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The Action Mechanism of several Ginsenosides in their Regulatory Action on the Activities of Adenylate Cyclase and Guanylate Cyclase

Kih Lim Seo, Jong Geon Moon, Mi Kyung Cha, Kwang Seung Lee*, Se Yong Lee, Youn Young Lee, and Inwon Park**
*Department of Chemistry, Seoul National University, *Ginseng and Tobacco Research Institute, **Department of Agricultural Chemistry, Korea University, Seoul, Korea*
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몇가지 진세노시드들의 아데닐산 고리화 효소와 구아닐산 고리화 효소의 활동성들에 대한 조절작용에 있어서의 작용 메카니즘

서기림 · 문종건 · 차미경 · 이광승* · 이세영** · 이윤영 · 박인원
서울대학교 화학과, *인삼연초연구소, **고려대학교 농화학과
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Abstract

The effects of the five ginsenosides on the activities of particulate adenylate cyclase and particulate guanylate cyclase of rat brain have been studied. The range of concentrations of ginsenosides were between 10 *ug* and 500 *ug* per 500 *ul* reaction mixture. Also, the effects of three ginsenosides on the activity of soluble guanylate cyclase have been studied in the same range of concentrations as in particulate guanylate cyclase. Only ginsenoside Re has shown the reciprocal effects when tested with particulate adenylate cyclase and particulate guanylate cyclase.

Regulatory action of the several mononucleotides on the activities of adenylate cyclase and guanylate cyclase was examined. Ginsenoside Rd-inhibited adenylate cyclase was activated in great extent by the addition of increasing amount of GMP. On the other hand, ginsenoside Rc-activated guanylate cyclase was inhibited by the addition of increasing amount of AMP and GMP. The fact that the stimulatory action of GMP is observed only with particulate adenylate cyclase but not with soluble guanylate cyclase suggests that the action is membrane-related one.

The competitive action was observed between ginsenoside Rb2 and dopamine in their binding to the receptors. This result is clear-cut evidence that the ginsenoside Rb2 binds specifically to β-adrenergic receptors.

I. Introduction

As we have presented elsewhere[1], some of the ginsenosides have shown the opposite effects on adenylate cyclase and guanylate cyclase of rat brain. Of the ginsenosides we had tested Ra, Rb1 and Rc had shown to have this property. We have concluded on the basis of our previous results that this kind of ginsenosides may play the role of metabolic modulators or effectors on these two enzymes.

One of the important features of biological action of cAMP and cGMP is that they are involved in the dual mechanism in cellular metabolisms. Many workers have studied the effects of nucleoside diphosphates and triphosphates on these enzymes [2,3]. It would be informative if nucleoside monophosphates have any effect on the activities of adenylate cyclase and guanylate cyclase in the presence of a particular ginsenoside. It was observed rather unexpectedly that adenylate cyclase was stimulated by GMP in the presence of lower concentration of ginsenoside Rd.

As we have suggested in our previous report[1] the internalization of some ginsenosides seems to occur. This possibility is examined by using an ^{125}I-labelled ginsenoside in the present work. Our results suggest that some ginsenosides penetrate into the cell after binding to the receptors.

We have also examined the effects of some ginsenosides on the two enzymes at very low concentration, that is, several microgrammes of ginsenosides per milliliter of reaction mixture, compared with the higher concentration used in our previous work, that is, several milligrammes of ginsenosides. Only one of five ginsenosides tested showed the reciprocal effect, while soluble guanylate cyclase was stimulated in great extent by three ginsenosides.

II. Materials and Methods

1. Materials

Dried ginseng root of six years old was purchased from the market. Cyclic AMP assay kit and cyclic GMP assay kit were the preparation of the Radiochemical center (Amersham, England). ATP (disodium salt form) and GTP (disodium salt form) were purchased from Merck (Germany). Theophylline, bovine serum albumine, dithiothreitol, creatine phosphate and creatine phosphokinase were purchased from Sigma (U.S.A.). EDTA and Tris (hydroxymethyl) aminomethane were purchased from Aldrich (U.S.A.). Milipore filter was purchased from Millipore Cooperation (U.S.A.), and wet cellulose dialysis tubing (M.W. Cut off 1,000) from Spectrum Medical Industries (U.S.A.).

2. Extraction and separation of saponins

Extraction of saponins was performed according to the method of Oura[4] except the following modifications. Before following Oura's procedure, the filterate extracted with Tris-HCl buffer (pH 7.6) was freeze-dried, and the dried powder was dissolved in methanol, and then the methanol solution was treated with ether. The resulting precipitate was dissolved in water and applied to dialysis. The solution in dialysis tubing was freeze-dried[5]

Freeze-dried extract was dissolved in the smallest volume of methanol and applied to the silica gel TLC plate. The developing solvent system was the upper phase of the following mixture: n-butanol-ethyl acetate-water (v/v 4:1:5). The plate was visualized with iodine vapor.

3. Identification of purified ginsenosides

Saponin fractions obtained by the procedures as described above were identified by performing the cochromatography of each saponin fraction together with authentic ginsenosides, described in the previous report[1].

4. Preparation of adenylate cyclase

Rats weighing 150-200g were sacrificed by decapitation. The whole cerebellum and cerebrum were

immediately removed and placed in ice-cold homogenizing medium containing 0.25M sucrose, 10mM Tris-HCl buffer (pH 7.5), 3mM dithiothreitol. The tissues were bleeded twice in the same medium to remove the blood as much as possible. Adenylate cyclase was prepared according to the method of Sutherland[6]. Fresh preparation of enzyme, kept at −70°C, was used throughout the experiments. The final enzyme preparation contained about 2.57mg of protein per milliliter. The protein content was determined by Lowry's method[7]. When the enzyme preparation was used in cAMP radioimmunoassay, it was diluted with 20 volumes of water.

5. Preparation of guanylate cyclase

Soluble guanylate cyclase was prepared according to the modified method of Nakazawa[8]. Rats weighing 150-200g were sacrificed by decapitation. The brain tissues were quickly removed, chilled in an ice bath, and homogenized in a Teflon-glass homogenizer with 10 volumes of 0.25M sucrose containing 0.02M Tris-HCl buffer (pH 7.4), 1mM EDTA, and 10mM 2-mercaptoethanol. The homogenate was centrifuged for 10 min at 4°C at 27,000xg and the pellet was placed in ice bath to prepare the particulate guanylate cyclase. The supernatant was centrifuged further for 60min at 4°C at 105,000xg. The supernatant thus obtained was employed as crude soluble guanylate cyclase preparation. This enzyme preparation contained about 195 *u*g of protein per milliliter, and used in cGMP radioimmunoassay without dilution. The reserved pellet was weighed and homogenized in 9 volumes of homogenizing medium containing 10mM NaCl, 10 mM KCl, 0.1 mM dithiothreitol, 5 *u*M EDTA and 2 mM Tris-HCl (pH 7.4). From this homogenate particulate guanylate cyclase was prepared according to the method of T.D. Chrisman[9]. The final enzyme preparation contained about 2.40mg of protein per milliliter, and used in cGMP radioimmunoassay after diluting with 4 volumes of water.

6. Radioimmunoassay of cAMP and cGMP

Formation reaction of cAMP and cGMP in the reaction mixtures was assayed as described before in our previous report[1], except that unbound cAMP or cGMP was albowed to adsorb on the active charcoal, and the radioactivity was counted with stillation counter.

7. Iodination of Rb_2 with ^{125}I

Iodination of Rb2 was performed using iodogen (1, 2, 4, 6-tetrasholor-3a, 6a-diphenylglycouril) as described by Fraker and Speck[10]. Iodogen film was prepared on the interior surface of small (ca. 3ml) polyproplylene centrifuge tube. The reaction mixture contained 100 *u*l of sodium phosphate buffer (pH 7.6, 0.05M), 100 *u*l of ^{125}I-sodium idodide solution (pH 8.2, 0.4mCi of ^{125}I), and 200 *u*l of Rb2 solution (2mg/ml.) Iodination was carried out by allowing the reaction mixture for 30 minutes, at 2-4°C. Iodinated Rb2 was purrified as follows. The reaction mixture was applied on the thin layer plate of silica gel, and developed with the following solvent system; chloroform: methanol: water (75:25:2.5 v/v). The separation of iodinated Rb2 is shown in Fig. 1. After the spot of labeled Rb2 on the thin layer was marked according to the autoradiograph, it was extracted with small volume of methanol.

Fig. 1. Autoradiogram of separated ^{125}I-labelled ginsenoside Rb2 →

After the methanol was evaporated, labeled Rb2 was dissolved into an appropriate volume (usually 3ml) of distilled water.

8. Assay of binding of ^{125}I-labeled Rb_2 on the cells in the absence into an appropriate

Both normal chick myoblast and fibroblast SCK tumor cells[11] were used to test the binding of labeled Rb2. Both cells were subcultured before transferring into the RPMI 1640 tissue culture medium containing fetal calf-serum and penicillin streptomycin (100 units/ml). The cultures were incubated for one week at 37°C before binding test. Binding test of ^{125}I-labelled Rb2 on the cells were carried out as follows. The cultured cells were harvested into 5ml of PBS buffer and about 30 *u*l of ^{125}I-labelled saponin solution (ca. 50,000cpm) was added. Cell numbers were usually kept to about 10^7. After incubating the cells at various time intervals (5, 10, and 30 minutes and sometimes upto one hour), cells were washed thoroughly with PBS buffer, or sometimes with PBS buffer containing 4M urea. The washed cells were counted for their radioactivities.

In several separate experiments, competitive inhibition of binding of ^{125}I-labelled Rb2 by dopamine was determined by carrying out the similar experiments as following: In one experiment, 5.5×10^7 cells were mixed with 0.445 *u*moles of ginsenoside Rb2 and incubated for various period. On the other hand, 0.445 *u*moles of dopamine were added into the incubation mixture and incubated for various period to examine the competition.

This binding experiment was performed with the kind help of Prof. Man-Sik Kang, Department of Zoology, Seoul National University.

III. Results and Discussion

1. The effects of ginsenoside on the activity of guanylate cyclase and adenylate cyclase

The effects of the five ginsenosides (Rd, Rc, Re, Rf and Rgi) on the activities of particulate adenylate cyclase and particulate guanylate cyclase of rat brain have been studied. The range of concentration of ginsenosides were between 10 *u*g and 500 *u*g per 500 *u*l of reaction mixture. Also, the effects of three ginsenosides (Rc, Rd, and Rg1) on the activity of soluble guanylate cyclase have been studied in the same range of concentrations as in particulate guanylate cyclase. The results are shown in Fig. 2. Only Re has shown the reciprocal effects when tested with particulate adenylate cyclase and particulate guanylate cyclase (see part C in Fig. 2).

As can be noted from part F,G,H. in Fig. 2, the activity of soluble guanylate cyclase increases in great extent at very low concentration of ginsenosides (up to 100 *u*g of Rc, Rd and Rg1 each), and then decrease more or less slowly. The marked increase of the activity of soluble guanylate cyclase by these three ginsenosides is of particular interest. It is generally accepted that soluble guanylate cyclase is not subject to any specific direct effect by substances of physiological importance[2]

It is also believed that nonionic detergents do not enhance the activity of soluble guanylate cyclase[3]. The marked decrease of activity by AMP and GMP of the activated enzyme (see curve B and C of Fig. 3) suggest that there exists a sort of competition between ginsenosides and nucleoside monophosphate. Thus, the inhibition of soluble guanylate cyclase by the nucleotides may be of a specific character. The enhanced activation by GMP of the ginsenoside-inhibited adenylate cyclase (see Fig. 3). may be argued in a similar manner, but, since particulate adenylate cyclase is present in a complexed form in the membrane, GMP may be involved here in a quite different and complicated manner.

2. Regulatory effects of nucleoside monophosphated on the activities of adenylate cyclase and guanylate cyclase

Regulatory action of the several mononucleotides on the activities of adenylate cyclase and guanylate

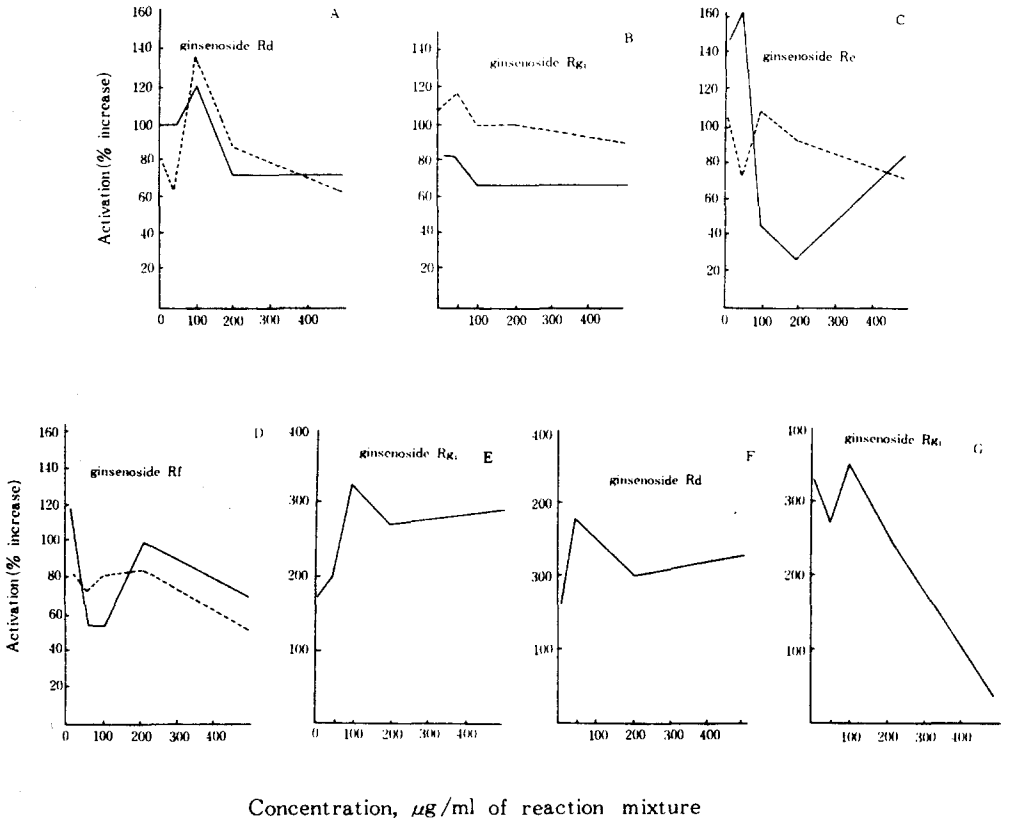

Fig. 2. Effects of ginsenosides on the activities of particulate guanylate cyclase, particulate adenylate cyclase, and soluble guanylate cyclase. ..... (particulate adenylate cyclase), _____ (particulate guanylate cyclase) for panels A, B, C, and D. _____ (soluble guanylate cyclase) for panels E, F, and G.

cyclase was examined to understand the inhibitory effect of ginsenosides on the adenylate cyclase and guanylate cyclase (cf. Fig. 2) in the range of low concentration (10 μg to 60 μg/500 μl of reaction mixture).

As shown in Fig. 3, ginsenoside Rd-inhibited adenylate cyclase increases greatly by the addition of increasing amount of GMP. On the contrary, ginsenoside Rc-activated guanylate cyclase was inhibited by the addition of increasing amount of AMP and GMP. The role of GMP in the stimulation of adenylate cyclase is somewhat surprising. So far, there is no report in the literature that GMP has any stimulatory effect on adenylate cyclase. We do not yet have any data to show whether the stimulatory effect is correlated with the formation of active complex between nucleotide regulatory protein and GMP, and thereby affecting the receptor-ginsenoside complex, so that ginsenoside which had an inhibitory action on adenylate cyclase is forced to be released from the complex. The fact that the stimulatory action of GMP is observed only with particulate adenylate cyclase but not with soluble guanylate cyclase suggests that the action is membrane-rrelated one.

The effects of cAMP on the activity of guanylate cyclase, and that of cGMP on that of adenylate cyclase are shown in Fig. 4. It is well known that the two exogenous cyclic nucleotide produce a stimulatory effect

on the counterpart nucleotide cyclases. As can be seen in Fig. 4, guanylate cyclase is activated by cyclic adenylate in the range of 5 to 8 phomles, while adenylate cyclase is activated by cyclic guanylate in the range of 5 to 12 pmoles. Beyond this range of concetration of two cyclic nucleotides, both adenylate cyclase and guanylate cyclase are inhibited.

3. Binding of the ^{125}I-labelled ginsenodide Rb_2 on the cells

We have measured the extent of binding of ^{125}I-labelled Rb2 both on the normal and tumor cells in the presence or in the absence of dopamine. The results are shown in Fig. 5 and Fig. 1. Fig. 5 shows that binding of the ginsenoside on the cell increased during the initial ten munutes of incubation and then reaches a plateau during the next twenty minutes. The plateau may represent either the saturation of the receptors on the cell surface or steady penetration of ginsenoside into the cells. The competition experiment during the short incubation time using one of β-adrenergic ligands, such as dopamine, may give a definitive answer.

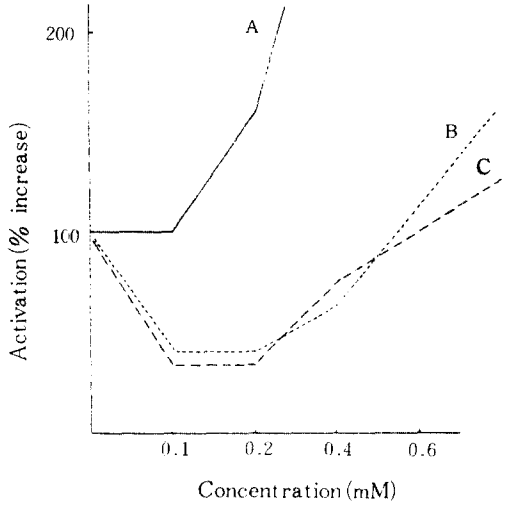

Fig. 3. The effect of nucleotides on the adenylate cyclase and guanylate cyclase in the presence of ginsenoside Rc or Rd. ..... Soluble guanylate cyclase-Rc plus AMP; ------ soluble guanylate cyclase-Rc plus GMP; ___ particulate adenylate cyclase-Rd plus GMP. Concentration of ginsenoside in each reaction mixture is 20 μg of Rc or Rd. Activities of adenylate cyclase and guanylate cyclase were assayed as described in Materials and Methods.

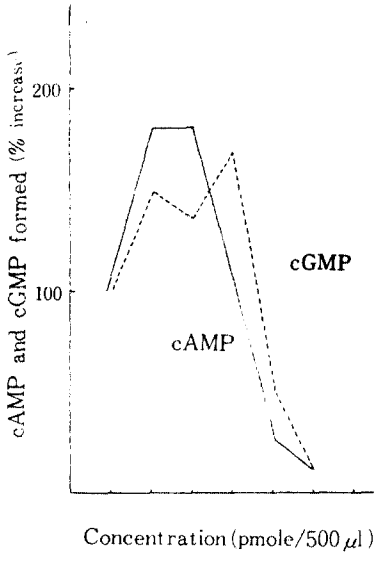

Fig. 4. Reciprocal effect of cyclic nucleotides on the counterpart nucleotide cyclase. ..... Adenylate cyclase plus cGMP, _____ guanylate cyclase plus cAMP.

The great extent of binding during the longer incubation time, 60 minutes, suggests that the bound ginsenoside somehow managed to penetrate into the cells during the plateau.

We have performed the competition experiment both at the initial binding stage and at the plateau range

(see Fig. 5.). As shown in Fig. 6, binding of ginsenoside was markedly decreased by the presence of dopamine at 15-minute (compare the data here with that in Fig. 5. 15-minute data is lacking in Fig. 5.). This result of competition is a clear-cut evidence that ginsenoside Rb2 binds specifically to β-adrenergic receptors on the cell membrane. It is still early to generalize that all the ginsenosides might behave as β-adrenergic ligands or agonists. It will be interesting and informative to test if other ginsenosides have β-adrenergic property or not.

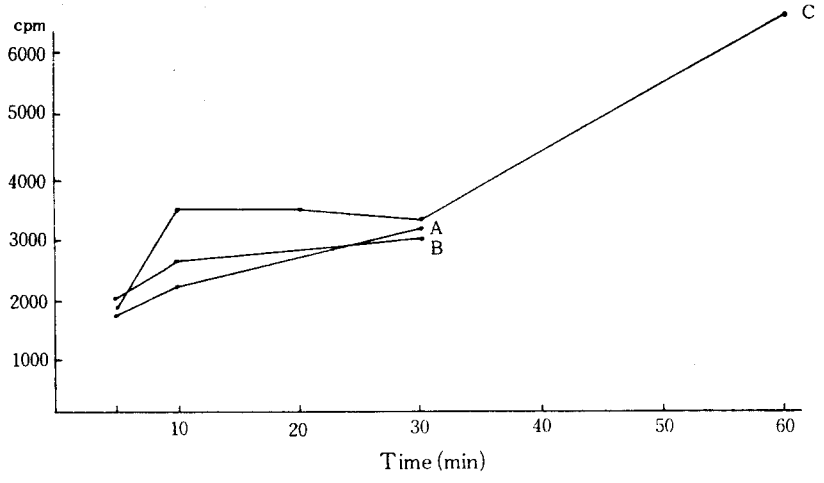

Fig. 5. Binding experiments of ^{125}I-labelled Rb2 on the cells. (A) Normal chick myoblasts; (B) fibroblast SCK tumor cells. In the cases of (A) and (B), incubated cells were washed three times with PBS buffer without urea. (C) Tumor cells; incubated cells were washed three times with PBS buffer with urea.

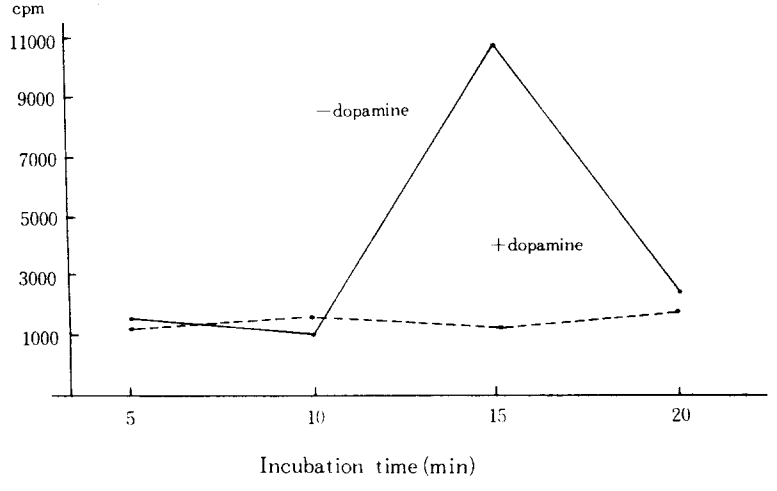

Fig. 6. Competition between ginsenoside Rb2 and dopamine in binding on the cell membrane. _____ binding of ^{125}I-Rb2 in the absence of dopamine, ... binding of ^{125}I-Rb2 in the presence of dopamine. The cells used for this experiment were SCK tumor cells which is orginated spontaneously, and characterized by Song *et al.*[11]

From theβ-adrenergic property of ginsenoside Rb2 it can be deduced that Rb2 acts as a modulator by binding to receptors on the adenylate cyclase system and/or on the guanylate cyclase system in the membrane.

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요 약

다섯 가지 진세노시드들이 쥐의 뇌에 있는 입자상의 아데닐산 고리화효소와 구아닐산 고리화효소의 활동성에 미치는 영향을 조사하였다. 진세노시드들의 농도범위는 500μl의 반응 혼합물에 대해서 10mg에서 500μg까지이었다. 또한 같은 농도 범위에서 세가지 진세노시드들이 가용성 구아닐산 고리화효소에 미치는 영향을 조사하였다. 입자상의 아데닐산 고리화효소와 구아닐 산 고리화효소에 미치는 영향을 조사하오다. 입자상의 아데닐산 고리화효소와 구아닐산 고리화효소에 대해서 시험한 진세노시드들 중 Re만이 두 효소들에 대해서 상반적으로 작용하였다.

아데닐산 고리와효소와 구아닐산 고리화효소에 미치는 몇가지 모노누클레오티드들의 조절성 작용을 조사하였다. 진세노시드 Rd로 방해된 아데닐산 고리화효소가 첨가하는 GMP의 양에 따라서 그 활동성이 크게 증가한다. 다른 한편, 진세노시드 Rc로 활성화된 구아닐산 고리화효소가 AMP와 GMP의 첨가하는 양에 따라서 방해되었다. GMP의 자극적 작용은 가용성 구아닐산 고리화효소가 아니라 입자상의 아데닐산 고리화효소에서 볼 수 있다는 것은 이 자극적 작용이 막과 관련된 것임을 암시한다.

막의 수용체에 대해서 진세노시드 Rb_2와 도파민 사이에 경쟁 반응이 있음을 관찰하였다. 이 결과는 진세노시드 Rb_2가 β-아드레날린성 수용체에 특이하게 결합한다는 명확한 증거가 된다.

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