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Cytochemical Study on Guanylyl Cyclase Activity in Rabbit Taste Bud Cells: Effects of i) cadmium, mercury and zinc ions, and ii) concentration of exogenous manganese

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Summary

Some characteristics of the membrane-bound guanylyl cyclase activity in taste bud cells of rabbit foliate papillae were cytochemically examined. The enzymatic activity was almost completely inhibited by 1 mM CdCl_2 , HgCl_2 or ZnCl_2 and recovered by simultaneous addition of 2 mM dithiothreitol, indicating the presence of sulfhydryl groups required for enzyme activity. The activity appeared to be the same whether 1 mM or 3 mM MnCl_2 was added. It was even observed to a similar extent in the absence of exogenous Mn^{2+} ; a formation of a 'metalloenzyme' during the experimental procedure was hinted at.

Introduction

Guanylyl cyclase activity has been demonstrated in the apical portion of rabbit foliate taste bud cells, suggesting the involvement of cyclic GMP in vertebrate taste transduction^{1~4)}. This enzymatic activity seemed to be that of a membrane-bound form of guanylyl cyclase^{3,4)} and was only slightly enhanced by soluble guanylyl cyclase activators (sodium azide, hydroxylamine or sodium nitroprusside)³⁾. The purpose of the present study is to further clarify the characteristics of this enzymatic activity by, firstly, adding Cd^{2+} , Hg^{2+} or Zn^{2+} in the cytochemical incubation medium. The effects of these ions alone had already been investigated⁴⁾, but in the present study, their effects were examined in the presence as well as in the absence of dithiothreitol, a potent sulfhydryl group protector⁵⁾, because those metal ions oxidize sulfhydryl groups and form mercaptides⁶⁾. Secondly, we examined the effect of a concentration of added manganese, which is a cation cofactor for guanylyl cyclase activation⁷⁾.

Materials and Methods

Foliate papillae were removed from male white domestic rabbits (1.6-3.3 kg), which had been killed by an intravenous injection of a fatal dose of sodium amobarbital (100-200 mg/kg body weight). After a brief rinse in an ice-cold physiological saline solution containing 0.9% NaCl and 5 mM HEPES, pH 7.2, the papillae were cut into several pieces and fixed for 30 min on ice in 2%

paraformaldehyde + 0.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.4, containing 0.15 M NaCl. After rinsing for several hours in ice-cold 0.05 M cacodylate buffer containing 0.15 M NaCl with several changes of the solution, the tissue was cut into 0.1-0.2 mm thick sections with a thin razor blade and stored overnight at 0-6°C in the same solution.

The incubation procedure was based on the strontium method, originally described by Ernst for ATPase cytochemistry⁹⁾. To examine the effects of Cd²⁺, Hg²⁺ and Zn²⁺, the tissue sections were preincubated for 10-20 min at room temperature in a medium containing 80 mM Tris-HCl, pH 9.1, 0.11 M NaCl, 5 mM theophylline (cyclic nucleotide phosphodiesterase inhibitor⁹⁾), 2.5 mM levamisole (nonspecific alkaline phosphatase inhibitor¹⁰⁾) and 10 mM SrCl₂, to which was added either 1 mM CdCl₂, HgCl₂ or ZnCl₂. The metals were added singly or together with 2 mM dithiothreitol. The media were clear except that a slight amount of precipitation was formed when HgCl₂ and dithiothreitol were added together. Incubation was initiated by adding to those media 1 mM MnCl₂ and 0.5 mM 5'-guanylylimidodiphosphate (substrate for guanylyl cyclase¹¹⁾) and warming the media to 37°C in a shaking water bath. Incubation without substrate was also carried out. Incubation was continued for 30 min at this temperature. To examine the effect of the concentration of exogenous manganese, the tissue sections were preincubated for 15-20 min at room temperature in a similar preincubation medium as above except that 5 mM dithiothreitol was added. Then, 0.5 mM 5'-guanylylimidodiphosphate was added to this medium without or with either 1 or 3 mM MnCl₂ or 4 mM MgCl₂, and the tissue was incubated at 37°C for 30 min. The final pH of all the incubation media ranged from 8.7-9.0.

Incubated sections were cooled on ice and rinsed three times in 80 mM Tris-HCl buffer, pH 9.1, containing 0.23 M sucrose; next, twice in 2% lead nitrate solution containing 0.15 M sucrose. The purpose of this treatment was to convert precipitated strontium phosphate, i.e. the cytochemical reaction product, into lead phosphate for better visualization in the electron microscope. The sections were then rinsed three times in 0.30 M sucrose solution and twice in 0.05 M cacodylate buffer, pH 7.4, containing 0.25 M sucrose. The rinses were carried out at room temperature and each rinse period was 5-10 min. The sections were postfixed for about 1 h on ice in 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 812. Ultrathin sections were cut from near-surface portions of the tissue sections using an LKB 2088 microtome and examined unstained in a JEOL 1200EX-II electron microscope operated at 80 kV.

5'-Guanylylimidodiphosphate (sodium salt), dithiothreitol, theophylline and levamisole were obtained from Sigma Chemical Co., St. Louis, MO, USA; paraformaldehyde, sodium cacodylate, Tris(hydroxymethyl)-aminomethane, HEPES, lead nitrate and propylene oxide (all specially prepared reagent grade) from Nacalai Tesque, Inc., Kyoto, Japan; glutaraldehyde, osmium tetroxide and Epon 812 from TAAB Laboratories Equipment, Ltd., Reading, UK. All other materials were of guaranteed reagent grade from Nacalai Tesque or Kanto Chemical Co., Inc., Tokyo, Japan.

Results

Figure 1 shows the electron-dense precipitate, i.e. the cytochemical reaction product of guanylyl cyclase activity, being attached to the apical plasma membrane of the taste bud cells. As previously reported⁹⁾, this activity was strongly inhibited by 1 mM CdCl₂, HgCl₂ or ZnCl₂ (Fig. 2a, c and e). However, when 2 mM dithiothreitol was added in the incubation medium together with these metals, the activity was almost completely recovered (Fig. 2b, d and f).

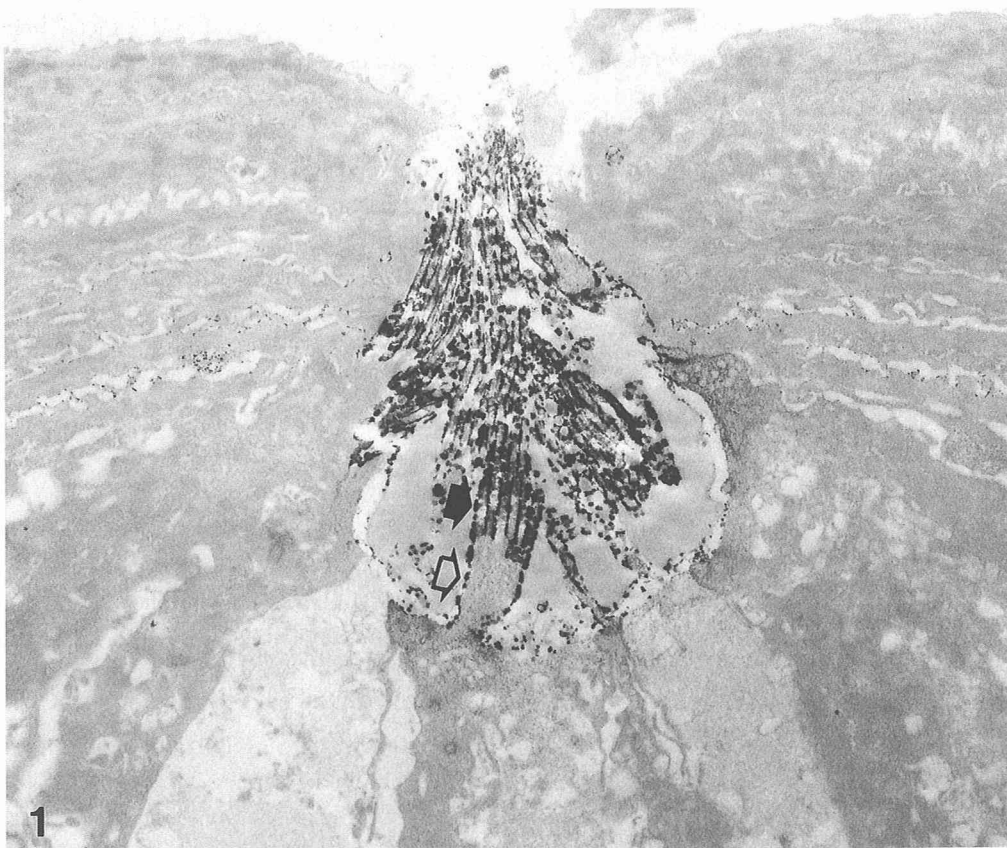


Fig. 1: Guanylyl cyclase activity in the apical region of rabbit foliate taste bud cells. Electron-dense precipitate, which represents the cytochemical reaction product of the enzymatic activity, is seen on the plasma membrane of the microvilli (filled arrow) and neck region (open arrow) of the cells. $\times 9,000$.

There was no difference in the amount of the cytochemical reaction product whether 1 mM or 3 mM MnCl_2 was added in the incubation medium (Fig. 3a and b). We could not test higher concentrations of manganese since white precipitate tended to form in the incubation medium. Replacing MnCl_2 by 4 mM MgCl_2 usually did not affect the amount of the reaction product (Fig. 3c). On some occasions, however, the reaction product decreased to some extent. To our surprise, incubation without exogenous Mn^{2+} or Mg^{2+} did not eliminate nor even reduce the reaction product (Fig. 3d). Incubation without exogenous substrate resulted in total absence of the reaction product (not shown).

Discussion

Cd^{2+} , Hg^{2+} and Zn^{2+} oxidize sulfhydryl groups of proteins and affect various enzymatic activities⁶⁾. The inhibitory effect of those metal ions on guanylyl cyclase activity and its prevention by sulfhydryl protectors such as dithiothreitol was already reported in early studies of this enzyme¹²⁾. Subsequently, it became elucidated that activation of a soluble form of guanylyl cyclase requires interactions between substrate and sulfhydryl groups at its catalytic site^{13~17)}. Compared to

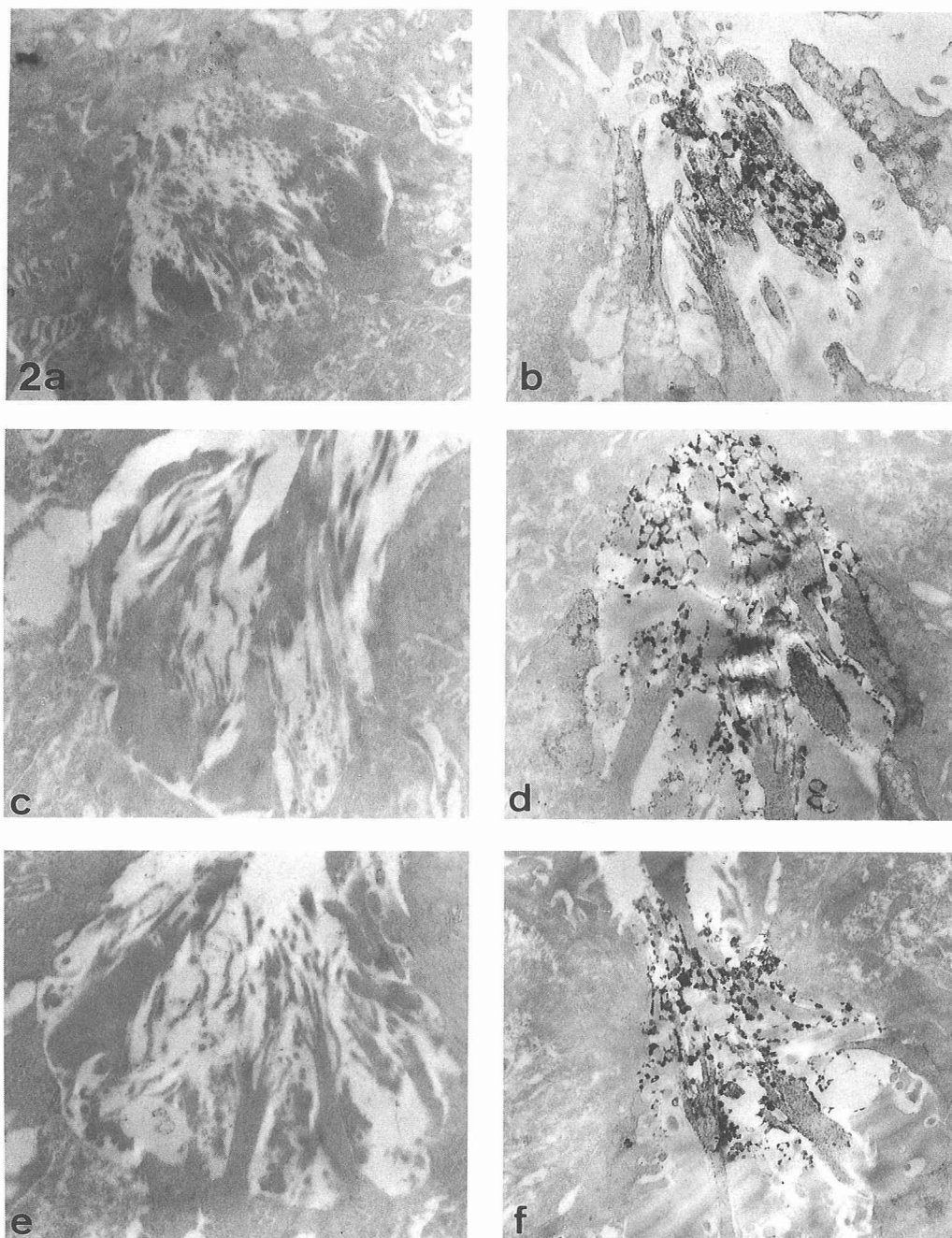


Fig. 2: The effect of Cd^{2+} , Hg^{2+} and Zn^{2+} on the guanylyl cyclase activity in the taste bud cells. (a) Incubated with 1 mM CdCl_2 . (b) Incubated with 1 mM CdCl_2 + 2 mM dithiothreitol. (c) Incubated with 1 mM HgCl_2 . (d) Incubated with 1 mM HgCl_2 + 2 mM dithiothreitol. (e) Incubated with 1 mM ZnCl_2 . (f) Incubated with 1 mM ZnCl_2 + 2 mM dithiothreitol. Note that the enzymatic activity is inhibited by the metal ions (a, c, and e) but recovers with the addition of dithiothreitol (b, d, and f). $\times 9,000$.

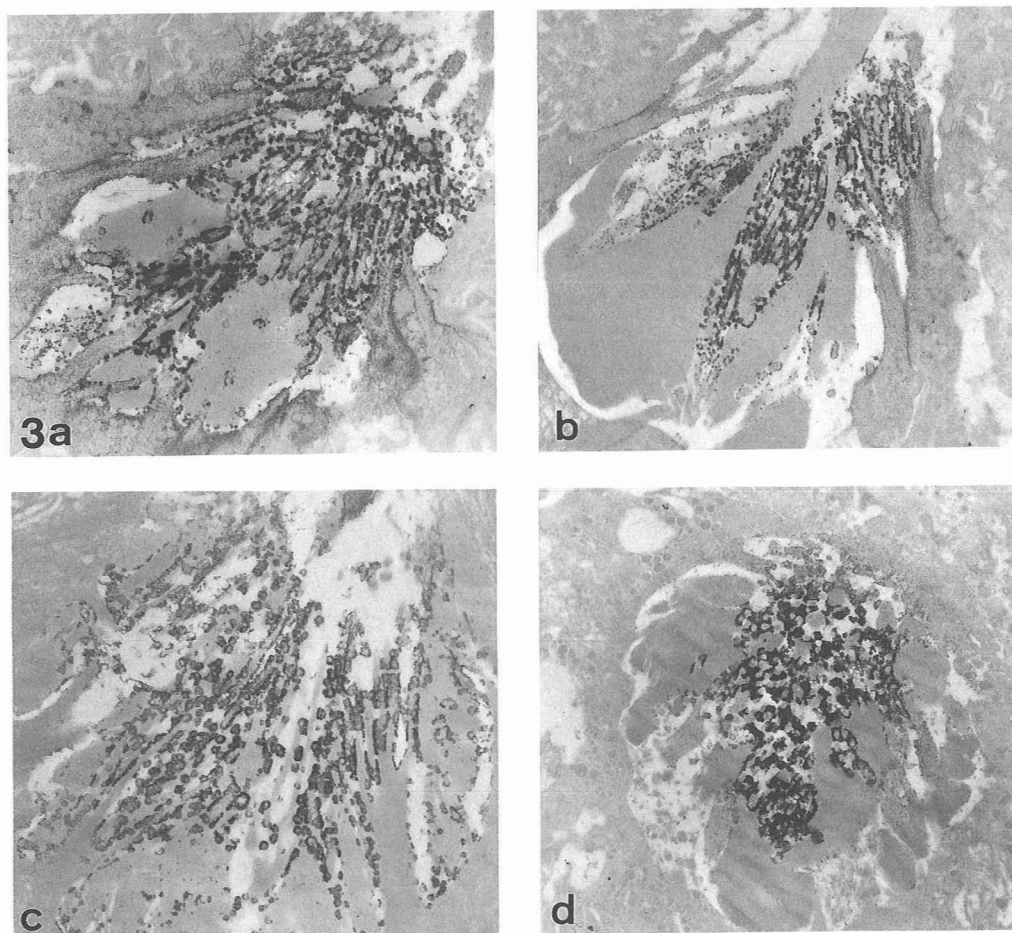


Fig. 3 : The effect of exogenous Mn^{2+} and Mg^{2+} on the guanylyl cyclase activity in the taste bud cells. (a) Incubated with 1 mM $MnCl_2$. (b) Incubated with 3 mM $MnCl_2$. (c) Incubated with 4 mM $MgCl_2$. (d) Incubated without exogenous Mn^{2+} or Mg^{2+} . Note that the amount of the cytochemical reaction product appears the same through (a) to (d). $\times 9,000$.

the soluble enzyme, the role of sulfhydryl groups in regulating membrane-bound guanylyl cyclase activity is less well understood¹⁷⁾. Some researchers have reported that $CdCl_2$ of the similar concentration as used in the present study did not inhibit or only slightly inhibited the membrane-bound enzyme in rat liver^{13,14)}.

The enzymatic activity demonstrated in the present study is considered that of membrane-bound guanylyl cyclase^{3,4)}. However, this activity was almost completely inhibited by 1 mM $CdCl_2$ in contrast with the enzyme in rat liver. One explanation for such a discrepancy is that the characteristics of the enzyme are different between the two tissues. Another possible explanation is that it is due to the difference in experimental conditions. Since the structure of the catalytic domain itself is similar in two different forms of guanylyl cyclase¹⁸⁾, it is not surprising that the membrane-bound enzyme lost its activity when its sulfhydryl groups were oxidized by Cd^{2+} . Those who did not observe the inhibition by Cd^{2+} might have conducted their experiments in such conditions that hindered the metal ion to oxidize sulfhydryl groups of the enzyme. In fact, Waldman

et al.¹⁹⁾ reported the inhibition of membrane-bound guanylyl cyclase from rat liver by mixed disulfide formation with cystamine, indicating the presence of sulfhydryl groups required for enzyme activity.

The enzymatic activity appeared the same whether 1 mM or 3 mM MnCl_2 was added. It was even observed to a similar extent in the absence of exogenous Mn^{2+} . This was a surprise since both membrane-bound and soluble guanylyl cyclases require Mn^{2+} as a cofactor of the enzyme activation²⁰⁾. The reason why exogenous Mn^{2+} was unnecessary is unknown but fixing the tissue might have tightly bound endogenous Mn^{2+} to the enzyme, thus making the enzyme a metalloenzyme, which no longer requires excess Mn^{2+} for catalytic activity²¹⁾. This may explain why substituting MgCl_2 for MnCl_2 did not affect the enzymatic activity even though Mg^{2+} cannot replace Mn^{2+} in activating membrane-bound guanylyl cyclase²¹⁾. Since the reaction product decreased at times by the addition of MgCl_2 , Mg^{2+} might rather inhibit the enzymatic activity.

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抄録：ウサギ味蕾細胞グアニル酸シクラーゼ活性の細胞化学的研究：

i) カドミウム、水銀、亜鉛イオンの影響、および ii) 添加マンガン濃度の影響

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ウサギ葉状乳頭味蕾細胞の膜結合性グアニル酸シクラーゼ活性の性質を細胞化学的手段により調べた。酵素活性は1 mM CdCl₂, HgCl₂またはZnCl₂の添加によって殆ど完全に阻害されたが、2 mM ジチオスライトールを同時に与えると回復した。このことから、活性にはSH 基が必要と思われる。グアニル酸シクラーゼ活性の発現にはMn²⁺が必要と言われるが、反応溶液に1 mM あるいは3 mM MnCl₂を添加した場合、両方で酵素活性に変化はなく、更にはMnCl₂を添加しなくとも同程度に見られた。この理由は不明であるが、試料を固定する等の実験操作が、酵素に内在性 Mn²⁺を結合させて metalloenzyme の状態を作り出した可能性もある。