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Sweet tastants stimulate adenylate cyclase coupled to GTP-binding protein in rat tongue membranes

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Sucrose and other saccharides, which produce an appealing taste in rats, were found to significantly stimulate the activity of adenylate cyclase in membranes derived from the anterior-dorsal region of rat tongue. In control membranes derived from either tongue muscle or tongue non-sensory epithelium, the effect of sugars on adenylate cyclase activity was either much smaller or absent. Sucrose enhanced adenylate cyclase activity in a dose-related manner, and this activation was dependent on the presence of guanine nucleotides, suggesting the involvement of a GTP-binding protein ('G-protein'). The activation of adenylate cyclase by various mono- and di-saccharides correlated with their electrophysiological potency. Among non-sugar sweeteners, sodium saccharin activated the enzyme, whereas aspartame and neohesperidin dihydrochalcone did not, in correlation with their sweet-taste effectiveness in the rat. Sucrose activation of the enzyme was partly inhibited by Cu^{2+} and Zn^{2+}, in agreement with their effect on electrophysiological sweet-taste responses. Our results are consistent with a sweet-taste transduction mechanism involving specific receptors, a guanine-nucleotide-binding protein and the cyclic AMP-generating enzyme adenylate cyclase.

INTRODUCTION

Although considerable information is available on sweet-taste responses [1–6], little is known about the transmembrane molecular transduction events in taste cells. It has been previously hypothesized that sweet- and bitter-taste transduction involves cyclic nucleotides as second messengers [1,7–13]. High adenylate cyclase activity has been found in the gustatory epithelium of vertebrates [14], and histochemical techniques have shown that both adenylate cyclase and phosphodiesterase activities are located in the microvillar membranes of cells in taste buds of rabbits [15]. These studies, however, did not provide direct evidence for the involvement of cyclic AMP as a second messenger in sweet-taste transduction, as it has not been conclusively demonstrated that tastants stimulate adenylate cyclase activity in taste-cell membranes. Recently it has been suggested that cyclic AMP is a second messenger in olfactory transduction, and that its odorant-dependent generation is mediated by a GTP-binding protein (G-protein) [16–18]. The present study addresses the question of similarity between the two chemosensory transduction mechanisms, providing evidence for the GTP-dependent stimulation of cyclic AMP production by sweet-tasting compounds in rat lingual membranes. The recent reports that cyclic AMP blocks transmembrane K^+ currents and that cyclic AMP and cyclic GMP increase resistance in taste cells [19,20], in addition to the fact that cyclic AMP-dependent protein kinase is involved in this process [21], lend further support to the participation of cyclic AMP in taste transduction. A preliminary account of our present work has already been presented [12,13].

MATERIALS AND METHODS

Reagents

Guanosine 5'-[γ-thio]triphosphate (GTP[S]) was purchased from Boehringer-Mannheim, and forskolin was from Calbiochem. Sucrose was purchased from J. T. Baker Co. All other reagents were obtained from Sigma. [α-^{32}P]ATP was bought from the Nuclear Research Center-Negev, Israel. Aspartame was a product of Searle (Arlington Heights, IL, U.S.A.). Neohesperidin dihydrochalcone (NHD) was supplied by Jaf-Ora, Rehovot, Israel.

Membrane preparations

Male and female Sprague–Dawley rats (5–6 weeks old) were obtained from the Weizmann Institute Animal Breeding Center. The animals were quickly decapitated, the tongues and skeletal muscle were removed and collected in an iso-osmotic 25 mM-Tris/HCl buffer, pH 8.0, containing 150 mM-NaCl and 1 mM-phenylmethanesulphonyl fluoride. This and all subsequent operations were carried out at 0–4 °C. Each tongue was separated into the following parts: (*a*) the anterior 4–5 mm, as well as an additional 4–5 mm of the upper-dorsal area immediately posterior to it ('anterior tongue taste epithelium', ATTE); (*b*) muscle tissue containing some epithelium with no taste buds from the ventral part of the tongue ('tongue muscle', TM); and (*c*) posterior-dorsal area of 4–5 mm^2, located next to the circumvallate papillum and not containing taste buds ('tongue non-sensory epithelium', TNSE). Skeletal muscle (SM) was processed in parallel. Pools containing each tissue from

Abbreviations used: G-protein, GTP- or guanine-nucleotide-binding protein; GTP[S], guanosine 5'-[γ-thio]triphosphate; ATTE, anterior tongue taste epithelium; TM, tongue muscle; TNSE, tongue non-sensory epithelium; SM, skeletal muscle; OC, olfactory cilia; G_s, stimulatory-type G-protein; NHD, neohesperidin dihydrochalcone.

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50–100 rats were homogenized with a Polytron tissue homogenizer (30 s, speed setting 6) in hypo-osmotic 25 mM-Tris/HCl (pH 8.0)/1 mM-phenylmethanesulphonyl fluoride (lysis buffer). Membranes were prepared in a two-step procedure essentially as described [22]. Following the removal of tissue pieces, intact cells and nuclei, by centrifugation at 1100 *g* for 15 min, the supernatant was centrifuged at 27000 *g* for 30 min. The sedimented membranes were resuspended in the lysis buffer at 500 μg of protein/ml, frozen in aliquots in liquid nitrogen, and stored at −70 °C. Samples were thawed once only, before determination of adenylate cyclase activity. These thawed membranes are presumed vesicles which are permeable to nucleotides and other charged molecules utilized in the adenylate cyclase assay. Owing to difficulties in rapid isolation of large quantities, no attempt was made to dissect individual lingual fungiform papillae or to utilize taste epithelium from other sweet-responding areas, such as the bone-attached soft palate [23,24]. Olfactory cilia (OC) were prepared as described by Chen *et al.* [25].

Adenylate cyclase assay

Adenylate cyclase was assayed by the method of Salomon [26], with slight modifications. The reaction mixture in each test tube (50 μl) included 10 μl of assay cocktail, 20 μl of effectors (tastants, guanine nucleotides, others) and 20 μl of membrane suspension. This allows for a relatively low dilution (×2.5) of all the tastant solutions, hence the attainment of high final sugar concentrations. All the data shown represent results of several experiments, each carried out in replicate, as indicated in the Figures. The protein content of samples was measured by the method of Bradford [27], with bovine serum albumin as a standard.

RESULTS

Membranes from the anterior-dorsal region of rat tongue (ATTE) were examined. This area contains > 90% of the tongue's fungiform taste papillae [24,28], which are known to respond well to sweet-taste stimuli [24]. It was found that adenylate cyclase in ATTE membranes was stimulated 3-, 8- and 102-fold by GTP, GTP[S] and NaF respectively (Fig. 1, open bars) and 11-fold by forskolin (results not shown). Such stimulation by effectors is reminiscent of that observed in other membrane preparations, suggesting the presence of a forskolin-sensitive catalytic subunit, coupled with a guanine nucleotide- and fluoride-activated GTP-binding protein.

In order to find out whether this enzyme cascade is coupled with chemosensory receptors, perhaps underlying sweet-taste transduction, we examined the effect of sucrose, a widely studied sweet compound, in the rat and other species, on adenylate cyclase activity. We utilized a concentration (1.0 M) previously shown to elicit maximal or near-maximal electrophysiological and behavioural responses in the rat [29–34]. Sucrose was found to activate adenylate cyclase to various extents depending on the effector added (GTP, GTP[S] and NaF, Fig. 1). On average, sucrose was found to activate adenylate cyclase by 27% when no effector was added or by 17% in the presence of NaF. In contrast, when the guanine nucleotides GTP and GTP[S] were present, respective activation values of 95 and 93% were observed (see the

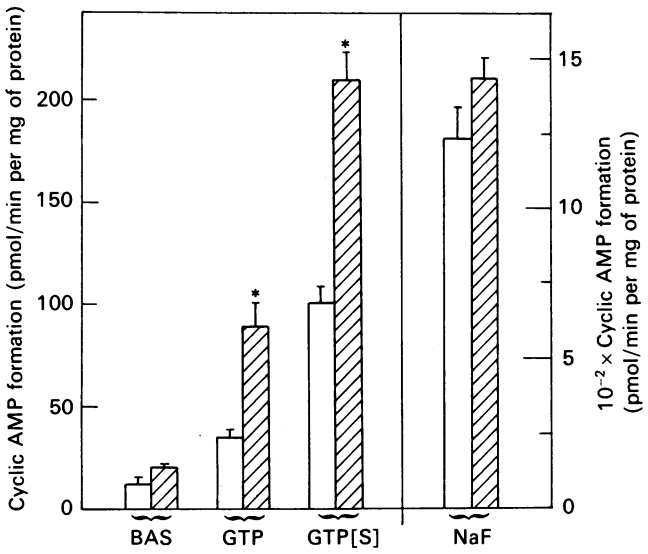

Fig. 1. Adenylate cyclase activity in rat tongue membrane (ATTE)

Basal activity (BAS) and stimulation by 10^{-5} M-GTP, 10^{-5} M-GTP[S], 10^{-2} M-NaF (open bars) and stimulation of this activity by 1.0 M-sucrose (shaded bars) are shown. Values are means ± S.E.M. of four triplicates in a representative experiment. The mean of the stimulation by sucrose in *n* experiments was: BAS, 27 ± 15% (S.E.M., $n = 11$); GTP, 95 ± 16% ($n = 10$); GTP[S], 93 ± 7% ($n = 26$); and NaF, 17 ± 9% ($n = 12$). * indicates a significant difference ($P < 0.05$) between the activities in the absence and in the presence of sucrose. NaF results refer to the right-hand ordinate scale, others refer to the left-hand one.

legend to Fig. 1), thus indicating that the activation of adenylate cyclase in ATTE membranes is dependent on guanine nucleotides. Such synergism between a physiological ligand and guanine nucleotides in adenylate cyclase activation suggests the coupling of receptors via a GTP-binding protein of the stimulatory type (G_s).

Subsequently, other sugars previously shown to elicit taste electrophysiological responses in the rat [29–31] were tested for their ability to stimulate adenylate cyclase in the ATTE membrane preparation. All of these sugars were also appealing to the rat in behavioural (sensory) experiments, suggesting sweet taste [32–34]. Activation was measured in the presence of GTP[S], which was found to be almost equal to GTP in its synergistic effect with sucrose, but gave rise to higher absolute activity values, thus minimizing experimental errors. Two disaccharides, sucrose and maltose, and two monosaccharides, D-glucose and D-fructose, were found to be effective adenylate cyclase activators in the presence of GTP[S] (Fig. 2*a*) or GTP (results not shown). Sucrose was the most effective, D-glucose and maltose gave intermediate activation values, and D-fructose was the least potent. These results resemble their effectiveness in electrophysiological recordings from the rat chorda tympani nerve [29–31].

The range of saccharides used in the present study was broadened by including two other sugars possessing high water solubility, so as to form the required stock concentration (3.0 M). Thus trehalose and melibiose were found to activate adenylate cyclase in the presence of GTP[S], and melibiose was even more effective than

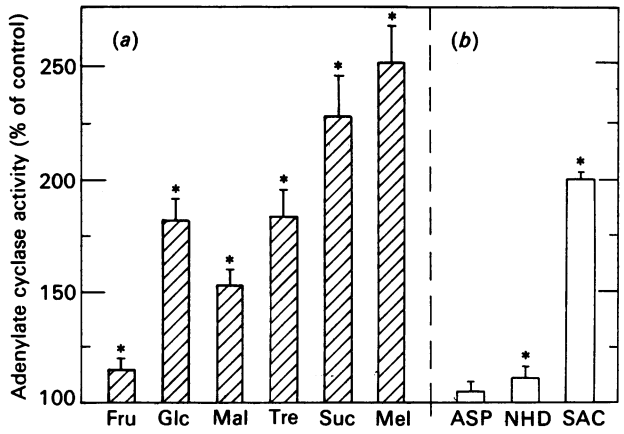

Fig. 2. Adenylate cyclase activity in ATTE membranes after stimulation by sweeteners

(*a*) Sugar sweeteners: D-fructose (Fru), D-glucose (Glc), maltose (Mal), trehalose (Tre), sucrose (Suc) and melibiose (Mel), at 1.0 M. (b) Non-sugar sweeteners: aspartame (ASP) at 10 mM, NHD at 1 mM and sodium saccharin (SAC) at 20 mM. Values are means ± S.E.M. for three or four triplicates for each data point. The reaction mixture contained 10^{-5} M-GTP[S] as the guanine nucleotide. * indicates a significant difference ($P < 0.05$) between the activity measured in the presence of tastant and that of control GTP[S] only.

sucrose in adenylate cyclase activation (Fig. 2*a*). Since no data were available on the taste properties of these two disaccharides, preference-aversion experiments were conducted, using a brief-exposure (10 min) two-choice preference test as described in [35]. The results (B. J. Striem, U. Zehavi and M. Naim, unpublished work) showed 90% preference level ($P < 0.05$) at concentrations above 0.3 M for both melibiose and trehalose.

In addition to sugar sweeteners, the non-sugar sweeteners sodium saccharin, aspartame and NHD were also tested. Sodium saccharin (20 mM), a potent appealing tastant in the rat [35,36], was found to stimulate adenylate cyclase in the rat ATTE membranes by 99% in the presence of GTP[S] (Fig. 2*b*) and 113% in the presence of GTP (result not shown). The other two sweeteners, aspartame and NHD, which appear to be tasteless to the rat [35,36], gave very small activation values at their respective physiological concentrations (Fig. 2*b*).

The activation of adenylate cyclase by sucrose in the presence of GTP[S] was measured at different sugar-tastant concentrations and was found to be dose-dependent over the range 0.2–1.0 M-sucrose (Fig. 3; ATTE). It was not possible to reach a plateau within the range tested, the highest feasible sucrose concentration (1.2 M) giving 142% activation (result not shown).

In order to further verify the specificity of the sucrose effect on adenylate cyclase in ATTE membranes, other membrane preparations were studied in parallel as controls. TM membranes gave considerably lower activation values (maximally 36%) by 1.0 M-sucrose (Fig. 3). Membranes from TNSE, OC and SM showed negligible activation, or even some inhibition, of adenylate cyclase (Fig. 3).

The same approach was employed to test the tissue specificity of the sodium saccharin response. Unexpectedly, sodium saccharin was also found to be a rather

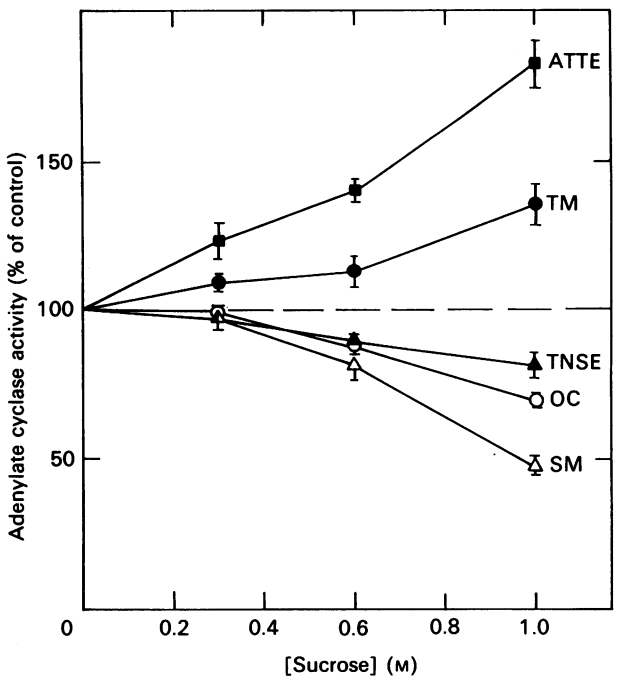

Fig. 3. Dose–response curve for sucrose-induced adenylate cyclase activity in membranes of different tissues (ATTE, TM, SM, TNSE and OC)

The reaction mixture contained 10^{-5} M-GTP[S] as the guanine nucleotide. The results are means ± S.E.M. for two triplicates for each data point for TNSE, OC and SM and of five triplicates for each data point for TM and ATTE. The total values (pmol of cyclic AMP/min per mg of protein) with no added sucrose were: ATTE, 122; TM, 97; SM, 172; TNSE, 96; and OC, 245535.

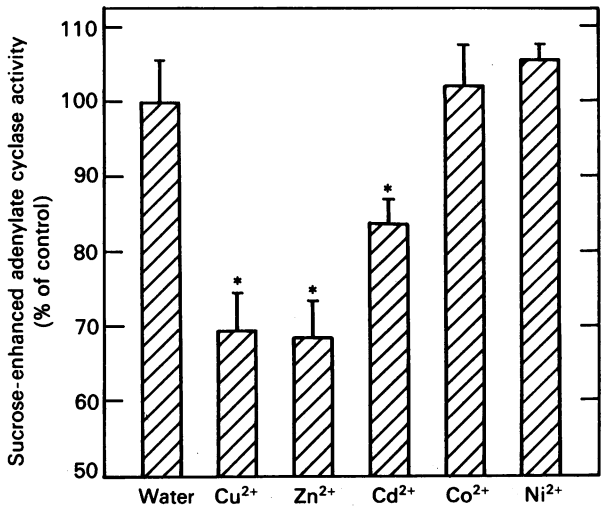

Fig. 4. Effect of bivalent-metal ions on sucrose activation of adenylate cyclase in the ATTE preparation

The sucrose-enhanced activity with no metal ions added (water) and the inhibitory effect of different metal ions (all at 10^{-4} M of their chloride salt). * indicates a significant difference ($P < 0.01$) between the activity measured in the absence of metal ions and their presence. Values are means ± S.E.M. for at least two triplicates for each data point. The reaction mixtures contained 10^{-5} M-GTP[S], and the concentration of sucrose employed was 1.0 M.

potent activator of adenylate cyclase in membranes from TM (67 % activation), whereas lower activation was seen in SM membranes (34 % activation), and practically none was observed in all other membrane preparations tested.

It has been shown that rat taste responses are inhibited by some bivalent cations, notably by Cu^{2+} and Zn^{2+} [3,5,37,38]. We found that $CuCl_2$ and $ZnCl_2$ at 10^{-4} M also inhibit the sucrose-enhanced component of adenylate cyclase activity (Fig. 4) and that this inhibition is dose-dependent in the range of 10^{-6}–10^{-4} M (results not shown). Importantly, the basal activity of the enzyme (in the absence of sucrose) remains unchanged within experimental error (±9 %). Cd^{2+} produced a slight inhibition effect, whereas Co^{2+} and Ni^{2+} were ineffective, in line with their effect on the electrophysiological taste responses in mice [38].

DISCUSSION

The present study deals with the molecular mechanism of rat sweet-taste transduction. This submodality was chosen because of ample evidence of receptor participation, including extensive data on structure–function relationships of sweet compound [39], information on the binding of sweet-tasting compounds to lingual membranes [40,41], as well as the existence of sweet-tasting proteins, sweet-taste modifiers, and sweet antagonists [1,5,42–45]. Such information could be employed in future biochemical studies. Although there is also evidence for receptor involvement in bitter taste [7,8], bitter compounds have not been included in the present study because many of them have pharmacological effects unrelated to taste (e.g. quinine is an inhibitor of voltage-dependent K^+ channels [46]). Salts and acids were not currently studied because of the expected side effects of pH and ionic strength on enzymes in a preparation of permeable membrane vesicles. The rat was chosen as a preferred experimental animal for biochemical studies because of the facile availability in large numbers and in defined strains, and because of the wide knowledge of rat taste behaviour and electrophysiology [29–36].

The present study demonstrates that some sweet-tasting compounds stimulate the formation of the second messenger cyclic AMP in a rat tongue membrane preparation. The activation of the enzyme adenylate cyclase by sugar sweeteners appears to be specific, since it is dose-dependent, it requires guanine nucleotides and it is tissue- and ligand-specific. The results are thus consistent with adenylate cyclase activation, which is mediated by specific protein receptors coupled through a G-protein.

The activation of adenylate cyclase in the rat tongue membranes can be used as an '*in vitro*' assay for chemosensory responses, since ATTE membranes are enriched in taste-cell membranes when compared with both TNSE and TM membranes, similarly to odour activation of the enzyme in olfactory membranes [16,17]. Such an assay could be refined through future development of improved taste membrane preparations, e.g. derived from isolated taste papillae or cells, or enriched in taste-cell microvilli, similar to the isolated olfactory cilia preparation [25]. Our results fullfil the criterion set by Robinson *et al.* [47] for cyclic AMP involvement in a receptor mechanism. Further support for cyclic AMP as a second messenger in taste transduction is derived from previous reports suggesting the presence of the enzyme adenylate cyclase in taste cells [10,14,15], as well as the modulation of taste responses by cyclic nucleotides and related compounds [9,48]. The suggested modulation of taste responses by adenosine receptors [49] and the identification of G_s protein in the fish taste membranes [50] are also consistent with cyclic AMP participation in taste transduction. Recently, Lindemann and co-workers [19,21] showed by means of patch-clamp ion-channel recordings, that K^+ conductance of the taste-cell membrane is decreased by cyclic AMP, thus providing evidence for cyclic AMP-dependent protein kinase involvement in this process. Decreased K^+ conductance induced by protein phosphorylation could underlie the observed tastant-induced taste-cell depolarization, leading to synaptic transmission [4]. Independently, intracellular injected cyclic AMP and cyclic GMP were shown to induce membrane depolarization and increased membrane resistance in mouse taste cells [20].

At present it is impossible to estimate the time course of the cyclic AMP-related response in intact cells. Such kinetics depend on many unknown parameters, such as phosphodiesterase activity, the cell compartment volume available to the second messenger, and the concentration, kinetics and dose-dependence of the cyclic AMP-responding elements. Yet, as in vision [51] and olfaction [52], it is conceivable that the small changes in cyclic nucleotide concentration necessary for sensory response could occur within a fraction of a second, consistent with the reported kinetics of tastant-related potential changes in taste-cell membranes [4].

The saccharide concentration range which gave rise to adenylate cyclase activation in ATTE membranes agrees well with the range reported to elicit taste responses as measured by electrophysiological methods [29–31]. The high concentrations necessary (0.1–1.2 M) suggest the existence of low-affinity receptors, probably evolved to accommodate the nutritionally significant sugar concentrations in many edible substrates. Such high concentrations of mono- and di-saccharides may present problems of non-specific effects on adenylate cyclase, owing to the high density, viscosity and osmotic pressure of the solutions. However, observations that different sugars at identical concentrations give widely different activations, which are tissue-specific and guanine nucleotide-dependent, are inconsistent with non-specific mechanisms related solely to physical parameters.

In the present study we were unable to demonstrate that the dose–response curve of adenylate cyclase activation by sucrose reaches saturation. Owing to experimental constraints related to sugar solubility, it was not feasible to attain final disaccharide concentrations higher than 1.2 M. It should nevertheless be pointed out that non-saturating dose–response curves have also been reported in electrophysiological studies [29,31], thus enabling one to estimate only the lower limit of the sucrose concentration required for half-maximal activation, which is about 0.8 M. This could provide an approximation of the apparent association constant of sucrose, characterizing the presumed receptor mechanism [53].

The potency of three different sugars in the activation of adenylate cyclase (sucrose > D-glucose > maltose) is in agreement with the reported strength of chorda tympani nerve responses [30,31]. Although this correlation needs to be further substantiated by parallel

electrophysiological and biochemical analyses for many sugars, the available agreement lends support to the use of adenylate cyclase measurement in studies of taste transduction.

An additional physiological correlation is the partial inhibition of sucrose activation of adenylate cyclase by Cu^{2+} and Zn^{2+}, in line with, although at different concentrations from those used in, the reported inhibition of electrophysiological sweet-taste responses [3,37,38]. Of most interest, the inhibition of sucrose activation of adenylate cyclase by bivalent cations such as Cu^{2+} and Zn^{2+}, but not by Cd^{2+}, Co^{2+} and Ni^{2+}, correlates well with the neural taste responses of rats and mice [37,38].

A correlation is also observed among the non-sugar sweeteners sodium saccharin, aspartame and NHD; sodium saccharin, the only compound which elicits a significant electrophysiological response in the rat [35,36], is also the only significant adenylate cyclase activator.

The stimulatory effect of sweeteners on the adenylate cyclase of tongue membranes is specific to this tissue, as several other membrane preparations showed much lower activation, or even inhibition. There is, however, one exception: considerable activation by sodium saccharin was also observed in membranes from TM, but in no other tested membrane preparations. This effect of sodium saccharin on muscle membranes could also contribute to the observed activation in ATTE membranes, which contain a sizeable amount of muscle membrane contamination. Additional studies will require clarification of the taste-related process of the effect of sodium saccharin on lingual adenylate cyclase.

Guanine nucleotides have been found to be required for sucrose activation in rat ATTE membranes. Both GTP and its non-hydrolysable analogue GTP[S] have been found to act in synergy with several sugar sweeteners as well as with sodium saccharin. The two guanine nucleotides, when applied alone, gave activation values which were roughly 10% of those attained with NaF. Thus both guanine nucleotides appear to activate only partially adenylate cyclase in rat ATTE membranes, allowing further activation by sweet-tasting ligands. Whereas GTP is known to be a universal partial activator of adenylate cyclase, GTP[S] is found to activate maximally in many membrane preparations [51], including olfactory cilia [16], and thus is not usually acting in synergy with receptor-binding ligands. In this respect the stimulatory GTP-binding protein (G_s) in ATTE membranes appears to differ from those of many other membrane preparations, but resembles rather the G_s of turkey erythrocyte membranes, where GTP[S] has been reported to act in synergy with isoproterenol [54].

It is important to note that no synergism with sucrose was seen in the ATTE membranes in which adenylate cyclase was fully activated with NaF. This further supports the claim that sucrose activation is a receptor-mediated process; a physically mediated activation due to viscosity or osmotic pressure would be also manifested in the fully activated enzyme.

Our results are consistent with the involvement of a GTP-binding protein in rat taste transduction, suggesting that taste may be a third sensory mechanism, parallel with vision and olfaction, which is mediated by a GTP-binding protein.

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