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Localization of phosphatidylinositol signaling components in rat taste cells: Role in bitter taste transduction

(denatonium/von Ebner's gland/cytidine diphosphate diacylglycerol/inositol 1,4,5-trisphosphate/circumvallate papillae)

PAUL M. HWANG, AJAY VERMA, DAVID S. BREDT, AND SOLOMON H. SNYDER*

The Johns Hopkins University School of Medicine, Departments of Neuroscience, Pharmacology and Molecular Sciences, and Psychiatry, Baltimore, MD 21205

Contributed by Solomon H. Snyder, July 2, 1990

ABSTRACT **To assess the role of phosphatidylinositol turnover in taste transduction we have visualized, in rat tongue, ATP-dependent endoplasmic reticular accumulation of $^{45}Ca^{2+}$, inositol 1,4,5-trisphosphate receptor binding sites, and phosphatidylinositol turnover monitored by autoradiography of [^{3}H]cytidine diphosphate diacylglycerol formed from [^{3}H]cytidine. Accumulated $^{45}Ca^{2+}$, inositol 1,4,5-trisphosphate receptors, and phosphatidylinositol turnover are selectively localized to apical areas of the taste buds of circumvallate papillae, which are associated with bitter taste. Further evidence for a role of phosphatidylinositol turnover in bitter taste is our observation of a rapid, selective increase in mass levels of inositol 1,4,5-trisphosphate elicited by low concentrations of denatonium, a potently bitter tastant.**

Different taste modalities employ diverse signaling mechanisms at taste buds in the tongue. Salty taste involves interactions of sodium with recognition sites resembling renal receptors for diuretics of the amiloride class (1, 2). Sour taste is mediated by hydrogen ions impinging on recognition sites associated with potassium channels in the apical membrane of taste cells (3, 4). Sweet substances stimulate adenylyl cyclase (5), analogous to the stimulation by odorants of an olfactory adenylyl cyclase (6, 7). Transduction mechanisms for bitter substances have been more elusive. Recently, Akabas *et al.* (8) described an increase in intracellular calcium elicited by the intensely bitter substance denatonium in a subpopulation of taste cells dissociated from rat tongue. Though specific signaling mechanisms were not elucidated, the increased levels of calcium appear to involve internal stores of calcium, consistent with a role for the phosphatidylinositol (PI) second messenger system.

One of the difficulties in molecular investigations of taste has been the sparsity of taste bud cells, with <1% of tongue surface area occupied by taste buds, which are diluted by the more abundant nontaste tissue. We have developed techniques to localize microscopically elements of the PI system, including autoradiography of inositol 1,4,5-trisphosphate (IP_3) receptors and $^{45}Ca^{2+}$ accumulated selectively by the endoplasmic reticulum calcium pump (9) as well as autoradiographic localization of PI turnover monitored as the accumulation of [^{3}H]cytidine diphosphate diacylglycerol ([^{3}H]CDP-DAG) (10) derived from [^{3}H]cytidine (11). In the present study we demonstrate an extraordinarily selective localization of calcium accumulation by endoplasmic reticulum, IP_3 receptors, and PI turnover in taste buds of the circumvallate papillae, which contain the cells mediating bitter taste. We also demonstrate a rapid, selective increase in IP_3 mass levels in response to low concentrations of the bitter tastant denatonium.

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MATERIALS AND METHODS

Materials. *myo*-[2-^{3}H]Inositol (17 Ci/mmol; 1 Ci = 37 GBq), D-[inositol-1-^{3}H]inositol 1,4,5-trisphosphate (10–30 Ci/mmol), [5-^{3}H]cytidine (27.8 Ci/mmol), and $^{45}CaCl_2$ (10–75 Ci/g) were obtained from DuPont/NEN. *myo*-Inositol 1,4,5-trisphosphate and other inositol phosphates were purchased from Calbiochem.

Tissue Preparation. Male Sprague–Dawley rats (150–200 g) were sacrificed by decapitation; circumvallate papillae were dissected and rapidly frozen in OCT medium (Tissue-Tek). Cryostat sections (14 μm) were cut, thawed on 0.5% gelatin/0.05% chrome alum-coated glass slides, and stored desiccated at −20°C. To bilaterally denervate the circumvallate papillae, rats were anesthetized with ether, a midline incision was made on the ventral surface of the neck, the glossopharyngeal nerve was visualized as it crossed the tympanic bulla, and a section of the nerve was avulsed (12). Circumvallate papillae taste buds were allowed to degenerate a minimum of 10 days before denervated rats were used.

$^{45}Ca^{2+}$ Uptake Assay. A recently developed method for the selective localization of intracellular Ca^{2+} stores in cryostat tissue sections was used (9). Circumvallate papilla cryostat sections were permeabilized in buffer containing 50 mM Hepes/Tris (pH 7.7), 140 mM KCl, 3% polyethylene glycol (PEG; average M_r = 8000), and 2.5 μM digitonin for 10 min at 25°C. Sections were then transferred into uptake buffer containing 30 mM Hepes/Tris (pH 7.7), 70 mM potassium oxalate, 1 mM $MgCl_2$, 25 μM $CaCl_2$, 50 μM *N*-hydroxyethylethylenediaminetriacetic acid (Sigma), 2 mM K_2ATP, 3% polyethylene glycol, 0.25 μM digitonin, and 0.5 μCi (0.1 μM) $^{45}CaCl_2$ per ml for 60–90 min at 25°C. Then the sections were washed in buffer containing 50 mM Hepes/Tris (pH 7.7), 1 mM EGTA, and 3% polyethylene glycol for 5 min at 4°C to remove nonsequestered $^{45}Ca^{2+}$; dried under a gentle stream of air; dipped in Kodak NTB2 emulsion diluted 1:1 in water; exposed 1–2 weeks; developed in Kodak D19; and stained with Mayer's hematoxylin/eosin. Sensitivity of accumulated $^{45}Ca^{2+}$ to IP_3 and other inositol phosphates was determined by including them in the uptake buffer at the desired concentrations.

Histochemical localization of Ca^{2+}-ATPase activity in taste buds was determined by the modified lead citrate method (13).

[^{3}H]IP_3 Autoradiography. [^{3}H]IP_3 autoradiography was performed as described (14). Cryostat circumvallate papillae tissue sections (14 μm), similar to those used for $^{45}Ca^{2+}$ uptake, were incubated in 20 nM [^{3}H]IP_3 alone for total binding and with 10 μM unlabeled IP_3 to displace specific binding. Sections were washed twice for 2 min in fresh buffer at 4°C, dried under a stream of cool air, and apposed to Kodak

Abbreviations: PI, phosphatidylinositol; IP_3, inositol 1,4,5-trisphosphate; CDP-DAG, cytidine diphosphate diacylglycerol.

*To whom reprint requests should be addressed.

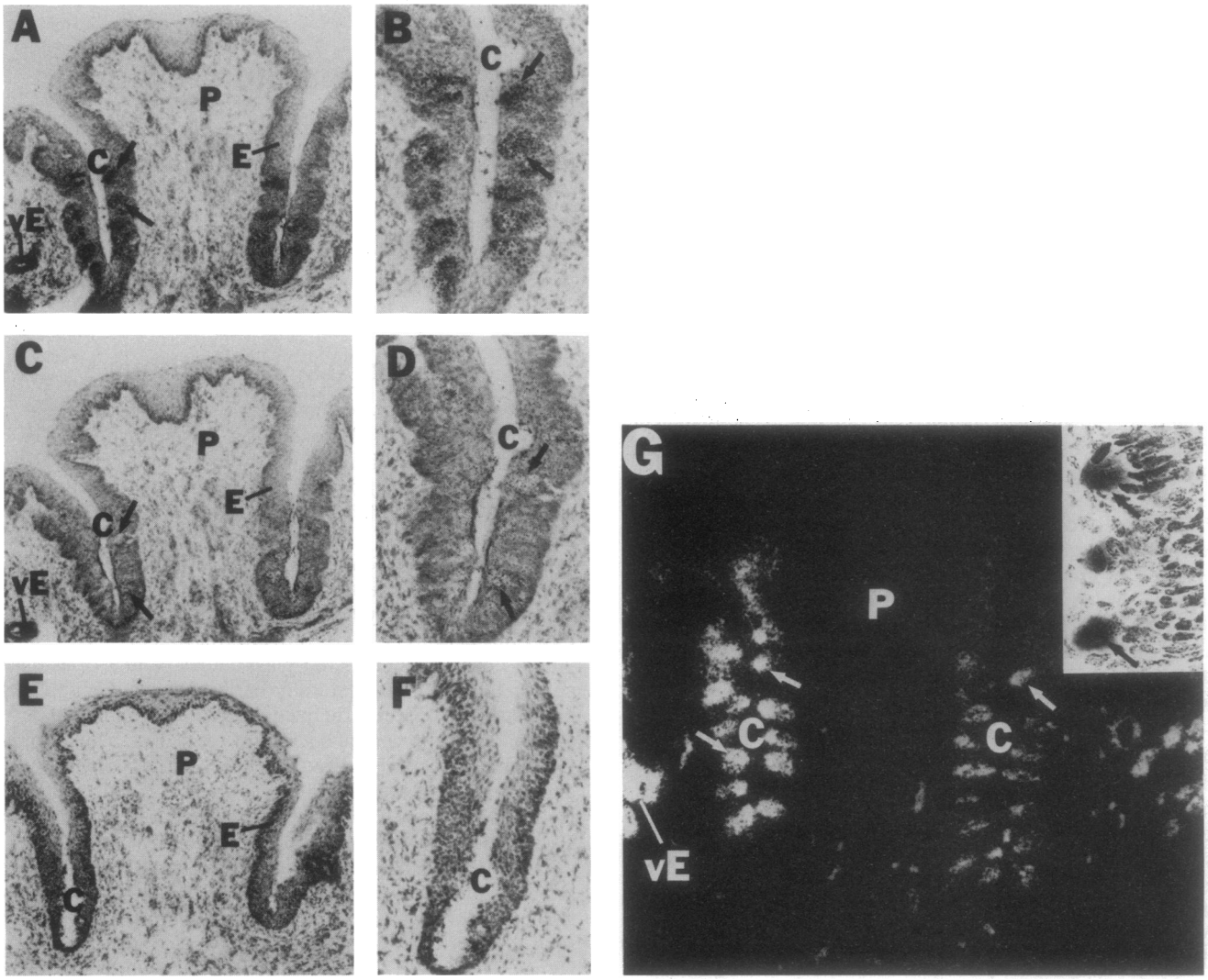

FIG. 1. Localization of $^{45}Ca^{2+}$ in the rat circumvallate papilla. (*A*, *B*, and *G*) Uptake of $^{45}Ca^{2+}$ visualized by autoradiographic emulsion silver grains in light-field (dense, black silver grains) and dark-field (white silver grains) images, respectively. (*G Inset*) ATPase stain localization to the apical part of taste buds. (*C* and *D*) Reduction in the net accumulation of $^{45}Ca^{2+}$ by 1 μM IP_3. (*E* and *F*) No uptake of $^{45}Ca^{2+}$ in denervated circumvallate papilla due to taste bud degeneration. P, circumvallate papilla; C, crypt; E, epidermal layer; vE, von Ebner's gland. Arrows indicate taste buds. (*A–F*, ×85; *G*, ×35 and ×280.)

NTB2 emulsion-coated coverslips for 3–6 months. Developed sections were stained with toluidine blue.

PI Turnover Measurements. PI turnover in circumvallate papillae was monitored by two techniques, using either [^{3}H]inositol (15) or [^{3}H]cytidine (11) as precursor. To facilitate entry of precursors into the taste cells, 0.25 ml of 0.25% (wt/vol) collagenase/0.25% (wt/vol) elastase (Sigma) in Hanks' balanced salts solution (HBSS) was injected between the epidermal–dermal junction surrounding the circumvallate papillae and incubated for 30 min at 37°C. Each circumvallate papilla was then carefully dissected free of adjacent tongue tissue, washed exhaustively in HBSS, and allowed to recover for 1 hr in Krebs/bicarbonate buffer exposed to 95% O_2/5% CO_2 atmosphere.

Individual circumvallate papillae were prelabeled in 0.05 ml of Krebs/bicarbonate buffer containing either 1.5 μM [^{3}H]inositol or 2 μM [^{3}H]cytidine for 1 hr at 30°C. Ten minutes prior to adding denatonium (Sigma) or carbachol, LiCl was added to a final concentration of 5 mM. [^{3}H]Inositol phosphate or [^{3}H]CDP-DAG accumulations were determined after 60 min of stimulation as described (11, 15).

IP_3 Mass Level Measurements. Individual rat circumvallate papillae were dissected free of surrounding tongue tissue and allowed to recover for 1 hr at 20°C in Krebs/bicarbonate buffer in an interface chamber (95% O_2/5% CO_2). They were then stimulated for 15–30 sec in the appropriate concentration of denatonium dissolved in Krebs/bicarbonate buffer, heat inactivated in 300 μl of hypotonic buffer [50 mM Tris·HCl (pH 7.7) and 1 mM EDTA at 95°C for 5 min], sonicated, centrifuged at 10,000 × *g* for 15 min (Sorvall Microspin 24S), and assayed for IP_3 mass levels by a sensitive and specific IP_3 radioreceptor assay (16). Protein content per circumvallate papilla was determined by the Coomassie protein assay (Pierce).

PI Turnover Imaging in Taste Buds. A method for autoradiographically imaging stimulated PI turnover, as reflected by the accumulation of [^{3}H]CDP-DAG, has recently been developed (10). Circumvallate papillae were stimulated as described in the previous section on PI turnover measurements except that the Krebs/bicarbonate buffer contained 20 μM [^{3}H]cytidine, actinomycin D (1 μg/ml; Boehringer Mannheim), and 50 mM hydroxyurea (Sigma). Labeled circumvallate papillae were transferred to plastic molds and embedded in OCT medium (Tissue-Tek). Cryostat sections (16 μm) were cut on gelatin-coated glass slides and quickly dried. These were treated in wash buffer [50 mM Tris·HCl (pH 7.4), 2 mM EDTA, 10 mM LiCl, 1 mM cytidine, 3% PEG, 0.005% saponin, and RNase A and DNase I (Sigma) each at 20

μg/ml] for 1–5 min at 37°C. The sections were dried under a stream of cool air and apposed to Kodak NTB2 emulsion-coated coverslips for 1–3 weeks. Developed sections were stained with toluidine blue.

RESULTS

Recently we developed a technique permitting the autoradiographic localization in brain slices of $^{45}Ca^{2+}$ accumulated by the ATP-dependent Ca^{2+} pump into endoplasmic reticulum stores of calcium (9). The oxalate-stimulated $^{45}Ca^{2+}$ uptake is localized to discrete areas of the brain, and a major portion is sensitive to IP_3. We have labeled endoplasmic reticulum stores of calcium in the tongue by a similar procedure (Fig. 1 *A* and *B*). Autoradiographic grains are most highly concentrated in the apical areas of taste buds along the crypt of the circumvallate papillae. We have observed some uptake of $^{45}Ca^{2+}$ in taste buds of the foliate papillae, though their density is much less than circumvallate papillae. Virtually no $^{45}Ca^{2+}$ accumulation is detected in any epithelial layers of the tongue not associated with taste transduction. The only other substantial $^{45}Ca^{2+}$ uptake occurs in von Ebner's glands, which are selectively associated with taste bud containing foliate and circumvallate papillae and which extend secretory ducts into the crypts of the circumvallate papillae (17).

Autoradiographic grains associated with $^{45}Ca^{2+}$ uptake in circumvallate papillae are abolished by *ortho*-vanadate, which inhibits the calcium pump of the endoplasmic reticulum, but are not influenced by rotenone, ruthenium red, or oligomycin, inhibitors of the mitochondrial calcium transporter (data not shown). The calcium accumulation is abolished in the absence of ATP or magnesium (data not shown).

Examination under higher magnification reveals accumulated calcium that is highly concentrated at the apex of taste buds in the area of the taste pore, which is exposed to the tastants and is thought to contain the highest density of taste receptors (Fig. 1 *B* and *G*). Direct histochemical staining of the calcium ATPase at high magnification also shows a similar selective localization to taste buds with an intense concentration in the area of the taste pore (Fig. 1*G Inset*).

The accumulated calcium is markedly depleted in sections treated with 100 nM IP_3 (Fig. 1 *C* and *D*), which also decreases calcium accumulated by endoplasmic reticulum in brain slices, presumably by opening Ca^{2+} channels that release accumulated $^{45}Ca^{2+}$. To ascertain whether this effect of IP_3 involves physiologic IP_3 receptors, we explored the inositol phosphate specificity of this effect. Partial decrease of net $^{45}Ca^{2+}$ accumulation is detectable with 10 nM IP_3; most $^{45}Ca^{2+}$ is depleted by 100 nM IP_3, whereas $^{45}Ca^{2+}$ accumulation is abolished at 1 μM IP_3. Inositol 2,4,5-trisphosphate, which has some affinity for IP_3 receptors (18), also decreases accumulated $^{45}Ca^{2+}$ but less potently than IP_3. At 1 μM, inositol 2,4,5-trisphosphate is as effective as 100 nM IP_3. Inositol phosphate, inositol bisphosphate, inositol pentaphosphate, inositol hexaphosphate, and inositol 1,3,4-trisphosphate fail to inhibit $^{45}Ca^{2+}$ accumulation at 10 μM (data not shown).

Heparin is a potent inhibitor of IP_3 receptor binding (19) and blocks IP_3 release of $^{45}Ca^{2+}$ in brain slices (20). Heparin (100 μg/ml) reverses the effect of IP_3 on $^{45}Ca^{2+}$ accumulation in the taste buds (data not shown).

Innervation of the circumvallate papillae is provided by the glossopharyngeal nerve, whose transection is followed by degeneration of taste buds (12, 21). To ensure that the observed $^{45}Ca^{2+}$ accumulation involves taste bud cells, we bilaterally transfected the glossopharyngeal nerve (Fig. 1 *E* and *F*). After denervation, accumulation of $^{45}Ca^{2+}$ by apparent taste buds is abolished coincident with the disappearance of taste buds from the circumvallate papillae.

The ability of IP_3 to release $^{45}Ca^{2+}$ from taste buds implies the existence of specific IP_3 receptors, which we verified by autoradiography with [^{3}H]IP_3 (Fig. 2). Autoradiographic grains are selectively associated with taste buds of circumvallate papillae. As with accumulated $^{45}Ca^{2+}$, [^{3}H]IP_3 binding sites are most concentrated in the apical region of taste buds. Except for taste buds of the circumvallate papillae, no selective concentrations of [^{3}H]IP_3-associated grains are observed throughout the tongue. Addition of 10 μM unlabeled IP_3 completely abolishes specific labeling of taste buds by [^{3}H]IP_3.

To assess whether tastants act by means of the PI cycle, we examined the influence of the bitter tastant denatonium upon

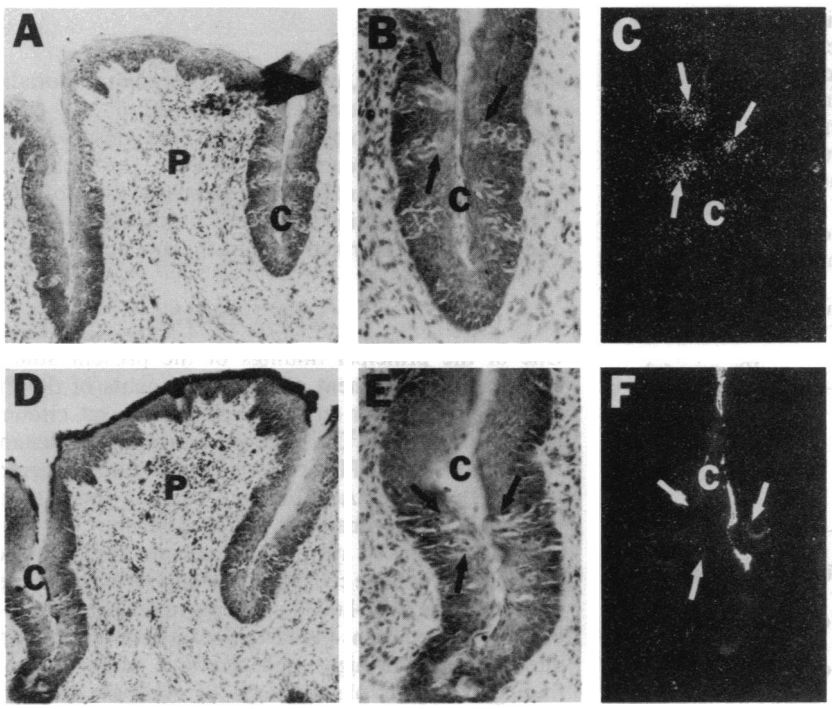

FIG. 2. Localization of IP_3 receptors in the circumvallate papilla. (*A–C*) A high density of specific [^{3}H]IP_3 binding sites in taste buds is revealed by dark-field silver grains selectively localized over taste buds. (*D–F*) Nonspecific binding with 10 μM unlabeled IP_3. P, circumvallate papilla; C, crypt; arrows indicate taste buds. (*A* and *D*, ×85; *B*, *C*, *E*, and *F*, ×170.)

Table 1. PI turnover in circumvallate papillae

Additions	[^{3}H]CDP-DAG (*n*)	[^{3}H]IP (*n*)
Li^+ + carbachol	1.32 ± 0.06* (4)	
Li^+ + denatonium	1.01 ± 0.05 (3)	0.99 ± 0.03 (3)

Data are expressed as the mean ± SEM of the ratios of the stimulated to the control accumulation. The concentrations used were 5 mM LiCl, 1 mM carbachol, and 10 μM denatonium. *n*, Number of experiments (each performed in duplicate). The mean control values, expressed in cpm per circumvallate papilla, were 524 for [^{3}H]CDP-DAG and 4340 for [^{3}H]IP.
*Differs from Li^+ samples in the absence of carbachol ($P < 0.01$).

PI turnover measured in dissected portions of the tongue enriched in circumvallate papillae dissected from rat tongue (Table 1). PI turnover was monitored by two techniques, utilizing either [^{3}H]inositol (15) or [^{3}H]cytidine (11) as precursor. Both of these techniques require the use of lithium to block inositol 1-phosphate phosphatase to permit the accumulation respectively of [^{3}H]inositol phosphates or [^{3}H]CDP-DAG. With the use of either technique for measuring PI turnover, 10 μM denatonium does not appear to alter PI turnover. Denatonium also fails to alter PI turnover when assessed at 10 nM or 1 mM concentrations (data not shown).

Stimulation of PI turnover by neurotransmitters, hormones, or other regulatory substances is secondary to activation of phospholipase C, producing increased mass levels of IP_3. Accordingly, we measured IP_3 mass levels in dissected circumvallate papillae utilizing a sensitive radioreceptor assay (16). Denatonium produces a rapid enhancement of IP_3 mass levels, which is detected 15–30 sec after treatment with the tastant (Table 2) but is no longer apparent after 1 min (data not shown). This very rapid time course resembles the influence of neurotransmitters on IP_3 mass levels in brain slices (22).

To examine whether the influence of denatonium is taste-specific, we examined its effects on IP_3 levels in epithelium adjacent to circumvallate papillae but which lacks taste buds. No effect on IP_3 mass levels was observed in these specimens. We have also examined the effects of sucrose and quinine at 1 mM concentration and have not observed an alteration in IP_3 mass levels.

Cholinesterase-staining nerve fibers have been described at the base of taste bud cells (23). Whether these neurons are afferent or efferent has not been established, and it is not known whether they are acetylcholine containing, since many noncholinergic neurons stain for cholinesterase (24). Indeed, no definitive evidence for efferent innervation of taste buds has been established. Muscarinic cholinergic stimulation enhances PI turnover in numerous tissues. Accordingly, we examined the influence of carbachol on PI turnover monitored with [^{3}H]cytidine (Table 1). A substantial enhancement of [^{3}H]CDP-DAG accumulation occurs with carbachol. To ascertain whether the influence of carbachol involves taste buds, we imaged PI turnover autoradiographically (Fig. 3). In the absence of carbachol, only diffuse, low levels of autoradiographic grains are observed over the epithelium of the circumvallate papillae. Carbachol treatment (1 mM) elicits a pronounced intensification of autoradiographic grain densities selectively associated with taste buds and highly concentrated toward the apical portion.

Table 2. IP_3 mass levels in response to denatonium

Tissue	Denatonium conc.	IP_3 mass (*n*)
Circumvallate papillae	0	42.9 ± 1.4 (29)
	10 μM	50.4 ± 2.9* (16)
	1 mM	53.9 ± 3.8† (12)
Nontaste epithelium	0	24.7 ± 2.2 (9)
	1 mM	24.4 ± 2.1 (9)

IP_3 concentrations are expressed as pmol per mg of protein ± SEM. *n*, Total number of samples in three to five experiments (each performed in triplicate).
*Differs from control samples in the absence of denatonium by the two-tailed *t* test ($P < 0.02$).
†Differs from control samples in the absence of denatonium by the two-tailed *t* test ($P < 0.002$).

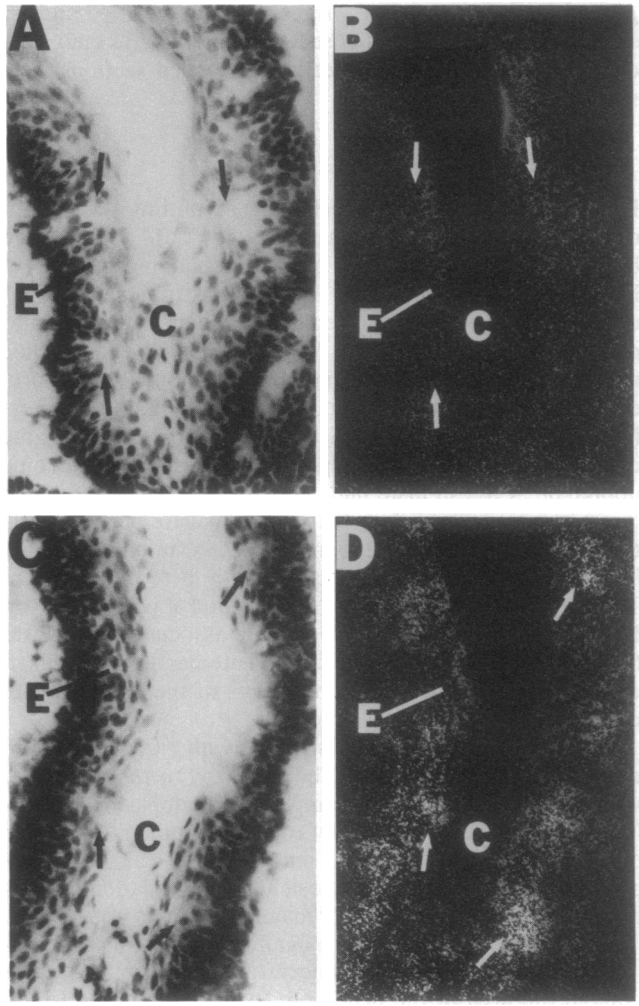

FIG. 3. Carbachol-stimulated PI turnover in the circumvallate papilla localizes to taste buds. (*A* and *B*) Respective light-field and dark-field images of the circumvallate papilla epithelium with 5 mM LiCl alone. (*C* and *D*) LiCl plus 1 mM carbachol elicits a pronounced intensification of silver grains in dark-field images selectively associated with taste buds. E, epidermal layer; C, crypt. Arrows indicate taste buds. (×170.)

DISCUSSION

One of the principal findings of the present study is the remarkable enrichment of the components of the PI signal transduction system in taste buds of the rat circumvallate papillae, including $^{45}Ca^{2+}$ accumulated by the calcium pump, IP_3 receptors, and PI turnover imaged autoradiographically with [^{3}H]cytidine. Accumulated calcium involves the IP_3-releasable pool primarily if not exclusively.

A role for the PI system and calcium in the transduction mechanisms of taste cells is further supported by the selective concentration of PI-linked elements in the apical area of taste buds, which contains the taste pore where tastants primarily interact with the high concentrations of receptors. These findings fit with observations of Akabas *et al.* (8) that

denatonium can elevate intracellular calcium in taste bud cells dissociated from circumvallate papillae.

Other than taste bud cells, the only substantial accumulation of $^{45}Ca^{2+}$ occurs in von Ebner's glands, which secrete into the crypts of the circumvallate papillae. These results are consistent with the considerable enrichment of the PI cycle in various glands including the salivary glands of the blow fly and the parotid gland (25) in which the PI cycle provides a signal transduction system for glandular secretion.

Direct evidence for a role of PI turnover in bitter taste transduction comes from the ability of denatonium to rapidly and transiently augment IP_3 levels in circumvallate papillae selectively. Interestingly, quinine does not augment IP_3 levels. Since the increases in IP_3 levels with denatonium are relatively small, the inactivity of quinine may relate to its substantially lesser potency than denatonium. Alternatively, quinine and denatonium may utilize different transduction mechanisms, as there is evidence for genetic determinants in the ability to taste different bitter substances. For instance, certain strains of inbred mice, which cannot detect particular bitter substances, still can respond to quinine (26).

The transient enhancement of IP_3 levels in response to denatonium resembles the transient rise in intracellular calcium with denatonium, which is detectable as early as 5 sec but which has substantially subsided by 30–60 sec (8). The transient augmentation of IP_3 may account for our failure to detect changes in PI turnover, which for technical reasons requires monitoring accumulation of [^{3}H]inositol phosphates or [^{3}H]CDP-DAG over a 60-min stimulation period.

A substantial enhancement of PI turnover was observed with the cholinergic stimulant carbachol. This strongly suggests that the cholinesterase-staining nerve fibers to taste buds contain efferent cholinergic neurons. Interestingly, in familial dysautonomia, taste discrimination is severely impaired and taste buds are absent (27), but both are restored by treatment with the muscarinic cholinergic agonist methacholine (28, 29).

Besides a role in taste transduction, the PI system may also mediate olfaction. Odorants rapidly and transiently increase mass levels of IP_3 in rat olfactory cilia (30), and amino acid odorants increase PI turnover in olfactory tissue of catfish (31). Other parallels also exist between taste and olfaction, such as a role for odorant- and tastant-sensitive adenylyl cyclases (5–7). Also, von Ebner's glands secrete a protein whose amino acid sequence (32) shows marked homology to that of the odorant binding protein (33).

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