Possible Novel Mechanism for Bitter Taste Mediated Through cGMP

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Rosenzweig, Sophia, Wentao Yan, Maximillian Dasso, and Andrew I. Spielman. Possible novel mechanism for bitter taste mediated through cGMP. J. Neurophysiol. 81: 1661–1665, 1999. Taste is the least understood among sensory systems, and bitter taste mechanisms pose a special challenge because they are elicited by a large variety of compounds. We studied bitter taste signal transduction with the quench-flow method and monitored the rapid kinetics of the second messenger guanosine 3′,5′-cyclic monophosphate (cGMP) production and degradation in mouse taste tissue. In response to the bitter stimulants, caffeine and theophylline but not strychnine or denatonium cGMP levels demonstrated a rapid and transient increase that peaked at 50 ms and gradually declined throughout the following 4.5 s. The theophylline- and caffeine-induced effect was rapid, transient, concentration dependent and gustatory tissue-specific. The effect could be partially suppressed in the presence of the soluble guanylyl cyclase (GC) inhibitor 10 μM ODQ and 30 μM methylene blue but not 50 μM LY 83583 and boosted by nitric oxide donors 25 μM NOR-3 or 100 μM sodium nitroprusside. The proposed mechanism for this novel cGMP-mediated bitter taste signal transduction is cGMP production partially by the soluble GC and caffeine-induced inhibition of one or several phosphodiesterases.

INTRODUCTION

Taste is an essential sensory system for food selection and in general provides a good quality of life. Over the past 2 decades many laboratories focused on understanding taste mechanisms with a variety of methods: electrophysiological, biochemical, biophysical, genetic, molecular biological, and behavioral (reviewed by Kinnamon and Margolskee 1996; Lindemann 1996a). The emerging consensus is that taste signal transduction is complex and mediated through a large variety of mechanisms. This should not be surprising considering that taste is activated by a very diverse group of chemicals. Among the basic taste qualities, the bitter taste modality stands out as a particularly difficult system to understand because of the large number and structurally varied bitter chemicals. Most likely, bitter taste evolved as a screening mechanism for potentially toxic compounds (Glendinning 1994).

Bitter compounds are proposed to be mediated by a variety of peripheral taste transduction mechanisms (Kinnamon and Margolskee 1996; Spielman et al. 1992). One of the simplest signaling mechanisms is a direct blockage of potassium channels. Bitter compounds such as tetraethyl ammonium, bromide, and quinine are known to inhibit potassium channels, leading to cell depolarization. Quinine may have an additional mechanism through the direct activation of G proteins (Naim et al. 1994). A further mechanism was proposed for transduction of sucrose octaacetate and denatonium. These compounds appear to be receptor mediated and coupled to the inositol phosphate pathway leading to formation of inositol-1,4,5-trisphosphate (IP₃) and release of calcium from intracellular stores (Akabas et al. 1988; Hwang et al. 1990; Spielman et al. 1994, 1996). After the discovery of a taste tissue-enriched, G-protein α subunit, gustducin (McLaughlin et al. 1992), an additional bitter taste mechanism was proposed. The similarity of this G protein to transducin led to the suggestion that gustducin-mediated taste transduction may decrease intracellular cyclic nucleotides such as cyclic adenosine 3′,5′ monophosphate (cAMP) or cyclic guanosine 3′,5′ monophosphate (cGMP), resulting in the opening a cyclic nucleotide-inhibited ion channel (Kolesnikov and Margolskee 1995). We report another possible mechanism for bitter taste, one that increases cGMP production in response to certain bitter stimuli such as caffeine and theophylline. This study was done by measuring real-time changes in the levels of cGMP with the quench-flow technique (QFM) (Breer et al. 1990).

METHODS

Tissue collection

Circumvallate and foliate taste papillae from 6- to 8-wk-old SWR mouse taste and nongustatory control tissue (Hilltop Lab Animals; Scottsdale, PA) were obtained (Spielman and Brand 1995). Control tissue was collected from the ventral side of the tongue adjacent to the lingual frenum. Our previous studies used the dorsal surface of the tongue as nongustatory tissue. No difference was observed between the two control tissues (data not shown). Tissue was kept at 4°C in the presence of a protease inhibitory cocktail (1 mg/ml, containing serine, cysteine, aspartic, and metalloproteinase inhibitors, Sigma Chemical; St. Louis, MO). For a typical experiment taste tissue from 60–80 mice were homogenized with a glass–glass homogenizer in 50 mM MOPS, pH 6.9, containing 100 mM NaCl, 2.5 mM MgCl₂, 1 mM dithiothreitol (DTT), 10 mM EGTA, and 81 μM CaCl₂ to give a calculated free Ca²⁺ concentration of 0.010 μM, spun at 2,000 × g for 10 min at 4°C. The supernatant (diluted to a protein concentration of 85–100 μg/ml) was retained. Free calcium levels were calculated with the Chelator software (shareware, Dr. T.J.M. Schoenmakers, Dept. Animal Physiology, Toernooiveld, Nijmegen, The Netherlands).

Rapid kinetics

A rapid quench-flow module (QFM5, BioLogic, France; Molecular Kinetics; Pullman, WA) equipped with five syringes was used to measure the subsecond kinetics of second-messenger formation. In the QFM5 system, syringe 1 was filled with MOPS buffer supplemented with freshly prepared 1 mM ATP, 1 μM GTP, and 0.05% sodium cholate (BASAL buffer). Bitter stimulants or pharmacological agents were dissolved into BASAL buffer. The following stimulants and concentrations were tested (in mM): caffeine 1, 5, 10, 25, and 50; theophylline 1, 2, and 10; strychnine 0.1, 2, and 10; and denatonium 0.1, 1, and 10. Syringe 3 contained taste tissue homogenate in MOPS.
FIG. 1. Guanosine 3′,5′-cyclic monophosphate (cGMP) accumulation in mouse taste tissue 50 ms after stimulation with 4 bitter compounds. Taste tissue was stimulated with either buffer (Bas) or 1 mM denatonium (Den), 2 mM strychnine (Str), 10 mM theophylline (Theo), or 25 mM caffeine (Caff) in gustatory or nongustatory (NG) tissue. Data are the means ± SE of 3–22 data points from independent triplicate experiments (for gustatory tissue n = Bas 9, Den 10, Str 7, Theo 3, Caff 6). Data are expressed as percentage of the basal value of the respective tissue. Data were analyzed with an analysis of variance followed by a Student-Newman-Kuels test. (* P < 0.001 compared with basal.)

buffer at a concentration of 85–100 μg/ml. Tissue was kept at 4°C at all times and loaded into syringe 3 in batches of 500–1,000 μl just seconds before injection at 22°C. Tissue sedimentation was prevented by rapidly moving the plunger just before injection. Syringe 4 contained 9% perchloric acid. Syringe 5 was used to collect the waste. The reaction was initiated by mixing 60 μl buffer or buffer-containing stimulants with 60 μl tissue. After 0, 25, 50, 100, 200, 500, 1,000, or 5,000 ms, the reaction was terminated by injection of 60 μl of 9% perchloric acid. The activation of all syringes was controlled by an IBM AT computer with software developed by the manufacturer (BioLogic; France) and drive sequences developed in our laboratory. At time 0 tissue was first quenched with perchloric acid and then stimulated. Variation among triplicates was usually between 1 and 7% but not >10%. When guanylyl cyclase (GC) inhibitors 10 μM ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), 30 μM methylene blue, or 50 μM LY 83583 (Calbiochem; La Jolla, CA) were used, taste tissue was preincubated with the inhibitor for 30–60 min at 4°C and subsequently stimulated with 25 mM caffeine. Nitric oxide (NO) donors, sodium nitroprusside (SNP), and (±)-(E)-ethyl-2-[(E)-hydroxymino]-5-nitro-hexenamide, NOR-3, also known as FK 409 (Calbiochem; La Jolla, CA), were freshly prepared 30 min before quenching. NOR-3 was dissolved first in DMSO, with a final DMSO concentration of <0.5%. Control experiments included the same amount of DMSO.

After quenching, 330 μl of each sample was stored at −20°C until used. On the day of the assay, samples were centrifuged at 1,000 × g for 5 min. cGMP was extracted from the supernatant by mixing with 82.5 μl 10 mM EDTA, pH 7.0. 10 μl of Universal indicator, and adjusted to pH 7.0 with 1.5 M KOH and 60 mM HEPES (Palmer and Wakelam 1990). The mix was centrifuged at 1,200 × g for 5 min at 4°C, and the supernatant was assayed for cGMP with [125I]-labeled RIA kit from NEN-DuPont (Boston, MA). Protein analysis followed the Bradford assay with bovine gamma globulin as a standard (Biorad; Hercules, CA).

R E S U L T S

By using a quench flow (QFM5) module, we screened several bitter compounds (denatonium, strychnine, caffeine, and theophylline) for their ability to generate cGMP in gustatory and nongustatory control tissue homogenates from SWR mice. The concentrations tested in this study were chosen based on their gustatory thresholds in mice and humans. For instance, denatonium has a detection threshold of 100 μM in mice and as low as 1–10 μM in humans. Caffeine in turn is tasted as bitter in humans and mice only in high millimolar concentrations. Some compounds, such as strychnine, theophylline, and denatonium, are not soluble above 10 mM or precipitate out in the presence of perchloric acid during quenching. Therefore we could not test all stimulants at the same concentrations. However, even at 10 mM, concentrations that exceed by 100-fold the physiologically relevant levels for denatonium or strychnine gustatory and nongustatory cGMP levels were no different from controls.

Caffeine (25 mM) and theophylline (10 mM) but not denatonium (1 mM) or strychnine (2 mM) induced cGMP production when monitored at 50 ms after stimulation (Fig. 1). Caffeine and theophylline alone induced an increase of 825 ± 73% (mean ± SE) and 953 ± 21%, respectively, compared with basal levels (P < 0.001). The response was primarily in the gustatory tissue, although as expected nongustatory tissue response to the same stimulants demonstrated a 486 ± 17% increase (theophylline, P = not significant) and 329 ± 19% (caffeine, P = not significant), respectively (Fig. 1).

To determine the time course of second-messenger activation, we monitored cGMP production over 5 s. As expected from data in Fig. 1, neither denatonium nor strychnine induced cGMP accumulation (Fig. 2). Even when the concentrations of denatonium and strychnine were raised to 10 mM, concentrations at 100 times above threshold levels, no accumulation of cGMP was seen. However, both caffeine and theophylline induced peak accumulation of cGMP within 50 ms of stimulation (P < 0.001 compared with basal); cGMP gradually declined over the following 5,000 ms (data shown only for the first 500 ms) (Fig. 2).

Further evidence that caffeine induced the cGMP accumulation is provided by a concentration-response curve in Fig. 3. Increasing caffeine concentrations (5, 25, and 50 mM) caused
Bitter Taste Mechanism

Our working hypothesis was that caffeine and theophylline inhibited one or more phosphodiesterases (PDEs) in taste tissue, which led to cGMP accumulation generated through the basal activity of one or several GCs. To test this hypothesis, we inhibited the soluble GC with ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) (10 μM) or methylene blue (30 μM). Both inhibitors significantly decreased caffeine-induced cGMP accumulation by 45 ± 8.6% (P < 0.05) and by 54 ± 3% (P < 0.01), respectively (Fig. 4). In contrast, LY-83583 (50 μM), a competitive inhibitor of soluble GC that lowers cGMP by blocking intracellular Ca2+ release (Pandol and Schoeffield-Payne 1990), as expected was ineffective (26 ± 12%, P = not significant) in our cell-disrupted system.

When NO donors were tested in the presence of caffeine (25 mM) or theophylline (10 mM), SNP (100 μM) and NOR-3 (25 μM) both specifically increased cGMP production in gustatory tissue. cGMP levels increased by 39% (SNP + caffeine) and 49% (NOR-3 and caffeine) (P < 0.006 and P < 0.001, respectively) over caffeine-induced levels but not in control nongustatory tissue (Fig. 5). Theophylline response was similarly enhanced (35%) in gustatory tissue by NOR-3 (P < 0.005) but only 21% by SNP (P < 0.07).

**DISCUSSION**

We used tissue homogenates derived from the circumvallate and foliate taste papillae of the mouse as well as nongustatory control tissue to test a new taste mechanism. We used the QFM, a powerful method to monitor peripheral events in taste. The major advantage of the QFM is the real-time measurement of second messengers that normally degrade in the subsecond time frame. The resolution of the QFM permits monitoring second-messenger formation as low as 7 ms. QFM, however, cannot work with intact cells, only tissue homogenates or membranes. Thus during quench flow taste tissue loses its polarity, and its interaction with the stimulant does not necessarily occur only at the apical end. Therefore, similar to other in vitro techniques, one needs further corroboration of quench-flow data by other methodologies.

On the basis of our results, we hypothesize that the possible mechanism for caffeine and theophylline bitter taste transduction is accumulation of cGMP because of inhibition of PDEs. Although not tested in this study cGMP accumulation may lead to activation of a cyclic nucleotide-gated channel (CNGgust). To support this hypothesis, there should be corroborating evidence at key levels of this signal transduction pathway: 1) PDE inhibition by bitter compounds, 2) presence of a GC and NO synthase (NOS) in taste tissue, 3) cGMP-induced cell depolarization in taste cells, and 4) presence of a taste-specific cyclic nucleotide-gated channel (CNGgust).
Caffeine or theophylline directly inhibit \( \geq 1 \) phosphodiesterases in tissue (1), leading to accumulation of cGMP (2). cGMP, which is generated at least partially by a soluble GC (3), possibly activates a cyclic nucleotide gated cation channel, CNGgust (4), and may lead to influx of cations into the cell (5).

Further evidence to support our hypothesis comes from studies of the role of cyclic nucleotides in taste. Injection of cyclic nucleotides (cGMP or cAMP) into intact frog or mouse taste cells caused depolarization (Okada et al. 1987; Tonosaki and Funakoshi 1988). Subsequently it was demonstrated that cGMP is involved in the sweet taste signal transduction of the fly and hamster (Amakawa et al. 1990; Cummings et al. 1993). Taken together these studies indicated that cGMP is an important second messenger in the transduction of sweet compounds. Our data extend the role of cGMP to possibly include bitter taste as well.

Finally, a crucial last component is needed to complete the proposed caffeine and theophylline signal transduction: CNGgust, which was previously identified in visual and olfactory transduction. CNGgust was recently cloned from the rat gustatory tissue (Misaka et al. 1997). This channel, when expressed, opened in response to changes in intracellular cAMP and cGMP. CNGgust is located close to the taste pore and is hypothesized to be a nonselective cation channel with a similar role to the one found in olfaction (Nakamura and Gold 1987). In our model for caffeine and theophylline signal transduction, this channel would have an important role in modulating influx of cations leading to cell depolarization or possibly release of neurotransmitters.

Preliminary evidence suggests that both theophylline and caffeine also induce cAMP production. Incidentally, several cAMP or cGMP PDE inhibitors (3-isobutyl-1-methylxantine, papaverine, RO-20-1724, and EHNA) also taste bitter to humans (personal observations). Experiments are in progress to elucidate those mechanisms, and further studies are needed to identify if PDE inhibition and activation of CNGgust are involved.

The mechanism proposed in this study would contrast with the one proposed for gustducin-mediated bitter taste signal transduction, where cyclic nucleotides maintain an open state and their degradation closes the ion channel (Kolesnikov and Margolskee 1995). Caffeine signal transduction is not mediated through gustducin because gustducin knockout mice were not deficient in their caffeine-tasting ability, and caffeine failed to activate transducin or gustducin in an in vitro assay (Ming et al. 1998). In fact, denatonium, which appears to be IP3 mediated in rodents and mudpuppy (Akabas et al. 1988; Hwang et al. 1998). In fact, denatonium, which appears to be IP3 mediated in rodents and mudpuppy (Akabas et al. 1988; Hwang et al. 1998; Ogura et al. 1997; Spielman et al. 1996) and modulated by gustducin, does not increase cGMP production (Fig. 2). Caffeine- and gustducin-mediated mechanisms may both exist in the same cell, and there could be cross talk between the two pathways proposed for the gustatory system (Glendinning and Hills 1997; Lindemann 1996b).

Further evidence that caffeine signal transduction is different from any bitter taste mechanism so far described comes from recordings in the rat nucleus tractus solitarius (Scott and Mark 1987) and chorda tympani (Dahl et al. 1997). Multidimensional scaling representation of the similarities of the neural inputs demonstrates caffeine as a very distinct compound, quite apart from any other bitter compound including strychnine and denatonium. Furthermore, the kinetics of cGMP formation in response to caffeine and theophylline contrasts with the profile of IP3 formation induced by denatonium and sucrose octaacetate (Spielman et al. 1996). Although IP3 is rapidly formed and degraded within 100–200 ms, cGMP is not readily degraded, lingering for \( \geq 1–5 \) s after formation. The differences in the kinetics of IP3 and cGMP may be due to stimulus-induced IP3 formation for denatonium and strychnine as op-
posed to caffeine- and theophylline-induced inhibition in cGMP degradation.

Although it remains to be established if all components of the proposed signal transduction pathway (PDE, GC, and CNGgust) exist in the same taste cell, this study demonstrates that in response to caffeine and theophylline there is rapid accumulation and gradual degradation of cGMP. If this mechanism exists in the mouse gustatory system, it is likely to be present only in a subset of gustatory cells. However, on the basis of our study, accumulation of cGMP occurs in a rapid, transitory fashion and within a physiologically relevant time frame, characteristic of a second messenger that usually precedes cellular depolarization or release of neurotransmitters.

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