Rapid entry of bitter and sweet tastants into liposomes and taste cells: implications for signal transduction

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IN CONTRAST TO THE INITIAL STAGE in sour and salty taste transduction, which appears to involve the entry of H+ and Na+ through specific membrane channels (26), signal transduction of bitter and sweet tastants appears to involve putative G protein-coupled receptors (GPCRs) (17, 28, 46). Activation of effecter enzymes such as adenylyl cyclase, phospholipase C, and phosphodiesterases has been proposed, and changes in the level of signal molecules such as cAMP and inositol trisphosphate following sweet and bitter taste stimulation (1, 6, 18, 10, 48) may, as in olfaction (4), be in the 25- to 500-ms time range (1, 40). However, the time-intensity relationships of many non-sugar sweeteners (though not sugars) and bitter tastants may be slow in onset and may linger, sometimes up to a few minutes (2, 33). The delay in onset and the lingering aftertaste seem to be a taste-peripheral phenomenon as shown in electrophysiological and behavioral studies with experimental animals (15, 35), but the molecular basis for these temporal properties is ill-defined.

Interestingly, although the chemical structures of bitter tastants and non-sugar sweeteners are very diverse, all of these tastants are amphipathic, i.e., contain both hydrophobic and hydrophilic domains. The amphipathic properties of these tastants allow them to interact with liposomes to affect membrane potentials (3, 22). Most interestingly, amphipathic bitter and sweet tastants may, under some circumstances, induce depolarization in the membranes of living cells and stimulate transduction pathways and physiological responses in a variety of cells and tissues that are not related to taste cells and are, therefore, unlikely to contain taste receptors (7, 21, 24, 44). A variety of amphipathic neuropeptides, venom peptides, and non-peptide substances have been found to be direct activators of G proteins and likely to activate transduction pathways due to their ability to permeate the plasma membrane, in addition to their action on specific receptors (16, 30). In line with these results, some amphipathic non-sugar sweeteners and bitter tastants are also direct activators of G proteins in vitro (31, 39).

For the amphipathic tastants to interact directly with membrane transduction components located downstream to the GPCRs, they need to be able to permeate deeply into the membrane and/or translocate to the cytosolic side of the plasma membrane. To address this possibility, uni- and multilamellar liposomes were used to determine possible effects of amphipathic tastants on liposomal membrane integrity and their translocation by using fluorescence measurements and selected fluorescent tastants. Concomitantly, translocation experiments were conducted with actual taste cells.

MATERIALS AND METHODS

Animals and tastants. Male Sprague-Dawley rats (Anilab, Tal Shahar, Israel) weighing 170–200 g were used for the isolation of circumvallate (CV) taste bud and nonsensory epithelial sheets (see Permeation of tastants into taste cells). Quinine hydrochloride (bitter) and sodium saccharin (sweet) were purchased from Sigma Chemical (St. Louis, MO). Cyclo-(Leu-Trp), a casein-hydrolysate-derived cyclodiodeptide (38), which is about two- to threefold more bitter than caffeine, was
K2SO4, and Na2SO4 were from Merck (Darmstadt, Germany). Other chemicals. Egg phosphatidylcholine (PC) from soybean lecithin and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Azoletin, HEPES, valinomycin, and melittin were purchased from Sigma; DMSO was from Fluka (Germany); 3-3′-diethylthiadicarbocyanine iodide (diS-C2(5)) was from Molecular Probes (Eugene, OR); and KI, K2SO4, and Na2SO4 were from Merck (Darmstadt, Germany).

Preparation of liposomes. Multilamellar vesicles (MLV) were prepared either from PC with cholesterol or from azoletin, based on the method described by Kumazawa et al. (22). Briefly, dry lipid and cholesterol were dissolved in a chloroform-methanol mixture (2:1 vol/vol), such that the solution contained 10% (wt/wt) cholesterol. The solvents were evaporated under a nitrogen stream, and the lipids were resuspended in appropriate buffer via vortex mixing. Except in “diffusion potential” experiments, the buffer was PBS. Small unilamellar vesicles (SUV) were prepared by sonication of MLV for 15–30 min in a bath-type sonicator (G1125SP1; Laboratory Supplies, NY) until the suspension became clear (36). For the preparation of large unilamellar vesicles (LUV), the MLV were extruded once through a 0.2-µm membrane and eight times through a 0.05-µm membrane (Nucleopore, Pleasanton, CA). A quick freeze-thaw step was included before the first extrusion. In most experiments, the lipid composition was PC:cholesterol (4.5:1 mol/mol). In few cases, the diffusion potential experiments, azoletin was employed as a stabilizer.

Fluorometric detection of small membrane pores (diffusion potential). Pore-mediated diffusion-potential collapse was detected fluorometrically as previously described (36). A liposome suspension (5 µl) prepared in K+ buffer (50 mM K2SO4, 25 mM HEPES, pH 6.8) was added to 1 ml of isotonic K+ free buffer (50 mM Na2SO4, 25 mM HEPES, pH 6.8) in a glass tube, and the dye diS-C2(5) was added. pH values were 6.8, 6.8, and 6.7 for cyclo(Leu-Trp), quinine, and saccharin-containing suspensions, respectively. The final lipid concentration was 40 µM and the final dye concentration was 1 µM. Ten micromolar 10−7 M valinomycin in solution was added to slowly create a negative diffusion potential inside the vesicles by selectively carrying K+ outside; this, in turn, caused quenching of the dye’s fluorescence. An SLM 4800 spectrophotometer coupled to a personal computer with fluorescence data acquisition was used. After the fluorescence intensity was stable (3–10 min), the liposomes were added, causing permeation of the other ions (influx of Na+ and efflux of SO42−). Dissipation of the diffusion potential and release of the dye caused an increase in fluorescence intensity. The peptide melittin, which is known to be a strong membrane-pore-forming activator (36), was used at 35.5 µM as a positive control. The excitation and emission wavelengths were 620 and 670 nm, respectively.

Translocation of amphipathic tastants through liposome membranes. The assay was based on fluorescence quenching by KI (23) whose penetration through liposomal membranes was extremely slow. The idea is that tastant molecules that translocate and either remain bound on the internal side of the membrane or distribute into the internal medium would be protected from KI. KI was added externally to a suspension containing relatively large concentrations of liposomes and tastants to maximize the effect of binding and translocation of tastants. Sodium thiosulfate and potassium phosphate were added to the 500 mM KI solution according to Ref. 23. pH values of solutions containing liposomes and tastants in buffer were 7.0, 7.25, and 6.8 for quinine, cyclo(Leu-Trp), and saccharin, respectively. The relevant pKa values for quinine (25) and saccharin (47) are 8.52 and 1.8, respectively. Cyclo(Leu-Trp) is uncharged under these conditions (29).

The suspension was diluted, e.g., 200-fold into PBS containing 500 mM KI final concentration. The dilution step was introduced to reduce the optical density, avoiding turbidity. The analysis was aimed at determining the fluorescence fraction, α, of tastant molecules protected from KI. If F is the fluorescence intensity of the molecules incubated with liposomes and diluted in KI solution and F5 are the fluorescence intensity of the incubated suspension after dilution in buffer while FKI denotes the fluorescence intensity of the suspension diluted in the KI solution without preincubation, then

\[ F = F_5 \alpha + F_K(1 - \alpha) \]  
\[ \alpha = \frac{F - F_K}{F_5 - F_K} \]

Effect of binding of cyclo(Leu-Trp), quinine, and saccharin to liposomes on the fluorescence intensities. MLV at lipid concentrations of 4.5, 9, and 18 mM were incubated with the tastants, and changes in the fluorescence intensities of cyclo(Leu-Trp), quinine, and saccharin in the presence of liposomes were determined. To perform measurements under lower optical density (<0.3), the samples were diluted after incubation, before fluorescence measurements. Fluorescence intensities were also measured for the tastants and lipid at the same final concentrations without incubation.

Association of tantant molecules with liposomes. The experiments employed a 5 mM stock solution of quinine or a 400 mM stock solution of saccharin in double-distilled water, or a 20 mM stock solution of cyclo(Leu-Trp) in 100% DMSO, and double-distilled water was added. The final content of DMSO in the tube did not exceed 2%. MLV PC:cholesterol (4:5:1) liposomes were prepared in 500 mM PBS. The final lipid concentration was 18 mM, and the extraliposomal tastant concentration at t = 0 was 0.5 mM for quinine and cyclo(Leu-Trp) and 40 mM for saccharin.

Association of tastants with liposomes was determined by three procedures. In all cases, a suspension of MLV liposomes and tantant molecules was filtered through 0.2-µm filters (Sartorius, Germany) after a 1-h incubation at room temperature, and the decline in tantant concentration in the filtrate was determined by fluorescence measurements. MLV liposomes could not pass through 0.2-µm pores. Thus the reduction in the tantant concentration was equated to the fraction of bound tantant.

For quinine and saccharin, in addition to the filter method just described, we employed a procedure based on centrifugation. After incubation for 5, 15, or 30 min, the samples were ultracentrifuged (Centrifron T-1170, 133,000 g) for 1 h. The supernatants were separated, and tantant concentrations were determined by fluorescence measurements. The fraction of depleted tantant corresponded to its fraction associated with the liposomes. As a test of mass conservation, the pellets were diluted 500-fold and the associated amount of tantant was determined. For the cyclo(Leu-Trp) peptide, association with liposomes was also determined by the following procedure. Cyclo(Leu-Trp) peptide (0.5 mM) was placed inside a dialysis tube (Medicell, London). The peptide was free to pass through the tube while MLV liposomes (18 mM final lipid concentration) were placed outside the tube. The first 2 h of incubation were at room temperature, and the incubation was continued at 4°C for an additional 36 h to allow the system to equilibrate. At periods before 24 h, a plateau was not reached.
No binding of cyclo(Leu-Trp) to the dialysis tube was observed.

Permeation of tastants into taste cells. Rats were anesthetized with ether and then decapitated. CV taste bud and nonsensory epithelial sheets were prepared from rat tongues by means of subepithelial collagenase treatment (4 mg/ml, collagenase Boehringer type D, 0.28 U/mg, and Sigma type I-S trypsin inhibitor, 4 mg/ml) (43) during a 35-min incubation in modified Tyrode’s solution [140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM glucose, 10 mM pyruvic acid, 10 µM phenylmethylsulfonyl fluoride, and 10 mM HEPES adjusted to pH 7.4 with Tris base]. Separated CV and epithelial sheets were incubated in the Tyrode’s solution with either quinine (2 mM), cyclo(Leu-Trp) (1 mM; in the presence of 0.2% DMSO, which was also present in the corresponding controls), or saccharin (20 mM) at 30°C for 30 s or 5 min. These concentrations of tastants are compatible with those that elicit taste sensation in rats (5, 12, 38). After incubation, using a fine forceps, taste bud sheets were transferred five times to different test tubes containing Tyrode’s solution to wash all tastant molecules from the extracellular medium. Cells were then permeabilized by freeze-thawing (~70°C) in deionized water, and membranes were removed by centrifugation (30 min, 28,000 g, 4°C). The intracellular content was collected and lyophilized, and the level of these tastants in CV and epithelial cells was determined with an L-7100 Merck-Hitachi HPLC equipped with F-1050 fluorescence and L-7455 ultraviolet (UV)-visible diode-array detectors set up in a sequence for concomitant determinations, using a LiChrospher 100 RP-18 column (5 µm, 250 mm, 4 mm; Merck) with an RP-18 precolumn. This setup allowed simultaneous tastant determinations by the fluorescence and UV-visible detectors. The mobile phase (isocratic separation) for the analysis of quinine and cyclo(Leu-Trp) consisted of an acetonitrile aqueous phosphate buffer (10 mM) mixture (50:50, vol/vol) containing 25 mM SDS and 3 mM tetrabutylammonium bromide, adjusted to pH 2.3 with orthophosphoric acid (50). Flow rates were 0.9 and 0.7 ml/min for quinine and cyclo(Leu-Trp), respectively. The mobile phase (also isocratic separation) for saccharin determination consisted of H2O (including 1.5% vol/vol acetic acid) and methanol (60:40 vol/vol) at a 0.5 ml/min flow rate. The fluorescence detector was set for the excitation and emission values applied in the liposome experiments. Samples (20-µl aliquots) were injected. Peaks of the three tastants were identified and quantitated with known markers [retention time (RT) = 5.1, 16.2, and 3.6 min for saccharin, quinine, and cyclo(Leu-Trp), respectively] injected alone and coinjected with unknown samples using the fluorescence detector. The UV-visible spectrum and the correlation of the unknown peaks with those of the saccharin and cyclo(Leu-Trp) standards were monitored for further identification using the diode-array detector.

Imaging of in situ permeation of tastants into CV taste cells using confocal microscopy. The CV sheets described above were subjected to additional collagenase treatment for 10 min, and then to Ca2+-free Tyrode’s solution containing 1 mM EGTA for 2–3 min (1). With the use of fine forceps, the tissue sheets were shaken slightly to detach the taste buds. Single CV taste buds were then adhered to polylysine-coated slides (Sigma).

A Zeiss LSM 410 confocal laser-scanning system attached to the Zeiss Axiovert 135M inverted microscope with ×63/1.2 C-Apochromat water immersion lens was utilized to monitor the continuous permeation of the three fluorescent tastants, cyclo(Leu-Trp), quinine, and saccharin, into the taste bud cells. The system was equipped with a 25-mW air-cooled argon laser (488-nm excitation line with 515-nm long pass barrier filter) for the excitation of green fluorescence. Red fluorescence was excited with the internal helium-neon laser (543-nm excitation line with 570-nm long pass emission filter). The differential interference contrast images according to Nomarski were collected using a transmitted light detector.

A slide containing isolated single taste buds was viewed under the microscope, and an appropriate taste bud (one showing natural shape with no deformation) was selected for the 10-min imaging experiment. At zero time, either cyclo(Leu-Trp) (1 mM), quinine (2 mM), or saccharin (20 mM) was added, and the autofluorescence of the tastant was set to background level. The increase in fluorescence in the tested taste bud was then monitored for 10 min. The volume of the tastant solutions was manifold higher than that of the taste buds. Therefore, one may assume no change in concentration in the extracellular medium during experiment. Viability of the cells was verified using Trypan blue staining at the end of each experiment.

To reduce the visual noise, each confocal optical section was examined in the fast-scan acquisition mode (512 pixels/line) by averaging eight images before the final image was produced on the monitor. In each experiment, exciting light intensity, background, aperture, contrast, and electronic zoom size were maintained at the same levels.

Confocal images were converted to TIFF format and transferred to the Zeiss imaging workstation for pseudocolor representation. Image brightness and contrast levels were processed using the Zeiss and Adobe Photoshop programs.

Localization of amphipathic tastants in the taste cells. To localize tastants within the cells, single taste buds were mounted on a slide coated with neutralized type I collagen (37). Taste bud cells were then incubated with one of the three tastants for 5 min at room temperature and then fixed with cold (~20°C) absolute methanol for 5 min. Nuclei were counterstained with propidium iodide (Sigma, 1 µg/ml) for 10 min. Finally, slides were mounted with 1,4-diazabicyclo[2.2.2]octane containing glycerol solution (to reduce fading) for confocal microscopy.

RESULTS

Fluorometric detection of enhanced permeability (diffusion potential). Tastants were mixed with SUV (at constant concentration), pretreated with the fluorescent dye and valinomycin (see MATERIALS AND METHODS). Melittin was used as a positive control. To control for the possible effect of osmotic pressure, we applied 30 mM sucrose and 30 mM NaCl, which resulted in no interaction with the liposomes. Application of the bitter tastants cyclo(Leu-Trp) and quinine and the sweetener saccharin (Fig. 1) each induced an immediate increase in fluorescence intensity in a concentration-dependent manner, indicating marked interactions of tastant molecules with membrane liposomes. About 70% fluorescence recovery was observed with the bitter tastants and 28% with saccharin. At physiological concentrations for taste sensations induced by the above tastants (5, 12, 38), the percentages of fluorescence recovery were ~40% for quinine and cyclo(Leu-Trp) and 10% for saccharin.

Effect of liposomes on fluorescence intensities of cyclo(Leu-Trp), quinine, and saccharin. Incubation of the amphipathic tastant quinine with liposomes resulted in decreased fluorescence intensities, whereas an in-
Rapid Permeation of Amphipathic Tastants into Taste Cells

The increase in the fluorescence intensity of the cyclo(Leu-Trp) is in accord with the increase in the fluorescence intensity of tryptophan in a hydrophilic environment (8). No significant changes in fluorescence intensity were observed in the case of saccharin. The decreased fluorescence intensity of quinine when bound to liposome membranes (MLV) could reflect its fluorescence in a hydrophobic medium (data not shown). This may also be due to enhanced self-quenching, reflecting the enhanced concentration of a tastant in the membrane relative to its concentration in buffer. Enhanced self-quenching might also reflect an aggregation of tastant molecules within the membrane. The results in Table 1 (200 µM quinine) demonstrate that the largest decrease in fluorescence intensity occurred in the case of the largest lipid concentration, i.e., at the largest lipid-to-tastant concentration ratio. In this case, the surface concentration of tastant molecules is expected to be the smallest. Hence it appears that the observed decrease in quinine fluorescence is due mainly to the effect of the hydrophobic environment in the membrane and not to enhanced self-quenching of tastant molecules in the membrane. Clearly, the fraction of quinine molecules bound to the liposomal membranes should increase with lipid concentration and decrease with transtant concentration. In the case of quinine, we tested this point further by using 18 mM lipid and fourfold less quinine (50 µM). In this case, there was no observed decrease in fluorescence within the experimental uncertainty. This implies that, in the case of quinine, self-quenching in the membrane also plays a role. Admittedly, the use of 50 µM quinine (followed by 200-fold dilution) is at the limit of reliable detection sensitivity.

The results in Table 1 cannot support or refute self-aggregation of the cyclo(Leu-Trp) within the membranes. Because no significant changes in fluorescence intensity of saccharin due to the presence of liposomes were observed, data for saccharin were not included in Table 1.

Kinetics of translocation of cyclo(Leu-Trp), quinine, and saccharin through MLV. The experimental procedure and determination of percent translocated tastant molecules are described in MATERIALS AND METHODS (see Eqs. 1 and 2). Figure 2 gives the percent of cyclo(Leu-Trp), quinine, and saccharin fluorescence protected from KI, at times varying from 0 to 30 min, and the kinetics of translocation over short duration, from 0 to 20 s. These results demonstrate that a remarkable fraction of the cyclo(Leu-Trp) peptide molecules translocated in a matter of seconds. After 30 min, the fraction of translocated peptide reached 27 ± 16%. The results of translocation through LUV were largely similar to those with MLV (not shown). The fraction of quinine

Table 1. Effect of liposomes on fluorescence intensities of quinine and cyclo(Leu-Trp)

<table>
<thead>
<tr>
<th>Lipid/Tastant Concentration</th>
<th>Changes in Fluorescence, %</th>
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<tbody>
<tr>
<td>18 mM lipid/400 µM quinine</td>
<td>-14 ± 1.5</td>
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<tr>
<td>18 mM lipid/200 µM quinine</td>
<td>-19 ± 2.0</td>
</tr>
<tr>
<td>9 mM lipid/200 µM quinine</td>
<td>-12 ± 1.5</td>
</tr>
<tr>
<td>4.5 mM lipid/200 µM quinine</td>
<td>-6 ± 1.5</td>
</tr>
<tr>
<td>18 mM lipid/0.5 mM cyclo(Leu-Trp)</td>
<td>+17 ± 5.0</td>
</tr>
<tr>
<td>18 mM lipid/0.25 mM cyclo(Leu-Trp)</td>
<td>+24 ± 6.0</td>
</tr>
<tr>
<td>9 mM lipid/0.25 mM cyclo(Leu-Trp)</td>
<td>+8 ± 2.0</td>
</tr>
<tr>
<td>4.5 mM lipid/0.25 mM cyclo(Leu-Trp)</td>
<td>+3 ± 0.5</td>
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Values are means ± SE of triplicate measurements. Saccharin fluorescence was not affected by the presence of liposomes.
translocated through MLV (external) membranes at 5 s was ~15% (0.5 mM quinine and 18 mM lipid), whereas with 9 mM lipid it was 7.8% (data not shown). After 30 min, the fraction of translocated quinine reached 60 ± 12%. The fraction of saccharin translocated after 30 min reached ~6%, but, despite the lower fraction of translocated molecules, the saccharin concentration inside the MLV liposomes may reach ~1 M due to the high extraliposomal concentration used (1.5 M). At a lower lipid concentration (4.5 mM), we did not observe saccharin translocation within the experimental uncertainty. With all three tastants a prolongation of the incubation time to 60 min yielded marginal increases in percent translocation.

Association of tastant molecules with liposomes. The percent association of tastants with MLV liposomes is shown in Table 2. The incubation of 0.5 mM quinine with 18 mM MLV yielded 68 ± 5% association by the filter method or 91 ± 6% by the centrifugation method. These fractions were obtained after 5 min. Further incubation for 15 or 30 min did not change the outcome. The association of 40 mM saccharin with liposomes was ~10%. In the centrifugation experiment, the samples were spun for 1 h in addition to the incubation time, which might give a somewhat higher outcome. With cyclo(Leu-Trp), the floating of the liposomes ruled out the centrifugation procedure. Instead, a dialysis tube method was employed. The percentages of peptide associated with the liposomes were 27 ± 2% when applying a filter and 25 ± 2% when the dialysis tube was used.

Permeation of saccharin, quinine, and cyclo(Leu-Trp) into CV and epithelial cells. Rats find the taste of saccharin appealing at normal concentrations and they reject the bitter tastant quinine (5, 12). Recent behavioral experiments (Krizhanovsky, unpublished observations) have also indicated that the bitter tastant cyclo(Leu-Trp) is as aversive to rats as it is bitter to humans (38) and that rats also respond to its taste electrophysiologically (T. Yamamoto, personal communication).

Estimated intracellular tastant concentrations after incubations of CV and epithelial tissue sheets with tastants for 30 and 300 s are shown in Table 3. Direct identification using fluorescence and UV-visible diode-array HPLC detectors, including UV spectral analysis, clearly indicated the presence of peaks corresponding to saccharin, quinine, and cyclo(Leu-Trp) in the CV and epithelial tissues incubated for 30 s with these tastants. Correlation of the unknown peaks with the appropriate standards in the UV-diode-array chromatograms were 99 and 98% for cyclo(Leu-Trp) and saccharin, respectively. The peaks for the respective tastants were missing in the corresponding control CV and epithelial tissues incubated without the added tastants. With the exception of saccharin, similar peak RTs were observed in the sample incubated for 5 min. However, in the 5-min saccharin CV-incubated and saccharin epithelial-incubated samples, no saccharin peak was found in the HPLC analysis. Rather, a new fluorescent peak appeared at a lower RT (4.14 min) than saccharin (RT = 5.10 min). This new peak had a very similar UV spectrum to that of saccharin, and it was absent in the HPLC chromatogram of control CV and epithelial samples incubated without saccharin. Thus, following the long (5-min) incubation period, saccharin was probably metabolized to a different, unidentified fluorescent molecule.

To calculate the intracellular contents of tastants, one can approximate the volume of a taste cell. We assume that the CV papilla of rats at the age of ~50 days contains ~400 taste buds (34) with 100 cells each, i.e., ~40,000 cells/CV papilla. Assuming that a taste cell is a 6-µm diameter globe (soma) and a 20 µm cylinder (dendrite), the volume of a single taste cell will be ~0.25 pl (no change in cell volume is further assumed). The cytosolic free solvent space is smaller than the total cell volume, but, for the purpose of these calculations, we use the 0.25-pl value as the cell cytosol volume of a taste cell and assume that the volume of a nonsensory epithelial cell is similar. Based on these calculations, it is evident that, during a 30-s incubation period, the intracellular concentrations of tastants in the CV cells increase 3.5-, 7.5-, and 7-fold for cyclo(Leu-Trp), quinine, and saccharin, respectively, relative to extracellular concentrations of these tastants (Table 3). The rate of tastant permeation was fast in the first 30 s and decreased significantly thereafter, although the accumulation process continued. Similar magnitudes of tastant permeation were observed in the epithelial cells, except for quinine, where the rate was two- to fourfold lower than that in the CV cells.

Dynamic accumulation of amphipathic tastants followed by confocal microscopy. The intracellular accumulation of these fluorescent tastants could be clearly seen...
under the microscope (Fig. 3). At zero time, weak fluorescence was observed when the three tastants were applied extracellularly. This level was set to zero (background). Thereafter, one taste bud under the focal plane was selected to monitor the increase in fluorescence due to tastant accumulation. To minimize possible fluorescence bleaching during measurements, a minimal number of fluorescent exposures was applied. The Trypan blue test was negative through the entire experiments (no cell death was observed), indicating that the cells remained viable. Some dead cells were detected only 1 h after the experiment was completed. Furthermore, the continuous tastant accumulation in taste cells also indicates that the plasma membrane of these cells was not leaking during the test period. Two minutes after application of cyclo(Leu-Trp), quinine, or saccharin (Fig. 3, panel 3, in A, B, and C), fluorescent accumulation could already be seen in selected areas but not in the entire taste bud cells. After 5 min, the fluorescence intensity of the three tastants (particularly that of quinine) increased, and, under all circumstances, after 10 min, the fluorescence spread over the entire taste bud.

Localization. In some experiments, we incubated each tastant for 10 min with single CV taste buds adhered to glass slides, and we fixed the cells followed by staining of the nuclei (Fig. 4). This procedure enabled multiple fluorescent exposures for each preparation without fluorescence bleaching. A focal plane of a few cells was selected under confocal microscopy where cell nuclei, cytosol, and cell borders could be clearly seen. In the control CV cells incubated without tastants (Fig. 4, panel 1), only red nuclei with no green fluorescence in the cytosol were observed. In contrast, strong green fluorescence in the cytosol with slight staining of the nuclei was seen in the taste cell samples incubated with either cyclo(Leu-Trp), quinine, or saccharin (Fig. 4, panels 2–4, respectively).
DISCUSSION

Amphipathic tastants, applied at concentrations compatible with those that elicit taste sensation, produced an immediate and concentration-dependent change in the diffusion potential of SUV liposomes. Thus, similar to the amphipathic peptide melittin, these amphipathic tastants form small pores in the membrane, in line with similar findings for some bitter and non-sugar sweeteners using liposomes and lipid bilayers (3, 22). However, the pore formation capacity of these tastants is significantly lower than amphipathic peptides with several (>20) amino acids. In contrast to the amphipathic tastants, hydrophilic ones, such as saccharin or NaCl, were not effective in dissipating the diffusion potential. The main purpose of employing liposomes was to monitor the process of tastant translocation. There are very few comparisons in the literature between the translocation rates or permeabilities of non-electrolytes through plasma membranes of living cells and liposomes. Indeed, in contrast to the very low permeabilities through liposomes known for cations, anions, glucose, and sucrose, the rates of this translocation are very rapid during the first 20 s (Fig. 2).

What is the mechanism of translocation or permeation of amphipathic tastants through liposomal membranes? It is widely accepted that the permeabilities of most non-electrolytes through lipid bilayer membranes can be adequately explained by the solubility-diffusion model (42). Only slight variation of pH values (6.8-7.25) was observed in all of the liposome experiments (Figs. 1 and 2; Tables 1 and 2). Under these conditions, quinine is a slightly cationic amphipathic tastant due to its \( pK_a \) (8.52). Hence significant fractions of quinine remain in their uncharged form, which indeed correlates with the solubility-diffusion model and the ability of quinine to intercalate into the liposomes. Saccharin (\( pK_a \) 1.8) is an anion under these conditions. However, saccharin appears to be more lipophilic than would be inferred from its dissociation constant (49). This may explain its moderate permeability through liposomes in the present experiments. The cyclo(Leu-Trp) peptide is uncharged under these conditions (29). Interestingly, the slightly cationic quinine translocates more extensively than the cyclo(Leu-Trp). The extent of the translocation of the tastants through liposomes (Fig. 2) follows the same sequence as that shown in their total association (Table 2) with the following order: quinine > cyclo(Leu-Trp) > saccharin. Nevertheless, in view of the capacity of the tastant to form small pores in liposomal membranes, it is instructive to consider another model for the translocation of amphipathic molecules.

In a recent study on the interaction of the amphipathic peptide GALA (30 amino acids), which forms pores in liposomes of a variety of compositions, its translocation did not occur, even after very long residence time in the liposomal membrane, unless at least four to six peptide molecules were bound per vesicle (32). The process of peptide translocation and pore formation, whereby the peptide’s orientation is perpendicular to the membrane, follows peptide aggregation in the membrane after an initial stage of binding parallel to the membrane. This mechanism of translocation is very different from the solubility-diffusion model, since it requires the binding of some critical number of amphipathic molecules in the liposomal membrane, and in the above example the process is based on cooperative action. The amphipathic peptides alamethicin and magainin-2 were reported to be adsorbed in a parallel orientation to the membrane surface at low peptide-to-lipid molar ratios, whereas at high ratios they were inserted perpendicularly into the membrane (14, 27). The amphipathic peptides alamethicin, magainin, and melittin are much less effective in forming pores than the peptide GALA, where a peptide-to-lipid ratio of 1:2,500 (or 1:5,000) was sufficient. Similarly, the amphipathic tastants quinine, cyclo(Leu-Trp), and saccharin are much less effective than melittin (Fig. 1). Hence tastant concentrations higher than that of melittin are required for formation of small pores. The tastant-to-lipid ratios used in the translocation experiments (Fig. 2) were much smaller than those in the demonstration of pore formation (Fig. 1). Under the former conditions, translocation could occur, but the entry of KI into the liposomes was largely prevented. This implies the absence of sufficiently large pores. Under these conditions the solubility-diffusion mechanism may be predominant.

It should be noted that both the induction of diffusion potential changes (22) (Fig. 1) and the translocation of tastants through liposomal membranes (Fig. 2) may be critically dependent on the lipid composition of those membranes. In the current experiments, saccharin, for example, induced changes in the diffusion potential and translocated through vesicles made of azolectin but not of PC, whereas quinine and cyclo(Leu-Trp) translocated effectively through the PC vesicles.

The results further demonstrate that these amphipathic tastants permeate very rapidly and accumulate inside living taste cells (Table 3, Figs. 3 and 4). It should be noted that, in the taste bud preparation sheets (Table 3), accumulation of tastants occurred under conditions where the structural organization of the taste buds within the CV papilla is likely to remain intact. Therefore, the contact between the tastants and taste cells was mainly through the taste pores. Even a relatively weakly fluorescent molecule, such as saccharin, could be detected inside the taste cells within 30 s of incubation of isolated taste buds by using a fluorescent HPLC detector. If this initial accumulation is assumed to increase linearly with time, then the average rate of saccharin accumulation in the CV cells during the first 30 s is 4.7 mM/s. Under the same conditions, the average accumulation rates for quinine and the cyclo(Leu-Trp) peptide are \( \sim 500 \) \( \mu \text{M/s} \) and \( \sim 117 \) \( \mu \text{M/s} \), respectively. This means that, within 1–2 s, the levels of these tastants inside the taste cells are compatible with those needed to elicit taste sensation (5, 12, 38). These extremely rapid rates of tastant accumulation in taste cells probably activated protective mechanisms of tastant elimination from inside to
the extracellular medium, as in the case of MDR1 found recently in rat CV cells (19). It remains to be further tested whether such mechanisms may explain, at least in part, the reduced accumulation rates after the first 30 s (see Table 3, 30 s vs. 300 s).

The results presented here are the first to show that such large amounts of amphipathic tastants rapidly permeate taste bud cells. Since rapid permeation occurred through liposomes, one needs to evaluate whether the mechanism of tant permeation involves accelerated diffusion or cooperative action, as elaborated in the discussion of liposome results, or whether perhaps active transporters are involved. Unless specific cytosolic proteins bind the amphipathic tastants, the dramatic accumulation of tastants in the cytosol of taste cells may not be solely due to accelerated diffusion (11, 42). Interestingly, the uptake of saccharin (activation energy was needed) into Streptococcus mutants led to a 30- to 40-fold higher concentration within cells than in the incubation medium (49). Recently, fatty acids were found to modulate K+ channels in taste cells, thus suggesting gustatory cues for dietary fat (10). The half-life of fatty acid movement from the outer to the inner phospholipid bilayer may be as short as 20 ms (20), and this may be relevant to the detection of fatty taste. However, such a mechanism may be challenged by the membrane-associated and cytoplasmic proteins that bind fatty acids in various tissues (11) and by the occurrence of fatty acid putative transporters in the CV taste cells (9). Further research is needed to explore the mechanism by which such a dramatic accumulation of amphipathic tastants in taste cells occurs.

Could the rapid permeation of these tastants into taste cells be related to taste signal transduction pathways activated by these tastants? A variety of amphipathic peptides (e.g., the wasp venom mastoparan) and amphipathic nonpeptide compounds (e.g., compound 48/80) have been shown to be direct activators of G proteins that mimic receptor activities and are likely to induce cellular responses by receptor-independent pathways (13, 16, 30). The evidence for the permeation of amphipathic tastants into taste cells within a time course corresponding to taste sensation is now provided.

A definitive test of the potential of all amphipathic bitter and sweet tastants to directly activate G proteins is not yet available, but it is well known that many drugs that are direct activators of G proteins are also bitter (e.g., procaine, tetracaine) (13). In fact, the tastants saccharin and quinine were potent direct activators of transducin and a purified mixture of Gi/Go proteins in vitro (all contained the intact α- and βγ-subunits), when applied at concentrations similar to those used here (31). Therefore, under such rapid permeation of amphipathic tastants into taste cells, tastant molecule access to membrane-transduction components, such as the α- and the βγ-subunits, or to effector enzymes (7) is very likely. Specificity is likely to be achieved by the large variety of G protein subunits, by various transduction components in different living cells, and by the composition of particular membranes.

Such mechanisms may indeed explain the ability of some amphipathic tastants to affect transduction pathways and cellular responses in cells not related to taste. These include depolarization of neuroblastoma cells by some bitter tastants (21), activation of adenyl cyclase cascades by saccharin in liver and muscle membranes (44) and in fat cell membranes (7), and stimulation of insulin release from isolated pancreas islets by the non-sugar sweetener acesulfame K (24). Alternatively, one may hypothesize that such tastants act on receptors not related to taste.

The permeation of amphipathic tastants into taste cells may further explain, at least in part, the slow taste onset and lingering aftertaste that is so salient in humans’ perception of non-sugar sweeteners and bitter tastants and that may result from concomitant activities of receptor-dependent and receptor-independent mechanisms. The present results may thus open the way for applied research aiming to modify these temporal properties, which are the main drawbacks in the use of many non-sugar sweeteners in the low-calorie soft drink industry.

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