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Cytochemical Examination of the AMP–PNP–Hydrolyzing Enzymatic Activity in Rabbit Taste Bud Cells : Effects of Inhibitors and Activators of ATP Pyrophosphatase and of Adenylyl Cyclase

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Summary

In the apical regions of rabbit taste bud cells, especially on the surface of the microvilli, there exists an enzymatic activity that hydrolyzes an ATP analogue, 5'–adenylylimidodiphosphate. Since our previous study had suggested that this activity was that of either ATP pyrophosphatase or adenylyl cyclase, the effects of inhibitors and activators of these enzymes on the enzymatic activity were examined cytochemically. ATP pyrophosphatase inhibitors (dithiothreitol, sodium fluoride and amiloride) reduced the enzymatic activity, while adenylyl cyclase inhibitors (p–chloromercuriphenylsulfonic acid and 5,5'–dithio–bis (2–nitrobenzoic acid))did not. The effect of a mild activator of ATP pyrophosphatase(sodium azide) was not generally clear. Forskolin, a potent activator of adenylyl cyclase, did not show any enhancing effect. Ca^{2+} enhanced the enzymatic activity. The results indicate that the enzymatic activity is that of ATP pyrophosphatase and probably that of Ca^{2+}–dependent type.

Introduction

5'–Adenylylimidodiphosphate (AMP–PNP) is an artificially produced ATP analogue in which an imino group replaces the terminal bridge oxygen of the triphosphate chain. Since this substance was reported to be hydrolyzed by adenylyl cyclase (EC 4.6.1.1) but not by ordinary membrane ATPases (EC 3.6.1.3) [16,29], it was once widely and routinely used as a substrate in enzyme histocytochemistry of adenylyl cyclase. Two decades ago, we demonstrated cytochemically on the surface of microvilli of taste bud cells in rabbits an enzymatic activity that could hydrolyze this substance and referred to it as an adenylyl cyclase activity[2]. However, researchers have recently become more cautious in accepting AMP–PNP as a specific substrate for adenylyl cyclase since it has been reported that the substance is hydrolyzed by some ATPases[30,32,33] and also by alkaline phosphatase (EC 3.1.3.1) [24,37] and ATP pyrophosphatase (EC 3.6.1.8) [13,19].

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Later, an X-ray microanalysis of the cytochemical reaction product of the enzyme in question showed that this enzyme hydrolyzed AMP-PNP at its α and β phosphate bonding[1]. Hence, the enzyme may be adenylyl cyclase or ATP pyrophosphatase rather than ATPase or alkaline phosphatase ; the former group of the enzymes change ATP into cyclic AMP or AMP and pyrophosphate, while the latter into ADP and orthophosphate. Furthermore, this enzyme was inhibited by dithiothreitol[4,6] and sodium fluoride (NaF) [4], which implied that it was ATP pyrophosphatase and not adenylyl cyclase[15,19,27].

ATP pyrophosphatase belongs in the ecto-nucleotidase group, some of which are now being paid particular attention for their role in controlling nucleotide-induced signal transduction[26,38,39]. We are now further examining the AMP-PNP-hydrolyzing enzyme on the microvilli of taste bud cells to identify it more precisely and clarify its role. In the present study, we examined the effects on this enzyme of various inhibitors and activators of ATP pyrophosphatase and of adenylyl cyclase, and reconfirmed the enzyme as being ATP pyrophosphatase. A brief account of some of this work has been presented elsewhere[4,6].

Materials and Methods

Foliate papillae of male white domestic rabbits (2–3 kg), which contained numerous taste buds, were used. The papillae were removed from the animals immediately after they had been killed by an intravenous injection of a fatal dose of sodium amobarbital (100–200 mg/kg body weight). 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 several pieces and fixed for 30–60 min on ice in a mixture of 2% paraformaldehyde and 0.25–1% glutaraldehyde dissolved in 0.05 M sodium cacodylate buffer, pH 7.4. The osmotic pressures of the fixing solutions were adjusted with sucrose or NaCl. After rinsing for several hours with six or seven changes of ice-cold 0.05 M cacodylate buffer containing 0.25 M sucrose or 0.15 M NaCl, the tissue was cut into 40 μm thick sections using a Microslicer DTK-1000 (Dosaka EM Co. Ltd., Kyoto, Japan) and stored overnight at 1–2℃ in the same solution. In the case of the tissue fixed with 2% paraformaldehyde and 0.25% glutaraldehyde, the tissue was cut into 100 μm thick since it was difficult to obtain 40 μm thick sections.

The incubation procedure was based on the strontium method, originally described by Ernst[12] for Na^+-K^+-ATPase cytochemistry. The tissue sections were preincubated for 15–30 min at room temperature in a medium that contained 80 mM Tris-HCl, pH 9.1, 0.2 M sucrose (or 0.1 M NaCl), 4 mM $MgCl_2$, 2 mM theophylline (cyclic nucleotide phosphodiesterase inhibitor[8]), 2.5 mM levamisole (non-specific alkaline phosphatase inhibitor[7]) and 10 mM $SrCl_2$ (capture agent of the cytochemical reaction product). Theophylline was sometimes omitted, which did not affect the results in the present study. Incubation was initiated by adding to this medium 0.5 mM AMP-PNP as the substrate of the enzyme and warming the medium to 37℃ (or 30℃ in some cases), and usually continued for 30 min at this temperature in a shaking water bath. In some experiments, AMP-PNP concentration was reduced to 0.1 or 0.2 mM.

The following experiments were run for comparison : (i) incubation in the absence of substrate, (ii) incubation without $MgCl_2$, (iii) incubation with 10 mM dithiothreitol, 10 mM NaF or 2 mM amiloride, which are reported to inhibit ATP pyrophosphatase [15,19,27,35], (iv) incubation with 20 mM sodium azide (NaN_3) as a possible activator of ATP pyrophosphatase[9,19] and an inhibitor of apyrase

(EC 3.6.1.5) [20,25], (v) incubation with 5 mM $CaCl_2$ as an activating metal ion for some ATP pyrophosphatase[13], (vi) incubation with 1 mM p-chloromercuriphenylsulfonic acid (PCMPS) or 1 mM 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), possible inhibitors of adenylyl cyclase[22,36] and (vii) incubation with 200 μM forskolin, a potent adenylyl cyclase activator[17,31]. Dithiothreitol and NaF do not only inhibit ATP pyrophosphatase but also activate adenylyl cyclase[15,19,27]. Chemicals in (iii) to (vii) were added at the preincubation stage. Forskolin was dissolved in dimethyl sulfoxide and added into the preincubation medium. The final concentration of dimethyl sulfoxide in the incubation medium was 5%, which was reported not to affect the activation of adenylyl cyclase[17]. The incubation medium that contained NaF was slightly turbid. The final pH of all the incubation media ranged from 8.8 to 9.1. Since the difference in prefixing conditions of the tissue should affect the activity of enzymes, the effects of the above chemicals were examined by comparison with the control tissue sections prefixed and incubated in a similar condition.

Incubated sections were cooled on ice to minimize further reaction and rinsed three times in 80 mM Tris-HCl buffer, pH 9.0, containing 0.23 M sucrose. Afterwards, they were rinsed twice in 2% lead nitrate solution with 0.23 M sucrose to convert strontium phosphate, the cytochemical reaction product, into lead phosphate for better visualization in the electron microscope. Then the sections were rinsed three times in 0.30 M sucrose and twice in 0.05 M cacodylate buffer, pH 7.4, containing 0.25 M sucrose. Those rinses were carried out at room temperature for 5-10 min each. The sections were then postfixed for 30-45 min on ice with 1% osmium tetroxide in 0.05 M cacodylate buffer, pH 7.4, containing 0.23 M sucrose, dehydrated in graded series of ethanol and propylene oxide and embedded in Epon-Araldite. Ultra-thin sections were cut on an LKB 2088 microtome and examined unstained in a JEOL 1200 EX-II electron microscope operated at 80 kV.

AMP-PNP (lithium salt), theophylline, levamisole, dithiothreitol, amiloride, NaN_3, PCMPS, DTNB and forskolin were purchased from Sigma Chemical Co. (St. Louis, MO, USA); paraformaldehyde, sodium cacodylate, Tris (hydroxymethyl) -aminomethane, HEPES, dimethyl sulfoxide, propylene oxide and lead nitrate (all specially prepared reagent grade) from Nacalai Tesque, Inc. (Kyoto, Japan); glutaraldehyde, osmium tetroxide and Epon 812 from TAAB Laboratories Equipment Ltd. (Reading, UK); Araldite 6005 from Ciba Products Corp. (Fair Lawn, NJ, USA). All other materials were of guaranteed reagent grade or EM grade.

Results

The foliate papillae are a pair of oval swellings on the lateral surface of the posterior part of the tongue, which consist of parallel folds of the epithelium. Taste buds exist in the lateral wall of these folds, facing each apex towards the surface of the epithelium. According to Murray's nomenclature[23], there are four types of cells (type I-IV) in the taste buds of rabbit foliate papillae. Except for type IV cells, which are basal cells, taste bud cells possess a slender shape and extend from the base to the apex of the taste bud. The apical portions of these cells are exposed to the oral cavity through a narrow taste pore and typically characterized by long microvilli connected to a neck in type I cells, short microvilli in type II cells and a long blunt process without microvilli in type III cells, although some variations are seen.

In the present study, electron-dense precipitate of the cytochemical reaction product of the AMP-PNP-hydrolyzing enzymatic activity was exclusively observed in the apical portions of type I-III cells (Figs.1 a, 2 a and 4 a). Type IV or basal cells showed no reaction product (not shown). The bulk

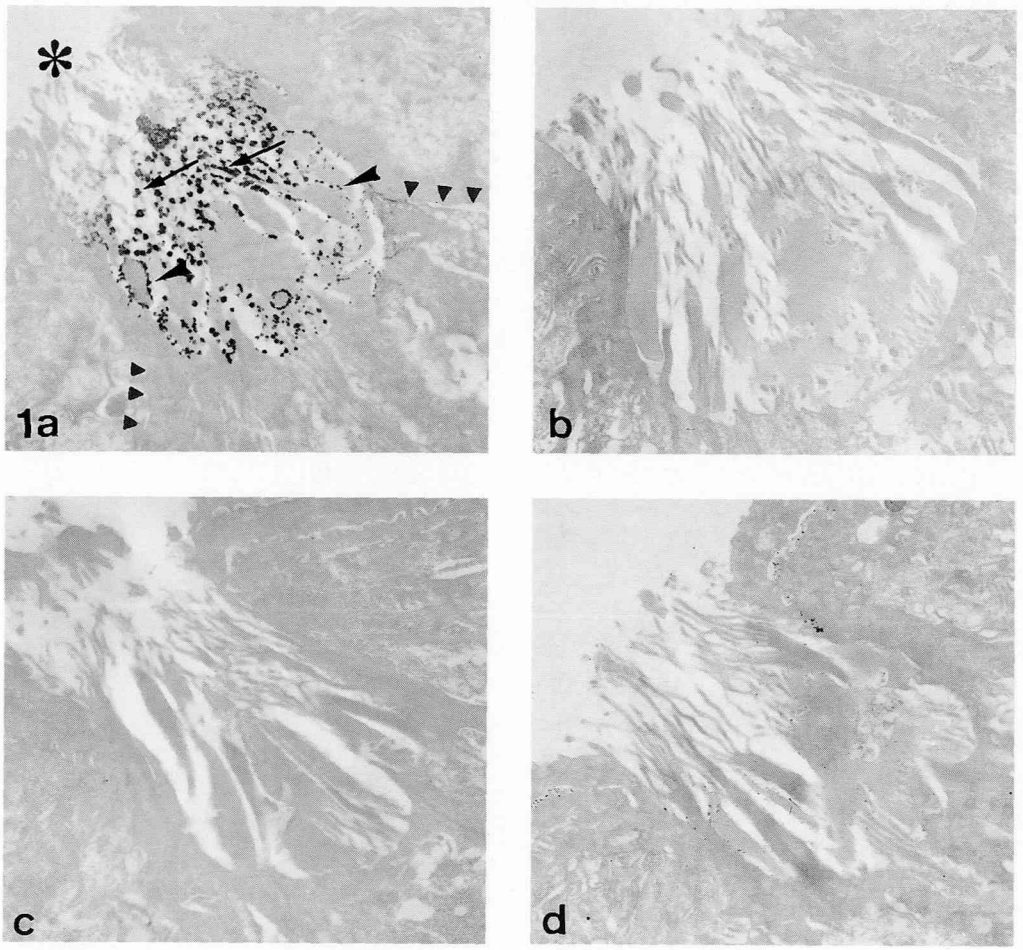

Fig.1 : AMP–PNP–hydrolyzing enzymatic activity in the apical portions of rabbit taste bud cells : the effect of ATP pyrophosphatase inhibitors. The tissue was prefixed with 2% paraformaldehyde and 1% glutaraldehyde for 60 min, and incubated with 0.5 mM AMP–PNP for 30 min at 37℃. (a) Control (incubated without any ATP pyrophosphatase inhibitors). Electron–dense precipitate of the cytochemical reaction product, which indicates the presence of the enzymatic activity, is seen on the microvilli (arrows) and somewhat on the neck of the cells (arrowheads). The asterisk shows the taste pore and the small triangles indicate the boundary of the taste bud and the surrounding epithelium. (b) Incubated with 10 mM dithiothreitol. (c) Incubated with 10 mM NaF. (d) Incubated with 2 mM amiloride. Almost no reaction product is seen in (b)–(d). ×8,000.

of the reaction product was attached to the plasma membranes of the microvilli. Long microvilli of type Ⅰ cells often showed a larger amount of the reaction product than short microvilli of type Ⅱ cells as previously reported[2] (Fig.2 a). The reaction product was also seen in the neck of type Ⅰ cells and a blunt process of type Ⅲ cells (Figs.1 a and 2 a). The epithelial cells surrounding taste buds showed no enzymatic activity (Fig.1 a). The tissue incubated without AMP–PNP showed no reaction product (not shown).

Dithiothreitol, NaF or amiloride inhibited the enzymatic activity (Fig.1 b–d). Although NaN_3 occasionally seemed to slightly enhance the enzymatic activity, its effect was not generally clear (Fig.2 b). Omitting $MgCl_2$ from the incubation medium did not affect the enzymatic activity (Fig.2 c). Addi-

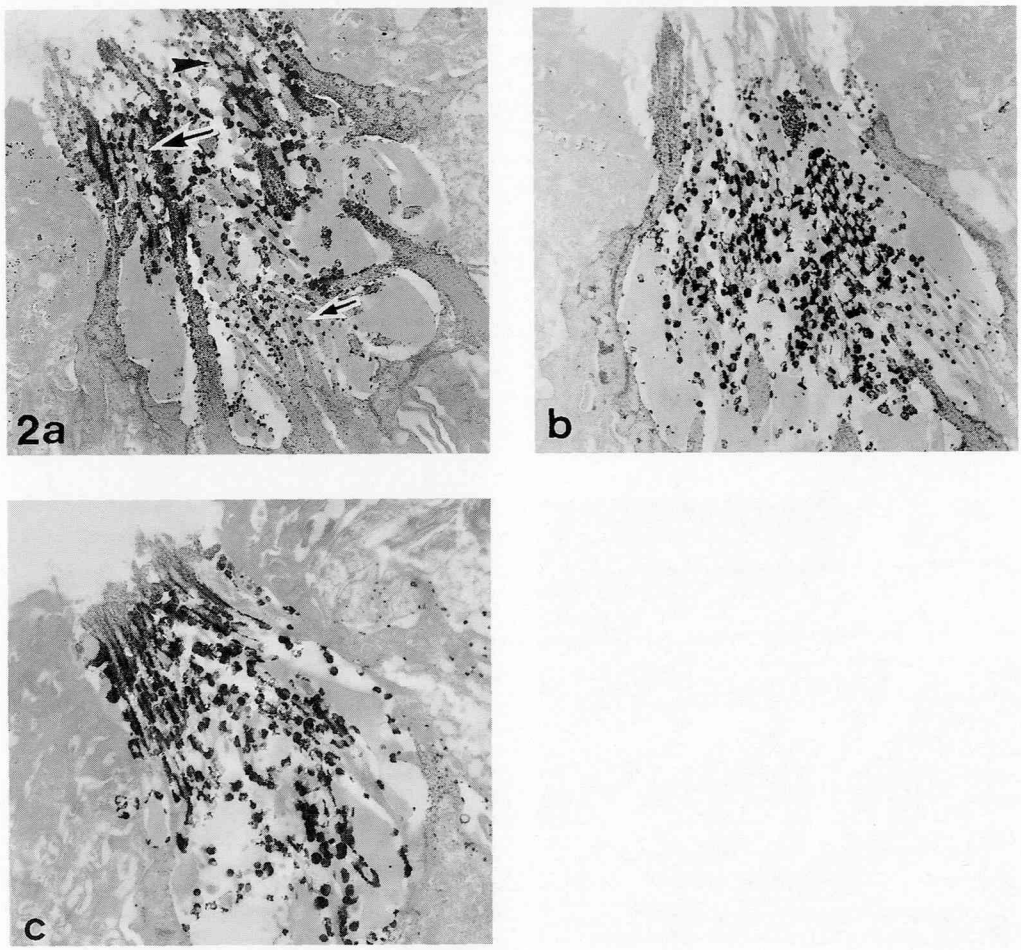

Fig.2 : The effects of NaN_3, and omission of Mg^{2+} on AMP–PNP–hydrolyzing enzymatic activity of rabbit taste bud cells. The tissue was prefixed with 2% paraformaldehyde and 0.5% glutaraldehyde for 40 min (a and b) or 30 min (c), and incubated with 0.5 mM AMP–PNP for 30 min (a and c) or 40 min (b) at 37℃. (a) Control (incubated in the standard incubation medium). The large and small arrows point the microvilli of a type Ⅰ cell and those of a type Ⅱ cell, respectively. The arrowhead indicates an apical portion of a type Ⅲ cell. (b) Incubated with 20 mM NaN_3. (c) Incubated without added $MgCl_2$. No obvious change of the enzymatic activity is seen with NaN_3 or without exogenous Mg^{2+}. ×8,000.

tion of 5 mM $CaCl_2$ in the incubation medium did not appear to affect the enzymatic activity in the tissue incubated with 0.5 mM AMP–PNP (not shown). However, when the concentration of AMP–PNP was reduced to 0.2 mM, which resulted in formation of less cytochemical reaction product (Fig.3 a), enhancement of the enzymatic activity by Ca^{2+} was clearly seen (Fig.3 b). Reducing the substrate concentration to 0.1 mM resulted in total absence of the reaction product, which was not recovered by Ca^{2+} (not shown). PCMPS, DTNB and forskolin did not affect the enzymatic activity (Fig.4). Differences between the cell types were not clearly noticed concerning the effects of the chemicals examined.

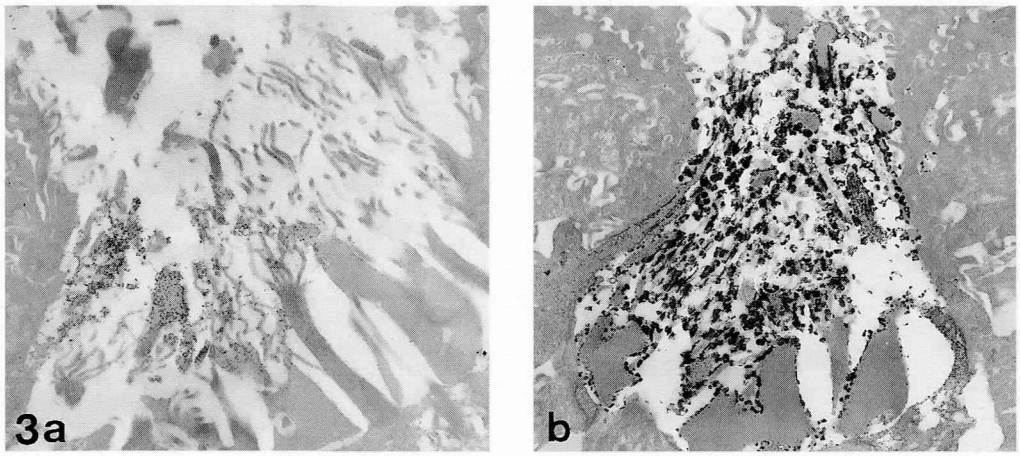

Fig.3 : The effect of Ca^{2+} on AMP–PNP–hydrolyzing enzymatic activity of rabbit taste bud cells. The tissue was prefixed with 2% paraformaldehyde and 1% glutaraldehyde for 60 min, and incubated with 0.2 mM AMP–PNP for 30 min at 37℃. (a) Control. Reducing the substrate concentration caused a considerable reduction of the reaction product (compare with Fig.1 a). (b) Addition of 5 mM $CaCl_2$ greatly increased the formation of the reaction product. ×8,000.

Discussion

Most of our previous studies on AMP–PNP–hydrolyzing enzyme used lead as a capture agent of phosphate released in the cytochemical reaction[1,2,5]. However, this metal formed precipitate in the incubation medium with dithiothreitol, an ATP pyrophosphatase inhibitor. Hence, in the present study, strontium was used as a capture metal ion, which basically gave the same localization pattern of the enzymatic activity as in the lead–based cytochemistry. The strontium–containing medium, on the other hand, tended to make precipitate with high concentration of NaF, so that 10 mM NaF could barely be used as in the present study. The strontium–based method has a disadvantage that it cannot be applied at a physiological pH of incubation media. However, its detection sensitivity to AMP–PNP–hydrolyzing enzymatic activity in rabbit taste buds is higher than the lead–based method and makes the enzymatic activity in the non–microvillous apical regions of the cells more observable[6].

AMP–PNP–hydrolyzing enzymatic activity was localized in the apical regions of taste bud cells, mainly on the microvilli. This is in contrast to ATPase activity, which is demonstrated both on the apical and the lateral membranes of the cells[1]. Our previous study using X–ray microanalysis of the cytochemical reaction product showed that the AMP–PNP–hydrolyzing enzyme in rabbit taste bud cells hydrolyzed AMP–PNP at the α and β bond of the phosphate chain, which led us to conclude that the enzyme was either adenylyl cyclase or ATP pyrophosphatase and not ATPase or alkaline phosphatase[1]. The catalytic site of adenylyl cyclase is known to exist on the inner surface of plasma membranes[21,28], while that of ATP pyrophosphatase on the outer surface[39]. In our previous study, which included an observation using a cross section of the microvilli[5], the cytochemical reaction product appeared to form on the outer surface of the plasma membrane. This implies that the enzyme is ATP pyrophosphatase.

In the present study, dithiothreitol and NaF inhibited AMP–PNP–hydrolyzing enzymatic activity.

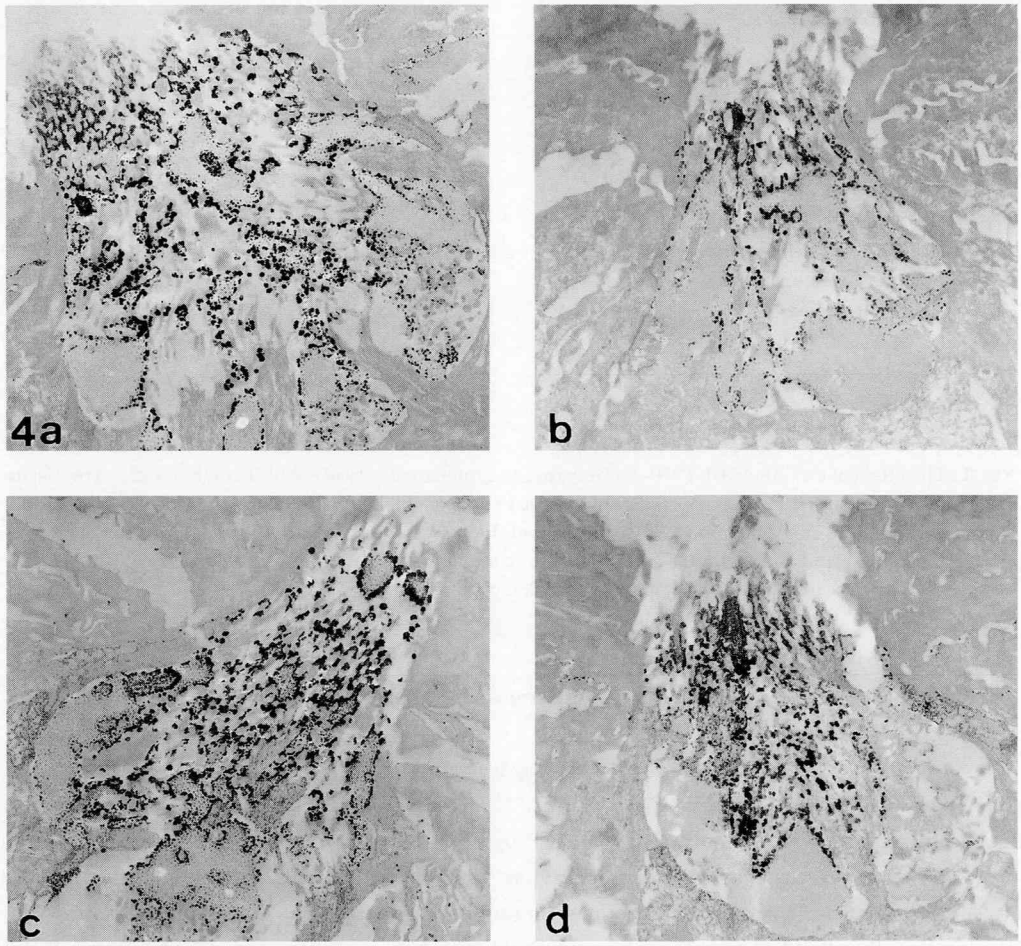

Fig.4 : The effects of adenylyl cyclase inhibitors and activator on AMP–PNP–hydrolyzing enzymatic activity of rabbit taste bud cells. The tissue was prefixed with 2% paraformaldehyde and 0.25% glutaraldehyde for 30 min, and incubated with 0.5 mM AMP–PNP for 30 min at 30℃. (a) Control(incubated in the standard incubation medium). (b) Incubated with 1 mM p–chloromercuriphenylsulfonic acid, an adenylyl cyclase inhibitor. (c) Incubated with 1 mM 5,5'–dithio–bis (2–nitrobenzoic acid), an adenylyl cyclase inhibitor. (d) Incubated with 200 μM forskolin, an adenylyl cyclase activator. The chemicals in (b)–(d) do not appear to affect the enzymatic activity. ×8,000.

These chemicals are reported not only to inhibit ATP pyrophosphatase but also activate adenylyl cyclase[15, 19, 27]. Although the incubation medium containing NaF was slightly turbid, the similar inhibition had been observed in the previous lead–based method, in which the medium was clear with NaF[4]. The AMP–PNP–hydrolyzing enzyme was also inhibited by amiloride, another inhibitor of ATP pyrophosphatase[35]. Hence, it is suggested that the enzyme in question is ATP pyrophosphatase. That inhibitors and an activator of adenylyl cyclase failed to affect the enzyme also supports this notion.

The AMP–PNP–hydrolyzing enzymatic activity was not prevented by NaN_3, which is known to inhibit apyrase[20, 25], an enzyme that hydrolyzes ATP into AMP and two orthophosphates. Hence, the activity is unlike that of apyrase. It is reported that NaN_3 mildly stimulates ATP pyrophos-

phatase[9,19] but this effect was not clear in the present study. This may be because its stimulating effect was not large enough (30–45% stimulation with 10–30 mM NaN_3 [9,19]) to be clearly demonstrated in cytochemistry.

Since we had previously been examining adenylyl cyclase activity in taste buds, we had usually added in the incubation medium Mg^{2+}, a metal ion required for adenylyl cyclase activation[10,18]. In the present study, omission of $MgCl_2$ in the incubation medium did not decrease the reaction product, which might suggest that Mg^{2+} is not needed for activation of the AMP–PNP–hydrolyzing enzyme. However, in our previous study by a lead–based method, preincubation of the tissue with EDTA, a chelating agent for divalent cations, resulted in inactivation of the enzyme even though the chemical had been rinsed out before incubation[3]. Therefore, although not identified yet, some divalent cation must be needed for activation of the enzyme. It is possible that some endogenous divalent cation is tightly bound to the enzyme, making the enzyme a metalloenzyme, which no longer requires excess metal ion for catalytic activity[11]. Since Ca^{2+} enhanced the enzymatic activity in the present study, this endogenous ion may be Ca^{2+}, and the enzyme may be Ca^{2+} –dependent ATP pyrophosphatase, as reported by Flodgaard and Torp–Pedersen[13].

Nucleotides such as ATP and ADP are extracellular signaling substances in a wide range of tissues, hence some ecto–nucleotidases such as ecto–ATPase and apyrase are now being intently studied as enzymes controlling such signaling[26,38,39]. ATP pyrophosphatase is also an ecto–nucleotidase but its physiological role is not yet understood despite its early discovery[14] and its broad distribution in various tissues[34]. It is possible that this enzyme controls nucleotide–mediated signaling but no evidence has been obtained. Although some of the enzymatic activity was observed in non–microvillous apical regions of taste bud cells, its major distribution on the microvilli is notable and we observed a similar enzymatic activity existing also on the microvilli of the duct of von Ebner's glands[3]. Torp–Pedersen et al.[34] reported, using biochemical methods, that high levels of activity of this enzyme were observed in the salivary gland, liver, kidney and small intestine. Since all of these tissues possess microvilli, it is also possible that this enzyme plays some unknown role in the functions of microvilli or is involved in metabolism of microvilli in general. Further study including cytochemistry in those tissues is, of course, needed to confirm such a possibility.

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抄録：ウサギ味蕾細胞における AMP-PNP 加水分解酵素の細胞化学的研究：ATP ピロホスファターゼおよびアデニル酸シクラーゼの抑制物質・賦活物質の効果

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ウサギ味蕾細胞の先端部，特に微絨毛表面には ATP 類似物質である 5'-アデニリルイミド 2 リン酸（AMP-PNP）を加水分解する酵素活性が見られる．以前の研究より，この酵素は ATP ピロホスファターゼまたはアデニル酸シクラーゼのいずれかと考えられるので，これらの酵素を抑制または賦活すると報告されている物質を添加して，ウサギ葉状乳頭切片上で AMP-PNP を基質とする細胞化学反応を行わせ，その効果を調べた．酵素活性は ATP ピロホスファターゼの抑制物質（ジチオトレイトール，フッ化ナトリウム，アミロライド）で抑制されたが，アデニル酸シクラーゼの抑制物質（p-クロロメルクリスルホン酸，5, 5'-ジチオビス（2-ニトロ安息香酸））や賦活物質（フォルスコリン）では変化しなかった．ATP ピロホスファターゼを若干賦活させるといわれるアジ化ナトリウムは明確な賦活効果が認められなかったが，今回の結果を総合すると，本酵素は ATP ピロホスファターゼと考えられる．さらに Ca^{2+} による活性増大が見られたことから，Ca^{2+} 依存性 ATP ピロホスファターゼではないかと思われる．