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Influence of Ginsenosides on the Kainic Acid-Induced Seizure Activity in Immature Rats

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We studied the effects of ginsenosides in immature rats based upon the previous results that ginseng has a suppressive or anticonvulsive activity. To examine the suppressive effect of ginsenosides on kainic acid-induced seizures, the severities and frequencies were observed for 4 h after injection of kainic acid (KA; i.p., 2 mg/kg b.w.) using 10-day-old male Sprague-Dawley rats (22 ± 2 g). Protopanaxadiol saponins such as ginsenoside-Rb1 (Rb1), ginsenoside-Rb2 (Rb2), ginsenoside-Rc (Rc), and ginsenoside-Rd (Rd) generally reduced the seizure activities while protopanaxatriol saponins such as ginsenoside-Rg1 (Rg1) and ginsenoside-Re (Re) rather increased stereotypic "paddling-like" movements. When vinyl-GABA (v-G) was injected together with Rb1 or Rc, KA-induced seizure severities were additionally reduced only by the injection of Rc, but not by Rb1. The level of gamma isozyme of protein kinase C (PKC-γ) in the hippocampus increased about three times as much as that of normal rats at 4 h after KA injection. The increased level of PKC-γ by KA was significantly reduced to about 35% by the coinjection with v-G alone, but it was not changed by v-G together with Rb1 or Rc. The increased level of PKC-γ at 4 h after injection of KA was not consistent with the reduction of seizure severities between Rb1 and Rc. These results suggest that Rc and Rb1 may reduce seizure severity independent of PKC-γ levels, and Rc may additionally act with v-G regarding the GABA metabolism during the stage of KA-induced seizures in the immature rats.

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Introduction

Convulsions and seizures during infancy may bring about the obstruction of the central nervous system including neurochemical changes, increased seizure susceptibility, and impediment of cognitive functions over long periods of time. Kainic acid (KA), an analogue of the naturally-occurring excitatory amino acid neurotransmitter glutamate results in a condition similar to human temporal lobe epilepsy, when given either systemically or intracerebrally to adult rats (Nadler and Cuthbertson, 1980; Ben-Ari, 1985). It was found that there were no pathological changes in the immature brain after a single set of KA-induced seizures unlike in adult rats (Pollard *et al.*, 1994), although KA is sufficient to induce status epilepticus in immature rats at lower dosage than adults. The mechanism underlying the age-dependent selective vulnerability of the developing brain to the consequences of early seizures is not yet clear. However, it may be possible to study seizure activity itself and actions with compounds so-called AED (Anti-Epileptic Drug) related to GABAergic metabolism, including vinyl-GABA (v-G), an irreversible inhibitor of gamma-amino-butyric acid transaminase (GABA-T). GABA (γ-amino butyric acid) is a major inhibitory neurotransmitter in the mammalian central nervous system (CNS) (Cooper, *et al.*, 1986). The release of GABA by nerve terminals and its subsequent binding to its receptor is followed by a rapid inactivation of the neurotransmitter. When the concentration of GABA in brain diminishes to below a threshold level, various neurological disorders including epilepsy, seizures, convulsions, Huntington's disease, and Parkinsonism may occur (Perry *et al.*, 1973;

De Biase *et al.*, 1991; Lloyd *et al.*, 1977). The concentration of GABA in the brain is controlled by two GABA shunt enzymes, glutamate decarboxylase (GAD) and GABA-T. The first enzyme catalyzes the synthesis of GABA, whereas the second enzyme catalyzes the conversion of GABA to succinic semialdehyde. The activation of GAD or the inactivation of the GABA-T in brain tissues increases the concentration of GABA.

It has been postulated that the regulation of GABAergic neurotransmission may be an important action of some ginsenosides which were purified from the root of *Panax ginseng C.A. Meyer* (James *et al.*, 1982; Kimura *et al.*, 1994). Our previous results have shown that Rb2 and Rc increased *in vitro* GAD activities in a dose-dependent manner in response to increasing concentrations of Rc (Choi *et al*, 1994; 1998). Among the GABA shunt enzymes, only *in vivo* GAD activities were increased after total ginsenosides treatment (Choi *et al.*, 1998).

The actions of GABA are mediated by at least two different receptor classes that have been defined pharmacologically: $GABA_A$ receptors and $GABA_B$ receptors. The $GABA_A$ receptor activates a Cl^- channel (Bormann, 1988) whereas $GABA_B$ receptor is not coupled to Cl^- channels but modulates Ca^{2+} or K^+ channels via second messenger systems (Bormann, 1988; Bowery, 1993). It has also been reported that the reduction of GABAergic inhibition is associated with only a small reduction in the binding of $GABA_A$-receptor ligands (Titulaer *et al.*, 1994), and Protein Kinase C (PKC) activity may be an important determinant for the $GABA_A$-mediated response *in vivo* (Krishek *et al.*, 1994).

The interactions of ginsenosides with ligand bindings of $GABA_A$ and $GABA_B$ receptors showed that Rb1, Rb2, Re, Rf, and Rg1 were associated with only $GABA_A$-receptors, but Rc was associated with both $GABA_A$ and $GABA_B$ receptors (Kimura *et al.*, 1994). On the other hand, Rb1, unlike Rc, has been reported to increase synapse number in the hippocampal CA3 region of mouse brain treated for 4 weeks (Ying *et al.*, 1994) and showed beneficial effects on the cholinergic and neurotrophic systems (Salim *et al.*, 1997). These results above and the GAD enzyme activation of Rc led us to question whether Rc and Rb1 could differently or additionally act on KA-induced seizure activities when coinjected with v-G, an inhibitor of GABA-T, and also the changes of PKC regarding signal transductions in this model system.

In the present work, we studied the effect of ginsenosides on KA-induced seizure activities associated with the influence of Rc regarding GAD enzyme activation. In particular, we examined the level of PKC-γ isozymes related to the reduction of seizure activities in the hippocampus of KA-injected immature rats.

Materials and Methods

Animal treatment and materials To examine the suppressive effect of compounds on the KA-induced seizures, the severities and frequencies were observed at a constantly-controlled temperature (25 ± 2°C) in individual chambers for 4 h after injection (i.p., 2 mg/kg b.w., solubilized in buffered saline, pH 7.4) of KA (Sigma Chemical Co., St. Louis, USA) in 10 day-old male Sprague-Dawley (SD) rats (22 ± 2g). The ginseng saponin fraction (GTS, 50 mg/kg b.w.) or ginsenosides (Rb1, Rb2, Rc, Rd, Re, and Rg1, 12.5 mg/kg b.w.) and/or v-G (20 mg/kg b.w.) were administered intraperitoneally 30 min before KA injection. Antibody against peptide unique to the PKC-γ isoform was from GIBCO BLR (Grand Island, USA). Peroxidase-labeled secondary antibody and ECL detecting reagent were from Amersham International. All other materials were obtained from commercial sources.

Classification of seizure activities and analysis The seizure activities (James *et al.*, 1982) were classified in five grades as follows: normal behavior (grade 0), "paddling-like" movement (grade 1), single opistotonic spasm and sporadic jerk (grade 2, grade 3), loss of balance and tonic clonus (grade 4), and final upset as an apparent lack of consciousness (grade 5). The behavioral characteristics of the seizures for each group were recorded with video camera and the durations and frequencies of the convulsions were then classified and summed up according to the above scoring criteria at 10 s intervals. Grades were checked at intervals of every 10 s.

Western analysis Animals were decapitated at 0, 0.5, 2, 4, 6, 12, 18, and 24 h after the KA injection. Hippocampi were rapidly dissected from the brains and frozen in liquid nitrogen and then stored at –70°C until use. The brain samples were homogenized in 200 μl of ice-cold homogenizing buffer (20 mM Hepes, pH 7.4, 1 mM EDTA, 2 mM DTT, 0.1 mM EGTA, 0.1 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 20 μg/ml aprotinin) using a teflon-glass homogenizer and centrifuged at 20,000 × *g* for 30 min at 4°C. The protein was determined by a Bio-Rad protein assay kit with bovine serum albumin as the standard. Equal amounts of protein (10–25 μg) were subjected to 10% SDS-PAGE and transferred to nitrocellulose (Hybond-ECL, Amersham). PKC-γ was immunoreacted with an appropriate dilution of primary antibodies to PKC-γ (1:500) at room temperature for 1 h and incubated with a proper dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG antibody as a secondary antibody. ECL was used to reveal antibody binding. Quantification of PKC isoforms was performed by measuring the band intensity on a film using a scanning densitometer (Hoefer Scientific Instruments, GS300).

Statistical analysis Data were analysed using the Student' *t*-test and one-way ANOVA followed by Mann Whitney U-test for individual comparisons. Significance level was defined as 0.05 for all comparisons.

Results

We tested whether individual ginsenosides could alleviate convulsive activities during KA-induced seizures in 10-day-old rats. As shown in Fig. 1, Rb1, Rb2, Rc, and Rd significantly reduced the seizure frequencies while Re and

Rg1 did not. Re and Rg1 rather increased the stereotypic paddling-like movements defined as grade of seizures (data not shown).

It has been reported that ginsenosides fractions inhibited the uptake of GABA, glutamate, dopamine, noradrenaline, and serotonin into rat brain synaptosomes (Tsang *et al.*, 1985). Among the ginsenosides tested, the fractions mainly containing Rc or Rd were postulated to inhibit the GABA transport system to a greater extent than other neurotransmitter uptake stystems, but those with Rb1 or Rb2 were not (Tsang *et al.*, 1985). Our previous results showed that Rb2 and Rc increased glutamate decarboxylase (GAD) activities in a dose-dependent manner *in vitro*, however, Rb1 did not show any significant effect on GAD activities (Choi *et al.*, 1998). Therefore, we decided to compare the differences between Rc and Rb1 on KA-induced seizure activities using v-G. Rc, when injected into the rats together with v-G, decreased more significantly than the v-G-injected control group or v-G plus Rb1 group (Fig. 2).

It was found that Rb2 and Rc cause a GTP-reversible inhibition of adenylate cyclase from rat brain *in vitro* although Rb1 does not (Park *et al.*, 1984). Recently, it has also been suggested that phosphodiesterases (PDE) inhibitions could be one of the molecular mechanisms which participate in ginsenosides effects, especially the effect of Protopanaxadiol (PPD) on blood vessels and CNS (Lugnier and Kim, 1998). Together with this, it would be important to elucidate the differences between Rb1 and Rc on the regulation of GABA metabolism in view of the additional anticonvulsive effect of Rc with v-G (Fig. 2). We investigated the level of PKC-γ isozymes associated

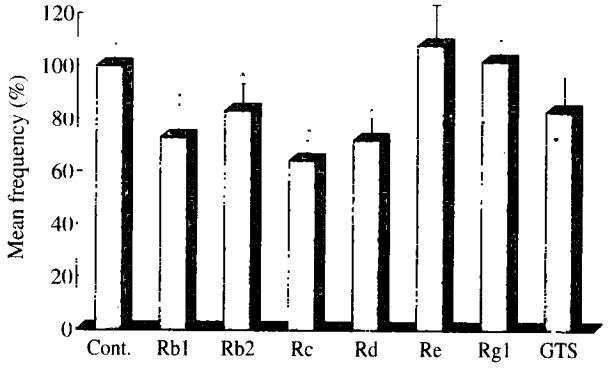

Fig. 1. Effect of ginsenosides on kainic acid-induced seizure activities in the immature rats. The grades of seizure activities were analyzed for 4 h after kainic acid (2 mg/kg, b.w., s.c.) injection in 10 day-old rats. Ginsenosides (12.5 mg/kg, b.w.) were injected intraperitoneally 30 min before kainic acid treatment. Data are expressed as mean % of the control values which summed up the scores of the 3rd, 4th, and 5th grade seizure activities (mean ± SE, n=10). GTS, Ginseng total saponin fraction (50 mg/kg b.w.). *, $p < 0.05$ vs control.

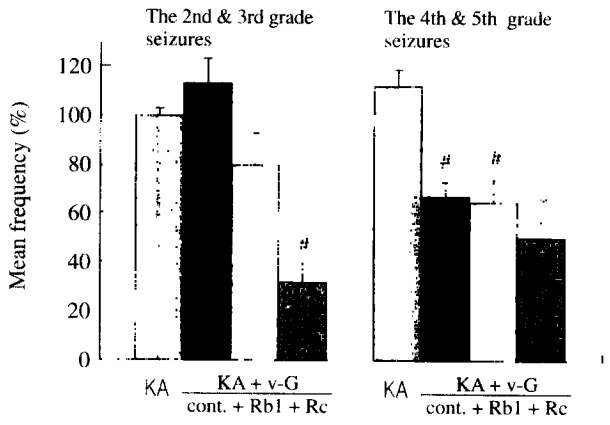

Fig. 2. Effect of ginsenosides (-Rb1 or -Rc) with vinyl-GABA on kainic acid-induced seizures in the immature rats. The seizure activities were classified and analyzed for 4 h after injection of kainic acid (2 mg/kg, b.w., s.c.) in 10-day-old rats. The vinyl-GABA(v-G, 20 mg/kg b.w., i.p.) and/or ginsenoside Rb1 and Rc (12.5 mg/kg, respectively) were injected intraperitoneally 30 min before kainic acid (KA) treatment. Data are expressed as mean ± S.E., n=5. $p < 0.05$ vs KA-injected control (#), KA + v-G control (*).

with the KA-induced hippocampal deficits. Among PKC isozymes currently studied, PKC-γ in the hippocampus showed very dramatic changes during and after KA-induced seizures. The level of PKC-γ was maximally increased for 4–6 h after KA injection, maintained until 12 h, and then significantly reduced to normal levels at 24 h (Fig. 3).

The level of PKC-γ in the hippocampus increased about 3-fold over normal levels at 4 h after KA injection (Fig. 4.) but there were no significant changes by injection of Rb1 or Rc as compared with the KA-injected control at 4 h (data not shown). The increase of PKC-γ was reduced to about 35% by injecting v-G in KA-induced seizures. On the other hand, when Rb1 or Rc was injected together with v-G, the level of PKC-γ showed significant increases as compared with the v-G-injected control group and there were no significant changes between Rb1 and Rc (Fig. 4).

These results suggest that Rc and Rb1 may reduce the severities of seizures (Fig. 2) and Rc may additionally act with v-G on the GABA metabolism.

Discussion

Among the GABA shunt-regulating enzymes, only GAD activities were increased after saponin treatment in rat brain, and Rc among all the ginsenosides had the lowest K_m value for GAD *in vitro* (Choi *et al.*, 1998). The enzymatic activation was thought to be due to the conformational change induced by the binding of ginsenoside to the enzyme (Choi *et al.*, 1998). We examined how Rb1 and Rc affect the KA-induced seizure activities regarding GABA shunt since Rc was effective in

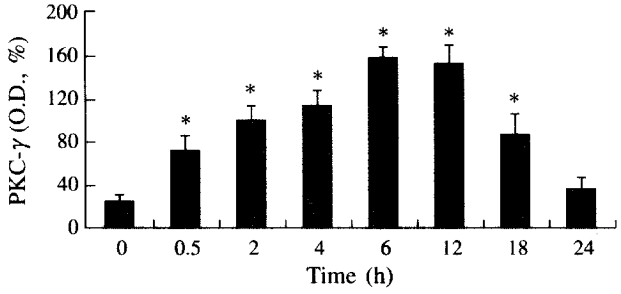

Fig. 3. Time-course differences of PKC-γ immunoblots in hippocampus after injection of kainic acid in 10-day-old rats. Animals were decapitated at various times after KA injection. PKC-γ was immunoreacted with an appropriate dilution of primary antibodies to PKC-γ (1:500) at room temperature for 1 h and incubated with a proper dilution of horseradish peroxidase conjugated goat anti-rabbit IgG antibody as a secondary antibody. ECL was used to reveal antibody binding. Quantification of PKC isoforms was performed using a scanning densitometer (Hoefer Scientific Instruments, GS300). Data are expressed as mean ± S.E., n=5. *, $p < 0.01$ vs zero time.

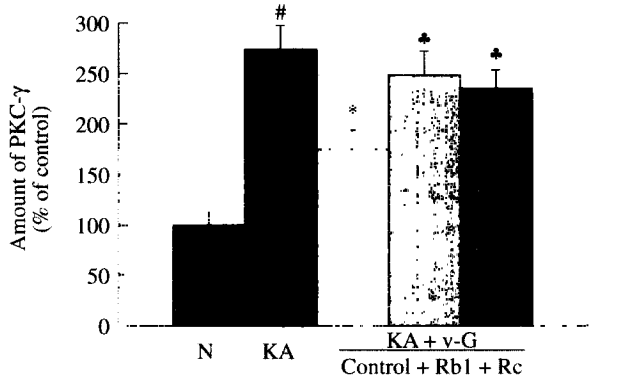

Fig. 4. Combining effect of vinyl-GABA with ginsenoside-Rb1 or -Rc on the hippocampal level of PKC-γ at 4 h after kainic acid injection in 10-day old rats. PKC-γ was immunoreacted with an appropriate dilution of primary antibodies to PKC-γ (1:500) at room temperature for 1 h and incubated with a proper dilution of horseradish peroxidase conjugated with goat anti-rabbit IgG antibody as a secondary antibody. ECL was used to reveal the level of bound antibody binding. Quantification of PKC isoforms was measured using a scanning densitometer (Hoefer Scientific Instruments, GS300). $P < 0.05$ vs normal (#), vs KA(*), vs KA+ v-G control(♣). N, normal; KA; kainic acid; v-G, vinyl-GABA; Rb1, ginsenoside-Rb1; Rc, ginsenoside-Rc.

reducing hyperthermia-induced seizures in immature rats (Park *et al.*, 1995), while Rb1 has been found to beneficially regulate certain aspects of the cholinergic and neurotrophic systems (Kenneth *et al.*, 1997). We studied 10-day-old rats which had shown the most severities in hypoxia (Park *et al.*, 1997) as well as in KA-induced seizure (Carl *et al.*, 1992). The protopanaxadiols such as Rb1, Rb2, and Rc generally reduced the KA-induced seizure activities in immature rats (Fig. 1). However, ptotopanaxatriols such as Re and Rg1 increased the grade 1 "paddling-like" stereotypic movements but decreased the grade 4 and 5 seizures. In the inhibition of grade 3 seizures (single opistotonic spasm and sporadic jerk), Rc was more effective than Rb1. The effect of v-G on grade 3 seizure activity was not significantly changed at a dosage of 20 mg/kg b.w. as compared with KA-injected control values, but the seizure frequencies were decreased by the systemic injection of v-G together with Rb1 or Rc before KA-injection (Fig. 2). In grades 4 and 5 seizures, v-G reduced the activities to about 35% as compared to KA-injected control (Fig. 2), and injection of Rb1 together with v-G did not show any additional inhibitory effect unlike the case of Rc. These results suggest that Rc might retard the GABA drain better than Rb1 during seizure activities when coinjected with v-G. It has been reported that v-G showed a neuroprotective effect in adult rat hippocampus after status epilepticus induced by KA (Halonen *et al.*, 1995). A single monotherapy trial comparing vigabatrin (v-G) with carbamazepine in patients with newly diagnosed epilepsy showed that similiar numbers of patients were treated successfully. Among patients who withdrew, those taking v-G withdrew more because of lack of efficacy, whereas those receiving carbamazepine withdrew more because of adverse effects (Kalviainen *et al.*, 1995). These results are thought to show that there is some dose-related efficacy reason in v-G treatment. Several studies have shown that v-G is particularly effective against infantile spasms, and a recent European retrospective survey of 250 infants concluded that v-G is sufficiently effective that it can be considered for initial treatment of infantile spasms (Aicard *et al.*, 1996). Therefore, the additional severity-reducing effect of Rc with v-G in KA-induced seizures could be effective as an auxiliary treatment. We studied further the level of PKC-γ at 4 h after KA-injection (Fig. 3) since PKC-γ level was greatly increased (about 3 times normal) at this time and maintained until 12 h after KA injection and then significantly reduced again to normal levels by 24 h (Fig. 3). PKC enhanced NMDA-receptor-mediated glutamate responses and the roles of NMDA receptors related with PKC are critical in generating and maintaining a variety of sustained neuronal responses in adult rats (Chen and Huang, 1992). However, the receptor functions and signal transduction mechnisms associated with the age-specific resistance on the KA excitotoxicity have not been clearly elucidated yet in immature rats. The increasing level of hippocampal PKC-γ at 4 h after KA-induced seizures did not significantly change with pretreatment of Rc or Rb1 at doses of 12.5 mg/kg b.w. This dosage is neither more nor less than when considering the adult rat dose, 10–20 mg/kg, for significant ischemic protection of both neuronal density and latency time in step-down passive avoidance task (Lim *et al.*, 1997). There was a small dose-dependent relationship between 10 and 20 mg/kg (b.w.). We also have preliminary evidence that 5

mg/kg/day i.p. injection of ginsenosides in immature rats was also effective in significantly ($p < 0.05$) reducing the frequencies and severities of KA-induced seizures (unpublished observations). The amounts (12.5 mg/kg) of Rb1 and Rc were enough to suppress as much as 30–40% on KA-induced seizure severities (Fig. 1), at least in 10-day-old rats. On the other hand, dose-response results of individual Rc with v-G injection in KA-induced seizures could not be determined. Regardless, the dosage of 12.5 mg/kg on Rc could bring about the results statistically significant in both cases of with/without v-G. Dose-response experiments involving the effects of diol ginsenosides on KA-induced seizures are now in progress employing tremor monitors (San Diego Instrument) in order to get more defined results. We now believe that one of the difficulties may come from the narrow dose-response range of KA, and some tolerance mechanism. In our experiment conditions, the maximal doses of KA needed to observe status epilepticus without death were limited to around 2 mg/kg b.w. This window might be linked to the lack of neuronal death following KA administration, with increased glutamate levels being neuroprotective rather than excitotoxic at some levels or at some developmental stages (Sperber *et al.*, 1991). It has been postulated that the immature brain is protected against the detrimential effects of KA because of an incomplete glutaminergic input pathway from dentate granule cells to CA3 pyramidal neurons and cells of the dentate hilus (Parent *et al.*, 1997). Direct injections of glutamate into the hippocampus of 10-day-old rats failed to produce cell loss (Liu *et al.*, 1996). Since KA acts in part by releasing glutamate from presynaptic stores (Chittajallu *et al.*, 1996), it is possible that excessive KA-released glutamate may play a protective role against seizure-induced damage in immature rats (Sarkisian *et al.*, 1997). Oh *et al.* (1995) reported that Rc strongly increased glutamate release and Ca^{2+} concentrations, where the level of glutamate release was about three times lower in culture treated with NMDA-plus-Rc than in cultures treated with Rc alone. However, Rb1 and Rg1, as contrasted with Rc, induced extracellular glutamate elevation without increase of $[Ca^{2+}]$ in cultured rat cerebella neurons (Oh *et al.*, 1995). It has been also postulated that ginsenoside Rb1 and Rg3 may be efficacious in protecting neurons from oxidative damage that is produced by exposure to excess glutamate (Kim *et al.*, 1998).

To sum up, Rc, unlike Rb1 and/or Rb2, strongly inhibited GABA and glutamate uptake (Tsang *et al.*, 1985), increased GAD activities (Choi *et al.*, 1998), and is associated with ligand binding of not only $GABA_A$-receptors but also $GABA_B$ receptors (Kimura *et al.*, 1994). Rc did not affect the levels of PKC-γ increased by KA. However, Rc increased the decreased PKC-γ level again when injected with v-G. Since there were no significant differences between Rb1 and Rc in their PKC-γ levels, the increment by v-G use (Fig. 4) seems rather to be more involved in $GABA_A$ receptors than in$GABA_B$ receptors.

Conclusively, it was suggested that Rc, unlike Rb1, may suppress GABA and glutamate uptake, and additively act together with v-G by increasing GAD activities, followed by retarding the GABA concentration rapidly dried up during the KA-induced seizure activities

The increase of PKC-γ isoform was also found in both pentylenetetrazole-induced chemoshocked and electro-shocked mouse brain (Chen, 1994). The increased level of PKC-γ by KA (Fig. 4.) was significantly reduced to about 35% by the coinjection of v-G but it was not changed by v-G together with Rb1 or Rc. The increased level of PKC-γ at 4 h after injection of KA was not consistent with the reduction of seizure severities between Rb1 and Rc. The dosage (20 mg/kg, b.w.) of v-G which we used in our experiment was not less than the lower limit of the usual adult dosage (17 mg/kg, b.w., 1000 mg/day/60 kg b.w.). In this study, we tried to understand the influence of Rc related to GAD enzyme activation (Choi *et al.*, 1998) and the increased level of PKC-γ associated with the reduction of KA-induced seizure activities in the immature rats. The results of the present study suggest that Rc and Rb1 may reduce the severities of seizures regardless of PKC-γ level, and Rc may additionally act with v-G regarding GABA metabolism during the stage of KA-induced seizures in the immature rats.

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