Changes in IP_3 and cytosolic Ca^{2+} in response to sugars and non-sugar sweeteners in transduction of sweet taste in the rat

Samuel J. Bernhardt, Michael Naim, Uri Zehavi and Bernd Lindemann * †

*Department of Biochemistry, Food Science and Nutrition, The Hebrew University of Jerusalem, Rehovot 76-100, Israel and *Department of Physiology, Universität des Saarlandes, D-66421 Homburg, Germany*

1. The transduction pathways of sweet-sensitive cells in rat circumvallate (CV) taste buds were investigated with assays for inositol 1,4,5-trisphosphate (IP_3) and with Ca^{2+} imaging. Stimulation with the non-sugar sweeteners SC-45647 and saccharin rapidly increased the cellular content of IP_3 by 400 pmol (mg protein)$^{-1}$, while sucrose had a much smaller effect on IP_3. As shown previously, sucrose, but not saccharin, increased the content of cyclic adenosine monophosphate (cAMP) of this preparation.

2. Stimulation of isolated CV taste buds with SC-45647 increased the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) by $56{\cdot}7 \pm 3{\cdot}2$ nM ($n = 181$). Due to the non-confocality of the measuring system, these concentrations are underestimates. The increase in $[Ca^{2+}]_i$ did not require the presence of extracellular Ca^{2+}, suggesting that the Ca^{2+} release was from intracellular stores.

3. Individual cells responding to the non-sugar sweeteners with Ca^{2+} release also responded to sucrose and to forskolin with an increase in $[Ca^{2+}]_i$. Such cells did not respond to the bitter tastant denatonium chloride.

4. Responses to sucrose were abolished by lowering the Ca^{2+} concentration of the stimulus solution, indicating Ca^{2+} uptake from the extracellular medium.

5. The responses of sweet-sensitive cells to forskolin were also abolished when Ca^{2+} ions were omitted from the stimulus solution. They were partially inhibited by the presence of Co^{2+}, Ni^{2+}, D600 (methoxyverapamil) and amiloride, indicating multiple pathways of Ca^{2+} uptake activated by cAMP.

6. In conclusion, a sweet-sensitive cell of the rat responds to sucrose with an increase in cAMP and Ca^{2+} uptake, but to non-sugar sweeteners with an increase in IP_3 and Ca^{2+} release. The increase in $[Ca^{2+}]_i$, common to both pathways, is presumably required for synaptic exocytosis and for signal termination.

The initial step in taste transduction is the interaction of chemical stimuli with the apical membrane of taste receptor cells. Thereby a transduction pathway is activated which initiates membrane depolarization and release of neurotransmitter at the synapse with a sensory nerve (Avenet & Kinnamon, 1991). It is known that sucrose, as well as other saccharides, has an appealing taste to rats and pigs and stimulates the activity of adenylate cyclase in membrane preparations derived from rat and pig tongues (Striem, Pace, Zehavi, Naim & Lancet, 1989; Naim, Ronen, Striem, Levinson & Zehavi, 1991). In intact taste bud cells of the rat, sucrose causes the concentration of cyclic adenosine monophosphate (cAMP) to increase (Striem, Naim & Lindemann, 1991). cAMP, in turn, elicits membrane depolarization in taste cells of frog, mouse and hamster (Avenet, Hofmann & Lindemann, 1988; Tonosaki & Funakoshi, 1988; Béhé, DeSimone, Avenet & Lindemann, 1990; Cummings, Powell & Kinnamon, 1993), by decreasing a potassium conductance (Avenet *et al.* 1988).

Saccharin (an anionic *N*-sulphonyl amide), however, does not stimulate cAMP formation in taste buds of rat circumvallate (CV) tissue (Striem *et al.* 1991). Furthermore, our preliminary experiments indicated that the guanidinium compound SC-45647, which is a sweetener to humans and rats (Nofre, Tinti & Chatzopoulos-Ouar, 1988; Hellekant & Walters, 1993), also does not stimulate cAMP formation in

† To whom correspondence should be addressed.

rat taste buds. This suggested that these compounds utilize transduction pathways different from that of sucrose.

Since sweet and bitter tastes are believed to be related and because bitter taste transduction can utilize the phosphoinositide (IP_3) pathway (Hwang, Verma, Bredt & Snyder, 1990; Spielman, Huque, Nagai, Whitney & Brand, 1994), the present study was designed to explore the possible involvement of the IP_3 pathway in sweet taste induced by the above non-sugar sweeteners. Sweetener-induced IP_3 formation in CV tissue sheets and tastant-induced Ca^{2+} release in isolated CV taste buds and in single taste cells were determined.

METHODS

Animals

Male Sprague–Dawley rats weighing 180–250 g were anaesthetized with ether and killed by decapitation. Circumvallate tissue sheets (CV) and sheets of non-sensory epithelium (EP) were prepared from rat tongues, using collagenase (5 mg ml^{-1}; Boehringer type 103586, lot 13027121-09, 0·65 U mg^{-1}) and trypsin inhibitor (2 mg ml^{-1}; Sigma) as described previously (Striem *et al.* 1991). From each rat one CV sheet was obtained.

Measurement of IP_3 content

Each CV sheet was cut into two halves. Four halves of CV (1 from each of 4 rats) were pooled and used for taste stimulation in one experiment. The other pool of four (matched) CV halves were used for obtaining the basal levels. EP sheets were treated identically and used as a control tissue. Each pool was transferred into polypropylene tubes containing 50 μl of ice-cold reaction saline (composition (mM): 140 NaCl, 5 KCl, 10 *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethane sulphonic acid (Hepes), 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose and 10 pyruvate; pH 7·4, saturated with phenylmethylsulphonyl fluoride). To insure accurate and rapid pipetting, a pressure-driven electronically controlled device containing two pipettes was built. One pipette contained 150 μl of the reaction mixture (reaction saline containing the tastant or not containing it for the basal control), the other 150 μl of the stop solution (ice-cold perchloric acid, 16% w/v). Each tissue pool was preincubated at 35 °C for 1 min. Upon activation of valve 1 and 2, stimulant and stop solution were consecutively injected into the reaction tube at a predetermined interval, allowing exposure times in multiples of 0·1 s (minimum, 0·5 s). Immediately afterwards, tubes were inserted into liquid nitrogen, then kept at −30 °C. For determination of IP_3, each tube was thawed on ice, 300 μl of EDTA buffer added (10 mM ethylendiamine tetraacetate, pH 7), then mixed. IP_3 was extracted and neutralized by the addition of 500 μl of a freon and tri-*N*-octylamine (1:1) mixture. Tubes were vigorously mixed with a Vortex (3 × 30 s), followed by centrifugation (1 min at 1000 *g*). The upper phase was removed, kept on ice and used for the IP_3 binding assay (Palmer & Wakelam, 1990). The permeabilized CV and EP sheets were used for protein determination (Striem *et al.* 1991). In some of the sucrose experiments, the sugar (300 mM) was added to the Tyrode solution (which thereby became hypertonic).

Imaging

The peeled CV sheets were exposed to the collagenase solution for 10 min, then to Ca^{2+}-free Tyrode solution containing 1 mM EGTA (ethyleneglycol-bis(β-aminoethylether)*N,N,N',N'*-tetraacetic acid) for 3–5 min. Using fine forceps, the tissue sheets were shaken slightly in a 100 μl chamber. This shaking detached the taste buds from the tissue, and adhered them to a coverslip which had been coated with a layer of adhesive (Cell-Tak, Labor-Schubert, Munich, Germany), forming the bottom of the chamber. In the course of isolation of the buds from the epithelium, the bud capsule, consisting of flat epithelial cells, became sufficiently permeable to allow loading with fura-2 AM (the acetoxymethyl ester form of fura-2). Buds were loaded with fura-2 (10 μM in Tyrode solution) for 30 min, then washed with Tyrode solution for another 30 min. Chambers with loaded taste buds were placed on an inverted microscope (IMT2-F, Olympus) and imaged with a ×40, 0·85 objective lens (Fluor 40, Nikon Düsseldorf, Germany). Single cells were occasionally imaged with a Fluor ×100, 1·3 oil-immersion objective lens. Pulses of light (340 and 380 nm) were generated with a galvanometric scanner (Till Photonics, Munich, Germany), which was coupled to the microscope with a light guide. Images were recorded with an integrating slow scan camera (Theta System, Munich, Germany) and digitized to 12 bits per pixel. The camera and scanner were controlled with a computer program written in the laboratory. Paired images (e.g. Fig. 2*B*), obtained with excitation at 340 and 380 nm, were used to calculate pixel ratios. Occasionally, images were deblurred with the algorithm of Monck, Oberhauser, Keating & Fernandez (1992) (see Fig. 2*C*). From them, apparent concentrations of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) were calculated with the formula of Grynkiewicz, Poenie & Tsien (1985) and displayed in a colour code of 240 shades. Calibration curves were obtained with calibration buffer kits (Molecular Probes). *In situ* Ca^{2+} calibration of the fluorescence ratio was not possible because the cells responded to ionophore-mediated Ca^{2+} uptake with rapid blebbing and lysis.

In a time series of images, each record consisted of three stored images of the same focal plane, i.e. one transmission image (Fig. 2*A*; needed for documenting the constancy of the focal plane) and two fluorescence images (at 340 and 380 nm excitation; see Fig. 2*B*). A chosen focal plane was an area containing well-defined and vital cells. Taste bud geometry was considered constant when small details at the periphery of the bud remained in focus throughout the image series. The hardware permitted image ratios to be taken at a maximal rate of 6 s^{-1}. However, initial trials showed that the Ca^{2+} responses were slow (for reasons of membrane accessibility and due to the slow superfusion used, see below). The fastest data rate used was one ratio every 1·5 s (e.g. Fig. 3*E*).

Imaged taste buds contained thirty to fifty cells, many of which were initially elongated but tended to round off within 1–2 h. Taste cells were stimulated by superfusion (driven by gravity) with the sweeteners and other agents dissolved in Tyrode solution. Since fast superfusion tended to deform or even wash away the buds, the rate was lowered to values which maintained the taste buds in place. In consequence, the onset of responses obtained was delayed by 2–5 s. In some cases longer delays were observed, probably due to blockage of the taste pore by the Cell-Tak or by its obstruction when the cells were rounded. Isolated taste cells and small clusters of cells were obtained by tissue printing (Barres, 1992), i.e. by gently pressing the interstitial surface of CV tissue sheets (exposed to nominally Ca^{2+}-free Tyrode solution) against the layer of Cell-Tak. Thereby some taste cells detached from the tissue and stuck to the bottom of the chamber.

Tyrode solution contained (mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, adjusted to pH 7·4 with NaOH, 10 glucose, and 10 sodium pyruvate. Amiloride was a gift from Merck Sharp & Dohme GmbH, Munich, Germany; denatonium chloride was a gift from Dr A. Saroli, Lyon, France; D600 (methoxyverapamil), forskolin and sodium saccharin were obtained from Sigma; SC-45647 was a gift from Drs C. Nofré, J.-M.Tinti, G. E. DuBois and the NutraSweet Co, Chicago, IL, USA. Means are given ± S.E.M.

RESULTS

IP_3 formation in taste buds in response to stimulation with sweeteners

To determine mass levels of the cytosolic messenger 1,4,5-IP_3, rat circumvallate tissue (CV), which is rich in taste buds, was isolated with collagenase (Striem *et al.* 1991) and incubated with sucrose or the non-sugar sweeteners saccharin and SC-45647. After incubation times between 0·5 and 60 s, the cells were fragmented with perchloric acid and the IP_3 content determined with a binding assay (Palmer & Wakelam, 1990). To increase the accuracy, the pipetting of stimulus and acid was controlled by an electronic timer. Stimulation with SC-45647 (1 mM) increased the IP_3 content significantly above basal levels ($P < 0·05$; Student's paired t test). The largest rise (430 ± 79 pmol (mg protein)$^{-1}$, $n = 5$) occurred at the shortest exposure time used (Fig. 1), indicating that the rate of generation of IP_3 within intact taste receptor cells exceeded the time resolution of our method. Responses to SC-45647 were also elicited by lower concentrations of the sweetener (at 20 μM: 68 ± 21 pmol (mg protein)$^{-1}$, $n = 5$; at 100 μM: 168 ± 37 pmol (mg protein)$^{-1}$, $n = 5$), measured at 0·5 s. Increases in IP_3 were observed with 20 mM sodium saccharin, while sucrose (300 mM added to Tyrode solution) caused a strikingly smaller rise in IP_3 (Fig. 1).

The IP_3 generation induced by the non-sugar sweeteners occurred in the CV tissue but not in the surrounding epithelial tissue (EP). Stimulation by the bitter tastants denatonium chloride (1 μM) and sucrose octaacetate (1 mM) was used as positive controls. Both increased IP_3 mass levels significantly (more than 600 pmol (mg protein)$^{-1}$)) in the CV but not in the EP tissue. Similar responses to bitter tastants were previously obtained by Hwang *et al.* (1990) and Spielman *et al.* (1994).

$[Ca^{2+}]_i$ changes in response to non-sugar sweeteners

Taste buds from the circumvallate papilla of the rat tongue were isolated from the surrounding tissue (Béhé *et al.* 1990; Striem *et al.* 1991), loaded with fura-2 AM (Grynkiewicz *et al.* 1985) and subjected to digital fluorescence ratio imaging. We recorded from only one focal plane per taste bud. Usually, as in Fig. 2, the transmitted light image shows about fifteen cellular cross-sections within the focal plane chosen. As shown in Fig. 2*A*, the exact location of the taste pore was not clearly visible. Only after removing stray light from the fluorescence image of Fig. 2*B* by deblurring, was the topography of the taste bud revealed. The deblurred image (Fig. 2*C*) shows the taste pore as a small dark area. From this point structures are seen to radiate outward which may be presumed to be the apical processes of taste cells. One of the taste cells, located in the lower part of the image, stained more strongly than the cellular neighbours. This cell, as shown in Fig. 2*D*, subsequently responded to SC-45647 with an increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ occurred not only in the soma, but also in the dendrite, which is visible in panels *C* and *D* as a colon-shaped structure pointing upwards.

In unstimulated taste cells the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was between 10 and 60 nM, but typically below 30 nM. An increase in $[Ca^{2+}]_i$ during exposure to a taste

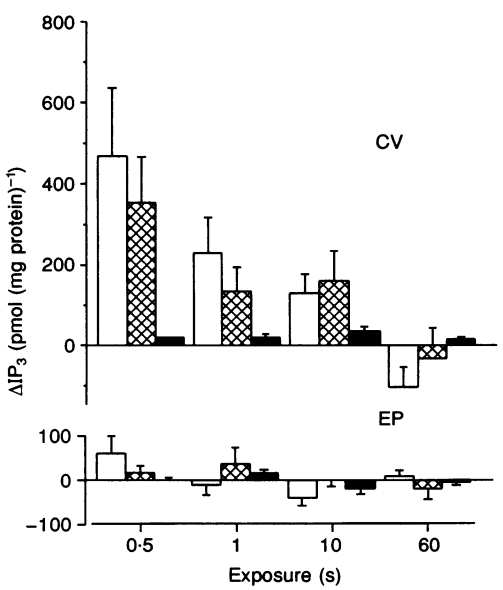

Figure 1. Time course of IP_3 formation in CV and EP cells
Saccharin (▩, 20 mM) and SC-45647 (□, 1 mM) induced IP_3 formation in circumvallate tissue (CV) but not in the non-sensory lingual epithelium (EP) surrounding the CV. Sucrose (■, 300 mM) also caused a significant increase in the mass level of IP_3 in CV tissue, but this response was much smaller. Results are given in picomoles of IP_3 formed above basal level. Each value represents the mean ± S.E.M. of 5 experiments.

Table 1. Peak responses of $\Delta[Ca^{2+}]_i$ (± S.E.M.) in individual taste cells (SC-45647-sensitive cells only)

Tastant or agent	Concentration	$\Delta[Ca^{2+}]_i$ (nM)	n
SC-45647	100 μM	56·7 ± 3·2	181
Saccharin	20 mM	41·4 ± 6·3	19
Sucrose	300 mM	72·3 ± 13·5	30
Forskolin	50 nM	37·3 ± 3·0	6
Denatonium	1 μM	—	61

Significance at $P < 0·01$.

agent was considered a response if it exceeded 30 nM. When buds were stimulated by superfusion with Tyrode solution containing 100 μM SC-45647, typically, within any two focal planes chosen, only one cell responded, following a delay of 5–15 s, with an increase in $[Ca^{2+}]_i$ of 30 to 200 nM. In most cases, $[Ca^{2+}]_i$ peaked and returned to lower or resting values during the exposure to the sweetener (Fig. 3*A*). In 27 out of 57 focal planes a response to 1 mM SC-45647 was seen and 181 out of 337 focal planes showed a response to 100 μM SC-45647. Nineteen cells responding to SC-45647 were challenged with saccharin (20 mM). All of them showed a transient increase in $[Ca^{2+}]_i$ which was, however, smaller than that elicited by SC-45647 (Table 1).

The increase of IP_3 and $[Ca^{2+}]_i$ caused by non-sugar sweeteners suggests release of Ca^{2+} from IP_3-sensitive intracellular stores. Ca^{2+} release was confirmed by testing the dependence on the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). Removal of $[Ca^{2+}]_o$ from the superfusion medium, shortly before addition of SC-45647, did not prevent the increase in $[Ca^{2+}]_i$ (Fig. 2*D*, Fig. 3*B* and Table 2), while exposure to the Ca^{2+}-free medium for more than 2 min did prevent it.

$[Ca^{2+}]_i$ changes in response to denatonium chloride

Denatonium chloride, as shown by two-bottle preference tests, is bitter to rats at a concentration of 1 μM (Akabas, Dodd & Al-Awqati, 1988; S. Bernhardt, personal communication). Stimulation with this concentration caused an increase in $[Ca^{2+}]_i$ in isolated rat taste buds (Akabas *et al.* 1988). In our experiments, superfusion of 1, 10 or 100 μM denatonium chloride over the taste buds rarely increased the cytosolic Ca^{2+} concentration of a cell. In sixty-one focal planes passing through a SC-45647-responsive cell only nine cells responded to 1 μM denatonium chloride. These nine cells did not respond to SC-45647 (Fig. 4).

$[Ca^{2+}]_i$ changes in response to sucrose

A sucrose concentration of 300 mM is a moderate concentration for the sweet taste of the rat (Ogawa, 1972). Yet, 300 mM sucrose added to Tyrode solution doubles the osmolarity of this medium. Superfused over isolated taste buds, the hypertonic solution caused significant cell shrinkage, noticed as a decrease in taste bud diameter and a downward displacement of morphological details with respect to the image plane of the microscope. The volume change made continued imaging from one focal plane difficult. Therefore, in most experiments of this kind, 300 mM sucrose was added by replacement of 140 mM NaCl, resulting only in a small increase in osmolarity but low Na^+ and Cl^- concentrations (10 mM).

Control experiments were carried out to check for effects of low extracellular Na^+ and Cl^- concentrations. On replacing 140 mM NaCl with 140 mM sodium gluconate, taste bud geometry remained stable and no increase in $[Ca^{2+}]_i$ was noticeable in imaging experiments. In binding assays, this change caused IP_3 to decrease by the small amount of

Table 2. $[Ca^{2+}]_i$ peak response to tastants: dependence on $[Ca^{2+}]_o$

Tastant	Concentration	$[Ca^{2+}]_o$	$\Delta[Ca^{2+}]_i$	n
SC-45647	0·1 mM	1	65·2 ± 8·5	
		0	68·9 ± 10·2	19
Saccharin	20 mM	1	50·0 ± 6·4	
		0	58·5 ± 4·0	11*
Sucrose	300 mM	1	75·0 ± 16·5	
		0	−2·0 ± 7·6	11*
Forskolin†	50 nM	1	37·3 ± 3·0	
		0	0·0‡	6*

* Cells responsive to SC-45647. † First $[Ca^{2+}]_o$ was removed, then forskolin was added, finally $[Ca^{2+}]_o$ was replenished. ‡ $P < 0·01$ in paired t test.

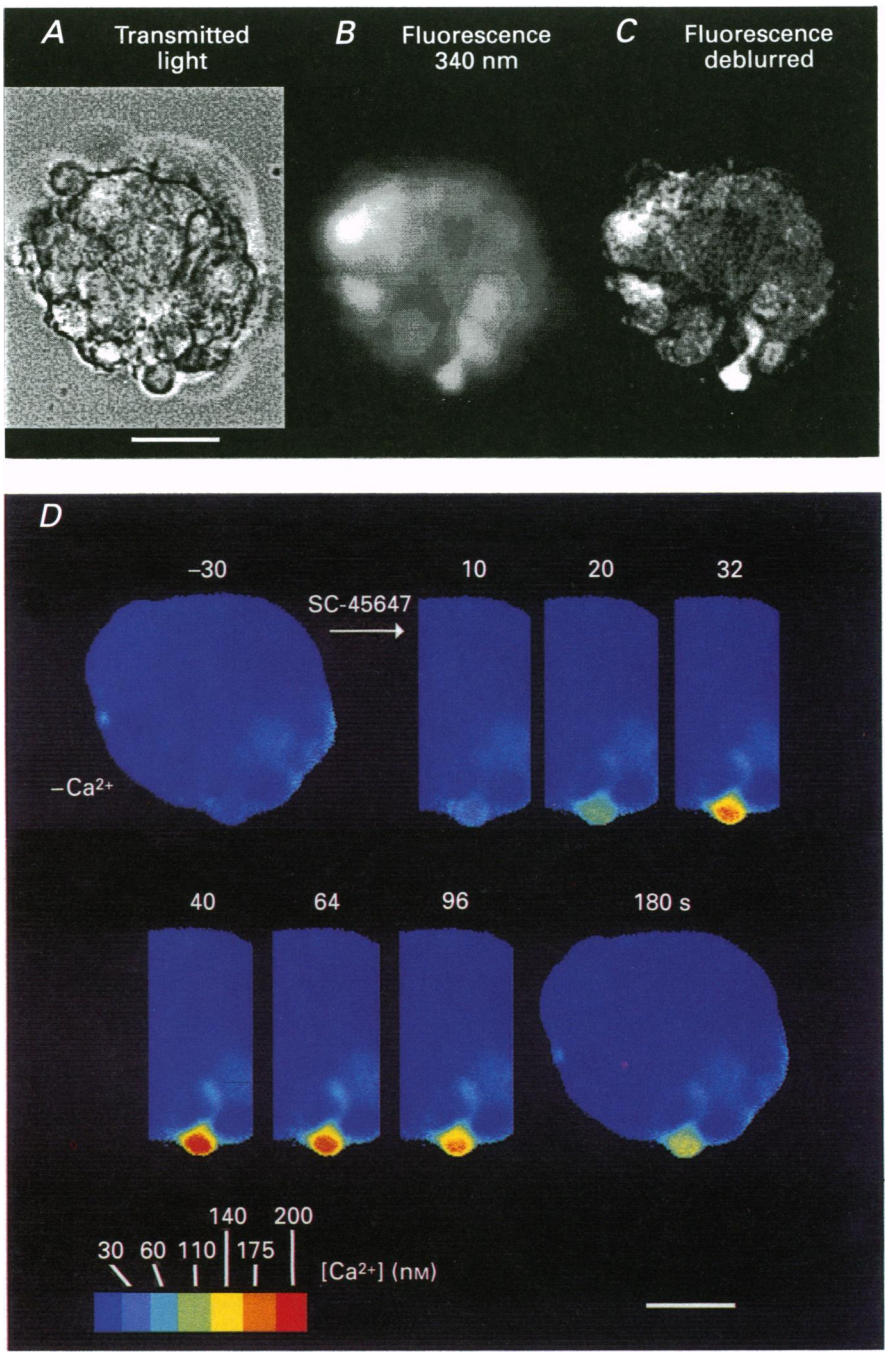

Figure 2. Optical sections of a taste bud

A, optical section through an isolated taste bud in transmitted light. *B*, the same section shown as a fura-2 fluorescence image, excited at 340 nm. *C*, the fluorescence image of *B* subjected to a no-neighbour deblurring procedure (Monck *et al.* 1992). From the taste pore, now noticeable as a dark area below the centre, a pattern of outward radiating structures is seen. These will be the apical portions (dendrites) of taste cells. One taste cell, which is stained more strongly, is visible in the lower part of the section, with the dendrite pointing upwards towards the taste pore. *D*, fura-2 ratio images of the optical section shown in panels *A*–*C*. The pseudocolour scale of the images has 240 shades, of which 7 were selected for the calibration bar on the lower left. Response to SC-45647 in the absence of Ca_o^{2+}. The taste bud was imaged at 28 consecutive times. Eight images of this set are shown. The time after addition of the stimulus is indicated in seconds. In 6 of the images, left and right parts were deleted to save space. $[Ca^{2+}]_o$ was lowered to values near 0 mM 30 s prior to stimulation. At this time, $[Ca^{2+}]_i$ was below 30 nM at most locations in the focal plane shown. At 0 time slow superfusion with SC-45647 (100 μM) began. An increase in $[Ca^{2+}]_i$ to about 200 nM occurred in a small peripheral area, corresponding to the soma of the heavily stained taste cell of panel *C*. The increase in $[Ca^{2+}]_i$ was followed by a decrease occurring in the continued presence of SC-45647 (the period of stimulus washout is not shown). In the upward-pointing dendrite of this cell (compare with panel *C*), $[Ca^{2+}]_i$ rose to apparent values below 100 nM in the presence of SC-45647. Scale bar, 20 μm.

45 ± 8 pmol (mg protein)$^{-1}$) ($n = 3$). In other experiments, 140 mM NaCl was replaced with 140 mM *N*-methyl-D-glucamine chloride, lowering $[Na^+]_o$ from 150 to 10 mM. Taste bud geometry remained stable, and merely small to negligible changes in $[Ca^{2+}]_i$ (less than 20 nM) were observed. Therefore, the increased $[Ca^{2+}]_i$ due to the reduction of $[Na^+]_o$ to a level of 10 mM (abolishing putative Na^+–Ca^{2+} exchange) was negligible compared with the level of $[Ca^{2+}]_i$ stimulated by sucrose. It remains possible, however, that cells stimulated with sucrose at 10 mM $[Na^+]_o$ (as described below), had impaired Ca^{2+} elimination and, therefore, elevated and prolonged responses of $[Ca^{2+}]_i$.

On superfusion with the sucrose solution of 300 mM (replacing 140 mM NaCl), taste bud geometry remained stable and $[Ca^{2+}]_i$ increased transiently in one or two cells of a bud (Fig. 3*C*, Fig. 5). In thirty-three focal planes passing through one or more cells responsive to SC-45647, all such cells also responded to sucrose with an increase in $[Ca^{2+}]_i$. Occasionally, a few additional cells responded to sucrose. The peak of the $[Ca^{2+}]_i$ response to sucrose tended to be larger than the peak response to non-sugar sweeteners found in the same cell (Table 1). The responses to sucrose had a pronounced transient component (Fig. 3*C*), showing that elimination of $[Ca^{2+}]_i$ was possible even at the decreased concentration of Na_o^+ used during exposure to sucrose.

When 300 mM sucrose was added to Tyrode solution without replacing NaCl, it was necessary to readjust the focal plane, compensating for its relative upward shift caused by the decrease in cell volume. When this could be done, an increase in $[Ca^{2+}]_i$ was observed in cells also responding in this way to sucrose replacing NaCl.

Removal of Ca_o^{2+} from the superfundate was used to check for a direct dependence of the $[Ca^{2+}]_i$ increase on extracellular Ca^{2+}. The removal was carried out shortly before addition of sucrose, in order to avoid depletion of intracellular Ca^{2+} stores prior to stimulation. Removal of Ca_o^{2+} prevented the increase in $[Ca^{2+}]_i$ in 8 out of 10 trials, implicating Ca^{2+} uptake in the response to sucrose (Fig. 5 and Table 2). Compatible with this conclusion is our observation that 300 mM sucrose increased the mass level of IP_3 much less than the non-sugar sweeteners (Fig. 1).

While the response to sucrose involved predominantly Ca^{2+} uptake rather than intracellular Ca^{2+} release, the cells responding appeared to contain IP_3-sensitive Ca^{2+} stores as well. This was suggested by their sensitivity to non-sugar sweeteners (Table 1). Furthermore, a small number of cells responding to SC-45647 and sucrose were exposed to 1 μM thapsigargin, a blocker of Ca^{2+} accumulation into IP_3-sensitive intracellular stores (Thastrup, Cullen, Drobak,

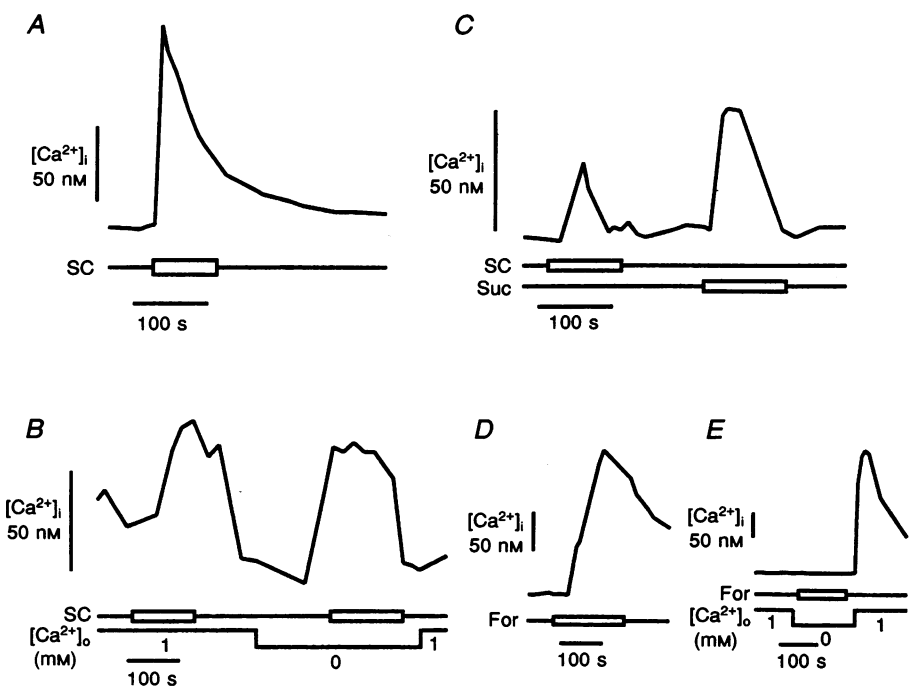

Figure 3. Time courses of local changes in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$)

Data are mean $[Ca^{2+}]_i$ values taken from small areas (e.g. 2 × 2 μm) of focal planes of isolated taste buds. *A*, response to SC-45647 (SC, 100 μM). The rectangle indicates the time of exposure. *B*, SC-45647 (100 μM) at 1 mM and nominally 0 mM $[Ca^{2+}]_o$ (1 mM of EGTA was added to the Tyrode solution instead of 1 mM $CaCl_2$). *C*, response of 1 cell to SC-45647 (100 μM) and to sucrose (Suc, 300 mM, replacing 140 mM NaCl) at 1 mM $[Ca^{2+}]_o$. *D*, forskolin (For, 100 nM) at 1 mM $[Ca^{2+}]_o$. *E*, forskolin (50 nM) at 1 mM and nominally 0 mM $[Ca^{2+}]_o$.

Table 3. $[Ca^{2+}]_i$ responses to forskolin (50 nM) with and without inhibitors of Ca^{2+} inflow (SC-45647-sensitive cells only)

Inhibitor	Concentration	Target	$[Ca^{2+}]_i$ (nM) Forskolin − inhibitor	$[Ca^{2+}]_i$ (nM) Forskolin + inhibitor	n
D600	50 μM	L	112·0 ± 4·7	47·0 ± 14·0*	6
Co^{2+}	2 mM	L,T	100·0 ± 7·2	78·1 ± 4·5**	9
Ni^{2+}	1 mM	T	108·1 ± 6·0	82·5 ± 3·8*	6
Mg^{2+}	1 mM	CNG	109·8 ± 4·2	56·8 ± 1·1*	4
Amiloride	50 μM	T,CNG	190·3 ± 45·1	97·6 ± 6·2**	7

L, L-type Ca^{2+} channel; T, T-type Ca^{2+} channel; CNG, cyclic nucleotide-gated channel. Forskolin was applied in the presence of 1 mM $[Ca^{2+}]_o$, to the same taste cell, first without, then with an inhibitor of Ca^{2+} uptake. * $P < 0·05$, ** $P < 0·01$, paired t tests.

Hanley & Dawson, 1990). Of twelve cells investigated, nine responded to thapsigargin with an increase of $[Ca^{2+}]_i$ exceeding 3 nM (e.g. Fig. 5). The mean $[Ca^{2+}]_i$ increase was 15·6 ± 3·8 nM ($n = 9$), suggesting the presence of IP_3-sensitive Ca^{2+} stores in sweet-responsive cells.

$[Ca^{2+}]_i$ changes in response to forskolin

As descibed above, sucrose could not be added to isolated taste buds without disturbing cell volume or Na^+ and Cl^- gradients. It was of considerable interest, therefore, to make use of agents which, like sucrose, increase the cellular concentration of cAMP, but which are effective at low, osmotically negligible concentrations. The membrane-permeant chlorphenylthio analogue of cAMP (cpt-cAMP), was used at an extracellular concentration of 1 mM. It was expected to cause an increase in the cytosolic cAMP concentration by slowly entering the cells. However, superfusion with this compound for 10 min rarely caused a significant increase in $[Ca^{2+}]_i$, and mean values based on eleven experiments did not show a significant change in $[Ca^{2+}]_i$ of sweet-responsive cells.

We therefore attempted to raise intracellular cAMP by stimulating the endogenous production. Forskolin, an activator of adenylate cyclase, increases cAMP levels in rat circumvallate tissue (Striem, 1990) and causes a pronounced depolarization in a subset of taste cells (Avenet *et al.* 1988; Cummings *et al.* 1993). At the high concentration of 1 μM, forskolin increased $[Ca^{2+}]_i$ strongly in many cells of a taste bud. At 50 and 100 nM a weaker increase involving fewer cells was seen (Fig. 3*D*, Fig. 6 and Table 1). Notably, a concentration of forskolin of 100 nM is far below that required for the direct effect of forskolin on voltage-gated K^+ channels (Hoshi, Garber & Aldrich, 1988).

Interestingly, in the isolated taste cell of Fig. 6, the increase in $[Ca^{2+}]_i$ in response to SC-45647 occurred not at the tip of the dendrite but in the soma, close to the base of the dendrite. This location may be a 'trigger zone' of Ca^{2+} release, as described for exocrine pancreas cells (Kasai & Petersen, 1994).

Cells responsive to SC-45647 were among those responding to thapsigargin and forskolin, and were the first to respond when forskolin was added. The increase in $[Ca^{2+}]_i$ in response to forskolin, like most $[Ca^{2+}]_i$ responses to sucrose, did not take place in the absence of $[Ca^{2+}]_o$ (Table 2), but was delayed until $[Ca^{2+}]_o$ was replenished (Fig. 3*E*). In conclusion, then, sucrose and forskolin, both agents which increase cellular cAMP, induced an increase in $[Ca^{2+}]_i$ driven by Ca^{2+} uptake from the extracellular medium.

Pathways of Ca^{2+} uptake

In preliminary experiments we attempted to identify the pathway of Ca^{2+} uptake in SC-45647-responsive cells. When the blockers of T-type Ca^{2+} channels Ni^{2+} (1 mM) and amiloride (50 μM) were added to the perfusion solution, the rise in $[Ca^{2+}]_i$ induced by forskolin was partially inhibited. The same result was obtained with 2 mM Co^{2+} (Fig. 6), which blocks both T- and L-type Ca^{2+} channels, and with D600 (50 μM), which blocks L-type Ca^{2+} channels only (Table 3). These observations indicate Ca^{2+} uptake in sweet-responsive cells by T- and L-type Ca^{2+} channels. Both channel types have been found previously in rat taste cells (Béhé *et al.* 1990).

Of the agents used, amiloride also blocks another Ca^{2+} uptake pathway, the cyclic nucleotide-gated channel (CNG channel) (Frings, Lynch & Lindemann, 1992). Since this channel is also blocked by Mg^{2+} ions (Colamartino, Menini & Torre, 1991; Weyand *et al.* 1994), we attempted to enhance the Ca^{2+} uptake by removal of Mg^{2+} (1 or 2 mM) from the superfundate. Indeed, brief Mg^{2+} depletion increased $[Ca^{2+}]_i$ in forskolin-treated cells (Table 3). These results are compatible with the presence of CNG channels in sweet-responsive cells, but do not constitute proof. Others recently reported that the cyclic nucleotide activated channels in rat taste cells (Herness, 1993).

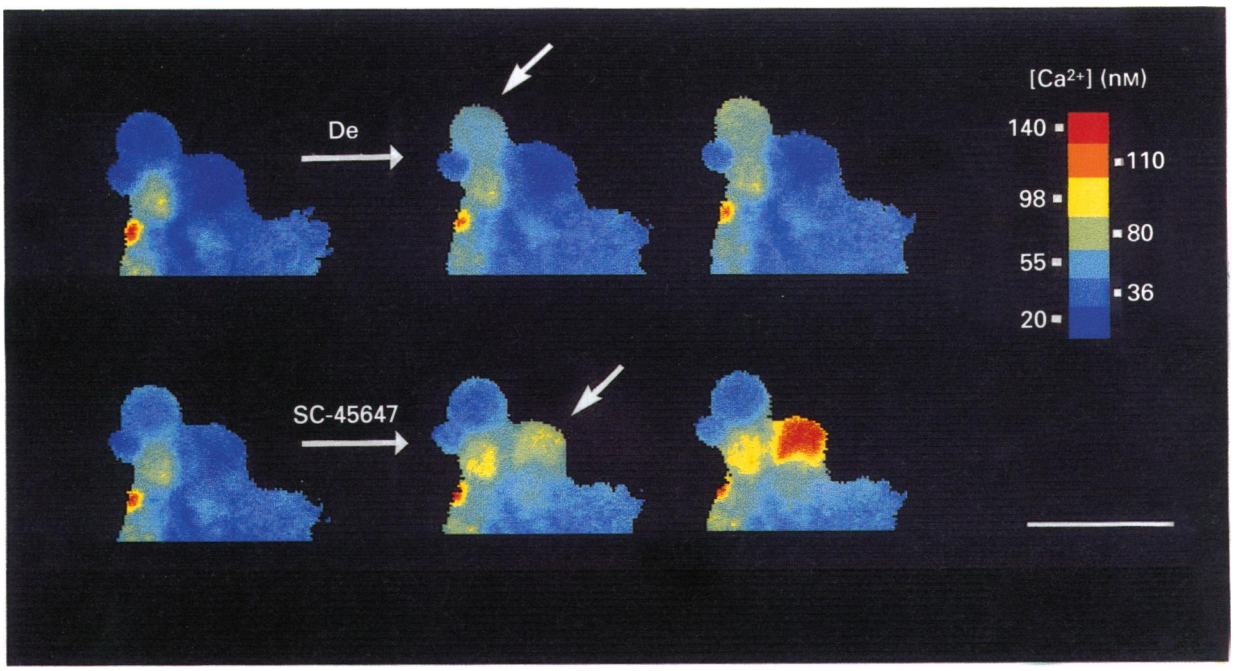

Figure 4. Pseudo-colour ratio images of a taste bud loaded with fura-2 (only part of one focal plane is shown)

Of two peripheral cells (oblique arrows), one responded to the bitter denatonium chloride (De, 1 μM), the other to the sweet SC-45647 (100 μM). Scale bar, 20 μm.

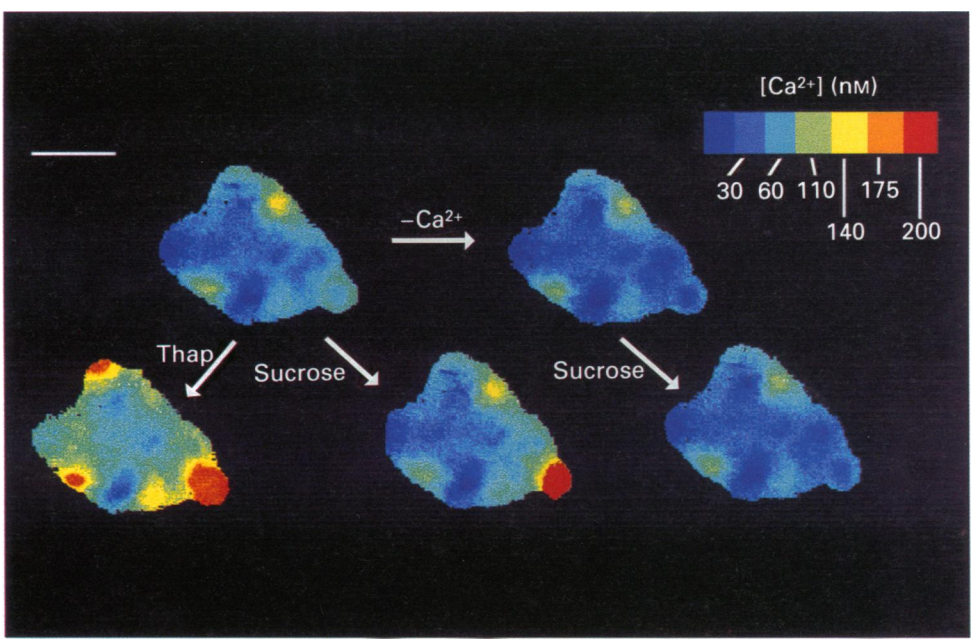

Figure 5. Pseudo-colour ratio images of a taste bud loaded with fura-2

In the focal plane shown, 1 peripheral cell responded to sucrose (300 mM, replacing 140 mM NaCl) in the presence of 1 mM $[Ca^{2+}]_i$, but not in the absence of $[Ca^{2+}]_o$. The same cell was foremost among those responding to thapsigargin (Thap, 1 μM). Scale bar, 20 μm.

DISCUSSION

Main observations

The present results indicate that stimulation by the non-sugar sweeteners saccharin and SC-45647 raised the cellular content of IP_3 of CV taste buds in a concentration-dependent manner. The response was tissue specific. Sucrose, however, which is known to stimulate the generation of cAMP, raised the IP_3 content only slightly. The comparatively small effect of sucrose upon the release of IP_3 leaves the physiological relevance of this increase questionable. Therefore sucrose and the non-sugar sweeteners trigger different transduction cascades, be they located in the same or in different subpopulations of taste cells.

As shown by imaging of isolated taste buds, both SC-45647 and sucrose increased the free cytosolic Ca^{2+} concentration of a small subset of cells. Notably, of the thirty-three taste cells tested with sucrose after responding to SC-45647, all responded to sucrose. Cells responding to SC-45647 were among those responding to forskolin.

While responses to forskolin were pronounced, those to cpt-cAMP, applied extracellularly, were insignificant. This result is in contrast to the clear effects of cAMP, also applied extracellularly, observed with taste cells of frog, hamster and gerbil (Avenet *et al.* 1988; Cummings *et al.* 1993; Kinnamon, Cummings & Daniels, 1994). The lack of response to extracellular cAMP suggests that in rat taste cells a transport system for organic anions (related to the renal *p*-aminohippurate transporter) was not present or was not functional due to a special metabolic situation. It is known that such transporters also mediate uptake of cyclic nucleotides, and that their affinity increases with the hydrophobicity of a cyclic nucleotide analogue (Ullrich, Rumrich, Papavassiliou, Klöss & Fritzsch, 1991).

Interestingly, those cells which responded to sweet tastants did not respond to the bitter tastant denatonium. This suggests that in the rat bitter and sweet tastes are transduced by different receptor cell populations. If such a separation were to hold for humans also, then the slight bitterness which accompanies the sweetness of saccharin

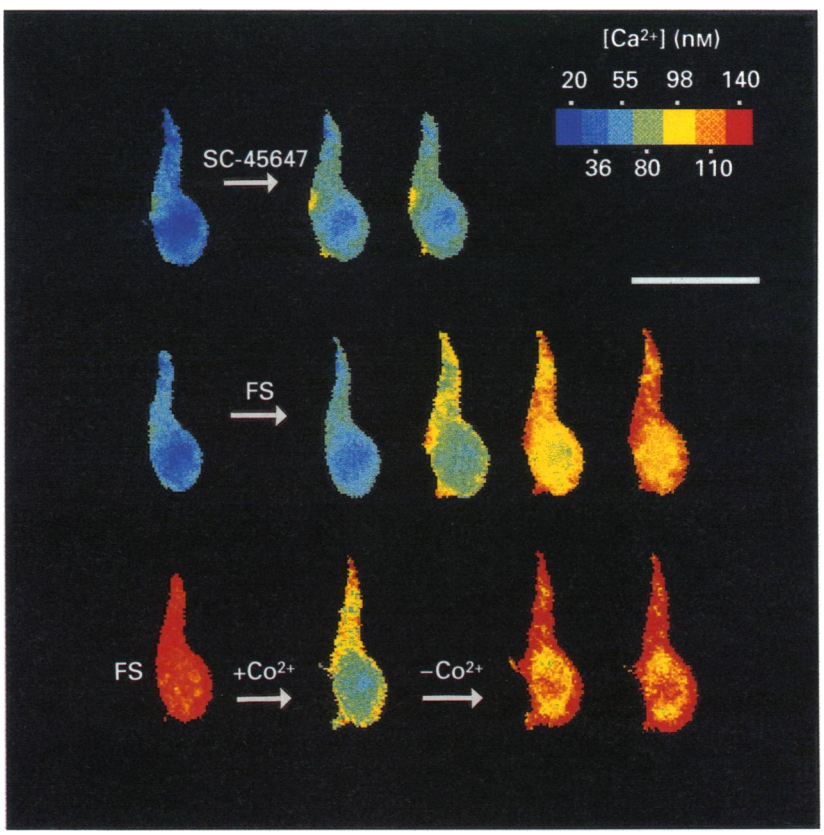

Figure 6. Pseudo-colour ratio images of a single taste cell loaded with fura-2

Responses to SC-45647 (100 μM) and to forskolin (FS, 100 nM) both in the presence of 1 mM $[Ca^{2+}]_o$. The increase in $[Ca^{2+}]_i$ in response to SC-45647 occurred not at the tip of the dendrite but in the soma close to the base of the dendrite. The Ca^{2+} response to forskolin became attenuated when Co^{2+} (2 mM) was added to the superfusion solution. Scale bar, 20 μm.

might be due to generation of IP_3 in bitter-responsive cells in addition to the IP_3 generation in sweet-responsive cells.

The $[Ca^{2+}]_i$ responses to SC-45647 and sucrose differed in their dependence on $[Ca^{2+}]_o$. Only the response to sucrose required the presence of $[Ca^{2+}]_o$, that to SC-45647 did not. This result is compatible with our observation that SC-45647 increases the mass level of IP_3 much more than sucrose does. It suggests further differences in transduction cascades of sweet-responsive cells. The one addressed by sucrose involves cAMP and Ca^{2+} uptake, while the one addressed by SC-45647 involves IP_3 and Ca^{2+} release from intracellular, IP_3-sensitive stores.

Yield of responding cells and mechanism of depolarization

The response of rat taste cells to saccharin was previously investigated with the patch clamp method (Béhé *et al.* 1990). Of the cells from which whole-cell recordings were made, 34% responded to saccharin with a decrease in outward K^+ current. In contrast, we found in this study that merely one cell per optical cross-section of a taste bud (i.e. roughly 1 in 15) responded to the artificial sweeteners, and that a responding cell was found in every second taste bud only. However, the fractions of responding cells, obtained with the two methods, cannot be expected to be the same: in the patch clamp experiments, the total yield of living, not-rounded single cells is very low, mainly because of the necessity to triturate the preparation. In contrast, in the imaging experiments every cell of an optical cross-section enters the statistics. Furthermore, the starting material for the patch clamp experiments (i.e. Béhé *et al.* 1990) was taste buds from fungiform papillae while the source for the present investigation was circumvallate tissue. The response to sweeteners need not be the same in these tissues.

In the patch clamp experiments with taste cells from rat fungiform papillae, saccharin caused depolarizations which were due to a decrease of a K^+ conductance which co-determined the resting potential (Béhé *et al.* 1990). A decrease in K^+ conductance was also noted in frog taste receptor cells (TRCs) in response to forskolin, cAMP and the catalytic subunit of protein kinase A (Avenet *et al.* 1988), in mouse TRCs in response to cGMP, cAMP and sucrose (Tonosaki & Funakoshi, 1988), and in hamster TRCs in response to cGMP and cAMP analogues and two artificial sweeteners (Cummings *et al.* 1993; Kinnamon *et al.* 1994). Thus the electrophysiological evidence permitted the hypothesis that sucrose and non-sugar sweeteners utilize the same transduction pathway, involving cyclic nucleotides, protein kinase A, closure of a leak-type K^+ conductance and depolarization. However, our present results with rat taste cells suggest that two non-sugar sweeteners utilize a different transduction pathway, involving IP_3 rather than cAMP. We expect that the IP_3 pathway will also lead to membrane depolarization, possibly via activation of protein kinase C and closure of the leak-type K^+ channels. More work will be required to elucidate this transduction pathway.

Evaluation of cytosolic Ca^{2+} signals

Synaptic exocytosis requires a local increase in Ca^{2+} by several hundred nanomolar, and transient Ca^{2+} values in excess of 50 μM have been observed (Augustine, Adler & Charlton, 1991; Llinas, Sugimori & Silver, 1992; von Gersdorf & Matthews, 1994). Therefore, the $\Delta[Ca^{2+}]_i$ values in the order of 50 nM found in the present experiments are too small to initiate release of transmitter. The same is true for the similar values obtained by others in taste buds responding to denatonium (Akabas *et al.* 1988). However, the $[Ca^{2+}]_i$ values obtained may be considered to be attenuated by blurring effects (Monck *et al.* 1992), which result from the non-confocality of the imaging system used and give rise to signal averaging across focal planes. The effect will be especially noticeable when, in a cluster of cells, all loaded with fura-2, only one cell responds with an increase in $[Ca^{2+}]_i$. Then the image of the responding cell will be contaminated with light from non-responding cells located in out-of-focus planes. In consequence, the $[Ca^{2+}]_i$ of the responding cell will be underestimated. For the same reason, a localized increase in $[Ca^{2+}]_i$, occurring somewhere within an isolated cell (as in the upper part of Fig. 6), will be underestimated because of contamination with light from volume elements surrounding the zone of elevated $[Ca^{2+}]_i$.

Therefore, the values for $[Ca^{2+}]_i$ obtained from fura-2-loaded taste buds by conventional, not confocal, microscopy are underestimates. Confocal methods will be required to correct them. In addition, temporal averaging might occur where long exposure times to fluorescent light are required. This would also lead to an underestimate of $[Ca^{2+}]_i$ values. However, the conclusions drawn in this study are not affected by the averaging effects, since the $[Ca^{2+}]_i$ changes are merely taken as indicators of activation of a given cell.

Two synergistic pathways

In vascular smooth muscle IP_3 and cAMP address antagonistic cellular functions, cAMP causing relaxation and IP_3 contraction (Adelstein, 1983). Yet, in many other cells, the two messenger pathways have *synergistic* actions, as in olfactory receptor cells, melanocytes, adrenal glomerulosa cells and exocrine pancreas cells (Boekhoff, Tareilus, Strottmann & Breer, 1990; Thremblay, Payet & Gallo-Payet, 1991; Graminski, Jayawickreme, Potenza & Lerner, 1993; Kasai & Petersen, 1994). We conclude that a cAMP–IP_3 synergism also holds for sweet-receptor cells.

Structural considerations have suggested for the rat that the ligands sucrose and SC-45647, notwithstanding their

chemical differences, might be accommodated by a *single* receptor binding site (Hellekant & Walters, 1993). Furthermore, it is known that heptahelical receptors often have the potency for *dual signalling*, i.e. to activate both the cAMP and the IP_3 pathway. Indeed, recent studies and reviews list a number of cloned, G protein-coupled receptors (for thyrotropin, luteinizing hormone, calcitonin, parathyroid hormone, vasopressin and catecholamines), which are able to activate both adenylyl cyclase and phospholipase C (Gierschik & Camps, 1993; Zhu, Gilbert, Birnbaumer & Birnbaumer, 1994). At least in some of these cases, the α-subunit of heterotrimeric G proteins activates the cyclase while, at higher agonist concentration, the β,γ-complex activates the phospholipase (Gierschik & Camps, 1993). A high receptor concentration also tends to favour activation of phospholipase C (Zhu *et al.* 1994), and it is not excluded that different ligands will predominantly activate one or the other of the two signalling pathways.

Therefore, our observation that different ligands, i.e. sugar and non-sugar sweeteners, activate separate transduction cascades in the same cell, does not force us to assume the involvement of more than one type of sweet receptor. Yet, suggestive psychophysical and chemical evidence for the existence of *more than one* sweet receptor (in humans) has also been found (Faurion, Saito & Mac Leod, 1980; Van der Heijden, Van der Wel & Peer, 1985). Nerve recordings in the gerbil are compatible with separate receptor sites for sucrose and saccharin (Jakinovich, 1982). Our present results indicate the coexistence of the cAMP and IP_3 pathways for sweet taste transduction for the rat in single receptor cells.

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