Inhibition of signal termination-related kinases by membrane-permeant bitter and sweet tastants: potential role in taste signal termination

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Zubare-Samuelov, Meirav, Merav E. Shaul, Irena Peri, Alexander Alliluko, Oren Tirosi, and Michael Naim. Inhibition of signal termination-related kinases by membrane-permeant bitter and sweet tastants: potential role in taste signal termination. Am J Physiol Cell Physiol 289: C483–C492, 2005. First published April 13, 2005; doi:10.1152/ajpcell.00547.2004.—Sweet and bitter taste sensations are elicited by the tastant-stimulated T1R and T2R G protein-coupled receptor (GPCR) subfamilies, respectively, which occur in taste cells. Although such tastants, with their significantly diverse chemical structures (e.g., sugar and nonsugar sweeteners, protein-coupled receptor (GPCR) subfamilies, respectively, which occur in taste cells. Although such tastants, with their significantly diverse chemical structures (e.g., sugar and nonsugar sweeteners, may share the same or similar T1Rs, some nonsugar sweeteners and many bitter tastants are amphipathic and produce a significant delay in taste termination (lingering aftertaste). We report that such tastants may permeate rat taste bud cells rapidly in vivo and inhibit known signal termination-related kinases in vitro, such as GPCR kinase (GRK)2, GRK5, and PKA. GRK5 and perhaps GRK2 and GRK6 are present in taste cells. A new hypothesis is proposed in which membrane-permeant tastants not only interact with taste GPCRs but also interact intracellularly with the receptors’ downstream shutoff components to inhibit signal termination.

amphipathic tastants; taste bud permeation; desensitization; lingering aftertaste

TASTE STIMULATION IS CHARACTERIZED by onset in less than a second and termination within a few seconds. However, some nonsugar sweeteners and many bitter tastants (most of them amphipathic, i.e., containing hydrophobic and hydrophilic domains) exhibit a significant delay in the extinction of taste sensation, which may last minutes, and is termed “lingering aftertaste” (6, 27). Although this particular delay in taste termination significantly affects the taste perception of many dietary constituents, its molecular basis is unknown.

The occurrence of G protein-coupled receptors (GPCRs) for both sweet and bitter tastes, as well as for the umami taste, is now incontestable (1, 3, 7–9, 22, 23, 26). In contrast, the nature of the cellular signals, essential components of taste transduction, is under considerable debate. A heterotrimeric G protein consisting of α-gustducin, Gβγ, and Gγ13 is involved in the cellular response to bitter stimuli, of which the Gβγ13 moiety activates PLCβ2 (21); moreover, changes in second messengers such as cAMP, cGMP and d-myo-inositol 1,4,5-triphosphate (IP3) in taste cells (5, 35, 40) in response to sweet and bitter taste stimulation have been proposed. Recent studies have indicated that mammalian transient receptor potential (TRPM)5, a PLC-dependent cationic channel that is believed to regulate Ca2+ entry into the cell, and PLCβ2 are present in taste cells (30). Interestingly, TRPM5- or PLCβ2-knockout mice almost completely lose their sensitivity to sugar, nonsugar sweetener, bitter, and umami taste stimulation, as evidenced by behavioral and electrophysiological experiments (46).

A few hypotheses may be proposed to explain the delay in taste signal termination induced by amphipathic tastants. Importantly, electrophysiological recordings of taste nerves at the periphery (16) have indicated a slow rise (onset) and a longer delay (lingering) in the sweet sensation response to stimulation by nonsugar sweeteners compared with that by sucrose. Biochemical time course measurements have shown that the transient onset and termination of IP3 release in taste tissue after stimulation by some bitter tastants known to possess lingering aftertaste is delayed >500 ms (38). These results suggest that the delay in taste signal termination induced by amphipathic tastants is at the taste cell level, at the periphery. Although sugar and nonsugar sweeteners appear to stimulate similar or identical sweet GPCRs, the lingering taste sensations are uniquely produced by nonsugar sweeteners (as well as bitter tastants). Because such tastants are direct activators of G proteins in vitro (25) and, unlike sucrose, rapidly permeate isolated taste bud cells in situ (31), they may interact with downstream transduction components that affect GPCR signal termination, in addition to their extracellular interaction with GPCRs.

GPCR signaling desensitizes rapidly as a consequence of receptor phosphorylation. GPCR phosphorylation is mediated by two families of protein kinases: the second messenger-dependent protein kinases (e.g., PKA) and the GPCR kinases (GRKs). In contrast to the former, GRKs specifically phosphorylate the agonist-activated form of GPCRs and promote the binding of arrestin proteins that further uncouple the receptors from G proteins (32). Thus, when ligands are involved, the phosphorylation of GPCRs by GRKs appears essential: deletion of GRK phosphorylation sites in the receptor results in stronger signaling responses to agonist stimulation in transfection studies (24). Inhibition of this phosphorylation can delay GPCR signal termination, as shown in vision (37) and other systems (39).

We previously found (31) that amphipathic tastants can translocate through multilamellar liposomes and rapidly permeate isolated circumvallate (CV) taste bud cells; intracellular concentrations of such tastants after 30 s of stimulation could reach millimolar levels. In line with previous conclusions (12), we hypothesize that such tastants, after permeation into the...
cells, have access to downstream transduction components that may be related to the delay in taste termination produced by such tastants. The present study was designed to further investigate whether amphipathic tastants can permeate taste cells under physiological conditions and interfere with GPCR signal termination, e.g., via inhibition of signal termination-related kinases.

MATERIALS AND METHODS

Animals and Tastants

Wistar female rats weighing 150–175 g were purchased from Harlan Laboratories (Jerusalem, Israel) and were kept in individual cages for a few days and supplied with rat laboratory chow and water ad libitum. The protocol for animal care and other procedures was approved by The Hebrew University of Jerusalem Animal Care Committee (NIH approval number: OPRR-A01-5011). The sweeteners sodium saccharin, neohesperidin dihydrochalcone (NH-D), sodium cyclamate, L-tryptophan, and acasculfame K (all from Sigma, St. Louis, MO) and the bitter stimuli cyclodeoxytrp (Bachem, Bubendorf, Switzerland), caffeine, quinine-HCl, L-tryptophan, limonin, and naringin (all from Sigma) were used.

Other Chemicals

Collagenase types D and A were purchased from Roche Diagnostics (Mannheim, Germany), and soybean trypsin inhibitor (type 1) and propidium iodide were from Sigma. Agarose was from Life Technologies (Paisley, UK), and ethidium bromide from Amresco (Solon, OH). Primary polyclonal antibodies for GRK2 (sc-562), GRK3 (sc-563), GRK5 (sc-565), and GRK6 (sc-566) and their respective blocking reagents (Mannheim, Germany), and soybean trypsin inhibitor (type 1) and paraformaldehyde were from Sigma, KH2PO4 from Merck (Darmstadt, Germany), HEPES, EGTA, tricine, and paraformaldehyde were purchased from Sigma. GRK2 and GRK5, kindly provided by R. J. Luftkowitz, Duke University, Durham, NC) were prepared from bovine heart, PKA inhibitor (fragment 6–22), and heparin were purchased from Sigma, GRK2 and GRK5, kindly provided by R. J. Luftkowitz, were purified from Sf9 insect cells (33). The three kinases were kept in 50% (vol/vol) glycerol in 2 mM EDTA, 20 mM Tris-HCl pH 7.4 buffer and stored at −12°C until use.

Permeation of Amphipathic Tastants into Taste Cells

Imaging of in situ permeation of tastants into CV taste papilla cells with confocal microscopy. During the experiments, animals were anesthetized with ether and then killed by decapitation. The CV papilla was identified under a dissection microscope as previously described (31, 40). The outer segment membranes of rod cells containing rhodopsin (kindly provided on and off into the oral cavity with a Pasteur pipette for a period of 90 s and could spill out from the oral cavity. Oral stimulation was conducted by administering 10 ml of 30 mM d-tryptophan, 2 mM quinine, 2 mM cyclodeoxytrp, or 10 mM caffeine. Because of the efflux of tastants during the subsequent collagenase treatment (see below), we selected tastants that are either fluorescent or strongly UV absorbing for HPLC-sensitive detection. Oral stimulation of control rats was carried out with solutions lacking the taste stimuli. The anesthetized animals were then immediately killed by decapitation. Tongues were rapidly removed and put into a beaker containing ice-cold Tyrode solution (40). Under a dissection microscope, a solution containing collagenase D (6 mg/ml) and trypsin inhibitor (4 mg/ml) in Tyrode solution was then injected submucosally at four or five locations around and under the single CV papilla. The whole tongue was then incubated in 20 ml of air-bubbled Tyrode solution at 30°C for 15 min. CV taste bud sheets were separated from the tongues under a dissection microscope as previously described (31, 40). The length of the entire process, from tongue removal, was ~25 min. Cells in taste bud sheets were then permeabilized by four freeze-thaw (~70°C) cycles in deionized water, and membranes were removed by centrifugation (30 min, 28,000 g, 4°C).

The intracellular content of various tastants was determined by HPLC equipped with UV-visible diode-array and fluorescent detectors. According to Ref. 31 with some modifications. The UV-visible diode-array detector was set at 270 nm for saccharin and caffeine determinations, and separation was conducted with a LiChrospher 100 RP-18 column (5 μm, 250 mm, 4 mm; Merck) with an RP-18 precolumn. The mobile phase (isocratic separation) for the analysis of saccharin consisted of 1.5% (vol/vol) acetic acid in H2O and methanol (80:20 vol/vol) at a 0.45 ml/min flow rate. For caffeine analysis it consisted of H2O and methanol (50:50 vol/vol) at a 0.85 ml/min flow rate. The fluorescence detector was used for quinine, cyclo(Leu-Trp), and d-Trp determinations, and separation was conducted with a
Purosphere 55 RP-18 column (3 μm, 55 mm, 4 mm; Merck) with an RP-18 precolumn. The mobile phase (isocratic separation) for the analysis of quinine consisted of a mixture of acetoniitrile and aqueous buffer containing (in mM) 10 KH₂PO₄, 25 SDS, and 3 tetrabutylammonium bromide, adjusted to pH 2.3 with orthophosphoric acid (47) (50:50 vol/vol) at a 0.9 ml/min flow rate. Acetonitrile with H₂O (40:60 vol/vol) at a 0.7 ml/min flow rate was used for cyclo(Leu-Trp). The mobile phase for the analysis of β-tryptophan (linear gradient) was as for saccharin, except that the methanol was increased from 5% to 25% during the first 0–4 min and then returned to 5% methanol for 4–8 min, at a 1 ml/min flow rate.

Excitation at 338 nm and emission at 388 nm were used for quinine, whereas 280 and 348 nm were used for β-tryptophan and cyclo(Leu-Trp). Peaks of the tasters were identified and quantitated with known markers [Retention Time = 6.7, 7.5, 7.1, 4.3, and 3 min for saccharin, caffeine, quinine, β-tryptophan, and cyclo(Leu-Trp), respectively].

One might assume that during the 25-min collagenase treatments (after the 90-s taste stimulation), at least some of the tasters would be likely to leave the cells, possibly via multidrug resistance P-glycoprotein (MDR1; Ref. 18) or by simple diffusion. To estimate the amount of efflux of each taster during the collagenase treatment, CV taste bud sheets were prepared from additional rats by collagenase separation. One-half of the CV tissue isolated from a single rat was then incubated with a taster solution (1 of the above 4 stimuli at the same concentrations) for 30 s at 30°C. The second half of the same CV tissue was incubated with the same taster for 30 s under the same experimental conditions. However, in this case, taste bud sheets were washed five times and left in Tyrode (at 30°C) for an additional 25 min. Taste bud sheets were then washed with Tyrode five times, cells were permeabilized (as already described), and the intracellular content of each taster was determined by HPLC. The difference in the content of each taster between the first half of the CV (initial intracellular concentration) and the second (final intracellular concentration) was used to estimate the efflux (calculated as percentage of the initial intracellular concentration) of tasters during the collagenase treatment. We assumed that the efflux of tasters from cells derived from isolated CV taste bud sheets is similar to that from taste cells of the CV before or during the collagenase treatment.

**Experiments Designed to Identify GRK1, GRK2, GRK3, GRK5, GRK6, T2R4, and T1R3 in Taste Bud Cells**

**RT-PCR.** CV taste bud and nonsensory epithelial sheets were prepared from rat tongues by subepithelial collagenase (type A) treatment (31, 40). Total RNA was isolated from pools of seven CV and nonsensory sheets with an RNasey Protect Starter kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. cDNA was synthesized from 0.5 μg of RNA using a Reverse-IT first-strand synthesis kit (ABgene, Epsom, UK) according to the manufacturer’s instructions. The cDNA was then amplified by PCR with a ReddyMix PCR Master Mix kit (ABgene), using specific primers for GRK1, GRK2, GRK3, GRK5, or GRK6 [believed to account for the regulation of most of the GPCRs found throughout the body (15)] and T2R4 or T1R3 taste receptors (all synthesized by the Oligonucleotide Synthesis Unit at the Weizmann Institute of Science, Rehovot, Israel). Specific primers for GAPDH (Metabion, Martinsried, Germany) were used as a control. GRK7 [apparently not present in the rat genome (44)] and GRK4 (thought to be expressed mainly in testes) were not analyzed.

The GRK1 primers were 5′-TTTCTCGCCGGTTTCTTCAGT-3′ (forward) and 5′-CACGGTCCCTCTCTGAGGTT-3′ (reverse); the expected size of the PCR product was 460 bp. The GRK2 primers were 5′-CTTTGACATTGGCTCCTTTGA-3′ (forward) and 5′-CTTTGACATTGGCTCCTTTGA-3′ (reverse); the expected size of the PCR product was 841 bp. The GRK6 primers were 5′-AGGTACCCAGATGAGAAA-3′ (forward) and 5′-TCTCAGCACATGAAAGACA-3′ (reverse); the expected size of the PCR product was 162 bp.

The T2R4 primers were 5′-CCACACATACCTTACCATTGA-3′ (forward) and 5′-GAGTTGGTGTGTTTTGGGCAG-3′ (reverse); the expected size of the PCR product was 560 bp. The T1R3 primers were 5′-CATGACCTTTACCCAAAAGGTA-3′ (forward) and 5′-CATGACCTTTACCCAAAAGGTA-3′ (reverse); the expected size of the PCR product was 705 bp. The GAPDH primers were 5′-TCCGCCTTCCCTCCTGTAGT-3′ (forward) and 5′-CAGCGAGGCCCAT-GCCAGTGA-3′ (reverse); the expected size of the PCR product was 350 bp.

PCR was performed with one cycle at 94°C for 5 min, then 30 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1 min, followed by one cycle at 72°C for 10 min. cDNA was electrophoresed on a 1.5% (wt/vol) agarose gel in the presence of ethidium bromide. In control experiments, in which PCR was carried out with GRK2 primers on RNA without including the reverse transcription step, no PCR product was observed, confirming that the total RNA was not contaminated with genomic DNA. The PCR products were purified with a PCR purification kit (Qiagen) and sequenced at the Sequencing Unit of the Weizmann Institute of Science.

**Immunoblot analysis for antibody specificity.** Rat brains were homogenized in RIPA buffer (20 mM Tris, 137 mM NaCl, 10% glycerol, 0.1% wt/vol SDS, 0.5% wt/vol deoxycholate, 1% vol/vol Triton X-100, 2 mM EDTA, 1 mM PMSF, 20 μM leupeptin) and centrifuged at 20,000 g for 15 min at 4°C. The supernatant was removed and incubated with sample buffer at 95°C for 5 min after a sample was taken for protein concentration. Total protein from the brain extract was loaded on a 12% SDS-PAGE (20 μg/lane) and transferred to a nitrocellulose membrane. The membrane was incubated with blocking solution [2% wt/vol BSA in Tris-buffered saline Tween (TBST)], and each lane was incubated at room temperature for 1 h with the appropriate antibody [GRK2 (1:5,000), GRK3 (1:500), GRK5 (1:1,000), or GRK6 (1:1,000)], with or without its blocking peptide (1 μg/ml). Peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L) was used as a secondary antibody.

**Immunohistochemistry.** Immunohistochemistry was performed essentially according to Ref. 10. Rats were deeply anesthetized with pentobarbital (60 mg/kg body wt) and perfused through the left ventricle, first with Hanks’ buffer (in mM: 137 NaCl, 5.3 KCl, 0.3 Na₂HPO₄, 0.8 MgSO₄, 1 MgCl₂, 5 HEPES, 6.7 tricine, 28 glucose, 0.3 NaHCO₃, and 0.96 EGTA, pH 7.3) and then with a fixative containing 3% (wt/vol) paraformaldehyde in a 0.1 M phosphate buffer (pH 7.3) for 10 min. After perfusion, tongues were removed and cryoprotected with 20% (wt/vol) sucrose in 0.1 M phosphate buffer overnight at 4°C. The tongues were then frozen in dry ice-cooled isopentane. Cryostat sections (10 μm) from CV papillae (visualized under a light microscope) were collected sequentially onto SuperFrost Plus slides (Menzel-Glazer, Braunschweig, Germany), air dried for 30 min, and kept at −20°C until use. Sections were distributed to different slides to ensure that controls and treated sections represented the same area of the CV papillae. Sections were incubated for 1 h at room temperature in a blocking solution containing 10% (vol/vol) goat serum (to reduce nonspecific binding) and 0.3% Triton X-100 in 0.1 M phosphate buffer (pH 7.3). Sections were then incubated in primary antibodies specific for GRK2, GRK5, or GRK6 (1:100 in blocking solution) in a closed, moist chamber for 24 h at 4°C. After being washed (3 times) with 1× PBS, the tissues were exposed to secondary FITC-conjugated goat anti-rabbit IgG (1:200 in PBS) for 1 h at room temperature. Sections were washed, mounted, and viewed under a Zeiss confocal fluorescent microscope with a Plan NoFluar ×40/1.3 NA lens. Control samples were treated similarly with mod-
Phosphorylation Assays

GRK2 (1.5–1.8 pmol/tube), GRK5 (20 pmol/tube) or PKA (5 U/tube) were preincubated with and without tastants (at the concentrations indicated in Figs. 4 and 5) in assay buffer (in mM: 20 Tris•HCl pH 7.4, 10 MgCl₂, 2 EDTA, and 1 DTT) for 15 min at 30°C. Samples containing cyclo(Leu-Trp) and limonin also contained 0.1% (vol/vol) DMSO. When tastants contained additional cations (e.g., sodium saccharin and sodium cyclamate) or potassium (acesulfame K), the same concentrations of either NaCl or KCl were used as specific controls. The reaction (15 min at 30°C) under light (when rhodopsin was used) in the same buffer was started by adding substrates (rod outer membrane segments containing rhodopsin to a final concentration of 0.5 μM or 20 μg casein) and 100 μM [γ-32P]ATP (1–2 μCi). After incubation, 12 μl of sample loading buffer (100 mM Tris•HCl pH 7.2, 4% SDS, 200 mM DTT, 0.25% wt/vol pyronin Y, and 20% glycerol) were added to terminate the 25-μl reaction. Samples were then subjected to SDS-PAGE. The gels were dried and exposed (14 h) to a PhosphorImager screen (Fuji Photo Film, Nakagawa, Japan). Phosphorylation was quantitated with Image J software and the mean of at least three independent experiment images, performed with and without tastants, which expressed the phosphorylation level as percentage of controls performed without any added tastants. Heparin was the positive control for inhibition of GRK2 and GRK5, and the PKA inhibitor amide, fragment 6–22, was the control for PKA.

Rhodopsin and ATP Kinetics

Rhodopsin and ATP concentration effects on NHD- and ß-tryptophan-inhibited phosphorylation produced by GRK2 were analyzed with two concentrations of each tastant. ATP concentration ranged from 0 to 1.5 mM. Rhodopsin concentration ranged from 0 to 2.1 μM. The increase in ATP concentration came from the addition of cold ATP, the level of labeled ATP being kept constant (3–4 μCi). To keep specific activity constant in the final quantitative determination, the phosphorylation results for each ATP concentration were multiplied by the dilution factor.

Data Analysis

Mean tests using JMP statistical software (SAS Institute, Cary, NC) were performed on the quantitative estimation values derived from the taste permeation data. Michaelis-Menten kinetics data were calculated with SigmaPlot 8.0 (SPSS, Chicago, IL), and ANOVA (JMP) was performed for the phosphorylation inhibition and kinetics data. Post hoc comparisons to determine the differences between the means were performed by either Tukey-Kramer honestly significant difference and Student’s t-tests (phosphorylation data) or nonoverlapping confidence intervals (kinetics).

RESULTS

Permeation of Amphipathic Tastants into Taste Bud Cells

Exposure of intact CV papillae to selected amphipathic tastants revealed the dynamics of tastant permeation in situ under confocal microscopy (Fig. 1). A dorsal view shows the increased fluorescence of tastants with time, located around the circular inner trench (Fig. 1, arrows) where the taste buds are found. Tastant fluorescence was also observed in the surrounding nonsensory epithelium. With this technique, an increase in fluorescence after tastant application was already visible after 1 min; however, the 0- to 6-min exposure range was selected to show the continuing increase in fluorescence intensity. Application of the membrane-impermeant fluorescence quencher KI had no effect on the fluorescence intensity contributed by saccharin, caffeine, or cyclo(Leu-Trp) (Fig. 1) or that contributed by ß-tryptophan, naringin, or quinine (data not shown). This indicated that tastant fluorescence was protected from quenching by KI because it occurred inside the CV cells. Such tastant permeation also occurred in epithelial tissue that did not contain taste buds. In contrast, fluorescence of the membrane-impermeant propidium iodide (PI) (fluorescent impermeant compound) was used as a positive control. Each image is of a representative experiment, replicated at least 4 times. Note that permeation of tastants also occurs in the surrounding nonsensory epithelial cells and that the addition of KI significantly reduced PI fluorescence without affecting tastant fluorescence.
Table 1. Estimated permeation of amphipathic tastants into CV taste bud cells via apical side

<table>
<thead>
<tr>
<th>Tastant</th>
<th>Oral Stimulation During Treatment, mM</th>
<th>Efflux During Collagenase Treatment, %</th>
<th>Intracellular Concentration After Oral Stimulation, mM</th>
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</thead>
<tbody>
<tr>
<td>D-Tryptophan</td>
<td>30</td>
<td>86 ± 9.5*</td>
<td>6.4 ± 1.0†</td>
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<tr>
<td>Quinine</td>
<td>2</td>
<td>75 ± 5.6*</td>
<td>5.5 ± 0.8†</td>
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<tr>
<td>Cyclo(Leu-Trp)</td>
<td>2</td>
<td>64 ± 3.0*</td>
<td>0.96 ± 0.1†</td>
</tr>
<tr>
<td>Caffeine</td>
<td>10</td>
<td>70 ± 2.5*</td>
<td>13.7 ± 3.6*</td>
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*Data are the means ± SE of 4 – 9 replicates, each derived from 1 or 2 rats. Mean tests were performed on the raw data. The oral cavity of anesthetized rats was on-and-off stimulated for 90 s by a tastant solution. Rats were then killed, tongues were removed, and the circumvallate (CV) papilla was isolated by a 25-min collagenase treatment and washed. The cells were permeabilized, and the intracellular concentration was determined by HPLC. Efflux was determined in parallel experiments (see MATERIALS AND METHODS). Significant values: *P < 0.03, †P < 0.001.

(Table 1). Tastant permeation was also evident in epithelial cells lacking taste buds (data not shown).

After the 90-s incubation period, the intracellular concentration of quinine was almost threefold that present in the extracellular medium, indicating permeation against a concentration gradient. The efflux (or the rate of tastant disappearance inside the taste cells) was significant for all tastants during the 25-min collagenase treatment, ranging between 64% and 86% (Table 1).

Presence of GRK2, GRK3, GRK5, GRK6, T2R4, and T1R3 in Taste Bud Cells

GRK5 has been shown to be present in CV papillae (33); however, expression of the other GRKs in taste bud cells was never verified. RT-PCR (Fig. 2) indicated that the signal termination-related kinases GRK2, GRK3, GRK5, and GRK6 (but not GRK1) are present in the CV taste bud sheet together with the bitter (T2R4) and sweet (T1R3) taste receptors. Specific primers for these genes were used for RT-PCR with RNA isolated from CV tissue, and agarose gel electrophoresis yielded PCR products of the expected sizes in all six tested proteins. Parallel PCR of RNA without the reverse transcriptase step did not show any bands, indicating no genomic DNA contamination. PCR products were sequenced and found identical to these four kinase genes in rats with the BLAST program [GenBank, National Center for Biotechnology Information]. Similar RT-PCR experiments indicated the presence of GRK2, GRK3, GRK5, and GRK6 in the nonsensory epithelial sheets but, as expected, no bands for the T2R4 and T1R3 receptors.

To further explore the presence of GRKs in CV taste cells, we examined the distribution of GRK2, GRK3, GRK5, and GRK6 antibodies in 10-μm sections prepared from CV papillae in an area where CV taste buds are clearly visible (Fig. 3A). Very weak immunostaining of GRK3 was noted (data not shown). However, strong immunostaining of GRK2, GRK5, and GRK6 was found: GRK5 was particularly noticeable in the taste bud cells, whereas GRK2 and GRK6 were essentially stained in the surrounding epithelium, and their presence in taste bud cells remains questionable.

To verify the specificity of each of the antibodies used for immunostaining, a Western blot was performed, using protein extract from brain homogenate. As shown in Fig. 3B, GRK2, GRK5, and GRK6 antibodies each showed one main band at the expected weight (GRK2 at ~80 kDa, GRK5 and GRK6 at ~65 kDa). These bands either disappeared or were significantly reduced when incubated with their respective blocking peptides. Thus the antibodies were specific enough to be used for immunostaining.

Inhibition of GRK2- and GRK5-Phosphorylated Rhodopsin and PKA-Phosphorylated Casein in Vitro

Initial experiments were run to determine the enzyme concentrations needed to carry out enzymatic activities in the linear range. Depending on the kinase tested, the various amphipathic nonsugar sweeteners cyclamate, saccharin, NHD, and D-tryptophan and the bitter tastants caffeine, quinine, limonin, and naringin were found to inhibit GRK2- and GRK5-phosphorylated rhodopsin (a GPCR) and PKA-phosphorylated casein in vitro (Figs. 4 and 5). Caffeine was most active in the inhibition of the three kinases (Fig. 4). Together with caffeine, L-tryptophan, cyclamate, and saccharin were very efficient at inhibiting GRK2, whereas caffeine, quinine, L-tryptophan, saccharin, NHD, and naringin were efficient inhibitors of GRK5. Caffeine, cyclamate, limonin, and NHD had the most efficient inhibitory effect on PKA. The sweetener acesulfame K and the bitter peptide cyclo(Leu-Trp) appeared to be the least effective inhibition could be shown. Inhibition could already be observed at relatively low concentrations, e.g., 2.5 mM caffeine, 600 μM NHD, 125 μM naringin (Fig. 5), and 100 μM limonin

Fig. 2. RT-PCR analysis of G protein-coupled receptor kinase (GRK)1, GRK2, GRK3, GRK5, GRK6, T2R4, and T1R3 mRNA in CV taste bud sheets and nonsensory epithelium (EP). cDNA was synthesized from CV and EP RNA and then amplified by PCR using specific primers for GRK1, GRK2, GRK3, GRK5, GRK6, T2R4, or T1R3; GAPDH was used as an internal reference gene. CON, parallel PCR omitting the RT step and using GRK2 primers. PCR products were separated on a 1.5% agarose gel stained with ethidium bromide. The expected PCR product sizes were 460 bp for GRK1, 559 bp for GRK2, 670 bp for GRK3, 841 bp for GRK5, 612 bp for GRK6, 560 bp for T2R4, 705 bp for T1R3, and 350 bp for GAPDH. M, 100-bp DNA marker ladder (Bioneer, Daejeon, Korea).
Kinetics of Phosphorylation

Inhibition of GRK2-phosphorylated rhodopsin by NHD and α-tryptophan produced saturation curves ($R^2 = 0.97$) when either rhodopsin (Fig. 6, A and B) or ATP (Fig. 6, C and D) concentrations were increased. NHD and α-tryptophan did not significantly modify the apparent $K_m$ values of GRK2 for rhodopsin (Fig. 6, A and B). In the NHD system, $K_m$ values ($\mu$M) were 0.71 ± 0.23, 0.79 ± 0.18, and 0.73 ± 0.22 for 0, 0.63, and 1.25 mM NHD, respectively. In the α-tryptophan system, $K_m$ values ($\mu$M) were 0.37 ± 0.08, 0.35 ± 0.05, and 0.43 ± 0.15 for 0, 5, and 15 mM α-tryptophan, respectively. On the other hand, both NHD and α-tryptophan reduced the $V_{max}$ values. In the NHD system, $V_{max}$ values (relative to controls monitoring phosphorylation level without the presence of a tautant) were 0.76 ± 0.08 and 0.35 ± 0.05 ($P < 0.05$) for 0.63 and 1.25 mM, respectively. In the α-tryptophan system, $V_{max}$ values were 0.73 ± 0.04 and 0.49 ± 0.06 ($P < 0.05$) for 5 and 15 mM, respectively.

Similarly, NHD and α-tryptophan did not significantly modify the apparent $K_m$ values of GRK2 for ATP (Fig. 6, C and D). In the NHD system, $K_m$ values (mM) were 0.30 ± 0.03, 0.11 ± 0.05, and 0.37 ± 0.18 for 0, 0.825, and 1.25 mM NHD, respectively (Fig. 6C). In the α-tryptophan system, $K_m$ values (mM) were 0.31 ± 0.03, 0.31 ± 0.12, and 0.35 ± 0.08 for 0, 3, and 6 mM α-tryptophan, respectively (Fig. 6D). Again, both NHD and α-tryptophan reduced the $V_{max}$ values. In the NHD system, $V_{max}$ values were 0.58 ± 0.06 and 0.48 ± 0.08 ($P < 0.05$) for 0.825 and 1.25 mM, respectively. In the α-tryptophan system, $V_{max}$ values were 0.60 ± 0.08 and 0.47 ± 0.03 ($P < 0.05$) for 3 and 6 mM, respectively.

**DISCUSSION**

Desensitization of GPCRs can be initiated when their cytosolic residues, containing either serine or threonine, are phosphorylated by selected kinases (32). Therefore, the hypothesis that amphipathic tastants interact with GRKs to affect signal termination requires that such tastants be able to permeate the cells under physiological conditions. In our previous experi-
ments (31), we showed that amphipathic tastants can permeate taste cells rapidly and against a concentration gradient when isolated taste bud preparations after a collagenase treatment are used. In that procedure, the basolateral side of the taste buds, not only their apical side (the route by which tastants interact with sensory taste cells), may have been exposed. In the present study, we took advantage of the autofluorescence of some of these tastants in two procedures designed to explore whether these tastants can permeate taste cells only through the apical side of the taste buds (taste pore). First, in situ imaging after the application of six amphipathic tastants to an intact CV papilla surgically removed without collagenase treatment indicated evident dynamics of tastant permeation into the CV taste bud cells located around the circular inner trench (Fig. 1, arrows). To observe the autofluorescence of these tastants more clearly (some are weak fluorescent compounds) under a confocal microscope, longer exposure times (i.e., up to 6 min) were used. Most important, the membrane-impermeant quencher KI had no effect on tastant fluorescence, indicating the presence of the tastant on the cytosolic side or in the cytosol of the taste bud cells.

In the second procedure, we stimulated the oral cavity of anesthetized rats with tastant solutions for 90 s, followed by immediate animal death and an HPLC determination of the tastants’ intracellular concentrations: millimolar levels of these tastants were found inside the taste cells. After oral stimulation, the entire separation process removed any tastant present on the extracellular surface of the cells. Analysis indicated only traces of tastant in the membranes: most was present in the cytosol. This indicates that tastants indeed permeated the membrane through to the cytosol and/or were released from the cytosolic side of the membrane during centrifugation. In either case, one can assume their potential access to GRKs or other membrane signal transduction components. Permeation of tastants into nonsensory epithelial cells was also observed (data not shown), as already reported when isolated taste bud sheets were used (31). The high sensitivity of the HPLC UV-visible diode-array and fluorescent detectors allowed us to detect the nanogram intracellular concentrations of these tastants in the 25-μg dry weight of a single CV taste bud sheet (40). The use of such a procedure was therefore appropriate for membrane-permeant tastants that show strong UV absorbance or fluorescence. Furthermore, one would assume that during the 25-min collagenase treatment after the oral stimulation, applied to separate the CV taste bud sheets from other epithelial, connective, and muscle tissues (40), tastants would leave the cells to the extracellular media, e.g., via MDR1 (18) or by simple diffusion. Indeed, parallel experiments (see MATERIALS AND METHODS) indicated significant (64–86%) efflux, which

Fig. 5. Tastants inhibit GRK2 (A and B)- and GRK5 (C and D)-induced phosphorylation of rhodopsin in a concentration-dependent manner. GRK2 and GRK5 were incubated with rhodopsin as indicated in Fig. 4 with the indicated concentrations of NHD, saccharin, caffeine, or naringin. Note that for saccharin phosphorylation, each concentration shows 2 bands, 1 for NaCl (control for sodium) and 1 for sodium saccharin. CON, phosphorylation level when tastants were not present. Results are means ± SE of 3 independent experiments.

Fig. 6. Michaelis-Menten equation curves: $K_m$ and relative $V_{max}$ values for the effect of rhodopsin (A and B) and ATP (C and D) concentrations on NHD and D-tryptophan inhibition of GRK2-phosphorylated rhodopsin. A and B: GRK2 was incubated with rhodopsin (from 0 to 2.1 μM) and NHD or D-tryptophan as indicated in MATERIALS AND METHODS. A: ●, control (no tastant added); ○, 0.63 mM NHD; ▼, 1.25 mM NHD. B: ●, control; ○, 5 mM D-tryptophan; ▼, 15 mM D-tryptophan. C and D: GRK2 was incubated with ATP (0–1.5 mM) and NHD or D-tryptophan as indicated in MATERIALS AND METHODS. C: ●, control (no tastant added); ○, 0.83 mM NHD; ▼, 1.25 mM NHD. D: ●, control; ○, 3 mM D-tryptophan; ▼, 6 mM D-tryptophan. Values are the means of 3 independent experiments for each data point.
was used to correct the estimated intracellular level of each tastant during the 90-s oral stimulation (Table 1). Together the results presented in Fig. 1 and Table 1 provide evidence that these and perhaps additional (though not necessarily all) amphipathic tastants rapidly permeate taste cells under physiological conditions, either through the taste bud pore or through the tight junction, and that such permeation also occurs in nonsensory lingual epithelial cells.

An additional requirement for the hypothesized interaction between membrane-permeant tastants and GRKs is the presence of GRKs in the same cells. GRK5 and potentially GRK2 have been previously identified in CV papilla (33), but there does not seem to be any evidence of their presence in taste cells. The presence of the bitter T2R1 (1) and the sweet (e.g., T1R3 and T1R2) (22, 26) taste receptors in subpopulations of cells present in rodent CV and other taste papillae has been demonstrated. Here, RT-PCR experiments (Fig. 2) suggested the presence of T2R4 and T1R3 along with GRK2, GRK3, GRK5, and GRK6 in isolated taste bud sheets of rat CV papillae. The same GRKs were found to be expressed in nonsensory lingual epithelial cells. Furthermore, antibodies against GRK2, GRK5, and GRK6 (with weak staining of GRK3) yielded clear, positive immunostaining in the 10-μm frozen sections of the CV papilla (Fig. 3). However, only GRK5 was clearly stained in the CV taste bud cells; GRK2 and GRK6 were notably stained in the surrounding epithelium, but their presence in the taste cells remains questionable. These immunostaining experiments cannot provide evidence of T2R4 and T1R3 receptor expression in the subpopulation of taste cells containing the GRKs. However, because GRK5 appeared to be expressed in almost all taste bud cells, it is likely that it coexpresses in cells expressing T2R4 and T1R3.

Subsequently, the effect of tastants on GRK2- and GRK5-phosphorylated rhodopsin, a well-studied model for GPCR phosphorylation (32), was investigated (Figs. 4–6). The amphipathic non sugar sweeteners cyclamate, saccharin, NHD, and D-tryptophan and the bitter tastants caffeine, quinine, limonin, and naringin, with diverse chemical structures, were found to inhibit GRK2- and GRK5-phosphorylated rhodopsin (a GPCR) and PKA-phosphorylated casein in vitro. Their effects depended on the kinase being tested. The nonamphipathic compounds sucrose and melibiose did not affect GRK activity when applied at concentrations of up to 200 mM. It should be noted that ~20% inhibition was found with these latter compounds at higher concentrations that are not relevant to intact mammalian cells; this minor inhibition was probably related to nonspecific osmotic factors.

Naringin (as well as some other flavonoids) has been found to inhibit phosphorylation, e.g., naringin inhibits AMP-activated protein kinase-activating kinase (20). Caffeine and theophylline have been recently found to be phosphoinositide 3-kinase inhibitors (14). However, nothing has been reported in relation to GRKs or PKA. In the present study, although inhibition could already be observed at relatively low concentrations, e.g., 2.5 mM caffeine, 600 μM NHD, 125 μM naringin (Fig. 5), and 100 μM limonin (Fig. 4), the tastant concentrations needed for kinase inhibition under the experimental conditions were at the millimolar level, higher than the micromolar levels usually used with other kinase inhibitors in vitro and in vivo medically (36) or experimentally in nontaste systems (43). Moreover, these tastants do not appear to be very specific because they inhibited PKA as well as the GRKs and may very well inhibit additional kinases. This suggests low tastant potency and raises the question of whether such inhibition has any physiological relevance. Nevertheless, the range of tastant concentrations applied here matches that used in taste stimulation during sensory and biochemical studies (16, 26, 35, 41, 45, 46), and the tastants were found inside the cells within seconds of their extracellular application (Table 1 and Ref. 31). The same tastants were also demonstrated recently to permeate other epithelial cells in situ, e.g., Xenopus laevis melanophores (48). Therefore, although these relatively high concentrations of amphipathic tastants suggest low potency, their physiological significance as kinase inhibitors in taste cells may result from their access to the cytosolic side of the taste cells and thus to GRK2 and GRK5, which are present in taste tissue (Figs. 2 and 3), and to PKA, which has been indirectly shown to be active in taste cells (42).

The mechanism(s) by which the tastants inhibit GRK-phosphorylated rhodopsin and PKA-phosphorylated casein is unclear. The inhibition of GRK2-phosphorylated rhodopsin by NHD and D-tryptphan produced a saturation curve with increasing concentrations of both rhodopsin and ATP (Fig. 6). NHD and D-tryptphan did not modify the apparent Km values of GRK2 for either substrate (rhodopsin or ATP), but the relative Vmax values were significantly reduced in the presence of both NHD and D-tryptphan. Therefore, the inhibition of GRK2 activity by both tastants appears to be noncompetitive, i.e., neither the ATP pocket nor the binding site for rhodopsin in the GRK2 molecule seems to be involved in the mechanism(s) of phosphorylation inhibition. Because tastants inhibited PKA when its catalytic unit was used (Fig. 4), such inhibition may be related to this part of the enzyme. Competitive, noncompetitive and uncompetitive types of inhibition have been found for various kinase inhibitors, and in many cases, the molecular events are unknown. When competitive inhibition is observed, it is usually related to interaction of the inhibitor with the ATP-binding pocket in the kinase and is noncompetitive with respect to the protein substrate (13, 29). Some inhibitors, depending on the kinase system, may act via two different mechanisms. The flavonoid genistein, for example, a known competitive inhibitor of ATP in some kinase systems (2, 43), can also induce both an increase in apparent Km values of adenylate kinase, suggesting uncompetitive inhibition (34). The slight variation in Km values for the controls in the NHD vs. D-tryptphan experiments (Fig. 6) was probably related to the different lots of GRK2 and rhodopsin used in the two experiments. The differences between the apparent Km values observed here for GRK2-rhodopsin and GRK2-ATP and those originally published (6 and 35 μM for rhodopsin and ATP, respectively; Ref. 4) may be related, in part, to the presence of glycerol (0.2%, 0.27 mM) in our experiments and its absence in the original studies (J. L. Benovic, personal communication). For example, we tested this level of glycerol in the PKA-casein incubation system and obtained ~30% inhibition of PKA activity. Therefore, although the presence of glycerol is important for kinase stability during storage, it may affect enzyme activity and perhaps its affinity for substrates.
Membrane-Permeant Sweet and Bitter Tastants Inhibit GRKs

In summary, a new hypothesis for the delayed taste termination caused by bitter tastants and nonsugar sweeteners is proposed. This hypothesis is based on results indicating that 1) such tastants permeate taste cells under physiological conditions, 2) GRKs are present in taste cells, and 3) such tastants inhibit the GRK- and PKA-induced phosphorylation of a GPCR in vitro. Importantly, transient concentrations were compatible with those used in taste stimulation and those permeating taste cells. Additional studies using the newly discovered taste GPCRs are needed to show their interaction with GRKs and possibly with other kinases, such as in intact cells in vivo, before anything can be unequivocally stated. The novelty of the proposed mechanism of signal termination may lie in the fact that the ligands themselves not only interact extracellularly with GPCRs to initiate the transduction chain, but may concomitantly interact intracellularly with downstream shutoff components to affect signal termination. The fact that tastants inhibit PKA and not just GRKs suggests that they inhibit other kinases as well. Because these tastants are components of our daily diets and may access other tissues along the gastrointestinal tract, these results may have implications for cellular signaling in tissues other than those involved in taste. Finally, the studied tastants, although not as potent as other kinase inhibitors, are membrane permeant, making them desirable for research purposes.

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