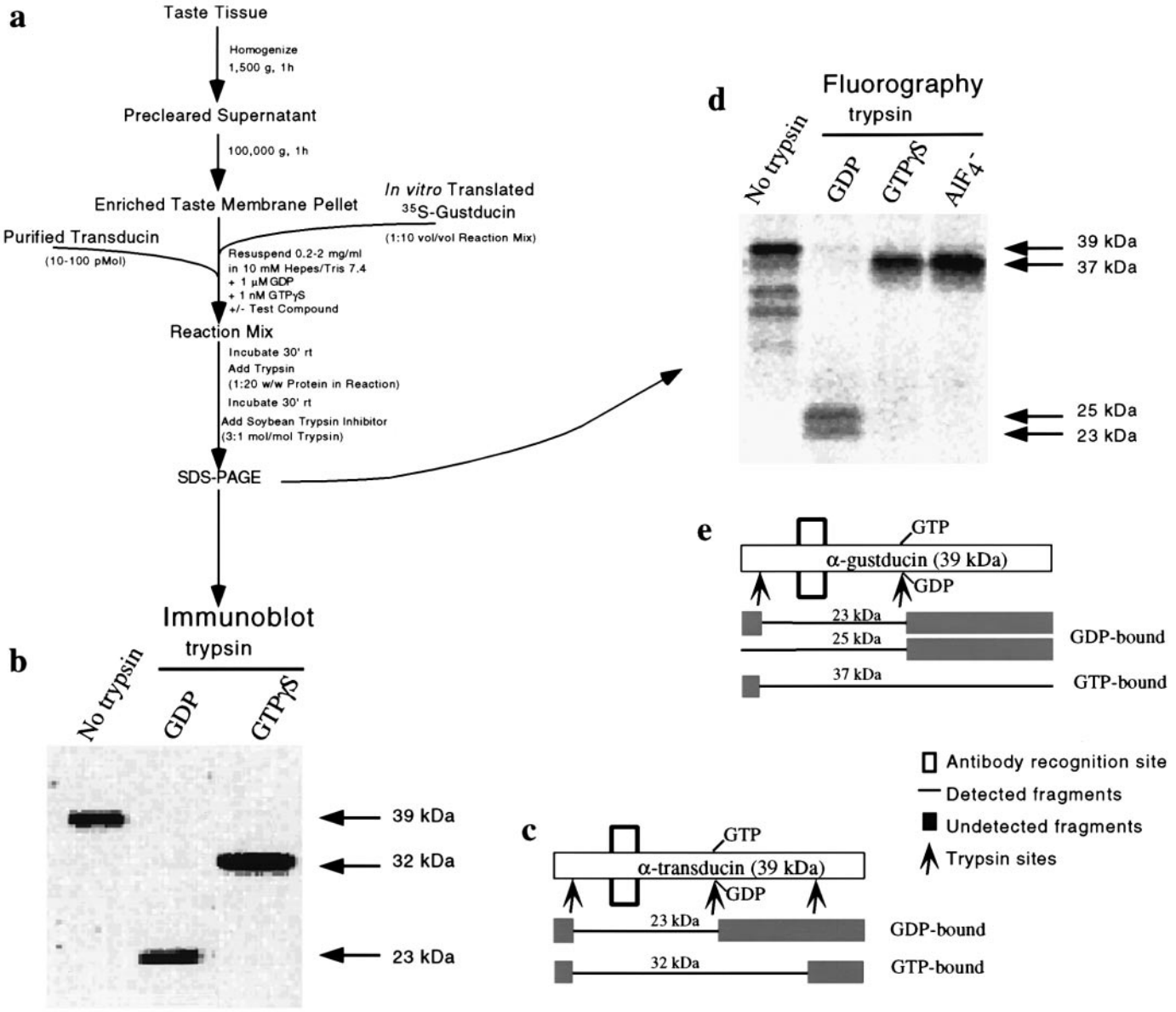


Figure 1 Activation of α-transducin and α-gustducin can be monitored by limited proteolysis with trypsin. **(a)** A flow chart of the *in vitro* G protein activation assay for analysis of bitter tastants. **(b)** The autoradiogram of the gel shows that undigested α-transducin runs at 39 kDa; trypsin cleavage of α-transducin yields a 32 kDa fragment from the activated/GTPγS-bound form of α-transducin and a 23 kDa fragment from the inactive/GDP-bound form of α-transducin. **(c)** Diagram of α-transducin showing locations of trypsin sites (up arrows), the antibody recognition site (open box), fragments detected by antibody (single lines) and fragments not detected by antibody (shaded boxes). **(d)** The autoradiogram of the gel shows that undigested *in vitro* translated α-gustducin runs at 39 kDa (with additional shorter translation products from internal initiation); trypsin cleavage of α-gustducin yields a 37 kDa fragment from the activated/GTPγS-bound form and 23 plus 25 kDa fragments from the inactive/GDP-bound form. **(e)** Diagram of α-gustducin showing locations of trypsin sites (up arrows), the antibody recognition site (open box), fragments detected by antibody (single lines) and fragments not detected by antibody (shaded boxes).

luminescent detection system from BioRad (Hercules, CA) and exposed to an X-ray film, which was quantitated by densitometry. For the gustducin assays, the gel was either dried directly or the separated proteins were transferred to a PVDF membrane, then exposed to a phosphorimager screen for analysis. The captured image was either used directly or digitized for presentation.

Results

G protein activation monitored by limited proteolysis

The activated (GTP-bound) forms of α-gustducin and α-transducin differ from their inactivate (GDP-bound) forms in their sensitivity to trypsin digestion (Ruiz-Avila *et al.*, 1995; Ming *et al.*, 1998), due to conformational changes

in these G proteins that occur upon activation (GTP binding) (Lambright *et al.*, 1994). Trypsin-treated GTP-bound α-transducin generates a 32 kDa fragment (detected by the anti-transducin antibody), while GTP-bound α-gustducin yields a 37 kDa fragment (detected by the anti-gustducin antibody, or by intrinsic radiolabel). Figure 1 diagrams the basis of the trypsin sensitivity assay for monitoring transducin and gustducin activation. In contrast, trypsin treatment of GDP-bound (inactive) α-gustducin or α-transducin generates 23 kDa (α-transducin) and 23 plus 25 kDa fragments (α-gustducin) (Figure 1). Undigested α-transducin and α-gustducin are of ~39 kDa mol. wt. Using this trypsin sensitivity assay the activation of transducin and gustducin by taste receptor-containing membranes plus tastants can be readily identified; non-taste membranes do not contain the bitter tastant-responsive receptors that couple to gustducin/transducin (Ruiz-Avila *et al.*, 1995; Ming *et al.*, 1998).

Only bitter compounds activate gustducin and/or transducin

We had initially shown that the bitter compound denatonium benzoate can activate α-transducin in the presence of bovine taste membranes (Ruiz-Avila *et al.*, 1995). Subsequently, we showed that many bitter stimuli (about half of all bitter compounds tested) can activate both transducin and gustducin, and that this activation depends upon taste membranes (Ming *et al.*, 1998). Denatonium benzoate and quinine hydrochloride in the presence of either bovine circumvallate or fungiform taste membranes gave robust activation of transducin (Figure 2a). Strychnine gave a more modest response (Figure 2b). Glycine gave no activation of transducin, nor did it competitively inhibit strychnine activation of transducin (Figure 2c), suggesting that the strychnine-activated taste receptor differs from strychnine-inhibited glycine receptors. Certain compounds bitter to humans, such as aristolochic acid (Figure 2d), sucrose octaacetate and caffeine (Table 1), did not activate transducin or gustducin at the tastant concentrations tested.

Other bitter tastants that potently activated transducin or gustducin in the presence of bovine taste membranes include sparteine, atropine, caffeic acid, naringin, yohimbine, (–)-epicatechin and nicotine (Table 1). The bitter compound phenylthiourea gave only a small response (Table 1). No activation of transducin or gustducin was observed with sucrose, glycine or SC45647 (compounds humans consider sweet), monosodium glutamate ('umami'), citric acid (sour), sodium chloride (salty) or potassium chloride (salty/bitter) taste stimuli (Table 1). These results demonstrate that this assay is responsive to certain compounds considered bitter by humans but unresponsive to other bitter compounds, or to the sweet, sour and umami compounds tested.

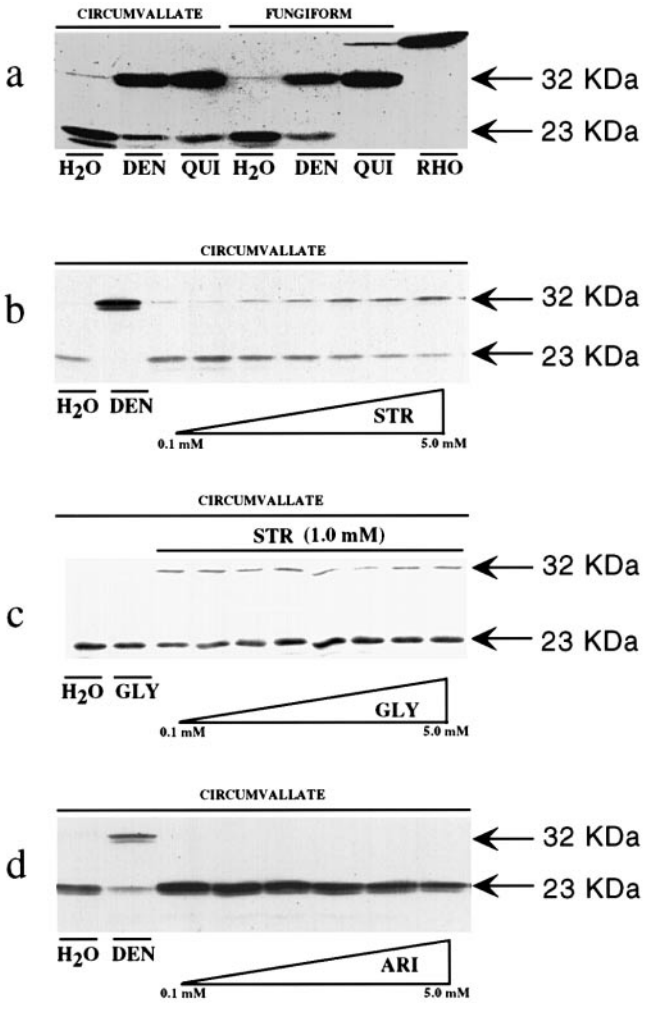
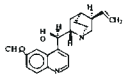
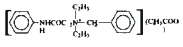
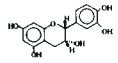
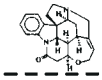
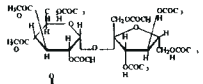
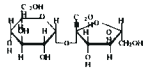
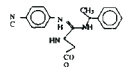
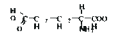


Figure 2 In the presence of bovine taste membranes, compounds that are bitter to humans activate α-transducin. **(a)** Autoradiogram of trypsin sensitivity assay showing that 2.0 mM denatonium (DEN) and 1.0 mM quinine–HCl (QUI) activate α-transducin in the presence of bovine circumvallate or fungiform membranes. Rhodopsin (RHO) served as a positive control. **(b)** Strychnine (STR) (0.1–5.0 mM) gave a low level of activation of α-transducin. **(c)** Activation of α-transducin by strychnine (1.0 mM) was not competitively inhibited by glycine (GLY) (0.1–5.0 mM). **(d)** Aristolochic acid (ARI) (0.1–5.0 mM) gave no activation of α-transducin.

Activation of transducin and/or gustducin depends on the concentration of the bitter agent

To quantitate G protein activation we have defined the activation index as the ratio of fragments derived from activated G proteins ('active fragments') to total fragments. For transducin this corresponds to the ratio of the 32 kDa ('active') band to the sum of 23 and 32 kDa bands. For gustducin the activation index is the ratio of the 37 kDa ('active') band to the sum of the 23, 25 and 37 kDa bands. Figure 3 shows that the activation of α-transducin and α-gustducin in the presence of taste receptor-containing membranes increased proportionally to the concentration of bitter tastants until a plateau was reached at which the G

Table 1 Sapid compounds tested *in vitro* using a G protein activation assay

Compound Name	MW (Daltons)	Taste to Humans	Chemical Structure	Transducin/Gustducin[a] Activation	Concentration[b] (mM)
Quinine	324.43	Bitter		+ +	0.1
Denatonium Benzoate	446.60	Bitter		+ +	1.0
Sparteine	234.37	Bitter		+ +	2.5
Atropine	289.40	Bitter		+ +	2.5
Caffeic Acid	180.16	Bitter		+ +	5.0
Naringin	580.53	Bitter		+ +	5.0
Yohimbine	354.43	Bitter		+ +	5.0
(-)-Epicatechin	296.08	Bitter		+ +	5.0
Nicotine	162.24	Bitter		+ +	15
Phenylthiourea	152.22	Bitter		+	5.0
Strychnine	334.42	Bitter		+	5.0
Sucrose Octaacetate	678.58	Bitter		-	1.0
Aristolochic Acid	341.28	Bitter		-	10
Caffeine	194.20	Bitter		-	10
Sucrose	342.30	Sweet		-	50
Glycine	75.05	Sweet		-	50
SC 45647	340.38	Sweet		-	1.0
Glutamic acid	147.13	Savory/Sour		-	50
Citric acid	192.12	Sour		-	50
Sodium Chloride	58.45	Salty	NaCl	-	50
Potasium Chloride	74.55	Salty/Bitter	KCl	-	50

[a] ++, compounds that elicited a high response; +, low response; –, no response.

[b] Minimum concentration to elicit a response from ++ and + compounds, or maximum concentration tested for – compounds.

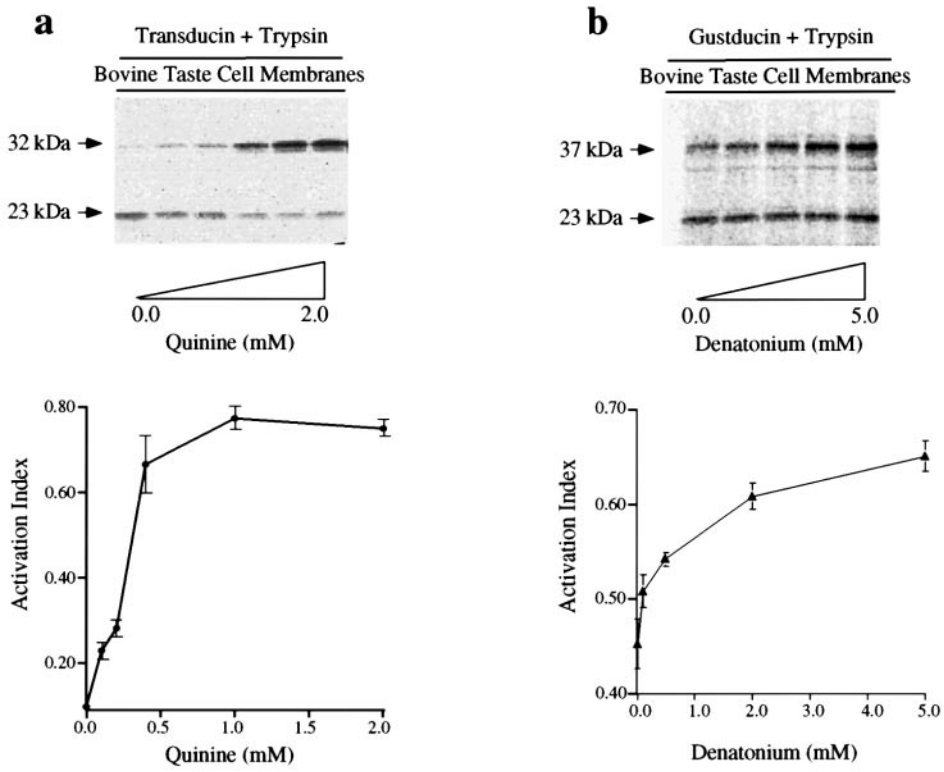


Figure 3 Quantitation of the activation of transducin and gustducin by bovine circumvallate taste membranes plus quinine or denatonium. **(a)** Upper panel: transducin is activated by taste receptor-containing membranes plus quinine (0.1–2.0 mM). Lower panel: quantitation indicates activation index of ~0.75 at 1.0–2.0 mM quinine. **(b)** Upper panel: gustducin is activated by taste receptor-containing membranes plus denatonium (0.1–5.0 mM). Lower panel: quantitation indicates activation index of ~0.65 at 5.0 mM denatonium.

proteins were maximally activated. Transducin was activated by quinine plus taste membranes to an activation index of 0.8; gustducin was activated by denatonium to an activation index of 0.65.

Taste membranes from cattle and mudpuppies are responsive to the bitter tastant quinine

To determine if responses to bitter tastants were generally conserved across species we tested taste membranes from mudpuppies and cattle. The mudpuppy taste membranes activated gustducin (data not shown) and transducin in response to quinine hydrochloride (Figure 4b). These results were comparable to those obtained with bovine taste membrane activated gustducin (Figure 3b) and activated transducin (Figures 2, 3a and 4a) in response to denatonium, quinine or strychnine. Taste membranes from fish, pigs and monkeys also showed similar responsiveness to quinine, denatonium and other compounds bitter to humans (data not shown).

Discussion

The development of a 'surrogate taste assay' that correlates with and/or substitutes for human psychophysical analysis has long been sought by chemical senses researchers and food product developers. Among various efforts, Toko developed an artificial taste sensor which utilized lipid membranes and measured the electric potential change of the membranes when taste substances were absorbed (Toko, 1996). The author stated that a specific output pattern could be defined for a given taste quality. However, this device could not measure the concentration–response function. Moreover, the relevance of this taste sensor is controversial, being based on the unsupported theory that taste signals are triggered by an electric potential change in the taste cell membrane caused by the physical absorption of a tastant molecule to the cell membrane.

Given the knowledge that gustducin is critically important to the transduction of responses to many compounds that humans consider bitter, we reasoned that we could use activation of gustducin as a measure for the initial events in bitter taste transduction, at least in those pathways that utilize gustducin. By using this *in vitro* biochemical assay, not only can we identify bitter tastants that activate the gustducin-mediated transduction pathways, but we can also measure the concentration–response function directly. In theory, this method could be used to screen for taste modifiers that block the bitter taste of compounds transduced via gustducin. In practice, this assay was used recently to identify small molecules that block behavioral and electrophysiological responses of mice to quinine,

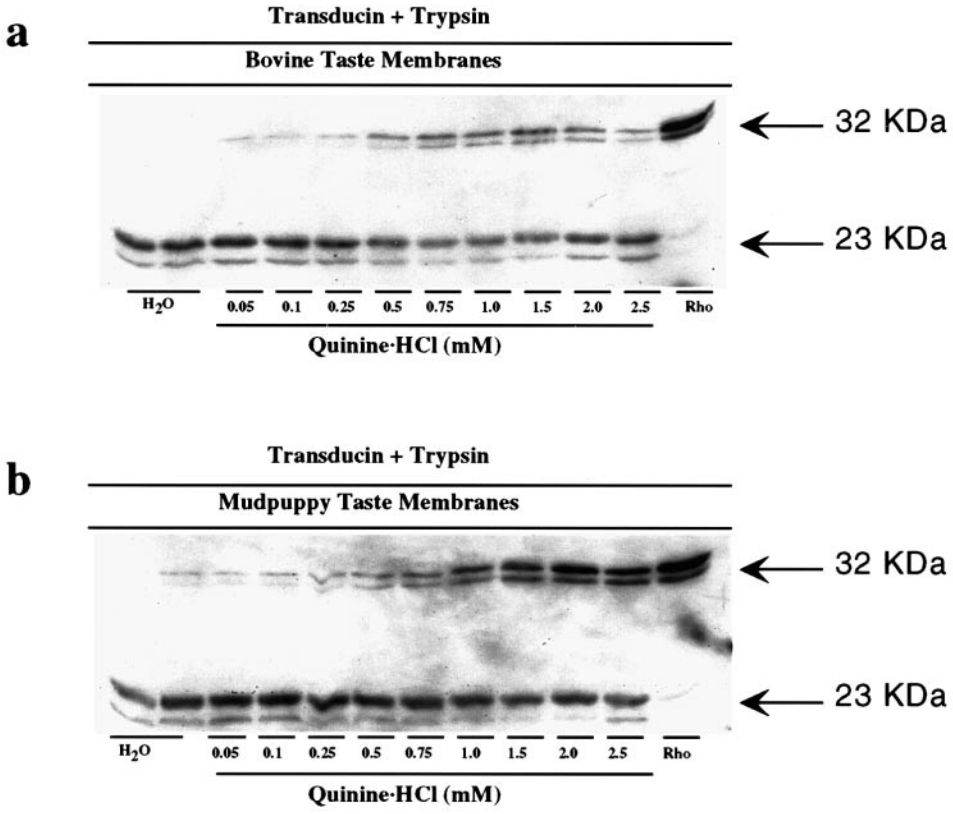


Figure 4 Taste membranes from mudpuppy and bovine activated transducin and gustducin in response to quinine. **(a)** Transducin is activated by bovine taste receptor-containing membranes plus quinine (0.05–2.5 mM). **(b)** Transducin is activated by mudpuppy taste receptor-containing membranes plus quinine (0.05–2.5 mM).

denatonium and other bitter compounds transduced by gustducin (Ming *et al.*, 1999). Based on the current set-up, 200 assays can easily be done by one person per day, and the consumption of tastants is in the microgram to milligram range. Modification of the assay using fluorescently or radioactively labeled G proteins and 96-well microplates should increase throughput to enable systematic screening for bitter inhibitors.

A limitation of this assay is its specificity: not all bitter or sweet compounds utilize the gustducin-dependent pathway(s). Many authors have speculated that multiple pathways underlie the transduction of responses to bitter compounds (Spielman *et al.*, 1992; Kinnamon and Margolskee, 1996; Lindemann, 1996; Gilbertson and Herness, 1999). Based on *in vivo* (Wong *et al.*, 1996a,b) and *in vitro* (Ming *et al.*, 1998) results, it is clear that the alpha subunit of gustducin is essential for transducing responses to denatonium and quinine. *In vitro* assays (such as we have used in the present study) argue that gustducin and/or transducin may be involved in transducing responses to strychnine, sparteine, nicotine, quinacrine, atropine, naringin, epicatechin, phenylthiourea and yohimbine (Ming *et al.*, 1998). Among the bitter tastants we have tested with this assay, aristolochic acid (up to 5 mM), caffeine (up to 5 mM) and SOA (up to 1 mM) did not give positive responses. It is possible that cattle differ from humans in sensitivity to these compounds, or that the transduction pathways for these compounds utilize other G proteins or are independent of G proteins (e.g. transduction via ligand-gated ion channels or direct action on other signal transduction components such as phosphodiesterases or K^+ channels). To go beyond the limitations of our present assay it will be necessary to elucidate additional transduction mechanisms for those bitter compounds that are not transduced by gustducin and reconstitute *in vitro* the key steps of these pathways as assays with which one can screen for gustducin-independent taste modifiers.

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