

# Cytochemical Study of the 5'-Adenylylimidodiphosphate-Hydrolyzing Enzyme Activity in the Microvilli of Taste Bud Cells in Rabbits : Estimation of the number of the catalytic sites

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## Summary

An enzymatic activity in the microvilli of rabbit taste bud cells, which is able to hydrolyze the ATP analogue, 5'-adenylylimidodiphosphate, was studied using a lead-based cytochemical method. The reaction product appeared as round electron-dense granules on the microvillous membrane. The size of the granules increased proportionally to the incubation period, while the number of the granules remained constant (approximately 20 granules/ $\mu\text{m}^2$  of the microvillous membrane). Varying the tissue-fixatives also had no effect on the number of the granules. These results indicate that a limited number of catalytic sites of the enzyme, which are presumably represented by the loci of granule formation, are evenly spaced in the membrane of the microvilli.

## Introduction

5'-Adenylylimidodiphosphate (AMP-PNP) is an ATP analogue in which an imino group replaces the terminal bridge oxygen of the triphosphate chain. Our previous cytochemical study<sup>1)</sup> demonstrated that in the microvilli of rabbit taste bud cells, there exists an enzymatic activity which is able to hydrolyze this ATP analogue. The reaction product was formed into round electron-dense granules, which were scattered on the microvillous membrane. Hence, we hypothesized that their location may represent the catalytic sites of the enzyme. If so, it is expected that the number of the granules should stay constant regardless of experimental conditions. In the present study, we examined the number and the size of those granules by varying the composition of the tissue-fixatives and the incubation period. We report that the size of the granules changes, while the number of the granules (granules/unit area of the membrane) remains unchanged regardless of the various experimental conditions.

In the previous study<sup>1)</sup>, we carried out our experiments using tissue blocks (0.5-1 mm thick).

Since it has been suggested that the use of tissue blocks may cause a false reaction<sup>2,3)</sup>, we used in the present study tissue sections (40  $\mu$ m thick) along with the tissue blocks and compared the results obtained with the previous results.

### Materials and Methods

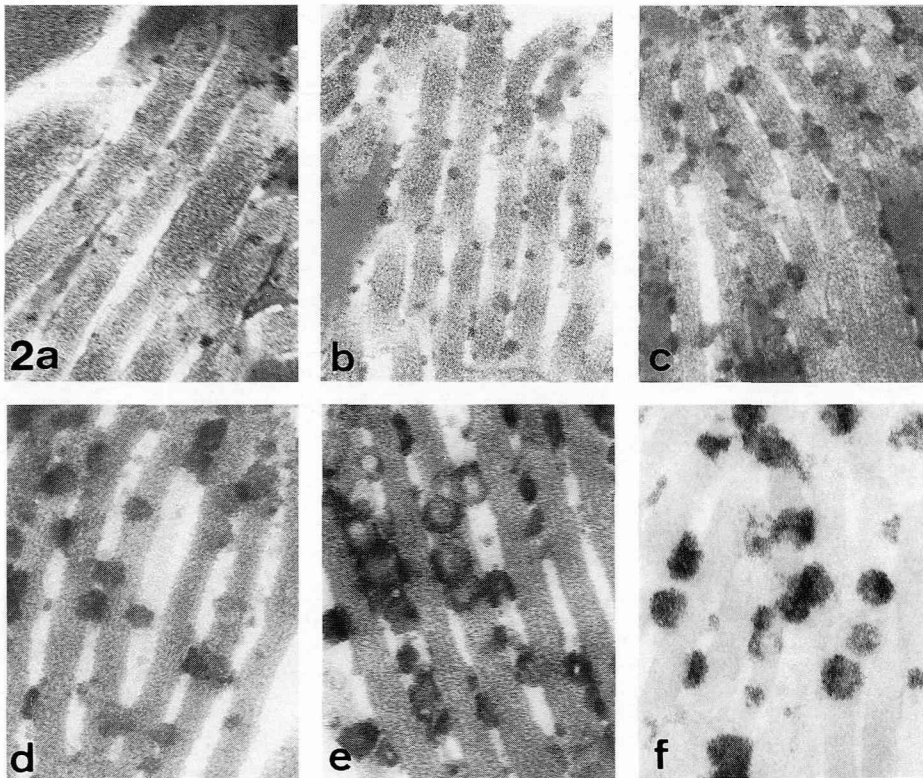
Foliate papillae were removed from white rabbits (2-3 kg) immediately after sacrificing by intravenous injection of an overdose of sodium amobarbital (100-200 mg/kg body wt.). They were rinsed briefly in a cold physiological saline solution (Hikari Seiyaku Co., Ltd., Tokyo, Japan) and fixed for 1 h at 5 °C in either 1% glutaraldehyde or a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde, both of which were dissolved in 0.1 M sodium cacodylate buffer, pH 7.4. Glucose (0.15 M) was added to the former solution to adjust the osmotic pressure. During the fixation, the papillae were cut into 0.5-1 mm thick blocks with a thin razor blade. Then, the blocks were rinsed overnight at 5 °C in 0.1 M sodium cacodylate buffer/0.25 M glucose and incubated for periods of 15, 30 or 60 min at 30 °C in a medium containing 80 mM Tris-maleate, pH 7.4, 0.45 M glucose, 2 mM theophylline (phosphodiesterase inhibitor), 4 mM magnesium sulfate, 1 or 0.5 mM L-p-bromotetramisole (nonspecific alkaline phosphatase inhibitor, Aldrich Chemical Co., Inc., Milwaukee, WI, USA), 0.5 mM AMP-PNP (tetralithium salt, Boehringer Mannheim GmbH, Mannheim,



**Fig. 1:** AMP-PNP hydrolyzing enzyme activity in the apical region of rabbit taste bud cells. After fixation with 2% paraformaldehyde + 2.5% glutaraldehyde for 1 h, the tissue was cut into 40  $\mu$ m thick sections and incubated for 30 min with AMP-PNP as substrate and lead as the capture agent of the released phosphate. The cytochemical reaction product is seen as round electron-dense granules on the microvilli of the taste bud cells, which is the same pattern as obtained in the previous study, in which tissue blocks were used (refer to Asanuma and Nomura, *Chem. Senses* 7, 1-9, 1982). Stained with uranyl acetate.  $\times 8,000$ .

Germany, or Sigma Chemical Co., St. Louis, MO, USA) and 4 mM lead nitrate. After incubation, the tissue blocks were rinsed briefly in 0.1 M cacodylate buffer/0.25 M glucose, postfixed for 2 h at 5 °C in 1% osmium tetroxide in the same buffer containing 0.25 M glucose, dehydrated in ethanol and propylene oxide, and embedded in Epon 812. Thin sections were cut on an LKB 4800 microtome, stained with uranyl acetate and lead acetate or uranyl acetate alone, and examined in a JEOL 100B electron microscope.

Some foliate papillae were cut into tissue sections and incubated. After fixation for 1 h at 5 °C in 2% paraformaldehyde + 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.4, or in 2% paraformaldehyde + 0.25% glutaraldehyde in the same buffer containing 0.25 M glucose, the papillae were rinsed for several hours at 5 °C in the cacodylate buffer/0.25 M glucose, and cut into 40 µm thick sections with a Microslicer (DTK 1000, Dosaka EM Co., Ltd., Kyoto, Japan). The sections were stored overnight at 5 °C in the above rinse solution. The sections were then incubated as mentioned above except that the concentrations of glucose and lead nitrate were reduced to 0.25 M and 2 mM, respectively. The tissue prefixed with 2% paraformaldehyde + 0.25% glutaralde-



**Fig. 2:** AMP-PNP hydrolyzing enzyme activity in the microvilli of rabbit taste bud cells. (a) The tissue was fixed with 2% paraformaldehyde + 2.5% glutaraldehyde and incubated for 15 min. (b) Fixed with 2% paraformaldehyde + 2.5% glutaraldehyde and incubated for 30 min. (c) Fixed with 2% paraformaldehyde + 2.5% glutaraldehyde and incubated for 60 min. (d) Fixed with 1% glutaraldehyde and incubated for 30 min. (e) Fixed with 1% glutaraldehyde and incubated for 60 min. (f) Fixed with 2% paraformaldehyde + 0.25% glutaraldehyde and incubated for 30 min. Fixation periods were all 1 h. (a)–(e) Stained with uranyl acetate and lead acetate. (f) Stained with uranyl acetate.  $\times 38,000$ .

hyde was further refixed in 3% glutaraldehyde in 0.05 M cacodylate buffer for 1 h at 5 °C after incubation and rinsed in the same buffer containing 0.25 M glucose. Postfixation of the 40  $\mu\text{m}$  thick sections was carried out for 30 min at 5 °C in 1% osmium tetroxide in 0.05 M cacodylate buffer/0.25 M glucose. The rest of the procedure was the same as above.

## Results

The cytochemical reaction product of the AMP-PNP hydrolyzing enzyme activity using the 40  $\mu\text{m}$  thick sections of the rabbit foliate papillae appeared in the same manner as in the previous study<sup>1)</sup> in which tissue blocks had been used: the reaction product was formed as round electron-dense granules on the microvillous membrane of the taste bud cells (Fig. 1).

When the tissue-fixatives or the incubation periods were varied, the size of the granules was also varied, but the distance between the granules seemed to be unchanged (Fig. 2).

Table 1 clearly shows how the size of the granules changed according to the tissue-fixatives and the incubation periods. The volumes of the granules were calculated from the diameters of the granules by assuming the granules being globose. It can be seen that the volume of the granules is in proportion to the period of incubation, i.e. the mean volume doubled when the incubation period was doubled. In the tissue fixed with 2% paraformaldehyde + 2.5% glutaraldehyde, however, the volume tripled when the incubation period was extended from 30 min to 60 min. This 'excess' increase is probably due to diffusion of the reaction product. With the same incubation period, the largest granules were observed in the tissue fixed with 2% paraformaldehyde + 0.25% glutaraldehyde, and the smallest in the tissue fixed with 2% paraformaldehyde + 2.5% glutaraldehyde. In contrast to the volume change of the granules, the distances between the granules, which were measured between the neighboring granules on the same side of a microvillus, remained unchanged. The mean distance was 0.14  $\mu\text{m}$  regardless of the difference in the prefixing solutions or in the incubation periods.

Fig. 3 is an electron micrograph of a semi-thin (light gold), transverse section of the microvilli on which the granules are seen. The number of the granules on each transversely-sectioned microvillus was counted as equal to the ratio of the number of the granules to the number of the microvilli in a micrograph. The mean number thus counted was  $0.62 \pm 0.29$  (mean  $\pm$  S.D.,  $n = 50$ ,

**Table 1:** The distance between and the size of the electron-dense granules on the microvilli of taste bud cells

Experimental condition		Distance between the granules* [ $\mu\text{m}$ ]	Diameter of the granules** [nm]	Volume of the granules** [ $\text{nm}^3$ ]
Fixation	Incubation			
2 % paraformaldehyde + 2.5% glutaraldehyde (1h)	15 min	$0.14 \pm 0.08$	$25 \pm 10$	$12,600 \pm 17,100$
	30 min	$0.14 \pm 0.07$	$33 \pm 12$	$26,400 \pm 29,600$
	60 min	$0.14 \pm 0.05$	$49 \pm 15$	$81,300 \pm 96,600$
1 % glutaraldehyde (1h)	30 min	$0.14 \pm 0.07$	$69 \pm 21$	$225,800 \pm 231,600$
	60 min	$0.14 \pm 0.06$	$89 \pm 28$	$479,900 \pm 492,900$
2 % paraformaldehyde + 0.25% glutaraldehyde (1h)	30 min <sup>§</sup>	$0.14 \pm 0.06$	$92 \pm 25$	$503,800 \pm 454,100$

Expressed as mean  $\pm$  S.D. \* $n=200$ ; \*\* $n=1,000$ ; <sup>§</sup> lead nitrate 2 mM.

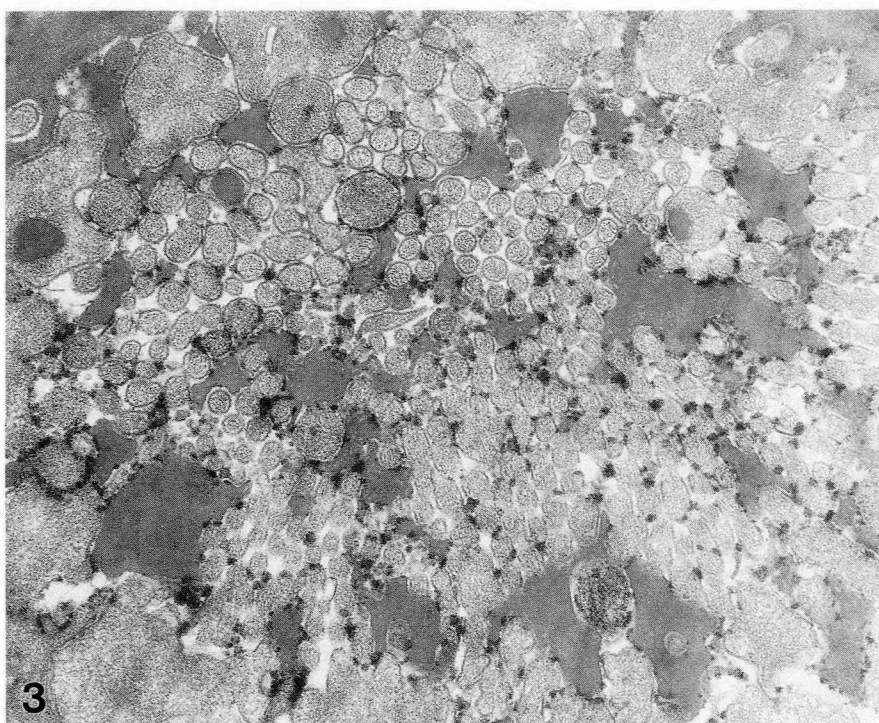
Volumes of the granules were calculated from the diameters by assuming the granules being globose.

i.e. counted in 50 micrographs). Since the thickness of each section can be assumed to be approximately  $0.1\ \mu\text{m}$ , this value indicates the mean number of the granules lying on  $0.1\ \mu\text{m}$  length of a microvillus. And since we found that the mean diameter of the microvilli was  $100 \pm 14\ \text{nm}$  (mean  $\pm$  S.D.,  $n = 2,000$ ), the number of the granules/unit area of the microvillous membrane was calculated to be approximately  $20/\mu\text{m}^2$ .

### Discussion

The present results concerning the size of the electron-dense granules, which represent the cytochemical reaction product, and the distance between the granules on the microvilli of the taste bud cells were obtained mostly using tissue blocks. It is often mentioned that the use of such tissue blocks for cytochemical incubation may result in a false reaction<sup>2,3)</sup>. However, since the AMP-PNP hydrolyzing enzyme activity gave the same localization pattern both by using tissue blocks ( $0.5\text{--}1\ \text{mm}$  thick) and tissue sections ( $40\ \mu\text{m}$  thick), the results obtained in the present study are considered to be reliable.

The volume of the granules increased proportionally to the incubation period. This suggests that the reaction product was constantly released and accumulated on the sites where the granules were formed and that these sites represent the catalytic sites of the enzyme. Higher concentration of glutaraldehyde in tissue-prefixation decreased the volume of the granules. This suggests that the remaining enzymatic activity was reflected on the volume of the granules, and again supports the



**Fig. 3:** AMP-PNP hydrolyzing enzyme activity in the transversely-sectioned microvilli of rabbit taste bud cells. The tissue was fixed for 1 h with 2% paraformaldehyde + 2.5% glutaraldehyde and incubated for 30 min. The thickness of the section is assumed ca.  $0.1\ \mu\text{m}$ . Stained with uranyl acetate and lead acetate.  $\times 38,000$ .

idea that the sites where the granules appeared represent the sites of the enzymatic reaction. In contrast to the volume change of the granules, the number of the granules remained unchanged regardless of the difference in the incubation period or in the tissue-fixing solution. This implies that a limited number of catalytic sites of the enzyme are evenly spaced in the microvillous membrane. The number of the granules, which was calculated to be approximately  $20/\mu\text{m}^2$  of the membrane, may be the number of the catalytic sites of the enzyme.

What is the AMP-PNP hydrolyzing enzyme? Ever since Rodbell et al.<sup>4)</sup> reported that AMP-PNP was hydrolyzed by adenylate cyclase but not by usual group of membrane-bound ATPases, this substance has been widely used as a specific substrate for adenylate cyclase studies. We also thought at first that the AMP-PNP hydrolyzing enzyme activity in the microvilli of the taste bud cells represented adenylate cyclase activity<sup>1)</sup>. However, it has recently been shown that various ATPases can hydrolyze AMP-PNP<sup>6-7)</sup>. Furthermore, ATP pyrophosphohydrolase (=ATP pyrophosphatase)<sup>8,9)</sup> and alkaline phosphatase<sup>10,11)</sup> also have been shown to hydrolyze the substance. Hence, one must be cautious in identifying enzymes that hydrolyze AMP-PNP. We performed an X-ray microanalysis of the cytochemical reaction product of the AMP-PNP hydrolyzing enzyme activity on the microvilli of rabbit taste bud cells, and found that the enzyme splits AMP-PNP into AMP (either cyclic AMP or 5'-AMP) and imidodiphosphate<sup>12)</sup>. Therefore, the enzyme is considered to be adenylate cyclase<sup>4)</sup> or ATP pyrophosphohydrolase<sup>8,9)</sup>, and not ATPase or alkaline phosphatase, both of which split AMP-PNP into ADP-NH<sub>2</sub> and orthophosphate<sup>6,7,10)</sup>. Although further identification of the enzyme has not been achieved yet, the discovery of the unique localization pattern of the enzyme is an attractive opening to the investigation of its function in taste bud cells.

### Conclusions

The cytochemical reaction product of the AMP-PNP hydrolyzing enzyme activity was scattered as round electron-dense granules on the microvilli of rabbit taste bud cells. The number of the granules (ca.  $20/\mu\text{m}^2$  of the microvillous membrane), which was constant regardless of the variation in the tissue-fixatives or in the cytochemical incubation periods, seems to represent the number of the catalytic sites of the enzyme.

### Acknowledgment

We thank Mr. M. Nol for his aid concerning the language.

抄録：ウサギ味蕾細胞微絨毛におけるアデニルイミド 2 リン酸加水分解酵素活性の細胞化学的研究：触媒部位数の算定

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ウサギ味蕾細胞の微絨毛には、ATP 類似物質であるアデニルイミド 2 リン酸を加水分解する酵素活性が見られる。今回、この酵素活性について、鉛を捕捉金属とする細胞化学により検討を加えた。

反応産物は、微絨毛膜上に電子不透過性の丸い顆粒として認められた。顆粒の大きさは細胞化学的浸漬時間に比例して増大したが、顆粒の数は、微絨毛表面積  $1 \mu\text{m}^2$  当たり約 20 個で、変化が見られなかった。顆粒の数は、組織の固定液の組成を変えても一定のままであった。これらの結果は、この酵素の触媒部位が微絨毛膜に一定の割合で存在し、おそらく顆粒の形成された場所に一致するだろうということを示している。

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