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Bitter taste transduced by PLC-β_2-dependent rise in IP_3 and α-gustducin-dependent fall in cyclic nucleotides

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Yan, Wentao, Gulshan Sunavala, Sophia Rosenzweig, Max Dasso, Joseph G. Brand, and Andrew I. Spielman. Bitter taste transduced by PLC-β_2-dependent rise in IP_3 and α-gustducin-dependent fall in cyclic nucleotides. *Am J Physiol Cell Physiol* 280: C742–C751, 2001.—Current evidence points to the existence of multiple processes for bitter taste transduction. Previous work demonstrated involvement of the polyphosphoinositide system and an α-gustducin ($G\alpha_{gust}$)-mediated stimulation of phosphodiesterase in bitter taste transduction. Additionally, a taste-enriched G protein γ-subunit, $G\gamma_{13}$, colocalizes with $G\alpha_{gust}$ and mediates the denatonium-stimulated production of inositol 1,4,5-trisphosphate (IP_3). Using quench-flow techniques, we show here that the bitter stimuli, denatonium and strychnine, induce rapid (50–100 ms) and transient reductions in cAMP and cGMP and increases in IP_3 in murine taste tissue. This decrease of cyclic nucleotides is inhibited by $G\alpha_{gust}$ antibodies, whereas the increase in IP_3 is not affected by antibodies to $G\alpha_{gust}$. IP_3 production is inhibited by antibodies specific to phospholipase C-β_2 (PLC-β_2), a PLC isoform known to be activated by $G\beta\gamma$-subunits. Antibodies to PLC-β_3 or to PLC-β_4 were without effect. These data suggest a transduction mechanism for bitter taste involving the rapid and transient metabolism of dual second messenger systems, both mediated through a taste cell G protein, likely composed of $G\alpha_{gust}/\beta/\gamma_{13}$, with both systems being simultaneously activated in the same bitter-sensitive taste receptor cell.

taste transduction; denatonium; second messenger; rapid kinetics; taste receptors; inositol 1,4,5-trisphosphate; phospholipase C

CONTINUING PROGRESS IS BEING made toward understanding the biochemical and molecular biological mechanisms of cellular taste transduction (7, 14, 20, 23, 25, 45). Several properties of the peripheral taste process make studying this chemical sense intriguing. For example, taste sensations can be elicited by a variety of structurally diverse compounds, some of which do not show absolute specificity toward a given modality (i.e., sweet, bitter, salty, sour, umami). It is also becoming clear that the taste system uses a variety of transduction mechanisms to signal the presence of taste-active compounds. Among gustatory stimuli, those that trigger the modality of bitterness are most diverse in structure (6, 48). Of the natural compounds that taste bitter, many are essential in plant defense mechanisms, with some being toxic. Indeed, bitter taste can be considered as a warning and defensive modality, providing a final analytical detector just before ingestion. A variety of bitter-tasting mechanisms may have evolved in response to this structural diversity and potential toxicity (7, 12, 48).

During the past two decades, bitter taste mechanisms have been the focus of an increasing number of studies. These studies used several techniques, including psychophysics (4, 10, 27, 59), behavioral genetics (e.g., Refs. 26 and 56), neurophysiology (13, 21, 35, 36, 44, 49, 55, 58), calcium imaging (2, 37), biochemistry (17, 29, 31, 39, 41, 43, 47, 51), and molecular biology (1, 11, 15, 16, 28–30, 42, 43, 58). These diverse studies support the assumption that several receptor/transduction processes are involved in bitter taste.

Several of these studies point to the possible involvement of receptor-second messenger pathways in bitter taste transduction. In particular, there is strong evidence from calcium imaging, biochemical, and neurophysiological studies that the polyphosphoinositol pathway is involved in mediating bitterness (2, 16, 17, 33, 34, 36, 41, 42, 47, 50, 51). Still other work convincingly demonstrates that a gustatory tissue-enriched G protein, $G\alpha_{gust}$, mediates bitter taste transduction. Because of $G\alpha_{gust}$'s sequence homology with the transducins, its activity is assumed to be via an activation of phosphodiesterases (PDE), thus implicating cyclic nucleotides as additional second messengers in bitter taste (21, 28, 29, 43, 45, 58).

We reasoned that both the polyphosphoinositol system and the cyclic nucleotide systems may be simultaneously activated in response to bitter compounds.

An important issue in supporting the relevance of several second messenger systems to bitter taste is

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measurement of the second messenger metabolism in real time. An outside limit to the initiation of a taste transduction response by most compounds can be set at ~250–500 ms after stimulus interaction with a receptor (19). Real-time assessment of biological processes in the millisecond time frame can be followed using stop-flow and quench-flow techniques. We and others have successfully developed and used these rapid kinetics procedures to measure second messenger metabolism in the millisecond time frame (8, 9, 16, 40–42, 51, 54).

Here we report second messenger metabolism in response to bitter stimuli in taste and control tissue from mouse SWR strain. Results indicate that bitter compounds such as denatonium and strychnine affect two second messenger systems simultaneously: they rapidly and reversibly elevate levels of inositol 1,4,5-trisphosphate (IP_3) and suppress levels of cAMP and cGMP. The G protein subunits activated by these bitter stimuli and the enzymes that mediate the metabolism of these second messengers are delineated using antibodies specific to each component.

MATERIALS AND METHODS

Tissue collection. Vallate and foliate taste papillae, along with control nongustatory lingual tissue, were collected from 6- to 8-wk-old SWR mice (Hilltop Lab Animals, Scottsdale, PA) by either carefully removing the papillae by a punch procedure (41, 46, 51) or peeling off the dorsal epithelium (5, 16). Peeling of the tissue was performed after subdermal injection of 4 mg/ml collagenase (type I; Boehringer Mannheim, Indianapolis, IN) and 1 mg/ml trypsin inhibitor (Worthington Biochemicals, Lakewood, NJ) in 50 mM MOPS buffer, pH 6.9, containing (in mM) 100 NaCl, 2.5 $CaCl_2$, and 2.5 $MgCl_2$ (MOPS I buffer). The tongue was incubated in this injection buffer for 20 min at 37°C, after which the entire posterior epithelium was peeled away under a dissecting microscope. From each peeled epithelium, one vallate and two foliate papillae and appropriate nongustatory control tissue were surgically isolated. Control tissue was removed from the dorsal eminence of the peeled epithelium, a region devoid of taste papillae. The tissue samples were washed in ice-cold MOPS II buffer [50 mM MOPS, pH 6.9, containing 100 mM NaCl, 2.5 mM $MgCl_2$, 1 mM 1,4 dithiothreitol (DTT), 10 mM EGTA, and 81 μM $CaCl_2$ to give a calculated free Ca^{2+} concentration of 0.010 μM] and a protease inhibitory cocktail [1 mg/ml, specific for serine, cysteine, aspartic and metallo-proteinases (Sigma, St. Louis, MO)]. In this study, tissue homogenates generated from the punch removal technique were used to collect data described in Figs. 1–4. Tissue homogenates generated from the epithelial peeling procedure were used for studies described by Figs. 5–7. Data from Figs. 1–4 were gathered early in this study. Subsequently (Figs. 5–7), we improved our tissue processing by removing some of the potential epithelial contaminants. This improvement also allowed us to use fewer mice per experiment. We checked to determine that the two preparations led to equivalent results.

For a typical experiment, taste and control tissue from 25–30 mice were homogenized using a glass-glass homogenizer in MOPS III buffer (equivalent to MOPS II buffer without enzyme inhibitors). The homogenate was centrifuged at 2,000 *g* for 10 min at 4°C. The supernatant (diluted to an appropriate protein concentration) was retained. Free calcium levels were calculated using the Chelator software (shareware, Dr. T. J. M. Schoenmakers, Dept. of Animal Physiology, Toernooiveld, Nijmegen 6525, The Netherlands). Protein analysis followed the Bradford assay using bovine gamma globulin as a standard (Bio-Rad, Hercules, CA).

Chemicals. Denatonium benzoate, strychnine HCl, and caffeine were purchased from Sigma. Rabbit polyclonal IgG antibodies to $G\alpha_{gust}$ (IgG), to phospholipase C-β_2 (PLC-β_2), PLC-β_3, and PLC-β_4 (and their respective blocking peptides), and normal (control) rabbit IgG (nIgG) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). [^{3}H]IP_3 and kits for assaying cyclic nucleotides (cAMP and cGMP) and IP_3 were purchased from NEN (Boston, MA). All other chemicals were of the highest purity available and were purchased from either Sigma or Calbiochem (San Diego, CA).

Quench-flow rapid kinetics. A quench-flow module (QFM5, BioLogic; Molecular Kinetics, Pullman, WA) equipped with five syringes was used to measure the subsecond kinetics of second messenger formation. (For description and operation details, see Refs. 41, 51, 54). In our QFM5 system, the first syringe was filled with MOPS III buffer supplemented with freshly prepared 1 mM ATP, 1 μM GTP, and 0.05% sodium cholate (hereafter called basal buffer). Bitter stimulants or pharmacological agents were dissolved into this basal buffer. The following stimulants and concentrations were tested (in mM): 2 and 10 strychnine and 1, 2, and 10 denatonium. For measures of cyclic nucleotides, the PDE inhibitor caffeine (25 mM) was added to the stimulus and control buffers for both taste and control tissues to raise the basal levels of the cyclic nucleotides (41).

The second syringe contained taste tissue or control homogenate in MOPS III buffer at a concentration of 35–50 μg/ml, except for studies shown in Figs. 5–7, where the protein concentration was 85–100 μg/ml. Tissue was maintained at 4°C at all times and loaded into the second syringe in small batches just seconds before injection at 22°C. The third syringe contained 9% perchloric acid to quench the reactions.

The reaction was initiated by mixing 60 μl basal buffer or basal buffer containing stimulants with 60 μl tissue. After 0, 25, 50, 100, 200, and 500 ms, the reaction was terminated by injection of 60 μl of 9% perchloric acid. For each time point this was repeated twice, so the collected volume for each time point was 360 μl. The activation of all syringes was controlled by a personal computer, using a software developed by the manufacturer (BioLogic) and drive sequences developed in our laboratory. For the zero time point, tissue was first quenched with ice-cold perchloric acid and then stimulated.

When antibodies to $G\alpha_{gust}$ or to the PLC-βs (10 μg/ml antibody per 35–100 μg protein/ml) or nIgG (same concentration) were used, the homogenate was preincubated with the antibodies for 90 min at 4°C and subsequently exposed to a particular buffer or stimulant. When blocking peptides were used, each blocking peptide was incubated with its appropriate antibody at 4°C overnight, and then the peptide-treated antibodies were incubated with the tissue under the conditions indicated above for antibodies not pretreated with blocking peptides. For studies involving blocking peptides alone, the same procedure was followed, except no antibody was added. After quenching, 330 μl (out of a total 360 μl) of the product was stored at −20°C until assayed.

Extraction and assay of cAMP and cGMP. On the day of the assay, samples were thawed and centrifuged at 1,000 *g* for 5 min. Cyclic nucleotides and IP_3 (shown in Figs. 6 and 7) were extracted from the supernatant by mixing with 82.5 μl of 10 mM EDTA, pH 7.0, and 10 μl of Universal Indicator, with pH adjusted further to pH 7.0 using 1.5M KOH and 60 mM HEPES (38). The mix was centrifuged at 1,200 *g* for 5

min at 4°C. The supernatant was assayed either for cGMP and cAMP (using a ^{125}I-labeled RIA kit from NEN) or for IP_3 (see below).

Extraction and assay of IP_3. Each sample was centrifuged at 1,000 *g* for 5 min, and the supernatant was mixed 5:1 with 10 mM Tris-EDTA, pH 9.0 (vol/vol), and further mixed with an equal volume of a freshly prepared mixture of 1:1 freon: tri-*n*-octylamine. Samples were vortex-mixed for 15 s and spun at 2,000 *g* for 1 min at 25°C. The upper phase (400 μl) was assayed for IP_3.

IP_3 was assayed using either a commercially available kit (NEN) or an IP_3-binding protein extracted from bovine adrenal gland. For studies using the adrenal extract, an ^{3}H-labeled IP_3 tracer was used. Extraction of this binding protein followed the protocol described by Palmer and Wakelam (38). Because the binding protein is contained within a partial membrane preparation of the adrenal cortex, a differential centrifugation procedure was employed. Briefly, bovine adrenal cortex was dissected from six glands at a time and homogenized in 200 mM $NaHCO_3$ and 1 mM DTT at 4°C. The homogenate was centrifuged at 5,000 *g* for 15 min. The supernatant was diluted to 40 ml with the $NaHCO_3$ homogenization buffer and spun at 35,000 *g* for 20 min. The pellet was resuspended in 40 ml $NaHCO_3$ buffer and centrifuged twice more. The final pellet from the final spin was resuspended in 12 ml of buffer at a protein concentration of 20–40 mg/ml. The binding protein was aliquoted at 30 mg/tube and frozen at −80°C until used. Each tube containing binding protein was thawed only once.

All steps for the IP_3 assay were performed in siliconized tubes. The assay followed a published procedure (38) with minor modifications. The assay volume was half of that described (38) to conserve binding protein. Centrifugation of the final pellet was increased to 12,000 *g* for 4 min to improve reproducibility between duplicates.

Data analyses. Data analyses were performed by comparing the areas under the time/concentration curves between the basal and stimulated levels (0–200 or 0–500 ms) of IP_3 anabolism or cyclic nucleotide catabolism. Six to eighteen measurements per point were made. Variation among triplicates was usually between 1 and 7% but not greater than 10%. It was possible to calculate the residual random error of the basal and stimulated regions. Two-sample (nonpaired) Student's *t*-tests were employed with statistical significance designated at the 95% level ($\alpha = 0.05$). The actual *P* values are reported in the figure legends.

RESULTS

Bitter stimulus-induced metabolism of second messengers. Stimulation of the mouse gustatory tissue with 1, 2, and 10 mM denatonium resulted in a rapid and transient increase in IP_3 production. Figure 1*A* shows that for 10 mM denatonium, levels of IP_3 were increased by the first time point measured, 25 ms, and peaked at 75–100 ms. These levels then declined, returning close to basal levels after 200 ms. IP_3 levels in gustatory tissue not stimulated by denatonium remained unchanged. When the same experiment using 10 mM denatonium was performed with nongustatory control tissue, IP_3 levels were not significantly affected (Fig. 1*B*).

Likewise, 2 or 10 mM strychnine HCl rapidly induced elevated levels of IP_3, which peaked at 100 ms. However, unlike the IP_3 levels seen for denatonium, those for strychnine showed a slower return to basal

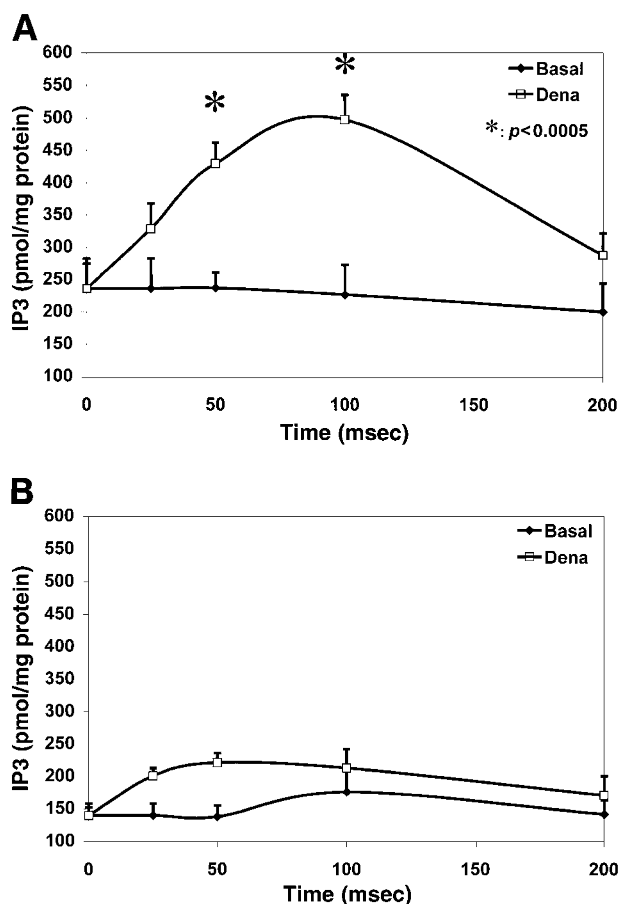

Fig. 1. Time course of denatonium benzoate (Dena, 10 mM)-induced inositol 1,4,5-trisphosphate (IP_3) production in taste tissue from SWR mice (*A*) and in control nongustatory tissue (*B*). The *x*-axis represents the time (in ms) at which quenching of the mixture occurred. Data for the basal (no Dena added) levels (◆) and Dena (□) are the means ± SE of 3 quadruplicate experiments. The Dena-stimulated elevation of IP_3 in the gustatory tissue was significantly different from that of the basal level (buffer); $^*P < 0.0005$.

levels (Fig. 2*A*, using 10 mM strychnine). The peak IP_3 levels doubled compared with basal IP_3 production when 10 mM strychnine was the stimulus. IP_3 levels of gustatory tissue not stimulated by strychnine did not fluctuate. With nongustatory control tissue, 10 mM strychnine induced an IP_3 increase that was about half of that seen in the gustatory tissue (Fig. 2*B*). From 50 to 100 ms, this increase was significantly different from the basal unstimulated controls.

On the other hand, the effect of strychnine and/or denatonium on cyclic nucleotide levels demonstrated the opposite effect. When gustatory tissue was stimulated by strychnine and/or denatonium, cyclic nucleotide levels were suppressed from 0 to 100 ms. Figure 3*A* shows the time course of the relative levels of cGMP when gustatory tissue was stimulated by a mixture of 2 mM denatonium and 2 mM strychnine. This mixture of denatonium and strychnine decreased cGMP levels to 54% of the prestimulated (caffeine alone) levels, with a maximum effect noticeable at about 50 ms (Fig. 3*A*). The time course for denatonium plus strychnine-in-

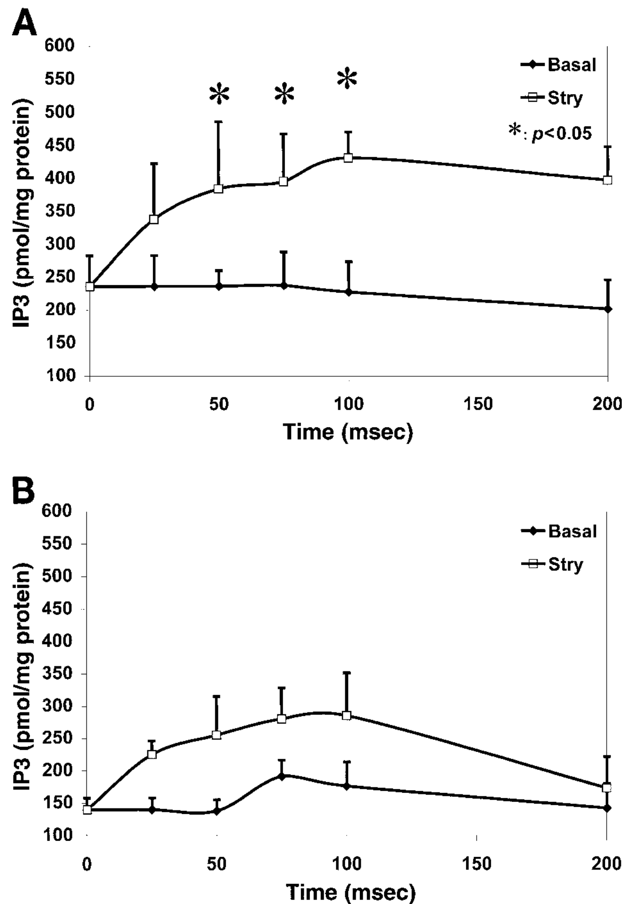

Fig. 2. Time course of strychnine (Stry, 10 mM)-induced IP_3 production in taste tissue from SWR mice (*A*) and in control nongustatory tissue (*B*). The *x*-axis represents the time (in ms) at which quenching of the mixture occurred. Data for the basal levels (◆) and strychnine (□) are the means ± SE of 3 quadruplicate experiments. The Stry-stimulated elevation of IP_3 in the gustatory tissue was significantly different from that of the basal levels (buffer); $^*P < 0.05$.

duced changes in the level of cAMP (data not shown) was similar to that seen for cGMP.

Figure 3*B* shows bitter-induced changes in cAMP levels at the 50-ms time point only. By 50 ms, denatonium alone at 2 mM suppressed cAMP levels to 55% of their prestimulated (caffeine only) value, whereas strychnine alone at 2 mM reduced this level to 40%. In Fig. 3*B*, as in Figs. 4 and 5, the zero value refers to the basal level of cyclic nucleotide in the homogenate before either caffeine and/or denatonium or strychnine addition. The 100% level is the normalized amount of cyclic nucleotide available after caffeine (alone) inhibition of the PDEs.

Role of $G\alpha_{gust}$ in bitter-stimulated second messenger metabolism. To understand the mechanism of cyclic nucleotide catabolism and IP_3 anabolism in taste tissue, we tested the possible role of $G\alpha_{gust}$ in generating these rapid changes in second messenger levels. Because specific antibodies to $G\alpha_{gust}$ exist, we preincubated taste and control tissues with antibodies to $G\alpha_{gust}$ or with a preimmune IgG fraction (see MATERIALS AND METHODS) in an attempt to rescue the bitter stimulus-mediated metabolism of cGMP, cAMP, and IP_3.

The results demonstrate that antibodies to $G\alpha_{gust}$, but not preimmune IgG, blocked the denatonium (1 mM)-induced suppression cyclic nucleotide levels to their prestimulated (i.e., caffeine stimulated) levels (Fig. 4, *A* and *B*). Figure 4*A* demonstrates that, at the 50-ms time point, denatonium suppresses cAMP levels (as shown also in Fig. 3) but that this decrease was blocked by preincubation of the tissue with an antibody to $G\alpha_{gust}$. The decrease was not blocked, however, by preincubating the taste tissue with preimmune IgG. The same pattern is seen when cGMP is measured (Fig. 4*B*). Nongustatory tissue did not demonstrate suppression of cAMP or cGMP by denatonium, and there was no effect of the $G\alpha_{gust}$ antibodies nor of preimmune IgG on the levels of these messengers after denatonium stimulation (data not shown).

Similar to denatonium, 2 mM strychnine suppressed levels of cAMP (Figs. 3*B* and 5*A*) and cGMP (Fig. 5*B*). In the case of strychnine, however, the suppression of cAMP was not rescued by the $G\alpha_{gust}$ antibodies (Fig.

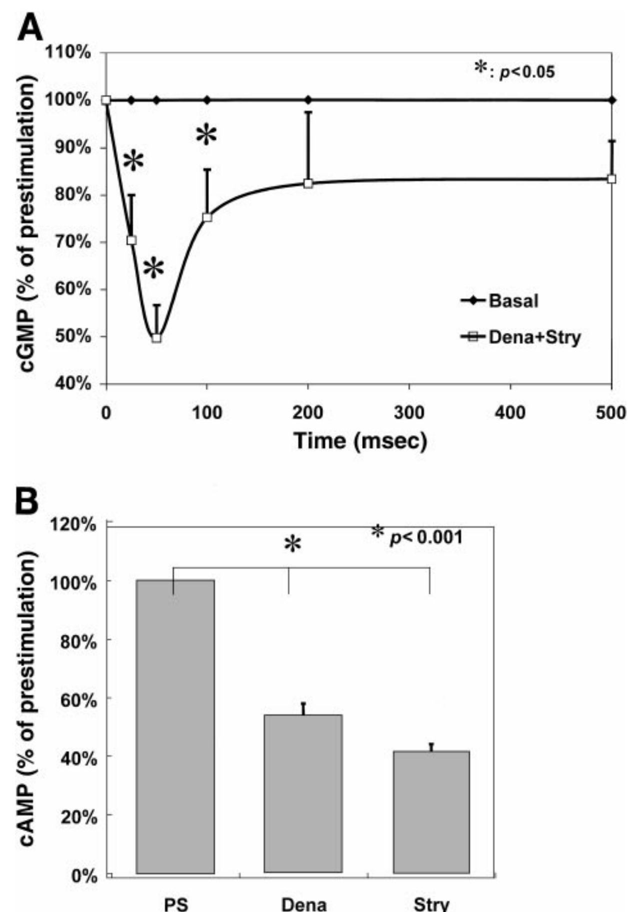

Fig. 3. *A*: time course of suppression of prestimulated (PS) levels (◆) of cGMP by the mixture of Dena and Stry (□, 2 mM each). Data are the means ± SE of 3 experiments performed in triplicate. The suppression at 50, 75, and 100 ms was statistically significant ($^*P < 0.05$) when compared with PS levels normalized to 100%. *B*: suppression of PS cAMP levels by Dena (2 mM) and Stry (2 mM). Data were collected at 50 ms and represent the mean of 3 triplicate experiments. Changes in cAMP levels due to Dena and/or Stry are statistically significant ($^*P < 0.001$ for each value) compared with PS levels.

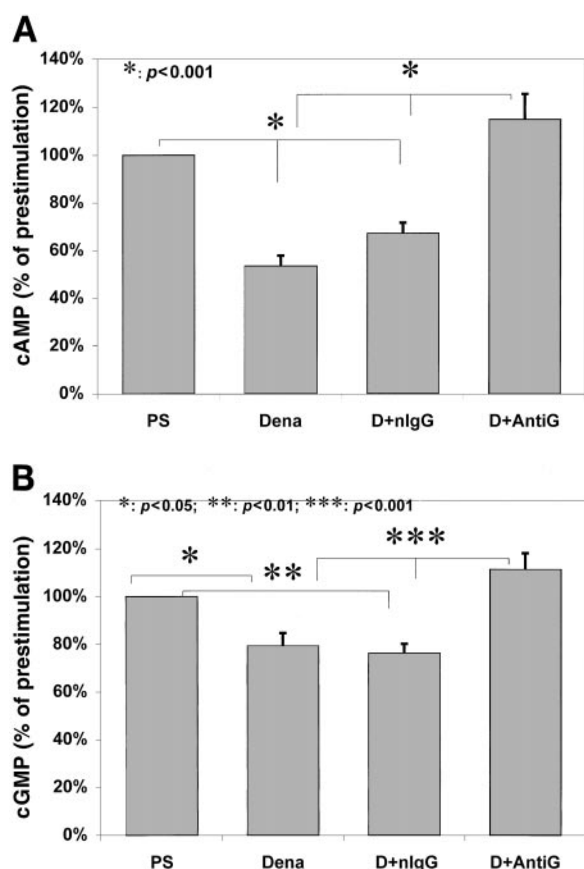

Fig. 4. *A*: the effect of antibodies to the taste-enriched G protein α-gustducin ($G\alpha_{gust}$) on Dena (D)-mediated drop in cAMP measured at 50 ms after stimulation by Dena in taste tissue homogenates. PS cAMP levels were reduced by 1 mM Dena (*$P < 0.001$). Suppression of cAMP was not affected by normal IgG (D+nIgG, 10 μg; $P < 0.001$ compared with PS) but was restored in the presence of antibodies to $G\alpha_{gust}$ (D+AntiG, 10 μg; $P < 0.001$ compared with Dena and D+nIgG). Data represent the means ± SE of 9–16 data points. *B*: the effect of AntiG on Dena-mediated drops in cGMP at 50 ms after stimulation by Dena in taste tissue homogenates. PS cGMP levels were reduced by 1 mM Dena (*$P < 0.05$). Suppression of cGMP was not affected by nIgG (D+nIgG, 10 μg; **$P < 0.01$ compared with PS) but was restored in the presence of AntiG (D+AntiG, 10 μg; ***$P < 0.001$ compared with Dena and D+nIgG). Data represent the means ± SE of 23–25 data points.

5*A*), whereas the strychnine suppression of cGMP was rescued by the $G\alpha_{gust}$ antibodies (Fig. 5*B*).

Data shown in Fig. 6 demonstrate that antibodies to $G\alpha_{gust}$ had no effect on the denatonium- (Fig. 6*A*) or strychnine (Fig. 6*B*)-induced increases in IP_3. Measurements made at the 50-ms time point show that these bitter-induced increases in IP_3 remained unchanged after the tissue was preincubated either with preimmune IgG or with antibodies specific to $G\alpha_{gust}$.

Role of PLC-β_2 in bitter stimulus-induced production of IP_3. Generation of IP_3 and diacylglycerol (DAG) is the result of the action of the enzyme PLC on the minor membrane lipid phosphatidylinositol 4,5-bisphosphate. There are several types of PLCs defined by their sensitivities to modulatory compounds and by their structural attributes (18, 42, 57). Antibodies to PLC-β_2, PLC-β_3, and to PLC-β_4 were used to determine if one or more of these inhibited the denatonium-induced rise in IP_3 levels in taste homogenates. Figure 7 shows data at the 50-ms time point for denatonium-induced changes in IP_3 levels in the absence of any antibodies or blocking peptides and in the presence of denatonium plus antibodies to PLC-β_2, PLC-β_3, and PLC-β_4. The data show that only in the presence of antibodies to PLC-β_2 was the denatonium-induced rise in IP_3 levels inhibited. This inhibition could be blocked when antibody to PLC-β_2 was preincubated with its blocking peptide. Blocking peptide-pretreated antibodies to PLC-β_3 and PLC-β_4 and blocking peptide alone did not affect denatonium-induced IP_3 increase. In addition, antibodies to PLC-β_2 did not affect cAMP or cGMP levels (Fig. 7*B*).

DISCUSSION

Because of the diverse chemical structures of substances that taste bitter, it has long been assumed that

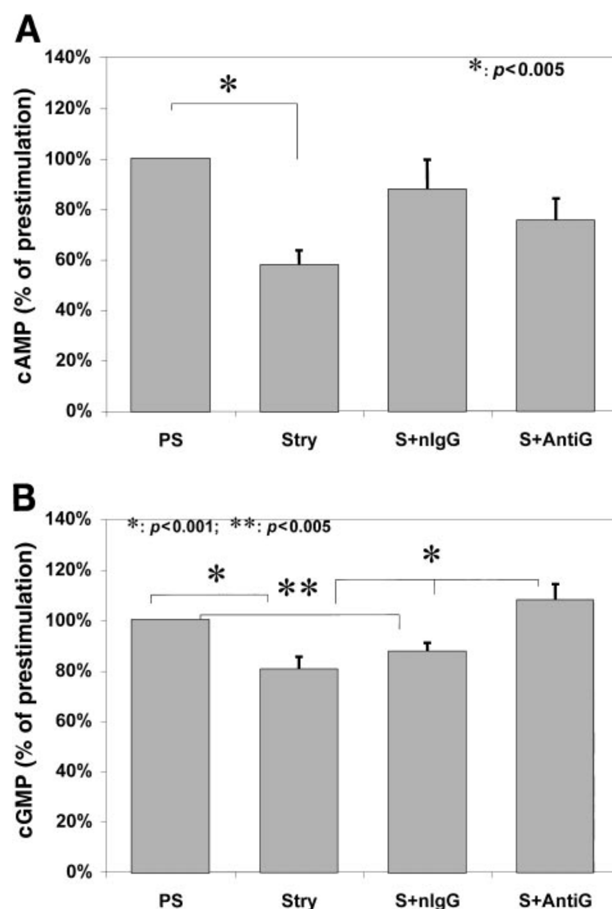

Fig. 5. *A*: the effect of AntiG on Stry (S)-mediated drop in cAMP measured at 50 ms after stimulation by Stry in taste tissue homogenates. PS cAMP levels were reduced by 2 mM Stry (*$P < 0.005$). Suppression of cAMP was affected neither by nIgG (S+nIgG, 10 μg) nor AntiG (D+AntiG). Data represent the means ± SE of 24 data points. *B*: the effect of AntiG on Stry-mediated drop in cGMP at 50 ms after stimulation by Stry in taste tissue homogenates. PS cGMP levels were reduced by 2 mM Stry (**$P < 0.005$). Suppression of cGMP was not affected by nIgG (S+nIgG, 10 μg; *$P < 0.001$ compared with PS) but was restored in the presence of AntiG (10 μg, $P < 0.005$ compared with Stry and S+nIgG). Data represent the means ± SE of 22–24 data points.

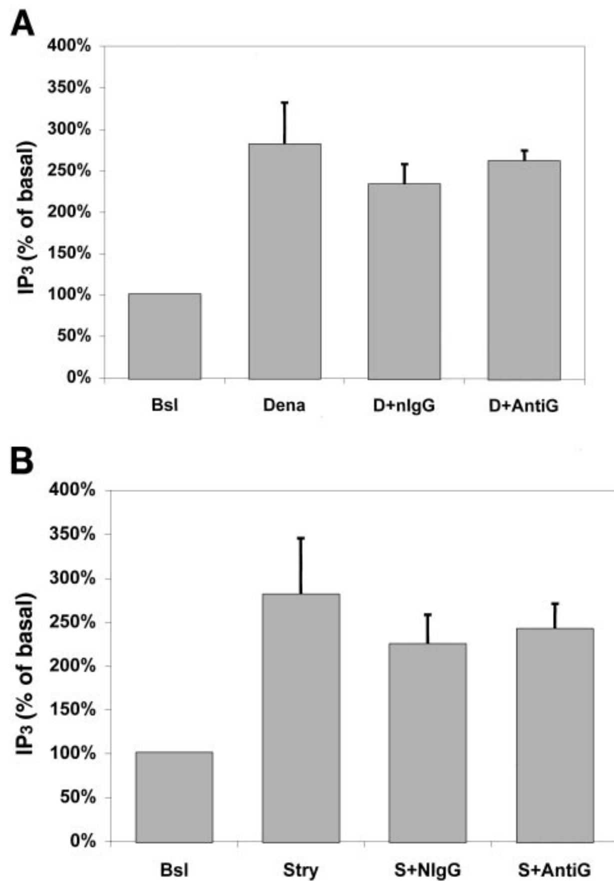

Fig. 6. *A*: the effect of AntiG on Dena-mediated increases in IP_3 at 50 ms after Dena (1 mM) stimulation of taste tissue homogenates. The elevation of IP_3 (Dena) was unaffected by either nIgG (D+nIgG) or AntiG (D+AntiG). *B*: the effect of AntiG on Stry-mediated increases in IP_3 at 50 ms after Stry (2 mM) stimulation of taste tissue homogenates. The elevation of IP_3 (Stry) was unaffected by either nIgG (S+nIgG) or AntiG (S+AntiG). Bsl, basal cyclic nucleotide level.

there are multiple receptor mechanisms for the modality of bitterness. The literature generally supports this suggestion (for reviews, see Refs. 7 and 48). For example, there is both direct and inferential evidence that bitterness may be transduced by direct interaction of bitter compounds with biophysical properties of the membrane (e.g., Refs. 23 and 32), by interaction with intracellular components, bypassing a receptor step (31, 39, 41, 48), by direct modulation of stimulus-gated plasma membrane ion channels (48, 49, 55), and by more traditional G protein-coupled receptor (GPCR) processes (1, 11). One such receptor/transduction mechanism using the GPCR route is one that leads to the metabolism of second messengers (for reviews, see Refs. 7, 20, 24, 25, 45). Our previous work suggests that both the polyphosphoinositides and/or the cyclic nucleotides act as second messengers in bitter taste GPCR transduction (41, 47, 51).

The involvement of IP_3 as a possible second messenger for bitter taste signal transduction has received support from several studies (2, 16, 17, 33, 34, 36, 41, 42, 47, 51). For example, we previously demonstrated that taste tissue homogenates from mice show a rapid and transient bitter stimulus-induced production in the second messenger IP_3 (51). This increase occurs within the physiologically relevant first 200 ms, making it likely that IP_3 could act as a second messenger in bitter taste. The finding that the gustatory-enriched G protein $G\alpha_{gust}$ (28) was involved in bitter taste transduction (58) and that this G protein showed high sequence homology with the transducins suggested that taste PDE (43), acting on cyclic nucleotide levels, may also be a messenger system for bitter taste (45). Our new findings, described in this current work, support the suggestion that some bitter-tasting compounds may simultaneously alter second messenger levels of both the polyphosphoinositol system and the cyclic nucleotide systems in a G protein-dependent manner.

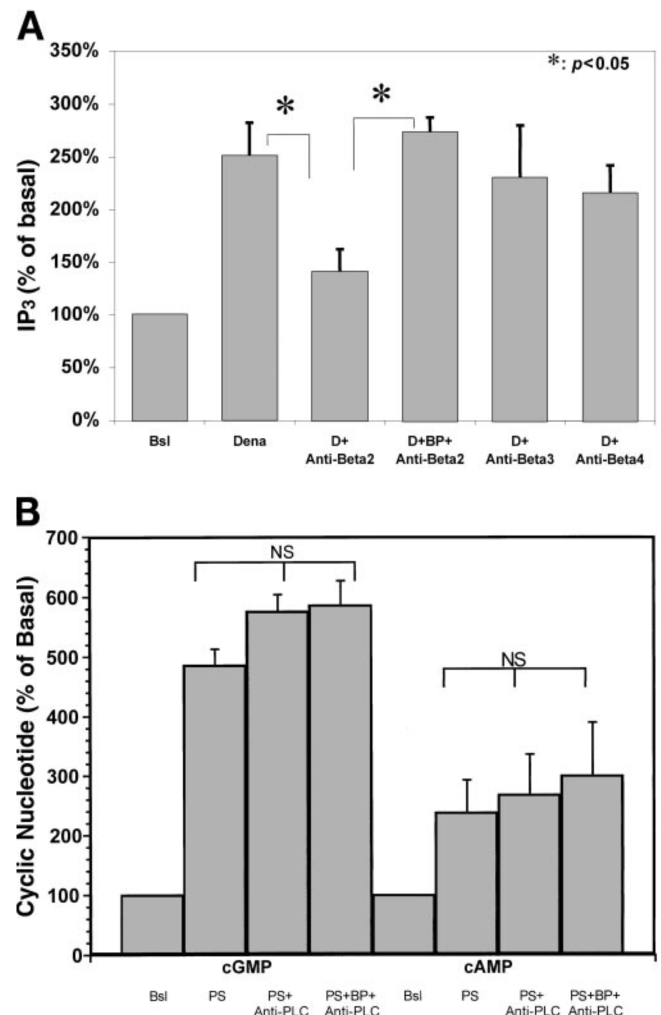

Fig. 7. *A*: the effect of antibodies to different phospholipase C (PLC) subtypes on Dena-mediated IP_3 increase. At 50 ms, the Dena (1 mM) induced IP_3 increase (Dena) was inhibited by antibodies to PLC-β_2 (D+Anti-Beta2, $P < 0.05$), although this increase was not affected by antibodies to either PLC-β_3 or PLC-β_4 (D+Anti-Beta3, D+Anti-Beta4). The inhibition by Anti-Beta2 was blocked by the blocking peptide for the antibodies (D+BP+Anti-Beta2, $^*P < 0.05$). *B*: the effect of Anti-Beta2 on cyclic nucleotide levels. At 50 ms, PS levels of cAMP or cGMP were not affected by PLC-β_2 antibodies (Anti-PLC) in the presence or absence of the specific blocking peptide (BP). NS, not significant. Data represent the means of 3–12 data points ± SE.

Second messenger metabolism in the millisecond time frame. The data presented here confirm that denatonium and strychnine can rapidly and transiently increase IP_3 levels in taste tissue from mice. Data shown in Figs. 1 and 2 demonstrate that IP_3 is increased after tissue stimulation by both denatonium and strychnine. Both of these responses were tissue specific, being significant at the $P < 0.005$ level. The data show rising IP_3 levels from the first point of measurement, 25 ms, to 100 ms, after which the values for IP_3 begin to decline rapidly for denatonium, but less rapidly for strychnine. Some increases in IP_3 levels are seen with nongustatory controls, although the magnitude of these is much less than that seen with gustatory tissues. Such activity in nongustatory tissue for some bitter compounds has been noted previously (33, 51) and is likely due to the fact that many bitter stimuli are pharmacologically active.

Data from this study plus those from previous ones (50, 51) show that rapid increases in IP_3 levels in rodent taste tissue are detected for the bitter-tasting stimuli, denatonium, strychnine, sucrose octaacetate, caffeine, quinine, naringin, and benzyltriethylammonium chloride (50, 51, 52, 53).

In addition to the bitter taste-induced changes in IP_3 levels, we show here, for the first time, that these same bitter stimuli can significantly reduce levels of both cAMP and cGMP within the millisecond time frame (Figs. 3–5). Data of Fig. 3*A* show that cGMP levels reach their minimum values by 50 ms, returning thereafter to near baseline levels by 150 ms. The decrease in nucleotide levels is likely due to the stimulation of a PDE by $G\alpha_{gust}$ (see below). The rapid increase in cyclic nucleotide levels after 50 ms may be due to the activation of several systems including a gradual decline in PDE activity and/or by a parallel increase in cyclic nucleotide synthesis. The data presented here cannot be used to distinguish between these possibilities, and further work is required to resolve this question.

One of the major hypotheses on bitter taste transduction states that bitter stimuli induce a receptor-mediated activation of a PDE through the action of the taste G protein, $G\alpha_{gust}$. If this were the case, then one would expect to observe a decrease in cyclic nucleotides due to stimulation by bitter compounds. Initial attempts to show such decreases using homogenates were only suggestive (34, 53). Only minimal lowering of cyclic nucleotides was seen, likely because the initial levels of cyclic nucleotides in the homogenate were very low, due to the very active PDEs. On the basis of our previous experimental work in taste, we knew that potent PDEs are present in these tissues, and that methyl xanthines were able to inhibit these, but only at high levels.

At the suggestion of a colleague (Dr. Heinz Breer, Stuttgart, Germany) we adopted the common procedure of adding a xanthine inhibitor of PDE [caffeine (41)] to raise the levels of cyclic nucleotides in our homogenate preparation. We chose to use 25 mM caffeine in these studies because at this concentration, consistently high and readily assayed concentrations of cyclic nucleotides could be sustained, allowing us to at least approach the testing of the hypothesis outlined above. Although 25 mM is a high level, it is not so high as to block the bitter stimulus-induced, $G\alpha_{gust}$-mediated activation of PDE to affect a rapid, and even reversible, drop in cyclic nucleotides. Although we cannot rule out the possibility that caffeine may affect other systems in the homogenate, the fact that a taste-specific, $G\alpha_{gust}$-mediated drop in cyclic nucleotides is seen in gustatory and not in control (nongustatory) tissue makes the use of this compound justified for this initial test of the gustducin hypothesis.

Just what the conditions are within the cell that might permit $G\alpha_{gust}$ signaling to effect a drop in cyclic nucleotides is unknown. But, unlike the homogenate, which, when being produced, likely quickly depletes cyclic nucleotides because no synthesis is occurring, in the cell, both synthesis and normal PDE-mediated degradation of cyclic nucleotides are at a steady state. Upon taste stimulation, the action of $G\alpha_{gust}$ further stimulates PDE to effect a transient drop in cyclic nucleotides. Our homogenate cannot accurately mimic these dynamic events. In the face of depleted cyclic nucleotides, we need to not only allow some synthesis to occur (by adding ATP at *time 0*) but also partially inhibit PDE to measure the drop in nucleotides.

Adding caffeine simultaneously with either denatonium or strychnine resulted in a taste tissue-specific decrease in cyclic nucleotide levels. In contrast to the ability of denatonium and strychnine to lower cyclic nucleotide levels in taste tissue, caffeine and other xanthines raise levels of cyclic nucleotides, suggesting an alternate mechanism for bitter taste transduction for these bitter tasting and membrane-permeable PDE inhibitors (e.g., Refs. 22 and 41).

We estimated the stimulus thresholds for both the IP_3 increases and the cyclic nucleotide decreases, for both denatonium and strychnine, to be in the range of 0.1 to 0.5 mM. These values are consistent with, though somewhat higher than, behavioral avoidance thresholds reported by Whitney et al. (56). The observation of slightly higher threshold values from the in vitro work presented here is not surprising given the likely weaker coupling of the major transduction constituents in the homogenate compared with their coupling in vivo.

G protein-mediated second messenger responses. Data in Figs. 4 and 5 demonstrate that the denatonium- and strychnine-induced decreases in cyclic nucleotides are dependent upon $G\alpha_{gust}$. When antibodies to $G\alpha_{gust}$ were preincubated with the taste homogenate, neither denatonium nor strychnine was able to induce a reduction in the levels of either cAMP or cGMP. Antibodies from nonimmune serum were ineffective. This block by antibodies to $G\alpha_{gust}$ in taste tissue homogenates was not seen with homogenates from nontaste tissue controls. The failure of the $G\alpha_{gust}$ antibodies to rescue the strychnine-induced decreases in cAMP (Fig. 5) may imply additional control elements for transducing signals from this and other bitter compounds.

In contrast, antibodies to $G\alpha_{gust}$ were ineffective at altering the denatonium- and strychnine-induced increases in IP_3 of taste homogenates (Fig. 6). These data, therefore, suggest that bitter stimuli alter levels of cyclic nucleotides by a $G\alpha_{gust}$ activation of a taste PDE but that stimulation of a PLC is $G\alpha_{gust}$ independent.

A recent study demonstrated that the bitter-induced increase in IP_3 levels was dependent upon the β/γ-subunit of a G protein (16). Antibodies to a taste $G\gamma_{13}$-subunit prevented the denatonium-stimulated rapid production of IP_3. This observation and the data of Rossler et al. (42) from rat taste tissue suggest that the PLC type responsible for taste-induced production of IP_3 is a PLC-β-type phospholipase. When antibodies to the PLC-β types 2, 3, and 4 were preincubated with taste tissue homogenates, only the antibodies to the PLC-β_2 subtype inhibited the denatonium-induced increase in IP_3. Use of blocking peptides confirmed that this effect was specific to the antibodies to PLC-β_2 (Fig. 7).

Bitter taste transduction mechanisms. From the new data presented here and from results of previous work, one can hypothesize that bitter taste signal transduction for denatonium and strychnine involve both a $G\alpha_{gust}$-dependent, PDE-mediated reduction in cyclic nucleotide levels and a $G\gamma_{13}$-dependent, PLC-β_2-mediated increase in IP_3. Both of these changes in second messenger metabolism occur within 100 ms, a time frame consistent with taste transduction. The data of Huang et al. (16) show that a taste cell-enriched $G\gamma_{13}$-subunit and the $G\alpha_{gust}$-subunit colocalize to the same cells, making it likely that the heterotrimeric G protein involved in bitter taste is composed of $G\alpha_{gust}$ and $G\beta\gamma_{13}$. In addition, the recently identified bitter taste receptors were found to colocalize with $G\alpha_{gust}$ (1).

These data lead to a comprehensive model of one mechanism for bitter taste transduction (Fig. 8). In this construct, the bitter stimuli interact with 7TM receptors, most likely those recently identified (1, 11). According to Chandrashekar et al. (11), it is very likely that the receptor for denatonium is molecularly dis-

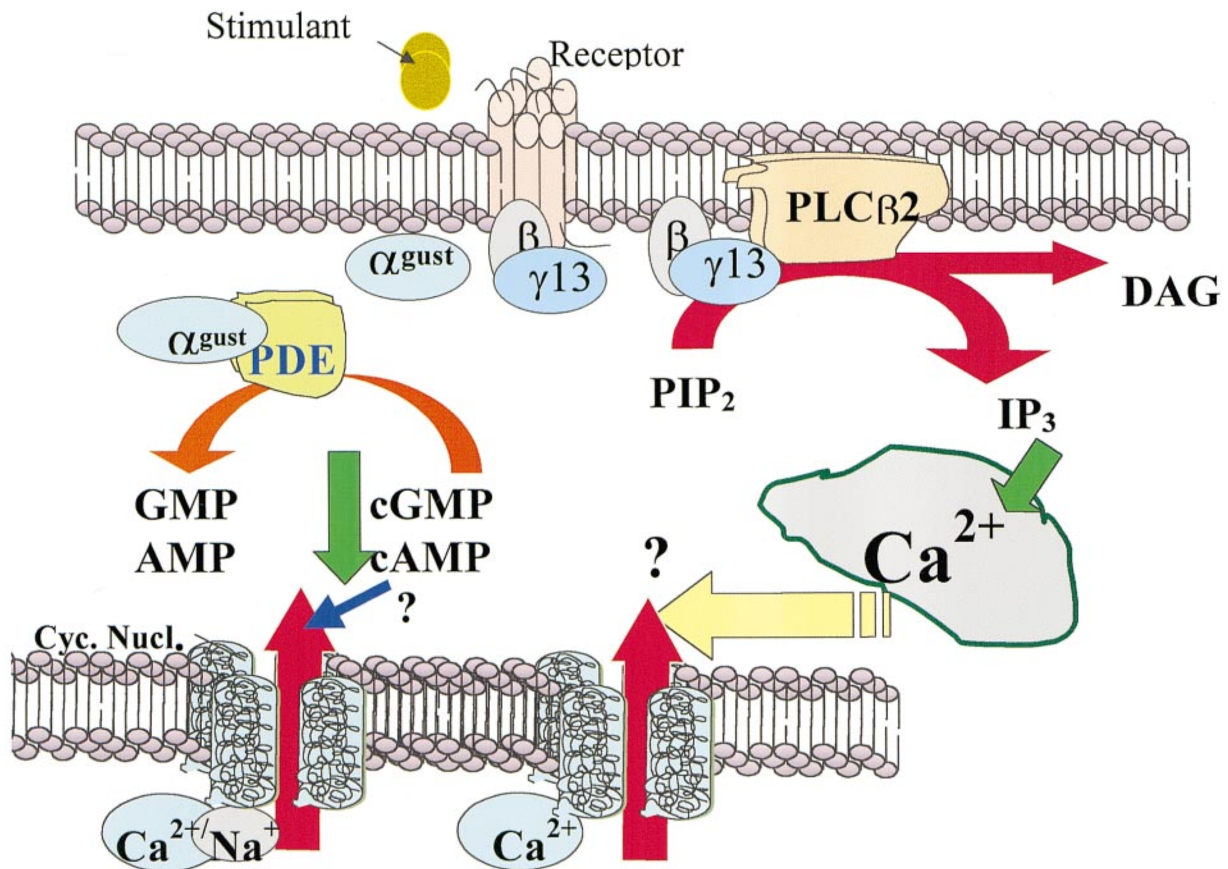

Fig. 8. Hypothesized G protein-coupled receptor model of Dena and Stry signal transduction in taste tissue. Dena and Stry bind to specific taste receptors. These receptors activate the taste-enriched G protein, gustducin. The activated G protein splits into the $G\alpha_{gust}$-subunit and a $\beta\gamma_{13}$-subunit. The $G\alpha_{gust}$ activates phosphodiesterases (PDEs) and enhances their ability to degrade cAMP and cGMP. Variation in cyclic nucleotide levels is rapid and transitory possibly due to an adaptation mechanism such as receptor or $G\alpha_{gust}$ inactivation, PDE reactivation, or stimulation of cyclic nucleotide synthesis. This decrease in cyclic nucleotide may open cyclic nucleotide suppressible cation channels, which normally bind cyclic nucleotides and remain closed during the resting state. The opening of these channels affects cellular conductance and depolarizes taste receptor cells. Concurrent with the changes in cyclic nucleotide levels, the βγ-subunit activates a taste-specific PLC-β_{13}, converting phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and IP_3. The increase in IP_3 releases calcium from intracellular stores and further depolarizes the cell.

tinct from that for strychnine. [This receptor step may not always be sufficient or necessary for initiating or sustaining the bitter taste response, because hydrophobic taste stimuli can cross the taste cell plasma membrane and possibly interact directly with intracellular targets (31, 39, 48)]. The binding of the bitter stimulus with its receptor(s) then activates the taste cell G protein, in this case, perhaps, $G\alpha_{gust}/G\beta\gamma_{13}$. The $G\alpha_{gust}$ activates one or more type of PDE, resulting in the breakdown of cyclic nucleotides within a time frame of 50–75 ms. This transitory nature of the cyclic nucleotide response may possibly be due to an adaptation mechanism such as phosphorylation of the cell-surface receptor(s), inactivation of $G\alpha_{gust}$, and/or stimulation of cyclic nucleotide synthesis, mechanisms that cannot be discerned from the current data. Simultaneously, the $G\beta\gamma_{13}$ activates a PLC-β_2, resulting in the production of the two second messengers, DAG and IP_3, within a time frame of 50–100 ms. The exact targets of these second messengers are not known. The cyclic nucleotides may, for example, stimulate the activity of a cyclic nucleotide-inhibitable ion channel (21) or a cyclic nucleotide-stimulated ion channel (30), altering cellular conductance. The messengers IP_3 and DAG may act to release calcium from intracellular stores and activate protein kinases, respectively. There is also evidence for a cyclic nucleotide-stimulated activation of a protein kinase in taste cells (3). The observation that the metabolism of these different second messenger pathways is stimulated by components of the same heterotrimeric G protein, i.e., $G\alpha_{gust}/G\beta\gamma_{13}$, leads us to hypothesize that the bitter stimulus-induced reductions in cyclic nucleotides and the bitter stimulus-induced increases in IP_3 occur within the same bitter sensitive cell.

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