Role of Glutamate Receptors and Voltage-Dependent Calcium Channels in Glutamate Toxicity in Energy-Compromised Cortical Neurons

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ABSTRACT—We have examined the effect of glutamate receptor antagonists and voltage-dependent calcium channel blockers on the neuronal injury induced by the combination of a low concentration of *N*-methyl-D-aspartate (NMDA) or kainate and energy compromise resulting from the use of glucose-free incubation buffer. Toxicity induced by NMDA or kainate was enhanced in the glucose-free buffer. NMDA- or non-NMDA-receptor antagonists added to the glucose-free buffer at the same time inhibited the neuronal cell death induced by each agonist. An NMDA-receptor antagonist, MK-801, but not non-NMDA-receptor antagonists, inhibited the toxicity when added to the culture medium after exposure of the cells to the agonists. P/Q-type calcium channel blockers, ω-agatoxin IVA and ω-agatoxin TK, and an N-type calcium channel blocker, ω-conotoxin GVIA, significantly attenuated the neuronal injury, although an L-type calcium channel blocker, nifedipine, showed little neuroprotective effect. A combination of calcium channel blockers of the three subtypes showed the most prominent neuroprotective effect. These observations suggest that the overactivation of NMDA and non-NMDA receptors and consequent activation of the voltage-dependent calcium channels lead to neuronal cell death in energy-compromised cortical neurons.

Keywords: Calcium channel, Cell culture, *N*-methyl-D-aspartate (NMDA), Kainate, Energy

Several studies have suggested that the pathogenesis of hypoxic-ischemic neuronal injury is partly due to excessive activation of glutamate receptors (1, 2). The activation of glutamate receptors causes an increase in intracellular calcium concentration, which is considered to trigger neuronal degeneration mediated by glutamate (3–5). This calcium neurotoxicity hypothesis is consistent with the observation of calcium accumulation in nervous tissues during ischemia (6), trauma (7) and epilepsy (8).

The major routes of calcium entry into neuronal cytoplasm under ischemic conditions may include voltage-dependent calcium channels in addition to excitatory amino acid receptor channels. We have previously demonstrated that under ischemic conditions, the cellular membrane can be depolarized by *N*-methyl-D-asparate (NMDA)-receptor activation, leading to calcium influx through voltage-dependent calcium channels (4). Recently it has been shown that P/Q-type and N-type calcium channel blockers are neuroprotective in both in vivo (9–13) and in vitro (4, 14, 15) models of ischemia, but L-type calcium channel blockers have little neuroprotective effect (4, 14–16). Activation of the P/Q-type and N-type calcium channels is predominantly coupled to the synaptic transmission mediated by excitatory amino acids and catecholamines in a physiological situation (17), and the release of neurotransmitter induced by high potassium-elicited depolarization was inhibited by ω-conotoxin and ω-agatoxins (18).

We previously examined the mechanisms of neuronal cell damage induced by deprivation of oxygen and glucose by means of pharmacological modulation of the enhanced glutamate efflux and the consequent cell loss, and we suggested that glutamate efflux is probably the primary cause of the neuronal injury in oxygen/glucose deprivation in vitro, though it is difficult to produce a sufficient neuroprotective effect only by reducing the extracellular glutamate level (4). The results were consistent with previous studies showing that the inhibition of glutamate release is not necessarily correlated to the blockade of neurotoxicity (19, 20). Based on these results, we hypothesized that the blockade of calcium influx through NMDA receptor-coupled channels and/or voltage-dependent calcium channels might be sufficient for neuronal protection. The effect of voltage-dependent calcium

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channel blockers on the neuronal injury induced by excessive activation of NMDA receptor has not been reported previously, although Longo et al. reported that ω-conotoxin GVIA failed significantly to affect the recovery from the CA1 electrical failure induced by NMDA (21). We have examined the toxicity of glutamate receptor agonists, NMDA and kainate, towards neurons under energy-compromised conditions and investigated whether the blockade of glutamate receptors or voltage-dependent calcium channels can protect neurons from the neurotoxicity induced by agonists.

MATERIALS AND METHODS

Chemicals and reagents

ω-Conotoxin GVIA, ω-agatoxin TK (ω-Aga-TK) and ω-agatoxin IVA (ω-Aga-IVA) were purchased from Peptide Institute, Inc. (Osaka). Fetal calf serum, heat-inactivated horse serum, trypsin solution, penicillin, streptomycin, Dulbecco's modified Eagle's medium (DMEM) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) were purchased from Life Technologies, Inc. (Grand Island, NY, USA); insulin, sodium selenite, putrescine, cytosine arabinofuranoside (Ara C), DNase I, kainate, NMDA, glycine and nifedipine, from Sigma Chemical Co. (St. Louis, MO, USA); MK-801, from Research Biochemicals, Inc. (Natick, MA, USA); and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), from Tocris Neuramin, Ltd. (Bristol, UK). NBQX (2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(F)quinoxaline) was synthesized in our laboratory. All other chemicals used were of reagent grade.

Cortical cell cultures

Cortical cell cultures were prepared from fetal rats of the Wistar strain (gestational age of 17 days) as described (22). Briefly, the cortex was dissected and kept in ice-cold Hanks' balanced salt solution (HBSS; 10 mM HEPES, pH 7.3) and then incubated at 37℃ for 15 min in Ca^{2+}/Mg^{2+}-free HBSS containing 0.25% trypsin and 0.2 mg/ml DNase I. The cortical tissues were dissociated to single cells by gentle trituration using a glass pipette with a fire-polished tip. The cell suspension was mixed with DMEM supplemented with 10% fetal calf serum, 10% heat-inactivated horse serum, 5 μg/ml insulin, 30 nM sodium selenite, 100 μM putrecine, 20 nM progesterone, 15 nM biotin, 100 units/ml penicillin, 100 μg/ml streptomycin and 1 mM sodium pyruvate, as described (23). The cell suspension was centrifuged and the resulting pellets were resuspended in the medium described above. The cortical cells were then pelleted again by centrifugation, suspended in the medium and plated onto poly-L-lysine-coated coverslips. The cells were cultured in a CO_2 incubator (5% CO_2) at 37℃ for 1 day and the coverslips were then transferred onto a confluent glial cell layer and cultured for 8 days in DMEM containing 2% fetal calf serum and the same supplements as described above. After 8 days, the cortical cells were cultured in DMEM containing 2% fetal calf serum and the same supplements, but without glutamine. The cortical cells were treated with 10 μM Ara C for 1 day (it was added to the culture medium 3 days after plating) to reduce the growth of contaminating non-neuronal cells. The culture medium was changed every 3–4 days. The glial cells used were obtained from postnatal day 1 rats of the Wistar strain. The cerebral cortex was dissected and triturated in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, and the glial cells were cultured in a CO_2 incubator (5% CO_2) at 37℃ for 11–18 days before use.

Cell death assay

The experiment was performed on 14- to 17-day-old cultures. Cortical neurons on coverslips cultured on a glial layer were washed with HBSS and the neurons were incubated in glucose-free HBSS at 37℃. For the NMDA toxicity assay, we used glucose-free HBSS without Mg^{2+} ions containing NMDA and 1 μM glycine; and for the kainate toxicity assay, we used glucose-free HBSS containing kainate. The plates were transferred to a 5% CO_2 incubator and incubated at 37℃ for 1–3 hr. After the incubation, the cells were washed twice with HBSS without Mg^{2+} ions and then cultured in normal medium for another 20 hr in a CO_2 incubator. The measurement of lactate dehydrogenase (LDH) efflux into the media as an indicator of neuronal injury was made 20 hr after the treatment with agonists (24). LDH release was calculated as % of total activity in the medium and the cells lysed with 0.5% Triton X-100 in phosphate buffer, pH 7.4. Receptor antagonists and calcium channel blockers were added to the incubation buffer during the exposure to agonists or to the culture medium for the subsequent culture for 20 hr.

Statistical analyses

All data are expressed as the mean±S.E.M. Statistical comparisons between two groups were carried out by Student's *t*-test. In order to the compare the drug-untreated group with more than two other treated groups, data were analyzed by means of ANOVA and Fisher's PLSD.

RESULTS

Effect of energy depletion on glutamate toxicity

Neuronal cell damage was examined after the incuba-

tion of cortical neurons with NMDA or kainate in the presence or absence of glucose. When the cultured cortical neurons were exposed to NMDA at 10 μM in the presence of glucose, no neuronal toxicity was induced after incubation for 3 hr. However, after incubation of cortical neurons with NMDA at the same concentration in glucose-free HBSS for 2 hr or more, the level of LDH measured after incubation of the cells in normal medium for 20 hr was significantly increased (Fig. 1). When cultured cortical neurons were exposed to the non-NMDA agonist kainate at 100 μM in the presence of glucose, no neuronal toxicity was observed within 3 hr. However, when they were incubated with kainate at the same concentration in the glucose-free HBSS for 2 hr or more, the level of LDH was again significantly increased (Fig. 2).

Effect of glutamate receptor antagonists on excitotoxicity in energy-compromised neurons

The incubation of cortical cell cultures with NMDA at 10 μM in the glucose-free HBSS for 3 hr evoked LDH efflux during incubation for 20 hr after return of the cells to normal medium. An NMDA-receptor antagonist, MK-801 (1 μM), inhibited the LDH efflux when added to the cortical neurons during the exposure to NMDA (Fig. 3). MK-801 is a non-competitive antagonist of the NMDA receptor and has no significant effect on other receptors including non-NMDA receptors (25). A non-NMDA antagonist, CNQX (10 μM), significantly attenuated the LDH efflux, although another non-NMDA antagonist, NBQX (10 μM), failed to protect the neurons (Fig. 3). It has been reported that high concentrations of CNQX antagonize NMDA receptor-mediated effects by blocking

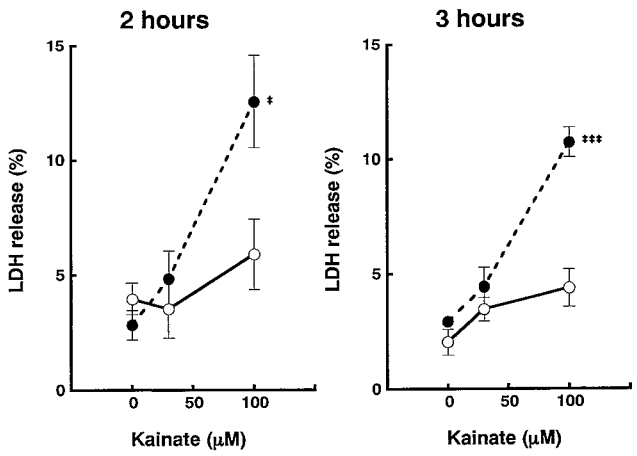

Fig. 2. Cell death induced by kainate in cortical neurons energy-compromised by depletion of glucose. Cultured cortical neurons were incubated with kainate in HBSS in the absence (●) or presence (○) of glucose. The buffer was then replaced with normal medium and the cortical neurons were incubated for a further 20 hr. The activity of LDH in the medium at 20 hr was measured. Each value represents the mean with S.E.M. (n=4). *P<0.05, ***P<0.001 vs cell death in the presence of glucose at the respective concentration of kainate (Student's *t*-test).

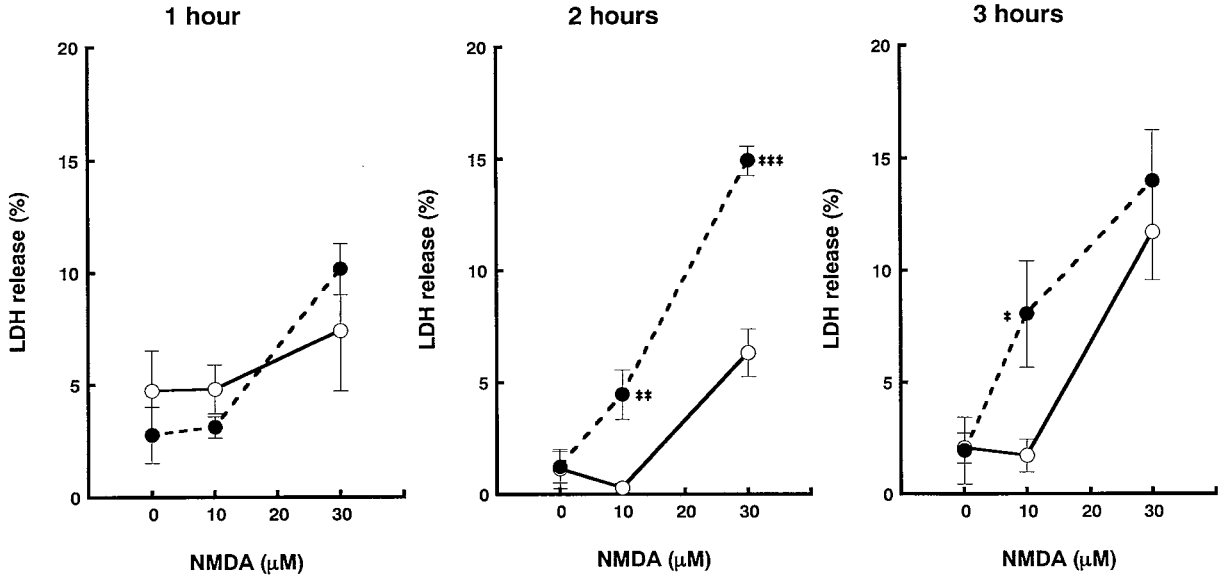

Fig. 1. Cell death induced by NMDA in cortical neurons energy-compromised by depletion of glucose. Cultured cortical neurons were incubated with NMDA and 1 μM glycine in HBSS in the absence (●) or presence (○) of glucose. The buffer was then replaced with normal medium and the cortical neurons were incubated for a further 20 hr. The activity of LDH in the medium at 20 hr was measured. Each value represents the mean with S.E.M. (n=4). *P<0.05, **P<0.01, ***P<0.001 vs cell death in the presence of glucose at the respective concentration of NMDA (Student's *t*-test).

the strychnine-insensitive glycine-binding site on the NMDA receptor (26). CNQX, therefore, might have dual effects as both an NMDA-receptor antagonist and a non-NMDA-receptor antagonist on the glutamate receptors. On the other hand, NBQX is reported to have selectivity for non-NMDA receptors (27). When MK-801 was added to the cultured cortical neurons after the exposure to NMDA, MK-801 at 1 μM did not show any neuroprotective effect, while MK-801 at 10 μM partially attenuated the LDH efflux (Fig. 3). When CNQX or NBQX was added to cultured cortical neurons after the exposure to NMDA for 3 hr, each of them failed to protect the neurons (Fig. 3).

The incubation of cortical cell cultures with kainate in the glucose-free HBSS for 2 hr evoked LDH efflux during incubation for 20 hr after return of the cells to normal medium. MK-801 at 1 μM attenuated the LDH efflux to 44% when added to the neurons during exposure to kainate (Fig. 4). CNQX (10 μM) and NBQX (10 μM) also inhibited the LDH efflux (Fig. 4). When MK-801 was added to the cultured cortical neurons after the exposure to kainate, MK-801 effectively attenuated the LDH efflux (Fig. 4). When CNQX or NBQX was added to the cultured cortical neurons after the exposure to kainate, neither of the non-NMDA receptor antagonists showed any neuroprotective effect (Fig. 4).

Effect of voltage-dependent calcium channel blockers on excitotoxicity in energy-compromised neurons

We examined the effect of voltage-dependent calcium channel blockers on the neuronal damage induced by NMDA in cells incubated in glucose-deprived buffer solution. P/Q-type calcium channel blockers, ω-aga-IVA and ω-aga-TK, and an N-type calcium channel blocker, ω-conotoxin GVIA, significantly attenuated the neuronal

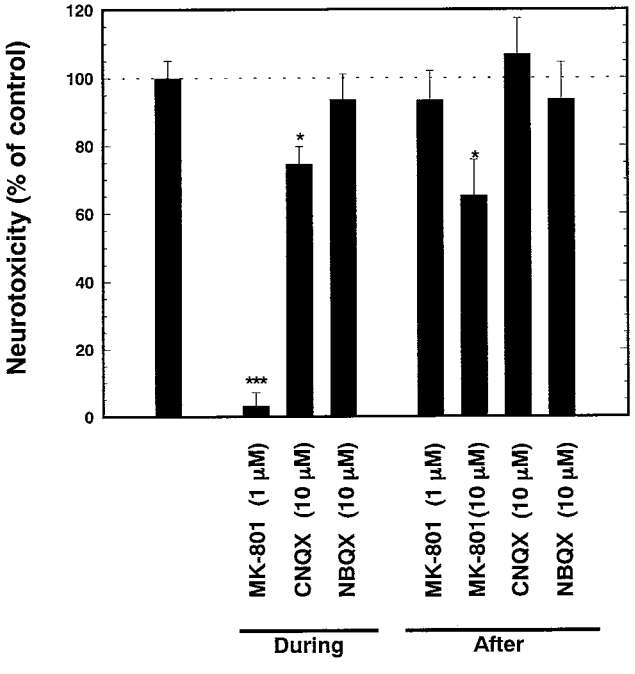

Fig. 3. Effect of glutamate receptor antagonists on LDH efflux induced by NMDA in the absence of glucose. Cultured cortical neurons were incubated with 10 μM NMDA and 1 μM glycine in HBSS in the absence of glucose for 3 hr. The buffer was then replaced with normal medium and the cortical neurons were incubated for a further 20 hr. The activity of LDH in the medium at 20 hr was measured. LDH efflux from neurons incubated in the absence of NMDA and glycine (basal) and that from neurons incubated with NMDA and glycine (injury control) were 6.8±1.1% and 20.9±2.3%, respectively. Neurotoxicity (% of control) is expressed as % of LDH efflux induced by the agonist. A glutamate receptor antagonist (MK-801, 1 or 10 μM; CNQX, 10 μM; NBQX, 10 μM) was added with NMDA for 3 hr (During) or added to normal medium for 20 hr after NMDA treatment (After). Each value represents the mean with S.E.M. (n=4–13). *P<0.05, ***P<0.001 vs control. Data were analyzed by means of ANOVA and Fisher's PLSD.

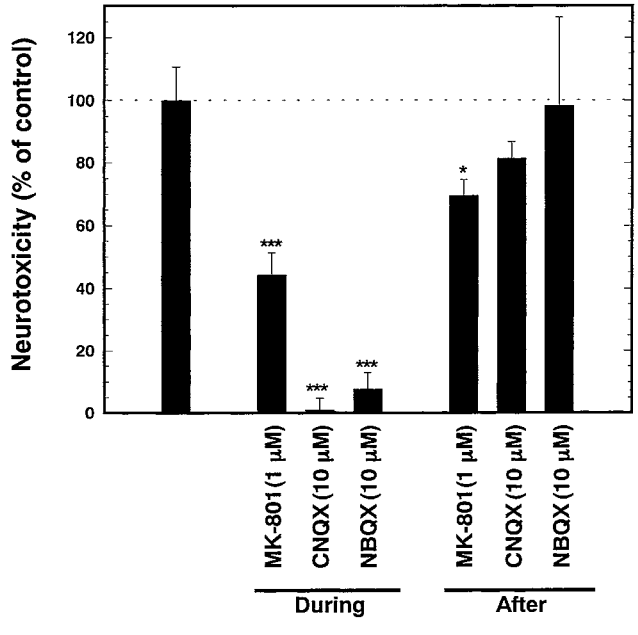

Fig. 4. Effect of glutamate receptor antagonists on LDH efflux induced by kainate in the absence of glucose. Cultured cortical neurons were incubated with 100 μM kainate in HBSS in the absence of glucose for 2 hr. The buffer was then replaced with normal medium and the cortical neurons were incubated for a further 20 hr. The activity of LDH in the medium at 20 hr was measured. LDH efflux from neurons incubated in the absence of kainate (basal) and that from neurons incubated with kainate (injury control) were 4.8±1.7% and 19.7±3.7%, respectively. Neurotoxicity (% of control) is expressed as % of LDH efflux induced by the agonist. A glutamate receptor antagonist (MK-801, 1 μM; CNQX, 10 μM; NBQX, 10 μM) was added with kainate for 2 hr (During) or added to normal medium for 20 hr after kainate treatment (After). Each value represents the mean with S.E.M. (n=4–9). *P<0.05, ***P<0.001 vs control. Data were analyzed by means of ANOVA and Fisher's PLSD.

injury, although an L-type calcium channel blocker, nifedipine, showed little neuroprotective effect (Fig. 5). A combination of calcium channel blockers of the three subtypes showed 51% inhibition of the LDH efflux (Fig. 5). These calcium channel blockers selectively block the respective calcium channels (18, 28–30).

DISCUSSION

We have established a culture system of rat cortical neurons in which the neurons are damaged by a low concentration of a glutamate receptor agonist, NMDA or kainate, in glucose-free buffer solution (Figs. 1 and 2). In our cell culture system, 10 μM NMDA was sufficient to induce neuronal cell loss in the glucose-free buffer solution, and this concentration of NMDA was far less than that needed to injure neurons in buffer solution containing a normal level of glucose (Fig. 1). Toxicity induced by kainate was also enhanced in the glucose-free buffer solution (Fig. 2). It was reported that glutamate becomes neurotoxic via NMDA receptors when intracellular energy levels are reduced (31, 32), and it was suggested that when the disturbance of ionic pumping at the cell membrane is sufficiently extensive due to the reduced energy production, partial cell depolarization may occur with subsequent alleviation of the voltage-dependent Mg^{2+} blockade of NMDA receptors, leading to the activation of the NMDA receptors by endogenous levels of glutamate. However, we used glucose-free HBSS without Mg^{2+} ions for the NMDA toxicity assay in our experiments (Materials and Methods, *Cell death assay*), so it is unlikely that the activation of NMDA receptors occurred by alleviation of the Mg^{2+} blockade of the receptors. Furthermore, this mechanism would not be applicable to explain the enhanced kainate toxicity in glucose-free buffer solution. An alternative explanation is as follows: The impairment of mitochondrial energy metabolism results in decreased ATP production, leading to various alterations in cellular physiology, including reduced Na^+/K^+-ATPase activity. When membrane depolarization elicited by an excitatory transmitter is propagated, cells lose K^+ and gain Na^+, and the cells must extrude Na^+ and accumulate K^+ to preserve excitability by using metabolic energy derived from oxidative metabolism.

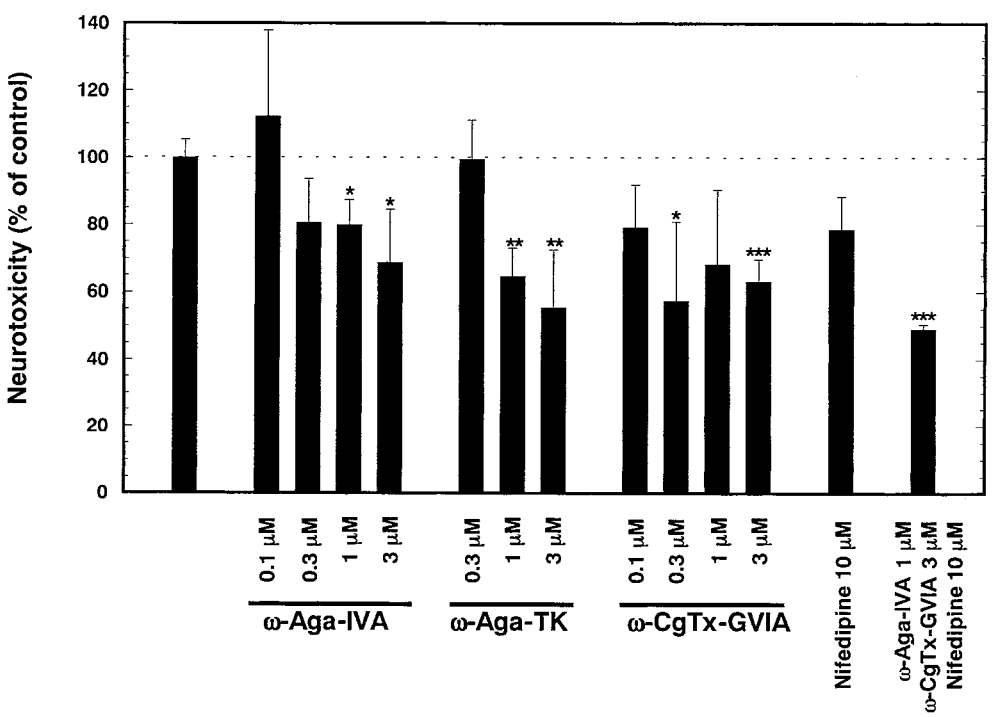

Fig. 5. Effect of voltage-dependent calcium channel blockers on LDH efflux induced by NMDA in the absence of glucose. Cultured cortical neurons were incubated with 10 μM NMDA and 1 μM glycine in HBSS in the absence of glucose for 3 hr. The buffer was then replaced with normal medium and the cortical neurons were incubated for 20 hr. LDH efflux from neurons incubated in the absence of NMDA and glycine (basal) and that from neurons incubated with NMDA and glycine (injury control) were 4.3±0.5% and 14.7±0.7%, respectively. Neurotoxicity (% of control) is expressed as % of LDH efflux induced by the agonist. A voltage-dependent calcium channel blocker (ω-Aga-IVA, ω-Aga-TK, ω-conotoxin GVIA (ω-CgTx-GVIA) or nifedipine) was added with NMDA for 3 hr. Each value represents the mean with S.E.M. (n=4–22). *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control. Data were analyzed by means of ANOVA and Fisher's PLSD.

If the availability of Na^+/K^+-ATPase is reduced, the excitability induced by the excitatory transmitter would be prolonged. This could facilitate the expression of neurotoxicity by relatively low levels of agonists to the NMDA and kainate receptors due to enhanced elevation of intracellular calcium concentration. The prolonged excitability might also enhance the calcium influx through the voltage-dependent calcium channels.

The NMDA-receptor antagonist inhibited glutamate toxicity (both NMDA and kainate toxicity) when added to the culture medium during or after the exposure of neurons to the agonist (Figs. 3 and 4). In contrast, the non-NMDA-receptor antagonist only inhibited the neuronal cell death induced by kainate when added simultaneously with the agonist (Figs. 3 and 4). Thus, the injury initially induced during exposure to kainate or NMDA should be subsequently augmented by the activation of NMDA receptors in response to endogenously released glutamate. From the result that an NMDA-receptor antagonist, MK-801, added after the NMDA or kainate exposure attenuated the LDH efflux (Figs. 3 and 4), it is possible that glutamate release was continuing after the exposure of neurons to NMDA or non-NMDA agonist, leading to neuronal injury through the activation of NMDA receptors. Although the mechanism of the late release of glutamate after the exposure of the cells to a glutamate receptor agonist is unclear, several possibilities exist: activation of the kainate receptor might be responsible since kainate has been suggested to induce glutamate release by activation of the presynaptic receptors on glutamatergic terminals (33). We have also confirmed that glutamate release is evoked by kainate (4). Other possible mechanisms include the activation of certain enzyme families, namely C kinases, calmodulin-regulated enzymes, calpains and phospholipase, which may produce a lasting enhancement of excitatory synaptic efficacy and circuit excitability (34–36).

We previously reported that non-NMDA receptor antagonists did not afford complete neuroprotection in oxygen/glucose-deprived conditions despite reducing the glutamate efflux to a large extent, although NMDA receptors are primarily responsible for the neuronal cell death (4). It was reported by Koh and Choi that the late neuronal degeneration induced by intense glutamate exposure is mediated mainly by NMDA receptors (37). They reported that selective antagonism of NMDA receptors with either competitive or non-competitive antagonists eliminated the late neuronal death induced by brief exposure to glutamate. In contrast, late death is not blocked by CNQX plus glycine (37). CNQX in the presence of 1 μM glycine exhibits good selectivity for α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) /kainate receptor-mediated toxicity. The degeneration of neurons by excitatory amino acids is thought to be dependent on the presence of extracellular Ca^{2+} and is probably triggered by excessive Ca^{2+} influx (38). Massive entry of calcium ions into cells might occur through NMDA receptors (5, 39, 40), which are not desensitized like non-NMDA receptors (41). Koh et al. reported that the induction of neuronal damage by AMPA or kainate requires exposure of the cells for hours (42), and this slowly triggered excitotoxicity may also involve excessive Ca^{2+} influx. However, the channels gated by non-NMDA receptors have limited permeability to calcium ions, and the main route of Ca^{2+} entry may be indirect; for example, involving voltage-gated Ca^{2+} channels and reverse operation of the Na^+-Ca^{2+} exchanger. The decline of the sodium gradient across the membranes due to the entry of sodium ions through non-NMDA receptors may degrade the ability of neurons to extrude Ca^{2+} ions through the sodium-calcium exchanger.

In the experiments with voltage-dependent calcium channel blockers, we clearly demonstrated the participation of P/Q-type and N-type calcium channels in NMDA toxicity (Fig. 5). Although an L-type calcium channel blocker alone did not effectively reduce the increase of LDH efflux, the concomitant blockade of all three subtypes of calcium channels significantly reduced the LDH efflux to 49% (Fig. 5). It therefore seems likely that the neuronal cell death induced by NMDA under energy-compromised conditions is caused at least in part by an increase in intracellular calcium concentration through multiple subtypes of voltage-dependent calcium channels.

In conclusion, glutamate receptor-mediated cell death under energy-compromised conditions was attenuated not only by the inhibition of NMDA and non-NMDA receptors but also by the blockade of voltage-dependent calcium channels. Indeed, it is well known that the depolarization of cell membrane in the brain is caused under ischemic conditions. Our results suggest that the activation of NMDA and non-NMDA receptors and voltage-dependent calcium channels might be involved in the elevation of cytosolic calcium in ischemia, leading to neuronal cell death, and that enhanced glutamate release after exposure to agonists might partly contributes to excitotoxic neuronal injury mediated by NMDA receptor-coupled channels. We suggest that the blockade of calcium influx through NMDA receptor-coupled channels and voltage-dependent calcium channels might be an effective approach for neuronal protection.

REFERENCES

1 Meldrum B: Possible therapeutic applications of antagonists of excitatory amino acid neurotransmitters. Clin Sci **68**, 113–122 (1985)

2 Rothman SM and Olney JW: Glutamate and the pathophysiology of hypoxic-ischemic brain damage. Ann Neurol **19**, 105–111 (1986)
3 Ellren K and Lehmann A: Calcium dependency of *N*-methyl-D-aspartate toxicity in slices from the immature rat hippocampus. Neuroscience **2**, 371–379 (1989)
4 Kimura M, Sawada K, Miyagawa T, Kuwada M, Katayama K and Nishizawa Y: Role of glutamate receptors and voltage-dependent calcium and sodium channels in the extracellular glutamate/aspartate accumulation and subsequent neuronal injury induced by oxygen/glucose deprivation in cultured hippocampal neurons. J Pharmacol Exp Ther **285**, 178–185 (1998)
5 MacDermott AB, Mayer ML, Westbrook GL, Smith SJ and Barker JL: NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurons. Nature **321**, 519–522 (1986)
6 Siesjo BK: Mechanisms of ischemic brain damage. Crit Care Med **16**, 954–963 (1988)
7 Balentine JD: Spinal cord trauma: in search of the meaning of granular axoplasm and vesicular myelin. J Neuropathol Exp Neurol **47**, 77–92 (1988)
8 Meyer FB: Calcium, neuronal hyperexcitability and ischemic injury. Brain Res Rev **14**, 227–243 (1989)
9 Asakura K, Matsuo Y, Kanemasa T and Ninomiya M: P/Q-type Ca^{2+} channel blocker ω-agatoxin IVA protects against brain injury after focal ischemia in rats. Brain Res **776**, 140–145 (1997)
10 Bowersox SS, Singh T and Luther RR: Selective blockade of N-type voltage-sensitive calcium channels protects against brain injury after transient focal cerebral ischemia in rats. Brain Res **747**, 343–347 (1997)
11 Buchan AM, Gertler SZ, Li H, Xue D, Huang ZG, Chaundy KE, Barnes K and Lesiuk HJ: A selective N-type Ca^{2+}-channel blocker prevents CA1 injury 24 hr following severe forebrain ischemia and reduces infarction following focal ischemia. J Cereb Blood Flow Metab **14**, 903–910 (1994)
12 Takizawa S, Matsushima K, Fujita H, Nanri K, Ogawa S and Shinohara Y: A selective N-type calcium channel antagonist reduces extracellular glutamate release and infarct volume in focal cerebral ischemia. J Cereb Blood Flow Metab **15**, 611–618 (1995)
13 Yenari MA, Palmer JT, Sun GH, Crespigny A, Moseley ME and Steinberg GK: Time-course and treatment response with SNX-111, an N-type calcium channel blocker, in a rodent model of focal cerebral ischemia using diffusion-weighted MRI. Brain Res **739**, 36–45 (1996)
14 Small DL, Monette R, Buchan AM and Morley P: Identification of calcium channels involved in neuronal injury in rat hippocampal slices subjected to oxygen and glucose deprivation. Brain Res **753**, 209–218 (1997)
15 Toner CC and Stamford JA: Involvement of N- and P/Q- but not L- or T-type voltage-gated calcium channels in ischemia-induced striatal dopamine release in vitro. Brain Res **748**, 85–92 (1997)
16 Bentué-Ferrer D, Decombe R, Saïag B, Allain H and Van den Driessche J: L-type voltage-dependent calcium channels do not modulate aminergic neurotransmitter release induced by transient global cerebral ischemia: an in vivo microdialysis study in rat. Exp Brain Res **93**, 288–292 (1993)
17 Tuner TJ, Adams ME and Dunlap K: Multiple Ca^{2+} channel types coexist to regulate synaptosomal neurotransmitter release. Proc Natl Acad Sci USA **90**, 9518–9522 (1993)
18 Kimura M, Yamanishi Y, Hanada T, Kagaya T, Kuwada M, Watanabe T, Katayama K and Nishizawa Y: Involvement of P-type calcium channels in high potassium-elicited release of neurotransmitters from rat brain slices. Neuroscience **66**, 609–615 (1995)
19 Lustig HS, Ahern KB and Greenberg DA: ω-Agatoxin IVA and excitotoxicity in cortical neuronal cultures. Neurosci Lett **213**, 142–144 (1996)
20 Valentino K, Newcomb R, Gadbois T, Singh T, Bowersox S, Bitner S, Justice A, Yamashiro D, Hoffman BB, Ciaranello R, Miljanich G and Ramachandran J: A selective N-type calcium channel antagonist protects against neuronal loss after global cerebral ischemia. Proc Natl Acad Sci USA **90**, 7894–7897 (1993)
21 Longo R, Sagratella S and Scotti de Carolis A: Effects of calcium antagonists on hypoxic and NMDA injury in rat hippocampal slices. Life Sci **55-6**, 455–462 (1994)
22 Teramoto T, Niidome T, Kimura M, Ohgoh M, Nishizawa Y, Katayama K, Mayumi T and Sawada K: A novel type of calcium channel sensitive to ω-agatoxin-TK in cultured rat cerebral cortical neurons. Brain Res **756**, 225–230 (1997)
23 Scholtz WK, Baitinger C, Schulman H and Kelly PT: Developmental changes in Ca^{2+}/calmodulin-dependent protein kinase II in cultures of hippocampal pyramidal neurons and astrocytes. J Neurosci **8**, 1039–1051 (1988)
24 Koh JY and Choi DW: Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. J Neurosci Methods **20**, 83–90 (1987)
25 Wong EHF, Kemp JA, Priestly T, Knight AR, Woodruff GN and Iversen LL: The anticonvulsant MK-801 is a potent *N*-methyl-D-aspartate antagonist. Proc Natl Acad Sci USA **83**, 7104–7108 (1986)
26 Birch PJ, Grossman CJ and Hayes AG: 6,7-Dinitroquinoxaline-2,3-dione antagonizes responses to NMDA in the rat spinal cord via an action at the strychinine-insensitive glycine receptor. Eur J Pharmacol **156**, 177–180 (1988)
27 Parsons CG, Gruner R and Rozental J: Comparative patch clamp studies on the kinetics and selectivity of glutamate receptor antagonism by 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo-(F)quinoxaline (NBQX) and 1-(4-amino-phenyl)-4-methyl-7,8-methyl-endioxyl-5*H*-2,3-benzodiazepine (GYKI 52466). Neuropharmacolgy **33**, 589–604 (1994)
28 Wisgirda ME and Dryer SE: Functional dependence of Ca^{2+}-activated K^+ current on L- and N-type Ca^{2+} channels: differences between chicken sympathetic and parasympathetic neurons suggest different regulatory mechanisms. Proc Natl Acad Sci USA **91**, 2858–2862 (1994)
29 Reynolds IJ, Wagner JA, Snyder SH, Thayer SA, Olivera BM and Miller RJ: Brain voltage-sensitive calcium channel subtypes differentiated by ω-conotoxin fraction GVIA. Proc Natl Acad Sci USA **83**, 8804–8807 (1986)
30 Teramoto T, Kuwada M, Niidome T, Sawada K, Nishizawa Y and Katayama K: A novel peptide from funnel web spider venom, ω-Aga-TK, selectively blocks P-type calcium channels. Biochem Biophys Res Commun **196**, 134–140 (1993)
31 Novelli A, Reilly JA, Lysko PG and Henneberry RC: Gluta-

mate becomes neurotoxic via the *N*-methyl-D-aspartate receptor when intracellular energy levels are reduced. Brain Res **451**, 205–212 (1988)

32 Schulz JB, Matthews RT, Henshaw DR and Beal MF: Neuroprotective strategies for treatment of lesions produced by mitochondrial toxins: implications for neurodegenerative diseases. Neuroscience **71**, 1043–1048 (1996)

33 Ferkany JW, Zaczek R and Coyle JT: Kainic acid stimulates excitatory amino acid neurotransmitter release at presynaptic receptors. Nature **298**, 757–759 (1982)

34 Fukunaga K, Muller D and Miyamoto E: CaM kinase II in long-term potentiation. Neurochem Int **28**, 343–358 (1996)

35 Kristian T and Siesjo BK: Calcium in ischemic cell death. Stroke **29**, 705–718 (1998)

36 Lu YM, Lu BF, Zhao FQ, Yan YL and Ho XP: Accumulation of glutamate is regulated by calcium and protein kinase C in rat hippocampal slices exposed to ischemic states. Hippocampus **3**, 221–227 (1993)

37 Koh JY and Choi DW: Selective blockade of non-NMDA receptors do not block rapidly triggered glutamate-induced neuronal death. Brain Res **548**, 318–321 (1991)

38 Rothman SM, Thurston JH and Hauhart RE: Delayed neurotoxicity of excitatory amino acids in vitro. Neuroscience **22**, 471–480 (1987)

39 Choi DW: Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. Trends Pharmacol Sci **11**, 465–469 (1988)

40 Choi DW: Glutamate neurotoxicity and diseases of the nervous system. Neuron **1**, 623–634 (1988)

41 Bateman MC, Yamada KA and Goldberg MP: AMPA receptor desensitization in hypoxic neuronal injury in vitro. Neurology **43**, A339 (1993)

42 Koh JY, Goldberg MP, Hartley DM and Choi DW: Non-NMDA receptor-mediated neurotoxicity in cortical culture. J Neurosci **10**, 693–705 (1990)