In Situ Ca^{2+} Imaging Reveals Neurotransmitter Receptors for Glutamate in Taste Receptor Cells

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The neurotransmitters at synapses in taste buds are not yet known with confidence. Here we report a new calcium-imaging technique for taste buds that allowed us to test for the presence of glutamate receptors (GluRs) in living isolated tissue preparations. Taste cells of rat foliate papillae were loaded with calcium green dextran (CaGD). Lingual slices containing CaGD-labeled taste cells were imaged with a scanning confocal microscope and superfused with glutamate (30 μM to 1 mM), kainate (30 and 100 μM), AMPA (30 and 100 μM), or NMDA (100 μM). Responses were observed in 26% of the cells that were tested with 300 μM glutamate. Responses to glutamate were localized to the basal processes and cell bodies, which are synaptic regions of taste cells. Glutamate responses were dose-dependent and were induced by concentrations as low as 30 μM . The non-NMDA receptor antagonists CNQX and GYKI 52466 reversibly blocked

responses to glutamate. Kainate, but not AMPA, also elicited Ca^{2+} responses. NMDA stimulated increases in $[\text{Ca}^{2+}]_i$ when the bath medium was modified to optimize for NMDA receptor activation. The subset of cells that responded to glutamate was either NMDA-unresponsive (54%) or NMDA-responsive (46%), suggesting that there are presumably two populations of glutamate-sensitive taste cells—one with NMDA receptors and the other without NMDA receptors. The function of GluRs in taste buds is not yet known, but the data suggest that glutamate is a neurotransmitter there. GluRs in taste cells might be presynaptic autoreceptors or postsynaptic receptors at afferent or efferent synapses.

Key words: calcium imaging; taste bud; gustatory system; glutamate receptors; tongue; foliate papilla; kainate; NMDA; calcium green dextran

Taste cells form synapses with axons of primary gustatory neurons and possibly with other cells in the mammalian taste bud. Taste cells also may receive efferent connections. However, the identity of neurotransmitters at these synapses in taste buds remains a key unanswered question in chemosensory neurobiology.

Glutamate is the major excitatory neurotransmitter in the CNS and in certain peripheral sensory organs (e.g., cochlea and retina). Glutamate also may be a neurotransmitter in taste buds. Chaudhari et al. (1996) identified ionotropic GluRs (iGluRs; NMDAR1, NMDAR2, KA2, and $\delta 1$) in lingual epithelium from foliate and vallate papillae in rats, implicating glutamatergic synaptic mechanisms in that tissue. Furthermore, primary gustatory neurons express iGluR subunits (Caicedo et al., 1999), raising the possibility that iGluRs are present on sensory axons that innervate taste buds. However, it is not known whether these iGluRs are functional synaptic receptors. Evidence that functional iGluRs are present on taste cells was shown by glutamate-induced Co^{2+} uptake studies (Caicedo et al., 2000). The iGluRs on taste cells may be receptors for glutamate as an efferent transmitter in taste buds, or they may act as presynaptic autoreceptors. For an analysis of these questions, functional experiments are needed to measure the activation of these receptors and correlate this information with the morphological studies. To date, it has not been possible to apply transmitter candidates focally to test and characterize synaptic responses in the intact tongue preparation, mostly because of technical limitations.

To address these questions, we have developed a new Ca^{2+} microfluorometric technique to measure changes in intracellular $[\text{Ca}^{2+}]_i$ induced by the activation of GluRs in taste cells *in situ*. This technique capitalizes on the ability of GluR activation to elicit changes in $[\text{Ca}^{2+}]_i$ in taste cells (Hayashi et al., 1996). Previous

Ca^{2+} imaging studies in taste have used ratiometric fluorescent Ca^{2+} indicators (e.g., fura-2) to measure responses to gustatory stimuli in isolated taste cells or isolated taste buds (Akabas et al., 1988; Bernhardt et al., 1996; Hayashi et al., 1996; Ogura et al., 1997; Ogura and Kinnamon, 1999). However, $[\text{Ca}^{2+}]_i$ measurements in taste buds have not been feasible *in situ* because, in the intact tissue, taste cells rapidly extrude indicators such as fura-2, presumably via powerful multidrug resistance transporters (Jakob et al., 1998). To overcome this problem and to measure $[\text{Ca}^{2+}]_i$ *in situ*, we have used the indicator calcium green 1 dextran (CaGD). Once incorporated into the cytoplasm, this indicator is not compartmentalized and is not extruded from the cell as much as other indicators (Haugland, 1996). With this approach we have found that $[\text{Ca}^{2+}]_i$ changes in well defined single cells within intact taste buds can be recorded accurately with little or no background fluorescence. Cellular relationships and the morphology of taste receptor cells are preserved in this preparation.

Our data show that a subset of taste cells responds to glutamate. The pharmacological profile of the responses to glutamate suggests that iGluRs of the non-NMDA and NMDA types are involved. These experiments provide the first demonstration of functional neurotransmitter receptors in taste cells in a semi-intact preparation.

MATERIALS AND METHODS

All experimental protocols were approved by the University of Miami Care and Use Committee.

CaGD injection and preparation of slices. Tongues were obtained from 67 young adult Sprague Dawley rats (150–200 gm) of both sexes. Rats were killed in a closed chamber containing CO_2 , followed by cervical dislocation. Tongues were removed quickly and immersed in cold, oxygenated Tyrode's solution (in mM: 135 NaCl, 5 KCl, 8 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 glucose, 10 Na-pyruvate, and 5 NaHCO_3 , pH 7.4, 320–330 mOsm). Blocks of tissue ($\sim 5 \times 5 \times 5$ mm) containing foliate papillae were removed from the tongue. CaGD (molecular weight 3000, $K_D = 259$ nM; Molecular Probes, Eugene, OR) was injected iontophoretically (5 mM in H_2O ; -3.5 μA ; 10 min) through a glass micropipette (20 μm tip) into the foliate papillae (cf. Krimm and Hill, 1998; Whitehead and Yao, 1998). Next, the block was sliced (100 μm) on a vibraslicer (Campden Instruments, Leicester, UK). Slices containing foliate taste buds were placed on a glass coverslip coated with adhesive protein (Sigma, St. Louis, MO), put in a recording chamber, and superfused with Tyrode's solution (room temper-

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Figure 1. Example of Ca^{2+} microfluorometric recordings, illustrating how data were processed in this study. *A*, Confocal image of a taste bud loaded with calcium green dextran (CaGD). The cell bodies of taste cells selected for measurements are encircled (*a–d*). *B*, Raw data from recordings of CaGD fluorescence from these four taste cells showing a response to KCl depolarization superimposed on a gradual decline in fluorescence over time (bleaching). The lowest trace (*d*) illustrates how an exponential fit was used to correct for this bleaching. The single exponential curve was fit to the first 120 sec and the last 30 sec of the recording. *C*, The exponential curve in the bottom trace in *B* (*d*) was subtracted from the recorded signal to correct for bleaching, and the fluorescence was expressed as $\Delta F/F$. Scale bar, 10 μm .

ature) at a rate of 2–3 ml/min. Once inside the cell, CaGD was neither extruded nor compartmentalized. This is in contrast with indicators such as fura-2, which are extruded rapidly by taste cells, presumably via powerful multidrug resistance transporters (Jakob et al., 1998). No photo damage was observed in most of the loaded cells. Thus, changes in $[\text{Ca}^{2+}]_i$ could be detected over several hours in response to chemical stimulation. We could monitor simultaneously the $[\text{Ca}^{2+}]_i$ changes in several taste cells and several taste buds in response to different pharmacological agents. Individual recordings lasted for as long as 30 min, during which dye bleaching occurred (from 0 to 70%; for instance, 60% in Fig. 1*B*). Fluorescence bleaching did not interfere with the experiments, and repeated measurements were possible (see below). We obtained similar results with Oregon green 488 BAPTA dextran (Molecular Probes). The improved tissue penetration and high sensitivity of confocal laser-scanning microscopy allowed us to record at a depth in the tissue slice at which the cells were affected minimally by the slicing procedure. We therefore used visible light-excitable Ca^{2+} indicators that are optimally suited for laser instrumentation (e.g., calcium green and Oregon green 488 BAPTA).

The mechanisms of CaGD uptake into taste cells are unknown. We have used a protocol that is used widely for the injection of tracer substances in the brain and has been used specifically in taste buds (Krimm and Hill, 1998; Whitehead et al., 1999). Current application was necessary; pressure injection of CaGD into the trench did not result in the uptake of indicator dye by the taste cells.

Drug application. All chemicals were bath-applied. Switching between solutions was achieved with electronically controlled small-volume solenoid valves (Lee Company, Westbrook, CT). Complete bath exchange was accomplished in ~20 sec when the tissue was in place. All experiments were performed at room temperature. Glutamate agonists were applied at ≥ 5 min intervals to avoid receptor desensitization. Antagonists (CNQX and GYKI 52466) were allowed to equilibrate with the receptors for 5 min before stimulation with an agonist. Antagonists were used at ~10 times their published IC_{50} values (Donevan and Rogawski, 1993; Hollmann and Heinemann, 1994). For stimulation with NMDA, the taste cells were superfused with an Mg^{2+} -free Tyrode's solution supplemented with 100 μM glycine.

The latencies of the responses were determined by the perfusion rate (2–3 ml/min) and the depth of the imaged cell within the slice. Slightly longer latencies in responses were observed for cells embedded deeper in the slice. Response latencies for a given cell were constant for sequential stimuli. We corrected for the differences in latencies when comparing responses between cells and when averaging responses (see below).

L-Glutamate, kainate, and GYKI 52466 were purchased from Sigma; NMDA, AMPA, and CNQX were obtained from Tocris (Ballwin, MO).

Confocal microscopy. CaGD-loaded cells were excited at 488 nm by using an argon laser attached to an Olympus Fluoview scanning confocal microscope. Foliate papillae were viewed with a 40 $\times$ water immersion objective. Images of single taste buds were magnified 5 $\times$ digitally. We acquired images without offset correction, additional gain, or filtering. We used a large confocal aperture (200–300 μm) to collect fluorescence from approximately the whole-cell thickness in a single image. This minimized artifacts associated with movement in the *z*-plane. To restrict photobleaching and phototoxicity, we reduced the laser intensity to 6% by a neutral density filter. Confocal images were collected at 5 sec intervals and processed with Fluoview software.

Fluorometric Ca^{2+} measurements. CaGD has a high affinity for Ca^{2+} ($K_D \sim 300$ nM), making it possible to measure small changes in $[\text{Ca}^{2+}]_i$. We measured the mean intensity of CaGD fluorescence in cell bodies, apical processes, and/or basal processes of individual taste cells by selecting a region 110% the area of the target cell or process and then measuring fluorescence changes every 5 sec. Changes in CaGD fluorescence over time were analyzed by Fluoview software. We recorded resting fluorescence levels for 2 min before applying stimuli (Fig. 1). Then stimuli were applied for 1 min and were followed by a 2 min washout. We corrected for fluorescence bleaching by fitting a single exponential curve to the resting fluorescence of each trace that was recorded before stimulation and for a 30 sec interval beginning 1.5 min after the stimulation (Fig. 1). We expressed the fluorometric signals as relative fluorescence change $\Delta F/F = (F - F_0)/F_0$, where F_0 denotes the resting fluorescence level corrected for bleaching. Using $\Delta F/F$ corrects for variations of baseline fluorescence, cell thickness, total dye concentration, and illumination. We did not attempt to estimate absolute values of $[\text{Ca}^{2+}]_i$.

To compare the responses between taste cells, we recorded from taste cells with similar resting fluorescence levels. In general, we recorded from cells with intermediate levels of CaGD loading. Taste cells heavily loaded with CaGD did not respond to stimulation and showed signs of phototoxicity (constantly increasing fluorescence and cell membrane blebbing).

Data analysis. Our criteria for accepting Ca^{2+} responses for analysis were (1) that responses could be elicited ≥ 2 times in the same cell by the same stimulus and (2) that peak $\Delta F/F$ was ≥ 2 times baseline fluctuation (with the exception of concentration–response relations; see Fig. 8). Baseline $\Delta F/F$ fluctuated ~1–2%. The peak $\Delta F/F$ constituted the response amplitude. Statistical tests of significance (Student's *t* test and ANOVA) were applied to determine whether the changes in the response amplitudes to a given treatment were significant. A Fisher Exact Test for comparing proportions was used to compare the incidence of responses between cell bodies and basal and apical processes of taste cells. For averaging, responses were aligned at the initiation of the rising phase. Curves were fit by using a Marquardt–Levenberg nonlinear least squares algorithm (SigmaPlot 5.0, SPSS, Chicago, IL).

RESULTS

CaGD loading in taste cells

Iontophoretic injection of CaGD into foliate papillae loaded up to 15 taste cells per taste bud with CaGD (Fig. 2*A,B*). However, not all taste buds were loaded. The nonsensory epithelium surrounding the taste buds showed little CaGD loading, suggesting that the outermost layers of the epithelium were relatively impermeable to CaGD and that the access of CaGD to taste buds was limited to the taste pore (see below). Consequently, there was little background fluorescence, and single taste cells could be imaged readily. Most if not all CaGD-loaded taste cells contacted the taste pore (Fig. 2*A–C*), suggesting that CaGD entered taste cells through their apical tips. CaGD-loaded taste cells were distinctly fluorescent at rest, and levels of loading differed from cell to cell (see Fig. 2*B,C*). CaGD filled the cytoplasm of the cells, making cell processes clearly distinguishable in many instances. Most cells had ovoid cell

Figure 2. Foliate taste bud cells can be loaded with CaGD and visualized in slices of lingual epithelium. This figure shows confocal images of tissue slices (100 μm thick) from a foliate papilla that had been injected iontophoretically with CaGD, as described in Materials and Methods. CaGD is present in some taste buds and as an adhering layer to the superficial epithelium. *A*, Superimposed fluorescence and Nomarski differential interference contrast image. *B*, Fluorescent image alone from a different preparation. *C*, A higher magnification of the boxed region in *B* shows that five taste cells are loaded with CaGD. Apical processes extend to and converge at the taste pore. *D*, Ca^{2+} response ($\Delta F/F$) in a single taste cell from another preparation to depolarization with elevated K^+ (50 mM) in the presence of 8 mM Ca^{2+} in the bath. Note that the response latency in this record and all subsequent figures is highly dependent on how deeply the imaged cell is situated within the tissue slice. In pseudocolor representations of raw images, red equals the highest fluorescence intensity. Scale bars: *A*, 50 μm ; *B*, 20 μm ; *C*, *D*, 10 μm .

bodies and long, thin processes extending to the apical and basal ends of the taste bud (Fig. 2*C*). Other CaGD-loaded cells had multiple processes and less-defined cell bodies. This is in agreement with the notion that different taste cell types (e.g., light cells, dark cells) contact the taste pore (Lindemann, 1996). More importantly, taste cells contacting the taste pore and loaded with CaGD presumably represent taste receptor cells, that is, taste cells that transduce taste stimuli at their apical tips and transmit this information at synapses within the taste bud (Lindemann, 1996).

Responses to depolarization

We first tested whether we could elicit Ca^{2+} responses in taste cells by depolarizing cells with Tyrode's solution containing 50 mM K^+ (equimolar substitution of NaCl with KCl). According to the

Nernst equation, a change of the extracellular $[\text{K}^+]$ from 5 to 50 mM (10-fold) might be expected to depolarize taste cells by ~ 60 mV. KCl depolarization increased CaGD fluorescence in 45% of the taste cells (50 of 110; Table 1) that were examined in the presence of 2 mM Ca^{2+} in the bath. The average peak $\Delta F/F$ in this series was $37.6\% \pm 4.5$ (mean $\pm$ SEM; range from 5 to 133%, $n = 50$; Figs. 1*C*, 3). Responses were obtained from cells located throughout the thickness of the slice, which rules out the possibility that diffusion barriers could limit the numbers of cells responding. These data indicate that not all taste cells responded to KCl depolarization, in agreement with studies showing that not all taste cells express voltage-gated K^+ channels and/or voltage-gated calcium channels (VGCC; Herness and Gilbertson, 1999).

Table 1. Proportions of taste cells responding to KCl depolarization and glutamate receptor agonists

Stimulus	Percentage of cells responding ^a	Percentage of glutamate-responsive cells responding ^b
K ⁺ (50 mM)	45 (50/110)	67 (18/27)
Glutamate (300 μ M)	26 (11/43)	—
Glutamate (1 mM)	35 (7/20)	—
Kainate	24 (13/53)	83 (5/6)
AMPA	0 (0/17)	0 (0/4)
NMDA	25 (15/61)	46 (6/13)

^aIn parentheses: number of cells responding/total number of cells examined.^bIn parentheses: number of cells responding/number of glutamate-responsive cells.

Figure 3. Ca^{2+} responses to KCl depolarization depend on extracellular Ca^{2+} . *A*, Ca^{2+} responses elicited by depolarization (elevated K^+ , 50 mM) were larger in 8 mM $[\text{Ca}^{2+}]_o$ than in 2 mM $[\text{Ca}^{2+}]_o$. *B*, Mean Ca^{2+} responses from cells bathed in 8 mM $[\text{Ca}^{2+}]_o$ were significantly larger than in cells bathed in 2 mM $[\text{Ca}^{2+}]_o$ (*Student's *t* test, $p < 0.01$). KCl responses were reversibly eliminated when Ca^{2+} was omitted from the bath. Numbers in parentheses equal the numbers of cells; error bars indicate SEM.

In cells that did respond to KCl depolarization, elevating Ca^{2+} in the bath to 8 mM significantly increased depolarization-induced responses (mean peak $\Delta F/F = 57.8\% \pm 8.6$; range from 8 to 120%, $n = 18$ cells; $p < 0.01$; Figs. 2*D*, 3). Responses were reversibly abolished when Ca^{2+} was omitted from the bath solution ($n = 4$). These findings indicate that the change in CaGD fluorescence elicited by depolarization depended on $[\text{Ca}^{2+}]_o$ and support the interpretation that these signals are triggered by Ca^{2+} flux through VGCC.

Responses to glutamate

To test whether taste cells responded to glutamate, we superfused slices with Tyrode's solution containing glutamate (30 μ M–1 mM) and measured changes in CaGD fluorescence. Between 26 and 35% of the cells that were examined responded to glutamate, under normal physiological conditions (when NMDA receptors would not be expected to be stimulated optimally; see below). The proportion of cells that responded to glutamate increased with increasing glutamate concentration (Table 1). Peak $\Delta F/F$ ranged from 4 to 18% when cells were stimulated with 300 μ M glutamate (mean = $8.7\% \pm 1.2$; $n = 11$). Response amplitudes may have been reduced by desensitization of the receptors because of the bath application of glutamate; thus these results provide a conservative estimate of the magnitude and frequency of glutamate responses. The responses generally were transient and decreased even in the continued presence of glutamate (Fig. 4). A fast rise phase was followed by a slower decay. In most cases the cells responding to glutamate showed changes in Ca^{2+} in the cell body or the basal process, but not in the apical process (Fig. 4; see below).

Glutamate could be eliciting Ca^{2+} responses directly by stimulating Ca^{2+} -permeable iGluRs, indirectly by depolarizing taste cells and activating VGCC, by inducing Ca^{2+} release from intracellular stores, or by a combination of the three. To begin to

Figure 4. Ca^{2+} response to glutamate (1 mM) in a taste cell. The cell body (bottom) and the apical process (top) were selected for measurements (circles in the top left panel). In the cell body the peak amplitude of the response was 18% $\Delta F/F$ (dark trace). Note that the response declined while glutamate was still present. By contrast, the apical process did not respond to glutamate (light trace). In pseudocolor representations of raw images, green equals the highest fluorescence intensity. Scale bar, 10 μ m.

discriminate among these alternatives, we used the next experiment to test whether both glutamate and KCl stimulation could evoke Ca^{2+} responses in any given taste cell. Eighteen percent of the cells responded to both glutamate and KCl depolarization (18 of 98; Fig. 5*Aa,B*). Most cells, however, responded only to depolarization (39%, 38 of 98; Fig. 5*Ab,B*). Importantly, a small number of taste cells responded only to glutamate (9%, 9 of 98; Fig. 5*Ac,B*). Of the cells that responded to glutamate, two-thirds also responded to KCl depolarization and one-third did not respond (Table 1). (34% of the cells in this experiment did not respond either to glutamate or to KCl.) These results show that, at least in certain taste cells (9%), glutamate elicited responses that might not be attributed to KCl depolarization and Ca^{2+} influx through VGCCs.

In the next experiment we assessed whether glutamate-induced Ca^{2+} responses were localized to regions of the taste cell where synapses are found. The majority of taste cell synapses in rodent taste buds occurs on the cell body and basal processes (Kinnamon et al., 1985). Thus, we examined Ca^{2+} changes in cell bodies and apical and basal processes. Taste cells were included in this experiment only if at least two regions (cell body, apical/basal process) were visible simultaneously and if at least one of the regions responded to glutamate. In a few cases all three regions could be imaged simultaneously (4 of 21 cells; Fig. 6). We found that responses to glutamate (300 μ M and 1 mM) occurred predominantly in basal processes and cell bodies (Figs. 4, 6*B*, 7). Ca^{2+} responses evoked by glutamate were observed in basal processes in all taste cells in which the basal process and the cell body could be visualized (100%; $n = 6$ taste cells; Fig. 7). Glutamate-evoked Ca^{2+} responses in cell bodies were observed in 9 of 11 taste cells (82%) in which the cell body was imaged either with the apical process or

Figure 5. Ca^{2+} responses to glutamate (1 mM) in KCl depolarization-sensitive and KCl depolarization-insensitive taste cells. *A*, Representative traces from three different taste cells stimulated sequentially with 1 mM glutamate (*glu*) and 50 mM KCl (K^+). *B*, Summary of data from 82 cells, showing incidence of responses to glutamate and KCl (*glu* + K^+) as in *a*, KCl alone (K^+) as in *b*, glutamate alone (*glu*) as in *c*, and cells that did not respond either to KCl or glutamate (*none*).

Figure 6. Ca^{2+} responses to glutamate are localized to basal processes and cell bodies. *A*, Representative responses from one cell to 1 mM glutamate (*glu*) and 50 mM KCl (K^+). This cell responded only to KCl depolarization; Ca^{2+} transients were recorded in the apical process, cell body, and basal process. *B*, Results from another cell that responded only to 1 mM glutamate and not to KCl depolarization. Note that responses to glutamate were restricted to the basal process and cell body in this cell.

the basal process. In contrast, glutamate evoked Ca^{2+} responses in apical processes in only two of seven taste cells (28%) that responded to glutamate in at least one of the three regions. The two taste cells having Ca^{2+} responses in the apical processes also produced Ca^{2+} responses in the cell body. That is, in no case did we observe Ca^{2+} responses in the apical processes alone. Comparisons of the proportions of the responses in the different compartments (Fisher Exact Test) showed that the proportion of the responses in the apical process was significantly lower than the proportions in the basal process ($p < 0.01$) and the cell body ($p < 0.05$; Fig. 7). Furthermore, response amplitudes appeared to be larger in the basal processes than in the cell bodies (see Fig. 6*B*). However, the differences in the amplitudes might result from differences in the surface-to-volume ratios among the regions, and we did not attempt to normalize for volumes.

We further tested whether responses to depolarization were localized similarly to one or another region of taste cells. KCl-evoked Ca^{2+} responses in all three regions were similar (Figs. 6*A*, 7). All basal processes (4 of 4), 90% of the cell bodies (9 of 10), and 88% of the apical processes (8 of 9) showed Ca^{2+} transients in response to KCl depolarization. The differences between these proportions were not significant.

Collectively, data from these experiments suggest that the GluRs responsible for Ca^{2+} responses are mainly on the cell body and basal processes of taste cells and that responses were produced by Ca^{2+} influx through iGluRs. That is, the observed compartmentalization of Ca^{2+} responses would not be expected if Ca^{2+} influx

Figure 7. Quantification and statistical analysis of results such as those illustrated in Figure 6. Taste cells were selected for this analysis according to the criteria described in Results. All basal processes (6 of 6) and 82% (2 of 7) of the apical processes responded to 300 μM glutamate (shaded bars), but only 28% (2 of 7) of the apical processes responded. The differences in the proportions of the responses between the apical process and the other cell regions were significant (Fisher Exact Test; * $p < 0.05$ for the difference between the apical process and the cell body and $p < 0.01$ for the difference between the apical process and the basal process). By contrast, KCl depolarization elicited Ca^{2+} transients in all compartments (filled bars).

Figure 8. Ca^{2+} responses to glutamate are concentration-dependent. *A*, Peak amplitudes increased with increasing glutamate (*glu*) concentrations (from 30 μM to 1 mM). Traces from four different cells are superimposed and aligned at the initiation of the rising phase. *B*, Summary of concentration–response data for several experiments. Dotted line is the maximum $\Delta\text{F}/\text{F}$ baseline fluctuation (i.e., noise). Numbers in parentheses equal the numbers of cells; error bars indicate SEM.

were secondary to cell depolarization, unless, of course, iGluR-induced depolarization was restricted spatially (e.g., decrements caused by changing surface-to-volume ratios). This is unlikely in such small, electrotonically compact cells as taste cells.

Pharmacological characterization of the responses to glutamate

To test further whether Ca^{2+} responses were elicited by activating GluRs, we examined glutamate concentration–response relationships and pharmacological specificity. Ca^{2+} responses elicited by glutamate were concentration-dependent in the range from 30 μM to 1 mM (Fig. 8). At concentrations ≥ 1 mM, glutamate often induced Ca^{2+} responses that did not recover and that resulted in prolonged $[\text{Ca}^{2+}]_i$ increases, resembling glutamate excitotoxicity in neurons (Tymianski et al., 1993).

To examine desensitization of the glutamate receptors, we tested cyclothiazide, an AMPA receptor-specific blocker of desensitization. However, the application of cyclothiazide (10 μM) alone induced large changes in $[\text{Ca}^{2+}]_i$. An accurate characterization of GluRs in the presence of cyclothiazide thus was not possible, and we did not continue with these experiments.

Application of the non-NMDA receptor agonist kainate (30 μM, $n = 9$; Fig. 9*A*, Table 1) elicited responses that were similar to those obtained with glutamate. The responses to 30 μM kainate had a mean amplitude of $5.8\% \pm 1.3$ (range from 3.8 to 10.9%) and

Figure 9. Taste cells responding to glutamate can be subdivided into NMDA-unresponsive and NMDA-responsive populations. *A, B*, Taste cells responded to kainate or to NMDA. Ca^{2+} responses to kainate (30 μ M) were different from those to NMDA (100 μ M with 100 μ M glycine and 0 mM Mg^{2+}). The response to kainate (*A*) was transient and recovered while kainate was still present. In another cell, NMDA induced a long-lasting response (*B*). *C*, Glutamate responses in NMDA-responsive cells were different from those in NMDA-unresponsive cells. NMDA-unresponsive cells (dark traces) showed large transient responses to glutamate (glu; 300 μ M) compared with glutamate responses in NMDA-responsive cells (light traces). *D*, Normalized and averaged glutamate (300 μ M) responses in NMDA-unresponsive cells (dark trace; $n = 4$ cells) and in NMDA-responsive cells (light trace; $n = 4$ cells). *E*, Glutamate responses in an NMDA-unresponsive cell were reversibly antagonized by the non-NMDA receptor antagonist CNQX (10 μ M). CNQX was applied 5 min before and during stimulation with glutamate (glu; 300 μ M). *F*, In an NMDA-responsive cell the glutamate responses (with 100 μ M glycine and without Mg^{2+}) were reversibly blocked by the NMDA receptor antagonist D-AP5 (50 μ M).

showed a sharp peak, followed by a rapid recovery. All of the kainate-responsive cells also responded to glutamate, and most glutamate-responsive cells responded to kainate (83%, 5 of 6 cells). The AMPA receptor-specific agonist AMPA (30 μ M), applied to cells that responded to kainate and glutamate ($n = 4$) as well as to other cells ($n = 17$), did not induce changes in $[\text{Ca}^{2+}]_i$. Last, NMDA (100 μ M) stimulated increases in $[\text{Ca}^{2+}]_i$ in 25% of taste cells (15 of 61) in a separate series of experiments when the bath medium was modified to optimize for NMDA receptor activation (Mg^{2+} -free, 100 μ M glycine; Table 1). Calcium responses elicited by NMDA usually were prolonged and had plateaus with a sustained amplitude (mean $\Delta F/F = 4.7\% \pm 0.7$; range from 3.3 to 8.1%; Fig. 9*B*).

Taste cells responding to glutamate under conditions favorable

for NMDA receptor activation could be subdivided into NMDA-unresponsive (54%, 7 of 13 cells) and NMDA-responsive cells (46%, 6 of 13 cells; see Table 1). Responses to glutamate (300 μ M) in NMDA-responsive cells were different from those of NMDA-unresponsive cells (Fig. 9*C, D*). Responses to glutamate of NMDA-unresponsive cells under these conditions were similar to those recorded in normal Tyrode's solution (sharp peak with a mean $\Delta F/F = 10.6\% \pm 2.5$; range from 6.5 to 15% for 300 μ M glutamate). In contrast, responses to glutamate in NMDA-responsive cells were smaller (mean $\Delta F/F = 3.5\% \pm 0.12$; range from 3.3 to 3.7% for 300 μ M glutamate; $p < 0.01$) and were prolonged, mimicking responses elicited by NMDA. The average responses show that the responses in NMDA-responsive cells had a prolonged plateau that lasted as long as the stimulus application (Fig. 9*D*), consistent with the agonist activation of NMDA receptors in neurons (Tymianski et al., 1993).

The non-NMDA receptor antagonist CNQX (10 μ M) reversibly abolished responses to glutamate (300 μ M; 3 of 3 cells; Fig. 9*E*). Similar results were obtained with the structurally unrelated non-NMDA antagonist GYKI 52466 (10 μ M; $n = 3$). Responses to NMDA and glutamate in NMDA-responsive cells could be reversibly blocked with the NMDA-specific antagonist D-AP5 (50 μ M; $n = 2$ of 2 cells; Fig. 9*F*).

Collectively, these data suggest that there are at least two populations of glutamate-sensitive taste cells—one with NMDA receptors and the other with non-NMDA receptors. The small sample size in our survey does not allow us to determine whether there is any substantial overlap in these two populations. We did not observe any Ca^{2+} transients that may have indicated the presence of NMDA and non-NMDA receptors on the same taste cell, but this possibility cannot be ruled out without more extensive testing.

DISCUSSION

We have developed a new preparation of rat taste buds and a new Ca^{2+} -imaging approach that enables us to record the activation of neurotransmitter receptors in taste buds *in situ*. The principal finding in this report is that a population of taste cells expresses functional glutamate receptors (GluRs), especially on their cell bodies and basal processes. The concentration–response relations and pharmacological characterization of the responses to glutamate indicate that ionotropic GluRs (iGluRs), similar to synaptic iGluRs in the brain, are present in taste cells. Both NMDA- and non-NMDA-type GluRs were observed. Taste epithelium indeed expresses neuronal kainate and NMDA receptor subunits (Chaudhari et al., 1996). Our present results suggest that iGluRs are involved in synaptic mechanisms in taste buds and that taste cells themselves are targets for transmitter action.

The present results are in agreement with our previous study that used glutamate-stimulated Co^{2+} uptake (Caicedo et al., 2000). In that study as well as in the present report, glutamate stimulated iGluRs on taste cells at concentrations from 30 μ M to 1 mM, i.e., below that which evokes taste responses (~ 100 μ M; Ninomiya et al., 1991; Yamamoto et al., 1991). Responses could be blocked with non-NMDA receptor antagonists (CNQX and GYKI 52466) and mimicked by kainate, a non-NMDA receptor agonist. AMPA was ineffective. Furthermore, only a subpopulation of cells in taste buds responded to glutamate. The proportion of taste cells expressing iGluRs is larger in the present study than in the Co^{2+} uptake study, but this might reflect methodological differences. For instance, in the present study we recorded from a restricted sample of taste cells (those for which the apical processes reached the taste pore) that might have a larger proportion of glutamate-responsive cells. Furthermore, the present studies were able to reveal NMDA responses, whereas NMDA receptors generally cannot be visualized by using the Co^{2+} technique [Caicedo et al. (2000), but see Nagy et al. (1994)].

Our studies reveal that non-NMDA iGluRs are present on taste cells. These receptors were activated by glutamate and kainate and were characterized by transient Ca^{2+} responses that declined even during the agonist application. The transient Ca^{2+} responses in-

Figure 10. Schematic drawings of the structure and putative synaptic connections of a taste bud. *A*, Fifty to 100 taste cells are grouped in a taste bud (only eight taste cells are shown). Taste receptor cells have apical processes that extend to and converge at the taste pore in the apical region. On their basal processes and cell bodies the taste cells form synaptic contacts with primary afferent fibers. *B*, Taste cells form synapses with primary sensory axons (*a*). Taste cells also may synapse with other taste cells within the taste buds (*b*). Furthermore, taste cells may receive efferent connections (*c*). The box shown in *B* is enlarged at the right (*a*, *c*). The GluRs reported in this study may function at each of these sites (see Discussion).

duced by kainate and glutamate in taste cells suggest that GluRs in taste cells desensitize rapidly, although other explanations are possible. In other tissues, kainate receptors, but not AMPA receptors, rapidly desensitize on exposure to kainate (Lerma et al., 1997). We could not observe responses to AMPA. In agreement with the lack of responses to bath-applied AMPA, AMPA receptor subunits GluR1–4 were not found in foliate papillae (Chaudhari et al., 1996). In contrast, at least one candidate kainate receptor subunit (KA2) is present in lingual tissue (Chaudhari et al., 1996). Although more information is needed about the localization in taste cells of this as well as of other glutamate receptor subunits, together these results suggest that the non-NMDA receptors on taste cells might be of the kainate type. However, we cannot discard that a different, as yet unknown, non-NMDA receptor is mediating responses to glutamate.

In addition, our results indicate that taste cells also express NMDA receptors. NMDA responses were prolonged and lasted the duration of agonist application. This may reflect a relative lack of receptor desensitization for NMDA receptors in taste buds, although other explanations are possible. Our results are consistent with the possibility that a population of taste cells expresses non-NMDA receptors and another population expresses NMDA receptors. Whether some taste cells express both NMDA and non-NMDA receptors remains a possibility.

In attempts to study glutamate as a *taste* stimulus, responses to glutamate, NMDA, and a metabotropic GluR agonist L-AP4 in isolated taste buds have been reported previously with Ca^{2+} imaging or patch-clamp recordings (Hayashi et al., 1996; Bigiani et al., 1997; Lin and Kinnamon, 1999). Neither AMPA nor kainate was tested extensively in those studies. In those reports, glutamate had multiple actions on taste cells. One action was mimicked by NMDA and characterized by a depolarization and an increased $[\text{Ca}^{2+}]_i$ in taste cells, consistent with the present findings. Another action was mimicked by L-AP4, but L-AP4 had mixed effects on $[\text{Ca}^{2+}]_i$. The recently discovered candidate taste receptor for umami, taste-mGluR4 (Chaudhari et al., 2000), is activated by glutamate at concentrations $\geq 100 \mu\text{M}$. In contrast, Ca^{2+} transients in the present study were elicited by glutamate at concentrations as low as $30 \mu\text{M}$. It is not clear whether the activation of taste-mGluR4 would elicit Ca^{2+} signals, although this might be inferred from the results reported in Hayashi et al. (1996). In general, however, the glutamate responses reported in the present study do not correspond well to taste responses, as described by these other reports, and our data are more consistent with the activation of neurotransmitter receptors.

Although we did not attempt to investigate Ca^{2+} mechanisms per se in taste cells, we can make some inferences from our results. In some cases the cells that responded to glutamate did not respond to KCl depolarization. This implies that these cells lack potassium channels, VGCCs, or both. Therefore, in these cells at least, the glutamate-induced Ca^{2+} responses resulted from Ca^{2+} entry through iGluRs. Alternatively, iGluRs also might interact with G-proteins, leading to activation of second messenger cascades and

of intracellular Ca^{2+} release mechanisms, as has been described for kainate receptors (Rodriguez-Moreno and Lerma, 1998) and AMPA receptors (Wang et al., 1997).

Responses to glutamate are localized to synaptic regions

We were able to measure changes in $[\text{Ca}^{2+}]_i$ in different compartments of taste cells. Responses to glutamate were localized to the basal processes and the cell bodies. We conclude that iGluRs are present at higher densities in the basal regions of taste cells. This preferential localization of iGluRs in the basal process and the cell bodies matches the distribution of synapses on murine taste cells (Kinnamon et al., 1985). Furthermore, the concentration–response relationships for glutamate in the present study, as well as for glutamate-stimulated Co^{2+} uptake (Caicedo et al., 2000), are consistent with the activation of synaptic iGluRs. Last, glutamate responses are blocked by ionotropic glutamate neurotransmitter receptor antagonists. Taken together, our results suggest that neurotransmitter receptors at synaptic sites on taste cells underlie the glutamate responses in this study.

Functional considerations

Glutamate receptors in taste cells might be presynaptic receptors (autoreceptors) at synapses between taste cells and sensory axons (Fig. 10). In this view, taste cells would release glutamate as a neurotransmitter to activate postsynaptic primary sensory axons. Primary gustatory neurons express GluRs (Caicedo et al., 1999; A. Caicedo, B. Zucchi, and S. D. Roper, unpublished results). Thus, GluRs might be present at postsynaptic sites on sensory axons, consistent with the possibility of glutamatergic afferent synapses in taste buds. Activation of iGluR autoreceptors on taste cells by synaptically released glutamate would provide feedback control of synaptic function (Parnas et al., 2000) or regulate other aspects of taste cell function. Evidence for the presence of non-NMDA and NMDA autoreceptors at presynaptic sites in the CNS is accumulating (McDermott et al., 1999). The expression of non-NMDA and NMDA receptors by taste cells fits into this scheme.

GluRs also might be postsynaptic receptors at efferent synapses between axons and taste cells (Fig. 10). Efferent synaptic regulation plays an important role in shaping incoming information in other sensory organs (e.g., cochlea). Efferent function of sensory neurons is well documented (for review, see Maggi, 1991), but evidence for efferent control of taste cells is sparse (for review, see Roper, 1989). Nonetheless, synapses in taste buds show some morphological features of efferent connections. For instance, clusters of vesicles are present in axons that innervate taste buds, and subsynaptic cisternae, similar to those of outer hair cells in the cochlea, can be seen in taste cells near some synapses in some species (Zahm and Munger, 1983). Alternatively, glutamate release by primary sensory axons themselves (axon reflex) has been reported (Jeftinija et al., 1991; Jackson et al., 1995; de Groot et al., 2000). According to this scheme, axon collaterals from taste afferents may exert local efferent synaptic feedback onto adjacent taste buds via axon re-

flexes (Murayama, 1988). Primary gustatory neurons release glutamate at their central terminals in the nucleus of the solitary tract (Bradley et al., 1996; Li and Smith, 1997) and thus have the potential to release glutamate at their peripheral processes. It is possible that gustatory sensory axons have a dual sensory–efferent function and exert efferent regulation of taste cells by releasing glutamate.

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