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# Ginsenosides Inhibit NMDA Receptor-Mediated Epileptic Discharges in Cultured Hippocampal Neurons

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Epilepsy or the occurrence of spontaneous recurrent epileptiform discharges (SREDs, seizures) is one of the most common neurological disorders. Shift in the balance of brain between excitatory and inhibitory functions due to different types of structural or functional alterations may cause epileptiform discharges. *N*-Methyl-D-aspartate (NMDA) receptor dysfunctions have been implicated in modulating seizure activities. Seizures and epilepsy are clearly dependent on elevated intracellular calcium concentration ($[Ca^{2+}]_i$) by NMDA receptor activation and can be prevented by NMDA antagonists. This perturbed $[Ca^{2+}]_i$ levels is forerunner of neuronal death. However, therapeutic tools of elevated $[Ca^{2+}]_i$ level during status epilepticus (SE) and SREDs have not been discovered yet. Our previous study showed fast inhibition of ginseng total saponins and ginsenoside $Rg_3$ on NMDA receptor-mediated $[Ca^{2+}]_i$ in cultured hippocampal neurons. We, therefore, examined the direct modulation of ginseng on hippocampal neuronal culture model of epilepsy using fura-2-based digital $Ca^{2+}$ imaging and neuronal viability assays. We found that ginseng total saponins and ginsenoside $Rg_3$ inhibited $Mg^{2+}$ free-induced increase of $[Ca^{2+}]_i$ and spontaneous $[Ca^{2+}]_i$ oscillations in cultured rat hippocampal neurons. These results suggest that ginseng may play a neuroprotective role in perturbed homeostasis of $[Ca^{2+}]_i$ and neuronal cell death *via* the inhibition of NMDA receptor-induced SE or SREDs.

**Key words:** Ginseng, Ginsenosides, Seizure, Epilepsy, NMDA receptor, Intracellular calcium, Hippocampal neuron

## INTRODUCTION

Seizures or the chronic condition of repetitive seizures (epilepsy) is one of the most common neurological disorders. Especially, status epilepticus (SE; continuous seizure activity for 30 min or longer) is a serious neurological disorder associated with significant morbidity and mortality because 30 or more minutes of continuous convulsive seizures leads to brain injury or even death. Although recent advances show that cellular and molecular mechanisms of epilepsy are diverse and may involve the complex interactions of ion channels (Burgess and Noebels, 1999; Scheffer and Berkovic, 2003; Kim *et al.*, 2001), receptors (Chapman, 2000; Wong *et al.*, 2003; Raol *et al.*, 2001), and genes (Noebels, 2003), our understanding of molecular basis of epilepsy is still limited. Despite the diverse and complex features of epilepsy, numerous experimental and clinical data strongly suggest a key role of excitatory glutamate neurotransmission in the generation and maintenance of SE (Chapman, 2000; Raol *et al.*, 2001; Sun *et al.*, 2001). Among subtypes of glutamate receptors such as ionotropic (NMDA and non-NMDA) and metabotropic glutamate receptors, NMDA receptor activation provides the widest spectrum in regulating neuronal functions such as neuronal excitability, neuronal signaling, long-term potentiation (LTP), and neuronal cell death (Bliss and Collingridge, 1993; Choi, 1988; Westbrook, 1994). These are probably due to high $Ca^{2+}$ permeability of NMDA receptors (Jahr and Stevens, 1993), which could lead to NMDA-$Ca^{2+}$ dependent changes in triggering $Ca^{2+}$-dependent signal pathways, gene expression, and synaptic plasticity. It has been also reported that the induction of epilepsy is strongly dependent on elevated intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) through NMDA receptor activation (DeLorenzo *et al.*, 1998; Rice and DeLorenzo, 1998; Pal *et al.*, 1999).

The effects of NMDA antagonists in several animal

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models support evidences for the role of NMDA receptors in the generation and maintenance of SE. Both kainic acid (KA) and pilocarpine models of SE have been known to produce neuronal damage similar to human temporal lobe epilepsy and also cause the development of spontaneous recurrent epileptiform discharges (SREDs) (Clifford *et al.*, 1987; Schwob *et al.*, 1980; Honchar *et al.*, 1983). Many effects of KA-induced SE are specifically linked to the NMDA subtype of glutamate receptors. NMDA antagonists prevent the development of SREDs and provide protection against SE-induced neuronal injury (Churn *et al.*, 1991; Clifford *et al.*, 1990; Rice and DeLorenzo, 1998). Along with these animal models, the hippocampal neuronal culture (HNC) model of SE provides a powerful tool to investigate the molecular mechanisms underlying the induction, maintenance, and termination of continuous epileptiform discharges in hippocampal neurons *in vitro* and demonstrates that neuronal networks in culture can be transformed to manifest continuous seizure-like activity. Recent studies have demonstrated that the induction of epileptogenesis in the HNC model of epilepsy was dependent on NMDA receptor activation (DeLorenzo *et al.*, 1998; Pal *et al.*, 1999; Gulyas-Kovacs *et al.*, 2002).

Our previous study has demonstrated the inhibitory effect of ginseng, a well-known oriental herbal medicine, on NMDA receptors (Kim *et al.*, 2002). Using fura-2-based imaging techniques, we previously showed that ginseng total saponins and ginsenoside $Rg_3$, one of active ingredients of ginseng, rapidly inhibited NMDA receptors in cultured hippocampal neurons. These results raise the possibility that therapeutic use of this herbal medicine as the blocking agent in the primary cause of neurological disorders. We, therefore, investigated the effects of ginseng and ginsenoside $Rg_3$ on the propagation of SE-induced neuronal cell death and the development of SREDs in the HNC model of SE using fura-2-based intracellular $Ca^{2+}$ imaging.

## MATERIALS AND METHODS

### Materials

Ginseng total saponins (GTS), ginsenosides $Rg_3$ and Re were obtained from the Korea Ginseng and Tobacco Research Institute (Taejon, Korea). GTS were dissolved in external recording solution and ginsenosides $Rg_3$ and Re were dissolved in DMSO as a concentrated stock and further diluted to its final concentration in external recording solution. All chemicals for cell preparation were purchased from Gibco (Grand Island, NY). *N*-Methyl-D-aspartate (NMDA), D(-)-2-amino-5 phosphonopentanoic acid (D-APV), (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*] cyclohept-5,10-imine maleate (MK-801), 7-chlorokynurenic acid (7-CK) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were from Tocris (Ellisville, MO). Nifedipine was purchased from Calbiochem (San Diego, CA). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

### Cell preparation

Cultured hippocampal neurons were prepared using the technique modified from Kim *et al.* (2002). Briefly, the hippocampi were isolated from 16~18-day-old fetal Sprague-Dawley rats and incubated with 0.25% trypsin Hanks Balanced Salt Solution (HBSS) at 37 °C for 15 min. Cells were then mechanically dissociated with fire-polished Pasteur pipettes by trituration and plated at a density of $2\times10^5$ cells/cm$^2$ on poly-L-lysine-coated coverslips in a 35-mm culture dish. Cells were maintained in Neurobasal/B27 medium containing 0.5 mM L-glutamine, 25 μM glutamate, 25 μM 2-mercaptoethanol, 100 U/mL penicillin and 100 μg/mL streptomycin under a humidified atmosphere of 95% air and 5% $CO_2$ at 37 °C. Cultures were fed twice a week with the same medium without glutamate. Experiments were carried out on neurons after 21-25 days in culture.

### Intracellular $Ca^{2+}$ imaging

The acetoxymethyl-ester form of fura-2 (fura-2/AM; Molecular probes, Eugene, OR) was used as the fluorescent $Ca^{2+}$ indicator. Cells were incubated for 60 min at room temperature with 5 μM fura-2/AM and 0.001% Pluronic F-127 in HEPES-buffered solution composed of (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose, pH adjusted to 7.4 with NaOH. The cells were then washed with HEPES-buffered solution and placed on an inverted microscope (Olympus, Japan). Cells were illuminated using a xenon arc lamp, and the required excitation wavelengths (340 and 380 nm) were selected using a computer-controlled filter wheel (Sutter Instruments, CA). Data were acquired every 1 sec and a shutter in the light path between exposures was interposed to protect cells from photo-toxicity. Emitter fluorescence light was reflected through a 515 nm long-pass filter to a frame transfer cooled CCD camera and ratios of emitted fluorescence were calculated using a digital fluorescence analyzer and converted to $[Ca^{2+}]_i$. All imaging data were collected and analyzed using Universal Imaging software (West Chester, PA). All the data are represented as mean±S.E..

### Electrophysiological recordings

Whole-cell voltage-clamp recordings were made using the perforated patch-clamp method (Horn and Marty, 1988). Patch electrodes with resistance of 3-4 MΩ were filled with the internal solution contained (in mM): 120 potassium gluconate, 20 NaCl, 2 $MgCl_2$, 10 HEPES, pH adjusted to 7.4 with NaOH. Nystatin was prepared as a

stock solution (25 mg/mL) in DMSO and diluted to a concentration of 250 μg/mL using the internal solution and back-filled into the pipette after the tip of the pipette was initially filled with the nystatin-free solution. The external solution contained (in mM): 150 NaCl, 5 KCl, 2 $CaCl_2$, 10 HEPES, 10 glucose and 0.001 glycine, pH adjusted to 7.4 with NaOH. To remove the blockade of NMDA channels by $Mg^{2+}$, $Mg^{2+}$ was not added to the external solution whenever it needed. Current recordings were obtained with an EPC-9 amplifier and Pulse/Pulsefit software (HEKA, Germany).

### Neuronal death assay

Neuronal viability was quantified by measuring dehydrogenase activity that was retained in living cells using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Briefly, aliquot (50 μL) of MTT solution (1 mg/mL) in PBS was added directly to the cultures, and the cultures were then incubated for 4 h to allow MTT to metabolize to a formazan. The supernatant was then aspirated, and 100 μL of dimethyl sulfoxide (DMSO) were added to dissolve the formazan. The optical densities (OD) were measured using an automated spectrophotometric plate reader at a wavelength of 560 nm. Data are expressed as the percentage of OD values relative to vehicle-treated control cultures. All numerical values are represented as mean±S.E. Statistic significance was performed on the data using unpaired Students *t*-test.

## RESULTS

### Calcium dynamics and levels during *in vitro* SE

Calcium ions and calcium-dependent systems have been implicated in the pathophysiology of SE. Recent advances have led to the development of *in vitro* models of SE (Pal *et al.*, 1999, DeLorenzo *et al.*, 1998). This model of epilepsy is well suited to study the role of calcium in epileptogenesis, since it utilizes an episode of continuous seizure activity to induce permanent plasticity changes in the neurons that results in SREDs. To induce *in vitro* SE, the cultured rat hippocampal cells were exposed to the HEPES solution without added $Mg^{2+}$. After exposure to $Mg^{2+}$ free HEPES-buffered solution, the long-term change of $[Ca^{2+}]_i$ was measured using fura-2-based intracellular $Ca^{2+}$ imaging technique (Fig. 1A). Within seconds of the initiation of $Mg^{2+}$ free HEPES-buffered solution, neurons immediately produced a transient increase of $[Ca^{2+}]_i$ and increased the basal level of $[Ca^{2+}]_i$ slowly. However, $[Ca^{2+}]_i$ levels were maintained at high steady level after more than 1 h duration of SE along with prominent oscillation of $[Ca^{2+}]_i$ (Fig. 1B). After 3-h SE, the level of $[Ca^{2+}]_i$ did not return to basal level within further 2-h washout time.

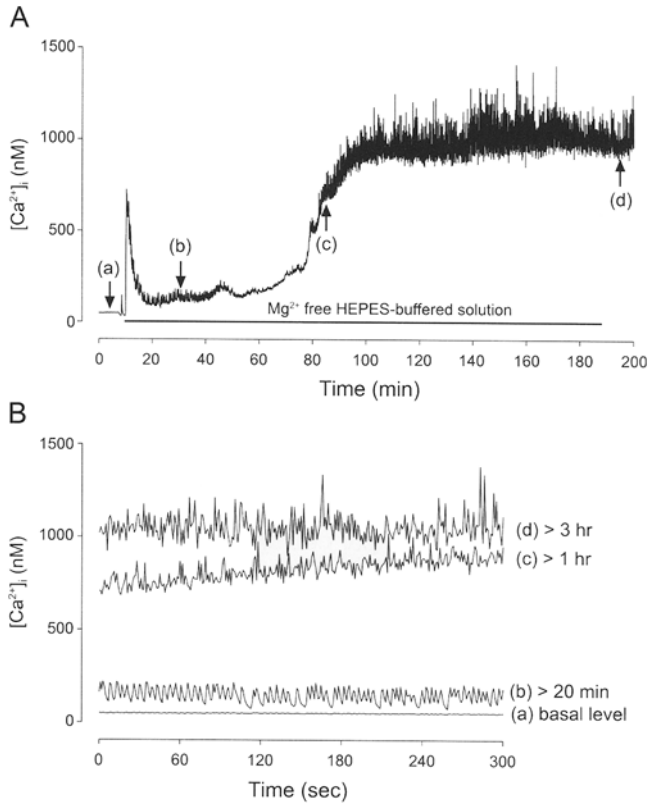


**Fig. 1.** Changes of $[Ca^{2+}]_i$ dynamics and levels during status epilepticus (SE) in the hippocampal neuronal culture (HNC) model. After exposure to $Mg^{2+}$ free HEPES-buffered solution, (A) illustrates the long-term change of $[Ca^{2+}]_i$ measured using fura-2-based intracellular $Ca^{2+}$ imaging technique. (B) Examples of time course of $[Ca^{2+}]_i$ dynamics and levels at labeled time points from (A). The level of $[Ca^{2+}]_i$ during SE was elevated and $[Ca^{2+}]_i$ spikes was higher in amplitude and frequency in neurons undergoing SE compared to the basal level.

### Effects of GTS and ginsenoside $Rg_3$ on NMDA receptors

We previously showed that ginseng inhibited NMDA-induced increase in $[Ca^{2+}]_i$ and ginsenoside $Rg_3$ is an active component for ginseng actions on NMDA receptors in cultured hippocampal neuron (Kim *et al.*, 2002). In order to confirm the direct modulation of NMDA by ginseng, we measured NMDA receptor-gated whole-cell currents using the nystatin perforated patch-clamp method. At the holding membrane potential of -60 mV, applied ginseng total saponins (GTS, 100 μg/mL) and ginsenoside $Rg_3$ (10 μM) rapidly inhibited NMDA-gated peak inward currents by 68.2±4.6% (n=6) and 75.9±1.8% (n=3), respectively (Fig. 2).

### Inhibitory effects of ginseng on induction of SE in the HNC model

It has been reported that the elevation in $[Ca^{2+}]_i$ during SE is dependent on increased entry of extracellular $[Ca^{2+}]_o$; NMDA-gated $Ca^{2+}$ entry accounts for main rise in $[Ca^{2+}]_i$

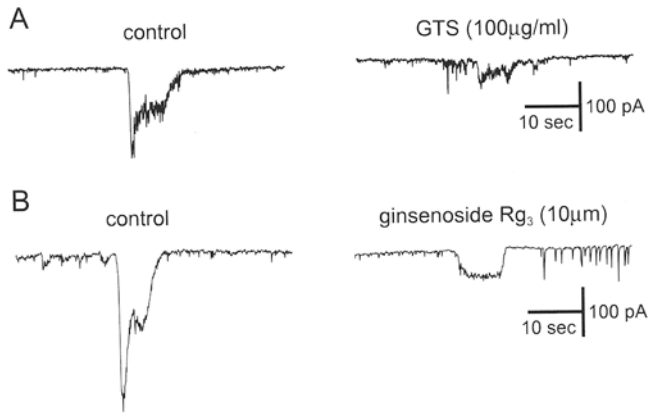


**Fig. 2.** Effects of ginseng total saponins (GTS) and ginsenoside $Rg_3$ on NMDA-gated currents. Using perforated whole-cell voltage-clamp recordings, acutely applied NMDA (100 μM, 10 sec) produced inward currents at the holding membrane potential of -60 mV. Application of GTS (100 μg/mL) and ginsenoside $Rg_3$ (10 μM) with 1 min pretreatment rapidly and reversibly inhibited NMDA-induced peak currents by 68.2±4.6% (n=6) and 75.9±1.8% (n=3), respectively.

during SE (Pal *et al.*, 1999). Because NMDA receptors can be blocked by various receptor antagonists, such as 2-amino-5-phosphonovaleric acid (D-APV), (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohept-5,10-imine maleate (MK-801), and 7-chlorokynurenic acid (7-CK), we were able to inhibit significantly 30 min SE-induced $[Ca^{2+}]_i$ dynamics using these antagonists (Fig. 3A). As shown in Fig. 2, GTS and ginsenoside $Rg_3$ can inhibit NMDA receptors in hippocampal neurons. We, therefore, examined effects of GTS and ginsenoside $Rg_3$ on SE-induced increase in $[Ca^{2+}]_i$. Treatment of GTS (100 μg/mL) or ginsenoside $Rg_3$ (10 μM) for 2 min significantly reduced and stopped SE-induced increase and oscillation of $[Ca^{2+}]_i$. However, the L-type channel inhibitor, nifedipine (5 μM), and the non-NMDA receptor blocker, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM), reduced the basal level slightly in some cells but produced an insignificant inhibition in SE-induced $[Ca^{2+}]_i$ increase. Fig. 3B showed the pooled results illustrating the mean percentage of SE-induced $[Ca^{2+}]_i$ increase by these various antagonists. Ginsenoside Re, having no significant effect on NMDA receptors in previous study (Kim *et al.*, 2002), had no effect on SE-induced $[Ca^{2+}]_i$ increase.

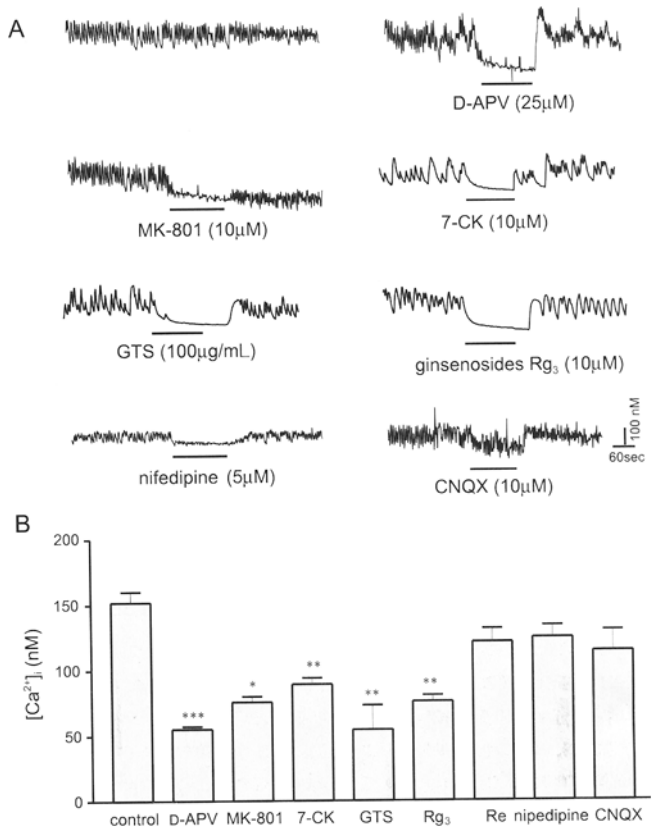


**Fig. 3.** Effects of ginseng and NMDA receptor antagonists on $[Ca^{2+}]_i$ level during SE. Hippocampal neurons were exposed to $Mg^{2+}$ free solution for 30 min and $[Ca^{2+}]_i$ level was measured before, during, and after 2 min treatment of drugs. Ginseng (GTS, ginsenoside $Rg_3$, ginsenoside Re), the competitive NMDA antagonists (D-APV for NMDA biding site, 7-CK for glycine binding site), NMDA open channel blocker, MK-801, the L-type channel inhibitor, nifedipine, and the non-NMDA receptor blocker, CNQX, were tested. (A) shows an example of single neuron and (B) shows the pooled results illustrating the mean of SE-induced $[Ca^{2+}]_i$ after treatment of these agents (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared to drug-untreated 30 min SE, control).

## Inhibitory effects of ginseng on development of SREDs in the HNC model

The $Mg^{2+}$-free (SE) treatment produced long-lasting plasticity changes in the neurons, resulting in the development of spontaneous representative epileptiform discharges (SREDs). It has been also reported that this epileptic state persisted for 2 days after treatment (DeLorenzo *et al.*, 1998). Following the procedure routinely performed in previous studies (Sombati and Delorenzo, 1995, DeLorenzo *et al.*, 1998), we measured hippocampal neuronal $[Ca^{2+}]_i$ 2 days after the 3-h exposure to SE. Control neurons having $Mg^{2+}$ free treatment significantly produced high level of $[Ca^{2+}]_i$ (from 57.1±13.8 to 837.5±55.4 nM, n=8). To evaluate the role of $[Ca^{2+}]_i$ in causing SREDs, we examined whether lowing in the extracellular medium during SE could inhibit the induction of epileptogenesis. When the NMDA receptor antagonists, D-APV, MK-801, and 7-CK, were applied during 3-h SE treatment, all completely blocked the SREDs-rised $[Ca^{2+}]_i$ (Fig. 4). GTS and ginsenoside $Rg_3$ were also effective in decreasing the rise in $[Ca^{2+}]_i$ as they were on SE-induced increase $[Ca^{2+}]_i$. However, in contrast to NMDA receptor antagonists, nifedipine, CNQX, and ginsenoside Re did not altered the SREDs-induced high level of $[Ca^{2+}]_i$. These results are

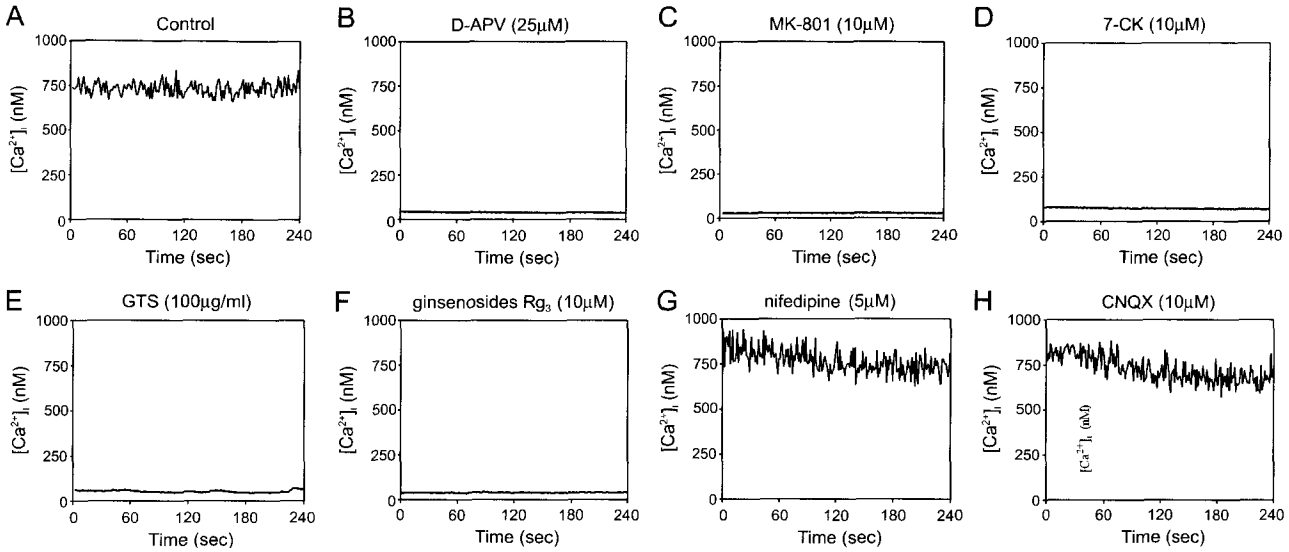


**Fig. 4.** Sample records comparing effects of various neuropharmacological agents on SREDs-induced $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ levels of hippocampal neurons were measured 2 days after the 3-h exposure to SE. Neuronal cultures were exposed to $Mg^{2+}$ free solution with or without the added drugs for 3 h.

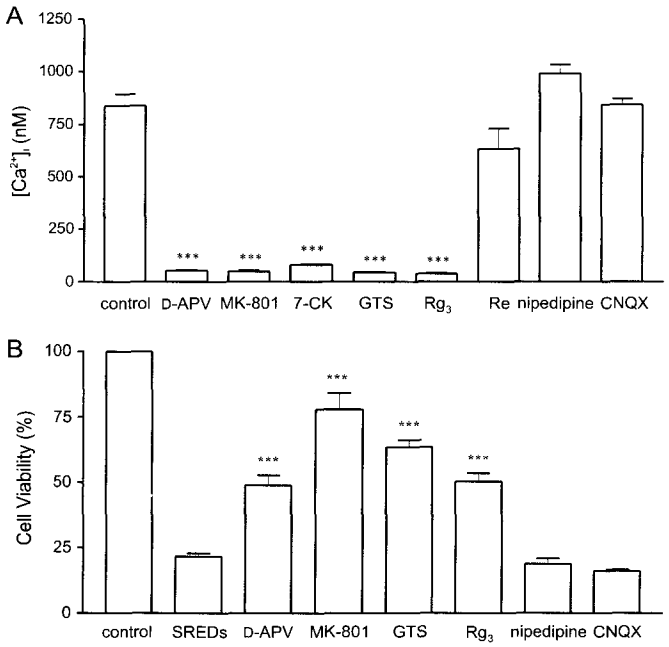


**Fig. 5.** Effects of ginseng and NMDA receptor antagonists on SREDs-induced $[Ca^{2+}]_i$ and neuronal death. After carried experiment like Fig. 4, the pooled results illustrated in (A), showing the mean SREDs-induced $[Ca^{2+}]_i$ after treatment of various neuropharmacological agents. (B) shows neuroprotective effects of ginseng and NMDA receptor antagonists on the HNC model developed SREDs. After 2 days incubation in normal culture medium, the cultures were assessed for the extent of neuronal death using the MTT assay. Data are expressed as the percentage of cell viability relative to the control cultures. The development of SREDs after 3-h SE treatment gave rise to considerable neuronal death when assessed 2 days later (21.7±1.3% cell viability compared to control). Results differ significantly from 3-h SE treatment alone (SREDs) at the *P* values indicated by asterisks (***$P < 0.001$).

also summarized from the pooled data in Fig. 5A.

Continuous convulsive seizures lead to brain injury or even death and a sustained increase in $[Ca^{2+}]_i$ is forerunner of neuronal death. Therefore, we examined neuroprotective effects of ginseng and NMDA receptor antagonists on the HNC model developed SREDs. The development of SREDs after 3-h SE treatment gave rise to considerable neuronal death when assessed 2 days later using the MTT assay (21.7±1.3% cell viability relative to the control cultures). However, it was significantly attenuated by 3-h SE treatment with NMDA receptor antagonists, ginseng or ginsenoside $Rg_3$ as shown in Fig. 5B (***$P<0.001$, compared to 3-h SE treatment alone). Nifedipine and CNQX were not attenuated SREDs-induced neuronal death. The neuroprotective effects of ginseng and NMDA receptor antagonists were correlated well with the inhibition of these drugs on the SREDs-induced $[Ca^{2+}]_i$.

## DISCUSSION

SE- or SREDs-induced brain damage results in death of susceptible cell types and fibillary gliosis, which occur typically in hippocampus but also in other structures including dentate gyrus, cerebellum, amygdala, and neocortex. It is increasingly clear that the neurochemical/molecular mechanisms of hippocampal epileptogenesis are diverse and may involve the complex interactions of ion channels, receptors, and genes (Burgess and Noebels, 1999; Scheffer and Berkovic, 2003; Kim *et al.*, 2001; Chapman, 2000; Wong *et al.*, 2003; Raol *et al.*, 2001; Noebels, 2003). Among them, NMDA receptor activation mainly contributes to induction of altered neuronal excit-

ability in the kindling (Behr *et al.*, 2001), hippocampal slice (Stasheff *et al.*, 1989), and cultured hippocampal neuron (DeLorenzo *et al.*, 1998; Pal *et al.*, 1999) models of epilepsy. Especially, the hippocampal neuronal culture (HNC) model of SE provides an ideal model system to study $[Ca^{2+}]_i$ in isolated neurons during continuous electrographic epileptiform discharges. This model of electrographic SE characterized by continuous 3-20 Hz epileptiform discharges can be sustained for prolonged periods of time in the HNC model. Recent studies have also demonstrated that the induction of epileptogenesis in the HNC model of epilepsy was mainly dependent on NMDA receptor activation (DeLorenzo *et al.*, 1998; Pal *et al.*, 1999; Gulyas-Kovacs *et al.*, 2002). Thus, the HNC model can serve as an excellent model to screen therapeutic agents preventing the induction of epileptogenesis, especially NMDA receptor-dependent propagation of SE with the subsequent development of SRED.

The widespread availability and use of herbal medicines raise the potential for adverse effects in the epilepsy population. Ginseng, the root of *Panax ginseng* C.A. Mayer (Aralicaceae), is a well-known traditional herbal medicine. Among the efficacies of ginseng, which produces an array of pharmacological responses, recent studies demonstrated beneficial effects of ginseng on the CNS. Further beneficial effects of ginseng were observed on neuronal cell death associated with ischemia or glutamate toxicity. As the detailed mechanism of ginseng-mediated neuroprotection, we recently demonstrated that ginseng, in particular by ginsenoside $Rg_3$, attenuates the influx of $Ca^{2+}$ *via* NMDA receptors in cultured hippocampal neurons (Kim *et al.*, 2002). These results raise the possibility that therapeutic use of this herbal medicine as the blocking agent for the NMDA receptor-mediated seizure and epilepsy in cultured hippocampal neurons.

Bases on our previous study investigating the inhibitory effects of ginseng on NMDA receptors, the present study examined the direct modulation of ginseng on the NMDA receptor-mediated epileptogenesis in cultured hippocampal neurons using fura-2-based digital $Ca^{2+}$ imaging and neuronal death assays. In the HNC model which we used, SE caused a sustained increase for the duration of SE, suggesting that elevated $[Ca^{2+}]_i$ may play a role in causing the long-lasting plasticity changes involved in developing SREDs. This paper also provides direct evidence that ginseng and the main active component of ginseng, ginsenoside $Rg_3$, inhibit SE-induced $[Ca^{2+}]_i$ with the subsequent development of SRED and further attenuate SREDs-induced neuronal death. These results are considered important because we first report the therapeutic effects of ginseng and ginsenoside $Rg_3$ on NMDA receptor-induced seizure and epilepsy in the HMC model and may provide insight into clinical seizure activity.

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