INTRODUCTION

Recent biochemical and electrophysiological studies have demonstrated that there are two intracellular pathways for sweet taste transduction: sugars stimulate an increase in the intracellular concentration of cyclic AMP (cAMP) (Bernhardt et al. 1996; Naim et al. 1991), whereas synthetic sweeteners and some amino acids stimulate the production of 1,4,5-inositol trisphosphate (IP$_3$) and diacylglycerol (DAG) (Bernhardt et al. 1996; Uchida and Sato 1997). Several lines of evidence suggest that both second-messenger pathways are expressed in the same sweet-sensitive taste cells. Calcium-imaging studies have shown that the same taste cells respond to both sucrose and the synthetic sweetener SC-45647 (Bernhardt et al. 1996), and electrophysiological studies have shown that the same taste cells respond to both cAMP and synthetic sweeteners (Cummins et al. 1996; Tonosaki and Funakoshi 1988). Presumably, these second messengers are activated when sweet stimuli bind to G protein–coupled receptors on the taste cell membrane. Recently, a putative sweet taste receptor was cloned (Hoon et al. 1999), but the receptor has not been characterized functionally.

Synthetic sweeteners and cAMP depolarize taste cells by closing a resting K$^+$ conductance, and these responses show cross adaptation (Cummins et al. 1996). Thus the cAMP and IP$_3$/DAG second-messenger pathways appear to target the same K$^+$ channels, but the mechanisms involved are not known. Taste cells have been shown to express both cyclic nucleotide–activated (Misaka et al. 1997) and cyclic nucleotide–suppressed (Kolesnikov and Margolskee 1995) ion channels, which suggests that nucleotides can interact directly with ion channels in taste cells. Yet, in studies of frog taste cells, K$^+$ channels are closed by cAMP-dependent protein kinase (Avenet et al. 1988), suggesting that protein kinase A (PKA)–mediated phosphorylation of K$^+$ channels can occur. For synthetic sweeteners, one consequence of elevating IP$_3$ is release of Ca$^{2+}$ from intracellular stores, which has been measured in rat taste buds (Bernhardt et al. 1996). An increase in intracellular Ca$^{2+}$ coupled with the production of DAG has been shown in other systems to activate protein kinases, specifically protein kinase C (PKC). However, whether PKC activation is required for K$^+$ channel closure in response to synthetic sweeteners is not known.

The loose-patch technique for recording from taste buds in situ has provided important information about transduction mechanisms in mammals for NaCl (Avenet and Lindemann 1991), acids (Gilbertson et al. 1992), and sweeteners (Cummins et al. 1993). The technique consists of recording action currents, reflecting taste cell action potentials, from single fungiform taste buds in situ. In hamsters, action currents are generated to NaCl, citric acid, sucrose, and several synthetic sweeteners, but not to most bitter compounds (P. Avenet and S. Kinnamon, unpublished data). Previous studies have shown that, whereas most hamster taste buds are NaCl sensitive, only ~35% are sweet sensitive. The responses to sweeteners in any single taste bud are reliable and repeatable for up to periods of 2 h. In addition, responses show dose dependency, with increased frequency of action currents at higher concentrations.
Sweet-sensitive taste buds also respond to membrane-permeant analogues of cAMP and cGMP, but sweet-insensitive taste buds are unresponsive to these second messengers. The responses to both sweeteners and second messengers show adaption at higher concentrations (Cummings et al. 1993).

In this study we used the loose-patch technique to investigate the role of protein kinases in sweet taste transduction. Specifically, we asked the following questions. 1) What is the role of PKC in the transduction of synthetic sweeteners? 2) What is the role of PKA in the transduction of sugars? 3) Do the sugar and sweetener transduction pathways interact with each other? Preliminary accounts of this work were published in abstract form (Varkevisser and Kinnamon 1998; Varkevisser et al. 1997).

Methods

Preparation and recording method

Golden Syrian hamsters ranging from 4 to 10 wk in age were killed by CO₂ asphyxiation and cervical dislocation. Their tongues were excised ~4 mm posterior to the field of the fungiform papilla, rinsed with distilled water, and mounted in a bipartitioned silicone elastomer (Sylgard) recording chamber. The dorsal surface of the exposed anterior half of the tongue was illuminated and viewed with a dissecting microscope at ×5–50 magnification. The loose-patch recording technique (Avenet and Lindemann 1991; Cummings et al. 1993; Gilbertson et al. 1992) was used to record from the fungiform papillae. The recording electrode, pulled on a Sachs-Flaming PC84 micropipette puller and fire polished to a tip diameter of 70–120 μm, was positioned over a fungiform papilla. Strong suction was applied to the pipette via a suction pump connected to the loose-patch device just above the pipette. Contained within this pipette were the perfusion pipette and a AgCl wire connected to the headstage ground.

Perfusion pipettes were fabricated from fused silica tubing (Polymicro Technologies, Phoenix, AZ; 224 μm OD, 100 μm ID) pulled by hand over a gas flame. The open end was fitted to polyethylene tubing that was connected to the pipette assembly. The tip was cut to a diameter of 40–50 μm. The tip of this inner pipette was positioned to within 500 μm of the tip of the recording pipette to allow the solution to reach the taste bud before removal by suction. Solutions were held in eight 50-mL syringes, each with a fitted stopcock, and were connected by polyethylene tubing to the pipette assembly. The solution reservoirs were pressurized such that a turn of one stopcock pushed the solution of choice through the perfusion pipette into the tip of the recording electrode and thus over the taste pore itself. Action currents, reflecting taste cell action potentials, were seen within 15–25 s after switching the stopcocks to deliver stimuli. Subthreshold responses usually could not be resolved. Because of variability in the stimulus delivery, response latencies could not be ascertained with this technique. The recording pipette, perfusion pipette, and ground electrode were all fitted into one end of the pipette assembly.

An agar bridge impregnated with Tyrode’s solution and connected to a AgCl wire was placed against the cut proximal end of the tongue. The lead was connected to the headstage of a patch-clamp amplifier (Axopatch 1D, Axon Instruments) with a 100-MΩ feedback resistor. A AgCl wire in the recording pipette served as the ground electrode. This reversal of leads minimized the power line interference (cf., Avenet and Lindemann 1991). Currents were recorded with the amplifier in voltage-clamp mode at a pipette potential of 0 mV. The amplifier was kept in tracking mode to minimize DC shifts resulting from junction potentials generated during solution changes. The signal was filtered with a low-pass Bessel eight-pole filter (Frequency Devices, 902 LPF) at a corner frequency of 270 Hz. The analogue signal from the amplifier was digitized at 125 Hz by an A/D board (Digidata 1200 interface, Axon Instruments), viewed on-line, and stored on a PC (Applied Computer Technology) using Axoscope software.

Solutions

The control solution, designed to mimic the low ionic strength of hamster saliva, consisted of 30 mM N-methyl-D-glucamine chloride (NMDG-Cl) and 5 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer (HEPES) titrated to a pH of 7.4 with HCl. Taste stimuli dissolved in this control solution were 100 mM NaCl, 200 mM sucrose, and 200 μM NC-00274–01 (NC-01), a synthetic sweetener. These concentrations were shown previously to be strongly preferred by hamsters and to elicit robust responses in loose-patch recordings of taste buds in situ (Cummings et al. 1993). The membrane-permeant drug, phorbol-12,13-dibutyrate (PDBu), diluted in the control solution to a concentration of 10 μM, was used as an activator of PKC. The inhibitors used were: bisindoylmaleimide I (Bis I; 0.15 μM), a specific inhibitor of PKC; H-89 dihydrochloride (10 and 19 μM), a specific inhibitor of PKA; and W-7 hydrochloride (W-7, 100 μM), a specific inhibitor of Ca²⁺-calmodulin dependent cAMP phosphodiesterase (CaM-PDE). Inhibitor concentrations were chosen based on the vendor’s specifications. For most experiments we chose the highest concentration that would ensure specificity. Inhibitors were perfused before and during the application of the test stimulus, after control stimuli were tested. Thus a typical experiment began with verifying that the taste bud was sweet sensitive, followed by the application of the inhibitor, then the simultaneous application of inhibitor with sweet stimuli. All inhibitors were obtained from Calbiochem (La Jolla, CA). NC-00274–01 was a generous gift of the NutraSweet Corporation. All other chemicals were obtained from Sigma Chemical Corporation (St. Louis, MO).

FIG. 1. Responses to the protein kinase C (PKC) activator in sweet-responsive (A) and sweet-unresponsive (B) taste buds. Summary of data (C) indicates that sweet responsivity is correlated with responses to phorbol-12,13-dibutyrate (PDBu). In this and subsequent figures, the bar above the trace represents the onset of the solution change. Thus the latency of the response involves both the stimulus delivery time (which can be several seconds) as well as the latency in response to the taste stimulus.
Data analysis

To determine the effects of various protein inhibitors on the response to sucrose and the synthetic sweetener NC-01, action currents were monitored in response to taste stimuli before and during application of the inhibitor. If spontaneous activity was not present, the response duration was defined as the interval between the first and last spikes of the response. In taste buds that exhibited spontaneous activity, a response was defined as a twofold increase in activity over basal (unstimulated) activity. Thus the beginning of a response was defined as the moment spike frequency increased to twice that of background activity; the end of the response occurred when the rate decreased to \(<50\%\) of the stimulated response. This response magnitude was chosen to ensure that responses could be delineated clearly from spontaneous activity. Because response rates tend to saturate at high concentrations of taste stimuli (Cummings et al. 1993), experiments with a high rate of spontaneous activity were omitted from analysis. Sweet stimulus response rates were analyzed by counting the number of spikes within the response and dividing by the total duration of the response. During experiments that involved using a protein inhibitor, a response was analyzed only if the effect of the inhibitor was reversible. For display purposes, response rates during application of the inhibitor were normalized by dividing the response rate during application of the inhibitor by the response rate in the absence of the inhibitor. Therefore a value of \(>1\) indicates an increase in the rate of response, whereas a value \(<1\) indicates a decrease in the interspike rate of the response to the taste stimulus. For statistical analysis, spike rates (spikes/s) were analyzed for each taste bud in the presence and absence of the inhibitor. Statistical analysis of all experiments was performed using a one-tailed Wilcoxon signed rank test.

RESULTS

Experiments were performed using the loose-patch technique for recording from taste buds in situ (Avenet and Lindemann 1991). By placing a recording electrode over the apical pore of fungiform papillae, we recorded population action currents from single taste buds in response to sweet taste stimuli in the presence and absence of protein inhibitors. The advantage of this approach is that the sweet stimulus could be restricted to the taste pore, as occurs in situ. This allowed us to use sucrose as a taste stimulus, which is sweet only at concentrations that would cause osmotic damage if applied to isolated taste cells. The disadvantage of the technique is that we are unable to distinguish which taste cells within the taste bud participate in a response, or if the same taste cells participate in each response during repetitive stimulation. In addition, responses to the same taste stimuli vary in different taste buds. Some taste buds exhibit robust responses to sweet stimuli,
whereas others show only weak responses or no response at all. Thus all responses to sweet stimuli in the presence of protein inhibitors were normalized to responses before application of the inhibitors.

Each taste bud was first tested by stimulation with 100 mM NaCl, because previous studies showed that most taste buds respond vigorously to NaCl (Avenet and Lindemann 1991; Cummings et al. 1993). This was done to ensure that cells within the taste bud were capable of generating action potentials. A total of 283 taste buds was tested in this manner. Taste buds that were responsive to NaCl were then tested for responses to NC-01 and sucrose. Of the total number of taste buds responding to NaCl (198) only a small fraction (<35%) responded to the sweet stimuli. Taste buds that were sweet sensitive usually responded to both sucrose and NC-01, as shown previously (Cummings et al. 1993).

Role of protein kinase C in sweet taste transduction

In rat membrane preparations, application of synthetic sweeteners leads to the production of DAG and IP$_3$ (Bernhardt et al. 1996), a second messenger that leads to the opening of IP$_3$-gated Ca$^{2+}$ channels in intracellular stores. One effect of elevated Ca$^{2+}$ in the presence of DAG is activation of protein kinases, specifically PKC. To determine whether PKC activation is required for generation of taste cell action potentials in response to synthetic sweeteners, we first examined whether activation of PKC mimicked the effect of sweet taste stimuli. Because not all taste buds are sweet responsive, we correlated responses to NC-01 and sucrose with responses to the membrane-permeant PKC activator, PDBu (10 $\mu$M).

Figure 1 depicts recordings from two fungiform taste buds. Both were first stimulated with NaCl to demonstrate viability of the taste bud. Following a wash of >3 min, each taste bud was stimulated sequentially with NC-01, sucrose, and PDBu, with intervening washes. PDBu elicited action currents in the sweet-sensitive taste bud (Fig. 1A) but had no effect on the sweet-insensitive taste bud (Fig. 1B). Of the 11 sweet-sensitive taste buds tested (Fig. 1C), all responded to PDBu with action currents. In contrast, only 4 of 19 sweet-insensitive taste buds tested responded to PDBu with action currents. Responses in sweet-insensitive taste buds may indicate a role for PKC in the transduction of taste qualities other than sweet.

The results described above suggest that PKC may be involved in the sweet transduction pathway, but whether it plays a role in the transduction of synthetic sweeteners or sugars cannot be determined from such correlative studies. To test more specifically PKC’s involvement in sweet taste transduction, Bis I (0.15 $\mu$M), a membrane-permeant inhibitor of PKC, was used to determine whether PKC activation is required for sweet taste transduction. Figure 2 illustrates the effect of Bis I on responses to sucrose and NC-01. In the taste bud illustrated in Fig. 2, A and B, responses to NC-01 before application of the inhibitor were more robust than responses to sucrose (Fig. 2A). However, in the presence of Bis I, responses to NC-01 decreased, whereas responses to sucrose showed a large increase (Fig. 2B). This large enhancement of the sucrose response occurred in only 2 of the 11 experiments, although there was a small increase in response to sucrose in several experiments. The effects of the inhibitor were reversible (Fig. 2C). Overall, the response to sucrose remained unchanged ($P = 0.1016$) during inhibition of PKC, whereas the response to NC-01 decreased to ~25% of the control response ($P = 0.0020; n = 11$, Fig. 2D). These data suggest that activation of PKC is directly involved in the transduction of synthetic sweeteners. In addition, the enhancement of the sucrose response in the presence of Bis I in some taste buds suggests that the transduction pathway for sugars may be negatively coupled to the transduction of synthetic sweeteners in these buds, possibly via activation of PKC.

Role of protein kinase A in sweet taste transduction

There is abundant evidence that sucrose stimulates cAMP (Bernhardt et al. 1996; Naim et al. 1991; Striem et al. 1989) and that cAMP elicits action potentials in taste cells (Cummings et al. 1993) by closure of a resting K$^+$ conductance (Cummings et al. 1996). What is not clear is whether phosphorylation is required for this response. cAMP-dependent protein kinase (PKA) has been shown to phosphorylate K$^+$ channels in frog taste cells (Avenet et al. 1988), but this response was not connected to sweet stimulation. To determine...
whether PKA is required for transduction of sugars in hamster taste buds, we perfused H-89 (10 and 19 \( \mu \)M), a membrane-permeant inhibitor of PKA, onto sweet-sensitive taste buds. Quite to our surprise, H-89 did not inhibit the response to either sucrose or NC-01 in any of the six taste buds tested. Instead, H-89 increased the response to sucrose and NC-01 in most taste buds. In the experiment shown in Fig. 3, the response to NC-01 increased \(-1.5\) fold in the presence of H-89, whereas the response to sucrose increased over 10-fold. The degree of increase was quite variable in magnitude, especially for sucrose. The increase in response rate to sucrose varied from 20\% to over 1,400\%, whereas the increase in response to NC-01 was somewhat less variable, ranging from little or no increase to approximately a 400\% increase. The effects of H-89 were reversible. Overall, the increase in response in the presence of H-89 was statistically significant (NC-01: \( P = 0.0313 \); sucrose: \( P = 0.0156 \)). What is very clear from these data are that PKA is not required for the transduction of sugars, because there was no decrease in the response to sucrose in the presence of H-89. These data suggest that cAMP may close K\(^+\) channels directly in response to sucrose stimulation.

Role of CaM-dependent phosphodiesterase in sweet transduction

Although not statistically significant, in a few of the taste buds tested with Bis I, there was an enhancement of the sucrose response coupled with the inhibition of the NC-01 response. This raises the possibility that the pathway for transduction for synthetic sweeteners inhibits the pathway for transduction of sugars. We have already demonstrated that PKC activation can inhibit the transduction of sucrose in some taste buds, because some taste buds showed increases in response to sucrose after inhibition of PKC (i.e., Fig. 2). In addition, further inhibition may result from the IP\(_3\) that is produced in response to stimulation with synthetic sweeteners. IP\(_3\) causes an increase in intracellular Ca\(^{2+}\) due to release of Ca\(^{2+}\) from intracellular stores. This increase in intracellular Ca\(^{2+}\) may activate CaM-PDE, resulting in a decrease in intracellular cAMP levels, which would inhibit the sucrose response. Thus we examined whether inhibition of CaM-PDE influences the response to sucrose. W-7 (100 \( \mu \)M), a specific membrane-permeant inhibitor of CaM-PDE (Itoh and Hidaka 1984), was perfused onto sweet-sensitive taste buds before and during sweet taste stimulation. Figure 4 illustrates the effect of W-7 on responses to sucrose and NC-01 in a sweet-responsive taste bud. In the presence of W-7 (Fig. 4B), there was no effect on the response to NC-01, whereas the response to sucrose increased fivefold. This effect of W-7 was reversible. Analysis of 10 taste buds showed an average 2-fold increase in the rate of response to sucrose (\( P = 0.0010 \)), whereas that to NC-01 remained unchanged (\( P = 0.2852 \)). Taken together, these data suggest that activation of CaM-PDE during stimulation with NC-01 may inhibit responses to sucrose by decreasing intracellular cAMP levels.

**FIG. 4.** Responses to sucrose and NC-01 before (A) and after (B) the application of the CaM-PDE inhibitor, W-7. C: responses from a different taste bud show that the effect of W-7 is reversible. D: mean responses of fungiform taste buds to sucrose and NC-01 in the presence of the inhibitor; responses are normalized to control responses.
PDBu, a potent activator of PKC, elicited action currents in PKC in the transduction pathway for synthetic sweeteners. sweet taste transduction. Lindemann 1991; Cummings et al. 1993; Gilbertson et al. technique for recording from taste buds in situ (Avenet and normalization of the taste cell. In this study, we used the loose-patch technique for recording from taste buds in situ (Avenet and Lindemann 1991; Cummings et al. 1993; Gilbertson et al. 1992) to examine the role of downstream regulatory proteins in sweet taste transduction.

Our study provides additional support for distinct, but interdependent pathways in the transduction of sugars and synthetic sweeteners. Moreover, we provide strong evidence for a role of PKC in the transduction pathway for synthetic sweeteners. PDBu, a potent activator of PKC, elicited action currents in sweet response taste cells. More direct evidence was obtained from inhibitor studies; responses to sweeteners during inhibition of PKC decreased 75% compared with control responses, whereas responses to sugars remained unaffected. It is possible that PKC phosphorylates the sweet-sensitive K+ channels, leading to closure and membrane depolarization, but this awaits confirmation by single-cell recording.

In contrast to sweeteners, our results do not support the need for phosphorylation of channels in the transduction pathway for sugars. Inhibition of PKA resulted instead in an enhancement of responses to both sugars and synthetic sweeteners. One caveat in this interpretation is whether H-89 is getting into the cells at a concentration sufficient to block PKA. We believe that the increase in the response to both sweeteners and sugars is evidence that the H-89 is getting into the cells. Responses were enhanced whether the inhibitor was in place for a few minutes or >1 h, which should be sufficient time for the inhibitor to permeate the taste cells. Further, we have used two different concentrations (10 and 19 μM) of the inhibitor and found no differences between the two concentrations. However, we cannot rule out the possibility that H-89 is not blocking all isoforms of PKA, although it is the most broad-spectrum membrane-permeant PKA inhibitor that is currently available.

The tendency for H-89 to increase the response to NC-01 was not totally unexpected, because several studies with other systems have shown that PKA-mediated phosphorylation can inhibit the production of IP3 when both second-messenger systems are expressed in the same cells (Campbell et al. 1990; Liu and Simon 1996). In taste cells, a recent study shows that some bitter compounds both increase IP3 and inhibit cAMP, suggesting that a decrease in cAMP may be required for full activation of the IP3 response (Yan et al. 1999). In contrast, the inhibitory effect of PKA on the response to sugars was unexpected, because a previous study had shown that PKA can phosphorylate and close K+ channels in taste cells (Avenet et al. 1988). It is likely that PKA plays a modulatory, rather than a mediatory role in the response to sugars. One possibility is that PKA phosphorylates components in the sugar transduction pathway, causing adaptation of the response. In support of this hypothesis, whole cell recordings show strong adaptation in response to membrane-permeant cAMP analogues (Cummings et al. 1996).

So, what is the role of cAMP in the transduction of sugars?


