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Effects of Taurine on Glutamate-induced Neurotoxicity and Interleukin-6 mRNA Expression in Astrocytes

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Taurine (2-aminoethanesulfonic acid), one of bioactive amino acid in the mammalian brain, is known to exert inhibitory effects on neurons via GABA receptor. In the present study, we examined effects of taurine on glutamate-induced neurotoxicity on hippocampal neuron cell culture using cell counting method and lactate dehydrogenase (LDH) assay. After 10 d of culture, cells were stimulated with appropriate drugs. Only 43% of cultured neuronal cells survived at one day after stimulation with 500 μM L-glutamate for 10 min. Survival rate was enhanced by 82% in the presence of 10 mM taurine. LDH activity from the culture supernatant incubated with a combination of L-glutamate and taurine was less than half of that with L-glutamate alone. In the next series of experiments, interleukin-6 (IL-6) mRNA expression in cultured astrocytes was investigated using reverse tanscription-PCR (RT-PCR). IL-6 mRNA was detected in the astrocytes stimulated with L-glutamate in a dose-dependent manner, while not detected in the unstimulated control astrocytes. The expression of IL-6 mRNA caused by 10 mM glutamate was inhibited by taurine, but not by GABA. These findings demonstrated a neuroprotective action of taurine against glutamate-induced toxicity.

Taurine (2-aminoethanesulfonic acid) is the second most abundant free amino acid after glutamate in the central nervous system (CNS) and its level varies significantly depending on the specific regions (Palkovits et al., 1986). Application of taurine produces γ-amino-butyric acid (GABA) and glycine-like inhibitory currents in neurons of brain slices (Horikoshi et al., 1988). Studies have demonstrated that taurine interacts with both $GABA_A$ and $GABA_B$ receptors (Kontro and Oja, 1990; Bureau and Olsen, 1991). Therefore, membrane-stabilizing effects of taurine, such as anticonvulsant activity, appear to be mediated through GABA receptors. However, a body of evidences have been accumulated that taurine possesses many specific actions, distinguishable from those of GABA, such as modulation of calcium flux and neurotransmitter release (Namima et al., 1983; Huxtable, 1992), regulation of osmolarity, and antioxidant (Wade et al., 1988).

It is particularly interesting that taurine has neuroprotective effects under pathological conditions, such as epilepsy (Durelli and Mutani, 1983), hypoxia/ischemia (Schurr et al., 1987), and excitotoxicity (French et al., 1986), all of which involve excitatory amino acids. Glutamate is the major excitatory neurotransmitter in the CNS, and is largely responsible for neuronal damages associated with hypoxia, ischemia, and hypoglycemia. Neurotoxic effects of glutamate has been well documented under various conditions and shown to be dependent on activation of the N-methyl-D-aspartate (NMDA) receptors, which are one of three subtypes of glutamate receptors; NMDA, kainate, and AMPA (Choi and Rothman, 1990). There are several reports that taurine may have a neuroprotective effect against glutamate-induced neurotoxicity (Menéndez et al., 1990). Increase in taurine level after stimulation with glutamate agonist in brain slices or whole animals have been also reported (French et al., 1986; Magnusson et al., 1991; Menéndez et al., 1993). Although the level of taurine has been detected to change following glutamate-induced excitotoxicity, there is no direct evidence that taurine enhances survival of hippocampal neurons against glutamate-induced toxicity or not. The primary goal of the present study is to test whether or not taurine can attenuate glutamate-induced neurotoxicity on rat hippocampal neurons using cell counting and lactate dehydrogenase (LDH) assay.

To further confirm that taurine can protect neurons against glutamate-induced neurotoxicity, we also investigated the effect of taurine on interleukin-6 (IL-6) mRNA expression. IL-6 is a multifunctional cytokine which plays roles in immune regulation, hematopoiesis, and neuronal survival/differentiation (Satoh et al., 1988; Kishimoto, 1989). During infection, inflammation, and

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injury, IL-6 is rapidly induced in peripheral tissues and the brain. IL-6 has been reported to influence many central neurotransmitters, such as GABA, 5-HT, and Ach (Rothwell et al., 1995) in the CNS. A number of second messenger systems in neurons are also affected by IL-6, including Ca^{2+} influx, activation of cAMP and protein kinase C (PKC), nitric oxide (NO) synthesis, and release of arachidonic acid (Kishimoto et al., 1989; Rothwell et al., 1995). Recent data suggests that IL-6 may have a protective role in brain injury as it promotes neuronal survival (Kishimoto et al., 1992), suppresses demyelination, and reduces glutamate-induced neurotoxicity (Toulmond et al., 1992) and ischemic damage (Rothwell and Hopkins, 1995; Hagberg et al., 1996). Production of IL-6 in the CNS may be mediated not only by infiltrating immuno-inflammatory cells, but also by astrocyte and microglia. It has been shown in an *in vitro* assay that both viral and bacterial infection resulted in the induction of IL-6 mRNA in astrocyte. Sciatic nerve cut, hypoxia, and ischemia also resulted in the increase in the IL-6 synthesis (Hagberg et al., 1996). These studies led us to hypothesize that if taurine has a neuroprotective action against glutamate toxicity, it would reduce IL-6 mRNA expression in astrocytes.

To address the hypothesis, we first demonstrated glutamate-induced mRNA expression, and then investigated the effect of taurine on glutamate-induced IL-6 mRNA expression in astrocyte, using RT-PCR.

Materials and Methods

Hippocampal neuron culture

Primary cultures of dissociated hippocampal neurons were prepared from the brains of 17-20 day old fetal Sprague Dawley rats as previously described (Akaneya et al., 1993). Briefly, tissue fragments were dissected in CMF-Hanks' balanced salt solution (CMF-HBSS; Gibco). The tissue was transferred into HBSS containing 0.125% trypsin and then incubated for 15 min at 37℃. After removal of the trypsin solution, the tissues were dissociated by slow trituration using a fire polished siliconized pasteur pipette. The neurons were resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 2 mM antibiotics(penicillin and streptomycin), 2 mM L-glutamine. Cell suspension was plated at a final cell density of 2×10^5 on a poly-D-lysine-laminin coated 24-well culture plates (Corning). After 1 d of culture in a humidified 5% CO_2/95% air incubator, the medium was replaced with fresh medium.

Astrocyte culture

Primary cultures of dissociated astrocytes were prepared from the brains of 1-3 day old Sprague Dawley rats. Cerebral hemispheres were carefully separated from meninges, and washed with ice-cold HBSS. Tissue was triturated in DMEM. Cells were resuspended in a DMEM supplemented with 5% FBS, 2 mM antibiotics, and 2 mM L-glutamine. Cells were then plated in 100 mm Falcon plastic culture dishes containing DMEM supplemented with 5% FBS and incubated in humidified atmosphere of 5% CO_2/95% air incubator at 37℃. Culture medium was replaced every third day.

Drug application

Cells were grown for 5-11 d in culture dishes containing DMEM supplemented with 5% FBS. Culture medium was replaced with serum-free medium at least 24 h before experiments. Astrocytes and neurons were incubated in the serum-free medium containing appropriate drugs for a given time. To test effects of taurine, taurine was pretreated for 15 min before exposure to L-glutamate. Glutamate, taurine, and GABA were freshly prepared daily. All drugs were purchased from Sigma.

Cell counting

Neuronal survival was quantified by counting viable neurons in premarked regions (4 areas of approximately 1 mm^2 each) of a culture dish at an initial time point, and at designated time points thereafter. Neuronal viability was assessed by established morphological criteria. Briefly, non-viable neurons had either degenerated completely and were absent upon inspection or, if still present, exhibited somal vacuolation and neurite fragmentation. Surviving cells was expressed as percent viable cells remaining from original counts. All values shown represent the mean$\pm$S.E. Statistical comparisons were independently performed using two-tailed Student's t-test.

LDH assay

Measurement of LDH in the culture supernatants was used as an indicator of neuronal cell death. A diagnostic kit (Promega, CytoTox 96 Non-radioactive cytotoxicity assay kit) was used to measure LDH activity. After 24 h of glutamate exposure, 50 μl of culture medium was combined with 50 μl of substrate Mix in 96 well flat bottomed microtiter plates. Following a 30 min incubation at room temperature, wells received 50 μl stop solution, and the absorbance was recorded at 490 nm using a Multiscan spectrophotometric plate reader. Accuracy of the assay was verified by periodically testing a standard enzyme, Enzyme Control E-2 (Sigma).

RNA extraction, cDNA synthesis, and PCR

Total RNA was isolated from astrocytes by the method of Chomczynski and Sacchi (1987). For synthesis of cDNA, the PreMix RT-PCR kit (Korea Bioneer) was used. Typically, 1 μg of total RNA was subjected to cDNA synthesis according to the manufacturer's

Table 1. Primers for PCR

	Primer	Sequence
IL-6	Sense	5′-TAGAGTCACAGAAGGAGTGG-3′
	Antisense	5′-GCCAGTTCTTCGTAGAGAAC-3′

instructions. cDNA synthesis was carried out at 42℃ for 60 min. The reaction was terminated by incubation at 72℃ for 5 min. Samples were stored at -80℃ for later use.

For IL-6, specific primers obtained from Korea Bioneer (Taejon, Korea) were designed as shown in Table 1. PCR was performed on a Coy thermocycler in a reaction volume of 20 μl in the presence of 5 μl cDNA, 0.5 U of Taq-polymerase (Korea Bioneer), and 25 pM primer mixture. The steps in the PCR cycle were 94℃ for 1 min, 55℃ for 1 min, and 72℃ for 1 min, for a total 30 cycles. Aliquots were separated on 2% Metaphor agarose (FMC Bioproducts) in TAE buffer containing ethidium bromide and photographed under UV light.

Whole cell patch clamp recording

Hippocampal neurons were acutely isolated from the 8-11 day old rats (Oh et al., 1995). Electrodes (6-8 MΩ resistance) were pulled on a Narishige PP-83 microelectrode puller using a two-stage pull and thin-walled borosilicate capillary glass. The intracellular (pipette) solution contained (in mM); Trizma phosphate (dibasic) 100, Trizma base 28, ethylene glycol bis-(β-aminoethylether)-N, N, N,' N'-tetraacetic acid 11, $MgCl_2$ 2, $CaCl_2$ 0.5, and Na^+-ATP 4 with pH adjusted to 7.35 with NaOH. The pipette solution also contained an intracellular ATP reconstitution consisting of 50 U/ml creatine phosphokinase and 22 mM phosphocreatine. Recordings were amplified using an Axopatch 200A amplifier and filtered at 5 kHz with a 4-pole Vessel filter before digitization. All data were displayed on a chart recorder on line, and stored on videotape. The applied drug concentrations were as follows: taurine (Sigma), 100 μM; bicuculline (Research Biochemicals Inc.), 20 μM; glutamate (Sigma), 100 μM. Solution changes were accomplished using the "sewer pipe" perfusion technique, in which several solutions flow out of parallel Teflon tubes in a laminar pattern.

Results

Current induced by taurine

Electrophysiological property of taurine was first studied in acutely isolated hippocampal neurons using whole cell patch clamp recording. Bath application of 100 μM taurine for 10 sec to hippocampal neuron elicited a current with a reversal potential of about -48 mV (Fig. 1A). The taurine-induced current showed outward rectification as GABA-induced Cl^- current, and was markedly reduced by 20 μM bicuculline (BIC) (Fig. 1B).

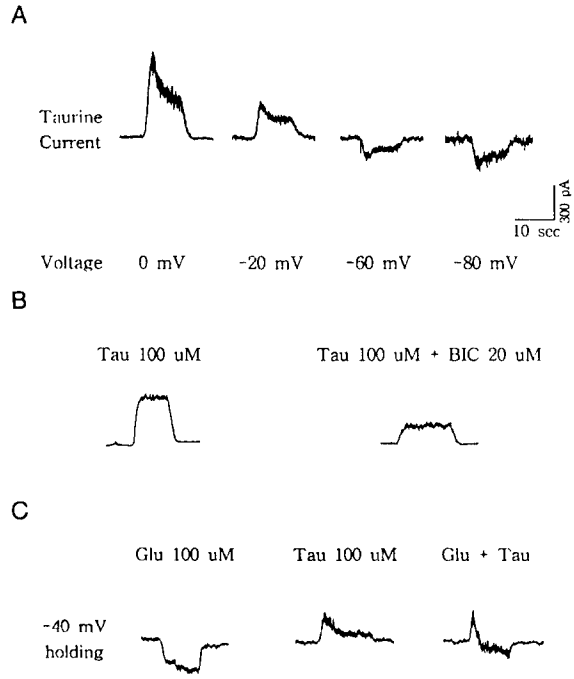

Fig. 1. Whole-cell patch recordings of taurine-induced current in acutely isolated hippocampal neurons. A. Voltage-current relationship of taurine-induced current. B. Taurine(100 uM)-induced outward (V_H=0 mV) current was blocked by 20 uM BIC. C. Glutamate (100 uM)-induced current was drastically attenuated in the presence of 100 uM taurine.

The responses were rapidly activated, and followed by desensitization toward a steady-state level during continuous application of taurine. In co-treatment of 100 μM of glutamate and taurine, glutamate-induced current was drastically attenuated (Fig. 1C). This result supports that taurine induces an increase in Cl^- conductance for exerting its inhibitory effect, and acts through $GABA_A$ receptor.

Taurine protects neuronal cell death

Exposure of cultured hippocampal neurons to 500 μM L-glutamate for 10 min resulted in neuronal cell death. Cell morphology was observed 24 h following the exposure of cultures to L-glutamate or both L-glutamate and 10 mM taurine. Neurons in control culture were healthy, exhibiting a smooth soma with extended processes (Fig. 2A), whereas neurons exposed to L-glutamate were degenerated with a rough cell surface. As shown in Fig. 2, neurites were lost and cell membranes were ruptured leaving only nuclei behind (Fig. 2B). The presence of taurine effectively reduced glutamate-induced neurotoxicity and prevented degeneration of neurons by glutamate (Fig. 2C). When morphological observation was made 10 min after L-glutamate treatment, cell swelling, an early indication of necrosis, was detected. Interestingly, taurine did not protect early cell swelling caused by glutamate. Therefore, protective effect of taurine appeared to be mainly associated with apoptosis, but not necrosis. In

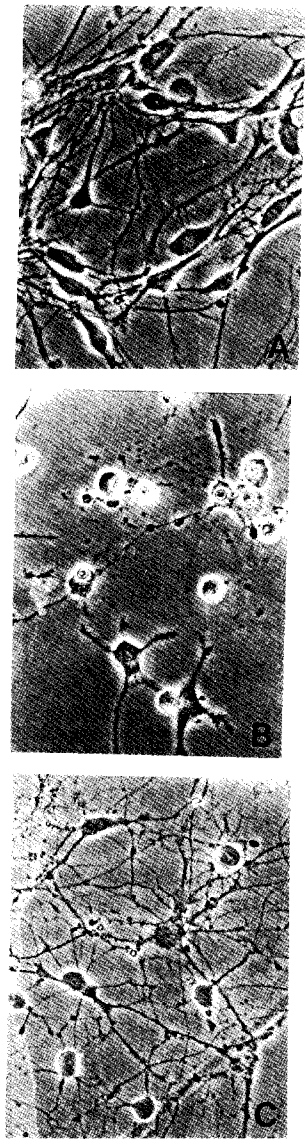

Fig. 2. Phase-contrast micrographs (×150) showing the protective effect of taurine on glutamate-induced neuronal cell death. A: control rat neuronal cells at 10 d after cell isolation were exposed to culture medium for 10 min and observed 24 h after exposure. B: rat neuronal cells were exposed to 500 μM L-glutamate for 10 min. C: the combined treatment with 500 μM L-glutamate and 10 mM taurine. Note that glutamate-induced neuronal cell death was prevented by the addition of taurine.

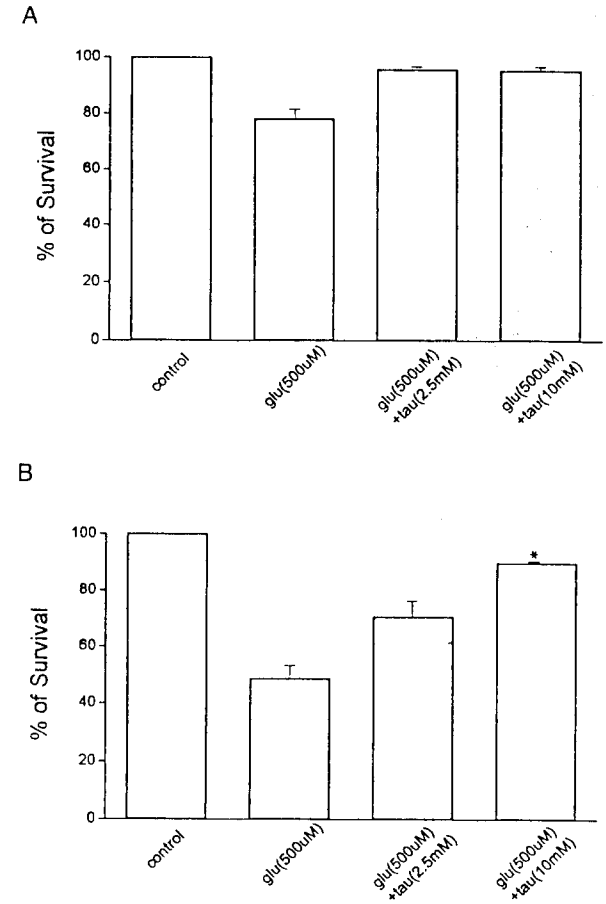

Fig. 3. Effect of taurine on glutamate-exposed neuronal viability. E17 rat neurons (<2×10^5 cells) were cultured in the walls of 24 well plates for 5 d (A) or 10 d (B). At one day after exposure to appropriate drugs for 10 min, viable neurons in premarked regions (3 areas of approximable 1 mm^2 each) were counted. Control was done with serum-free normal media during the exposure to drug. Values expressed as a percent of control and represented the means ± S.E. (n=3). * $P<0.05$, compared with the values for neurons exposed to glutamate alone.

the cell counting study, control cultures did not exhibit a significant loss of viable neurons, which were characterized by exclusion of trypan blue. However, L-glutamate exposure resulted in 20% loss and 57% loss in young cells (5-6 d) and adult cells (10-11 d), respectively (Fig. 3). Because immature neurons have fewer membrane receptors for glutamate than mature neurons, glutamate-induced excitotoxicity was expected to have a mild effect on young cells (Fig. 3A). The treatment with both L-glutamate and 10 mM taurine showed only 5% loss in young cells and 21% loss in adult cells, which made it more evident that the effects of L-glutamate were significantly reduced by addition of taurine.

To further study the protective action of taurine, the effect of taurine on glutamate-induced cell death was evaluated by measuring LDH activity in hippocampal neuronal culture supernatants (Fig. 4). A 10 min exposure of hippocampal neurons to 500 μM glutamate elicited a significant 2.5 fold increase in the level of LDH in the extracellular media, an index of cell damage. In contrast, a preceeding treatment of MK-801, a NMDA receptor antagonist, had a prominent suppressing effect on the glutamate-induced LDH release, suggesting the dominant role of NMDA receptor activation in many forms of glutamate neurotoxicity. Taurine administrated to the cultures for 15 min prior to glutamate also reduced LDH release by glutamate in a concentration-dependent manner. In the presence of 10 mM taurine, LDH release by glutamate was reduced by 36%.

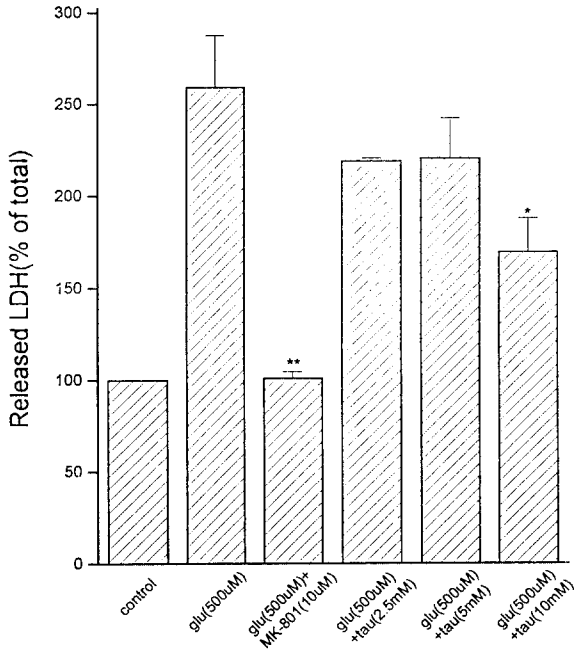

Fig. 4. The effect of taurine on glutamate-induced LDH release. E17 rat hippocampal neurons ($<2\times10^5$ cells) were cultured in the wells of 24 well plates for 10 d. At one day after exposure to appropriate drugs for 10 min, the supernatant was collected and its LDH activity was determined. Control was done with serum-free normal media during the process of drug-exposure. Values were expressed as a percent of control and represented the means ± S.E. (n=3). * $P<0.05$ and ** $P<0.01$, compared with the values for neurons exposed to glutamate alone.

Taurine inhibits IL-6 mRNA expression in astrocytes

Since IL-6 mRNA has been known to be inducible in the CNS by various stressful stimuli, such as infection and injury, it was tested whether glutamate, a possible neurotoxin, causes IL-6 mRNA expression and, if it does, whether taurine modulates glutamate-induced IL-6 mRNA expression. Since astrocytes were more resistant to glutamate treatment than neuronal cells, higher concentration and longer treatment were required to induce any detectable damage in astrocytes (data not shown). Astrocytes were isolated from rat of embryonic day 18 and 10 mM of glutamate was treated for 1 h in astrocytes cultured for 7 d. IL-6 mRNA was not detectable in unstimulated astrocytes. Since IL-6 mRNA was detected highest at 6 h after the stimulation, the extraction of IL-6 mRNA was performed at 6 h in all experiments.

As shown in Fig. 5, IL-6 mRNA was not detected in control culture cells. A clearly distinguishable IL-6 mRNA PCR product band was detected from astrocytes stimulated with 10 mM L-glutamate (Fig. 5A). In combined treatment of L-glutamate and 10 mM taurine, only a faint band was detected (Fig. 5B). In contrast, GABA, a major inhibitory neurotransmitter, did not inhibit glutamate-induced IL-6 mRNA expression (Fig. 5C). DNA sequence of the PCR product revealed by Sanger method was homologous to that of the *Rattus norvergicus* IL-6 cDNA.

Discussion

This study provides supporting evidence for the protective action of taurine against neuronal cell death caused by glutamate neurotoxicity in cultured rat hippocampal neurons. In addition, glutamate-induced IL-6 mRNA expression was shown to be inhibited by 10 mM taurine, but not by 10 mM GABA.

Interaction of glutamate receptor with taurine has been reported in various studies. Activation of NMDA receptors causes an increase in extracellular taurine levels in various brain regions both *in vivo* (Lehmann et al., 1984, 1985a, b; Menéndez et al., 1989, 1990) and *in vitro* (Magnusson et al., 1991). Intraperitoneal injection of NMDA to the rat also resulted in a significant increase in the taurine concentration in the hippocampal CA1 area, whereas the levels of aspartate and alanine were not substantially changed (Shibanoki et al., 1993). The release of taurine by kainate which activates non-NMDA receptor has been demonstrated to be lower than that by NMDA (Magnusson et al., 1991). Since NMDA receptor is permeable to Ca^{2+} ion, these results suggest that an increase of the intracellular Ca^{2+} concentration is a signal for taurine release, which in turn blocks NMDA-evoked Ca^{2+} entry. The increase of the extracellular taurine level induced by the activation of NMDA receptor might thus serve as a neuromodulator that protects against cell damage from the Ca^{2+} toxicity caused by glutamate. Glutamate neurotoxicity demonstrated here might be interpreted to be largely mediated by a toxic influx of extracellular Ca^{2+}, since it was blocked by a potent blocker of NMDA receptor, MK-801 (Fig. 3). The interpretation that glutamate toxicity was a result of NMDA receptor activation was further supported by the fact that no significant cell loss by glutamate was detected in neuronal cells at culture day 5 (Fig. 3). It has been reported that NMDA receptor is not fully expressed at early stage of development. This result is compatible with the previous reports that selective blockade of the NMDA receptor during glutamate exposure resulted in preservation of neuronal viability. Taurine did not completely prevent the glutamate-induced LDH release, while MK-801 did so. This indicates that the mechanism by which taurine prevents the neurotoxic effects of glutamate is not solely attributable to a direct antagonism at the level of the NMDA receptor complex.

Glutamate-induced neurotoxicity appears to be due to an increase of intracellular Ca^{2+} level (Connor and Wadman, 1988). Many of the effects of taurine on the CNS may be related to Ca^{2+} redistribution. Taurine forms ionic bonds with phospholipids in membranes, thereby altering membrane structure and the affinity of Ca^{2+} for its binding sites on the membranes. Especially, taurine affects both Ca^{2+} binding and uptake in excitable tissues. Thus, taurine inhibits both Ca^{2+} accumulation and calmodulin activity, which is associated with

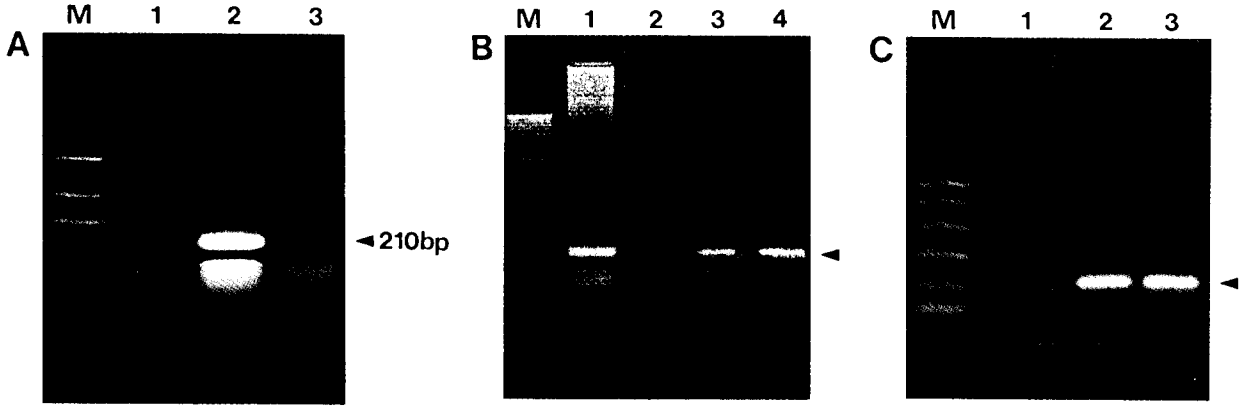

Fig. 5. A. The effect of taurine on glutamate-induced IL-6 mRNA expression. RNA extracted from co-cultured rat astrocytes exposed to appropriate drugs for 1 h was processed as described and amplified by IL-6 primers. Line M; PCR marker (Promega), 1. control (IL-6), 2. 10 mM L-glutamate (IL-6), 3. 10 mM L-glutamate+10 mM taurine (IL-6). B. The dose dependent effect of taurine on glutamate-induced IL-6 mRNA expression. Line M; PCR marker (TaKaRa, 1 Kb ladder), 1; 10 mM L-glutamate (IL-6), 2; 10 mM L-glutamate+10 mM taurine (IL-6), 3; 10 mM L-glutamate+5 mM taurine (IL-6), 4; 10 mM L-glutamate+1 mM taurine (IL-6). C. The effect of GABA on glutamate-induced IL-6 mRNA expression. Line M; PCR marker (Promega), 1; control (IL-6), 2; 10 mM L-glutamate (IL-6), 3; 10 mM L-glutamate+10 mM GABA (IL-6).

calmodulin-dependent protein kinase C (PKC). PKC is, in turn, related to both phosphorylation of membrane protein and Ca^{2+} release from the intracellular Ca^{2+} store. Recently, Trenkner (1994) reported that when taurine was added together with glutamate, taurine induced down-regulation or inhibition of protein kinase C activity. Thus, the inhibition of PKC activity by taurine may lead to an inhibition of Ca^{2+} release from intracellular Ca^{2+} store through the IP_3 receptors, plasma membrane Ca^{2+} channels including voltage-operated channels, receptor-operated channels, and Ca^{2+}/ATPase-dependent Ca^{2+} pump. From the aspects of the inhibitory action of taurine on neuronal activity, an interesting possibility has been raised that glutamate-induced neurotoxicity is due to influx of Ca^{2+}, followed by influx of Cl^- resulting in cell swelling and eventually cell death (Tang et al., 1996). According to this result, both taurine and GABA potentiated glutamate-induced neurotoxicity at low concentration (0.1-1.0 mM), probably resulting from the opening of GABA receptor which increases the influx of Cl^-. In contrast, at high concentration (10-50 mM), taurine but not GABA, protected glutamate-induced neurotoxicity. This report was consistent with our results that glutamate-induced IL-6 mRNA was not suppressed under combined treatment of 10 mM L-glutamate and 10 mM GABA.

IL-6 gene is not constitutively expressed in immune cells or glial cells. Endogenous signaling molecules, such as IL-1β and TNF-α are required to activate gene expression in astrocytes. Bacterial endotoxin and viral infection also cause an increase in the level of IL-6 mRNA in brain cells. To our knowledge, this is the first study to demonstrate that an endogenous neurotransmitter, L-glutamate, can induce IL-6 mRNA expression. Since mRNA expression does not always result in a release of biologically active molecules, further studies on IL-6 release are required before a definitive statement on the functional role of glutamate can be made.

Since the mechanisms for IL-6 mRNA expression induced by glutamate are not currently known, our study does not reveal the cellular mechanism by which taurine reduces IL-6 mRNA expression. It may be simply due to the cell protective effect of taurine; that is, the inhibition of IL-6 mRNA expression is accompanied by the reduced cell damage. We cannot rule out the possibility, however, that taurine blocks specific pathway(s) of glutamate-induced IL-6 mRNA expression, which is to be elucidated by further studies.

Based on our results, it is apparent that the protective role of taurine is not due to the reduction of neural activity by the increased hyperpolarizing current through $GABA_A$ receptor. It is more likely that taurine modifies the cell signal processing via unknown mechanisms that are unrelated to GABA receptor activation.

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