Sucrose-stimulated subsecond transient increase in cGMP level in rat intact circumvallate taste bud cells

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The initial stage of sour and salty taste transduction appears to involve the entry of H+ and Na+ ions through specific membrane channels (13). In contrast, the initial signal transduction of bitter and sweet tastants is believed to involve putative G protein-coupled receptors (9, 15, 28). Intracellular administration of cyclic nucleotides such as cAMP and cGMP into taste cells of frogs, mice, and hamsters produces cell depolarization (3, 7, 29); some of these cells are also depolarized in response to stimulation by sucrose and some nonsugar sweeteners. A 10-s stimulation of a crude preparation of whole fungiform papillae from mice by sucrose resulted in increased cGMP content (18). In rats, sugars have been found to stimulate adenylyl cyclase activity in crude lingual taste membrane preparations and cAMP formation in isolated circumvallate (CV) taste bud sheets (25, 26). Nonsugar sweeteners, but not sucrose, stimulated the formation of d-myo-inositol 1,4,5-trisphosphate in taste cells of rats and mice (4, 21). Identification of the G proteins transducin and gustducin (closely related to transducin) in taste cells (16, 17) has led to the suggestion that during taste transduction, these G proteins activate phosphodiesterase(s) (PDEs) in a manner similar to the activation of rod transducin in vision (11, 24).

Evidence has accumulated indicating close cross talk among different signal pathways in various tissues: i.e., a signal(s) may be important at the initial stage of taste stimulation, whereas others may be involved in later events. Thus time course experiments are needed to identify this sequence of events in sensory “real time” transduction. Such biochemical experiments in olfaction and taste systems have been elegantly conducted with cell-membrane preparations or cell homogenates of the target tissues using a fast Quench Flow Module (5, 23). However, this technique appears to be limited to in vitro measurements because responses in intact cells cannot be determined (23). We previously employed a fast pipetting machine with an electronic timer to monitor signal-molecule formation in intact taste cells at 500-ms stimulation (4). In the present study, to monitor cyclic nucleotide formation in intact taste cells in response to sucrose stimulation in a time range below 500 ms, we used an improved, computerized version of this fast-pipetting system (FPS).
MATERIALS AND METHODS

Animals, tasteants, and chemicals. Male Sprague-Dawley rats (Anilab, Tal Shahar, Israel) weighing 170–200 g were used for the isolation of CV taste bud and nonsensory epithelial (EP) sheets. The following chemicals were used: HEPEs and DMSO (Fluka); collagenase (type D, Boehringer); per-chloric acid, glucose, and sucrose (Merck); soybean trypsin inhibitor (type I), phenylmethylsulfonyl fluoride (PMSF), IBMX, acetic anhydride, triethylamine, pyruvic acid, and forskolin (all from Sigma); 125I-cAMP and 125I-cGMP (American); vanadyl riboflavin (Calbiochem); and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Biomol).

Solution preparation. Stock solutions of the following inhibitors were dissolved in DMSO. These solutions were used to prepare final concentrations of 40 μM zaprinast, 20 μM denbufylline, or 10 μM ODQ in Tyrode solution (140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM glucose, 10 mM pyruvic acid, 10 μM PMSF, and 10 mM HEPEs) adjusted to pH 7.4 with Tris base) during incubation. Final DMSO concentration was 0.2%. A stock solution of forskolin was prepared in ethanol. Forskolin was then prepared in Tyrode solution to reach a final concentration of 1 μM during incubation. Final ethanol concentration was 0.1%. IBMX was dissolved in Tyrode solution.

Isolation of taste bud sheets and incubation. Rats were anesthetized with ether and then decapitated. CV taste bud and nonsensory EP sheets were prepared from rat tongue by means of subepithelial collagenase treatment (4 mg/ml, 0.28 U/mg collagenase; and trypsin inhibitor, 4 mg/ml) for 30 min in Tyrode solution (25). The dissected sheet of CV tissue was cut into symmetrical halves. Each half was transferred to an Eppendorf tube containing 10 μl of ice-cold Tyrode solution. EP sheets were treated identically and used as control tissue. Ice-cold Tyrode solution (10 μl) was added to each tube in the presence or absence of the appropriate amount of a given inhibitor or forskolin. Tubes were then incubated at 30°C for 10 (0.5 mM IBMX, 40 μM zaprinast, 20 μM denbufylline, or 1 μM forskolin) or 20 min (10 μM ODQ) as specified. Then, 50 μl of a sucrose solution reaching a final concentration of 500 mM (“start” solution) and 18 μl of 60% (vol/vol) perchloric acid (“stop” solution) were injected consecutively via the FFS (see Time course changes in cyclic nucleotide levels during subsecond stimulation). In one experiment, stimulation with final concentrations of 100 and 300 mM sucrose was also tested. The selected concentrations of sucrose were compatible with the level used to elicit sweet-taste sensation in rats (6). Samples were then frozen at −70°C for at least 30 min, thawed on ice, and centrifuged (4°C, 10,000 g, 10 min). Supernatants were removed, neutralized to pH 6.2 with 2 M potassium carbonate solution, and recentrifuged as before. Supernatants were removed, kept on ice, acetylated, and used for second messenger determination by RIA (8). Membranes of the permeabilized CV and EP cell sheets were used for protein determination by the Bradford procedure with modifications (25).

Time course changes in cyclic nucleotide levels during subsecond stimulation. Based on a previous instrument (4), an improved FPS was built by Gertron Ltd. (Petach-Tikva, Israel) to monitor responses in intact cells during subsecond time periods. This instrument delivers start and stop solutions consecutively into an Eppendorf test tube at predetermined, short time intervals. The system contains: 1) a controller, a PC (ACER laptop model Acernote 350 PC) with application software; 2) pump-induced air pressure with an electronic timer that transfers signals to two miniature three-way valves (LFAA1201610H or equivalent); 3) valves that are driven with a “spike and hold” driver, reducing the turn-on time to 1.3 ms and turn-off time to 3.2 ms; and 4) two Gilson micropipettes (P-200) with sustaining accuracy of ± 1% down to 50 μl.

No loss in accuracy was found after the air tubes were connected to the micropipette tip holder. The application software was a timing generator for 1) start valve opening time, 2) stop valve opening time, and 3) delay (between end of start to beginning of stop). The function of the time (20–300 ms) that it takes for the start/stop solutions to depart from the pipette tips, depending on a given volume (10–150 μl), was calculated based on measurements using a Kodak motion analyzer (model 1000) at a rate of 400 frames/s. To record the actual time needed for the start/stop solutions to fully leave the pipette tip and reach the Eppendorf tube (injection time), air pressure was set to 13 mmHg above atmospheric pressure. These calculations resulted in the formula: T (ms) = 2.15 × v (μl) + 5.3 for nonviscous solutions and T (ms) = 2.58 × v (μl) + 5.3 for a 700 mM sucrose solution (a final concentration of 500 mM in the incubation mixture). The delay between start and stop (interval between start valve closing and start valve opening) was also verified by the aforementioned technique. Regression analysis of the volume of the solutions injected vs. injection time resulted in $R^2 > 0.95$.

The two micropipettes were positioned in the Eppendorf tube to achieve a strong vortex with fast homogeneous mixing, as evidenced by filming (with the above Kodak analyzer) the mixing process with a colored 50-μl (methylene blue) start solution injected into an Eppendorf tube containing 20 μl of Tyrode solution (the volume of the incubation mixture used in this study). Mixing time was defined from the time at which the colored solution started to enter the tube to the time at which, upon termination of injection, the liquid inside the Eppendorf tube completely settled, i.e., there was no detected turbulence. A very fast mixing value, essentially overlapping the injection time, was observed, and therefore, this value was ignored for the above calculations. Thus when setting different incubation times (delay between start and stop solutions) of 75, 150, 250, and 350 μs (e.g., Fig. 1), we defined these time periods as the length of time the taste cells were exposed to a predetermined concentration of taste stimulus.

Data analysis. ANOVA was performed on the data using the JMP statistics program (Statistical Analysis Systems Institute), and the paired t-test was used to determine the difference between the basal and stimulated levels. Significance level was accepted at least at the P < 0.05 level.

RESULTS

Stimulation of intact CV taste bud cells with 500 mM sucrose produced an immediate and transient elevation in the cellular level of cGMP (Fig. 1). A significant elevation was observed at 75, 150, and 250 μs, whereas no elevation in cGMP was observed at 350 μs. No changes in cGMP content were observed in the nonsensory EP cells during this period. Results are given as amount over the unstimulated basal levels of cGMP, the latter being 7.2, 4.4, 6.1, and 6.0 fmol/μg protein for CV, and 6.7, 4.3, 5.8, and 4.8 fmol/μg protein for EP cells, at 75, 150, 250, and 350 μs, respectively. Under the same conditions, no elevation or reduction in cellular levels of cAMP was observed. However, a strong trend (P < 0.07) toward reduced
cAMP levels in CV sheets was seen at 250 ms. Stimulation of CV taste bud sheets with 100 and 300 mM sucrose resulted in a 0.34 ± 0.06 fmol/μg protein cGMP increase (n = 9, P > 0.2, not significant) and a 0.75 ± 0.33 fmol/μg protein cGMP increase (n = 9, P < 0.033), for 100 and 300 mM, respectively.

The effects of guanylyl cyclase and PDE inhibitors and an adenylyl cyclase activator (all membrane permeant) on the 150-ms sucrose-stimulated changes in cGMP and cAMP are shown in Fig. 2. Pretreatment of CV taste cells with the potent, highly selective, soluble guanylyl cyclase inhibitor ODQ reduced, but did not abolish, the sucrose-stimulated elevation in cGMP. In contrast, pretreatment with either the nonselective PDE inhibitor IBMX, adenylyl cyclase activator, forskolin, or a specific cAMP-PDE inhibitor, denbufylline, abolished the sucrose-stimulated cGMP increase. Interestingly, pretreatment of the CV taste cells with a specific cGMP-PDE inhibitor (zaprinast) also abolished the sucrose-stimulated cGMP increase (Fig. 2). Basal levels of cGMP were 4.4, 6.5, 5.3, 5.8, 6.2, and 6.5 for no stimulation and pretreatments with ODQ, IBMX, forskolin, zaprinast, and denbufylline, respectively. A separate experiment indicated that a 10-min pretreatment with zaprinast did not in itself modify the basal cGMP level in CV taste bud cells (6.75 ± 0.41 and 6.61 ± 0.54 fmol/μg protein cGMP for basal and zaprinast-stimulated levels, respectively, P < 0.3, n = 5).

As already indicated, there was no elevation in cAMP levels of CV or EP cells in the 75- to 350-ms time range in response to sucrose stimulation (Fig. 1). Results are given as amount over unstimulated basal levels of cAMP, the latter being 10.9, 13.7, 10.7, and 10.8 fmol/μg protein for CV, and 4.2, 6.3, 3.5, and 4.8 fmol/μg protein for EP sheets, at 75, 150, 250, and 350 ms, respectively. When the CV cells were pretreated with IBMX, sucrose stimulated an increase in cAMP at 150 ms. No such increase occurred at 75, 250, or 350 ms, and no changes in cAMP level were found in the EP cells under these conditions (data not shown). Furthermore, pretreatment with ODQ, forskolin, zaprinast, and denbufylline did not affect the cAMP level under sucrose-stimulation conditions, though an elevated (but not statistically significant) cAMP level was seen in the case of pretreatment with denbufylline (Fig. 2).

![Fig. 1. Time course of changes in cGMP (A) and cAMP levels (B) in circumvallate (CV) taste bud cells (solid bars) and in nonsensory EP cells (open bars) during subsecond stimulation with 500 mM sucrose. Results are above the unstimulated basal level. Values are the mean ± SE derived from 15–20 rats from 2 independent experiments. *Significant (P < 0.05) difference between the basal and stimulated levels.](http://ajpcell.physiology.org/)

![Fig. 2. Effects of soluble guanylyl cyclase inhibitor, 1H-[1,2,4]oxidiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM), nonselective phosphodiesterase (PDE) inhibitor (IBMX, 0.5 mM), specific cGMP-PDE inhibitor [zaprinast (Zap), 40 μM], specific cGMP-insensitive CAMP-PDE inhibitor [denbufylline (Den), 20 μM], and adenylyl cyclase activator [forskolin (Fors), 1 μM] on changes in cGMP (A) and cAMP levels (B) in CV taste cells after 150-ms stimulation with sucrose. Results are above the unstimulated basal level. Values are the mean ± SE derived from 15–20 rats from 2 independent experiments. *Significant (P < 0.05) difference between the basal and stimulated levels. Suc, sucrose.](http://ajpcell.physiology.org/)

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Basal levels of cAMP were 13.2, 10.3, 22.5, 24.9, 13, and 30.1 for no stimulation and pretreatment with ODQ, IBMX, forskolin, zaprinast, and denbufylline, respectively. The significant increase in basal cAMP level in the presence of denbufylline suggests the presence of PDE4 in the CV taste cells.

**DISCUSSION**

An immediate and transient elevation of cGMP was observed in rat intact CV taste cells in response to sucrose stimulation. The procedure employed for the isolation of CV taste buds enabled the biochemical monitoring of the cGMP response and verification of the tissue-specific nature of the effect because the isolated taste tissue is free of muscle- or connective-tissue contamination. These results provide evidence of a sucrose (500 mM)-induced elevation in cGMP in intact taste cells within a real time range of 75–250 ms of sugar taste sensation and are, therefore, of physiological significance. The rate of cGMP accumulation (2–3 fmol/150 ms) appears to be greater than that reported for cGMP accumulation in other tissues. For example, accumulation of 68 fmol min⁻¹ μg⁻¹ protein was reported in vascular smooth muscle cells in response to stimulation of soluble guanylyl cyclase by S-nitroso-N-acetyl-DL-penicillamine (19). Compared with stimulation with 500 mM sucrose, a lower (but significant) cGMP increase was observed in response to 150-ms stimulation with 300 mM sucrose, whereas stimulation with 100 mM only slightly (not significantly) increased cGMP level. Interestingly, in contrast to sucrose, stimulation with the nonsugar sweetener saccharin (20 mM) did not affect cGMP level at the 150-ms time period (data not shown). The cGMP data support previous electrophysiological studies indicating that intracellular administration of cGMP and cAMP depolarizes sweet-responsive cells (7, 29) in rodents as well as in flies (1). An increase in cellular cGMP may result from the activity of membrane-bound guanylyl cyclase, from nitric oxide synthase-stimulated soluble guanylyl cyclases, and/or from inhibition of cGMP-PDE, all of which have been identified in taste cells (2, 12, 23). If the transient increase in cGMP level after sugar-taste stimulation was solely the result of sucrose-stimulated guanylyl cyclases, then the introduction of a specific cGMP-PDE inhibitor (zaprinast) during sucrose stimulation would inhibit cGMP degradation, thereby enhancing the cGMP increase. Instead, as shown in Fig. 2, the sucrose-stimulated cGMP increase was abolished when zaprinast was present. Therefore, it is possible that in the presence of zaprinast, cGMP-PDE was already inhibited, making further inhibition of this enzyme during sucrose stimulation impossible. It should be noted that pretreatment of taste bud cells with zaprinast or with IBMX (although affecting basal cAMP) did not elevate the basal level of cGMP. There is no explanation currently available for this phenomenon. It is possible that homeostatic processes occurring in intact cells during the 10-min pretreatment with these drugs prevented significant changes in cGMP level. Nevertheless, the inhibition by zaprinast of the 150-ms sucrose-stimulated cGMP increase may not be related to an elevated basal level of cGMP. The introduction of a potent, selective inhibitor of soluble guanylyl cyclase (ODQ) during sucrose stimulation, although reducing the sucrose-induced cGMP increase, did not abolish this effect. Both activation of guanylyl cyclase and inhibition of cGMP-PDE may be involved in the transient, sucrose-induced elevation of cGMP in taste cells.

Manipulations designed to increase the cellular level of cAMP in taste cells, such as pretreatment with forskolin (from 13–25 fmol/μg protein) and the cGMP-insensitive cAMP-PDE4 inhibitor denbufylline (from 13–30 fmol/μg protein) also abolished the sucrose-induced increase in cGMP content. Indeed, the significant increase in basal cAMP level in the presence of denbufylline strongly suggests the presence of PDE4 in CV taste cells. Thus, during the initial 75–250 ms after sucrose stimulation, cAMP may antagonize cGMP elevation. In fact, one can see that during this initial period when no inhibitor was used, there was a trend, although not statistically significant, toward lower cAMP content, possibly allowing cGMP level to be elevated. Only when IBMX was applied did cAMP content increase after 150 ms of sucrose stimulation (Fig. 2). Due to the presence of very potent PDEs in various tissues, IBMX is added in most cases to detect adenylyl cyclase activities. Therefore, it remains to be tested whether cAMP elevation is due to sucrose stimulation or whether the pharmacological effect of IBMX is a crucial factor. The increased cAMP content at a later stage, i.e., at periods above 500 ms (data not shown) and during stimulation periods lasting minutes (25), which was shown to be GTP dependent and inhibited by sweet-taste inhibitors (25, 27), supports cAMP’s role in sugar-taste transduction. However, cAMP may play a role in the extinction (e.g., taste adaptation) rather than in the initial stage of signal transduction. These biochemical results are in line with recent observations (10) indicating that the presence of adenylyl cyclase inhibitors enhances sucrose-stimulated electrophysiological responses in taste cells. Interestingly, an elevation in cGMP due (at least in part) to direct inhibition of cGMP-PDE has been recently proposed to transduce the bitter taste induced by the membrane-permeant xanthines caffeine and theophylline (23). It should be noted that in contrast to most tissues, where the cellular level of cAMP is about 10- to 100-fold higher than that of cGMP (8, 20), our results show that in taste cells, the basal level of cAMP is only two- to threefold higher than that of cGMP. This phenomenon further supports the notion that cGMP plays a role in taste transduction.

The pathway through which sucrose stimulation increased cGMP level in taste cells is currently unknown. The pharmacological effects (Fig. 2) suggest that both guanylyl cyclase and, interestingly, inhibition of cGMP-PDE, may be involved in sucrose-taste transduction. If so, the inhibition of cGMP-PDE may suggest that a novel mechanism such as sucrose is membrane...
impermeable and that its direct interaction with cGMP-PDE, as proposed for caffeine and theophylline (23), is highly unlikely. Thus elucidation of a mechanism of receptor-inhibited cGMP-PDE activity may be of general significance. In vision, upon absorption of a photon, photoexcited rhodopsin activates the G protein transducin, which in turn activates cGMP-PDE to reduce the level of cGMP, thereby closing cGMP-gated cationic channels (Ca2+) in the rod plasma membrane to reduce the inward current. Transducin and its G protein homologue gustducin, which are present in taste cells (16, 24), may be involved in sweet- and bitter-taste transduction (30) and are known to activate PDE derived from taste cells (24). However, the existence of PDE that is inhibited by transducin has also been reported (31), and preliminary data suggest the presence of PDE in bovine taste and nonsensory epithelia that was inhibited by transducin and gustducin (R. F. Margolskee and co-workers, personal communication). Recently, a novel mechanism for PDE activation as well as deactivation by transducin has been proposed (14). The α-subunit of transducin is composed of a GTP-binding Ras-like domain and an α-helical domain (HDα). Data for both HDα-mediated attenuation and stimulation of PDE indicate that the HDα and the PDE inhibitory subunit (Pγ) interact with PDE at independent sites. The HD proteins derived from visual transducin or taste gustducin α-subunits each have been found to attenuate the PDE catalytic core (Pαβγ) and synergize Gαstimulation of holoPDE (Pαβγγ) (14). The relevance of the hypothesis that sucrose-taste stimulation may activate transducin and/or gustducin or other G proteins (22) to inhibit cGMP-PDE remains to be explored.

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