Muscimol Prevents Neuronal Injury Induced by NMDA

Seitaro Ohkuma, Shi-Hu Chen, Masashi Katsura, Da-Zhi Chen and Kinya Kuriyama*

Department of Pharmacology, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamikyo-ku, Kyoto 602, Japan

Received September 2, 1993 Accepted December 14, 1993

ABSTRACT—The effect of muscimol on *N*-methyl-D-aspartate (NMDA)-induced injury of primary cultured cerebral cortical neurons was examined. NMDA induced a dose-dependent leakage of LDH activity, which was significantly inhibited by (±)-5-methyl-10,11-dihydro-5*H*-dibenzo-[*a*,*d*]cyclopentan-5,10-imine (MK-801). Muscimol significantly reduced the NMDA-induced increase of lactic dehydrogenase (LDH) leakage, and bicuculline abolished this protective effect of muscimol. Similarly, muscimol reduced the NMDA-induced increase in trypan blue staining of the cells, and bicuculline suppressed this inhibitory action of muscimol. These results suggest that GABAA-receptor stimulation exerts a protective action against the neuronal injury induced by NMDA-receptor activation.

Keywords: GABAA-receptor agonist, NMDA receptor, Neuronal injury

The *N*-methyl-D-aspartate (NMDA) receptor, a subtype of glutamate receptors, has been supposed to participate in the neuronal cell damage observed in central nervous system disorders such as Huntington's disease, epilepsy, parkinsonism, ischemia and trauma (1-4). The excessive accumulation of Ca^{2+} in neuronal cells induced by over-activation of NMDA receptors under the pathological conditions results in the neuronal injury (1, 2). On the other hand, an inhibitory receptor, GABAA receptor, has been reported to negatively regulate excitatory functions of NMDA receptors (5-7). This suggests that GABAA-receptor stimulation may suppress the excitatory actions of NMDA receptors, especially NMDA-induced neuronal toxicity. In the present study, we examined the effect of GABAA-receptor activation on NMDA-induced neuronal injury by measuring the leakage of lactic dehydrogenase (LDH) activity from primary cultured mouse cerebral cortical neurons and the staining of the neurons with trypan blue dye.

Cell isolation and primary culture were carried out by the previously reported procedures (8). In brief, the neopallium free of meninges dissected from 15-day-old fetuses of ddY strain mice were minced, trypsinized and centrifuged. Collected cells were incubated in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal calf serum at 37℃ for 3 days in humidified 95% air-5% CO_2, followed by the exposure to 10 μM cytosine arabinoside for 24 hr to suppress the proliferation of non-neuronal cells. Neurons were then cultured in DMEM containing 10% horse serum under the culture conditions described above until they were used for the experiments. The culture medium was exchanged every 4 days. All neurons used in the experiments were from 13-day-old cell cultures.

For measuring the leakage of LDH activity from neurons, the neurons were exposed to NMDA by the previously reported method (9) with a minor modification. Neurons were washed 3 times with ice-cold, Mg^{2+}-free Krebs-Ringer bicarbonate buffer (KRB: pH 7.4) and then preincubated with Mg^{2+}-free KRB at 37℃ for 10 min. Thereafter, the neurons were incubated with NMDA and other agents for 15 min. Following this incubation, the incubation medium was discarded, and the neurons were subsequently rinsed with ice-cold KRB. Fresh KRB (37℃) was added and further incubation was carried out. After the incubation for 60 min, the incubation medium from each culture was removed and pooled, and the neurons were scraped off with ice-cold sodium-potassium phosphate buffer (0.1 M, pH 7.4), homogenized and centrifuged (100,000×*g*, 4℃, 60 min). The LDH activity of the resultant supernatant prepared from the neurons and that of the pooled incubation medium were measured by the method of Cabaud and Wrobrewski (10). The leakage of LDH activity from the cells to the incubation medium was expressed as the percent of total LDH activity (intracellular LDH activity plus LDH activity determined in

*To whom correspondence should be addressed.

the incubation medium). In the present study, we checked the leakage of LDH activity 1 hr after the treatment with agents including NMDA, because it has been confirmed that glutamate- and NMDA-induced neuronal injury occurs after 30–75 min of the treatment with these agents (11–13).

Neurons were stained with trypan blue to examine the neuronal injury after the treatment with NMDA. The procedure to incubate neurons with NMDA and other agents was the same as that to examine the leakage of LDH activity. After the final incubation with KRB without any agents for 60 min, the neurons were further incubated with trypan blue dye (final concentration of 0.1%) at 37℃ for 2 min and then washed 3 times with ice-cold KRB. Then the neurons were directly observed under a phase-contrast microscope (Nikon Diaphoto-TMD; Nikon Co., Tokyo) following the addition of fresh KRB into a culture dish.

When neurons were exposed to various concentrations of NMDA, the leakage of LDH activity into the incubation medium increased with increasing NMDA concentration (Fig. 1). This increase of leaked LDH activity was significantly inhibited by MK-801, a non-competitive antagonist for NMDA receptors, while MK-801 alone showed no alteration in the leakage of LDH activity (Fig. 1). These data indicate that the cerebral cortical neurons used in this study are susceptible to the neurotoxicity induced by NMDA as previously reported (14, 15). In the cases of the control and the group treated with MK-801 alone, LDH leakage was also observed (Figs. 1 and 2). This leakage may be due to the effect of endogenous glutamate, because the neurons used in this study possess glutamate, and this glutamate released from cells may, in turn, activate other subtypes of glutamate receptors other than NMDA receptors to induce neuronal injury. Indeed, glutamate receptor subtypes other than NMDA receptors

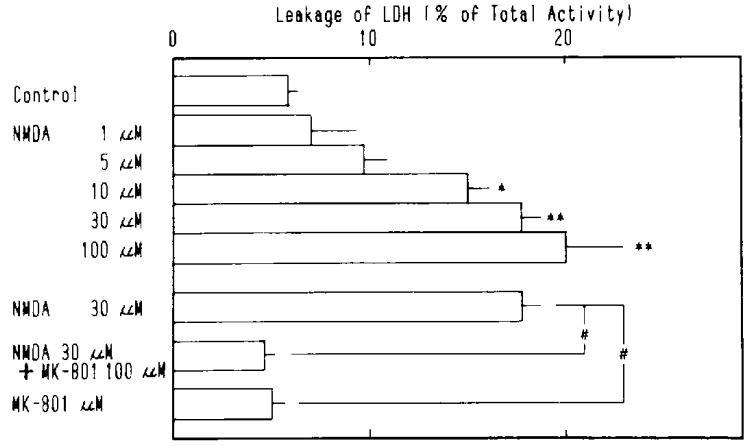

Fig. 1. Effect of *N*-methyl-D-aspartate (NMDA) on leakage of lactic dehydrogenase (LDH) activity from cerebral cortical neurons. Each value represents the mean±S.E. obtained from 4 separate experiments. *$P<0.05$, **$P<0.01$ vs. Control (Dunnett's test); #$P<0.01$ (Scheffe's test).

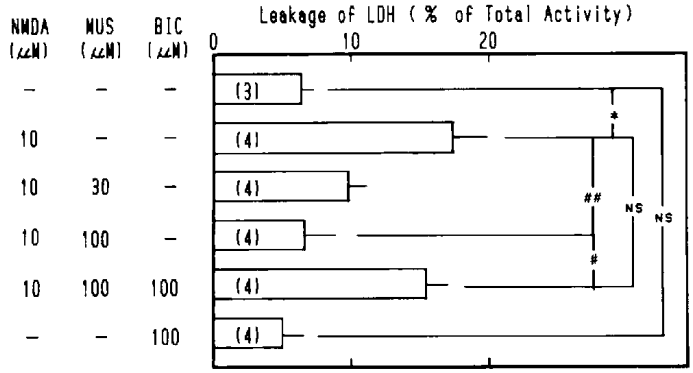

Fig. 2. Effects of muscimol (MUS) and bicuculline (BIC) on NMDA-induced leakage of LDH activity from cerebral cortical neurons. The number in parenthesis in the bar shows the number of experiments. *$P<0.05$ (Scheffe's test); #$P<0.05$, ##$P<0.01$ (Bonferroni's test). NS: not significant.

have also been reported to be involved in neuronal toxicity (16). However, the exact reason for such LDH leakage is not clear at present.

The simultaneous exposure to 10 μM NMDA and 100 μM muscimol, an agonist selective for GABAA receptors, significantly suppressed the NMDA-induced increase of the leakage of LDH activity, although muscimol at 30 μM showed a tendency to reduce the NMDA-induced leakage of LDH activity (Fig. 2). Muscimol (100 μM) alone induced no alteration in the leakage of LDH activity (data not shown). This suppressive effect of muscimol on the NMDA-induced leakage of LDH activity was significantly inhibited in the presence of bicuculline, an antagonist for GABAA receptors (Fig. 2). These results seem to lead to the conclusion that the protective effect of muscimol against NMDA-induced neuronal damage is mediated via GABAA-receptor stimulation by muscimol itself. In a previous report (16), GABA showed enhancement of NMDA-induced neuronal injury in a primary culture of rat cerebral cortical neurons, which is opposite to the data reported here. This difference in the effect of GABAA-receptor stimulation on NMDA-induced neuronal death may be attributed to the differences in the species of animals from which neurons have been separated and/or in the duration from the point of drug addition through that of the measurement of leaked LDH activity, although the exact reason is unclear.

The protective action of muscimol against NMDA-induced neuronal injury was also confirmed by the decrease in the trypan blue staining of neurons. Figure 3 shows the staining of neurons with trypan blue dye after the exposure to NMDA. Ten micromolar NMDA clearly increased the number of cells stained by trypan blue dye (Fig. 3B). This NMDA-induced increase in the staining of cells with the dye was reduced when the neurons were incubated with muscimol (Fig. 3C) and MK-801 (data not shown). On the other hand, the inhibitory effect of muscimol on the NMDA-induced increase in trypan blue staining of cells was abolished by the concomitant incubation with bicuculline (Fig. 3D).

In ischemic brain injury, neuronal death has been proposed to be caused by glutamate excessively released into the extracellular space (17). Under such conditions, GABAergic agonists have been reported to be ineffective for protecting against neuronal death in hippocampal CA1 cells (18). In contrast, Lyden and Hedges (19) have

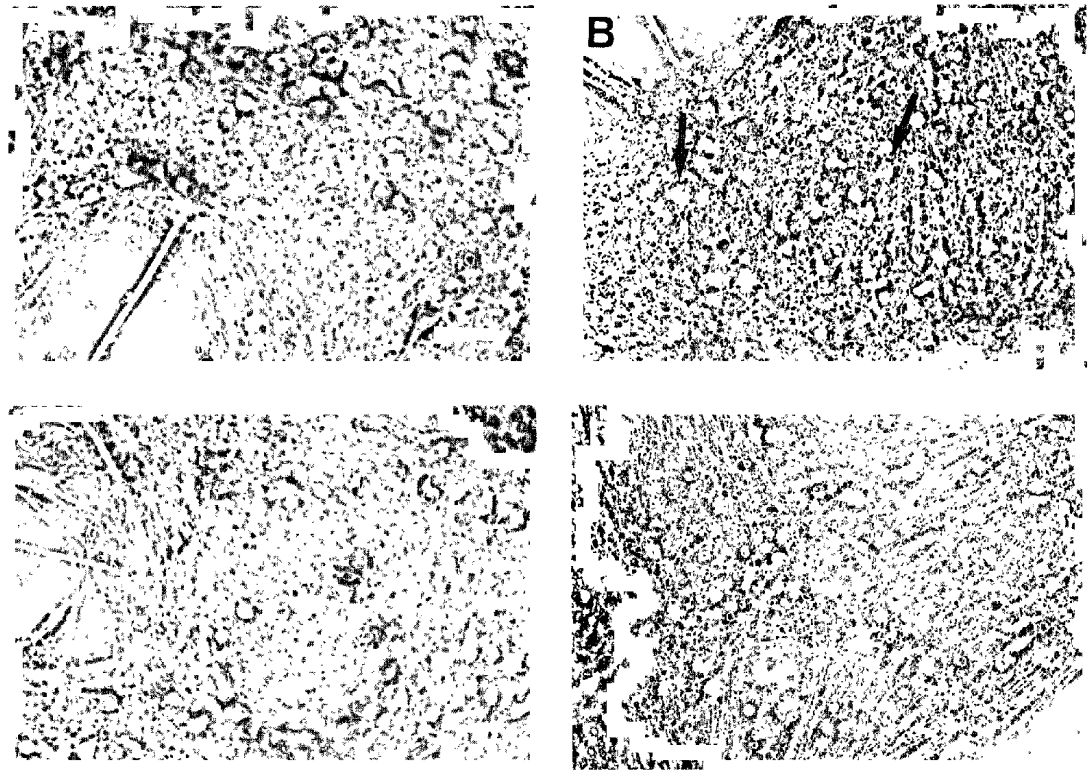

Fig. 3. Effect of muscimol and bicuculline on NMDA-induced trypan blue staining of cerebral cortical neurons. Arrows in the figures indicate the typical cells stained by trypan blue dye. The magnification of all figures is the same. Bar: 50 μm. (A) Control, (B) 10 μM NMDA, (C) 10 μM NMDA plus 100 μM muscimol, (D) 10 μM NMDA plus 100 μM muscimol and 100 μM bicuculline. Each photograph was a typical one observed in 4 separate experiments.

reported that muscimol as well as MK-801 protected against cell death during cerebral ischemia in rats and rabbits. The present study has also demonstrated that muscimol significantly suppresses the NMDA-induced neuronal injury using a primary culture of mouse cerebral cortical neurons. Such differences in the effectiveness of GABAergic agonists on neuronal injury may be due to differences in the experimental models used in each study and/or differences in neuronal cell types, although the reason for these differences is not clear at present.

As demonstrated above, GABAA-receptor activation is involved in the suppression of the NMDA-produced injury of cerebral cortical neurons in primary culture. Several mechanisms for the protective action of muscimol against the NMDA-induced neurotoxicity can be considered. First, the activation of GABAA receptors produces the hyperpolarization of neuronal membrane, which may counteract the hyperexcitation of neurons induced by NMDA-receptor activation. Namely, the hyperpolarization of neuronal membrane by muscimol may depress the NMDA-induced influx of calcium ion that is supposed to be the primary cause of neuronal injury induced by glutamate and/or NMDA. Secondly, unknown intracellular biochemical processes induced by NMDA-receptor activation may also be affected by GABAA-receptor stimulation. The exact mechanism for the protective action produced by GABAA-receptor activation on the NMDA-induced neuronal toxicity, however, remains to be elucidated.

In conclusion, the present study has confirmed that muscimol, a GABAA-receptor agonist, suppresses neuronal injury induced by NMDA, and this inhibitory action of muscimol is abolished by bicuculline, an antagonist selective for GABAA receptors. These data indicate that muscimol displays a protective effect against NMDA-induced neuronal injury through GABAA-receptor activation. Although the exact mechanisms for the protective action of muscimol are not clear at present, the possible usefulness of GABAA-receptor agonists to protect against glutamate- and/or NMDA-induced neuronal injury should be emphasized.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research from The Ministry of Education, Science and Culture of Japan.

REFERENCES

1 Rothman SM and Olney JW: Excitotoxicity and the NMDA receptor. Trends Neurosci **10**, 299–302 (1987)
2 Choi DW: Glutamate neurotoxicity and diseases of the nervous system. Neuron **1**, 623–634 (1988)
3 Meldrum B and Garthwaite J: Excitatory amino acid neurotoxicity and neurodegenerative disease. Trends Pharmacol Sci **11**, 379–387 (1990)
4 McGeer EG: Excitatory amino acid neurotransmission and its disorders. Curr Opin Neurol Neurosurg **4**, 548–554 (1991)
5 Aanonsen LM and Wilcox GL: Muscimol, γ-aminobutyric acidA receptors and excitatory amino acids in the mouse spinal cord. J Pharmacol Exp Ther **248**, 1034–1038 (1989)
6 Soltis RP and DiMicco JA: Interaction of hypothalamic GABAA and excitatory amino acid receptors controlling heart rate in rats. Am J Physiol **261**, R427–R433 (1991)
7 Rage F, Benyassi A, Aranciba S and Tapia-Arancibia L: γ-Aminobutyric acid-glutamate interaction in the control of somatostatin release from hypothalamic neurons in primary culture: In vivo corroboration. Endocrinology **130**, 1056–1062 (1992)
8 Ohkuma S, Tomono S, Tanaka Y, Kuriyama K and Mukainaka T: Development of taurine biosynthesizing system in cerebral cortical neurons in primary culture. Int J Dev Neurosci **4**, 383–395 (1986)
9 Choi DW, Koh J-Y and Peters S: Pharmacology of glutamate neurotoxicity in cortical cell culture: Attenuation by NMDA antagonists. J Neurosci **8**, 185–196 (1988)
10 Cabaud PG and Wrobrewski F: Colorimetric measurement of lactic dehydrogenase activity of body fluid. Am J Clin Pathol **30**, 234–236 (1958)
11 Novelli A, Reilly JA, Lysko PG and Henneberry RC: Glutamate becomes neurotoxic via the *N*-methyl-D-aspartate receptor when intracellular energy levels are reduced. Brain Res **451**, 205–212 (1988)
12 Lysko PG, Cox JA, Vigano MA and Henneberry RC: Excitatory amino acid neurotoxicity at the *N*-methyl-D-aspartate receptor in cultured neurons: pharmacological characterization. Brain Res **499**, 258–266 (1989)
13 Schramm M, Eimerl S and Costa E: Serum and depolarization agents cause acute neurotoxicity in cultured cerebellar granule cells: Role of the glutamate receptor responsive to *N*-methyl-D-aspartate. Proc Natl Acad Sci USA **87**, 1193–1197 (1990)
14 Frandsen A, Krogsgaars-Larsen P and Schousboe A: Novel glutamate receptor antagonists selectively protect against kainic acid neurotoxicity in cultured cerebral cortical neurons. J Neurochem **55**, 1821–1823 (1990)
15 Takadera T, Shimada Y and Mohri T: Extracellular pH modulates *N*-methyl-D-aspartate receptor-mediated neurotoxicity and calcium accumulation in rat cortical cultures. Brain Res **572**, 126–131 (1992)
16 Erdo SL, Michler A and Wolff JR: GABA accelerates excitotoxic cell death in cortical cultures: protection by blockers of GABA-gated chloride channels. Brain Res **542**, 254–258 (1991)
17 Auer RN and Siesjo BK: Biological differences between ischemia, hypoglycemia, and epilepsy. Ann Neurol **24**, 699–704 (1988)
18 Sternau LL, Lust WD, Ricca AJ and Ratcheson R: Role for gamma-aminobutyric acid in selective vulnerability in gerbils. Stroke **20**, 281–287 (1989)
19 Lyden PD and Hedges B: Protective effect of synaptic inhibition during cerebral ischemia in rats and rabbits. Stroke **23**, 1463–1470 (1992)