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CYTOCHEMICAL STUDY ON CYCLIC NUCLEOTIDE PRODUCING- AND
HYDROLYZING-ENZYMATIC ACTIVITIES IN
OLFACTORY AND TASTE CELLS

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TERMS

In this paper, the term 'olfactory cell' is defined as an olfactory receptor cell and the term 'taste cell' as a cell which exists within the taste bud and protrudes its apical end into the taste pore (refer to the Introduction section).

The term 'phosphodiesterase' exclusively represents cyclic 3',5'-nucleotide phosphodiesterase (EC 3.1.4.17).

'Nucleoside triphosphate pyrophosphohydrolase (NTP pyrophosphohydrolase)' is the same enzyme as ATP pyrophosphatase (EC 3.6.1.8), which hydrolyzes not only ATP but also other nucleoside triphosphates into nucleoside monophosphate and inorganic pyrophosphate.

ABBREVIATIONS

Following abbreviations were used in this paper:

cyclic AMP = adenosine 3',5'-cyclic monophosphate

cyclic GMP = guanosine 3',5'-cyclic monophosphate

IP₃ = inositol 1,4,5-triphosphate

G-protein = guanine nucleotide-binding protein

AMP-PNP = 5'-adenylylimidodiphosphate

GMP-PNP = 5'-guanylylimidodiphosphate

GTPγS = guanosine 5'-O-(3-thiotriphosphate)

NTP pyrophosphohydrolase = nucleoside triphosphate pyrophosphohydrolase

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ABSTRACT

The localization of adenylate cyclase, guanylate cyclase and phosphodiesterase activities was examined cytochemically in rat olfactory cells and rabbit taste cells. In olfactory cells, adenylate cyclase activity was observed in the cilia, axon and to some extent in the dendritic knob. The activity in the cilia and dendritic knob was enhanced by nonhydrolyzable GTP analogues and forskolin, and inhibited by Ca^{2+} , all in agreement with biochemical reports of the odorant-sensitive adenylate cyclase. Strong cyclic AMP-hydrolyzing phosphodiesterase activity was observed in the cilia, dendritic knob and axon and weaker activity in the dendritic shaft and cell body. Phosphodiesterase in olfactory cells hydrolyzed cyclic GMP to a smaller extent than cyclic AMP. Guanylate cyclase activity was detected only in the axon. These results support the hypothesis of cyclic AMP being involved as a second messenger in olfactory transduction and imply that the transduction sites exist not only in the cilia but also in the dendritic knob. It is also implied that the cyclic AMP produced in the dendritic terminal acts mainly in its original site but some of it may diffuse and act upon the membrane of the dendritic shaft and cell body. By treating the tissue with a low concentration of Triton X-100, an enzymatic activity presumed to be a different type of adenylate cyclase activity from that in the dendritic terminal was observed in the dendritic shaft, although the role of this enzyme is unknown.

In taste cells, both guanylate cyclase and phosphodiesterase activities were observed in the apical portion of cells, the most likely site of interaction with taste stimuli. Sucrose sometimes enhanced the guanylate cyclase activity. The phosphodiesterase hydrolyzed cyclic AMP and cyclic GMP to a similar extent. Adenylate cyclase activity,

however, was not detected in taste cells in the present study. The results suggest that cyclic GMP serves as a second messenger in taste transduction, particularly for sweet taste, and that the cyclic GMP produced in the apical portion of taste cells acts in its original site without diffusing into the cell body.

1. INTRODUCTION

Both olfactory and taste cells are well-known chemosensory cells which transduce chemical information provided by odor or taste substances into electrical signals. The electrical signals are transmitted to the brain and eventually provoke the sense of smell or taste. The mechanisms involved in those transducing processes are now becoming elucidated, especially in olfaction.

Olfactory cells of vertebrates are bipolar sensory neurons, which project an unmyelinated axon to the olfactory bulb and a long, single dendrite to the epithelial surface. The terminal of the dendrite, which is a dendritic knob with a variable number of cilia, protrudes into the mucus layer and comes in direct contact with odorants. Initial stages of olfactory signal transduction are thought to take place in the olfactory cilia (Rhein & Cagan, 1981; Getchell, 1986; Lancet, 1986, 1988) and at present, two pathways are proposed for this transduction. One is a pathway which involves cyclic AMP as an intracellular second messenger. The evidence in support of this pathway includes an odorant-stimulated increase of cyclic AMP in olfactory cells, especially in olfactory cilia (Pace et al., 1985; Shirley et al., 1986, 1987; Sklar et al., 1986; Lazard et al., 1989; Boekhoff & Breer, 1990; Boekhoff et al., 1990; Breer et al., 1990; Breer, 1991; Breer & Boekhoff, 1991; Ronnett et al., 1991); identification of a guanine nucleotide-binding protein (G-protein) in olfactory cilia, which mediates stimulation of adenylate cyclase by odorants (Pace & Lancet, 1986); identification of an olfactory cell-specific adenylate cyclase (Bakalyar & Reed, 1990); identification of an olfactory cell-specific G-protein, which is capable of stimulating adenylate cyclase (Jones & Reed, 1989); discovery of cyclic nucleotide-gated cation channels in olfactory cells (Nakamura &

Gold, 1987; Trotier & MacLeod, 1987; Persaud et al., 1988; Suzuki, 1989; Dhallan et al., 1990; Kurahashi, 1990; Firestein et al., 1991a,b; Vodyanoy, 1991; Zufall et al., 1991; Frings et al., 1992; Miyamoto et al., 1992); and positive correlation between the magnitude of the stimulation of adenylate cyclase and the summated electrical response of the olfactory epithelium (electro-olfactogram) evoked by individual odorants (Gold et al., 1989; Lowe et al., 1989). The other pathway involves inositol 1,4,5-triphosphate (IP_3) as a second messenger (biochemical evidence, Boekhoff & Breer, 1990; Boekhoff et al., 1990; Breer, 1991; Breer & Boekhoff, 1991; electrophysiological evidence, Restrepo et al., 1990; Miyamoto et al., 1992; Frings, 1993). In the cyclic AMP system, an odorant-receptor complex is presumed to activate adenylate cyclase in a G-protein-dependent manner and produce cyclic AMP, which, in turn, opens cyclic nucleotide-gated channels permitting inward cation flux (reviewed by Ronnett & Snyder, 1992). In the IP_3 system, it is presumed that an odorant-receptor complex activates phospholipase C via G-protein and produces IP_3 , which, in turn, triggers an influx of extracellular calcium ions (see Ronnett & Snyder, 1992, for review). The receptor sites and the G-proteins involved appear to be different in the two systems (Boekhoff et al., 1990; Breer, 1991).

Compared to olfaction, studies of taste sensation seem more complex. One reason for this complexity is that there are different modalities in taste (sweet, salty, sour and bitter); another reason is that there are different types of cells observed in taste buds.

Four types of cells (epithelial cells in origin) are generally reported in vertebrate taste buds (reviewed by Roper, 1989). One is a basal cell which has an oblate shape and lies at the base of the taste

bud. The other types of cells have a slender shape and extend from the base of the taste bud to its apex, the taste pore. These cells are often termed the dark cell (also termed the type I cell), the light cell (type II) and the intermediate cell (type III) based on the electron opacity of their cytoplasm. The first step of transduction is believed to be the interaction of taste substances with the apical membrane of these cells, although there is still controversy as to which type(s) of cell represents an authentic receptor cell (see Roper, 1989, for review). In the present paper, the term "taste cell" is defined as a cell which exists within the taste bud and possesses an apical end, to which direct access of taste stimuli is allowed.

Taste cells or, more precisely, taste receptor cells appear to utilize diverse transduction mechanisms for different taste modalities. 1) Salt taste is likely to be, in part, transduced by direct passage of sodium salts through apical amiloride-blockable sodium channels of taste cell membrane (Schiffman et al., 1983, 1990; Heck et al., 1984; Avenet & Lindemann, 1988). 2) Sour taste may be transduced by closure of voltage-dependent potassium channels densely localized on the apical membrane of taste cells (Kinnamon & Roper, 1988a,b; Kinnamon, S. C. et al., 1988). The opening of chloride channels (Simon & Garvin, 1985) or opening of calcium channels (Miyamoto et al., 1988) may also be a transduction mechanism for sour taste. 3) Transduction of bitter taste may be mediated via a blockade of potassium channels (Ozeki, 1971; Kinnamon & Roper, 1988b; Spielman et al., 1989) or an active secretion of chloride ions (Okada et al., 1988), or via a process involving an intracellular messenger such as IP_3 (Hwang et al., 1990; Spielman et al., 1992). 4) Transduction mechanism for sweet taste may involve

amiloride-sensitive sodium channels (Schiffman et al., 1983; Mierson et al., 1988; Simon et al., 1989) but an involvement of cyclic AMP or cyclic GMP is also a promising possibility. Sweet substances were found to increase cyclic AMP in lingual membrane or taste papillae (Lancet et al., 1987; Striem et al., 1989, 1990, 1991; Naim et al., 1991) and cyclic GMP in taste papillae (Uebayashi & Tonosaki, 1994); an injection of cyclic AMP or cyclic GMP in taste cells produced a membrane depolarization similar to that induced by sucrose stimulus to those cells (Tonosaki & Funakoshi, 1988).

Thus, there is a strong possibility that cyclic AMP or cyclic GMP works as an intracellular messenger in certain transduction pathways both in olfactory and taste cells. Most evidence which supports this hypothesis has been brought forward by using biochemical or electrophysiological techniques. An advantage of using biochemical techniques is that one is capable of quantitatively examining the increase or decrease of cyclic nucleotides produced in tissues in response to odorous or taste stimuli. Electrophysiological techniques are useful in examining electrical responses of cells to the stimuli, in other words, the results of the signal transduction. Both techniques, however, are insufficient for giving a whole view of in which sites of the sensory cells the cyclic nucleotides are formed, act and are decomposed. Biochemical studies of olfactory transduction have been exclusively performed by using olfactory cilia, which are thought to be the initial transduction site and are easily isolated from the olfactory mucosa (Rhein & Cagan, 1980; Anholt et al., 1986; Chen et al., 1986; Sklar et al., 1986). For lack of a sufficient amount of taste cells to be collected, the lingual epithelium or, at best, the lingual papillae

containing taste cells have been used for biochemical studies on taste (Kurihara & Koyama, 1972; Law & Henkin, 1982; Kalinoski et al., 1987; Lancet et al., 1987; Striem et al., 1989, 1990, 1991; Naim et al., 1991). Electrophysiologists have succeeded in patch-clamping various portions of olfactory cells including fine sensory cilia (Nakamura & Gold, 1987) but the apical portion of taste cells is still too fine an object for such a study. In addition, discrimination between different types of cells isolated from taste buds for the patch-clamp study has not yet been achieved. In order to examine the role of cyclic nucleotides in relation to the structure of sensory cells, I believe that cytochemistry (electron microscopic histochemistry) is a useful technique. However, only a few cytochemical studies concerning the formation and decomposition of the nucleotides have been reported in vertebrate olfactory cells (Asanuma & Nomura, 1991, 1993; Menco et al., 1992) or taste cells (Yamamoto & Ozawa, 1977; Pevzner & Tikhonova, 1980; Asanuma & Nomura, 1982, 1992; Nomura & Asanuma, 1982).

If cyclic AMP or cyclic GMP is involved in sensory transduction, it is expected that the cyclic nucleotide-producing enzyme (adenylate cyclase or guanylate cyclase) is located near the receptor sites in the plasma membrane and, for the control of sensory signals including the termination of signals, the cyclic nucleotide-degrading enzyme (phosphodiesterase) near the sites where the nucleotides act. So far, I have demonstrated cytochemically adenylate cyclase activity in the cilia and the dendritic knob of olfactory cells of rats (Asanuma & Nomura, 1991) and phosphodiesterase activity in the same regions (Asanuma & Nomura, 1993). As for taste sensation, adenylate cyclase and phosphodiesterase activities were reported in taste cells of rabbits and

cats (Asanuma & Nomura, 1982; Nomura & Asanuma, 1982). However, more recent study has shown that most, if not all, of the enzymatic activity previously observed in those taste cells was not adenylate cyclase but nucleoside triphosphate pyrophosphohydrolase (NTP pyrophosphohydrolase) activity (Asanuma & Nomura, 1986). Instead of adenylate cyclase, guanylate cyclase activity was demonstrated in rabbit taste cells (Asanuma & Nomura, 1992).

In this paper, the localization of adenylate cyclase, guanylate cyclase and phosphodiesterase activities in olfactory and taste cells is elaborated. The purpose of the study is to examine from the cytochemical point of view whether or not cyclic AMP or cyclic GMP may work in the sensory transduction in those cells and, if it does, how it may work. Part of this work has already been presented (Asanuma & Nomura, 1982, 1986, 1991, 1992, 1993).

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Animals

Male Wistar rats (200-360 g) were used for the study of olfactory cells because numerous biochemical and electrophysiological studies on olfaction had been done using rats, hence much information was available concerning the animals.

For the study of taste cells, male white domestic rabbits (1.6-3.3 kg) were used, since rabbits possess large foliate papillae enriched with taste buds. An additional reason for the selection of these animals was that they were easily available in the area where the present study was carried out.

2.1.2. Chemicals

5'-Adenylylimidodiphosphate (AMP-PNP, tetralithium salt), 5'-guanylylimidodiphosphate (GMP-PNP, sodium salt), guanosine 5'-O-(3-thiotriphosphate) (GTP γ S, tetralithium salt), adenosine 3',5'-cyclic monophosphate (cyclic AMP, sodium salt), guanosine 3',5'-cyclic monophosphate (cyclic GMP, sodium salt), adenosine 5'-monophosphate (5'-AMP, sodium salt), snake venoms (*Crotalus atrox* and *Ophiophagus hannah*), forskolin, 3-isobutyl-1-methylxanthine, theophylline, levamisole, sodium azide, Triton X-100 and EGTA were purchased from Sigma Chemical Co., St. Louis, MO, USA; paraformaldehyde, sodium cacodylate, Tris(hydroxymethyl)-aminomethane, HEPES, lead nitrate and sucrose (all specially prepared reagent grade) from Nacalai Tesque,

Inc., Kyoto, Japan; glutaraldehyde, osmium tetroxide and epoxy resin (Epon 812) from TAAB Laboratories Equipment, Ltd., Reading, UK; Ringer's solution from Hikari Seiyaku Co., Ltd., Tokyo, Japan. Dithiothreitol was obtained either from Sigma or Nacalai Tesque (the above grade). All other materials were of guaranteed reagent-grade or EM grade.

2.2. Methods

2.2.1. Cytochemistry of adenylate cyclase and guanylate cyclase activities in rat olfactory cells

2.2.1.1. Tissue preparation

Rats were sacrificed by decapitation and olfactory mucosae were removed. The mucosae were rinsed briefly in an ice-cold saline solution containing 0.9% NaCl, 1 mM EGTA and 5 mM HEPES, pH 7.2, and fixed for 1 h on ice in 4% paraformaldehyde in 0.05 M sodium cacodylate buffer, pH 7.4, containing 0.25 M sucrose + 1 mM EGTA. EGTA was employed to remove endogenous calcium, since calcium ion is reported to inhibit odorant-sensitive adenylate cyclase (Shirley et al., 1986, 1987; Sklar et al., 1986). Since in the preliminary experiments, glutaraldehyde had been found inhibitory to adenylate cyclase activity in olfactory cells except in the axonal region, only paraformaldehyde was used for prefixing the tissue unless otherwise indicated. After rinsing for several hours in ice-cold 0.05 M cacodylate buffer containing 0.25 M sucrose and 1 mM EGTA with more than six changes of the solution, the mucosae were cut into 0.2-0.3 mm thick sections with a thin razor blade

and stored overnight at 0 °C in the same solution.

In order to observe adenylate cyclase activity in the axonal region, the tissue was fixed for 1 h in 2% paraformaldehyde + 0.1% glutaraldehyde in 0.05 M cacodylate buffer containing 0.24 M sucrose and 1 mM EGTA, since 4% paraformaldehyde was insufficient to preserve the axonal ultrastructure. The above solution preserved both the structure and some of the enzymatic activity in that region. The fixed tissue was rinsed as above and cut into sections 80 μm thick using a DTK-1000 Microslicer (Dosaka EM Co. Ltd., Kyoto, Japan) and stored overnight.

2.2.1.2. Cytochemical incubation for adenylate cyclase activity

The strontium method, described by Ernst (1972) for Na^+/K^+ -ATPase cytochemistry and later applied by others for adenylate cyclase demonstration (Schulze et al., 1977; Poeggel et al., 1982) was employed with modification. The tissue sections were preincubated in a substrate-free incubation medium for 20-30 min at room temperature and then incubated for 1 h at 37 °C in a medium containing 80 mM Tris-HCl, pH 9.1, 0.18 M sucrose, 4 mM MgCl_2 , 2 mM theophylline (cyclic nucleotide phosphodiesterase inhibitor, Appleman et al., 1973), 2.5 mM levamisole (nonspecific alkaline phosphatase inhibitor, Borgers, 1973), 20 mM SrCl_2 and 0.5 mM AMP-PNP. AMP-PNP was employed instead of ATP as the exogenous substrate for adenylate cyclase, since it is hydrolyzed by adenylate cyclase but not by ordinary membrane ATPases (Rodbell et al., 1971; Howell & Whitfield, 1972).

The following experiments were run for comparison: 1) incubation in the absence of substrate (either with or without 30 μM GMP-PNP or $\text{GTP}\gamma\text{S}$), 2) preheating the tissue sections for 15 min at 80 °C in 0.30 M

sucrose before preincubation, and 3) incubation in the presence of either 10 μM forskolin (adenylate cyclase activator, Bender et al., 1984; Daly, 1984), 30 μM GMP-PNP, 30 μM GTP γ S, 10 mM CaCl_2 + 30 μM GTP γ S, or 5 mM dithiothreitol + 30 μM GTP γ S. Dithiothreitol was employed as an inhibitor of NTP pyrophosphohydrolase, which is an AMP-PNP-hydrolyzing enzyme in addition to adenylate cyclase (Johnson & Welden, 1977). GTP γ S was added to the CaCl_2 - or dithiothreitol-containing medium in order to observe clear effects of calcium and dithiothreitol by ensuring the cytochemical reaction (see Results section). Forskolin, CaCl_2 and dithiothreitol were added to the preincubation media as well. In the case of preincubation with CaCl_2 , the tissue was first placed in a medium containing 1 mM CaCl_2 , and then transferred into a 2 mM and finally a 10 mM CaCl_2 -containing medium in order to avoid sudden application of high-concentration calcium and lessen calcium shock (Anholt et al., 1986; Chen et al., 1986; Sklar et al., 1986). In some experiments, 0.0002% Triton X-100 was added to the fixing, rinsing (for the first 2-3 h), preincubation and incubation solutions.

The pH's of the media were readjusted to 9.1 with sodium hydroxide before adding the substrate and control chemicals. The final pH of all the incubation media ranged from 8.9-9.0, which was optimal for adenylate cyclase activity with AMP-PNP as substrate (Birnbaumer & Yang, 1974; Maguire & Gilman, 1974; Kempen et al., 1978).

2.2.1.3. Cytochemical incubation for guanylate cyclase activity

The fundamental procedure was similar to the adenylate cyclase cytochemistry described above. The tissue sections were preincubated in

a substrate-free incubation medium for 30 min at room temperature or on ice and incubated for 1 h at 37 °C in a medium containing 80 mM Tris-HCl, pH 9.1 or pH 8.5, 0.18 M sucrose, 3 mM MnCl₂, 2 mM theophylline, 2.5 mM levamisole, 20 mM SrCl₂ and 0.5 mM GMP-PNP. GMP-PNP was employed as substrate in order to distinguish guanylate cyclase activity from GTPase activity (Saito, 1977). The final pH of the incubation medium was 8.9 or 8.5. Incubation at pH 8.5 or higher was necessary to avoid inefficient trapping of hydrolyzed phosphate and solubilization of strontium phosphate (Ernst, 1972).

2.2.1.4. Postincubation procedure

Incubated tissue sections were rinsed three times in 80 mM Tris-HCl buffer, pH 9.0, containing 0.23 M sucrose, and then twice in 2% lead nitrate solution with 0.23 M sucrose to convert precipitated strontium phosphate into lead phosphate for better visualization in the electron microscope. The sections were then rinsed three times in 0.30 M sucrose and twice in 0.05 M cacodylate buffer, pH 7.4, containing 0.25 M sucrose. All rinses were carried out at room temperature and each rinse period was 5-10 min.

The tissue was then postfixed for 30 min on ice with 1% osmium tetroxide in 0.05 M cacodylate buffer, pH 7.4, containing 0.21 M sucrose, dehydrated in graded series of ethanol and propylene oxide and embedded in Epon 812. Ultrathin sections were cut on an LKB 2088 microtome and unstained sections were examined in a JEOL 1200EX-II or 100B electron microscope operated at 80 kV. In cytochemistry, adequate penetration of incubation mixture components is assumed in tissue sections of up to 40 µm thickness (Mayahara & Ogawa, 1968). Since weak

fixations did not allow such desirable sections in the present study, only a surface-portion of the 0.2-0.3 mm or 80 μ m-thick sections was examined instead.

2.2.2. Cytochemistry of phosphodiesterase activity in rat olfactory cells

2.2.2.1. Tissue preparation

Olfactory mucosae dissected from decapitated rats were rinsed briefly in an ice-cold Ringer's solution and fixed for 30 min on ice in 1% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.4, containing 0.15 M sucrose. After rinsing for several hours in ice-cold 0.05 M cacodylate buffer containing 0.25 M sucrose with at least five changes of the solution, the tissue was cut into 40 μ m thick sections using a Microslicer and stored overnight at 0 °C in the same solution.

2.2.2.2. Cytochemical incubation for phosphodiesterase activity

The tissue sections were incubated according to Florendo et al. (1971). First, the sections were preincubated for 30-50 min at room temperature in 60 mM Tris-maleate buffer, pH 7.4, 2 mM $MgCl_2$ and 0.25 M sucrose (TMS buffer) containing 5 mg/ml snake venom (*Crotalus atrox*). The sections were then incubated in a shaking water bath for 30 min at 37 °C in TMS buffer containing 2 mM lead nitrate, 3 mg/ml snake venom and 3 mM cyclic AMP (or cyclic GMP). The basis of the procedure is that the phosphodiesterase in the tissue converts cyclic AMP to 5'-AMP, which, in turn, is cleaved into adenosine and inorganic phosphate by

5'-nucleotidase in snake venom. The inorganic phosphate thus formed is captured by lead ions and forms an electron-dense reaction product at or near the site of phosphodiesterase activity. Preincubation was employed to promote the penetration of 5'-nucleotidase into the tissue.

The following experiments were run for comparison: 1) incubation in the absence of exogenous substrate, 2) preheating the tissue sections for 20-30 min at 70 °C before the preincubation step, 3) incubation in the absence of snake venom (preincubation omitted), 4) incubation with a cyclic nucleotide phosphodiesterase inhibitor, 5 mM 3-isobutyl-1-methylxanthine (Aschcroft et al., 1972; Peytreman et al., 1973) or 50 mM theophylline, and 5) penetration test of the exogenous enzyme. Since 5'-nucleotidase is a large molecule, the extent of its penetration into the tissue sections was checked by first preincubating the sections in TMS buffer containing 5 mg/ml snake venom, then incubating them with 1 mM 5'-AMP in venom-free TMS buffer containing 2 mM lead nitrate. The final pH of the above incubation media ranged from 7.1 to 7.3.

2.2.2.3. Postincubation procedure

After incubation, the sections were rinsed in TMS buffer, then postfixed for 30 min on ice with 1% osmium tetroxide in 0.05 M cacodylate buffer, pH 7.4, containing 0.22 M sucrose, then processed for electron microscopy in a similar manner as described before (2.2.1.4) and unstained ultrathin sections were examined.

2.2.3. Cytochemistry of adenylate cyclase and guanylate cyclase activities in rabbit taste cells

2.2.3.1. Tissue preparation

Rabbits were sacrificed by intravenous injection of a fatal dose of sodium amobarbital (100-200 mg/kg body weight) and foliate papillae were removed. After the papillae were rinsed briefly in an ice-cold saline solution containing 0.9% NaCl and 5 mM HEPES, pH 7.2, the muscles and glands lining the papillae were cut off. The papillae were then cut into 1-2 mm thick pieces with fine scissors and fixed for 30 min on ice in 2% paraformaldehyde + 0.5% or 0.1% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.4, containing 0.15 M NaCl or 0.25 M sucrose. Fixation using only paraformaldehyde was abandoned since it resulted in insufficient morphological preservation of microvilli of taste cells. After rinsing for several hours in ice-cold 0.05 M cacodylate buffer containing 0.15 M NaCl (or 0.25 M sucrose) with at least seven changes of the solution, the tissue was cut into 40 μ m thick sections using a Microslicer or into 0.1-0.2 mm thick sections with a thin razor blade and stored overnight at 0 °C in the same solution.

2.2.3.2. Cytochemical incubation for adenylate cyclase activity

The strontium method was employed as in 2.2.1.2 with further reformation. The tissue sections were preincubated for 10-30 min at room temperature in a medium containing 80 mM Tris-HCl, pH 9.1, 0.11 M NaCl (or 0.20 M sucrose for the tissue fixed and rinsed in the sucrose-containing solutions), 5 mM theophylline, 2.5 mM levamisole and 10 mM SrCl₂. Incubation was then initiated by adding to this medium 4

mM MgCl_2 and 0.5 mM AMP-PNP and warming the medium to 37 °C in a shaking water bath. Incubation was continued for 30 min at 37 °C. Incubation with either 2 or 5 mM dithiothreitol or 2 mM dithiothreitol + 100 μM forskolin was run for comparison. These chemicals were added at the preincubation step. The pH of the incubation media ranged from 8.9-9.0.

2.2.3.3. Cytochemical incubation for guanylate cyclase activity

The fundamental procedure was similar to that for adenylate cyclase cytochemistry in 2.2.3.2 except for the addition of 1 mM MnCl_2 and 0.5 mM GMP-PNP to the incubation medium instead of MgCl_2 and AMP-PNP.

The following experiments were run for comparison: 1) incubation in the absence of substrate, 2) incubation with 2-10 mM dithiothreitol, 3) incubation with a guanylate cyclase activator, i. e. 1-10 mM concentration of either sodium azide (Kimura et al., 1975a,b, 1976; Katsuki et al., 1977), hydroxylamine (Kimura et al., 1975b; Katsuki et al., 1977) or sodium nitroprusside (Katsuki et al., 1977), 4) incubation with either 1 mM CdCl_2 , ZnCl_2 or HgCl_2 , which are reported to inhibit guanylate cyclase activity (Hardman & Sutherland, 1969), 5) incubation with 1% Triton X-100 as a guanylate cyclase activator (Kimura & Murad, 1974, 1975; Kimura et al., 1975a; Durham, 1976), 6) incubation with 1 M sucrose, 7) incubation in the absence of MnCl_2 , and 8) incubation in the presence of 3 mM MnCl_2 . Dithiothreitol was employed as an inhibitor of NTP pyrophosphohydrolase, which hydrolyzes GMP-PNP as well as AMP-PNP (Johnson & Welden, 1977). Chemicals mentioned in 2)-6) were added at the preincubation step. The effects of guanylate cyclase activators in 3) were also examined in the tissue incubated for a shorter period of time (10 min). The final pH of the above incubation media ranged from

8.7-8.9.

2.2.3.4. Postincubation procedure

Incubated sections were cooled on ice to minimize further reaction and soon rinsed and processed for strontium-lead substitution as previously described (2.2.1.4). They were then postfixed for 1 h on ice with 1% osmium tetroxide in 0.05 M cacodylate buffer, pH 7.4, containing 0.23 M sucrose. The subsequent procedure for electron microscopy was the same as in 2.2.1.4.

2.2.4. Cytochemistry of phosphodiesterase activity in rabbit taste cells

2.2.4.1. Tissue preparation

Foliate papillae obtained from rabbits as previously described (2.2.3.1) were rinsed briefly in an ice-cold Ringer's solution, cut into 1-2 mm thick pieces and fixed for 50-60 min on ice in 1% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.4, containing 0.20 M sucrose. After rinsing for several hours in ice-cold 0.05 M cacodylate buffer containing 0.25 M sucrose with nine changes of the solution, the tissue was cut into 40 μ m thick sections using a Microslicer and stored overnight at 0-5 °C in the same solution.

2.2.4.2. Cytochemical incubation for phosphodiesterase activity

The incubation procedure was the same as for rat olfactory mucosae (2.2.2.2) except that 5 mM levamisole was added to the preincubation and incubation media in order to prevent disturbance caused by alkaline

phosphatase activity found in rabbit foliate papillae (Nomura, 1978). Snake venom employed was either from rattlesnakes (*Crotalus atrox*) or cobras (*Ophiophagus hannah*), both of which gave a similar localization of the enzymatic activity and a similar morphological preservation of the tissue. The control experiments were also the same as those in 2.2.2.2.

2.2.4.3. Postincubation procedure

Same procedure was employed as in 2.2.2.3 except that the postfixation period was from 40-50 min.

2.2.5. Electron microscopy for the observation of general structure of olfactory and taste cells

Some of the olfactory mucosae fixed with 2% paraformaldehyde + 0.1% glutaraldehyde and postfixed with 1% osmium tetroxide, originally prepared for adenylate cyclase cytochemistry, were used for observing the general structure of olfactory cells. Ultrathin sections obtained as in 2.2.1.4 were stained with uranyl acetate and examined in a JEOL 1200EX-II electron microscope.

To observe the structure of taste cells, rabbit foliate papillae were fixed for 1 h on ice with 2% paraformaldehyde + 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and postfixed for 30 min on ice with 1% osmium tetroxide in the same buffer containing 0.25 M glucose. Ultrathin sections were obtained as described in 2.2.1.4, stained with uranyl acetate and lead citrate and examined in a JEOL 100B

electron microscope.

3. RESULTS

3.1. Olfactory cells

3.1.1. General structure

Three types of cells were observed in the olfactory epithelium of rats (Fig. 1). The upper third of the epithelium was occupied by columnar supporting cell bodies and the middle or part of the lower third by olfactory cell bodies. Prismatic or irregular-shaped basal cells were seen close to the basal lamina. In addition to these three cells, duct cells of the Bowman's glands were also seen in some places in the epithelium (not shown).

Olfactory cells (sensory neurons) showed a bipolar, narrow-necked bottle shape with an axon projecting toward the basal lamina and a long, single dendrite toward the epithelial surface (Fig. 1). The dendrite was divided into two portions: the dendritic shaft, a long, rod-like portion, which was surrounded by supporting cells, and the dendritic knob, a swollen terminal which usually protruded from the epithelial surface (Fig. 2). The diameter of the dendritic shaft was 1-1.5 μm , while the length varied from one cell to the other depending on the thickness of the epithelium and the location of the cell body in the epithelium. The dendritic shaft was usually filled with mitochondria (Fig. 2; also see Figs. 18 and 19). The dendritic knob had a diameter of 1-2 μm and was provided with a variable number of cilia (Figs. 1 and 2). The cilium had a diameter of approximately 0.2 μm at the proximal portion and tapered off into the distal end.

The olfactory cell body had a diameter of 4-6 μm and was characterized by a large, somewhat lobate nucleus (Figs. 1 and 3). The

proximal pole of the cell body tapered and originated into an unmyelinated axon, the diameter of which was approximately 0.1 μm (Figs. 1 and 3). The axon ran crossing the basal lamina and formed a bundle with other axons, enwrapped by the Schwann cell cytoplasm (Fig. 4). In the tissue fixed using paraformaldehyde alone for the cytochemical examination, cells were often separated from each other. However, the cellular and nuclear outlines described above as well as the topographical location of cells in the epithelium gave enough information to discriminate olfactory cells from other cells even in unstained sections.

3.1.2. Adenylate cyclase activity

The cytochemical reaction product of adenylate cyclase activity, shown as a fine electron-dense precipitate, was observed mostly in the cilia and in smaller amounts in the dendritic knob of olfactory cells (Fig. 5). A larger amount of the reaction product was observed in the ciliary portion somewhat distal to the dendritic knob than in the proximal portion (Fig. 5a). The reaction product was scattered inside the whole cilium in most cases; however, in some transverse cut ends of cilia, it precipitated near the plasma membrane (Fig. 5b). In the dendritic knob, the reaction product was dense at the basal bodies, from which the cilia originated (Fig. 5b). The amount of the reaction product varied from cell to cell and cells which showed no reaction product were common (see Fig. 5b). By adding 10 μM forskolin to the incubation medium, the reaction product increased both in the cilia and

the dendritic knob (Fig. 6). Addition of nonhydrolyzable GTP analogues, 30 μ M GMP-PNP or GTP γ S, showed a similar enhancement of the enzymatic activity (Figs. 7 and 8). Addition of forskolin or GTP analogues not only increased the amount of reaction product in each cell but also increased the number of cells showing the enzymatic activity to a large extent. CaCl₂ (10 mM), on the other hand, inhibited the enzymatic activity in the cilia and dendritic knob (Fig. 9). Dithiothreitol (5 mM) did not inhibit the enzymatic activity (Fig. 10).

The reaction product was not usually seen in the dendritic shaft and cell body (Figs. 11 and 13) except for a very occasional appearance of the product in the dendritic shaft. It seemed, though not very conclusive, that forskolin facilitated this appearance of the product in the shaft (Fig. 12). The effects of GMP-PNP and GTP γ S were not apparent in this region. Fixation using paraformaldehyde alone was insufficient for morphological preservation of axons and it was difficult to sufficiently investigate the enzymatic activity in this region using a weakly-fixed mucosa. Fortunately, however, some of the enzymatic activity persisted in the axonal region after one hour fixation with 2% paraformaldehyde + 0.1% glutaraldehyde (Fig. 14). This activity was enhanced by 30 μ M GMP-PNP (Fig. 15).

Incubation in the absence of substrate resulted in no reaction product (Fig. 16). Addition of 30 μ M GMP-PNP or GTP γ S to this substrate-free medium also resulted in no precipitate of the product, confirming that the increase of the reaction product by the GTP analogues seen in the substrate-containing incubation was not due to direct release of phosphate from the chemicals (data not shown). Preheating the tissue at 80 °C eliminated the enzymatic activity (Fig.

17).

When the tissue was fixed and incubated with 0.0002% Triton X-100, much reaction product appeared inside the dendritic shaft (Fig. 18). The enzymatic activity in this region was not affected by 10 mM CaCl_2 (Fig. 19) but was inhibited by 5 mM dithiothreitol (Fig. 20). Forskolin (10 μM) and GMP-PNP (30 μM) did not further increase the reaction product (data not shown). Triton X-100, on the other hand, inhibited the enzymatic activity in the olfactory cilia and knob (Fig. 21); the activity was restored by 10 μM forskolin or 30 μM GMP-PNP (Figs. 22 and 23). Tissue-preheating or substrate-free incubation resulted in no reaction product as in the tissue untreated with Triton X-100 (data not shown).

As will be described in 3.1.4, phosphodiesterase activity was observed in basal cells. This indicates the possibility that a cyclic nucleotide producing-enzymatic activity also exists in these cells. Unfortunately, the structure of basal cells was not sufficiently preserved by fixation using paraformaldehyde alone, so adenylate cyclase activity could not be investigated in this region. Although some electron-dense precipitate was observed in this region, it was difficult to determine whether it was a cytochemical reaction product formed in basal cells or one diffused from other regions such as axons. In the tissue fixed with 2% paraformaldehyde + 0.1% glutaraldehyde, no reaction product was seen in basal cells (data not shown).

3.1.3. Guanylate cyclase activity

Guanylate cyclase activity was not usually detected from the olfactory cilia to the cell body (Figs. 24 and 25), although slight electron-dense precipitate was occasionally seen (especially in the cilia and dendritic knob, data not shown). The axonal region showed some reaction product (Fig. 26).

3.1.4. Phosphodiesterase activity

With cyclic AMP as substrate, a large amount of the reaction product of phosphodiesterase activity appeared in the cilia, dendritic knob and axon of olfactory cells (Figs. 27 and 29). Most of the reaction product was located on the plasma membrane of the cells. The membrane of the dendritic shaft showed less of the product (Fig. 27). The reaction product was also observed, though not always, on the membrane of the olfactory cell body (Fig. 28). Phosphodiesterase inhibitors, 5 mM 3-isobutyl-1-methylxanthine and 50 mM theophylline, reduced the reaction product (Figs. 30 and 31).

No reaction product was seen when the tissue was incubated in the absence of exogenous substrate (Fig. 32) or preheated at 70 °C (Fig. 33). Incubation in the absence of snake venom resulted in no reaction product in olfactory cells (Fig. 34). Interestingly, the reaction product was also observed in basal cells (Fig. 35) and the product in those cells still appeared in the incubation without snake venom (Fig. 36). The enzymatic activity in basal cells was not inhibited by 2.5 mM levamisole (Fig. 36).

When cyclic GMP was employed as substrate, the phosphodiesterase

activity was seen in the olfactory cilia and dendritic knob but the amount of the reaction product was usually smaller than that of cyclic AMP hydrolysis (Fig. 37). The dendritic shaft and cell body usually did not show the enzymatic activity (Figs. 37 and 38), although very slight reaction product was occasionally observed in the dendritic shaft. The axon showed some enzymatic activity (Fig. 39). The reaction product was seen in basal cells similar to the case of cyclic AMP hydrolysis (Fig. 40).

In the tissue preincubated with snake venom and later incubated with 5'-AMP in the absence of the venom, deposition of the reaction product took place in the whole section (Fig. 41).

3.2. Taste cells

3.2.1. General structure

Four types of cells were observed in taste buds of rabbit foliate papillae (Figs. 42-45). Three types of cells, defined as taste cells in the present study, were slender in shape (3-7 μm in diameter at the thickest portion and 30-40 μm in length) extending from the base of the taste bud to the taste pore (Fig. 42). The most abundant type among the three corresponded to the commonly named dark cell and was characterized by its electron-dense cytoplasm, dense and irregular-shaped nucleus and dark granules seen at the upper portion in the cytoplasm (Figs. 42 and 43a). The apex of the dark cell was a neck (1-4 μm in length) capped by microvilli (approximately 0.1 μm in diameter and 2-3 μm in length) (Fig. 43a). Oftentimes the dark granules were also seen in the neck region. Occasionally, dark cells were observed whose apex was a blunt process tied to a few microvilli at the side, which was a mixed feature of the dark cell and the intermediate cell (Fig. 43b).

The next abundant type of cell was the commonly named light cell, which was characterized by its vesiculated, electron-lucent cytoplasm and round, less-dense nucleus (Fig. 42). No dark granules were observed in the cytoplasm. No neck region was seen at the apex and short microvilli (usually less than 1.5 μm) protruded directly from the trunk of cell body (Figs. 42 and 43a). Some light cells lacked microvilli or possessed only a few of them (Fig. 43a). Oftentimes the cytoplasm appeared to be degenerating, showing numerous vacuoles (Fig. 42).

The third type of cell, often termed the intermediate or type III

cell by researchers, was characteristic of abundant dark-cored vesicles around the nucleus (Figs. 42 and 44a). The cytoplasm of this cell showed density similar to that of the light cell or intermediate between those of the dark cell and the light cell; its nucleus was also similar to that of the light cell, i. e. round and less-dense in most cases but some cells possessed a dense and somewhat irregular-shaped nucleus. Its apex was a long, blunt process with no microvilli (Fig. 44b).

The taste cells were connected by tight junctions at the apical portions, immediately beneath the taste pore (Figs. 42 and 43a). In addition to the above three types of taste cells, taste buds included a few basal cells of an oblate shape at the base (Fig. 45). Numerous nerve processes were seen attached to taste cells (Fig. 42). Morphological differences between three taste cells were also recognized in unstained sections.

3.2.2. Adenylate cyclase activity

Either with AMP-PNP or GMP-PNP as substrate, fixation using 2% paraformaldehyde + 0.5% glutaraldehyde gave a better morphological preservation of the tissue than using 2% paraformaldehyde + 0.1% glutaraldehyde, while both fixing solutions gave a similar localization of the enzymatic activity (compare Fig. 49 and Fig. 57, for example). Similarly, though the reason is unknown, osmolarity adjustment of fixing and incubation solutions using NaCl gave a similar localization of the enzymatic activity but a better preservation of the tissue structure than using sucrose (data not shown). For these reasons and also for the

reason that sucrose might affect the enzymatic activities as a sweet tastant, the foliate papillae fixed with 2% paraformaldehyde + 0.5% glutaraldehyde were used for the incubation and the osmolarity of the solutions was adjusted using NaCl through fixation to incubation, unless otherwise indicated.

Granular deposit of the reaction product was observed in the apical portion (above the tight junctions) of three types of taste cells (Fig. 46). Most of the deposit appeared on the plasma membrane of the cells. However, all or most of this reaction product disappeared when 2 or 5 mM dithiothreitol was added to the incubation medium (Fig. 47) and it was not restored by 100 μ M forskolin (Fig. 48). Therefore, the reaction product was considered that of NTP pyrophosphohydrolase activity and not adenylate cyclase activity. No other portion of taste cells showed enzymatic activity either with or without dithiothreitol.

3.2.3. Guanylate cyclase activity

For the reasons mentioned in 3.2.2, the tissue fixed with 2% paraformaldehyde + 0.5% glutaraldehyde was used for the cytochemical incubation and the osmolarity of the solutions was adjusted with NaCl through fixation to incubation, unless otherwise indicated.

Electron-dense precipitate of the cytochemical reaction product appeared in the apical portion of all types of taste cells (Fig. 49a). The reaction product seemed more closely attached to the plasma membrane than in the case of AMP-PNP hydrolysis (compare Fig.49a,b with Fig. 46). The microvillous membrane of dark cells showed an especially large

amount of the reaction product. The reaction product appeared also in the neck region of these cells. The long blunt process of intermediate cells often showed much reaction product (Fig. 49a; also see Fig. 51). Only a small amount of the product appeared in the microvilli of light cells (Fig. 49a; also see Fig. 51). The regions below the tight junctions were devoid of the reaction product (Figs. 49a and 50). Dithiothreitol (2-10 mM) did not reduce the reaction product (Fig. 51). It sometimes even enhanced the product. Incubation without exogenous manganese or with increased concentration of $MnCl_2$ (up to 3 mM) did not affect the amount of the reaction product (Figs. 52 and 53).

Sodium azide, hydroxylamine and sodium nitroprusside (all examined at the concentration 1-10 mM) appeared to enhance the reaction product to some extent but their effects were not clear in many cases (Figs. 54-56). Difference in the effects among the three chemicals was not clear. The enhancing effects of these guanylate cyclase activators seemed a little more apparent in the tissue fixed with a milder fixative but still not very decisive (Figs. 57-60). Since a shorter period of incubation would result in a smaller amount of the reaction product and might make the effects of the above chemicals more distinct, an incubation for 10 min was carried out. However, this still did not clearly demonstrate the effects of the chemicals (Figs. 61-64).

Compared to the above guanylate cyclase activators, 1% Triton X-100 caused a drastic enhancement of the enzymatic activity (Fig. 65a). With Triton X-100, much reaction product appeared not only on the plasma membrane but also inside the membrane of apical portions of the cells (Fig. 65a). Unlike in olfactory cells, the detergent did not affect the localization of the reaction product in taste cells. Triton X-100

usually enhanced the reaction product but in one experiment out of four, it inhibited the enzymatic activity in some tissue sections (Fig. 65b).

Incubation with 1 mM CdCl_2 , ZnCl_2 or HgCl_2 all resulted in strong inhibition of the enzymatic activity (Figs. 66-68).

Since the sweet taste transduction is reported to possibly involve cyclic GMP (see Introduction) and rabbits taste sucrose (Contreras et al., 1985), the effect of sucrose was examined. With 1 M sucrose in the incubation medium, a moderate enhancement of the reaction product was seen once in a while, though not always obvious (Fig. 69).

Incubation without exogenous substrate resulted in total absence of the reaction product (Fig. 70).

3.2.4. Phosphodiesterase activity

With either cyclic AMP or cyclic GMP as substrate, the cytochemical reaction product of phosphodiesterase activity appeared in the apical portion of taste cells, although the amount of the product was much smaller than in the case of cyclic AMP hydrolysis in olfactory cells (Figs. 71 and 72). The reaction product was seen in the microvilli and neck of dark cells, the microvilli of light cells and the blunt process of intermediate cells, mostly on the plasma membrane. It was not decisive as to which type of cells possessed the strongest enzymatic activity. Most reaction product was observed above the tight junctions but some was seen a little below that level. The amount of the reaction product was similar either with cyclic AMP or cyclic GMP as substrate. The cell body region showed no reaction product (Figs. 73 and 74). The

reaction product was reduced by 5 mM 3-isobutyl-1-methylxanthine (Figs. 75 and 76) or 50 mM theophylline (Figs. 77 and 78). No reaction product appeared when the tissue was preheated at 70 °C (Figs. 79 and 80) or incubated in the absence of snake venom (Figs. 81 and 82). Incubation with no exogenous substrate also resulted in no reaction product (Fig. 83). In the tissue preincubated with snake venom and incubated with 5'-AMP in the absence of the venom, deposition of the reaction product was observed in the whole region of taste buds, although it was not always evenly scattered (Fig. 84). This reaction product was also observed in the region below taste buds (data not shown) but was not seen inside the squamous epithelial cells surrounding taste buds (Fig. 84).

4. DISCUSSION

4.1. Olfactory cells

4.1.1. General structure

Olfactory cells of rats showed a bipolar, flask-like shape with an unmyelinated axon and a long and narrow single dendrite which ended in a ciliated apical swelling, the dendritic knob. All of these features are those commonly described of olfactory cells of vertebrates (Graziadei, 1971). Olfactory cilia and most dendritic knobs protruded from the surface of the epithelium. This seemed to be well-adapted for the adsorption of odorous particles. In olfactory mucosae which were fixed with paraformaldehyde alone, cells were often found to be separated from each other. However, the above features of olfactory cells, in addition to the existence of numerous mitochondria in the dendritic shaft and a large, somewhat lobate nucleus, provided useful information in differentiating olfactory cells from supporting cells and basal cells.

4.1.2. Adenylate cyclase activity

In order to fix olfactory mucosae for adenylate cyclase cytochemistry in the present study, only paraformaldehyde could be employed despite its poor morphological preservation qualities, because in the preliminary experiment, fixation with a mixture of 2% paraformaldehyde and 0.1% glutaraldehyde for 1 h resulted in no reaction product except in the axons. The effect of glutaraldehyde on adenylate cyclase activity in the olfactory cells was not biochemically examined

but similar inhibition has been reported in other tissues (Ogawa et al., 1986, 1987).

A fine, electron-dense precipitate was observed inside the cilia and somewhat in the dendritic knob of the olfactory cells. Absence of the precipitate in the preheated tissue confirms that the precipitate is a genuine cytochemical reaction product of enzymatic hydrolysis of the substrate and not a result of nonspecific substrate splitting. The ineffectiveness of dithiothreitol suggests that the activity is not that of NTP pyrophosphohydrolase, another AMP-PNP-hydrolyzing enzyme (Johnson & Welden, 1977; Poeggel et al, 1981). Although adenylate cyclase is a membrane-associated enzyme (Gilman, 1984), a clear membranous localization of the reaction product was not obtained in the present study. This is probably because the membrane structures had been insufficiently preserved for keeping the reaction product in place. That the reaction product was found to be precipitated near the plasma membrane in some transversely-cut cilia may suggest that the product had diffused from the plasma membrane. Fujimoto et al. (1981) pointed out that prolonged postfixation using osmium tetroxide may result in diffusion of cytochemical reaction products. This is a less likely cause of the diffusion in the present study because the precipitate was always observed inside the cells, which is a different diffusion pattern from that observed by Fujimoto et al. (1981). Whether or not olfactory cells contain soluble adenylate cyclase as is reported in the exceptional case of the testis (Braun & Dods, 1975) is undetermined.

The enzymatic activity in both the cilia and the dendritic knob was enhanced by nonhydrolyzable GTP analogues and forskolin, and inhibited by calcium ion. These are the same properties as those of the

odorant-sensitive, G-protein-dependent adenylate cyclase found in olfactory cilia (Pace et al., 1985; Sklar et al., 1986) and in sonicated material from ethmoturbinates (Shirley et al., 1986, 1987). In view of this similarity in both property and localization in the cells, it is suggested that the enzymatic activity observed in the olfactory cilia and dendritic knob in the present study is of the same adenylate cyclase as in those biochemical reports. The observed gradient in the strength of enzymatic activity from the distal portion of the cilia down to the dendritic knob further indicates that the activity is that of the type III adenylate cyclase (Menco et al., 1992). By using an immunocytochemical technique, Menco et al. (1992) localized this olfactory neuron-specific adenylate cyclase (Bakalyar & Reed, 1990) in the cilia and dendritic knob of rat olfactory cells with the similar gradient in the enzyme density and agreed that the enzyme they observed must be the same adenylate cyclase as in our study (Asanuma & Nomura, 1991). If cyclic AMP is involved in olfactory transduction, it is expected that adenylate cyclase is located near the receptor sites of odorous stimuli. Hence, if the above adenylate cyclase plays a role in olfactory transduction, it can be inferred that the olfactory receptor sites are located not only in the ciliary membrane but also in the membrane of the dendritic knob to some extent. Furthermore, together with the localization of phosphodiesterase activity, which will be discussed later in 4.1.4, it is implied that the transduction events occur both in the olfactory cilia and the dendritic knob.

The role of the olfactory cilia in olfactory reception has long been a matter of controversy. Tucker (1967) and Kashiwayanagi et al. (1988) reported that olfactory responses remained after the cilia had

been removed from olfactory cells of box turtles and carps, respectively. However, the micrographs of Kashiwayanagi et al. (1988) show the remainder of short proximal portions of the cilia. Tucker (1967) mentioned that his preparation contained the basal bodies of the olfactory knob, where relatively high adenylate cyclase activity was observed in the present study. Hence, it is probable that the above researchers' preparations still contained the olfactory transduction sites in the remaining ciliary portions and the dendritic knob. Adenylate cyclase activity in the proximal portion of the cilia and the dendritic knob appeared to be lower than that in the distal portion of the cilia, but if one considers that adenylate cyclase coupled with a stimulatory G-protein is an excellent amplification system of a signal transduction (Gilman, 1987), it is very possible that the above 'deciliated' preparations produce enough cyclic AMP for olfactory transduction and respond to odorous stimulation. In contrast to Tucker (1967) and Kashiwayanagi et al. (1988), Adamek et al. (1984) observed disappearance of olfactory responses in deciliated frog olfactory cells, in which proximal portions of the cilia and the dendritic knobs were still left. However, they applied 0.05% or 0.025% Triton X-100 for 5 min for deciliation. This probably caused damage to the remaining receptor-adenylate cyclase system in the apical dendritic portion, since lower concentration of the detergent depressed the enzymatic activity in the present study. In fact, Tucker (1967) observed 50% reduction of olfactory responses after deciliation by applying 0.1% or less concentration of Triton X-100 for 1-3 min. Thus, if the hypothesis that an adenylate cyclase-cyclic AMP system in the olfactory cilia and the dendritic knob plays a role in olfactory transduction is accepted, the

present results concur well with all of the above apparently discrepant observations. Therefore, conversely, the present results support this hypothesis. The results do not only explain signal transducibility in deciliated cells but also urge one to speculate that similar adenylate cyclase-cyclic AMP system might be involved in the signal transduction in nonciliated olfactory cells such as seen in some primitive teleosts (Bannister, 1965).

The enzymatic activity was also observed in the olfactory dendritic shaft when the tissue was treated with Triton X-100. This activity was not inhibited by calcium ion in contrast to the enzymatic activity in the cilia and dendritic knob. That the reaction product was reduced by dithiothreitol might imply that this enzymatic activity is that of NTP pyrophosphohydrolase. However, the characteristic of NTP pyrophosphohydrolase as an ecto-enzyme, whose catalytic site is located on the external surface of cells (Evance, 1974; Abney et al., 1976; Mårdh & Vega, 1980; Howell et al., 1984; Ryan et al., 1984, 1985; Caswell & Russell, 1985, 1988) contradicts the present observation that the reaction product was exclusively seen inside the cells. In addition, NTP pyrophosphohydrolase activity in fat cells is reported to be inhibited by low concentrations of Triton X-100 (Mårdh & Vega, 1980). Furthermore, forskolin seemed to facilitate a rare appearance of the reaction product in this region in the tissue untreated with the detergent. Those results suggest that the enzymatic activity found in the dendritic shaft is also an adenylate cyclase activity. Different responses to calcium ion and the detergent imply that it is a different type of adenylate cyclase from that in the cilia and dendritic knob. Absence of the type III adenylate cyclase in the dendritic shaft (Menco

et al, 1992) also supports this idea. It is possible that the adenylate cyclase in the dendritic shaft is usually in an inhibited state and structural changes in the membrane caused by the detergent treatment reduced this inhibited state (Perkins & Moore, 1971; Salesse & Garnier, 1984). The role of the enzyme in the dendritic shaft is yet undetermined.

Adenylate cyclase activity was also seen in the olfactory axon. The properties of the enzyme in this region have not been examined in detail but since the enzymatic activity was enhanced by GMP-PNP, the enzyme may be coupled with the adenylate cyclase-stimulating G-protein found in the same region (Jones & Reed, 1989). It is probably the same adenylate cyclase as that found by Anholt et al. (1989) in homogenates of the olfactory nerve. Anholt et al. (1989) reported that the axonal enzyme was stimulated by GTP γ S but was insensitive to odorants, thus it seems to be a different type from the ciliary enzyme. Cyclic AMP may act in the axons as a modulator in the transfer of odor information to the brain as mentioned by Anholt et al. (1989).

4.1.3. Guanylate cyclase activity

Until recently, odorants were reported to neither increase nor decrease cyclic GMP in olfactory cilia in vertebrates (Steinlen et al., 1990; Breer & Boekhoff, 1991). However, the recent study by Breer et al. (1992) has shown that relatively high concentrations of odorants elicit a small but significant increase of cyclic GMP in olfactory cilia of rats. Thus, there is a possibility that in addition to cyclic AMP,

cyclic GMP may play a role in olfactory signalling in vertebrates as in insects (Ziegelberger et al., 1990; Zufall & Hatt, 1991; Stengl et al., 1992). This elevation of the cyclic GMP concentration is considered to be due to soluble guanylate cyclase (Breer et al., 1992; Breer & Shepherd, 1993).

In the present study, guanylate cyclase activity was detected only in the axons. This may be because soluble guanylate cyclase was lost from the cells in the experimental process. Also, guanylate cyclase activity is low in rat olfactory cilia (more than ten times lower than adenylate cyclase activity in terms of the amount of cyclic nucleotides produced) (Steinlen et al., 1990; Breer et al., 1992). However, slight electron-dense precipitate was occasionally observed in the olfactory cilia and dendritic knob in the present study. This may be a reaction product due to the soluble enzyme, some of which may have remained inside the cells or due to a membrane-bound guanylate cyclase, if it exists in olfactory dendritic terminals. Since part of soluble guanylate cyclase may be left inside cells under the present experimental condition as will be discussed in 4.2.3, further improvement of the method may enable us to demonstrate the enzymatic activity in olfactory cells.

4.1.4. Phosphodiesterase activity

In the present study, snake venom was employed as a source of 5'-nucleotidase to hydrolyze 5'-AMP (or 5'-GMP) which was produced from cyclic AMP (or cyclic GMP) by the action of phosphodiesterase. Although

the use of snake venom is known to result in poor morphological preservation of tissues (Sugimura & Mizutani, 1978; Ueno & Ueck, 1988) probably due to proteinase in the venom (Ueno & Ueck, 1988), it was shown in the preliminary experiment that both snake venom and partially purified 5'-nucleotidase (purchased from Sigma Chemical Co.) gave a similar morphology of the tissue and a similar localization of the phosphodiesterase activity. In addition, snake venom is reported to stimulate phosphodiesterase activity (Cheung, 1967, 1969; Hidaka et al., 1975). Therefore, snake venom was used as a source of the exogenous 5'-nucleotidase. Ueno & Ueck (1988) pointed out the difficulty in uniform penetration of a large molecule of 5'-nucleotidase into tissues. However, a penetration test indicated that the enzyme from the snake venom penetrated the entire tissue section and hydrolyzed the exogenously-given 5'-AMP all over.

Judging from the amount of the reaction product, strong cyclic AMP-hydrolyzing phosphodiesterase activity was observed on the plasma membrane of the olfactory cilia and dendritic knob, and weaker activity on the membrane of the dendritic shaft and cell body. For control of the cyclic nucleotide-mediated olfactory signals, phosphodiesterase is expected to exist near the sites where cyclic nucleotides act. Therefore, the above finding implies, together with the present observation of adenylate cyclase activity in the cilia and somewhat in the dendritic knob, that cyclic AMP is produced in the dendritic terminal (mainly in the cilia) and acts predominantly in its original site; some of it, however, may also reach and act upon the membrane of the dendritic shaft and cell body. This idea is consistent with the electrophysiological studies which indicate that the cyclic

nucleotide-gated channels of olfactory cells exist densely in the membrane of the cilia but also at a low density in the membranes of the dendrite and the cell body (Firestein et al., 1991b; Kurahashi & Kaneko, 1991). Firestein et al. (1991b) mentioned that the latency between the odorous stimulation and the current recording from the dendrite (150 ms - 1.5 s in their recordings from salamanders) appears to be a reasonable time for cyclic AMP to diffuse from the cilia to the dendrite.

As for the dendritic knob region, Frings et al. (1992) found high density of cyclic nucleotide-gated channels in the membrane of the dendritic knob in rats and frogs. This suggests that a rather large amount of cyclic AMP, either produced in the knob itself or diffused from the cilia, may work in this region. The present finding that strong phosphodiesterase activity was observed in the dendritic knob is also supportive of this possibility. Furthermore, if cyclic AMP works exclusively in the cilia, the results of the deciliation experiments by Tucker (1967) and Kashiwayanagi et al. (1988) (see 4.1.2) would not be explained. Frings et al. (1992) mentioned that the distal part of the dendritic knob may form a continuum with the cilia, with essentially the same membrane proteins. In contrast to Frings et al. (1992), Firestein et al. (1991b) reported that the density of cyclic nucleotide-gated channels was low in the dendrite, including the dendritic knob, of salamander olfactory cells. The cause of this discrepancy is unknown but it may be due to a difference in animal species used. Kurahashi & Kaneko (1991) did not clearly describe whether or not the olfactory dendrites they examined in newts and toads included the knob region. Phosphodiesterase in the dendritic shaft may play some unknown role by balancing the Triton X-100-stimulated adenylate cyclase observed in the

same region, in addition to the hydrolysis of cyclic AMP diffused from the dendritic terminal.

Compared to cyclic AMP, cyclic GMP was less hydrolyzed. Although cyclic AMP and cyclic GMP similarly activate cyclic nucleotide-gated channels in olfactory cells (Nakamura & Gold, 1987; Suzuki, 1989; Dhallan et al., 1990; Kurahashi, 1990; Firestein et al., 1991a; Frings et al., 1992), Firestein et al. (1991a) found that only the current induced by cyclic AMP was desensitized, i. e. decayed with time, in their whole-cell patch clamp experiments using salamander olfactory cells. If this desensitization is caused by degradation of cyclic nucleotides, the above difference in the extent of hydrolysis between the two cyclic nucleotides concurs with their finding. Since guanylate cyclase activity in vertebrate olfactory cilia seems to be low (Steinlen et al., 1990; Breer et al., 1992; also indicated in the present study) and either odorant-insensitive (Steinlen et al., 1990) or requires high concentration of odorants for activation (Breer et al., 1992, compare with adenylate cyclase activation in Boekhoff & Breer, 1992), the primary transduction in olfaction may be mediated by cyclic AMP but not by cyclic GMP despite their similar effects on the cyclic nucleotide-gated channels. Phosphodiesterase should play a role in this transduction by modulating intracellular concentration of cyclic AMP. Involvement of phosphodiesterase in olfactory signalling, including termination of the signals, is further indicated by the finding by Firestein et al. (1991a) that the odor-induced electric current was prolonged by 3-isobutyl-1-methylxanthine, a membrane-permeable phosphodiesterase inhibitor. Rapid decay of the cyclic AMP concentration in olfactory cilia after odorous stimulation was also

blocked by 3-isobutyl-1-methylxanthine and an elevated cyclic AMP level was maintained for a long period of time (Boekhoff & Breer, 1992).

In contrast to the whole-cell patch preparations (Firestein et al., 1991a), no cyclic AMP-induced currents were desensitized in excised patch preparations (Nakamura & Gold, 1987; Dhallan et al., 1990; Frings et al., 1992). Since Dhallan et al. (1990) used plasma membranes excised from human embryonic kidney cells transfected with the rat-derived olfactory channel clone, no phosphodiesterase system in normal olfactory cells should exist in their preparation. Nakamura & Gold (1987) and Frings et al. (1992) used membrane patches excised from real olfactory cells. Hence, it may be possible that their preparations contained at least membrane-bound phosphodiesterase. Assuming that those excised patches contain intact phosphodiesterase, there must be some factor missing to activate this enzyme. Calmodulin seems to be a promising candidate for such a factor. Borisy et al. (1992) found high calcium/calmodulin-dependent phosphodiesterase activity in olfactory cells of rats. In contrast to calcium/calmodulin-dependent phosphodiesterase in many other tissues, which shows low affinity for cyclic AMP (Beavo, 1988), this enzyme possesses high affinity for cyclic AMP (Borisy et al., 1992). Its activity was predominant in the membrane fraction from the olfactory mucosa (Borisy et al., 1992). Furthermore, immunohistochemical staining localized this enzyme in the cilia, dendritic knob, axon and to some degree in the cell body of olfactory cells (Borisy et al., 1992). Considering its properties and localization, this enzyme is very likely to be the same enzyme as that observed in the present study. Influx of external calcium ions into olfactory cells is known to decay the membrane conductance increased by

odorous stimulation (Kurahashi & Shibuya, 1990; Kurahashi et al., 1990). This decay of olfactory response may be mediated, at least in part, by the above calcium/calmodulin-dependent phosphodiesterase.

The axon of olfactory cells showed strong phosphodiesterase activity. This activity probably balances the adenylate cyclase activity observed in the same region, the role of which is still undetermined (refer to 4.1.2). The phosphodiesterase observed in this region may also be the same calmodulin-dependent enzyme found by Borisy et al. (1992) for the same reason described above.

Interestingly, the cytochemical reaction product was also observed on the plasma membrane of basal cells. The reaction product in these cells was observed even when the tissue was incubated without snake venom. The enzymatic activity was not inhibited by levamisole, hence the possibility of it being alkaline phosphatase activity (Nomura, 1978) was refuted. Since 5'-nucleotidase activity was observed on the plasma membrane of the same cells (unpublished data), the above reaction product is considered to be a result of phosphodiesterase activity demonstrated with the help of intrinsic 5'-nucleotidase. The basal cells are considered to be the stem cells which differentiate into olfactory cells (Graziadei, 1971, 1973; Snyder et al., 1988; Costanzo, 1991). Therefore, it may not be surprising that a common enzymatic activity is observed in basal and olfactory cells.

4.2. Taste cells

4.2.1. General structure

In the taste bud of rabbit foliate papillae, four types of cells were identified: basal, dark, intermediate and light cells. The latter three types, which are defined as taste cells in the present study, all possessed a slender shape and extended from the base to the apex (the taste pore) of the taste bud. Although it can hardly be expected to obtain a whole picture of such a long, slender cell, the density and contents of the cytoplasm as well as the shape and density of the nucleus usually provided useful information to differentiate cell types. In particular, the morphological uniqueness in the apical region of each cell type, i. e. long microvilli connected to a neck in dark cells, short microvilli in light cells and a long blunt process in intermediate cells, was a prominent marker in distinguishing one cell type from another.

Tight junctions were found to connect taste cells at the level beneath the taste pore, thus separating the apical portion from the trunk of cells morphologically and functionally. Since the apical portion of taste cells is directly exposed to the oral cavity and the penetration of taste substances into the taste bud seems to be restricted by these tight junctions, the first interaction of taste stimuli is most likely to occur on the apical membrane of taste cells. In some species such as dogs, tight junctions in taste buds may be somewhat permeable to small molecules (Simon & Verbrugge, 1990; Holland et al., 1991). However, even such somewhat 'loose' junctions should not

allow large molecules, e. g. monellin and thaumatin, which are sweet tasting proteins (Morris & Cagan, 1972; Van der Wel & Loeve, 1972) to permeate into intercellular spaces.

There has been considerable controversy as to whether different types of cells in taste buds originate from separate cell lines or if they only represent different stages in the lifespan of a single population of taste cells (see Roper, 1989, for review). This problem is also concerned with the question of whether only a certain cell type is a taste receptor or if all three types are receptor cells. Early studies by Murray and his coworkers on taste buds of rabbit foliate and circumvallate papillae suggest that three types differentiate independently from basal cells and represent different cell lines: 1) In regenerating taste buds after severing the glossopharyngeal nerve, basal cells appeared first and then the other three types of cells appeared simultaneously (Fujimoto & Murray, 1970; Murray & Murray, 1971). 2) Intravenously injected ^3H -thymidine first appeared in basal cells and then in the other three types (Murray & Murray, 1971). 3) Type III cells, or intermediate cells, alone were found to make typical synaptic contacts with nerve endings, which they thought indicated that only this type was a primary receptor cell (Fujimoto & Murray, 1970; Murray & Murray, 1971; Murray, 1973). Recent studies by Kinnamon and his coworkers on taste buds of mouse foliate and circumvallate papillae using a high voltage electron microscope have provided totally different results: 1) Cells in taste buds displayed a continuous range of morphologies from typical dark cells to typical light cells (Royer & Kinnamon, 1988). 2) Peak of injected ^3H -thymidine appeared in taste bud cells in the order: basal, dark, intermediate and light cells (Delay et

al., 1986). 3) Three types of cells all formed synaptic connections with sensory nerve fibers (Kinnamon, J. C. et al., 1985, 1988; Royer & Kinnamon, 1988). Thus, they suggest that all taste bud cells are in a single cell line which undergoes morphological changes in its lifespan and that the three types of cells are all receptor cells. The cause of the above discrepancy between the two groups is yet unknown. Difference in animal species used is not likely to be the cause of this discrepancy because the rabbit and mouse species are too close to show a complete difference in such a primary biological process as differentiation of taste cells. Therefore, the cause of the discrepancy is most likely due to the difference in experimental conditions employed, including the method of data analysis. In addition to a conventional transmission electron microscope, Kinnamon's group used a high voltage electron microscope and utilized computer-assisted three-dimensional reconstructions of the taste bud from serial sections (Kinnamon, J. C. et al., 1985, 1988; Royer & Kinnamon, 1988). This seems to have sufficiently improved the visualization of synaptic contacts between taste cells and nerve fibers. To categorize cell types, Kinnamon's group adopted a double-blind analysis and at least three of the four researchers had to agree independently on the classification of each cell (Delay et al., 1986). This should have produced a fair classification of taste cells. Otherwise, especially in regenerating taste buds as in Fujimoto & Murray (1970) and Murray & Murray (1971), it seems difficult to interpret precisely which cell corresponds to a certain cell type. Furthermore, Kinnamon's group was cautious in focusing on cells from the first division after incorporation of the ³H-thymidine by judging from the grain density in nuclei of the labeled

cells (Delay et al., 1986). Murray & Murray (1971) admitted that after the labeling of basal cells with ^3H -thymidine, most of the labeled cells were dark cells, although some labeled light and intermediate cells were seen. This could be interpreted as either ^3H -thymidine appearing simultaneously in three types of cells or that it appeared mainly in dark cells after appearing in basal cells. Thus, although it is not yet conclusive, the single-cell-line hypothesis seems more probable for the present. A few observations obtained in the present study also seem to support this hypothesis: 1) Cells with an apex of a mixed feature of the dark cell and the intermediate cell were seen. 2) Light cells often showed degenerating aspect, suggesting that they were on the final stage of the lifespan. Early study by Farbman (1965) on morphogenesis of rat taste buds, which showed that in developing taste buds, basal cells and dark cells appeared earlier than light cells and that some cells appeared to be intermediate cells between basal and dark cells, also seems to favor the single-cell-line hypothesis. Therefore, it seems highly possible that the three types of cells are all taste receptor cells. Even Murray (1973), who proposed the separate-cell-line hypothesis, mentioned that extensive contact of nerves with the dark and light cells might imply an existence of different transduction pathways through these cells rather than only through intermediate cells. The finding of electrical coupling between taste cells (Akisaka & Oda, 1978; Yang & Roper, 1987) further broadens the possibility of more than one cell type playing a role in sensory transduction, although it is not yet ascertained which types of cells are electrically coupled.

4.2.2. Adenylate cyclase activity

Since Kurihara & Koyama (1972) found high adenylate cyclase activity in bovine fungiform and circumvallate papillae, several lines of evidence have appeared which suggest an involvement of cyclic AMP in vertebrate taste transduction: 1) Taste substances enhanced adenylate cyclase activity in lingual membranes of various vertebrates, which contained taste cells (Kalinowski et al., 1987, 1990; Lancet et al., 1987; Striem et al., 1989; Naim et al., 1991). 2) A cholera toxin substrate corresponding to the adenylate cyclase-stimulating G-protein was identified in the taste plasma membrane of catfish (Bruch & Kalinoski, 1987). 3) High cyclic AMP-hydrolyzing phosphodiesterase activity was found in homogenates from bovine taste papillae (Law & Henkin, 1982). 4) Injection of cyclic AMP into taste cells of frogs and mice caused a reversible depolarization in these cells (Avenet & Lindemann, 1987; Avenet et al., 1988; Tonosaki & Funakoshi, 1988).

Unfortunately, adenylate cyclase activity was not detected in taste cells in the present cytochemical study. An enzymatic activity which hydrolyzed the exogenous substrate, AMP-PNP, was undeniably observed in the apical region of taste cells. The inhibitory effect of dithiothreitol on this enzymatic activity and ineffectiveness of forskolin, however, suggested that it was not adenylate cyclase but NTP pyrophosphohydrolase activity (Johnson & Welden, 1977; Poeggel et al., 1981).

A few cytochemical reports on adenylate cyclase activity in taste cells have already appeared (Yamamoto & Ozawa, 1977; Asanuma & Nomura, 1982; Nomura & Asanuma, 1982). All of these studies, however, employed

lead, the most commonly-used capture metal, to capture released inorganic pyrophosphate. A great disadvantage of using lead in adenylate cyclase (and also guanylate cyclase) cytochemistry is that this metal forms a chelate compound with dithiothreitol (Toei & Yamamoto, 1966) and does not permit the use of this NTP pyrophosphohydrolase inhibitor. Among heavy metals which are not chelated by dithiothreitol, cerium itself inhibited the AMP-PNP-hydrolyzing enzymatic activity in taste cells, while barium and strontium did not affect the enzymatic activity and could be employed as a capture agent (Asanuma & Nomura, 1986). The inhibitory effect of dithiothreitol was also demonstrated by the barium method (Asanuma & Nomura, 1986). It is not yet proven that the enzymatic activity previously demonstrated by the lead method was caused by the same enzyme as that shown by the strontium or barium method. However, the fact that the activity was inhibited by NaF (Asanuma & Nomura, 1986) strongly implies that it is also NTP pyrophosphohydrolase activity because NaF is reported to activate adenylate cyclase and inhibit NTP pyrophosphohydrolase (House et al., 1972).

Although adenylate cyclase activity was not demonstrated in the present study, it does not necessarily mean that there is no adenylate cyclase activity in taste cells. The studies cited in the first paragraph indicate a strong possibility of cyclic AMP, as well as cyclic GMP (see 4.2.3), working in taste cells. The use of glutaraldehyde, which had to be employed for the morphological preservation of the microvilli of taste cells, may have caused the failure to detect the enzyme (see 2.2.1.1 and 4.1.2). Further reformation in the tissue fixation will be needed to demonstrate adenylate cyclase activity in

taste cells.

Apart from adenylate cyclase activity, the unique localization of NTP pyrophosphohydrolase activity in taste cells was an interesting finding. Although this enzyme has been described since long past (Hitchings & Fuller, 1939; Zeller, 1950) and is seen in various tissues (Torp-Pedersen et al., 1979), its role still seems ambiguous. Salvage of purines which escape from damaged cells as suggested by Tran-Thi et al. (1981) and Ryan et al. (1985) may be one of the possible roles of this ecto-enzyme in taste buds, since taste cells have only a short lifespan (Beidler & Smallman, 1965).

4.2.3. Guanylate cyclase activity

The enzymatic activity which hydrolyzed GMP-PNP was localized in the apical portion of taste cells. Unlike the AMP-PNP-hydrolyzing enzymatic activity, this activity was not inhibited by dithiothreitol, suggesting that it is not NTP pyrophosphohydrolase activity (Johnson & Welden, 1977). Furthermore, dithiothreitol sometimes even stimulated the enzymatic activity. This positively suggests that it is guanylate cyclase activity (Hardman & Sutherland, 1969). Inhibition by cadmium, zinc and mercury ions and stimulation by Triton X-100 are also congruent with the reported nature of guanylate cyclase (Hardman & Sutherland, 1969; Kimura & Murad, 1974, 1975; Kimura et al., 1975a; Durham, 1976).

Sodium azide, hydroxylamine and sodium nitroprusside appeared to enhance the GMP-PNP-hydrolyzing enzymatic activity once in a while, which is supportive of the enzyme being guanylate cyclase, but their

effects were not as great as expected. This may be because the tissue fixation made the enzyme less flexible and reduced the responsiveness to the enzyme activators, since the effects of the chemicals seemed a little more apparent in the tissue fixed using 0.1% glutaraldehyde than using 0.5% glutaraldehyde. Another possibility is that activating factors for sodium azide (Mittal et al., 1975) and hydroxylamine (Deguchi, 1977) may have been lost from the tissue during the process of fixation and rinse. In the case of sodium nitroprusside, the fixed tissue may have not been fit for the formation from the chemical of nitric oxide, an actual activator of guanylate cyclase (Katsuki et al., 1977; Murad et al., 1979).

In contrast to the above chemicals, Triton X-100 caused a prominent enhancement of the enzymatic activity. This implies that the detergent activated the enzyme in a different manner from that of the above guanylate cyclase activators. Neer & Sukiennik (1975) reported that Lubrol PX, a non-ionic detergent like Triton X-100, activated guanylate cyclase by changing its conformation. Triton X-100 once showed an inhibitory effect in the present study. This may indicate that Triton X-100 also affected the enzymatic activity by changing the enzyme conformation rather than by acting on the catalytic site, since it seems possible that some variation in the conformational change leads the enzyme into both an activated state and an inhibited state. In the tissue incubated with Triton X-100, much reaction product was seen both on the plasma membrane and inside the cytoplasm. This may suggest that some soluble guanylate cyclase remained inside the cells even after the fixing and rinsing process because Triton X-100 stimulates both membrane-bound and soluble guanylate cyclases (Kimura & Murad, 1975;

Kimura et al., 1975a; Durham, 1976).

The enzymatic activity was not influenced by the concentration of added $MnCl_2$; it was even observed in the absence of exogenous manganese. This was a surprise since both membrane-bound and soluble guanylate cyclases are known to require manganese ion as a cofactor of the enzyme activation (Kimura & Murad, 1974). The reason why exogenous manganese was unnecessary is unknown but fixing the tissue might have tightly bound endogenous manganese ion to the enzyme, thus making the enzyme a metalloenzyme, which no longer requires excess manganese ion for catalytic activity (Chrisman et al., 1975).

Compared to the case of cyclic AMP (see 4.2.2), evidence which indicates an involvement of cyclic GMP in vertebrate taste transduction is less readily available: 1) Sucrose taste increased the cyclic GMP concentration in taste papillae of mice (Uebayashi & Tonosaki, 1994). 2) Cyclic GMP enhanced the glossopharyngeal nerve responses to several taste stimuli (Nagahama et al., 1982). 3) Injection of cyclic GMP, as well as cyclic AMP, into taste cells of mice induced membrane depolarization in a similar manner as sucrose stimulus to those cells (Tonosaki & Funakoshi, 1988). The present observation that guanylate cyclase activity is localized in the apical portion of taste cells, i. e. above the tight junctions, is a new finding which further supports the idea of cyclic GMP involvement in taste transduction because the apical portion is a most likely site of interaction between taste stimuli and taste cells (see 4.2.1).

Much of the reaction product of guanylate cyclase activity seemed to closely attach to the apical membrane of the cells. This may indicate that the enzyme demonstrated was mostly membrane-bound

guanylate cyclase, at least in the absence of Triton X-100. That form of the enzyme might interact more efficiently with the taste receptor site than soluble enzyme, although a mediator between the receptor and the enzyme is not yet identified. There is also a possibility that guanylate cyclase itself is a taste receptor. Chinkers et al. (1989) suggested from their study of complementary DNA clone encoding guanylate cyclase that the membrane-bound guanylate cyclase that is activated by atrial natriuretic peptide may be a cell-surface receptor itself, which contains an extracellular ligand-binding domain and an intracellular catalytic domain. If guanylate cyclase acts as a cell-surface receptor of a certain taste modality, the extracellular domain is expected to show some variation from cell to cell to discriminate the taste, since the enzymatic activity was observed in all taste cells. Such a possibility is seen in peptide-activated guanylate cyclase whose extracellular domains show diversity as receptors interacting with different peptides, while its intracellular catalytic domains are highly conserved (Schulz et al., 1989).

Sucrose appeared to enhance the reaction product once in a while. This concurs with the idea that cyclic GMP may be involved in sweet taste transduction (Tonosaki & Funakoshi, 1988; Uebayashi & Tonosaki, 1994). Unfortunately, however, such enzyme-stimulating effect of sucrose was not large and not always obvious in the present study. This may be because the experimental process, especially tissue fixation, caused some damage to the enzyme or to a presumed mediator between the receptor and the catalytic site of the enzyme.

The enzymatic activity was usually most prominent in the microvilli of dark cells, followed by the blunt process of intermediate cells. The

microvilli of light cells showed only slight activity. The finding that a common enzymatic activity was seen in the apical portion of three types of cells is supportive of the single-cell-line hypothesis of taste cells (see 4.2.1). Furthermore, the above difference in the strength of the enzymatic activity agrees with the idea that taste cells change from dark cells, via intermediate cells, to light cells (Delay et al., 1986).

4.2.4. Phosphodiesterase activity

Similarly as in the cytochemistry in olfactory cells, snake venom was employed as a source of 5'-nucleotidase to hydrolyze 5'-AMP or 5'-GMP produced from cyclic nucleotides. The penetration test of the snake venom showed that 5'-nucleotidase penetrated into the cells within taste buds but not into squamous epithelial cells surrounding taste buds. This penetration was not always constant even in taste buds but seemed sufficient at least to help localize phosphodiesterase activity in taste cells.

Phosphodiesterase activity was observed in all types of taste cells, in good agreement with the finding that guanylate cyclase activity was seen in all taste cells. That phosphodiesterase activity was localized on the apical membrane of taste cells is consistent with findings in previous reports (Pevzner & Tikhonova, 1980; Asanuma & Nomura, 1982). Since there was not a difference in the extent of the hydrolysis between cyclic AMP and cyclic GMP, the phosphodiesterase in taste cells may possess a different characteristic from that in olfactory cells, which showed a preference for cyclic AMP. The amount

of the reaction product in taste cells was much less compared to that in olfactory cells. It is unknown whether this is due to a difference in the strength of the enzymatic activity between the two cells or due to a difference in the resistance of the enzyme to the experimental procedure.

It is not well understood how cyclic nucleotides work in taste cells as a second messenger. For example, an existence of cyclic nucleotide-gated channels such as those identified in olfactory cells is not reported. However, Avenet et al. (1988) and Tonosaki & Funakoshi (1988) found that cyclic AMP and cyclic GMP close potassium channels of taste cells in frogs and mice. Interestingly, potassium channels in taste cells, at least in mudpuppies, are usually open (Kinnamon & Roper, 1987) and are predominantly located on the apical membrane (Kinnamon, S. C. et al., 1988; McBride & Roper, 1988). If such a localization of potassium channels is seen in vertebrates in general, the mechanism for taste transduction involving cyclic nucleotides may be hypothesized as follows: Taste stimuli activate guanylate (or adenylate) cyclase in taste cells in some way and increase cyclic GMP (or cyclic AMP). Increased cyclic nucleotides somehow close potassium channels on the apical membrane and depolarize the cells. This will open voltage-dependent calcium channels, which are identified in taste cells (Avenet & Lindemann, 1987; Kinnamon & Roper, 1987, 1988b; McBride & Roper, 1988; Sugimoto & Teeter, 1990). Influx of calcium ions will then cause transmitter release at synapses (Avenet & Lindemann, 1989; Roper, 1989). The present finding that both guanylate cyclase and phosphodiesterase activities were observed exclusively on the apical portion of taste cells concurs with this hypothesis. In particular,

such localization of phosphodiesterase activity implies that cyclic nucleotides produced in the apical portion work in this original region without diffusing into the cell body. This corresponds nicely with the localization of potassium channels in taste cells, on which cyclic nucleotides are thought to, directly or indirectly, act.

5. CONCLUSIONS

In order to know whether or not, and how cyclic AMP or cyclic GMP works as a second messenger in olfactory and taste transductions, the localization of adenylate cyclase, guanylate cyclase and cyclic 3',5'-nucleotide phosphodiesterase activities was examined cytochemically in rat olfactory cells and rabbit taste cells. From this study, the following conclusions were obtained:

- 1) Three types of cells were observed in the olfactory epithelium of rats: olfactory cells (olfactory neurons), supporting cells and basal cells. The cilia and dendritic knob of olfactory cells protruded from the surface of the epithelium, which appeared to be fit for the adsorption of odor particles to those regions.
- 2) Adenylate cyclase activity was observed in the cilia and axon and to some extent in the dendritic knob of olfactory cells. In the dendritic knob, the enzymatic activity was mainly observed at the basal bodies from which the cilia originated.
- 3) Adenylate cyclase activity in the olfactory cilia and dendritic knob was enhanced by forskolin and nonhydrolyzable GTP analogues and inhibited by calcium ion. These are the same properties as those of the previously-reported, odorant-sensitive, G-protein-dependent adenylate cyclase in olfactory cilia; hence the enzymatic activity observed in the olfactory dendritic terminal in the present study is likely to be due to the same adenylate cyclase as in those previous biochemical reports.
- 4) From the localization of adenylate cyclase activity, it is implied that cyclic AMP works as a second messenger in olfactory transduction and that olfactory receptor sites are located not only in the olfactory cilia but also in the olfactory dendritic knob to some extent. This explains the other researchers' findings that olfactory responses were

obtained in deciliated olfactory cells.

5) An enzymatic activity which seemed to be due to a different type of adenylate cyclase from that in the olfactory cilia and dendritic knob was observed in the olfactory dendritic shaft. This enzymatic activity was not inhibited by calcium ion and enhanced by a low concentration of Triton X-100. The role of this enzyme is yet unknown.

6) Guanylate cyclase activity was not detected in olfactory cells except in the axonal region.

7) Strong activity of cyclic AMP-hydrolyzing phosphodiesterase was observed in the cilia, dendritic knob and axon of olfactory cells. Weaker activity was observed in the dendritic shaft and cell body. Cytochemical reaction product was seen mostly on the plasma membrane. From the localization of phosphodiesterase activity, it is implied that cyclic AMP produced in the dendritic terminal (mainly in the cilia) of olfactory cells acts predominantly in its original site but some of it may diffuse and act upon the membrane of the dendritic shaft and cell body.

8) Cyclic GMP-hydrolyzing activity of the phosphodiesterase in olfactory cells was observed in the cilia, dendritic knob and axon but the activity was lower than cyclic AMP-hydrolyzing activity. This may explain the finding by Firestein et al. (1991a) that cyclic AMP-induced current in olfactory cells decayed with time, while cyclic GMP-induced current did not show such a desensitization.

9) Phosphodiesterase activity was also observed in basal cells in the olfactory epithelium, which are considered to be the stem cells differentiating into olfactory cells.

10) In the taste bud of rabbit foliate papillae, four types of cells

were observed: basal, dark, intermediate and light cells. The latter three types of cells, defined as taste cells in the present study, extended from the base to the apex (the taste pore) of the taste bud. Tight junctions connected taste cells at the apical portion, immediately beneath the taste pore, and seemed to restrict the penetration of taste substances into the taste bud. In view of its direct exposure to the oral cavity and its being separated by tight junctions from the lower portion, the apical membrane of taste cells is most likely to be the site of the first interaction with taste stimuli.

11) Occasionally, taste cells possessing an apex of a mixed feature of dark cells and intermediate cells were observed. Light cells often showed a degenerating aspect. These observations are supportive of the hypothesis that taste cells change from dark cells, via intermediate cells, to light cells.

12) Adenylate cyclase activity was not detected in taste cells. It is yet undetermined, however, whether taste cells lack adenylate cyclase or the experimental procedure caused damage to the enzyme.

13) Nucleoside triphosphate pyrophosphohydrolase activity was localized in the apical portion of taste cells, although its role is unknown.

14) Guanylate cyclase activity was localized in the apical portion of three types of taste cells. The microvillous membrane of dark cells showed an especially strong activity. Microvilli of light cells showed only slight enzymatic activity. The above localization of guanylate cyclase activity implies that cyclic GMP serves as a second messenger in taste transduction.

15) Sucrose sometimes appeared to enhance the guanylate cyclase activity in taste cells. This is supportive of the hypothesis that cyclic GMP is

involved in sweet taste transduction.

16) Phosphodiesterase activity was localized in the apical portion of three types of taste cells. This implies that cyclic GMP produced in the apical portion of taste cells acts in its original site without diffusing into the cell body.

17) Phosphodiesterase in taste cells hydrolyzed cyclic AMP and cyclic GMP to a similar extent.

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FIGURES

Fig. 1. Longitudinal section of an olfactory cell of the rat. Olfactory cell (O) is a bipolar neuron with an axon (ax) projecting toward the basal lamina and a long single dendrite (sh = dendritic shaft) toward the epithelial surface. Dendritic knob (kn), a swollen terminal of the dendrite was provided with a variable number of cilia (ci). S = supporting cell, B = basal cell, mv = microvilli of the supporting cell. x8,000.

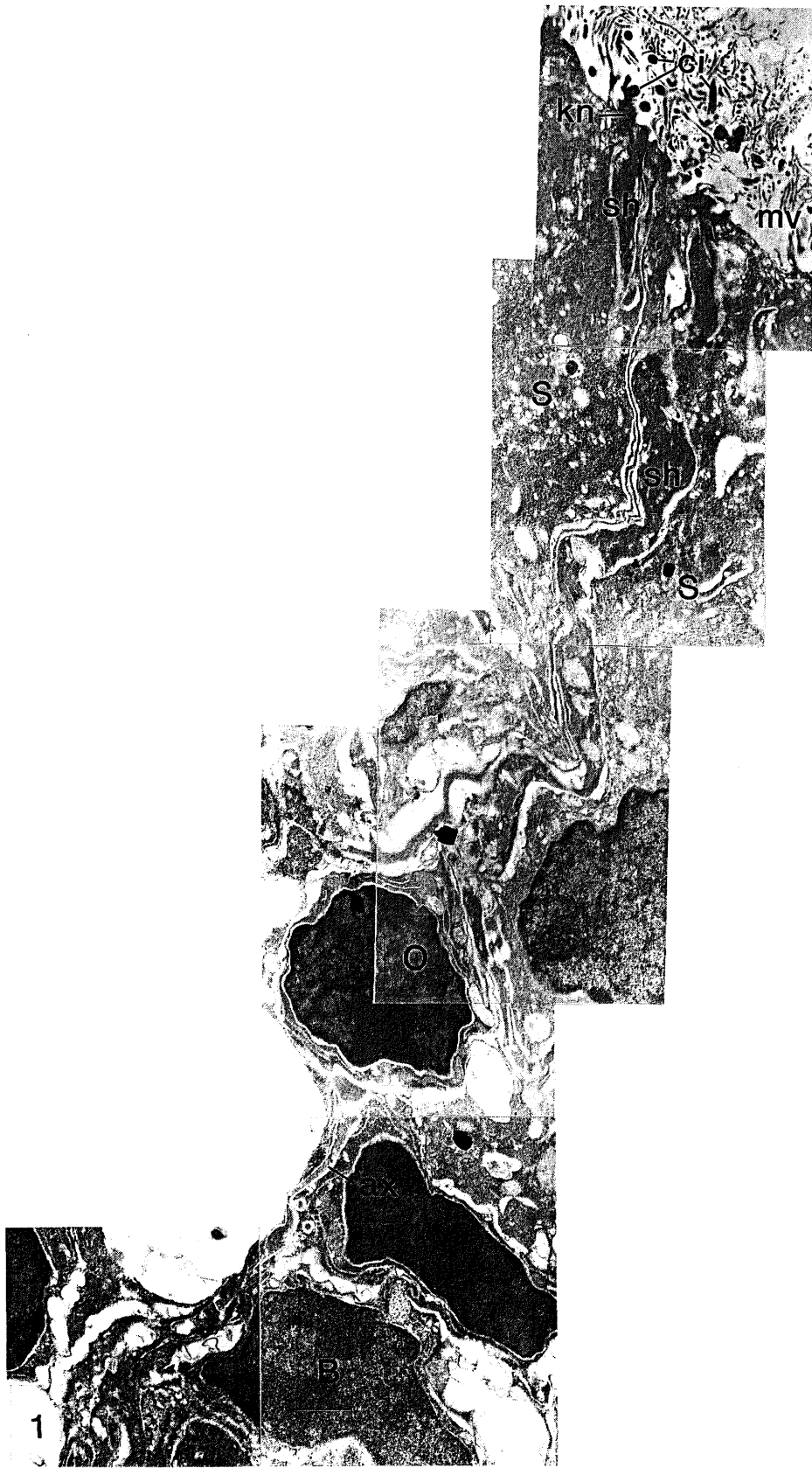


Fig. 2. Dendrite of the olfactory cell consisted of the dendritic shaft (sh) and the dendritic knob (kn) with cilia (ci). Note that the knob protrudes from the epithelial surface. mi = mitochondria, S = supporting cell, mv = microvilli of the supporting cell. x18,000.

Fig. 3. Longitudinal section of an olfactory cell body. A large, lobate nucleus (nO) was prominent. Compare with a round nucleus of the supporting cell (nS). x13,000.

Fig. 4. Transverse section of olfactory axon bundles. Unmyelinated axons (ax) were enwrapped by the Schwann cell (Sch) cytoplasm (arrowheads). x15,000.

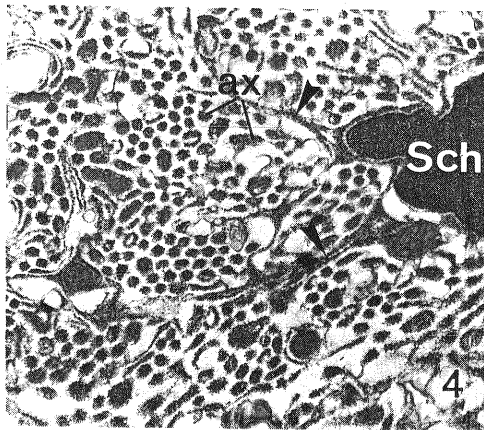
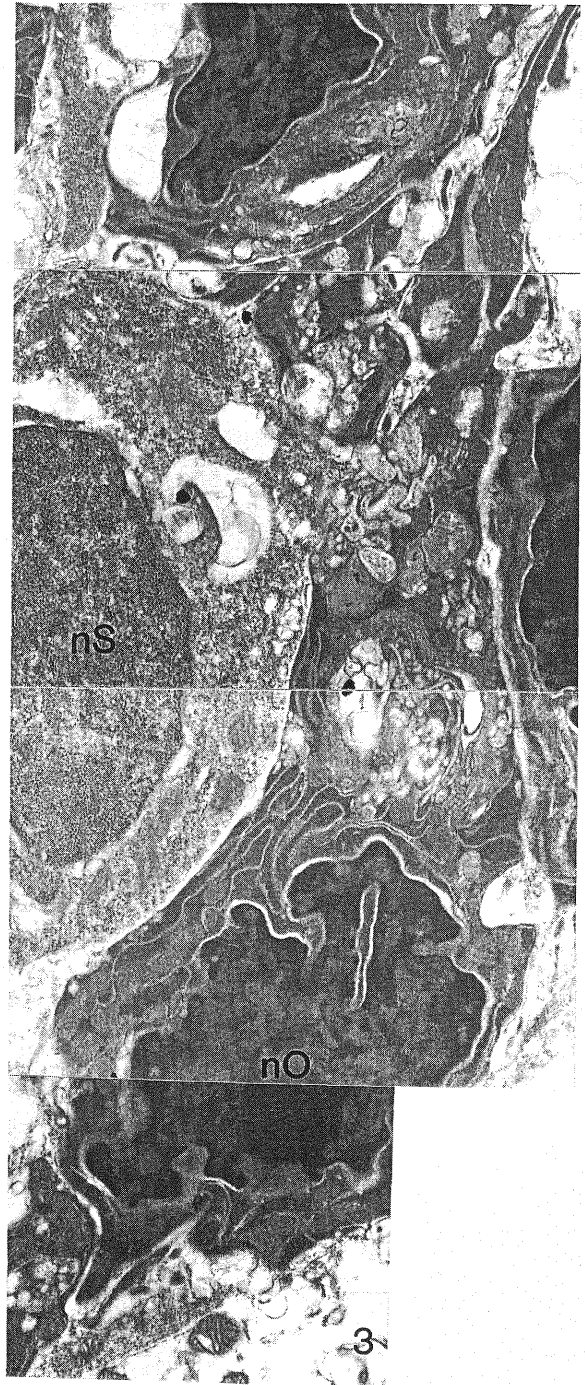
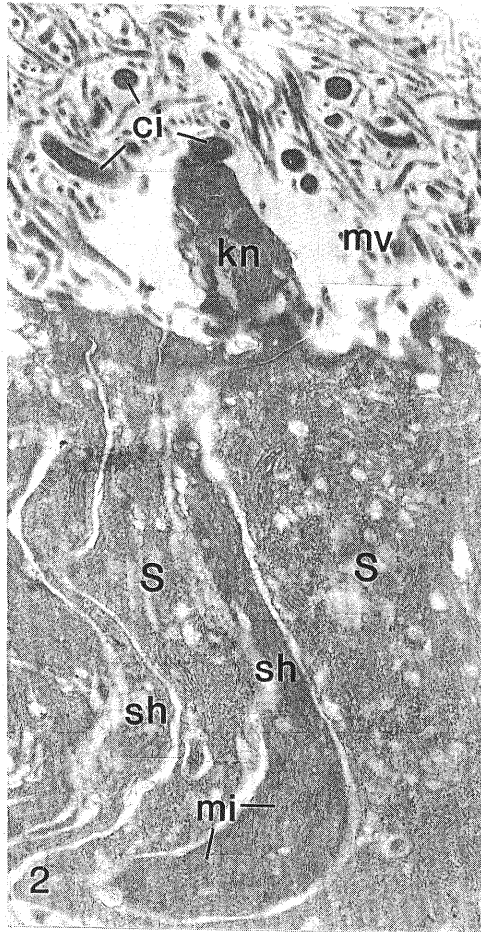
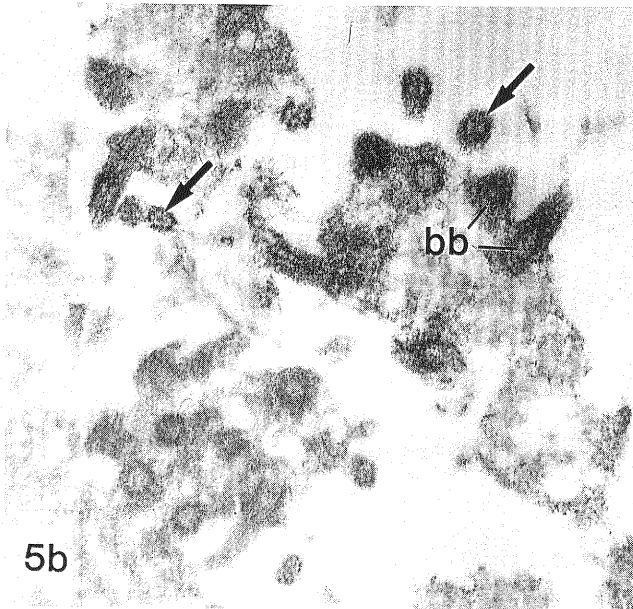
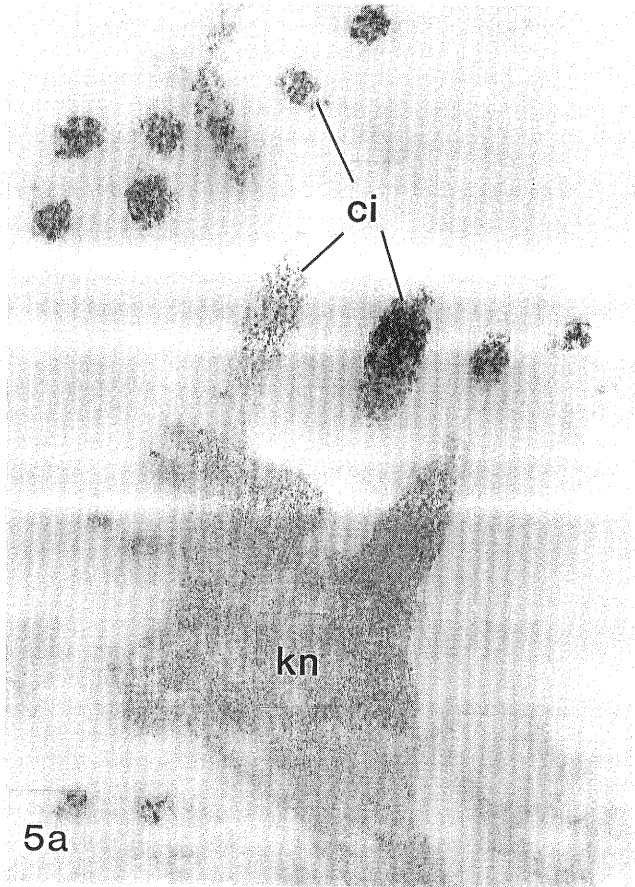


Fig. 5. Adenylate cyclase activity in the apical dendritic portion of the rat olfactory cell. (a) Cytochemical reaction product was seen as a fine electron-dense precipitate in the olfactory cilia (ci) and in smaller amounts in the dendritic knob (kn). A larger amount of the reaction product was seen in the distal portion of the cilia than in the proximal portion. x40,000. (b) In some transversely-cut cilia, the reaction product precipitated near the plasma membrane (arrows), which implies that the product originally formed at the plasma membrane. In dendritic knobs, the reaction product was dense at the basal bodies (bb) from which the cilia originated. The amount of the reaction product varied from cell to cell. In the cilia and dendritic knob at the bottom of the micrograph, the reaction product is scarcely seen. x25,000.

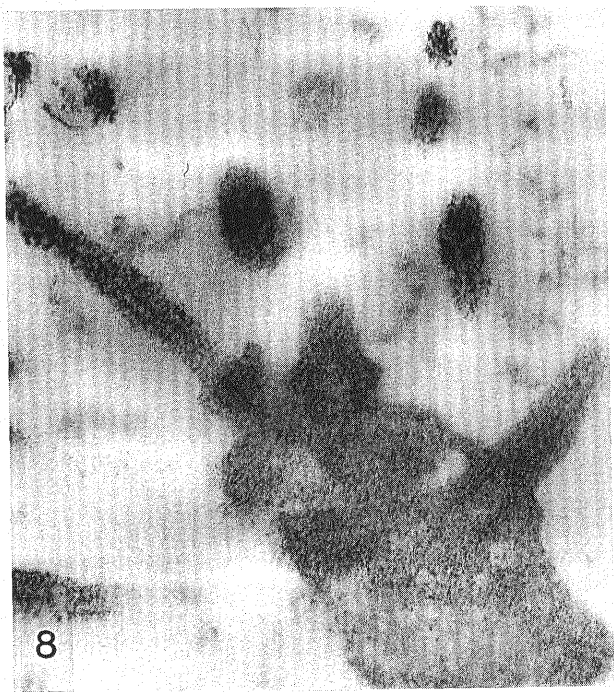
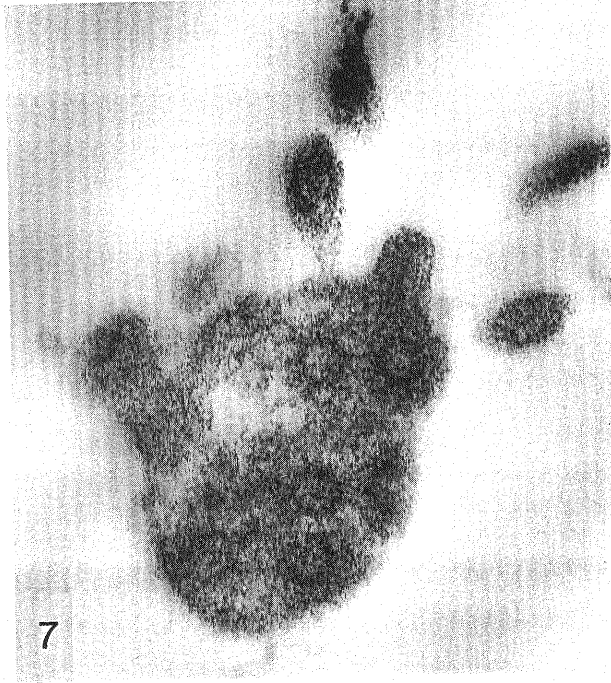
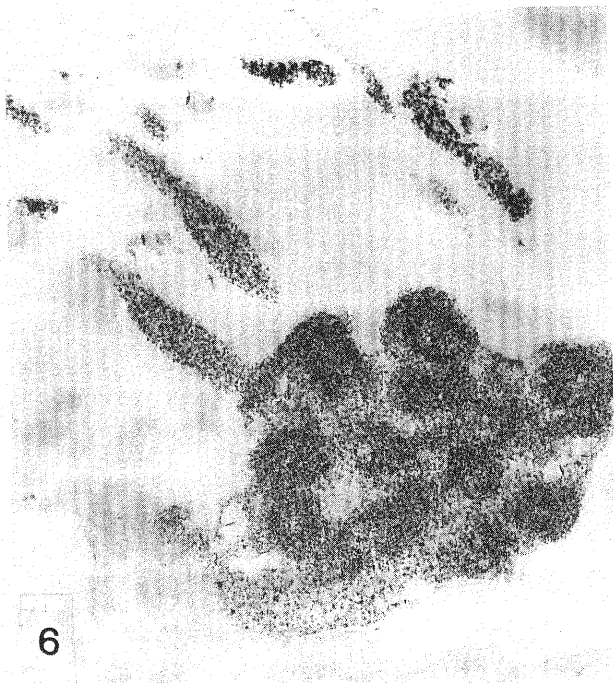


Figs. 6-8. Adenylate cyclase activity in the cilia and the dendritic knob of rat olfactory cells. Addition of forskolin or nonhydrolyzable GTP analogues resulted in a great increase of both the number of cells showing the enzymatic activity and the amount of the reaction product in each cell.

Fig. 6. The activity was enhanced by 10 μM forskolin. x36,000.

Fig. 7. The activity was enhanced by 30 μM GMP-PNP. x36,000.

Fig. 8. The activity was enhanced by 30 μM $\text{GTP}\gamma\text{S}$. x36,000.

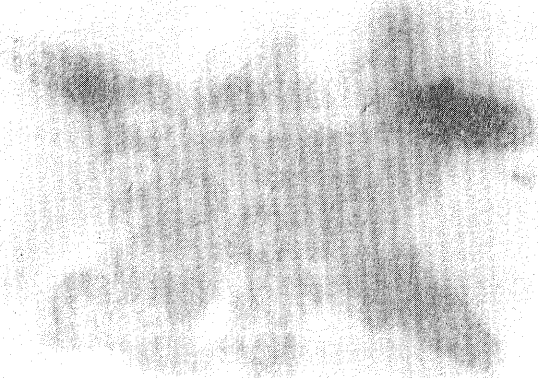


Figs. 9 and 10. Adenylate cyclase activity in the olfactory cilia and the dendritic knob.

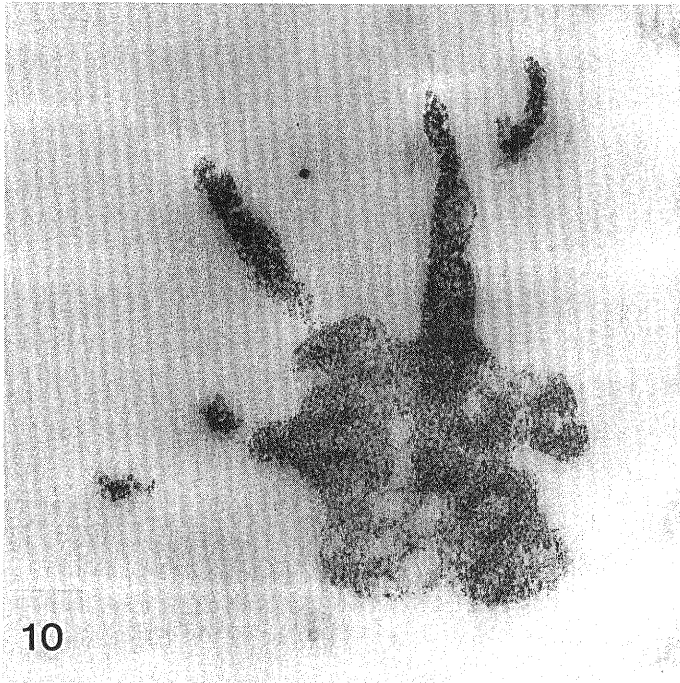
Fig. 9. The activity was inhibited by 10 mM CaCl_2 . Samples were incubated in the presence of 30 μM $\text{GTP}\gamma\text{S}$. x36,000.

Fig. 10. The activity was not inhibited by 5 mM dithiothreitol, which suggests that the activity was not caused by NTP pyrophosphohydrolase. Samples were incubated in the presence of 30 μM $\text{GTP}\gamma\text{S}$. x36,000.

9



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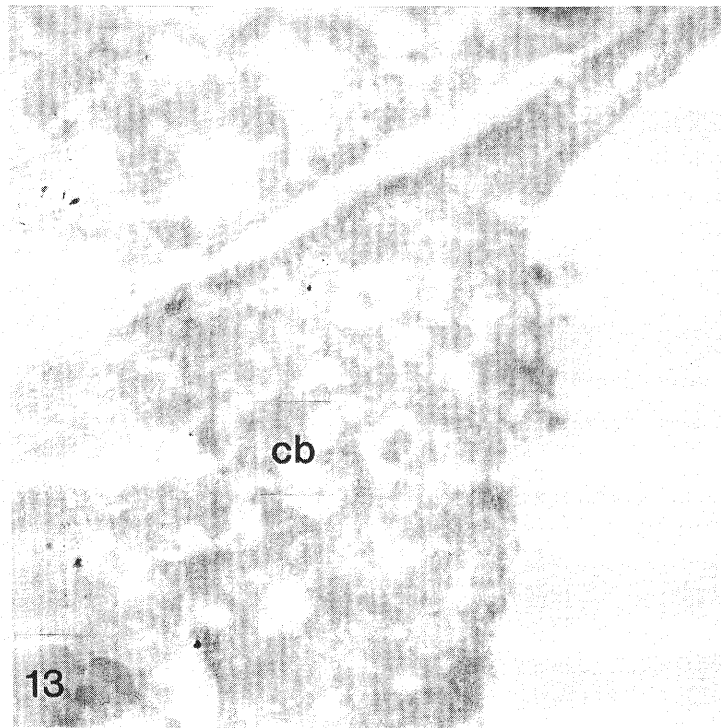
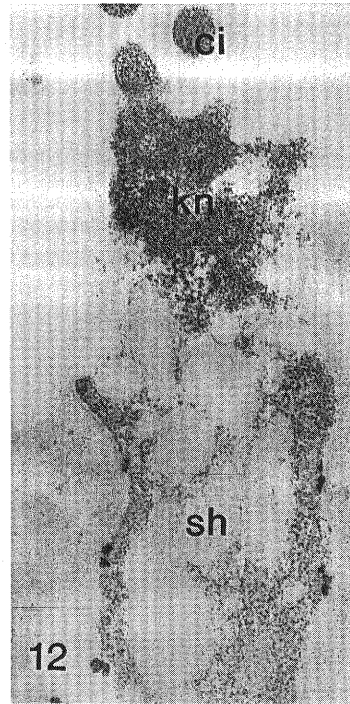
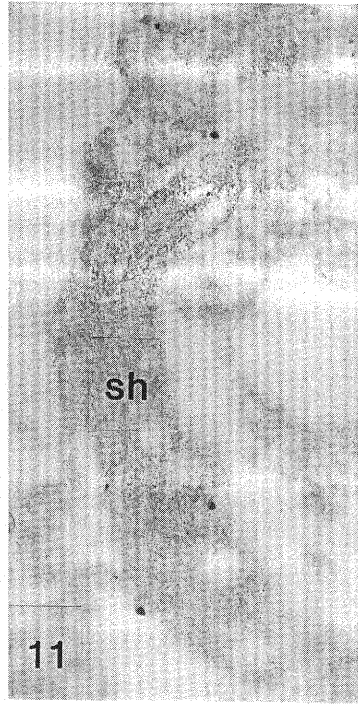


Figs. 11-13. Adenylate cyclase activity in the dendritic shaft and the cell body of olfactory cells.

Fig. 11. The activity was not usually seen in the dendritic shaft (sh).
x30,000.

Fig. 12. Samples were incubated with 10 μ M forskolin. Very occasionally, a rather large amount of the cytochemical reaction product appeared in the dendritic shaft (sh); forskolin seemed to facilitate the appearance of this reaction product. ci = olfactory cilia, kn = dendritic knob. x30,000.

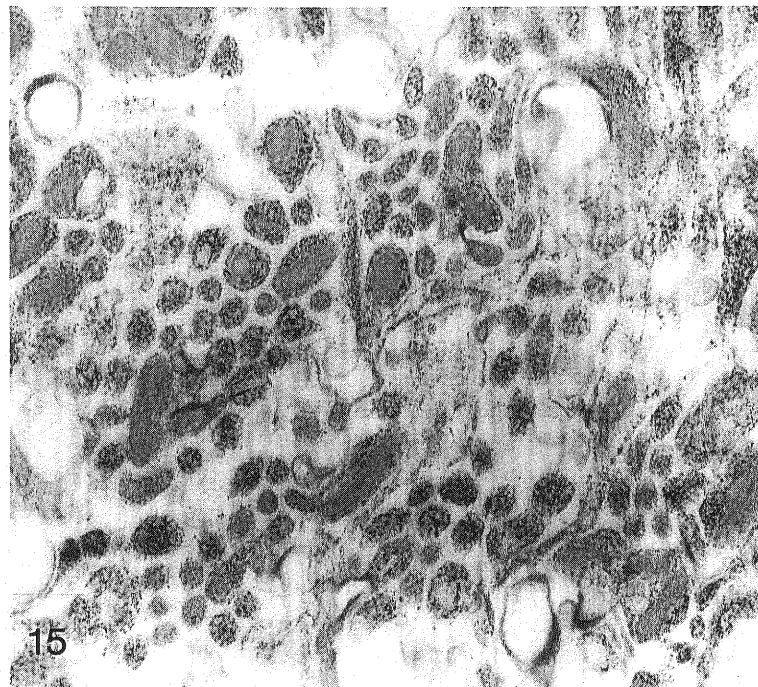
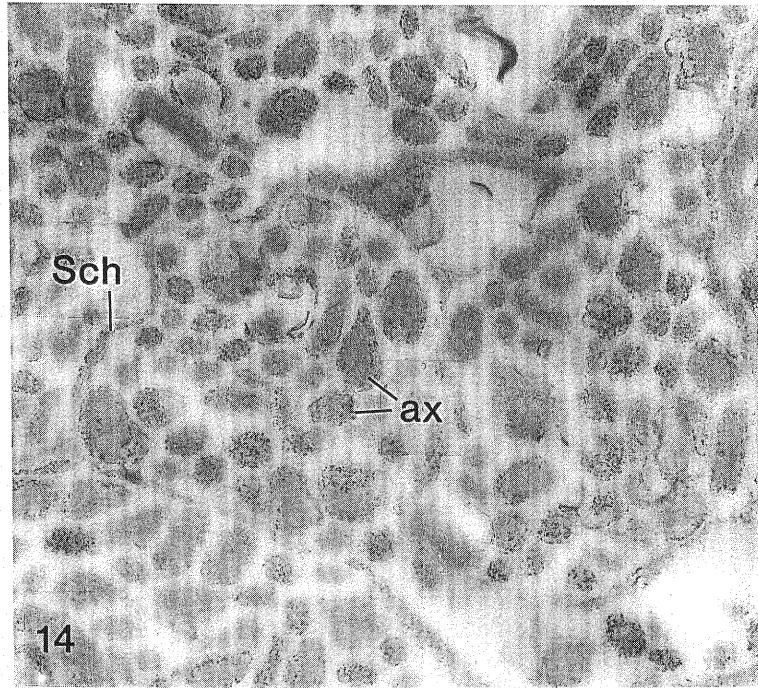
Fig. 13. No adenylate cyclase activity was observed in the cell body (cb). The incubation medium contained 30 μ M GTP γ S and 5 mM dithiothreitol. x20,000.



Figs. 14 and 15. Adenylate cyclase activity in the olfactory axon bundles (transverse section) fixed with 2% paraformaldehyde + 0.1% glutaraldehyde for 1 h.

Fig. 14. The activity was seen in the axons (ax) and the Schwann cell profiles (Sch). x36,000.

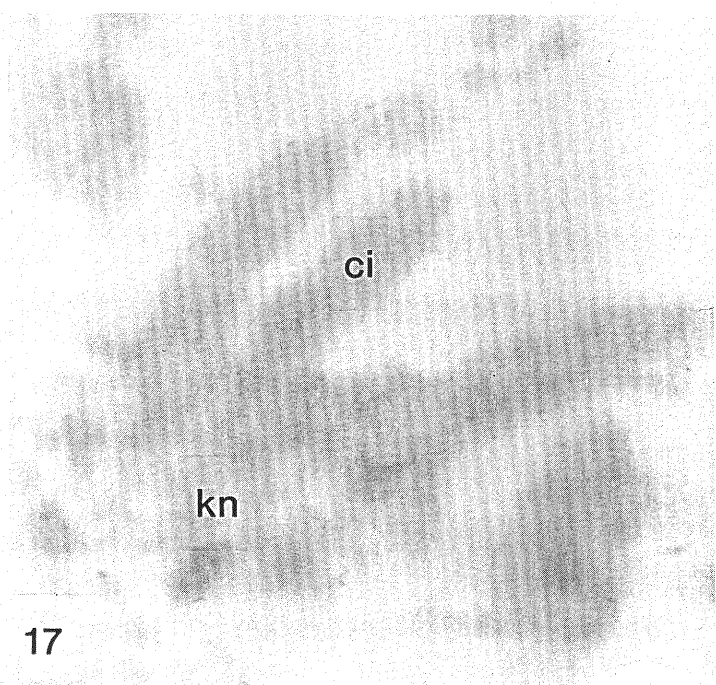
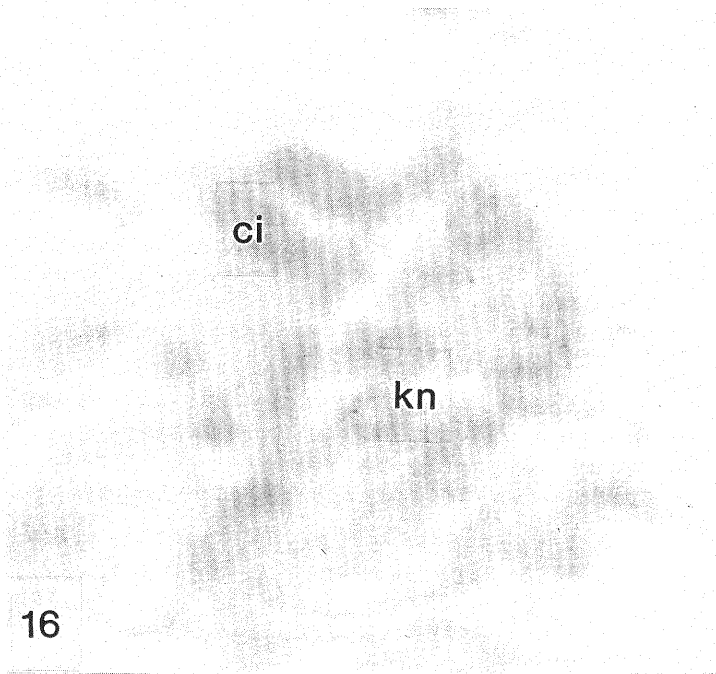
Fig. 15. The activity was enhanced by 30 μ M GMP-PNP. x36,000.



Figs. 16 and 17. Adenylate cyclase activity in the olfactory cilia (ci) and the dendritic knob (kn).

Fig. 16. Samples were incubated without exogenous substrate. No reaction product was seen. x36,000.

Fig. 17. Samples were preheated for 15 min at 80 °C before preincubation. No reaction product appeared. x36,000.



Figs. 18-20. An enzymatic activity, probably caused by adenylate cyclase, was observed in the olfactory dendritic shaft by treating the tissue with 0.0002% Triton X-100. Triton X-100 was added to the fixing, rinsing, preincubation and incubation solutions.

Fig. 18. Samples were incubated in the standard incubation medium with Triton X-100. The enzymatic activity in the dendritic shaft is considered to be caused by a different type of adenylate cyclase from that found in the dendritic terminal (see Discussion). The reaction product was absent in the mitochondria (mi). x30,000.

Fig. 19. The activity was not inhibited by 10 mM CaCl_2 . x30,000.

Fig. 20. The activity was inhibited by 5 mM dithiothreitol. sh = dendritic shaft. x30,000.

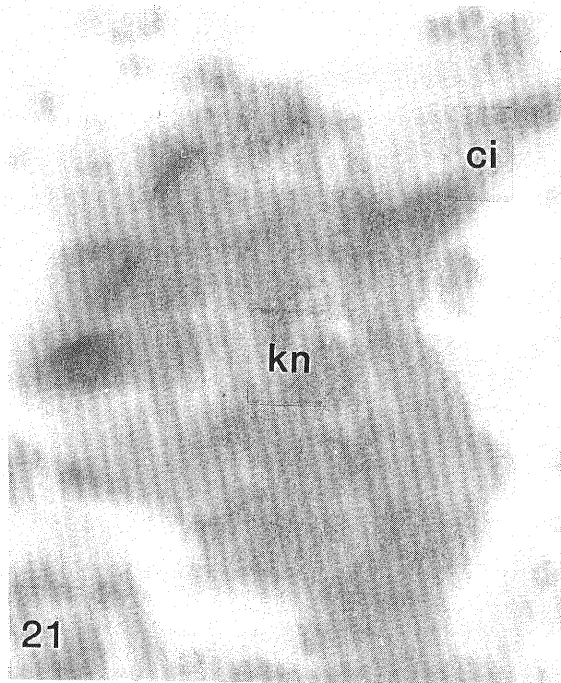


Figs. 21-23. Adenylate cyclase activity in the olfactory cilia and the dendritic knob treated with 0.0002% Triton X-100. The detergent was added to the fixing, rinsing, preincubation and incubation solutions.

Fig. 21. The activity was inhibited by Triton X-100. ci = olfactory cilia, kn = dendritic knob. x36,000.

Fig. 22. The activity was restored by 10 μ M forskolin. x36,000.

Fig. 23. The activity was restored by 30 μ M GMP-PNP. x36,000.

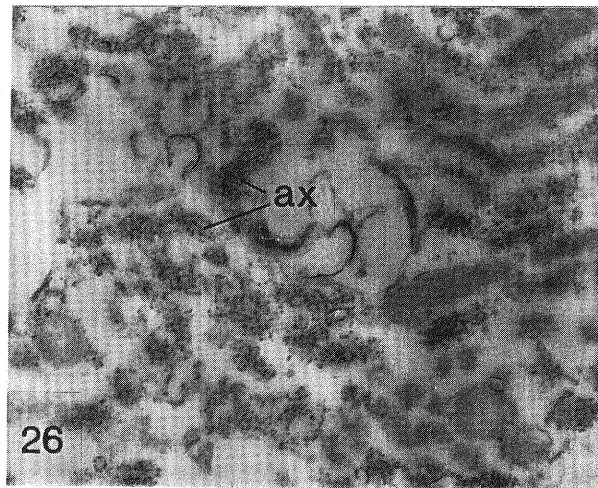
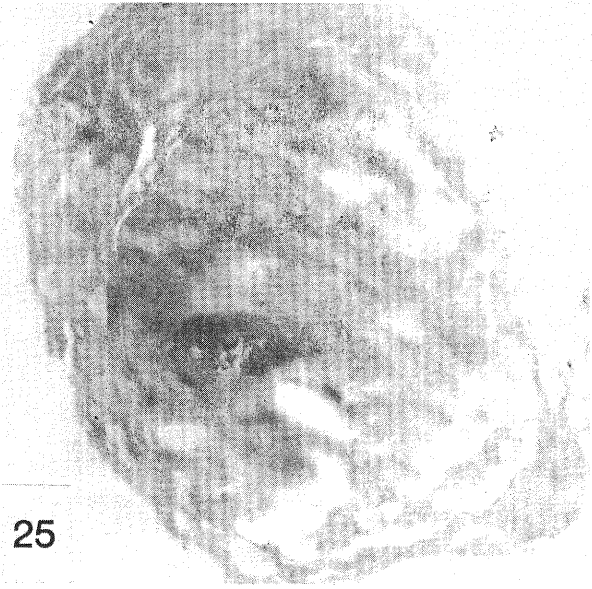
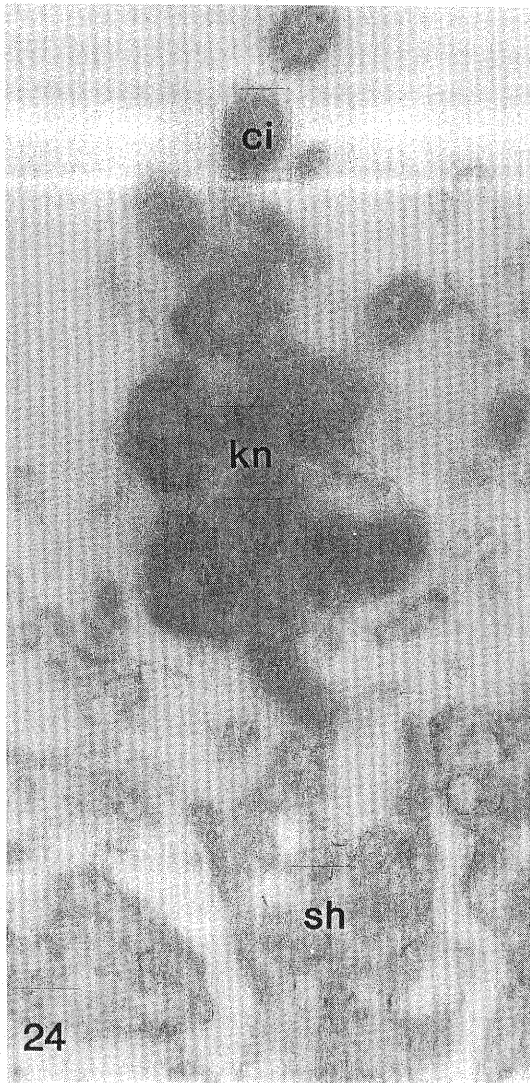


Figs. 24-26. Guanylate cyclase activity in olfactory cells of rats.

Fig. 24. The activity was not seen in the cilia (ci), dendritic knob (kn) and dendritic shaft (sh). x40,000.

Fig. 25. The activity was not seen in the cell body. x15,000.

Fig. 26. Some activity was seen in the axons (ax). x30,000.

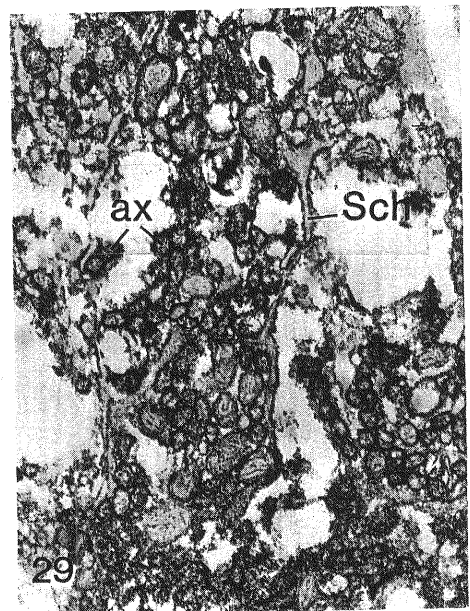
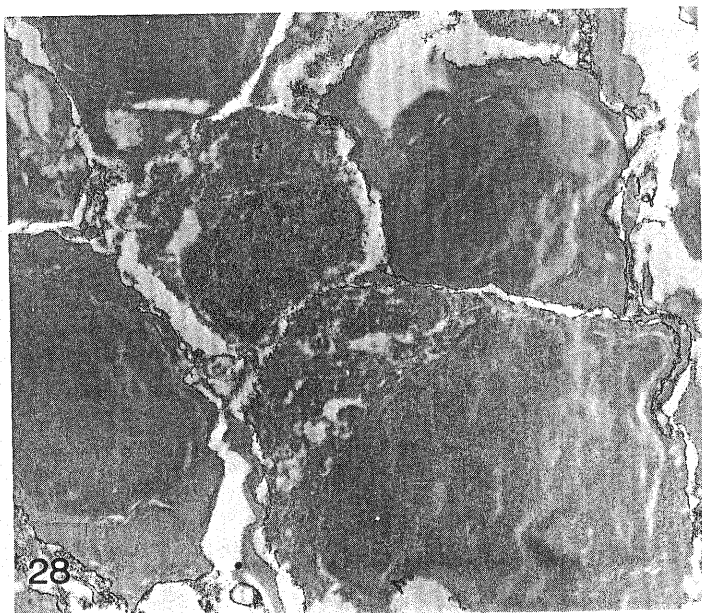
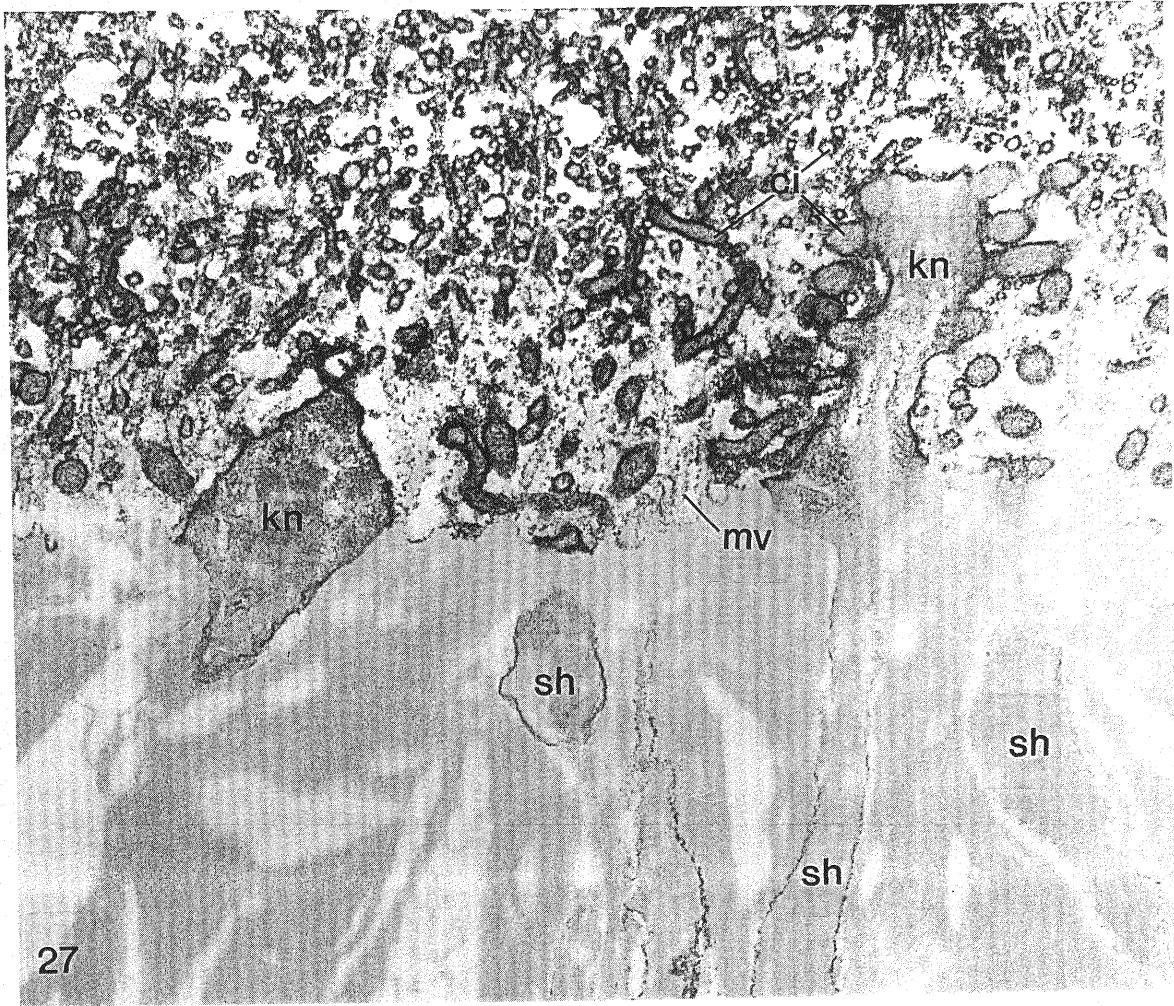


Figs. 27-29. Cyclic AMP phosphodiesterase activity in rat olfactory cells.

Fig. 27. A large amount of the reaction product was seen in the cilia (ci) and the dendritic knob (kn) mainly on the plasma membrane. A smaller amount of the reaction product was seen on the membrane of the olfactory dendritic shaft (sh) and of the microvilli (mv) of supporting cells. x20,000.

Fig. 28. The enzymatic activity was also seen, though not always, on the plasma membrane of olfactory cell bodies. x9,000.

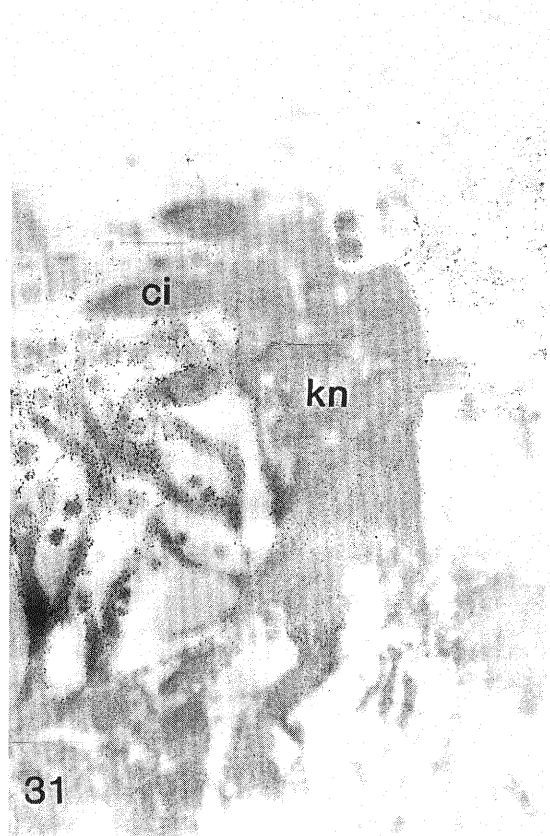
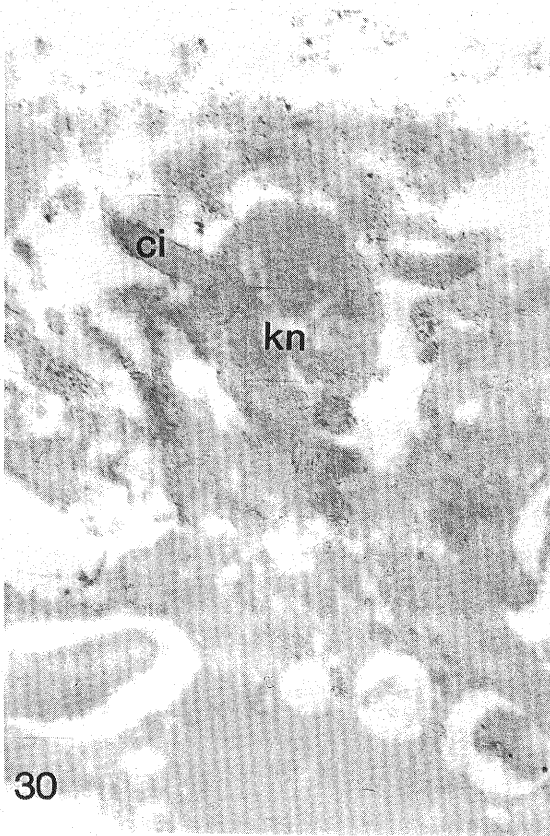
Fig. 29. Strong enzymatic activity was seen in the olfactory axons (ax) mainly on the plasma membrane. Schwann cell profiles (Sch) also showed the reaction product. x20,000.



Figs. 30 and 31. Cyclic AMP phosphodiesterase activity in the cilia (ci) and dendritic knob (kn) of olfactory cells.

Fig. 30. The activity was reduced by 5 mM 3-isobutyl-1-methylxanthine. x20,000.

Fig. 31. The activity was reduced by 50 mM theophylline. x20,000.

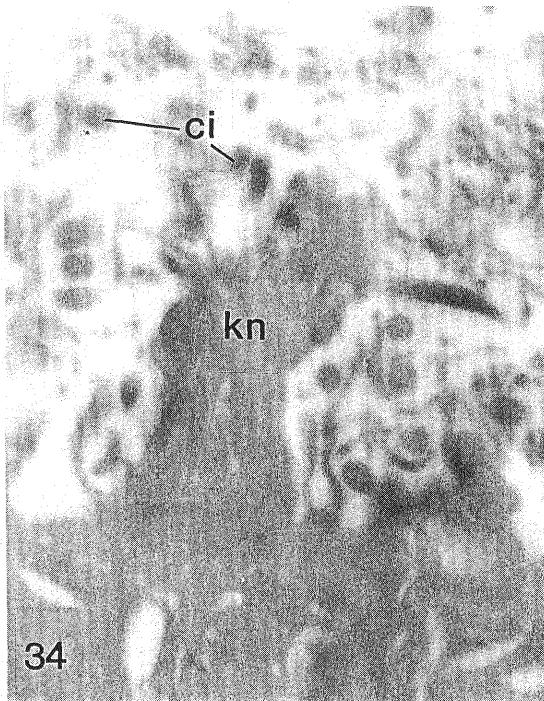
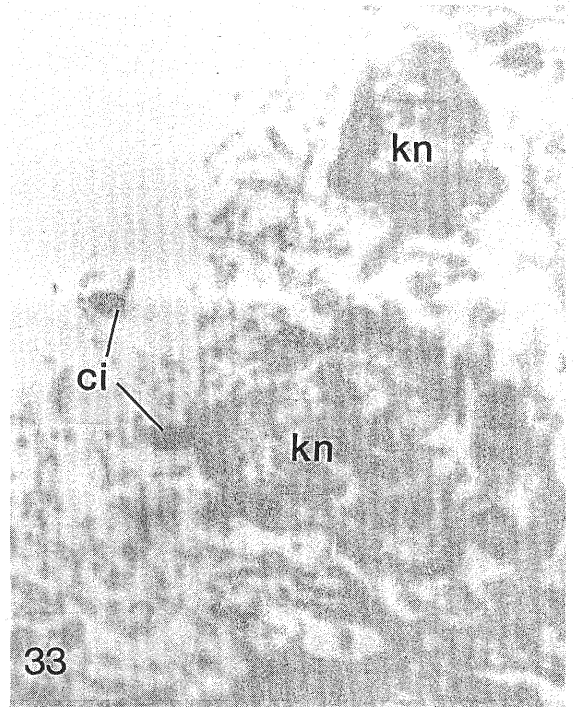
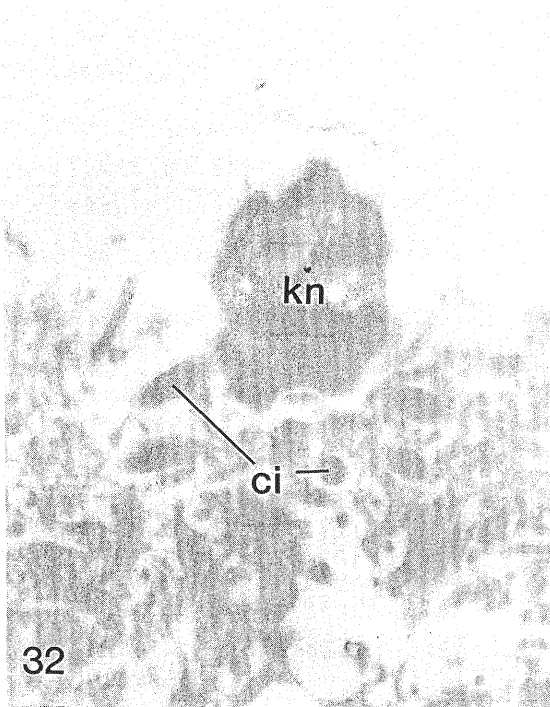


Figs. 32-34. Cyclic AMP phosphodiesterase activity in the cilia (ci) and dendritic knob (kn) of olfactory cells.

Fig. 32. Samples were incubated without exogenous substrate. No reaction product was seen. x20,000.

Fig. 33. Samples were preheated for 30 min at 70 °C before preincubation. No reaction product appeared. x20,000.

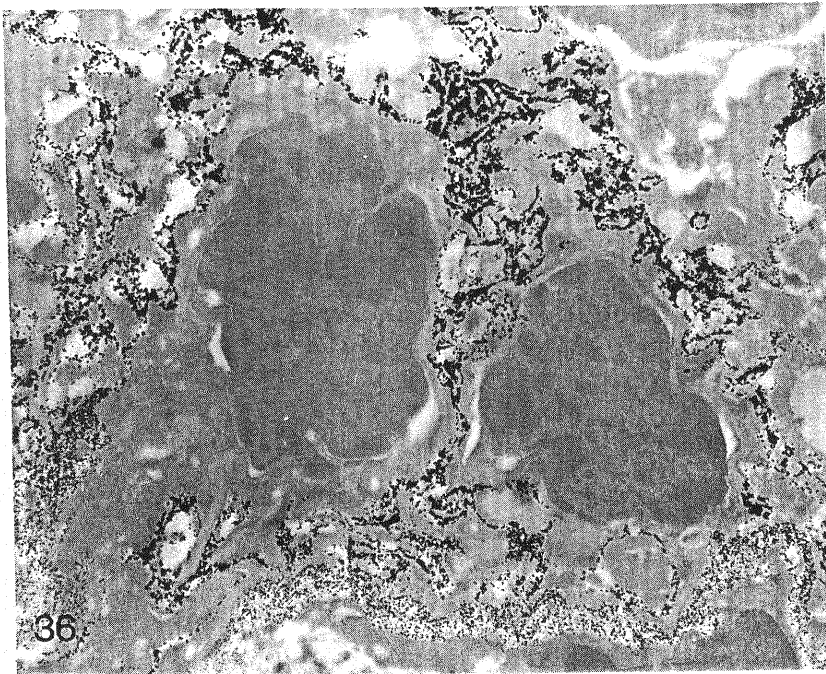
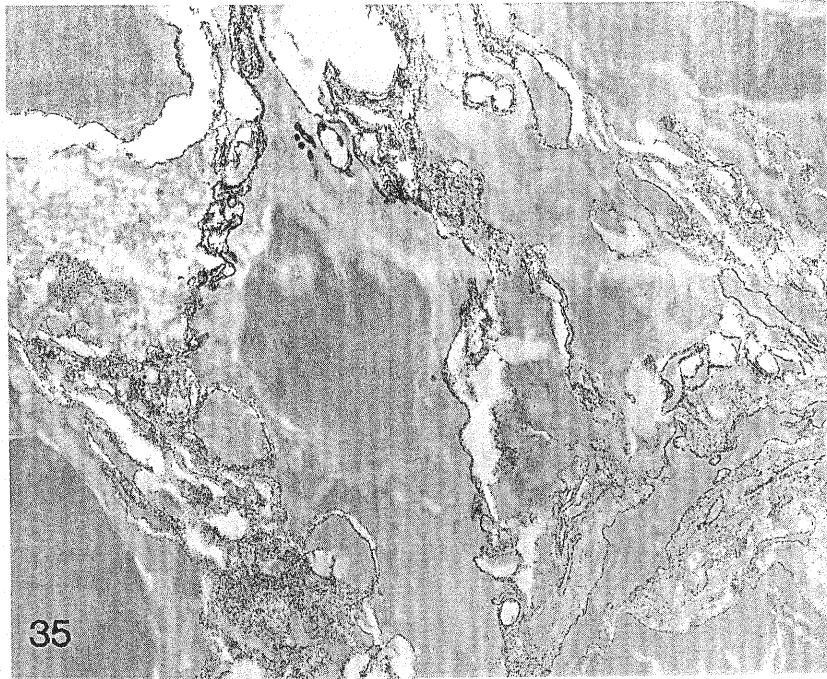
Fig. 34. Samples were incubated in the absence of snake venom. No reaction product appeared. x20,000.



Figs. 35 and 36. Cyclic AMP phosphodiesterase activity on the plasma membrane of basal cells in the olfactory epithelium.

Fig. 35. Samples were incubated in the standard incubation medium. x10,000.

Fig. 36. Samples were incubated in the snake venom-free incubation medium containing 2.5 mM levamisole. Preincubation was omitted. The enzymatic activity was presumably demonstrated with the help of intrinsic 5'-nucleotidase. x10,000.



Figs. 37-39. Cyclic GMP phosphodiesterase activity in rat olfactory cells.

Fig. 37. The enzymatic activity was seen in the cilia (ci) and dendritic knob (kn) of olfactory cells and the microvilli (mv) of supporting cells. The amount of the reaction product was smaller than that of cyclic AMP hydrolysis (compare with Fig. 27). The dendritic shaft (sh) usually showed no cyclic GMP phosphodiesterase activity. x20,000.

Fig. 38. No activity was seen in olfactory cell bodies. x9,000.

Fig. 39. Olfactory axons (ax) showed some enzymatic activity. x20,000.

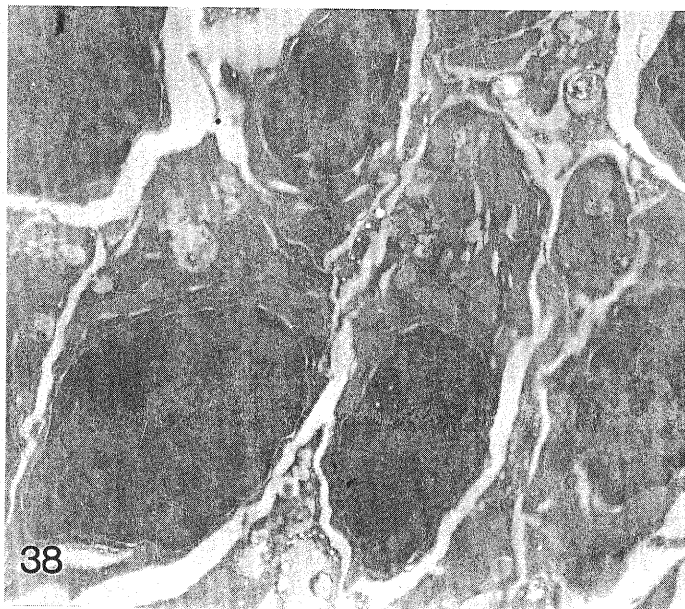
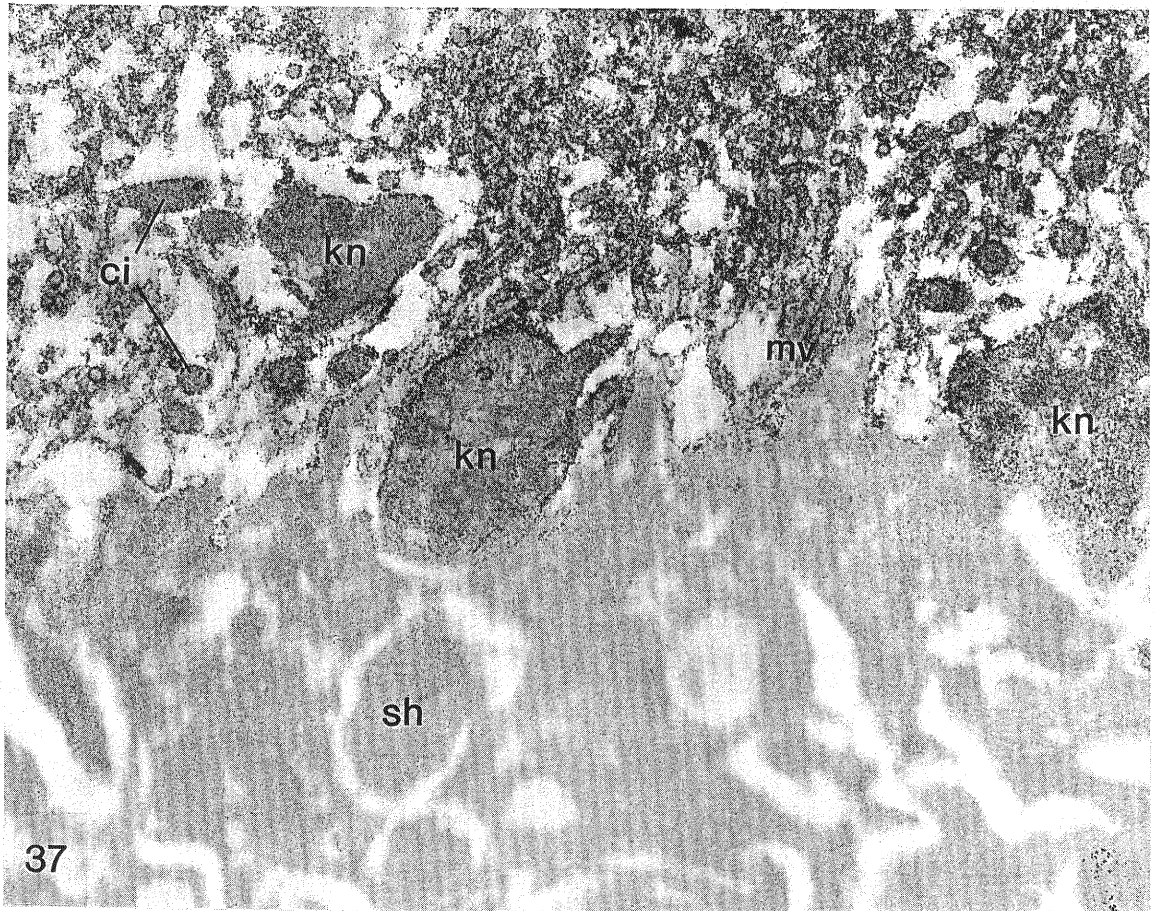


Fig. 40. Cyclic GMP phosphodiesterase activity on the plasma membrane of basal cells in the olfactory epithelium. x10,000.



Fig. 41. Olfactory mucosa sections were preincubated with snake venom (5 mg/ml), then incubated with 1 mM 5'-AMP in the absence of the venom. Reaction product was seen in the whole section, indicating entire penetration of 5'-nucleotidase from snake venom into the section. ci = olfactory cilia, kn = dendritic knob, sh = dendritic shaft, S = supporting cell, mv = microvilli of supporting cells. x12,500.

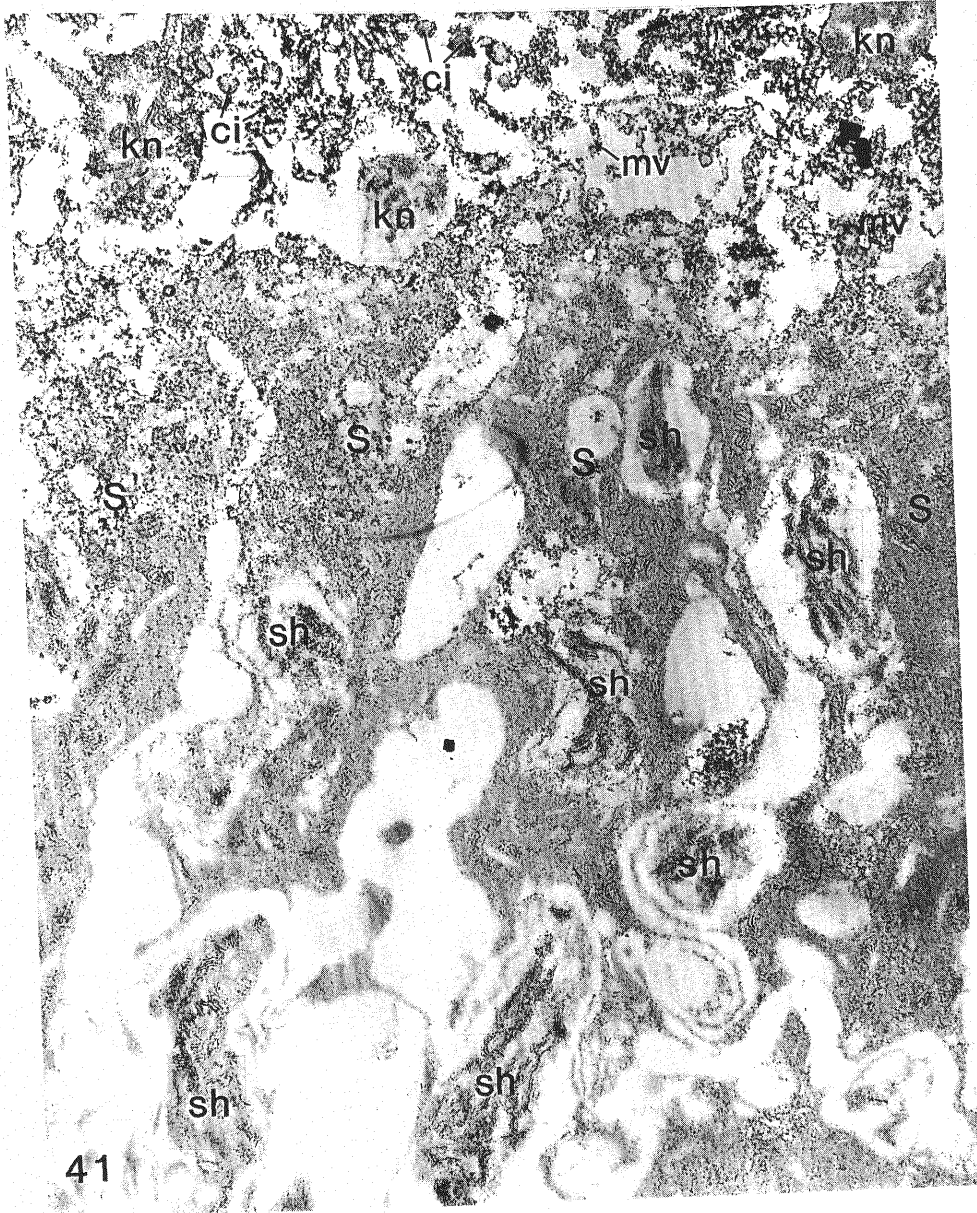


Fig. 42. Longitudinal section of a taste bud of the foliate papilla in rabbits. Three types of slender taste cells extended from the base to the apex of the taste bud. Dark cells (D) were characterized by electron-dense cytoplasm, an irregular-shaped nucleus and dark granules at the upper portion. Light cells (L) possessed vesiculated, electron-lucent cytoplasm and a round nucleus. Light cells often showed a degenerating aspect with numerous vacuoles (vc). Intermediate cells (I) were characteristic of abundant dark-cored vesicles around the nucleus. Tight junctions (tj) connected taste cells at the apical portion. N = nerve process. x4,400.



Fig. 43. Higher magnification of the apical portion of a rabbit taste bud. (a) The apex of dark cells (D) consisted of a neck and long microvilli, while that of light cells (L) showed short microvilli directly protruding from the trunk of cell body. Some light cells possessed only a few or no microvilli. The apex of intermediate cells (I) was a blunt process. Taste cells were connected by tight junctions (tj). x12,000. (b) Occasionally, dark cells showed an apex with a blunt process tied to a few microvilli at the side (arrows), which was a mixed feature of the dark cell and the intermediate cell. x17,500.

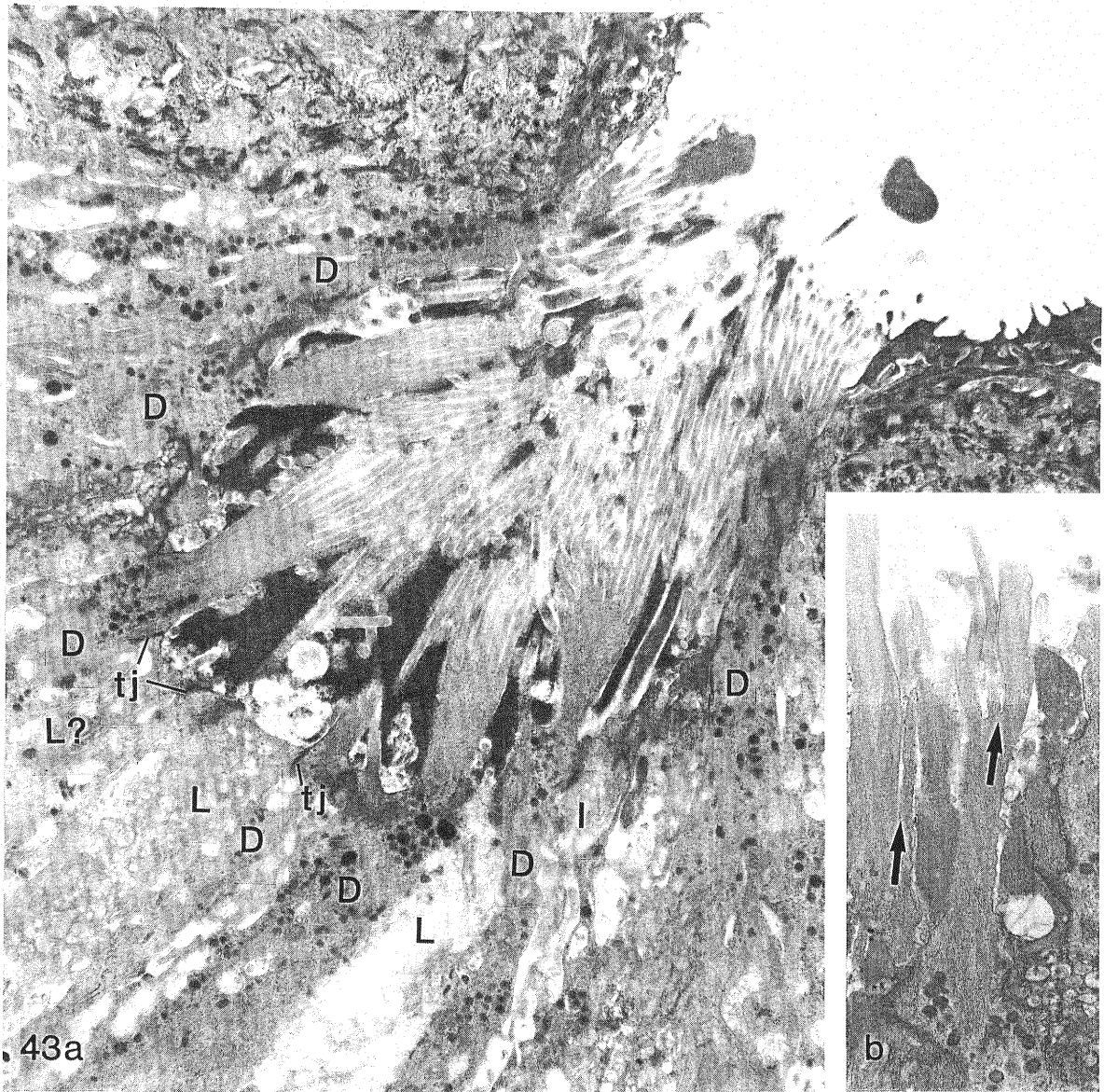


Fig. 44. An intermediate cell in the taste bud of rabbits. (a) Middle portion of an intermediate cell. Dark-cored vesicles were seen around the nucleus. x12,500. (b) Blunt process of an intermediate cell (I). This tissue was fixed with 2% potassium pyroantimonate + 1% osmium tetroxide for use in an experiment irrelevant to the present study. x12,500.

Fig. 45. A basal cell (B) seen at the base of a taste bud. x10,000.

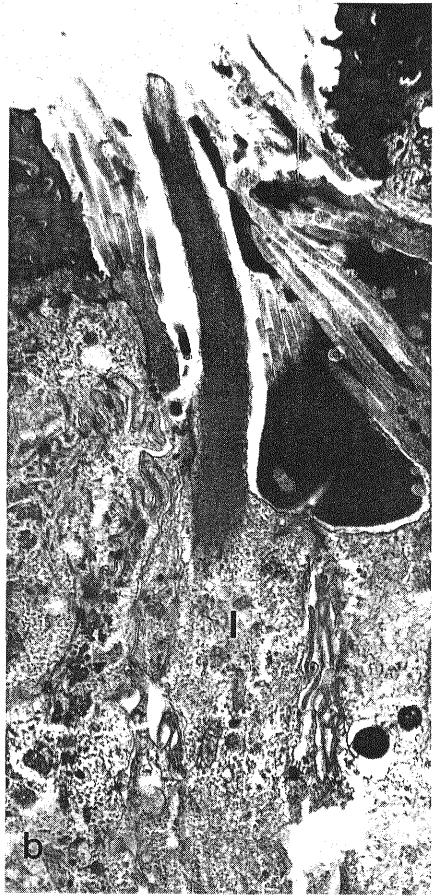
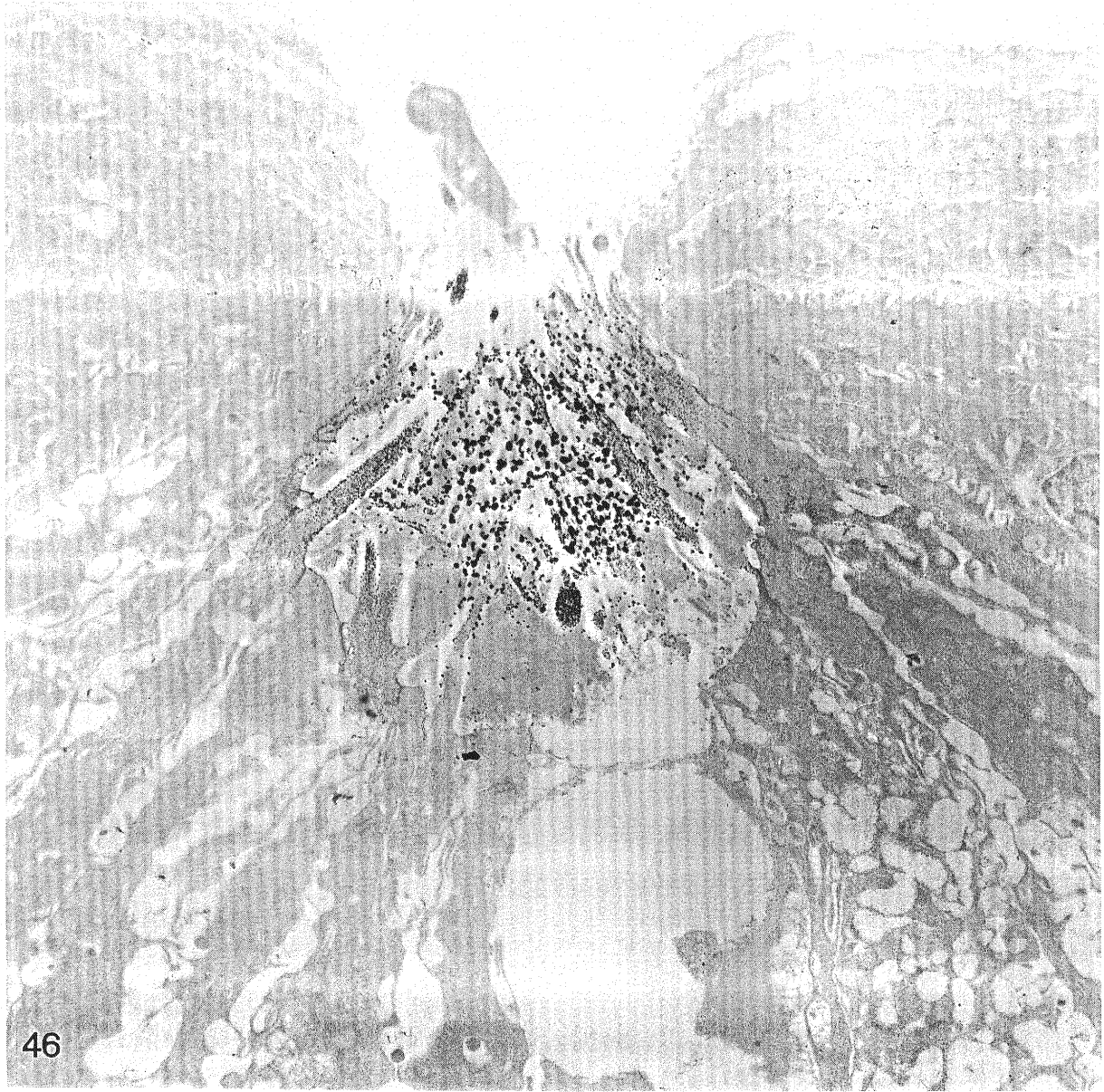


Fig. 46. AMP-PNP-hydrolyzing enzymatic activity in rabbit taste cells. Granular deposit of the reaction product was observed in the apical portion of all taste cells. x9,000.



Figs. 47 and 48. AMP-PNP-hydrolyzing enzymatic activity in the apical portion of taste cells.

Fig. 47. The activity was inhibited by 5 mM dithiothreitol, indicating that the activity was caused by NTP pyrophosphohydrolase. x10,000.

Fig. 48. Samples were incubated with 2 mM dithiothreitol and 100 μ M forskolin. Forskolin did not restore the enzymatic activity, confirming that the activity is not that of adenylate cyclase. The tissue was fixed with 2% paraformaldehyde + 0.1% glutaraldehyde. x10,000.

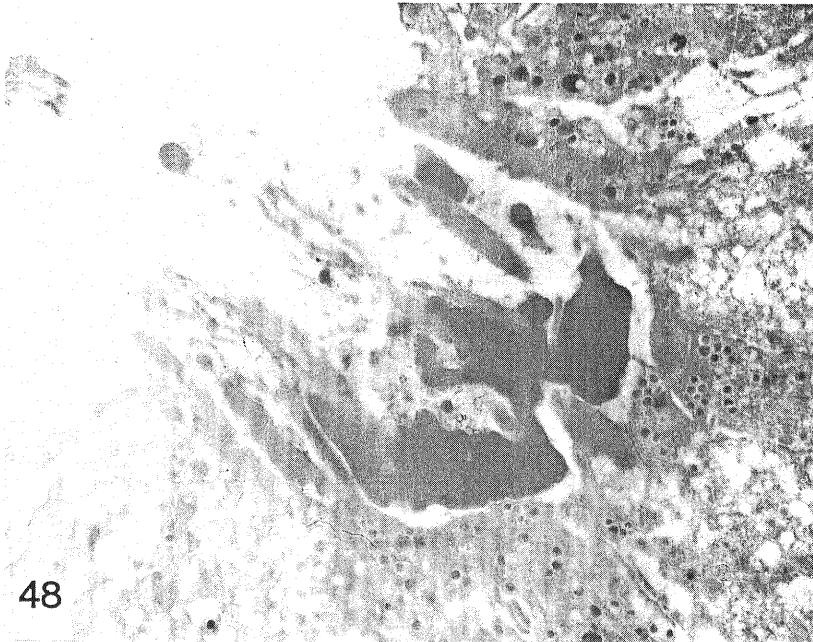


Fig. 49. Guanylate cyclase activity in the apical portion of rabbit taste cells. (a) Longitudinal section. An especially large amount of the reaction product was seen in the microvilli of dark cells (D). Only a small amount of the reaction product was seen in the microvilli of light cells (L). The blunt process of intermediate cells (I) often showed much reaction product. The region below the tight junctions (tj) was devoid of the reaction product. x9,000. (b) Transverse section of the microvilli. The reaction product was seen attached to the plasma membrane. x20,000.

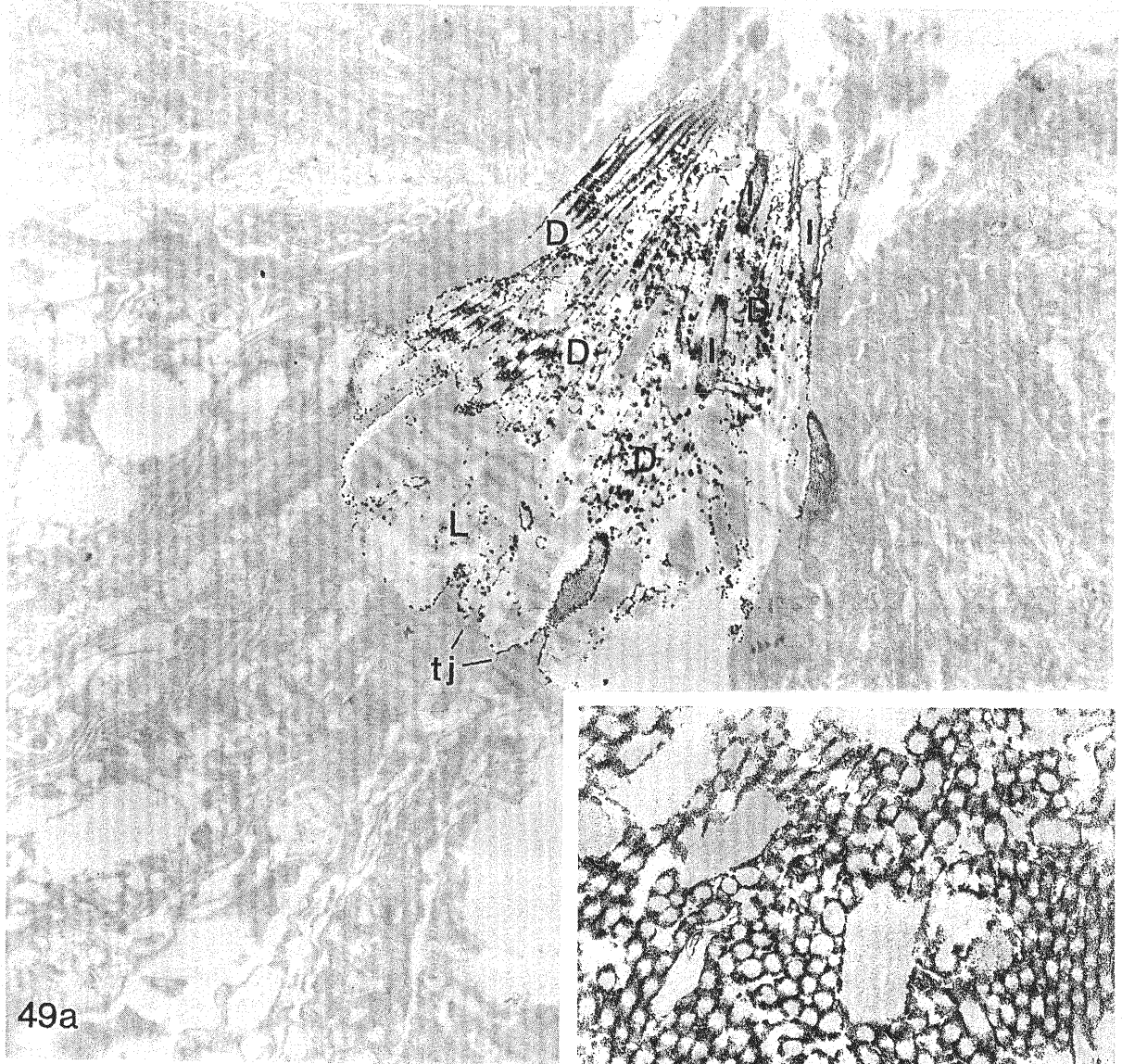


Fig. 50. No guanylate cyclase activity was seen in the middle portion of taste cells. x9,000.

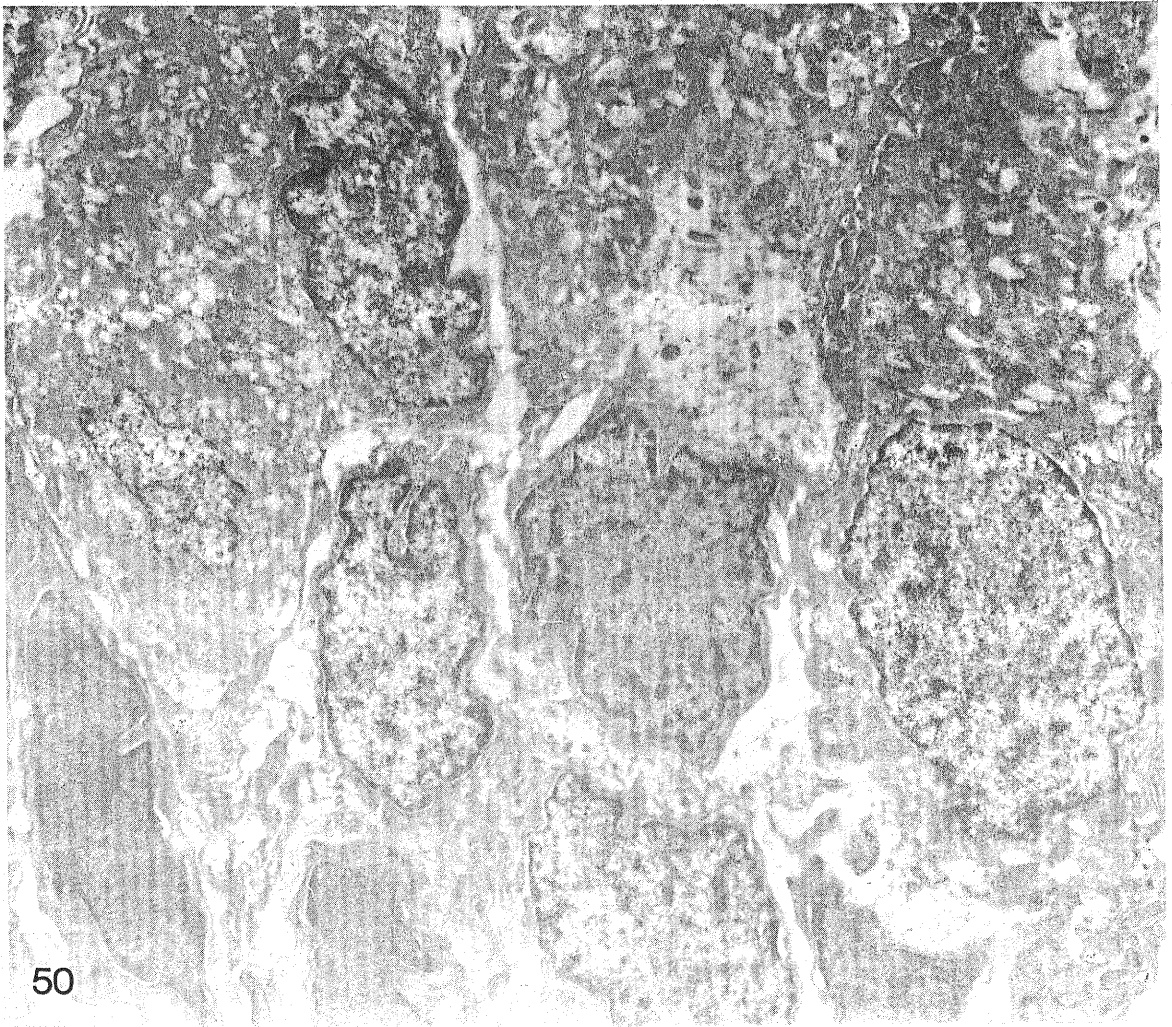
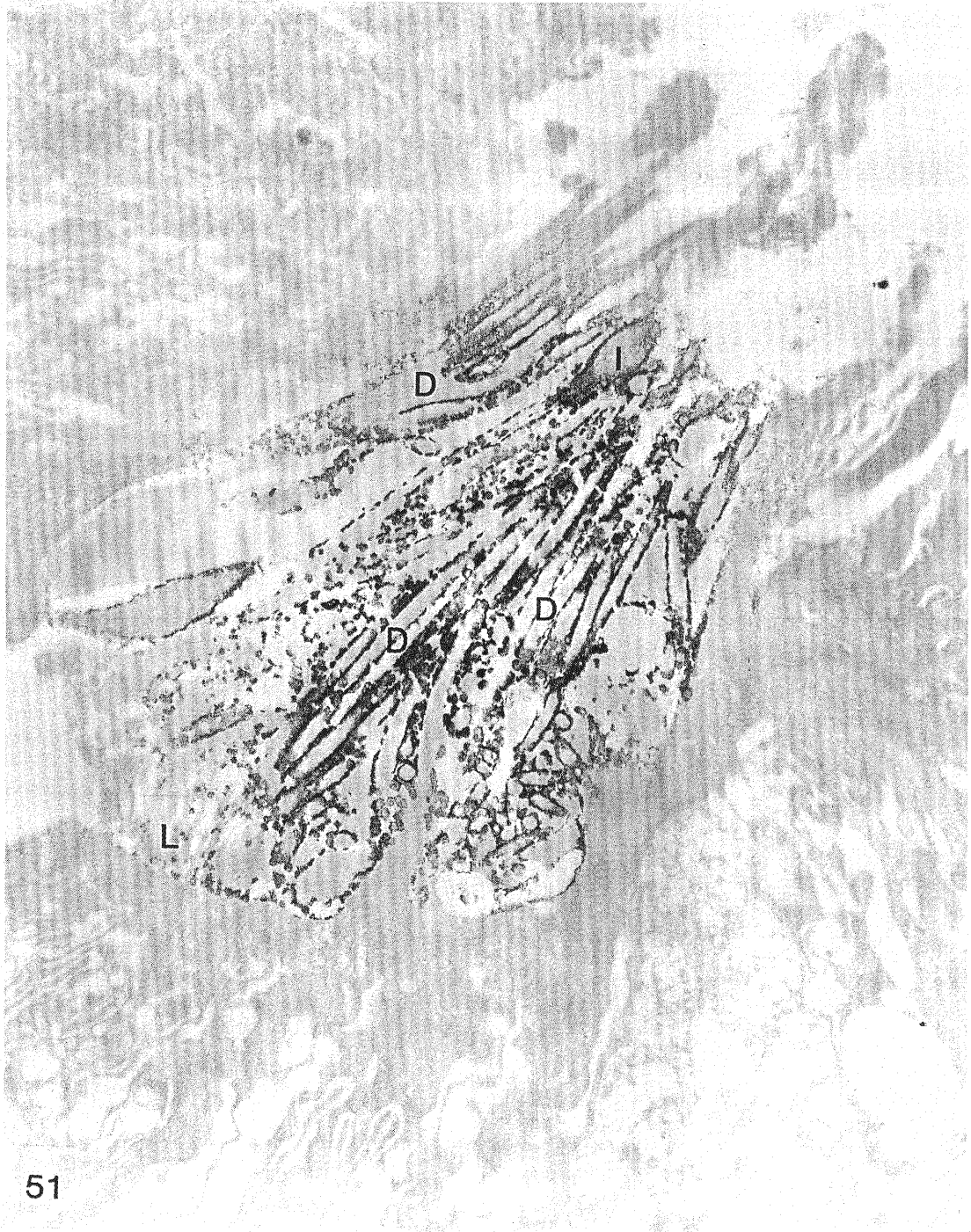


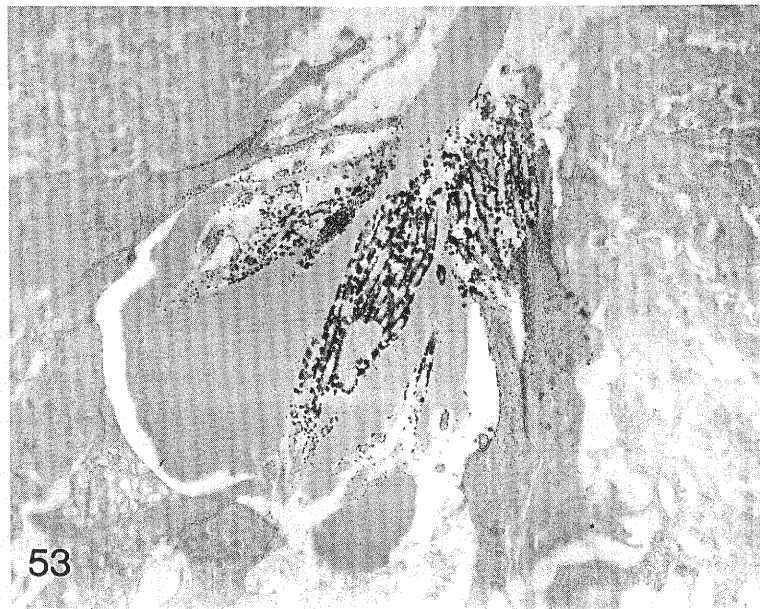
Fig. 51. Dithiothreitol (5 mM) did not inhibit the GMP-PNP-hydrolyzing enzymatic activity in the apical portion of taste cells. The chemical sometimes even enhanced the activity. This suggests that the enzymatic activity is that of guanylate cyclase and not NTP pyrophosphohydrolase. D = dark cell, L = light cell, I = intermediate cell. x15,000.



Figs. 52 and 53. The effect of the concentration of exogenous manganese on the guanylate cyclase activity in the apical portion of taste cells.

Fig. 52. Samples were incubated in the absence of added manganese. The amount of the reaction product was not reduced. $\times 10,000$.

Fig. 53. Increasing the concentration of MnCl_2 in the incubation medium from 1 mM to 3 mM did not affect the amount of the reaction product. $\times 10,000$.

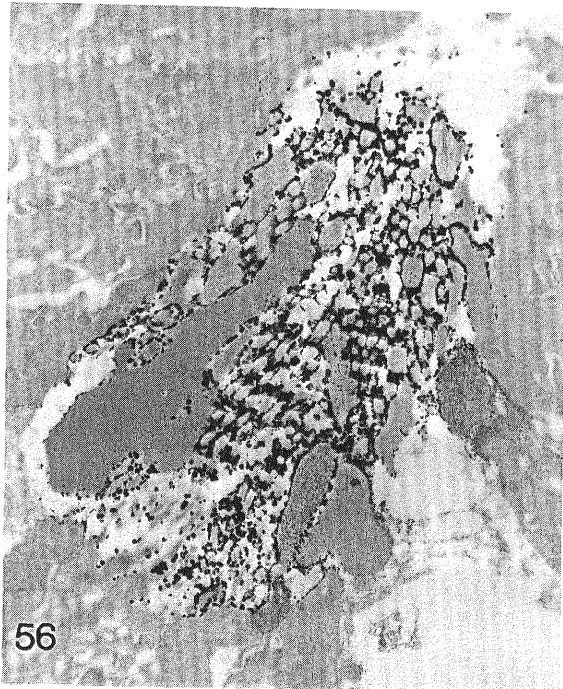
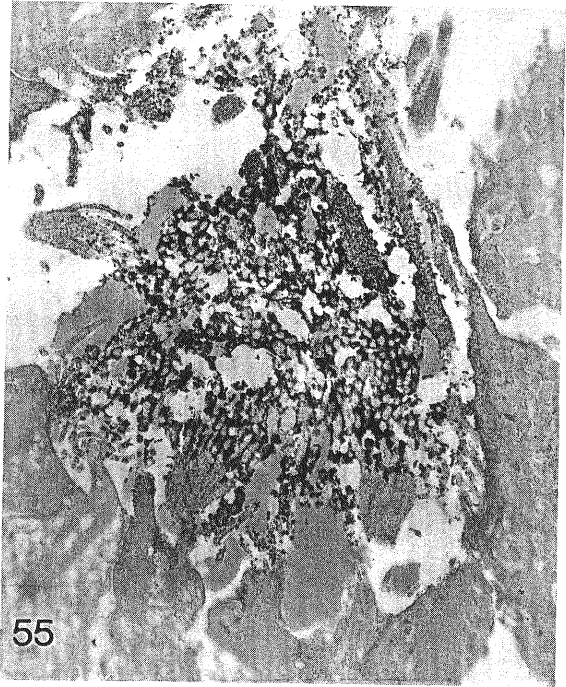


Figs. 54-56. The effects of guanylate cyclase activators on the guanylate cyclase activity in the apical portion of taste cells. All the three chemicals examined enhanced the enzymatic activity to a slight extent, although their effects were not always clear. Compare with Fig. 49a.

Fig. 54. Samples were incubated with 5 mM sodium azide. x10,000.

Fig. 55. Samples were incubated with 5 mM hydroxylamine. x10,000.

Fig. 56. Samples were incubated with 5 mM sodium nitroprusside. x10,000.



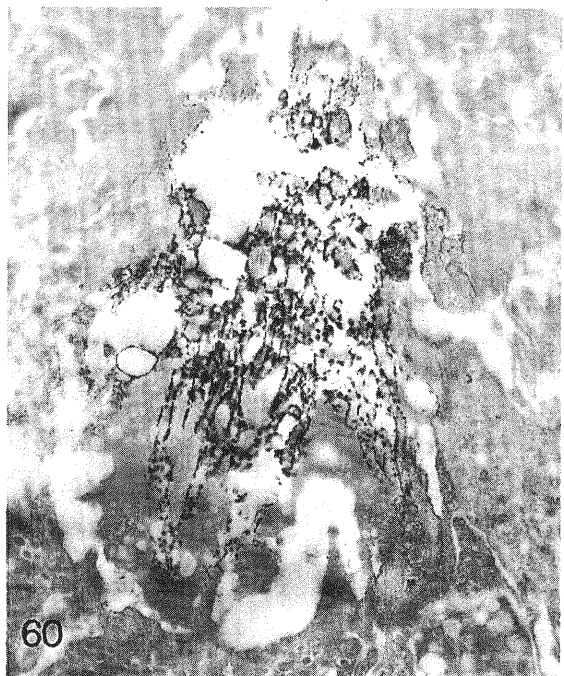
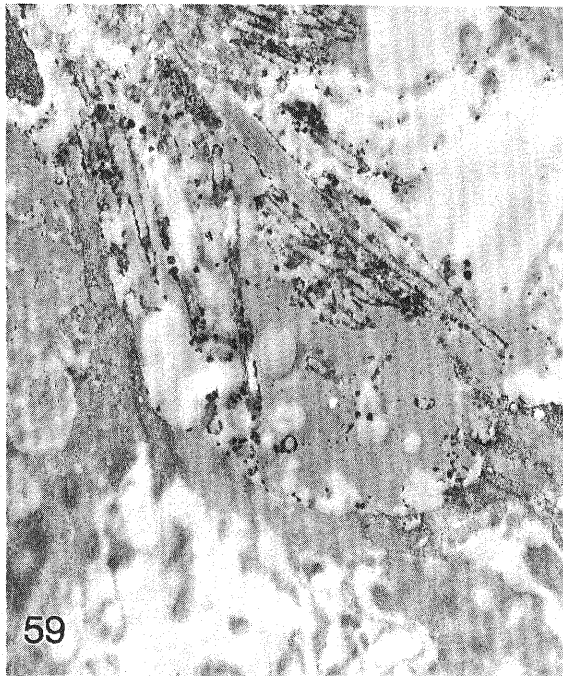
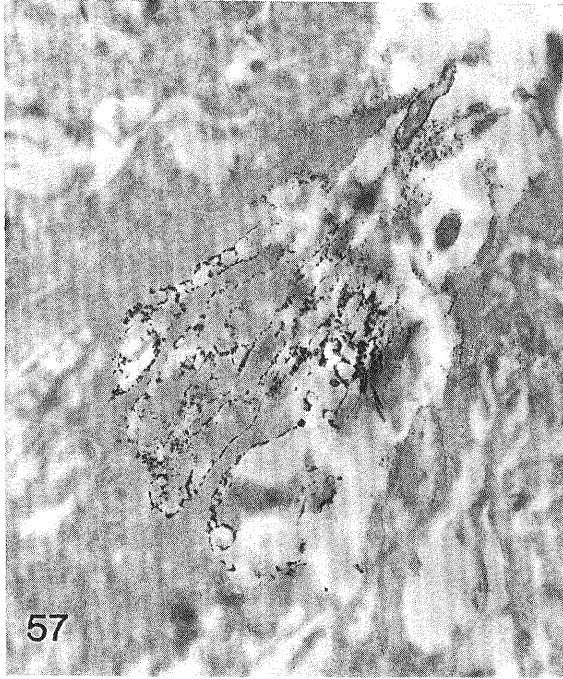
Figs. 57-60. The effects of guanylate cyclase activators on the guanylate cyclase activity in the apical portion of taste cells fixed with 2% paraformaldehyde + 0.1% glutaraldehyde. The stimulating effects of the chemicals seemed to be a little more apparent than in the tissue more strongly fixed.

Fig. 57. Samples were incubated in the absence of guanylate cyclase activators. x10,000.

Fig. 58. Samples were incubated with 1 mM sodium azide. x10,000.

Fig. 59. Samples were incubated with 1 mM hydroxylamine. x10,000.

Fig. 60. Samples were incubated with 1 mM sodium nitroprusside. x10,000.



Figs. 61-64. The effects of guanylate cyclase activators on the guanylate cyclase activity in the apical portion of taste cells. The incubation period was reduced from 30 min to 10 min with expectations that a shorter period of incubation would result in a smaller amount of the reaction product, which then would make the effects of the chemicals more distinct. Unfortunately, however, the effects of the chemicals were not clear.

Fig. 61. Samples were incubated in the absence of guanylate cyclase activators. x10,000.

Fig. 62. Samples were incubated with 10 mM sodium azide. x10,000.

Fig. 63. Samples were incubated with 10 mM hydroxylamine. x10,000.

Fig. 64. Samples were incubated with 10 mM sodium nitroprusside. x10,000.

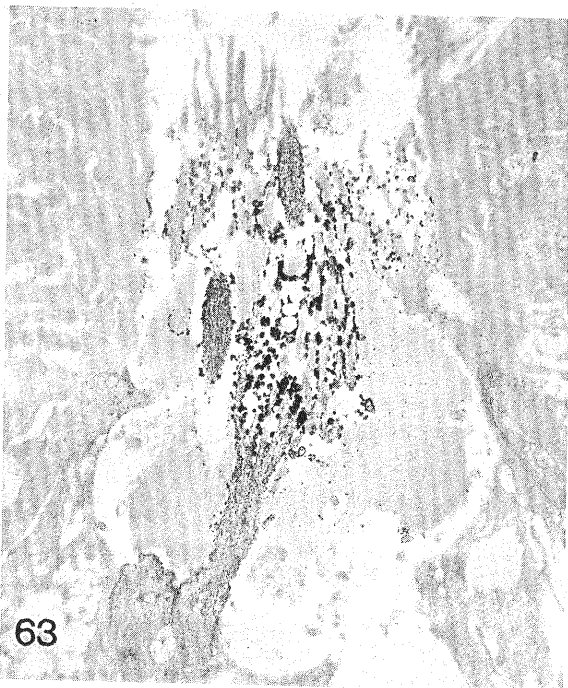
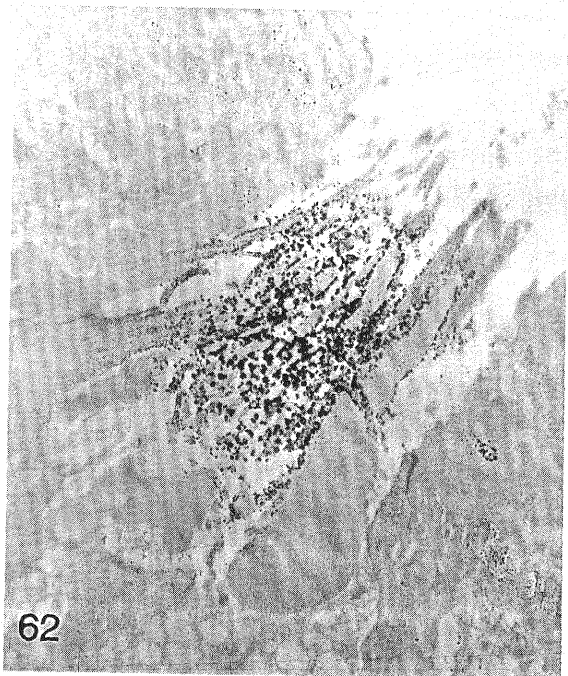
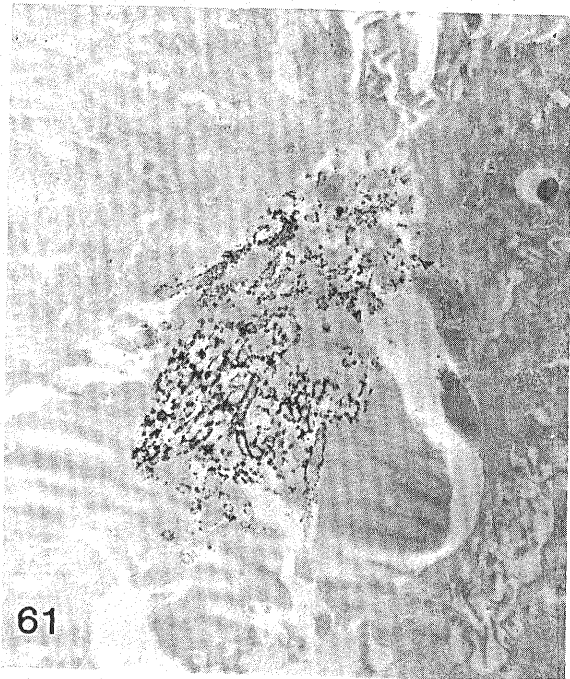
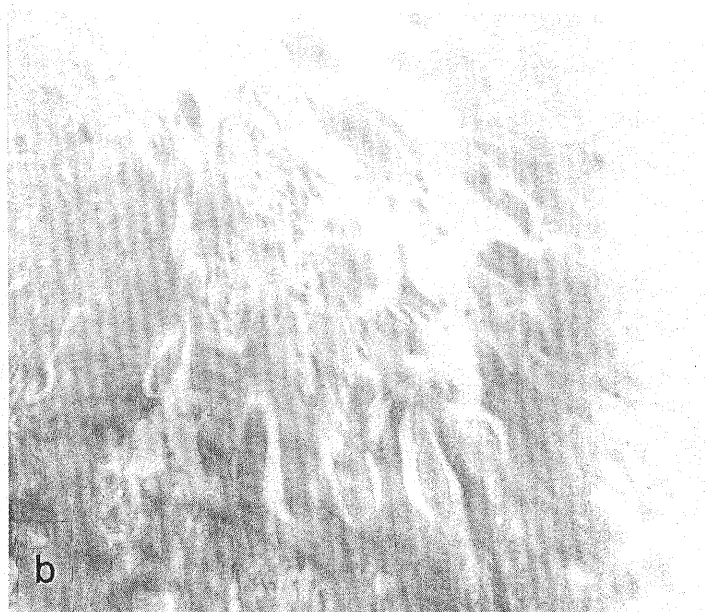


Fig. 65. The effect of 1% Triton X-100 on the guanylate cyclase activity in the apical portion of taste cells. (a) The detergent usually enhanced the enzymatic activity to a great extent. Much reaction product appeared both on the plasma membrane and inside the membrane. $\times 10,000$. (b) In some tissue sections in one experiment out of four, Triton X-100 inhibited the enzymatic activity. $\times 10,000$.

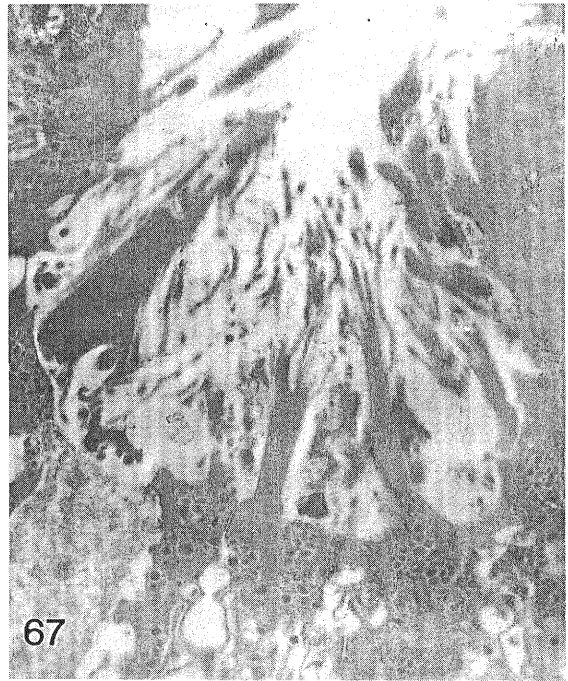
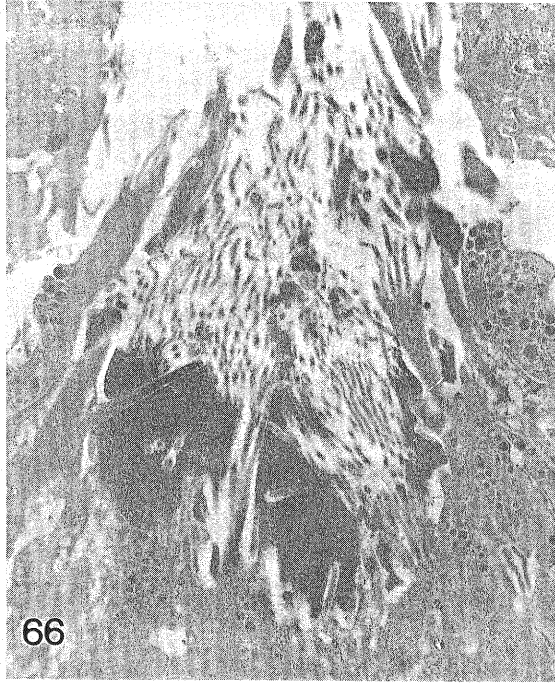


Figs. 66-68. The effects of Cd^{2+} , Zn^{2+} and Hg^{2+} on the guanylate cyclase activity in the apical portion of taste cells. These metals all inhibited the enzymatic activity.

Fig. 66. Samples were incubated with 1 mM CdCl_2 . x10,000.

Fig. 67. Samples were incubated with 1 mM ZnCl_2 . x10,000.

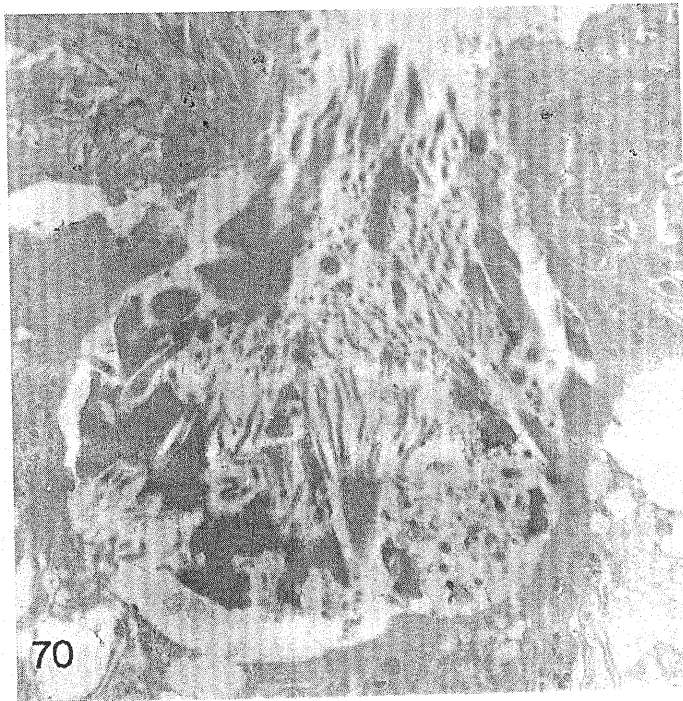
Fig. 68. Samples were incubated with 1 mM HgCl_2 . x10,000.



Figs. 69 and 70. Guanylate cyclase activity in the apical portion of rabbit taste cells.

Fig. 69. Sucrose (1 M) sometimes enhanced the enzymatic activity. x10,000.

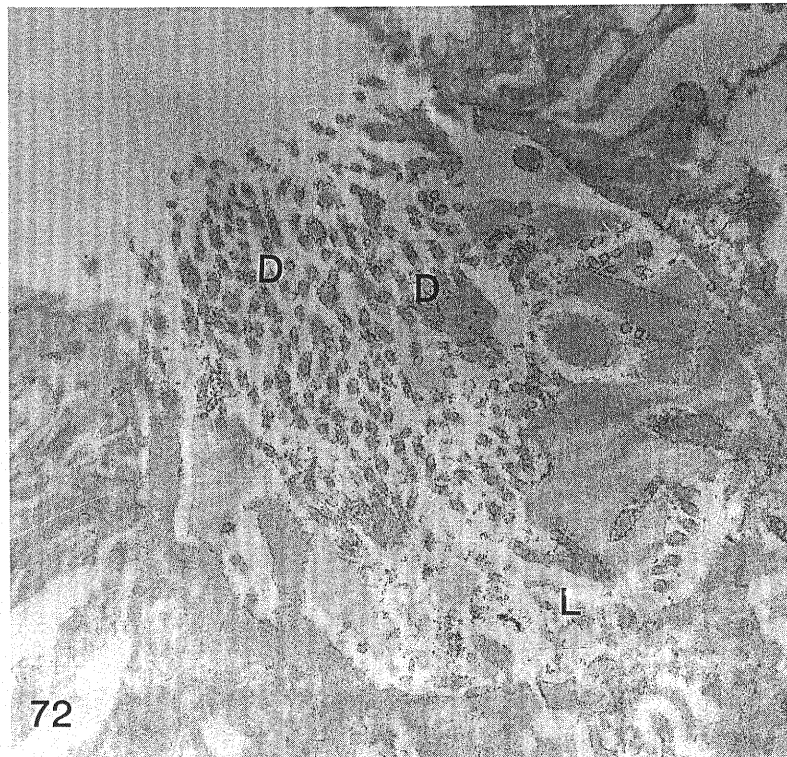
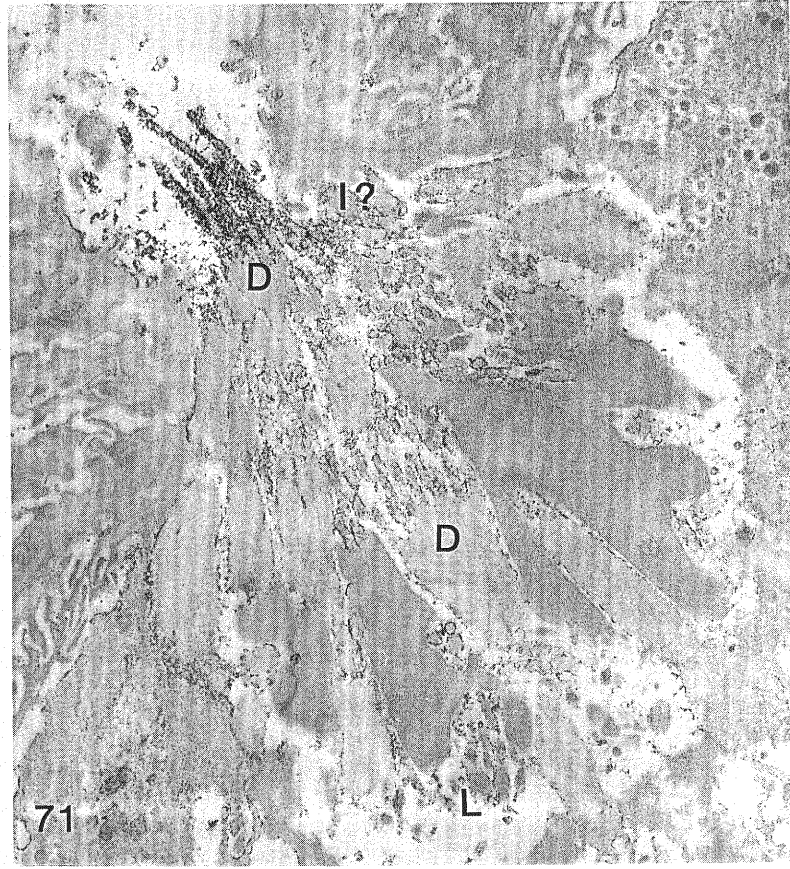
Fig. 70. Samples were incubated without exogenous substrate. No reaction product was seen. x10,000.



Figs. 71 and 72. Phosphodiesterase activity in the apical portion of rabbit taste cells. A fine electron-dense precipitate of the reaction product was seen mostly attached to the apical membrane. It was not decisive as to which type of cells showed the strongest enzymatic activity. D = dark cell, L = light cell, I? = presumably intermediate cell.

Fig. 71. Samples were incubated with 3 mM cyclic AMP as substrate. x12,500.

Fig. 72. Samples were incubated with 3 mM cyclic GMP as substrate. The amount of the reaction product was similar to that of the cyclic AMP hydrolysis. x12,500.



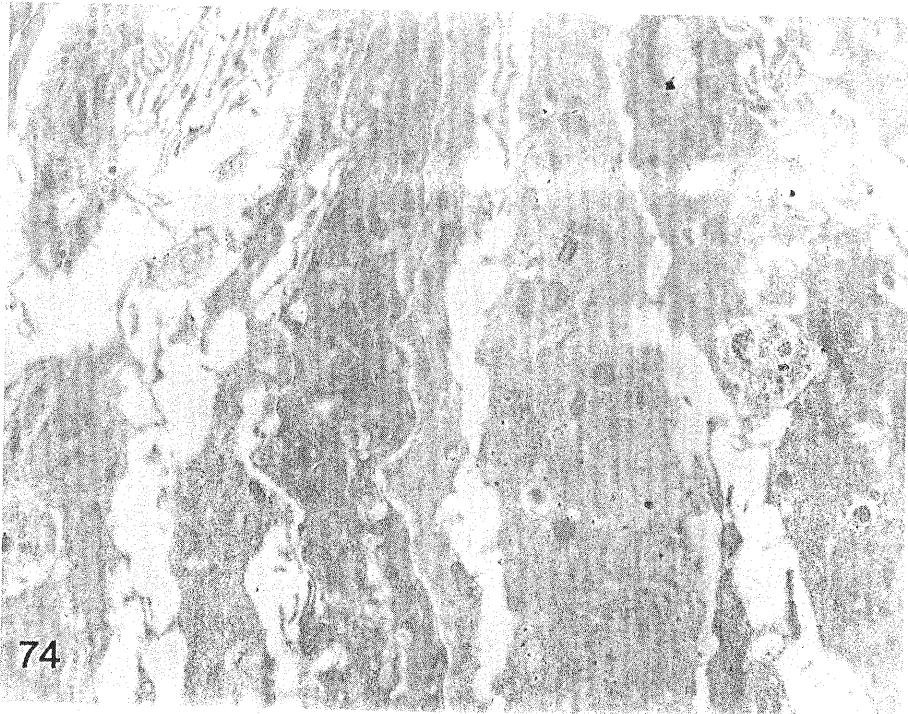
Figs. 73 and 74. No phosphodiesterase activity was seen in the middle portion of taste cells.

Fig. 73. Samples were incubated with 3 mM cyclic AMP as substrate.
x9,000.

Fig. 74. Samples were incubated with 3 mM cyclic GMP as substrate.
x9,000.



73



74

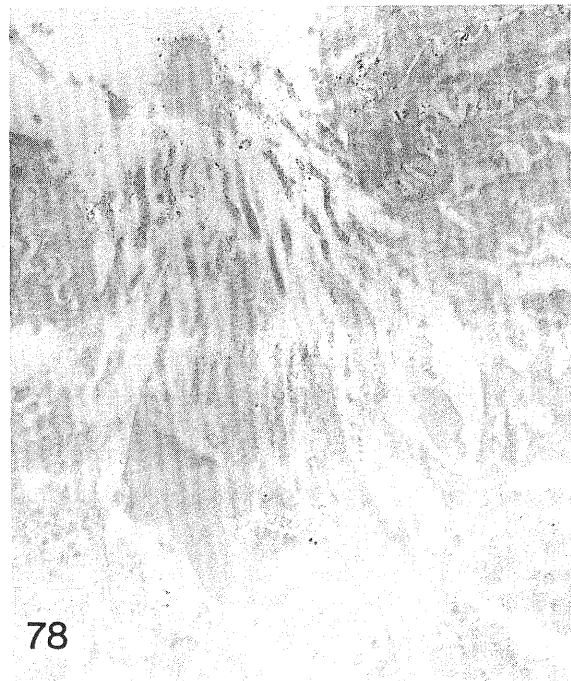
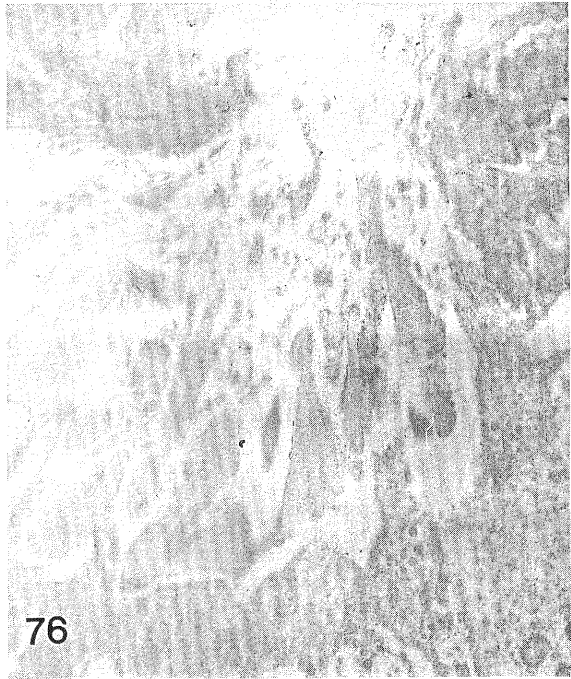
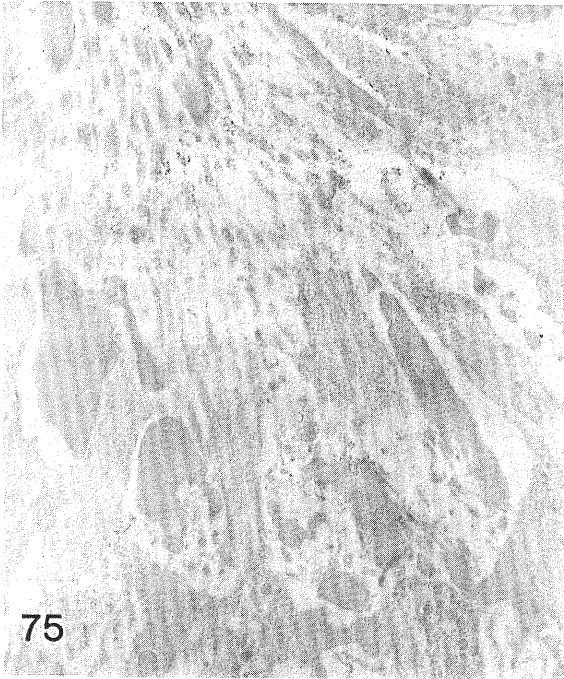
Figs. 75-78. Phosphodiesterase activity in the apical portion of taste cells.

Fig. 75. The activity was inhibited by 5 mM 3-isobutyl-1-methylxanthine. Substrate: 3 mM cyclic AMP. x10,000

Fig. 76. The activity was inhibited by 5 mM 3-isobutyl-1-methylxanthine. Substrate: 3 mM cyclic GMP. x10,000

Fig. 77. The activity was inhibited by 50 mM theophylline. Substrate: 3 mM cyclic AMP. x10,000.

Fig. 78. The activity was inhibited by 50 mM theophylline. Substrate: 3 mM cyclic GMP. x10,000.



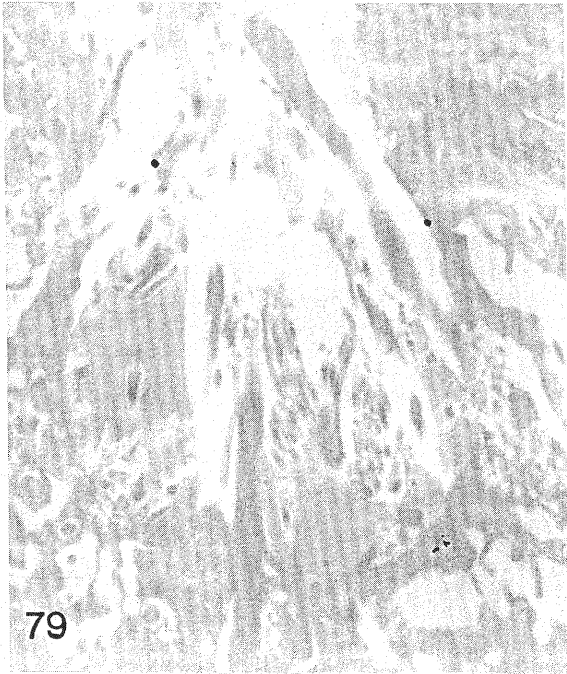
Figs. 79-82. Phosphodiesterase activity in the apical portion of taste cells.

Fig. 79. Samples were preheated for 20 min at 70 °C before preincubation. Substrate: 3 mM cyclic AMP. No reaction product appeared. x10,000.

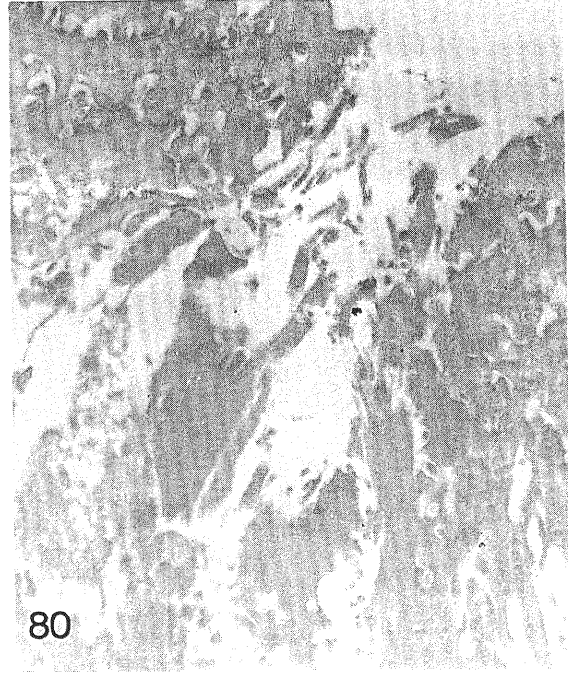
Fig. 80. Samples were preheated for 20 min at 70 °C before preincubation. Substrate: 3 mM cyclic GMP. No reaction product appeared. x10,000.

Fig. 81. Samples were incubated in the absence of snake venom. Substrate: 3 mM cyclic AMP. No reaction product appeared. x10,000.

Fig. 82. Samples were incubated in the absence of snake venom. Substrate: 3 mM cyclic GMP. No reaction product appeared. x10,000.



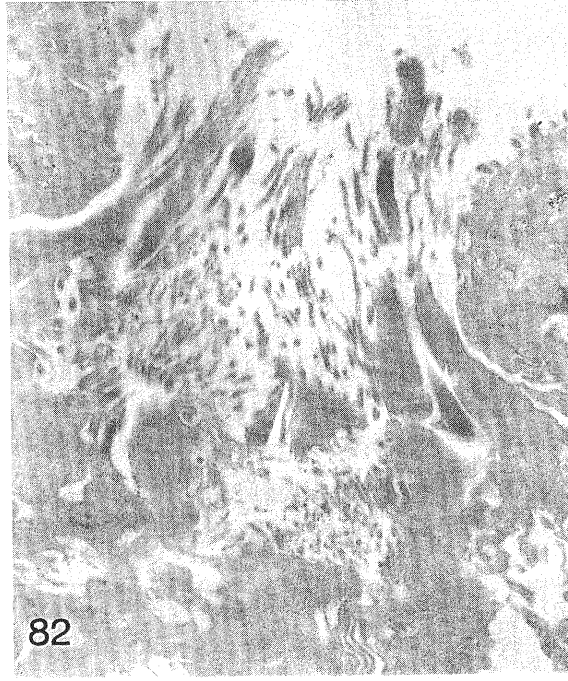
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80



81



82

Fig. 83. Samples were incubated without exogenous substrate as a control for the phosphodiesterase cytochemistry in taste cells. No reaction product was seen. x10,000.

Fig. 84. Foliate papilla sections were preincubated with snake venom (5 mg/ml), then incubated with 1 mM 5'-AMP in the absence of the venom. Reaction product was scattered in the whole region of the taste bud, indicating sufficient penetration of 5'-nucleotidase from snake venom into taste cells. The reaction product, however, was not seen inside the squamous epithelium cells surrounding the taste bud. x6,000.

