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A Study on the Influence of Ginseng Components On cAMP-cGMP Regulation Mechanism

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cAMP-cGMP 조절 메카니즘에 미치는 인삼 성분의 영향에 관한 연구

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Abstract

The effect of ginsenosides on the adenylate cyclase and guanylate cyclase of rat brain has been studied. We have found that Rb_1, Rc and one unknown ginsenoside (probably Ra) exerted reciprocal effects on adenylate cyclase and guanylate cyclase. This dual effect of ginsenosides leads us to speculate that some ginsenosides may act as regulatory agents and modulate the activities of these two enzyme systems.

I. Introduction

A number of papers have been published over past fifty years on the chemical, physiological and pharmacological studies on the chemical components of ginseng. Most of the papers published in the early period were concerned with physiological or morphological changes which appeared after the administration of ginseng extracts or ginseng powder into the animal body[1]. New biochemical or molecular biological approaches to study the specific effects of ginseng components, such as saponins, on particular metabolic pathways were also adopted by many workers[2-8].

As reported in our previous work[19], we have demonstrated, using the membrane bound adenylate cyclase of rat brain, that two kinds of ginsenosides enhanced the adenylate cyclase activity.

There are many reports showing that ginseng extracts sometimes exert the opposite physiological effects in different individuals[10]. These findings made us to speculate that some of the ginseng components might behave like regulators affecting, specifically, the membrane bound enzymes, such as adenylate cyclase and/or guanylate cyclase.

One of the important features of biological action of cAMP is that cAMP with cGMP participates

in dual regulation mechanism of the cells. cGMP is a unique component in a complex network of biological regulation that is distinct from the system in which cAMP appears to play the role of a second messenger. Guanylate cyclase, the enzyme that catalyze generation of cGMP from GTP, exhibits a number of properties that distinguish it from adenylate cyclase. A variety of cellular functions have been shown to be affected upon exposure of intact cells to exogeneous cGMP[11]. They include secretion, cardiac contractility, neuronal excitability, selectively stimulating the activity of enzymes and many of the process

In a majority of cases, the effect produced by exogeneous cGMP is opposite of the effect produced by exogeneous cAMP.

We report in this paper that some ginsenosides have, interestingly, dual regulatory property on cAMP-cGMP regulatory mechanism. The saponins which we have tested with up to now are all 20S- protopanaxadiol.

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 Rediochemical 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 (USA). EDTA and tris (hydroxymethyl) aminomethane were purchased from Aldrich (USA). Milipore filter was purchased from Millipore Cooperation (USA), and wet cellulose dialysis tubing (M.W. Cut off 1,000) from Spectrum Medical Industries (USA).

2. Methods

1) Extraction and separation of saponins

Extraction of saponins was performed according to the method of Oura[12] except the following modifications. Before following Oura's procedure, the filterate extracted with Tris-HCI buffer (pH 7.6) was freeze-dried, and the dried power 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[19].

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 5:1:5). The plate was visualized with iodine vapor and the spots were numbered as W_0, W_1, W_2, W_3, W_4, W_5, W_6, W_7, W_8 and W_9 in the increasing order of Rf values. Fraction W_2, W_3 and W_5 were rechromatographed on silica gel TLC employing the lower phase of the following mixture: chloroform-methanol-water (v/v 65:35:10).

2) 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; fraction to be identified was loaded on TLC plate, 2cm×5cm, silica gel, in such a manner so the strip of sample and that of authentic ginsenoside overlap partly each other at the ends of strips. The plates were developed with following solvent system: $CHCl_3$-CH_3OH-H_2O (v/v 75:25:2.5).

The authentic ginsenosides were kindly donated by Dr. Byung Hoon Han, Natural Products Research, Institute, Seoul National University.

3) 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[13]. 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[14]. When the enzyme preparation was used in cAMP radioimmunoassay, it was diluted with 20 volumes of water.

4) Preparation of guanylate cyclase

Soluble guanylate cyclase was prepared according to the modified method of Nakazawa[15]. 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 10min 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 60 min at 4°C at 105,000xg. The supernatant thus obtained was employed as crude soluble guanylate cyclase preparation. This enzyme preparation contained about 195 μg 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, 10mM KCl, 0.1mM dithiothreitol, 5uM EDTA and 2mM Tris-HCl (pH 7.4). From this homogenate particulate guanylate cyclase was prepared according to the method of T.D. Chrisman[19]. The final enzyme preparation contained about 2.40mg of protein per milliliter, and used in cGMP radioimmunoassay after diluting with 4 volumes of water.

5) Radioimmunoassay of cAMP

Formation reaction of cAMP was performed as described by Sutherland[13]. The reaction buffer was consisted of 4mM ATP, 8mM $MgCl_2$, 10mM theophylline (an inhibitor against phosphodiesterase), 0.5mg/ml bovine serum albumin, 1mM dithiothreitol (DTT), 50mM Tris-HCl (pH 7.5) plus 50 μl of purified ginsenoside solution. After chilling the buffer in ice-water bath and vortexing, 50 μl of prepared adenylate cyclase was added. The final reaction volume was adjusted to 500 μl. The control experimental condition was the same as above except that the saponin fraction was omitted from the reaction mixture, and adjusted the reaction volume with 50 μl of 50mM Tris-HCl buffer. The reaction mixture was vortex-mixed and allowed to incubate for eight minutes at 37°C. The reaction was stopped by immediately dipping the reaction mixture into the hot water (95°C) for three minutes. After the precipitation of the denatured enzyme, 50 μl of ^{3}H-cAMP (18 pmoles/ml, 0.5 μCi) and 100 μl of binding protein solution were added to 50 μl of the resulting supernatant.

After mixing the above solution thoroughly for a few seconds, it was allowed to stand for two hours in ice-cold water. Being reached at equilibrium, the unreacted cAMP was removed by passing through millipore filter (0.45 μm pore size)[17].

Millipore filter retaining the protein was dissolved in ethyl cellosolve and the radioactivity was counted with Packard Liquid Scientillation Counter, Tri-carb Model 240 CL/D.

6) Radioimmunoassay of cGMP

Formation reaction of cGMP was performed as described by Garbers[18]. To the test tube was added 100 μl of a freshly prepared mixture containing 80 ul of 25mM theophylline, 10 μl of 1M Tris-HCl (pH 7.6), 10 μl creatine kinase mixture, then 60 μl ginsenoside solution was added. The tube was chilled in an ice

bath and 20 μl enzyme preparation was added. The incubation in a final volume of 200 μl were initiated with the addition of 20 μl of a solution containing 10mM GTP and 40mM $MnCl_2$. The control experimental condition was similar to cAMP radioimmunoassay. After incubation (10 minutes at 37°C) the reaction was terminated by adding 1.8ml of 50mM Na-acetate buffer (pH 4.0) and heating for three minutes at 95°C. The denatured enzyme was centrifuged in the Eppendorf tube, and 50 μl of ^{3}H-cGMP (8 pmole/ml, 0.16 uCi) and 50 μl of antiserum solution were added to 100 μl of the resulting supernatant. After vortexing for a few secons, it was placed in a refrigerator at 2-4°C for 2 hours. After being reached at equilibrium, 1ml of ice-cold ammonium sulfate solution was added to the tube and vortexed for a few seconds. It was allowed to stand for five minutes in a ice bath, and then centrifuged for two minutes by Eppendorf Micro Centrifuge Model 5412. After decanting the supernatant liquid, 1.1ml distilled water was added. After vortexing until all the precipitate was dissolved, the sample was transfered to the counting vial and water-miscible scintillator was added. The radioactivity was counted as in the case of cAMP assay.

III. Results and Discussion

The effects of the four ginsenosides on the activity of adenylate cyclase and guanylate cyclase of rat brain tissue have been studied. Membrane bound enzyme was extracted and used for the experiment. The range of concentration of ginsenosides was between 0.25mg and 4mg per milliliter of reaction mixture.

These data were represented graphically in Fig. 1. The results show that, except for Rd, three ginsenosides W_2, Rb_1, and Rc affect the two enzymes in reciprocal manner, although there are some differences in the extents of effects on two enzymes. For instance, up to the concentration of 0.5mg/ml of reaction mixture starting from zero concentration, W_2 shows negative effect on the activity of adenylate cyclase, whereas, in the same concentration range, it shows positive effect on the activity of guanylate cyclase. However, in the concentration range higher than 0.5mg/ml the effects of W_2 on the two enzymes are inversed. That is to say, 0.5mg/ml of W_2 is a kind of transition point or threshold level. With increasing, concentration of W_2, the activity of adenylate cyclase is enhanced rather rapidly, while the activity of guanylate cyclase is inhibited. The similar pattern of ginsenoside effects are observed also with ginsenoside Rb_1 and Rc, although there is a ginsenoside-specific deviation in the threshold in each case. The transition points of threshold were in the low range of concentration (0.5mg/ml for W_2; 0.5 to 1mg/ml for Rb_1; 0.1mg/ml for Rc). Rd behaves rather uniquely (see Fig. 1. (d)). The result seems to show that Rd does not exert reciprocal effect as W_2, Rb_1, or Rc do. However, Rd also shows a reciprocal action on the two enzymes in small concentration range between 1mg/ml and 2mg/ml.

From the results we have obtained with ginsenoside W_2 (Ra), Rb_1, Rc and Rd, it is supposed that some ginsenosides exert, in reciprocal manner, on the activity of adenylate cyclase and guanylate cyclase. This mode of action of ginsenosides allows us to formulate a notion on the reaction mechanism of the ginsenosides with cAMP-cGMP regulatory machinary. Although it might be a precocious generalization, the reciprocity of the effects of ginsenosides may be a sufficient evidence to presume that certain ginsenosides resemble to modulator or effectors of many irreversible metabolic steps.

The quantitative differences in the extent of the effect of individual ginsenoside may be the reflection of the structural differences among ginsenosides. The four ginsenosides studies have same chemical moieties at position 3 of sapogenins. They have different number and different kind of sugar residues on position 20. The unique action of ginsenoside Rd may be caused by the short structural element instead of two sugar residues. The types, the positions of glycosylation, and the number of suger residues attached to sapogenin moiety seems to contribute an essential role in betermining the effects of ginsenosides and a whole and may be very important in determining biological action of ginsenosides.

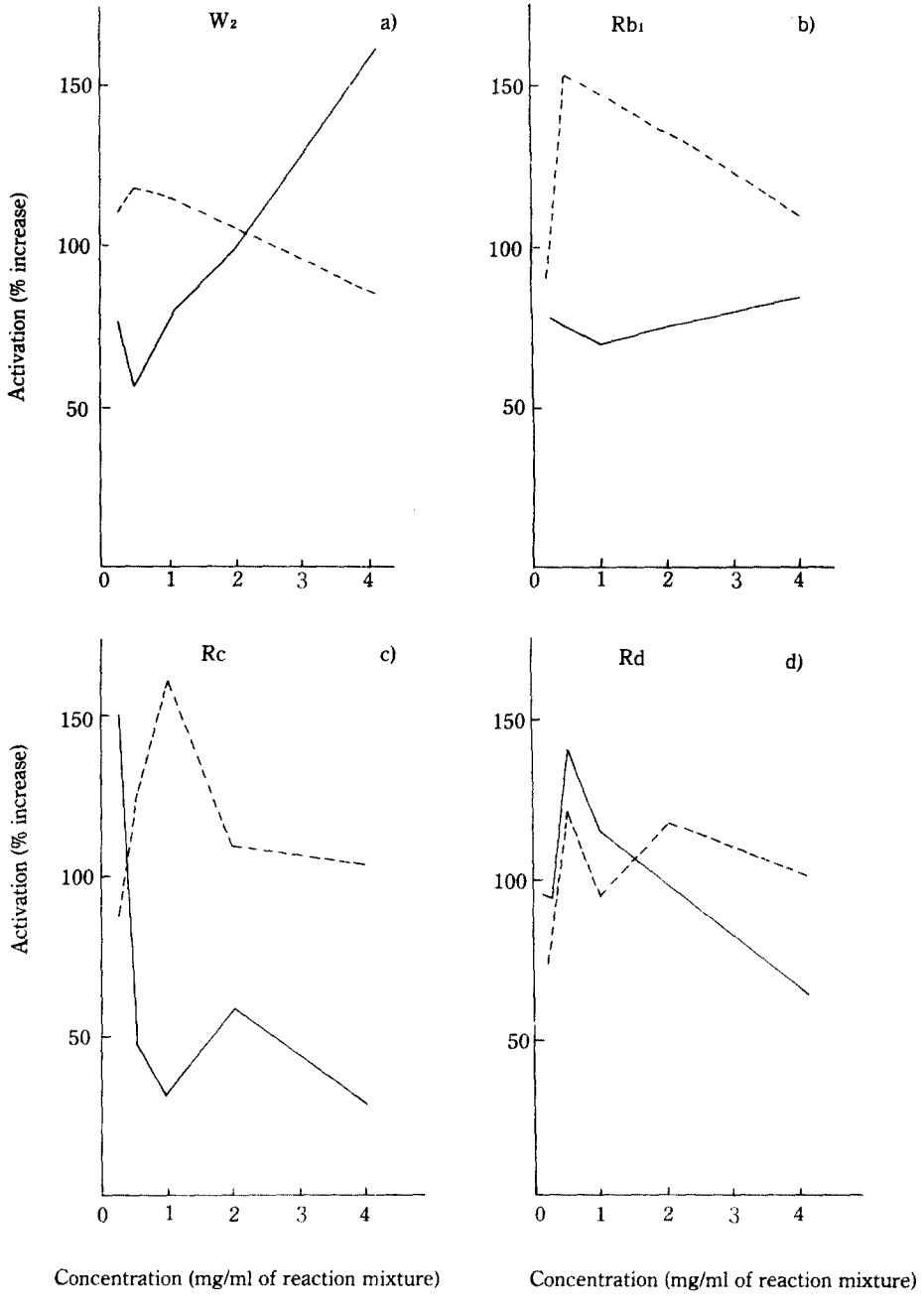

Fig. 1. Effects of ginsenoside on the activity of adenylate cyclase (——) and particulte guanylate cyclase (----)

If the sugar moiety may play an important role in determining the action of ginsenosides, as we have reasoned above, it is possible that the effect of ginsenosides are exerted on the surface of membrane, acting as ligands to the specific receptor(s) of adenylate cyclase and/or guanylate cyclase. But, since our

experiment was performed with membrane-bound enzymes, it is also possible that the ginsenosides react directly on the enzyme surface. Our assumption of this possibility was in further supported by our observation of reciprocity of the effects of ginsenosides. If we demonstrate that ginsenosides penetrate into the cell our notion that ginsenosides react directly with enzymes, thus acting like a modulator will be proved. Our experiment with soluble guanylate cyclase shows that the soluble enzyme can be affected directly with ginsenosides (see Table 1). But, out data are not sufficient to argue for our assumption. We should have more observations at various concentrations of ginsenosides. At the present time, we did not obtain any evidence concerning in this aspect. In this context, Pastan and Willingham's receptosome hypothesis[22] is very interesting. In fact, we have a preliminary results which show that ginsenoside-receptor binding is decreased in the presence of dopamine, one of the specific ligand to the recepton on the adenylate cyclase[20]. This aspect is a challenging problem to tackle in the further work. If our ginsenosides act as modulator for adenylate cyclase and guanylate cyclase enzyme system, the determination of the change of level of cAMP and cGMP in intact cells with the various administration of the ginsenosides will give a clue to characterize the action mechanism.

Table 1. Effects of ginsenosides on the activity of soluble guanylate cyclase.

Ginsenosides	Activation
control	100%
Rb_1	226
Rc	150
Rd	115

Goldberg and others[11] observed that role of cGMP in tissues could be increased by many agents that acted antagonistically to those promoting accumulation of cAMP, and put forth the Yin Yang hypothesis[11, 21] that cGMP and cAMP promote opposing regulatory influence on a number of bidirectionally controlled systems. In this respect, the fact demonstrated here that ginsenosides have opposite activity to adenylate cyclase and guanylate cyclase is very interesting and meaningful. As we have discussed already, some ginsenosides may be the naturally occurring exogenous modulators, at least, to rats.

It has been reported by a number of workers that 20S-protopanazadiols and 20S-protopanaxatriols have opposite or competitive biological action[10]. Tagaki[22] reported that the crude saponin fraction of ginseng contains ginsenosides exhibiting opposite effects in several respects. For example, central nervous system stimulating action was observed with ginsenoside Rg_1 in one hand, while central nervous system depressing action, on the other hand, was observed with ginsenoside Rb_1, Rg_1 showed transient blood pressure elevation, while Rb_1 exhibited prolonged blood pressure fall. There are number of similar reports on the opposite effect of various ginsenosides[10]. Most of the works on this line are, however, interested mainly in the different actions between 20S-protopanaxadiols and 20S-protopanaxatriols using whole body animal. Nevertheless, we have the impression, after examining this kind of reports, that also the individual ginsenosides might act differently on the samy enzyme in different tissues or in different cell organells. To verify this, we are investigating the effect of ginsenoside on adenylate cyclase-guanylate cyclase system of other tissues, such as heart and kidney.

요 약

몇가지 ginsenoside들이 쥐의 뇌의 adenylate cyclase와 guanylate cyclase에 미치는 영향을 조사하였다. Rb 1, Rc 및 한가지 미확인 ginsenoside (Ra로 추정됨)가 adenylate cyclase와 guanylate cyclase에 상반되는 영향을 미침을 알았다. 이러한 ginsenoside들의 이중 효과에 의하여 몇가지의 ginsenoside들이 이 두가지 효소계에서 조절인자처럼 행동할 것이라는 결론을 얻었다.

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