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Ginsenoside Rg_3 inhibits NMDA receptors in rat cultured hippocampal neurons: possible involvement of a glycine-binding site

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We previously reported that ginseng inhibited NMDA receptors in cultured hippocampal neurons. Here, we further examined the detailed mechanism of ginseng-mediated inhibition using its main active ingredient, ginsenoside Rg_3. Co-application of ginsenoside Rg_3 with increasing concentrations of NMDA did not change the EC_{50} of NMDA to the receptor, suggesting ginsenoside Rg_3 inhibits NMDA receptors without competing with the NMDA-binding site. Ginsenoside Rg_3-mediated inhibition also occurred in a distinctive manner from the well-characterized NMDA receptor open channel blocker, MK-801. However, ginsenoside Rg_3 produced its effect in a glycine concentration-dependent manner and shifted the glycine concentration-response curve to the right without changing the maximal response, suggesting the role of ginsenoside Rg_3 as a competitive NMDA receptor antagonist. We also demonstrated that ginsenoside Rg_3 significantly protected neurons against NMDA insults. Therefore, these results suggest that ginsenoside Rg_3 protects NMDA-induced neuronal death via a competitive interaction with the glycine-binding site of NMDA receptors in cultured hippocampal neurons.

Introduction

In spite of beneficial effects of ginseng on the CNS [1-3], little scientific evidence shows at the cellular level. In an effort to elucidate the mechanism of ginseng's action at the cellular level and the identity of the active substance, we investigated the modulation of ginseng on the activation of NMDA receptors due to the significant role of NMDA receptors in both acute and delayed forms of neuronal death. We recently reported that ginseng significantly attenuated the activation of NMDA receptors, and ginsenoside Rg_3 is the most potent component of this ginseng's action in cultured hippocampal neurons [4]. However, it remains to be clarified the detailed mechanism by which ginseng modulates the NMDA receptor channel complex in the brain. We, therefore, examined the involvement of ginsenoside Rg_3 in main modulatory sites of NMDA receptors and subsequent neuroprotective effect of ginsenoside Rg_3 in primary cultures of rat hippocampal neurons.

Materials and Methods

Cell preparation. Cell preparation and whole-cell recordings using perforated patch-clamp methods were done as previously described [4]. 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 7-15 days in culture.

Intracellular Ca^{2+} imaging Fura-2/AM was used as the fluorescent Ca^{2+} indicator. Cells were illuminated using a xenon arc lamp and excitation wavelengths (340 and 380 nm) were selected by a computer-controlled filter wheel. Data were acquired every 2 s 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 intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). All imaging data were collected and analyzed using Universal Imaging software. All the data are represented as means ± S.E.M.

Results and Discussion

Uncompetitive block on the NMDA-binding site. The NMDA receptor channel complex has a number of regulatory sites that are targets for modulation by endogenous as well as exogenous compounds. The main regulatory sites include agonist NMDA-, co-agonist glycine-binding sites, and sites within the channel lumen. We, therefore, investigated the involvement of ginsenoside Rg_3 in these regulatory sites. Firstly, we examined the possible involvement of ginsenoside Rg_3 in the NMDA-binding site using different concentrations of NMDA. Fig. 1A shows the effect of ginsenoside Rg_3 on variable concentrations of NMDA. For equilibrium concentration-response curves to NMDA, a half maximum concentration of ginsenoside Rg_3 (3 μM) was pretreated for 1 min before the application of NMDA. Ginsenoside Rg_3-mediated inhibition seems to be unchanged by changing concentrations of NMDA. For the full range of NMDA concentrations (1 μM ~ 1 mM), concentration-response curves to NMDA were obtained in the absence and presence of 3 μM ginsenoside Rg_3. As shown in Fig. 1B, concentration-response curves to NMDA showed a depressed maximum in the presence of 3 μM ginsenoside Rg_3 without shifting any significant change in the EC_{50} values. With a fixed concentration of glycine (1 μM), the calculated EC_{50} values were 20.4±5.1 μM in the absence and 17.7 ± 6.3 μM in the presence of 3 μM

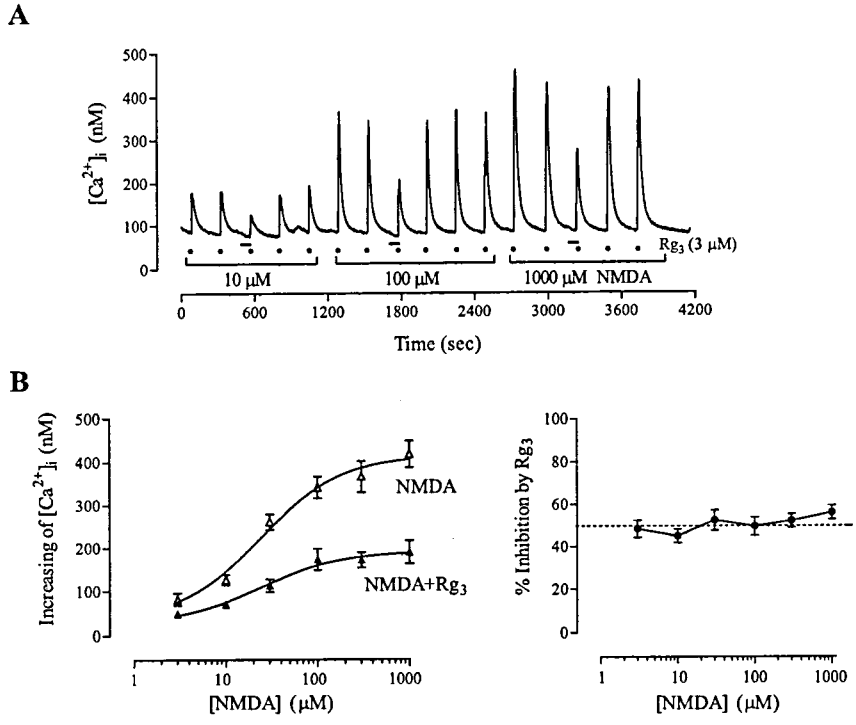
ginsenoside Rg_3 ($n > 9$). When the saturated concentration of ginsenoside Rg_3 (30 μM) was treated without pretreatment, the similar results were also observed; a depressed maximum but no significant change in the EC_{50} values by ginsenoside Rg_3. These results indicate that ginsenoside Rg_3 blocks NMDA receptors without competing with the NMDA-binding site in hippocampal neurons.

Effects of ginsenoside Rg_3 on the glycine-binding site of NMDA receptors. Since NMDA receptors require both NMDA and glycine for activation, we next made an attempt to check whether ginsenoside Rg_3 competes with the glycine-binding site of NMDA receptors. The effects of ginsenoside Rg_3 were examined using variable concentrations of glycine and compared with those of 7-chlorokynurenic acid, a well-known competitive NMDA receptor glycine site antagonist. We firstly used only two concentrations of glycine; in the absence of glycine (-Gly) and 100 μM glycine (+Gly). Although glycine acts as a co-agonist with NMDA to induce NMDA responses, the increase of $[Ca^{2+}]_i$ by NMDA can be detectable in the absence of glycine. Under this condition, application of ginsenoside Rg_3 with NMDA produced 91.9% inhibition of NMDA-induced $[Ca^{2+}]_i$ increase (Fig. 2A). However, when ginsenoside Rg_3 was applied in the presence of 100 μM glycine, ginsenoside Rg_3-mediated inhibition was significantly attenuated (19.5% inhibition, Fig. 2A inset). This pattern of glycine-concentration dependence was also observed when we tested with 7-chlorokynurenic acid (10 μM) in hippocampal neurons. Application of 7-chlorokynurenic acid completely inhibited NMDA-induced $[Ca^{2+}]_i$ increase in the absence of glycine, but this inhibition was almost diminished in the presence of 100 μM glycine (Fig. 2B). In order to know whether ginsenoside Rg_3 could produce its inhibitory effect by competitive interaction with the glycine-binding site, we further carried these experiments in the full range of glycine concentrations. As shown in Fig. 2C, the concentration-response curve to glycine was shifted to the right in the presence of ginsenoside Rg_3 (30 μM), without changing the maximal response significantly. In the presence of 100 μM NMDA, the glycine-dependent activation of NMDA receptors exhibited EC_{50} of 7.6 ± 3.9 nM that was significantly changed by the presence of 30 μM ginsenoside Rg_3 to EC_{50} of 222.3 ± 52.8 nM ($n > 5$, $P<0.01$). When this data were represented as the relationship between ginsenoside Rg_3-mediated inhibition and glycine concentration (Fig. 2D), ginsenoside Rg_3-mediated inhibition was diminished as the concentration of glycine was increased.

Neuroprotective effects of ginsenoside Rg_3 on NMDA neurotoxicity. Selective blockers for the glycine site on the NMDA receptor complex are considered as promising therapeutics leads to reduce the devastating effects of excitotoxic neuronal death. We, therefore, assessed whether ginsenoside Rg_3 can protect hippocampal cultures against NMDA insults via the antagonized action on the glycine-binding site.

A 15-min exposure to 100 μM NMDA gave rise to considerable neuronal death in hippocampal cultures. The optical density (OD) of NMDA-treated cultures was significantly reduced to 50.9 ± 3.2% relative to the control cultures when assessed 24 h later (P<0.001). When exposed for 1 min before the NMDA insult, ginsenoside Rg_3 dose-dependently inhibited the NMDA-induced neuronal death. At the concentration of 10 μM, ginsenoside Rg_3 attenuated NMDA-induce cell death by 77.3 ± 1.5% (n = 36), and itself did not produce any cytotoxicity (data not shown). Under the same condition, the well-known NMDA receptor antagonists were also tested as positive controls. D-(-)-2-amino-5-phosphonopentanoic acid (D-APV), the competitive antagonist of the NMDA-binding site and 7-chlorokynurenic acid (7-CK), the NMDA receptor glycine site antagonist, attenuated NMDA-induced cell death by 72.9 ± 2.6% (n = 19) and 92.7 ± 1.7% (n = 35), respectively. These results suggest that ginsenoside Rg_3 could reduce the long-tem exposed NMDA response and consequently protect neuronal cells against this NMDA insult.

Fig. 1. Effect of ginsenoside Rg_3 on the NMDA-binding site of NMDA receptors. (A) shows the effect of



ginsenoside Rg_3 on variable concentrations of NMDA (10, 100, and 1000 μM). (B) Uncompetitive block on the NMDA-binding site by ginsenoside Rg_3. Left, NMDA-induced $[Ca^{2+}]_i$ increase was measured with the indicated concentration of NMDA in the absence (△) or presence (▲) of 3 μM ginsenoside Rg_3.

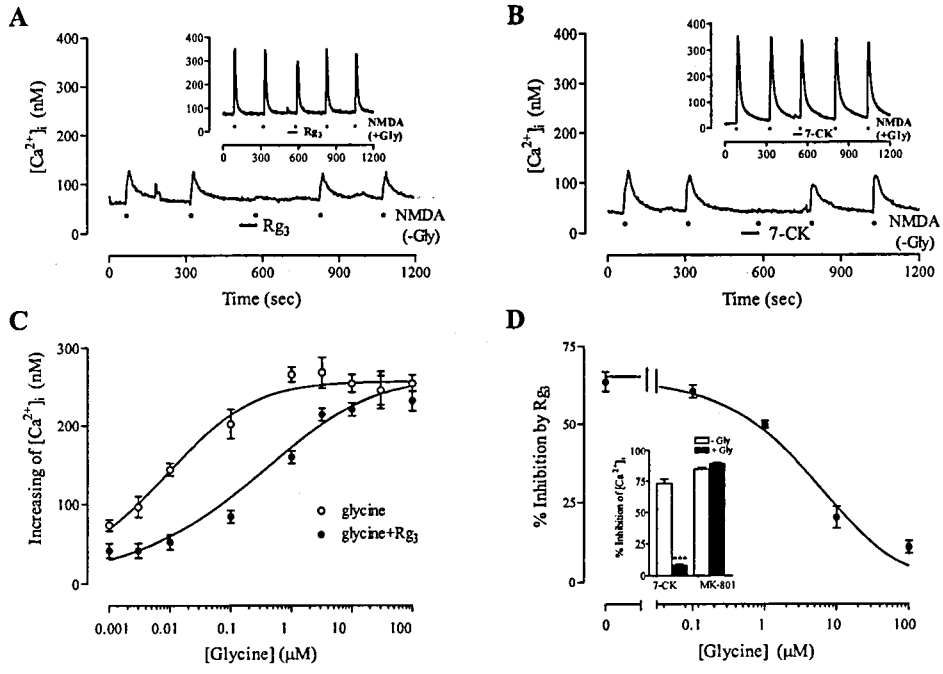


Fig. 2 Effect of ginsenoside Rg_3 on the glycine-binding site of NMDA receptors. (A) & (B) Sample records comparing the effects of ginsenoside Rg_3 and 7-chlorokynurenic acid (7-CK) in different concentrations of glycine. (C) Competitive interaction of ginsenoside Rg_3 on the glycine-binding site of NMDA receptors. 100 μM NMDA-induced $[Ca^{2+}]_i$ increase was measured with the indicated concentrations of glycine in the absence (○) or presence (●) of 30 μM ginsenoside Rg_3. (D) shows the relationship between ginsenoside Rg_3-mediated inhibition and the concentration of glycine.

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