Inhibitory Effect of Fangchinoline on Excitatory Amino Acids-Induced Neurotoxicity in Cultured Rat Cerebellar Granule Cells

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Glutamate receptors-mediated excitotoxicity is believed to play a role in the pathophysiology of neurodegenerative diseases. The present study was performed to evaluate the inhibitory effect of fangchinoline, a *bis*-benzylisoquinoline alkaloid, which has a characteristic as a Ca^{2+} channel blocker, on excitatory amino acids (EAAs)-induced neurotoxicity in cultured rat cerebellar granule neuron. Fangchinoline (1 and 5 μM) inhibited glutamate (1 mM), N-methyl-D-aspartate (NMDA; 1 mM) and kainate (100 μM)-induced neuronal cell death which was measured by trypan blue exclusion test. Fangchinoline (1 and 5 μM) inhibited glutamate release into medium induced by NMDA (1 mM) and kainate (100 μM), which was measured by HPLC. And fangchinoline (5 μM) inhibited glutamate (1 mM)-induced elevation of intracellular calcium concentration. These results suggest that inhibition of Ca^{2+} influx by fangchinoline may contribute to the beneficial effects on neurodegenerative effect of glutamate in pathophysiological conditions.

Key words: Fangchinoline, Excitatory amino acids, Neurotoxicity, Cerebellar granule cells

INTRODUCTION

Glutamate is the main excitatory transmitter in mammals. However, excessive activation of glutamate receptors leads to neuronal degeneration and death. Excitatory amino acids (EAAs) neurotoxicity has been proposed to contribute to the pathogenesis of different neurodegenerative diseases, including amyotrophic lateral sclerosis and Huntington disease. Glutamate neurotoxicity is also involved in the neuronal damage found in cerebral ischemia and it has been proposed to be involved in the origin of Alzheimer's and Parkinson's diseases.

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. 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 (Hanev at al., 1989).

Fangchinoline (6,6',12-trimethoxy-2,2'-dimethyl-berbaman-7-ol), together with 7-methylated derivative, tetrandrine, is a *bis*-benzylisoquinoline alkaloid derived from the Chinese herb *Radix Stephania tetrandra*. These *bis*-benzylisoquinoline alkaloids have been characterized pharmacologically to exhibit Ca^{2+} antagonistic, hypotensive, immunosuppressive and anti-inflammatory properties, etc (Ivanovska *et al.*, 1999a and 1999b; Jiangsu College of New Medicine, 1975; Kawashima *et al.*, 1990; Li *et al.*, 1995). Recent studies have shown that fangchinoline inhibits histamine release, platelet aggregation and thromboxane formation, and dopamine biosynthesis in *in vitro* assays, and lowers blood pressure as a non-specific Ca^{2+} channel antagonist (Kim *et al.*, 1997, 1998 and 1999; Nakamura *et al.*, 1992).

In the present study, on the basis of the fact that some

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Ca^{2+} channel blockers have protective effect on EAAs-induced neurotoxicity (Weiss *et al.*, 1990), we investigated the inhibitory effect of fangchinoline on EAAs-induced neuronal cell death in cultured cerebellar granule cells. Furthermore, in order to determine the contributable mechanism, inhibitory effect of fangchinoline on EAAs-induced glutamate release and $[Ca^{2+}]_i$ elevation were studied. And these effects of fangchinoline were compared to MK-801, NMDA-receptor antagonist, DNQX, kainate antagonist, and Ca^{2+} channel blockers.

MATERIALS AND METHODS

Materials

Fangchinoline was isolated from the creeper *Stephania tetrandra* S. Moore (or *fenfangji*), conformed by comparing the physical and chemical properties and ^{1}H-NMR spectra with previous reports (Lin *et al.*, 1993). MK-801, NMDA, Kainate and DNQX were purchased from Research Biochemicals International (Natick, MA, USA). Fluo-3/acetoxymethyl ester (Fluo-3, AM) was from Molecular probes (Eugene, OR, USA). Fetal bovine serum was from Hyclone (Logan, Utah, USA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cerebellar granule cell culture

Cerebellar granule cells were cultured as described in previous report (Seong *et al.*, 2000). Briefly, 7 to 8-day old rat pups (Sprague-Dawley) were decapitated, and the heads were partially sterilized by dipping in 95% ethanol. The cerebellum was dissected from the tissue and placed in medium which lacks serum and bicarbonate, and contains trypsin (0.25 mg/ml). Dissociated cells by trypsinization and slight trituration were collected by centrifugation and resuspended in 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) and 10% fetal bovine serum. Cells were seeded at a density of about 2×10^6 cells/ml into poly-L-lysine coated 12 well- or 24 well-plates or cover glass for $[Ca^{2+}]_i$ measurement. After 2 days incubation, growth medium was aspirated from the cultures and new growth medium containing 25 mM KCl and 20 μM cytosine arabinoside to prevent proliferation of nonneuronal cells was added. Cultures were kept at 37°C in a 7% CO_2 atmosphere. Cells were used after 8 to 10 days in culture.

Measurement of neuronal cell death induced by EAAs

The neurotoxic effects of EAAs were quantitatively assessed by the trypan blue exclusion method as described (Kashii *et al.*, 1994). For the experiment, the growth medium was replaced with an incubation buffer consisting of (in mM): NaCl 154, KCl 5.6, $CaCl_2$ 2.3, $MgCl_2$ 1, glucose 5.6 and HEPES 8.6 adjusted to pH 7.4 with NaOH. This is referred to as complete incubation buffer; variations included omitting Mg^{2+} and glucose (for glutamate and NMDA exposure), or glucose only (for kainate exposure). Cells were incubated at 37°C in the incubation buffer for 40 min prior to the addition of EAAs. Fangchinoline and other inhibitors were added during this preincubation period for the last 20 min. After an additional 30 min (for kainate, 20 min) incubation with EAAs, the buffer was replaced with EAAs-free and serum-free growth medium, cells were further incubated for 24 h (for kainate, 2 h). At the end of the incubation, cells were stained with 0.4% trypan blue solution at room temperature for 10 min. Only dead cells with a damaged cell membrane are permeable to trypan blue. The number of trypan blue-permeable blue cells and viable white cells was counted in six randomly chosen fields/culture well. Experiments were performed in triplicate with at least 3 different batches.

Measurement of glutamate

Growth medium was removed and cells were washed with the incubation buffer and equilibrated in the buffer for 40 min prior to the addition of EAAs. Fangchinoline and other inhibitors were added during this preincubation period for the last 20 min. After an additional 60 min and 20 min incubation with NMDA and kainate, respectively, a sample was taken from the buffer for the determination of glutamate secreted into the buffer. Glutamate was quantified by a high performance liquid chromatography (HPLC) with an electrochemical detector (Ellison *et al.*, 1987). Briefly, after a small aliquots was collected from the culture plates, glutamate was separated on an analytical column (ODS2; particle size, 5 μm; 4.6 × 100 mm) after prederivatization with *o*-phthaldialdehyde/2-mercaptoethanol. Derivatives were detected by electrochemistry at 0.1 μA/V, and the reference electrode was set at 0.7 V. The column was eluted with mobile phase (pH 5.20) containing 0.1 M sodium phosphate buffer with 37% (v/v) HPLC-grade methanol at a flow rate of 0.5 ml/min.

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

$[Ca^{2+}]_i$ was measured using a laser scanning confocal microscope with the Ca^{2+} indicator dye, fluo-3, AM. Cells, grown on coverslips and serum-starved for 1 h, were incubated with 5 μM fluo-3, AM in serum-free DMEM for 40 min and washed with the incubation buffer. Each coverslip containing fluo-3, AM labeled cells was mounted on a perfusion chamber, subjected to a confocal laser scanning microscope (Carl Zeiss LSM 410), and then scanned every second with a 488 nm excitation argon

laser and a 515 nm longpass emission filter. Glutamate was added to the cells by using an automatic pumping system. In order to test the effect of MK-801 or fangchinoline on glutamate-induced $[Ca^{2+}]_i$ change, cells were pretreated with the compounds for 20 min. All images from the scanning were processed to analyze changes of $[Ca^{2+}]_i$ in a single cell level. The results were expressed as the relative fluorescence intensity (Lee *et al.*, 1998).

Statistical analysis

Data were expressed as Mean ± SEM and statistical significance was assessed by one-way analysis of variance (ANOVA). Pairwise comparisons for the ANOVAs were made using Tukey test. Differences were accepted as significant at $P<0.05$ or less.

RESULTS

Inhibitory effect of fangchinoline on EAAs-induced neuronal cell death

The morphological changes of cerebellar granule neurons after exposure to EAAs were characterized by decomposition of neuronal aggregates, fragmentation of neurite and loss of soma. The neurotoxic effect of EAAs on the granule cells was quantitatively assessed using the trypan blue exclusion method. In a Mg^{2+}-free incubation buffer, cell death induced by glutamate and the inhibition afforded by pretreatment with fangchinoline or MK-801 were shown in Fig. 1. Treatment of cells with 1 mM glutamate showed the decrease of cell viability to approximately 55.3% from about 89.6% of control cells. Fangchinoline (1 and 5 μM) significantly inhibited the cell death, but fangchinoline alone was not toxic. Maximal inhibitory effect of fangchinoline was observed at a concentration of 5 μM showing 86.8% viability. MK-801 (1 μM) and verapamil (10 μM) also significantly inhibited neuronal cell death induced by glutamate (83.8 and 81.8%, respectively). Cell viability after treatment with 1 mM NMDA was reduced to 71.0% from 91.0% of control cells. Fangchinoline (1 and 5 μM) significantly inhibited neuronal cell death induced by NMDA, showing 89.1% cell viability by 5 μM. L-type Ca^{2+} channel blocker, verapamil (10 μM), and MK-801 (1 μM) also caused a marked inhibition of NMDA-induced neuronal cell death, showing 88.2% and 83.8% of cell viability, respectively (Fig. 2). The cell viability after treatment with kainate (100 μM) also showed marked decrease to 62.8% compared to 94.8% of control cells. Meanwhile, the cells pretreated with fangchinoline showed a significant decrease of the kainate-induced neuronal cell death, yielding 78.0% and 89.9% of cell viability at the concentration of 1 and 5 μM, respectively. Kainate

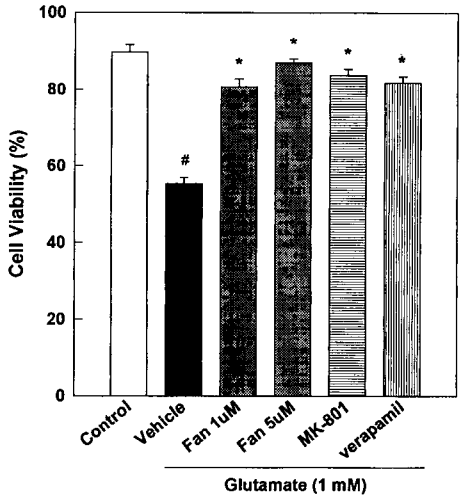

Fig. 1. Effects of fangchinoline, MK-801 and verapamil on glutamate-induced neuronal cell death. After washing and equilibration of 40 min at 37°C with incubation buffer, cells were incubated with glutamate (1 mM) for 30 min in the presence or absence of fangchinoline (Fan), MK-801 (1 μM) or verapamil (10 μM), and then further incubated in the compounds- and serum-free growth medium for 24 h. Inhibitors were added during the preincubation period for the last 20 min. At the end of the incubation, trypan blue exclusion test was performed. Values represent the mean ± SEM. *$p<0.05$ compared with control cultures exposed to glutamate alone; #$p<0.05$ compared with control cultures not exposed to glutamate by ANOVA followed by Tukey test.

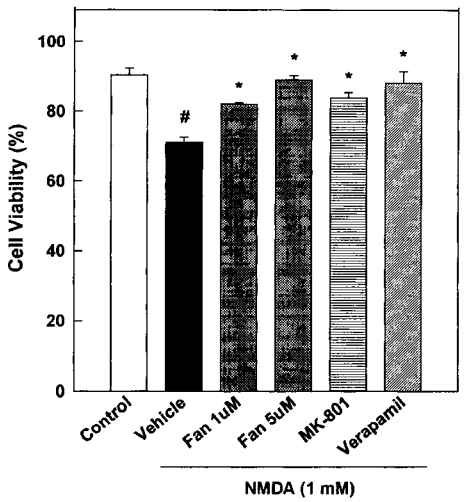

Fig. 2. Effects of fangchinoline, MK-801 and verapamil on NMDA-induced neuronal cell death. After washing and equilibration of 40 min at 37°C with incubation buffer, cells were incubated with NMDA (1 mM) for 30 min in the presence or absence of fangchinoline (Fan), MK-801 (1 μM) or verapamil (10 μM), and then further incubated in the compounds- and serum-free growth medium for 24 h. Inhibitors were added during the preincubation period for the last 20 min. At the end of the incubation, trypan blue exclusion test was performed. Values represent the mean ± SEM. *$p<0.05$ compared with control cultures exposed to NMDA alone; #$p<0.05$ compared with control cultures not exposed to NMDA by ANOVA followed by Tukey test.

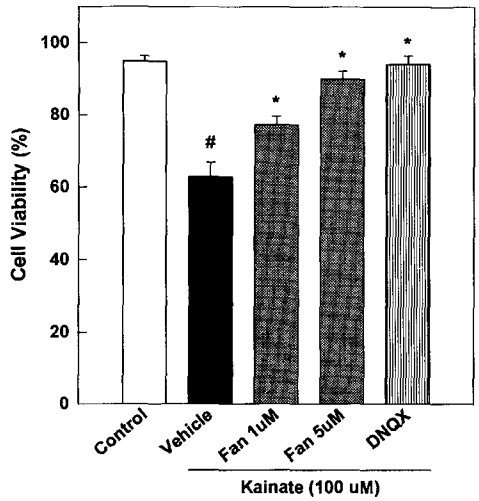

Fig. 3. Effects of fangchinoline and DNQX on kainate-induced neuronal cell death. After washing and equilibration of 40 min at 37°C with incubation buffer, cells were incubated with kainate (100 μM) for 20 min in the presence or absence of fangchinoline (Fan) or DNQX (5 μM) and then further incubated in the compounds- and serum-free growth medium for 2 h. Inhibitors were added during the preincubation period for the last 20 min. At the end of the incubation, trypan blue exclusion test was performed. Values represent the mean ± SEM. *$p<0.05$ compared with control cultures exposed to kainate alone; #$p<0.05$ compared with control cultures not exposed to kainate by ANOVA followed by Tukey test.

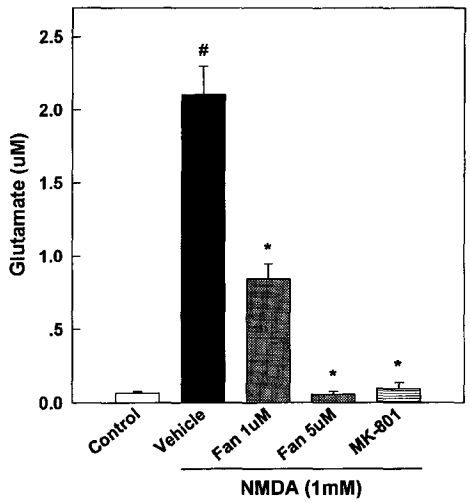

Fig. 4. Effects of fangchinoline and MK-801 on NMDA-induced elevation of extracellular glutamate. After washing and equilibration of 40 min at 37°C with incubation buffer, cells were incubated with NMDA (1 mM) for 60 min in the presence or absence of fangchinoline (Fan) and MK-801 (1 μM). Inhibitors were added during the preincubation period for the last 20 min. At the end of the incubation, the amount of glutamate secreted into the buffer was quantified by HPLC with EC detector. Values represent the mean ± SEM. *$p<0.05$ compared with control cultures exposed to NMDA alone; #$p<0.05$ compared with control cultures not exposed to NMDA by ANOVA followed by Tukey test.

receptor antagonist, DNQX (5 μM), also showed complete inhibition of neuronal cell death induced by kainate (94.1%) (Fig. 3).

Effect of fangchinoline on EAAs-induced elevation of extracellular glutamate

After washing with the incubation buffer, cells were incubated with the buffer containing 1 mM NMDA for 60 min, and fangchinoline or MK-801 was pretreated 20 min before NMDA treatment. As shown in Fig. 4, 1 mM NMDA markedly elevated extracellular glutamate level to 2.10 μM from 0.07 μM of control. Fangchinoline strongly blocked the NMDA-induced elevation of glutamate release showing 0.06 μM at the concentration of 5 μM. MK-801 (1 μM) also significantly inhibited the elevation of glutamate release showing 0.09 μM. To study kainate-induced elevation of glutamate release, cultured cerebellar granule cells were incubated with 100 μM kainate for 15 min. As shown in Fig. 5, kainate alone induced about 1.2 μM glutamate release showing 10 fold increase of basal level. Meanwhile, fangchinoline significantly inhibited kainate-induced glutamate release showing 0.8 μM and 0.6 μM of glutamate level at the concentration of 1 μM and 5 μM, respectively. DNQX (5 μM) completely inhibited glutamate release (0.18 μM) and, surprisingly, MK-801 (1 μM) showed slight, but significant, inhibition of kainate-

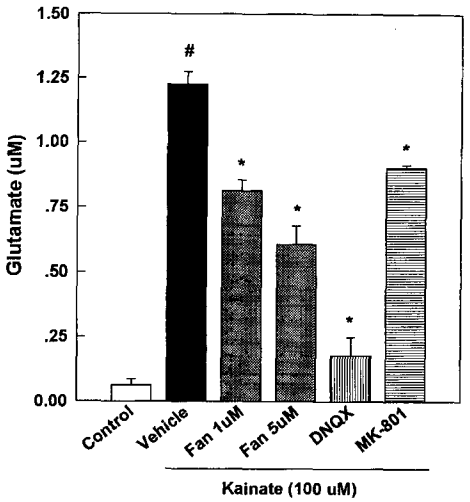

Fig. 5. Effects of fangchinoline, DNQX and MK-801 on kainate-induced elevation of extracellular glutamate. After washing and equilibration of 40 min at 37°C with incubation buffer, cells were incubated with kainate (100 μM) for 15 min in the presence or absence of fangchinoline (Fan), DNQX (5 μM) and MK-801 (1 μM). Inhibitors were added during the preincubation period for the last 20 min. At the end of the incubation, the amount of glutamate secreted into the buffer was quantified by HPLC with EC detector. Values represent the mean ± SEM. *$p<0.05$ compared with control cultures exposed to kainate alone; #$p<0.05$ compared with control cultures not exposed to kainate by ANOVA followed by Tukey test.

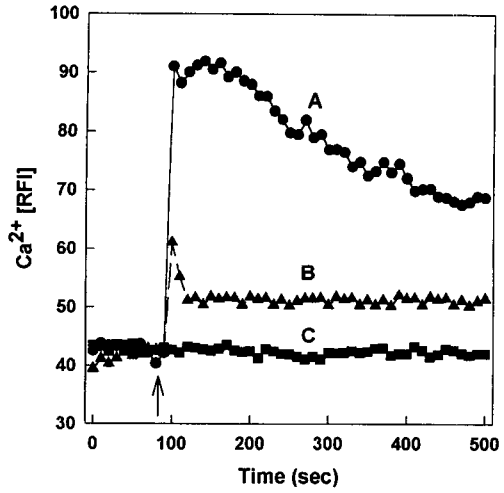

Fig. 6. Effects of fangchinoline and MK-801 on glutamate-induced elevation of $[Ca^{2+}]_i$. After equilibrium of 1 h with serum-free DMEM, cells were labelled with 5 μM fluo-3, AM for 40 min, and treated with glutamate for the indicated times in the presence or absence of inhibitors. Fangchinoline and MK-801 were treated 20 min prior to glutamate. $[Ca^{2+}]_i$ was monitored using laser scanning confocal microscope. Results are expressed as the relative fluorescence intensity (RFI). Each trace is a single cell representative. Allow shows the times of glutamate addition. (A) 1 mM Glutamate alone (B) Glutamate + 5 μM fangchinoline (C) Glutamate + 1 μM MK-801.

induced elevation of glutamate release (0.9 μM). This result suggest that kainate-induced neurotoxicity is partially related to NMDA receptor.

Effect of fangchinoline on glutamate-induced elevation of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was immediately elevated after the addition of glutamate (1 mM), showing 2.5 fold increase of basal fluorescence level, and the elevated $[Ca^{2+}]_i$ was maintained for over 10 min (Fig. 6). It was demonstrated that any response was not shown by glutamate when external Ca^{2+} was eliminated by EGTA (5 mM) in the present study (data not shown). This result suggest that Ca^{2+} influx from extracellular buffer is essential for glutamate-induced $[Ca^{2+}]_i$ elevation. On the other hand, glutamate application in the presence of MK-801 (1 μM) failed to increase of $[Ca^{2+}]_i$ through all the measurement time. Fangchinoline (5 μM) also showed almost complete inhibition of $[Ca^{2+}]_i$ elevation induced by glutamate.

DISCUSSION

The current findings (Kashii *et al.*, 1994; Malcolm *et al.*, 1996) demonstrated that brief exposure to glutamate can cause delayed cell death in the hippocampus, retina and cerebellum. In addition, glutamate receptor is a major cause of the delayed death induced by to glutamate, as the cerebellar glutamate neurotoxicity is prevented by NMDA receptor antagonist (Rothman and Olney, 1986; Tecoma *et al.*, 1989). Cerebellar granule cells in culture may provide a useful model for screening potential drugs for use in the treatment of such neurological disorders as amyotrophic lateral sclerosis, ischemic-hypoxic injury, epilepsy, dementia, Parkinson's disease and huntington's disease and for screening suspected environmental poisons for neurotoxicity (Choi, 1989; Gerlach *et al.*, 1995; Greenamyre and Young, 1989; Weiss and Choi, 1991).

Fangchinoline is structurally related to papaverine, which seems to involve a combination of phosphodiesterase inhibition and block of calcium channels in the pharmacological activity, and known to inhibit histamine release *in vitro* assay (Nakamura *et al.*, 1992) and lowers blood pressure as a non-specific calcium channel antagonist (Kim *et al.*, 1997). The availability of Ca^{2+} channel blockers on EAA-induced neurotoxicity has been confirmed by many studies (Freund and Reddig, 1994; Weiss *et al.*, 1990). The present study was designed to investigate the inhibition and its mechanism of fangchinoline on EAAs-induced neurotoxicity. For this purpose, neuronal cell death by EAAs was first studied. The present experiments confirmed Choi *et al.*'s finding (1987) that there is a delayed, as well as rapid, neurotoxicity when cultures of neuron are exposed to EAAs and Kashii *et al.*'s results that EAAs produce marked neuronal cell death in Mg^{2+}- and glucose-free buffer. Cell viability after glutamate treatment (55.3%) was lower than that after NMDA treatment (71.0%) in the Mg^{2+}- and glucose-free buffer. This indicates glutamate acts on not only NMDA receptor but also other receptors (AMPA/kainate, metabotropic receptor). We also identified previous results (Albers *et al.*, 1992; Cox *et al.*, 1989) that MK-801 inhibited neuronal cell death induced by glutamate and NMDA. Kainate also produced marked neuronal cell death, and the kainate receptor antagonist, DNQX, inhibited the cell death in the present studies as previous reported (McCaslin and Smith, 1988; Oh and McCaslin, 1996). Fangchinoline (1 and 5 μM) also significantly inhibited neuronal cell death induced by EAAs showing maximal effect at 5 μM. And, L-type Ca^{2+} channel antagonist, verapamil, showed similar inhibitory effects on cell death induced by EAAs. These results suggest a possibility that fangchinoline seems to have beneficial effect on neuronal cell death induced by EAAs as a nonspecific Ca^{2+} channel antagonist.

Most of the previous hypotheses dealing with glutamate-induced neurotoxicity have invoked abnormal release and/or decreased uptake of glutamate as playing a key role in the process of excitotoxicity: for example, glutamate release is increased by ischemic and insulin-induced hypoglycemia (Coyle and Puttfarcken, 1993). Release of endogenous glutamate, which acts on glutamate receptors, secondly trigger sodium influx and neuronal depolarization. This

leads to chloride influx down its electrochemical gradient, further cationic influx and osmotic lysis of the neuron, resulting in neuronal cell death (Van Vliet *et al.*, 1989). In accordance with previous reports (Choi, 1987; Watkins and Evans, 1981), NMDA and kainate induced significant elevation of glutamate release into buffer in the present study. Fangchinoline significantly inhibited glutamate release induced by EAAs (NMDA, kainate) in Mg^{2+}- and/or glucose-free conditions, mimicking the effects of MK-801 and DNQX. Meanwhile, MK-801 also slightly but significantly inhibited kainate-induced glutamate release elevation. This result is consistent with previous study (Oh and McCaslin, 1996) that kainate acts not only kainate receptors but NMDA receptors in neurotoxicity.

Ca^{2+} may also pass directly through channels activated by EAAs. Choi (1987) has provided evidence that this is the likely route by which extracellular Ca^{2+} enters neurons which are exposed to glutamate. And many studies (Freund and Reddig, 1994; Hanev *et al.*, 1989; Mody and MacDonald, 1995) showed that excitatory amino acids-induced elevation of $[Ca^{2+}]_i$ plays a fundamental role in the regulation of many cellular processes, including vesicular exocytosis, synaptic transmission and neurotoxicity. Transient increases in $[Ca^{2+}]_i$ by EAAs can result from the entry of Ca^{2+} from receptor-operated channels, voltage-activated channels, or from the release of Ca^{2+} from internal stores. A sustained increase in $[Ca^{2+}]_i$ triggers a series of events including the elevation of cGMP, the glutamate release and the activation of NOS. Released glutamate secondly acts on glutamate receptors and therefore potentiates the neurotoxicity. This conditions are reversed by using Ca^{2+} channel antagonists and MK-801. In the present experiments, glutamate produced sustained $[Ca^{2+}]_i$ elevation which was completely prevented by MK-801. It was demonstrated that extracellular Ca^{2+} was essential for glutamate-induced elevation of $[Ca^{2+}]_i$, because removal of extracellular Ca^{2+} by adding EGTA failed to produce the increase of $[Ca^{2+}]_i$. Fangchinoline, known to have vasodilative effect and inhibitory effect on high K^+- and NE-induced Ca^{2+} influx in rat aorta strips (Kim *et al.*, 1997), markedly inhibited the dramatic elevation of $[Ca^{2+}]_i$ induced by glutamate. This result suggest that fangchinoline prevented EAAs-induced neurotoxicity via Ca^{2+}-channel antagonistic activity. These results suggest a new possible pharmacological profile of fangchinoline in brain. Tetrandrine, a derivative of fangchinoline, has protective effect against ischemia-reperfusion brain damages in gerbils (Sun and Liu; 1995). In this regard, it was hoped that fangchinoline might be of value in preventing neurodegenerative pathophysiological condition.

In summary, fangchinoline, which mainly acts on Ca^{2+} channels, effectively prevented the *in vitro* neuronal cell death induced by EAAs, via the inhibition of Ca^{2+} entry and related glutamate release caused by glutamate receptors activation.

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