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# Effects of Ginsenosides on the Glutamate Release and Intracellular Calcium Levels in Cultured Rat Cerebellar Neuronal Cells

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These studies were designed to examine the effects of ginsenosides on glutamate neurotransmission. In primary cultures of rat cerebellar granule cells, ginsenosides (Rb1, Rc and Rg1, 500 μg/ml) increased glutamate release which was measured by HPLC, but Re did not show an elevation of glutamate release. However, all of these ginsenosides down-regulated N-methyl-D-aspartate (NMDA)-induced glutamate release. Rc strongly increased glutamate release and elevated intracellular calcium concentrations ($[Ca^{2+}]_i$) which was measured by ratio fluorometry with FURA-2 AM. These results indicate that ginsenosides have a homeostatic effect on glutamate neurotransmission, and there is a structure-function relationship among the ginsenosides tested.

**Key words :** Ginsenosides, Cerebellar granule cells, Glutamate, N-methyl-D-aspartate (NMDA)

## INTRODUCTION

Panax ginseng root has been commonly used for thousands of years in China, Korea and Japan. In general, ginseng was considered to have tonic, stimulant and sedative properties. Ginsenosides isolated and purified from ginseng saponin fraction were steroid-derivatives containing carbohydrates (Kata *et al.*, 1975). It was reported that ginsenosides, Rb and Rc, exhibited a sedative effect and Rg showed stimulative actions on CNS (Takagi *et al.*, 1972). Saito *et al.* (1977) observed that ginsenoside, Rb1, enhanced the stimulatory effect of nerve growth factor on neurite outgrowth in organ cultures of chick embryonic dorsal root ganglia. Ginsenoside, Rg1, inhibits the rat brain cAMP phosphodiesterase activity (Stancheva and Alova, 1993). Several studies have indicated that excitatory amino acids are involved in neuronal survival, synaptogenesis, neuronal plasticity, and learning and memory processes (Balazs *et al.*, 1988; Collingridge and Bliss, 1987; Muller *et al.*, 1988). This consideration prompted us to investigate the effect of ginsenosides on glutamate neurotransmission.

Glutamate is a major excitatory transmitter in the mammalian central nervous system. This amino acid produces its effect by acting on at least 4 receptor subtypes designated as N-methyl-D-aspartate (NMDA), kainic acid, amino-3-hydroxy-5-methylisoxazole-4-propionic acid and metabotropic receptors. Abnormalities in glutamate neurotransmitter systems were believed to be involved in neurological disorders such as epilepsy (Sloviter and Dempster, 1985; Wyler *et al.*, 1987), and certain neurodegenerative diseases such as Alzheimer's disease (Greenamyre and Young, 1989), and brain and spinal cord damage following ischemia and spinal cord trauma (McIntosh *et al.*, 1990; Panter *et al.*, 1990).

The cytotoxicity of glutamate in cultured cerebral cortical neurons as well as in cerebellar slices was apparently related to increased intracellular free $Ca^{2+}$ ($[Ca^{2+}]_i$) which stimulated and regulated phospholipase, proteases, protein kinases and endonucleases (Choi, 1988; Meyer, 1989). Under pathological situations, the increased extracellular glutamate concentration stimulated excitatory amino acids receptors which resulted in a persistent increase in $[Ca^{2+}]_i$. This increased $[Ca^{2+}]_i$ may be responsible for the abnormal metabolism of neural membrane phospholipids and generation of high levels of free fatty acids, diacylglycerols and lipid peroxidation.

The effects of several ginsenosides (Rb1, Rc, Re and Rg1) on both glutamate release and NMDA receptor function were investigated using cultured cerebellar

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granule cells. An effect of one of the ginsenosides examined namely, Rc, on the intracellular calcium elevation was presented.

## MATERIALS AND METHODS

### Materials

NMDA and MK-801 were purchased from RBI (Natick, MA, U.S.A.). Fura-2/acetoxymethyl ester (FURA-2 AM) was purchased from Molecular Probes (Eugene, OR, U.S.A.). Ginsenosides were supplied by the Korea Ginseng and Tobacco Research Institute. All other chemicals used were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

### Cerebellar granule cell culture

Cerebellar granule cells are cultured as described (McCaslin and Morgan, 1987) with slight modifications. Briefly, 8-day-old rat pups (Sprague-Dawley) are decapitated, and the heads are partially sterilized by dipping in 95 % ethanol. The cerebellum is dissected from the tissue and placed in culture medium which lacks serum and bicarbonate. Dissociated cells are collected at a density of about $2\times10^6$ cells/ml. Growth medium (5 ml/60 mm dish) is Dulbecco's modified Eagle's medium (DMEM) supplemented with sodium pyruvate (0.9 mM), glutamine (3.64 mM), sodium bicarbonate (40 mM), glucose (22.73 mM), 6% bovine calf serum (Hyclone, Logan, UT) and 6 % fetal bovine serum (JRH Bioscience, Lenexa, KS). After 2 day incubation, growth medium is aspirated from the cultures and new growth medium (5 ml/dish) containing 25 mM KCl is added with 5 μM cytosine arabinoside to prevent proliferation of nonneuronal cells. Primary cultures of cerebellar granule cells from neonatal rat brain represent a virtually homogeneous population of glutamatergic neurons.

### Measurements of amino acids

Experiments were performed using cultures grown for 10-14 days after plating. Cells were washed to remove medium and placed in a physiological saline HEPES (PSH) buffer containing 135 mM NaCl, 3.6 mM KCl, 2.5 mM $CaCl_2$, 40 mM bicarbonate, 10 mM glucose and 5 mM HEPES (pH 7.4, 300 mOsm). Cells were incubated in the presence of various amounts of compounds at 37°C for 1 h after preincubation for 30 min in the dark with PSH buffer. A sample was taken from buffer for the determination of amino acids secreted into the buffer. Amino acids were quantified by high performance liquid chromatography (HPLC) with an electrochemical detector (Ellison *et al.*, 1987). Briefly, after a small aliquot was collected from the culture dish, amino acids were separated on a Rainin (particle size, 5 μm; 4.6×150 nm) C18 column (reverse-phase) after prederivatization with o-phthaldialdehyde/2-mercaptoethanol. Derivatives were detected by electrochemistry at 20 nA/V, and the reference electrode was set at 0.70 V. The column was eluted with mobile phase (pH 5.25) containing 0.1 M sodium phosphate buffer with 37% (v/v) HPLC-grade methanol at a flow rate of 1.0 ml/min.

### Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined by ratio fluorometry as described (Tsien, 1989; Cai and McCaslin, 1992). Cells grown on glass cover slides were loaded with 10 μM FURA-2 AM for 1 hr in PSH buffer at 37°C in the $CO_2$ incubator, and washed with PSH buffer. Cell culture slides were cut and mounted into spectrophotometer cuvettes containing 2.5 ml PSH buffer (without bicarbonate). Fluorescence was measured with a FLUOROLOG-2 spectrophotometer (SPEX Ind. Inc., Edison, NJ) by exciting cells at 340 and 380 nm and measuring light emission at 505 nm. Baseline of $[Ca^{2+}]_i$ was measured for 60 sec prior to the addition of various experimental compounds. In case of Rc, the change of $[Ca^{2+}]_i$ was measured 10 min after the treatment with Rc. Ionomycin and EGTA (final concentration 10 μM and 20 mM, respectively) were added at the end of experiments to determine the emission of dye saturated with $Ca^{2+}$ and free of $Ca^{2+}$, respectively. Calcium concentrations were calculated according to the method of Grynkiewicz *et al.* (1985) using a KD of 224 nM by using TM 3000 software (SPEX).

### Data analysis

Statistical differences were analyzed by analysis of variance (ANOVA) followed by the Duncan's test. A $p<0.05$ was considered as a statistical significance.

## RESULTS

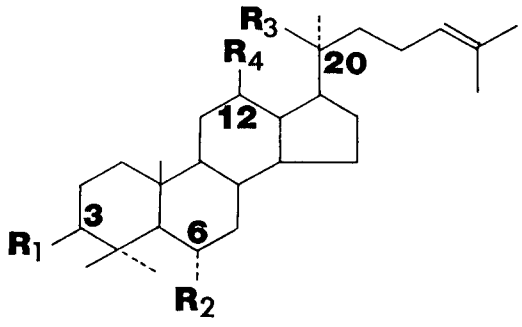
### Effects of ginsenosides on glutamate release

Each ginsenoside dissolved in PSH buffer (500 μg/ml) was added to the culture and the culture was incubated for 1 h in the $CO_2$ incubator. The elevation of extracellular glutamate was not significant in short-term incubation (10-20 min) with ginsenosides, but it was highly significant after 1 h incubation in our previous experiments. At the end of incubation, glutamate and other amino acids were analyzed by HPLC. All of the ginsenosides caused significant increase of glutamate release (extracellular glutamate levels). Rc strongly (13 times higher than control) elevated glutamate and slightly (1.5 times) elevated glycine release (Table 1). The elevated level of glutamate release by Rc responded to an increase in its concentration (Fig. 2).

**Table I.** Effects of ginsenosides on amino acids release in cultured cerebellar granule cells

| | Gln | Glu | Arg | Gly | Tau |
|---|---|---|---|---|---|
| Concentration (μM) | | | | | |
| Control | 0.97±0.05 | 0.38±0.07 | 0.24±0.11 | 0.86±0.07 | 2.08±0.06 |
| Rb1 | 0.93±0.03 | 1.07±0.22* | 0.22±0.03 | 0.92±0.04 | 1.88±0.10 |
| Rc | 1.10±0.04 | 5.22±0.75* | 0.24±0.02 | 1.29±0.04* | 1.95±0.11 |
| Re | 1.07±0.15 | 0.29±0.01 | 0.24±0.02 | 0.75±0.06 | 2.07±0.23 |
| Rg1 | 1.11±0.11 | 0.95±0.07* | 0.21±0.02 | 0.90±0.09 | 2.09±0.06 |

Cerebellar granule cells grown for 10-14 days were incubated with ginsenosides (500 μg/ml) for 60 min. The buffer was then collected, and the concentrations of amino acids were measured. Data are means±S.E.M. of 8-10 different cultures. *p<0.05 from the corresponding amino acid in the respective control group




| | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| Ginsenoside-Rb1 | -O-Glc$^{2}$-Glc | -H | -O-Glc$^{6}$-Glc | -OH |
| Ginsenoside-Rc | -O-Glc$^{2}$-Glc | -H | -O-Glc$^{6}$-Ara (fur) | -OH |
| Ginsenoside-Re | -OH | -O-Glc$^{2}$-Rha | -O-Glc | -OH |
| Ginsenoside-Rg$^{1}$ | -OH | -O-Glc | -O-Glc | -OH |

**Fig. 1.** The structures of purified ginsenosides. Glc: glucopyranoside, Ara (fur): Arabinofuranoside, Ara (pyr): Arabinopyranoside, Rha: Rhamnopyranoside

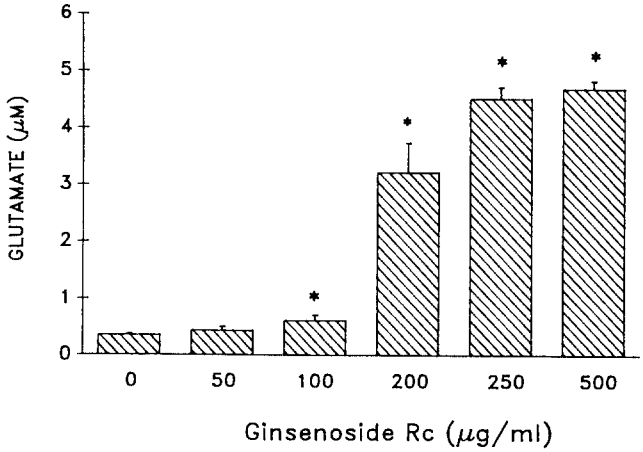



**Fig. 2.** Concentration-response of the augmentative effects of Rc on glutamate release. Cultured cerebellar granule cells were incubated with each concentration of Rc for 60 min. Values represent the means±S.E.M. of 8-10 different cultures. *p<0.05 from the control group.

## Effects of ginsenosides on NMDA-induced glutamate release

To see the modulating effects of ginsenosides on NMDA receptor, each ginsenoside (500 μg/ml) was added with NMDA (100 μM) and incubated for 1 h in the $CO_2$ incubator. Surprisingly, Rb1, Re and Rg1 blocked NMDA-induced glutamate release significantly (Fig. 3). Even though Rc did not block NMDA-induced glutamate release as observed with Rb1, Re and Rg1, the level of glutamate release was lower in culture treated with NMDA plus Rc than in culture treated with Rc alone (Fig. 2 and Fig. 3).

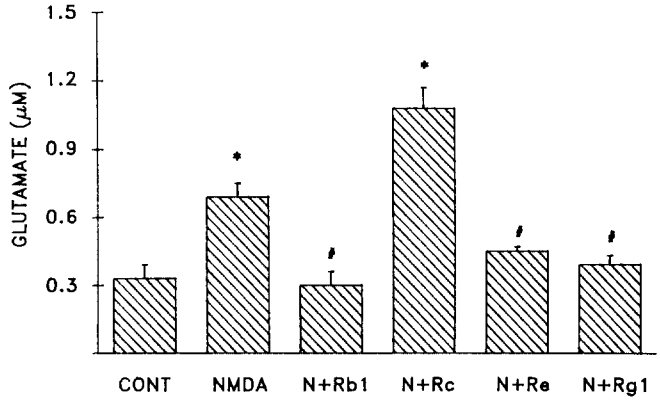



**Fig. 3.** Effects of ginsenosides on NMDA-induced glutamate release. Cultured cerebellar granule cells were co-incubated with NMDA (100 μM) and each ginsenoside (500 μg/ ml) for 60 min. Values represent the means±S.E.M. of 8-10 different cultures. *p<0.05 from the control group, #p<0.05 from the NMDA treatment group.

## Effects of Rc on elevation of $[Ca^{2+}]_i$

To determine what caused ginsenosides-induced glutamate release, $[Ca^{2+}]_i$ was measured by ratio fluorometry with fluorescence dyes, FURA-2 AM. The resting level of $[Ca^{2+}]_i$ was 30-40 nM. Rb1 and Rg1 at the concentration of 500 μg/ml did not change the $[Ca^{2+}]_i$ (data not shown), while Rc increased $[Ca^{2+}]_i$. In preliminary experiments, the $[Ca^{2+}]_i$ was increased gradually, and reached plateau level 10 min after the treatment with Rc. Thus, the $[Ca^{2+}]_i$ was measured with Rc (250 μg/ml) after 10 min exposure, and to determine whether Rc directly activated NMDA receptor, NMDA receptor antagonist, MK-801 (10 μM),

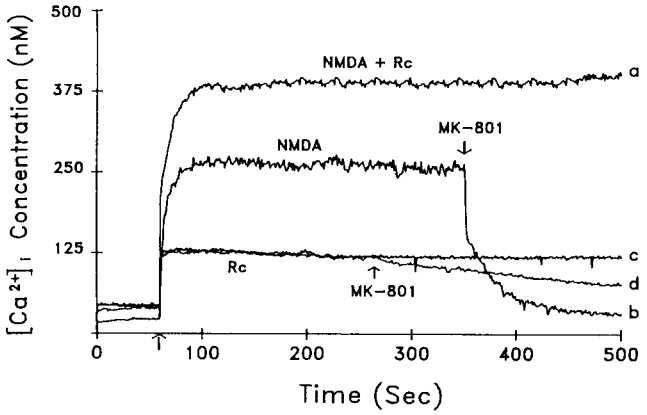



**Fig. 4.** Intracellular calcium levels in cerebellar granule cells induced by NMDA or Rc and reduced by MK-801. Experiments were performed as described in the text. Cells were loaded with FURA-2 AM for 60 min, washed with PSH buffer, and NMDA (50 μM) and/or Rc (250 μg/ml) and were applied as arrow indicated. In case of Rc treatment group (curve c and d), 10 min was paused after adding the ginsenoside to obtain a plateau calcium curve. Each curve represents the average of 3-4 experiments.

was added during the plateau level of $[Ca^{2+}]_i$ after Rc treatment. Rc elevated the $[Ca^{2+}]_i$ (127.00±27.83 nM, n=4) (curve c in Fig. 4) and MK-801 slightly blocked the Rc-induced elevation (31.32±6.97%, n=4) (curve d in Fig. 4), but pretreatment of MK-801 did not affect Rc-induced elevation of $[Ca^{2+}]_i$. However, the concentration of MK-801, 10 μM, was quite enough to block 50 μM NMDA-induced $[Ca^{2+}]_i$ elevation (251.90±10.77 nM, n=4) (curve b in Fig. 4). On the other hand, Rc did not show any inhibitory effect on NMDA-induced $[Ca^{2+}]_i$ elevation, rather it augmented NMDA effect (381.00±44.66 nM, n=3) (curve a in Fig. 4).

## DISCUSSION

In the present study, ginsenosides isolated and purified from ginseng saponin fraction affected glutamate release with different intensities. Since a lot of experiments were performed using ginseng total saponin, a mixture of several components, it was not easy to define the mechanism of actions.

Ginsenosides (Rb1, Rc and Rg1) increased extracellular glutamate concentration in our cultured cells. Especially, Rc produced a strong increase in extracellular glutamate level. This result may not be due to saponin effect, since LDH test of ginsenosides with their concentration up to 500 μg/ml did not show significant difference compared to control after 1 h incubation (data not shown). If neurons were damaged by ginsenosides, many kinds of amino acids would be leaked from cytosol to extracellular medium through damaged cell membrane. However, there was no significant increase in many amino acids concentration in extracellular medium after incubation with ginsenosides (Table 1).

Interestingly, ginsenosides inhibited NMDA-induced glutamate release, while ginsenoside alone increased the glutamate release. An excitatory amino acid, glutamate, is a major neurotransmitter in the mammalian central nervous system and the concentration is high (approximately 100 mM) in glutamatergic synaptic vesicles (Tayler *et al.*, 1992). Moreover, glutamate plays a major role in some amino acids metabolism in cytoplasm and its cytoplasmic concentration in neurons is about 10 mM (Taylor *et al.*, 1992). One metabolic pathway for glutamate involves its decarboxylation to γ-aminobutyric acid. In another reaction, it may undergo transamination with oxaloacetate to form α-ketoglutarate and aspartate. A further aspect of the metabolism of glutamate is its conversion into glutamine by the enzyme glutamine synthetase (Kurk and Pycock, 1991). The glutamate level found in rat plasma, cerebrospinal fluid and hippocampus extracellular fluid is 160, 11 and 3 μM respectively (Lerma *et al.*, 1986). This amino acid is responsible not only for normal excitatory transmission but also for potential neurotoxicity. Therefore the concentration of glutamate around its receptor is regulated by several different mechanisms (Mayer and Westbrook, 1987; Flott and Seifert, 1991), predominantly, glutamate is removed by reuptake into both neurons and glia by high affinity transport systems. Abnormally low level of glutamate can compromise normal level of excitation, whereas excessive level can produce toxic effects (Rothman and Olney, 1986). As mentioned previously, ginsenoside stimulated nerve growth in cultured cell (Saito *et al.*, 1977), and glutamate was involved in neuronal survival and learning processes via elevation of $[Ca^{2+}]_i$, nitric oxide generation and/or cGMP elevation (Balazs *et al.*, 1988; Collingridge and Bliss, 1987). In this regard, ginsenoside-induced glutamate release may be related to the stimulation of nerve growth and improvement of the cognitive processes. On the other hand, ginsenosides down-regulated the NMDA-induced glutamate release, suggesting that ginsenosides decrease the excitotoxicity. Although Rc did not block NMDA-induced glutamate release as observed with Rb1, Re and Rg1, the level of glutamate release was lower in culture treated with NMDA plus Rc than in culture treated with Rc alone. These results indicate the homeostatic effects of ginsenosides; ginsenoside by itself works positively but under a pathological condition it works negatively to maintain homeostasis.

Ginsenoside Rc strongly increased the glutamate release and $[Ca^{2+}]_i$. There are two possibilities to explain the Rc-induced $[Ca^{2+}]_i$ elevation. One possibility is that Rc changed/increased membrane potential, which caused an opening of voltage sensitive cal-

cium channel (VSCC). The other possibility is that Rc directly activated NMDA receptor (ionotropic calcium channel), resulting in elevation of $[Ca^{2+}]_i$. To differentiate these two possibilities, a specific NMDA receptor antagonist, MK-801 (10 μM), was applied during plateau level of $[Ca^{2+}]_i$ after Rc exposure. Although MK-801 slightly blocked Rc-induced increase of $[Ca^{2+}]_i$, the possibility of activation of VSCC still existed. It seems that partial blocking effect of MK-801 on Rc-induced elevation of $[Ca^{2+}]_i$ is due to inhibitory effect on released glutamate. This means that Rc induces a fixed amount of glutamate release during its exposure period (10 min) and the released glutamate can activate NMDA receptor as well as non-NMDA receptor. In terms of glutamate release, Rc (250 μg/ml) showed nearly 6.5 times higher activity than NMDA (100 μM), while the elevation of Rc-induced $[Ca^{2+}]_i$ was much lower than NMDA-induced effect. And Rb1 and Rg1 induced extracellular glutamate elevation without increase of $[Ca^{2+}]_i$. These facts suggest a possibility that ginsenosides inhibit glutamate reuptake resulting in the elevation of extracellulr glutamate level. However, further experiments would be helpful to clear the precise mechanism. Structure of ginsenosides is shown in Fig. 1. Interestingly, ginsenoside Rc is a steroid-derivative containing furanoside (penta-ring) among the ginsenosides. Similarly one of cardiotonic compounds, ouabain (selective $Na^+,K^+$-ATPase inhibitor) has a steroid backbone containing sugar and penta-ring and it can increase $[Ca^{2+}]_i$ (Scheufler *et al.*, 1992). This structure similarity raise an intriguing possibility that Rc may have an inhibitory effect on $Na^+,K^+$-ATPase.

In summary, the data presented here showed that ginsenosides, Rb1, Rc and Rg1, increased the glutamate release but Re did not. However, all of the ginsenosides down-regulated NMDA-induced glutamate release. Only Rc elevated $[Ca^{2+}]_i$ which was slightly blocked by NMDA antagonist, MK-801.

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