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Pungent Chemicals Increase Cytoplasmic Free Calcium Concentration in Mammalian Taste Receptor Cells

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Pungent compounds are widely used for seasoning. Hot sensation evoked by pungent chemicals including capsaicin, has been known that it is the result of stimulation of free nerve endings in sensory neurons. However, there has not been rigorous study of its direct effects on the taste receptor cells(TRCs). In the present study, we investigated direct effect of two kinds of pungent chemicals, capsaicin and piperine, on the cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) in the isolated rat taste receptor cells using micrspectrofluorimetry technique. The effect of bitter taste and sweets on $[Ca^{2+}]_i$ was also examined for comparision with those of pungent chemicals. Epithelial sheets rich in taste buds and free of muscle tissue were isolated from the circumvallate papilla of the rat tongue by mixed enzyme treatment following microdissection. After exposure to Ca^{2+}-free Tyrode solution, we could get taste buds or single TRCs from the epithelial sheets. In the high K^+ bath solution, $[Ca^{2+}]_i$ transient was observed, suggesting existence of voltage dependent Ca^{2+} channels in TRCs. Capsaicin increased $[Ca^{2+}]_i$ in a dose dependent manner in these cells. 10 μM capsaicin little affected on $[Ca^{2+}]_i$, but 50 μM capsaicin increased a small amount of $[Ca^{2+}]_i$. 100 μM capsaicin increased a large amount of $[Ca^{2+}]_i$, from 1.04 ± 0.05 (Mean ± S.E.) to 1.40 ± 0.059(n=28). The effect of piperine on $[Ca^{2+}]_i$ responses was similar to those of capsacin, but piperine was more potent than capsaicin. 1 μM piperine little affected on $[Ca^{2+}]_i$ responses, but 5 μM piperine increased a small amount of $[Ca^{2+}]_i$. 10 μM piperine increased a large amount of $[Ca^{2+}]$i, from 0.99 ± 0.03 (Mean ± S.E.) to 1.43 ± 0.05(n=20). The increase of $[Ca^{2+}]_i$ evoked by both capsaicin and piperine appears to be due to Ca^{2+} influx from the extracelllular medium, since the capsaicin induced $[Ca^{2+}]_i$ increase was not observed in Ca^{2+} free bath solution. Furthermore, 10 mM ruthenium red also inhibited this capsaicin induced $[Ca^{2+}]_i$ increase, suggesting it's mediated by vanilloid receptors. We also confirmed that denatonium, one of bitter taste and sucrose, sweet compound, both increase $[Ca^{2+}]_i$ in these cells. But the Ca^{2+} responses to these two substances were quite different to those evoked by pungent chemicals; Ca^{2+} response was even observed in the Ca^{2+} free bath solution. All our results suggest that pungent chemicals including capsaicin and piperine could increase $[Ca^{2+}]_i$ responses of TRCs, presumably via vanilloid receptors.

Keywords: taste receptor cells, calcium, microspectrofluorimetry, capsaicin, piperine.

Introduction

Although the mechanism of taste transduction has been studied for a long time, very little is known about the physiology of the taste cells or the molecular mechanisms involved in the gustatory transduction process. One of the difficulties in molecular investigations of taste has been the sparsity of the taste bud cells, with less than 1% of tongue surface area occupied by taste buds (Hwang *et al.*, 1990). Also the organization and morphology of the taste buds and papillae determine that only the apical 1-2% of the taste cell's plasma membrane is exposed to the oral environment (Spielman *et al.*, 1996). Thus, the lack of knowledge about taste transduction might be due to their relative inaccessibility in situ.

Recently the various enzymes to dissociate taste receptor

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cells has been successfully applied to mammals (Akabas *et al.*, 1988; Spielman *et al.*, 1989). Recent research on cellular mechanisms of peripheral taste sensation has elucidated transduction pathways involving membrane receptors, G proteins, second messengers and ion channels (Lindemann, 1996). Based on up-to-date study, taste can be divided into four primary and two further sensations : salty, sour, sweet, bitter, umami and water. Each taste submodality has its own specific mechanism: salty taste seems to be due to Na^+ flux through apical Na^+ channels (Avenet *et al.*, 1991). Sour seems to be mediated by H^+ ion blockade of K^+ or Na^+ channels (Gilbertson *et al.*, 1992). Bitter (Spielman *et al.*, 1996) and sweet (Bernhardt *et al.*, 1996) seem to be mediated by G-protein dependent mechanisms. Recently, two further classes were found, which relates to the pleasant taste called 'umami' (Chaudhari *et al.*, 1995) and a receptor mechanism for water (Okada *et al.*, 1993).

Many pungent compounds are familiar to us as seasonings. Although their structures have been manifested, their action mechanism as seasonings remain unknown since few physiological experiments have focused on this oral, gustatory aspects of pungent stimuli. In contrast, pharmacological actions of the pungent compounds, especially those of capsaicin (Holzer, 1991), have been extensively investigated in sensory neurons. Capsaicin exerts a peculiar action on a subpopulation of sensory neurons with a possible nociceptive action (Fusco *et al.*, 1997). Hence capsaicin and other compounds have been supposed to stimulate free nerve endings, so it has been categorized as one of the pain sensation caused by irritation of free nerve endings in mouth.

However, the threshold concentration of capsaicin on human tongues for producing a simple warm sensation, was lower than that for producing definite painful burning sensation (Gilmore and Green, 1993). It is also unlikely that many spices act only as irritants when added to dishes. In the present study, we investigated whether pungent compounds could increase $[Ca^{2+}]_i$ in isolated rat taste cells directly using microspectrofluorimetry technique.

Materials and Methods

Taste cell preparation

Male Sprague-Dawley rats weighing around 200 g were sacrificed by cervical dislocation after ether anesthesia, and the tongue was removed. Cell preparation was done according to the method of Striem *et al.* (1991). The enzyme mixture containing collagenase 5 mg/ml and trypsin inhibitor 2 mg/ml was injected submucosally at 3-4 locations around and under the single CV papilla found back section of the tongue. The enzyme treated tongue was incubated for 30 minutes in 37°C oxygenated Tyrode solution. The CV tissue was identified under a dissection microscope (10-12× magni-fiication). Iris scissors were used to make a circular or square cut around the CV. Then epithelium containing the CV was peeled off from the underlying muscle. The peeled epithelium was treated again with enzyme containing collagenase and trypsin inhibitor to dissolve the remaining basal membrane for 30 minutes. After exposure to Ca^{2+}-free Tyrode solution for about 10 minutes, taste buds from the papilla could be separated. By controlling the exposure time of Ca^{2+}-free Tyrode solution, more single taste receptor cells(TRCs) than taste buds were obtained. Taste buds or dissociated TRCs could be printed (attached) on to the surface of the several coverslips which were pre-coated with Cell-Tak (Collabo-rative Biomedical Products Co.).

Fluorescent dye loading and $[Ca^{2+}]_i$ measurement

A microscope photometry technique with Ca^{2+} sensitive dye, fura-2 acetoxymethyl ester (fura-2) was used to determine $[Ca^{2+}]_i$ Isolated TRCs were loaded by 2 μM fura-2 in normal tyrode solution of 2 ml for 30 minutes at room temperature(25°C). TRCs are quite different from the epithelial cells in shape, so they were identified by cell morphology in our experiment. TRCs were looked as spindle-shaped with one or two elongated process contrary to the spherical epithelial cells. They were visualized using an epifluorescence microscope (Axiovert 10, Zeiss, Germany). For excitation of the fluorescent probe fura-2, a collimated beam of light from a 75W Xenon arc lamp was passed through a dual spectrophotometer (Photon Technology International, U.S.A.). This altered wavelengths from 340 to 380 nm by means of a spinning chopper (60 Hz). The emitted light, selected at 510 nm, was collected by a photomultiplier.

Experimental solutions and drugs

The $[Ca^{2+}]_i$ measurement was carried out in the bath when the solution was perfused at 37°C. The flow rate was 1 ml/min. Capsaicin and piperine were added to normal Tyrode solution and the concentration mentioned in this experiment is the final concentration. The composition of control Tyrode solution was(mM/L); NaCl 120, KCl 5, $CaCl_2$ 5, $MgCl_2$ 1, glucose 10, HEPES(free acid) 5. The pH was adjusted to 7.4 with NaOH. Calcium free Tyrode solution was made by omitting $CaCl_2$ and adding 1 mM EGTA. All the reagents of capsaicin, piperine, ruthenium red, denatonium, sucrose and trypsin inhibitor were purchased from Sigma. Collagenase from Beringer Manheim(Germany) and fura-2 from Molecular probe.

Results

Capsaicin effect on $[Ca^{2+}]_i$.

Fig. 1A shows a $[Ca^{2+}]_i$ transient in a single TRC evoked by high K bath solution (60 mM KCl). KCl is replaced with an equimolar amount of NaCl in the normal Tyrode solution. High K bath solution increased $[Ca^{2+}]_i$ from 1.12 ± 0.03 in

the resting state to 2.24± 0.15(n=14). The Y axis means values of 340/380 nm ratio fluorescence of $[Ca^{2+}]_i$. Under the Ca^{2+}-free bath solution, the increase of $[Ca^{2+}]_i$ was not observed (n=4). Our result suggests that there are volatage-dependent Ca^{2+} channels in TRCs. A $[Ca^{2+}]_i$ transient evoked by depolarization is due to Ca^{2+} influx from the extracellular medium via these channels. Fig. 1B shows that capsaicin increase $[Ca^{2+}]_i$ in a dose dependent manner. 10 μM capsaicin hardly increased $[Ca^{2+}]_i$, but 50 μM capsaicin increased a small amount of $[Ca^{2+}]_i$. 100 μM capsaicin increased a large amount of $[Ca^{2+}]_i$, from 1.04±0.05 (Mean ± S.E.) to 1.40± 0.059 (n=28).

Fig. 2A shows a source of $[Ca^{2+}]_i$ evoked by 100 μM capsicin. In the Ca^{2+}-free bath solution, an increase of $[Ca^{2+}]_i$ was not observed. When bath solution was changed again to normal bath solution, $[Ca^{2+}]_i$ was increased. The transient increase of $[Ca^{2+}]_i$ evoked by capsaicin (n=28) was from 1.04± 0.05 (Mean± S.E.) to 1.40± 0.05. As shown in this figure, capsaicin induced $[Ca^{2+}]_i$ seems to be due to extracellular Ca^{2+} influx. Fig. 2B shows the antagonizing effect of 10 μM ruthenium red on the capsicin induced $[Ca^{2+}]_i$ increase(n=4). Ruthenium red has been known as a capsaicin-sensitive cation channel blocker.

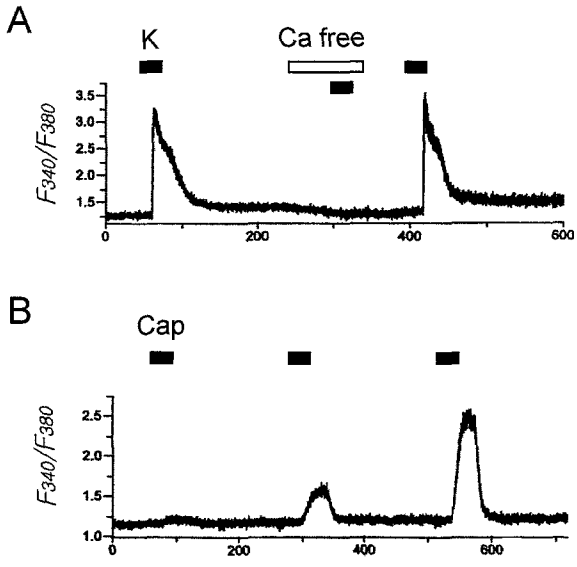

Fig. 1. A. $[Ca^{2+}]_i$ transient in a single TRC evoked by high K bath solution (60 mM KCl). The Y axis means values of 340/380 nm ratio fluorescence of $[Ca^{2+}]_i$. High K bath solution increased $[Ca^{2+}]_i$ from 1.12± 0.03 in the resting state to 2.24± 0.15(n=14). Under the Ca^{2+}-free bath solution, the increase of $[Ca^{2+}]_i$ was not observed (n=4). B. Capsaicin increase $[Ca^{2+}]_i$ in a dose dependent manner.

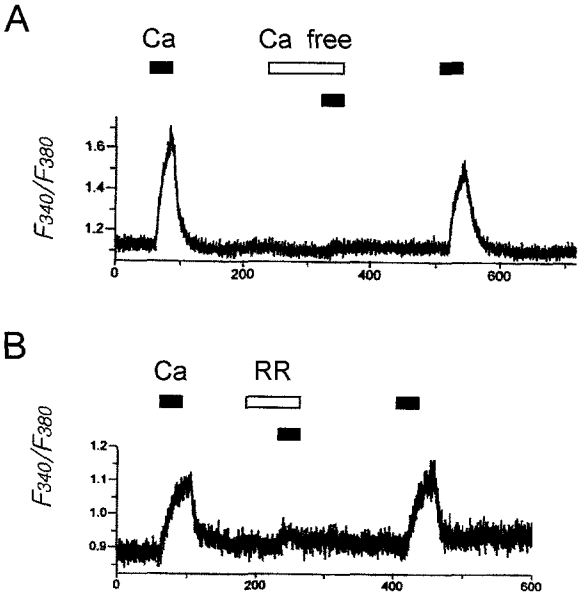

Fig. 2. A. $[Ca^{2+}]_i$ transient evoked by 100 μM capsicin only observed in the bath solution containing Ca^{2+}. B. The antagonizing effect of 10 μM ruthenium red on the capsicin induced $[Ca^{2+}]_i$ increase(n=4).

Piperine effect on $[Ca^{2+}]_i$

Fig. 3A shows a dose-response relationship of piperine on $[Ca^{2+}]_i$. 1, 5 and 10 μM piperine was added to normal

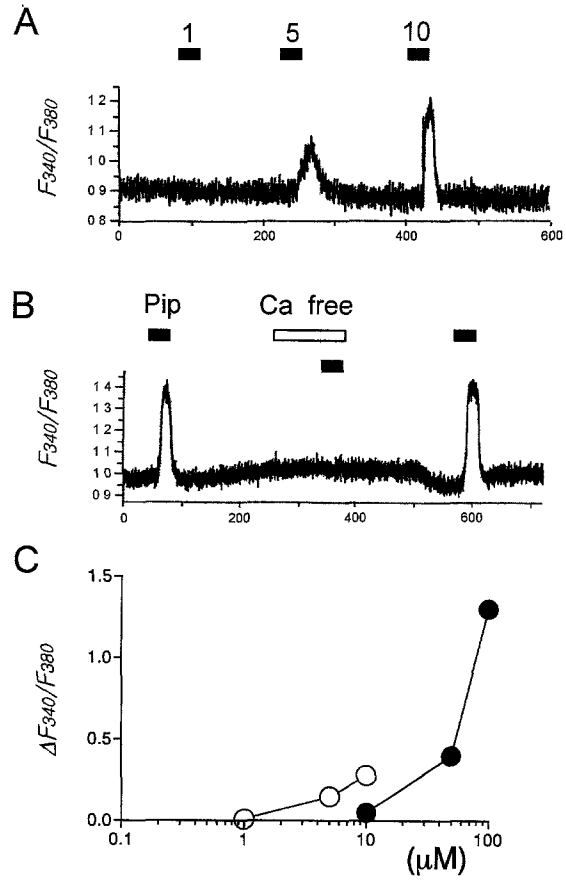

Fig. 3. A. Dose-response relationship of piperine on $[Ca^{2+}]_i$. B. $[Ca^{2+}]_i$ transient evoked by 10 μM piperine also only observed in the bath solution containing Ca^{2+}. C. Comparision of dose response curves for capsaicin(dark circles) and piperidine(white circles). The graph shows that piperine is more potent than capsaicin roughly by one magnitude.

Tyrode solution. In the lower concentration of 1 μM, the elevation of $[Ca^{2+}]_i$ induced by piperine was not found, but 5 μM piperine increased a small amount of $[Ca^{2+}]_i$. 10 μM piperine increased a large amount of $[Ca^{2+}]_i$, from 0.99± 0.03 to 1.43± 0.05(n=20). Fig. 3B shows that a $[Ca^{2+}]_i$ increase evoked by 10 μM piperidine is also due to $[Ca^{2+}]_i$ influx from the extracellular medium. In the Ca^{2+}-free bath solution, increase of $[Ca^{2+}]_i$ was not observed. When bath solution was changed again to normal bath solution, $[Ca^{2+}]_i$ increased. The result suggests that extracellular Ca^{2+} is indispensable for TRCs to respond to piperine. Fig. 3C shows a dose response curve for capsaicin(dark circles) and piperidine(white circles). The graph shows that piperine is more potent than capsaicin roughly by one magnitude. However, the amount of $[Ca^{2+}]_i$ release is much higher in capsaicin than piperine.

Effect of denatonium and sucrose on $[Ca^{2+}]_i$

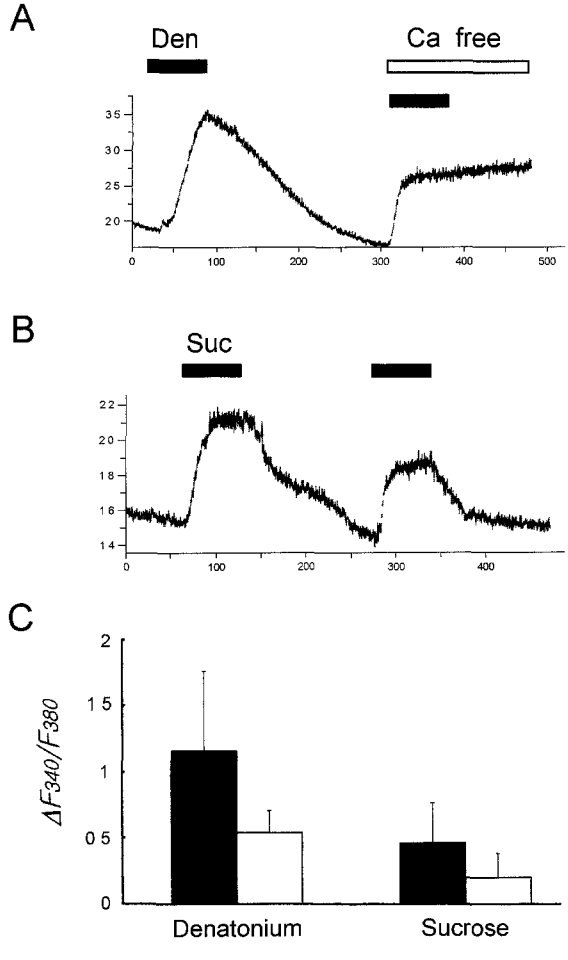

Fig. 4. The effect of two kinds of sapid substances, denatonium, one of bitter tastants, and sucrose, sweet substance, on the $[Ca^{2+}]_i$. A. 10 mM Denatonium evoked a $[Ca^{2+}]_i$ increase even in the Ca^{2+} free bath solution. B. A reversible effect of 300 mM sucrose on $[Ca^{2+}]_i$. C. The average amount of $[Ca^{2+}]_i$ increase induced by denatonium and sucrose in the presence (dark bars, n=3-4) and absence of extracellular Ca^{2+} (white circles, n =3-4).

Fig. 4 shows the effect of two kinds of sapid substances, 10 mM denatonium, one of bitter tastants, and 300 mM sucrose, sweet substance, on the $[Ca^{2+}]_i$. Both denatonium and sucrose increased $[Ca^{2+}]_i$. In contrast, denatonium evoked a $[Ca^{2+}]_i$ increase even in the Ca^{2+} free bath solution, suggesting that a $[Ca^{2+}]_i$ increase induced by denatnium is due to the Ca^{2+} release from intracellar stores(Fig. 4A). Fig. 4B shows a reversible effect of sucrose on $[Ca^{2+}]_i$ of TRCs. Fig. 4C shows the amount of $[Ca^{2+}]_i$ increase(dark bars) is much higher in denatonium than sucrose and both sapid substance evoked $[Ca^{2+}]_i$ increase in the absence of Ca^{2+} in extracellula medium(white bars). The amount of $[Ca^{2+}]_i$ increase was 1.16± 0.54 and 0.6± 0.31(n=3-4) by denatonium and sucrose, respectively. While, the amount of $[Ca^{2+}]_i$ increase by these sapid substances in the Ca^{2+} free medium was 0.46± 0.2 and 0.17± 0.18(n=3-4), respectively.

Discussions

Capsaicin, a pungent ingredient of chili peppers of the genus *Capsicum*, is eaten daily by over a third of the world's population (Rozin, 1990). Piperine, which is found in black pepper (*Piper nigrum*) is considered natural analogue of capsaicin. Capsaicin elicits responses in a subset of nociceptive C and Aδ-fibers, including the polymodal nociceptive fibers (Winter *et al.*, 1995). Acute excitation of sensory neurons by peripheral application of capsaicin causes a burning painful sensation (Wood *et al.*, 1993), which is associated with depolarization of sensory neurons by opening of nonselective cation channels (Oh *et al.*, 1996). Capsaicin interact at a specific membrane recognition site expressed almost exclusively by primary sensory neurons involved in nociception and neurogenic inflammation (Szallasi, 1994: Szallasi and Blumberg, 1996). Capsaicin stimulates a vanilloid receptor on a particular population of sensory neurons with unmyelinated C-fiber-type process, which are polymodal nociceptive detectors with free nerve endings peripherally (Buck and Burks, 1986). Based on up-to-now researches, pungent compounds have been assumed to stimulate the free nerve endings of the sensory neurons contained in taste nerve bundles rather than TRCs.

The increasing rate of $[Ca^{2+}]_i$ to reach the peak was in proportion to the capaicin concentration. Application with high concentration of capsaicin(100 μM) accerelated increasing rate of $[Ca^{2+}]_i$ to the peak. An increase of $[Ca^{2+}]_i$ induced by capsaicin was reproducible. After $[Ca^{2+}]_i$ rapidly increased following application of capsaicin, the $[Ca^{2+}]_i$ gradually declined to the prestimulus level within 50 seconds. The taste responses to capsaicin were transient and were not shown the biphasic states seen from other research experimented in sensory neuron (Park *et al.*, 1997). The capsaicin-sensitive cation channel blocker, ruthenium red antagonized the increase of $[Ca^{2+}]_i$ evoked by capsaicin effectively. All above results suggest that capsicin induced

increase of $[Ca^{2+}]_i$ in TRCs is mediated by vanilloid receptors, which is quite a different intracellular transduction mechanism compared with those of a bitter or sweet tastant. It was dubious that threshold concentration of capsaicin was in the range of 10-100 M, which was extremely higher than we expected. When placed on the human tongue, capsaicin induces a warm sensation(at 0.6 μM), a burning and sometimes a stinging/pricking sensation(at 3 μM), and a painful sensation(at 15 mM) (Gilmore and Green, 1993; Karrer and Bartoshuck, 1991; Lawless and Stevens, 1984). But in this experiment, the threshold concentration for capsaicin (50 μM) was not comparable with the above capsaicin concentration which caused warm or burning sensation. On the contrary, threshold concentration for piperine (5 μM) was comparable with that obtained from dose-response curve (3 μM) [15]. In cultured trigeminal ganglion neurons, capsaicin is more potent than piperine(Liu and Simon, 1996). However, our results revealed that piperine was more potent than capsaicin especially in eliciting gustatory responses.

It has been suggested that external Ca^{2+} may be important in regulating the responsiveness of neurons to capsaicin (Santicioli *et al.*, 1987). In TRCs, external Ca^{2+} was important in eliciting taste responses to capsaicin, too. An increases of $[Ca^{2+}]_i$ evoked by pungent stimuli were not observed in the Ca^{2+} free bath solution. When Ca^{2+} was added to the bath, $[Ca^{2+}]_i$ was increased again by pungent chemicals. These data indicated that the $[Ca^{2+}]_i$ increases induced by depolarization or pungent stimuli were due to Ca^{2+} influx. Thus there are high possibilities that receptors for capsaicin and piperine are existing in TRCs and they involve in elevating $[Ca^{2+}]_i$, probably by Ca^{2+} influx via voltage dependent Ca^{2+} channels and/or nonselective cation channels.These findings, which were quite different from the those in the neuron, suggest that capsaicin may stimulate the taste cells directly and thus elicit taste sensations, as supported by other researcher (Yoshi and Matui, 1994).

In summary, gustatory effects of capsaicin appears to be different from its pharmacological action on sensory neurons. The increase of $[Ca^{2+}]_i$ evoked by pungent chemicals seems to be mediated by vanilloid receptors, which is quite a different mechanism to those elicited by other tastants, sweetner or a bitter tastant.

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