Arch. Pharm. Res. Vol.18, No.6, pp. 391-395, 1995

Kainate-induced Elevations of Intracellular Ca^{2+} and Extracellular Glutamate are Partially Decreased by NMDA Receptor Antagonists in Cultured Cerebellar Granule Neurons

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(Received August 16, 1995)

Several lines of evidence indicate that physiological activity of N-methyl-D-aspartate (NMDA) receptor was blocked by physiological concentration of Mg^{2+} (1.2 mM). However, the activity of NMDA receptor may not be blocked totally with this concentration of Mg^{2+} under elevated membrane potential by kainate. Here, we described the effect of Mg^{2+} on NMDA receptor and how much of NMDA receptor functions could be activated by kainate. Effects of NMDA receptor antagonist on kainate-induced elevation of intracellualr Ca^{2+} levels ($[Ca^{2+}]_i$) and extracellular glutamate level were examined in cultured rat cerebellar granule neurons. Kainate-induced elevation of $[Ca^{2+}]_i$ was not affected by physiological concentration of Mg^{2+} though NMDA-induced elevation was blocked by the same concentration of Mg^{2+}. Kainate-induced elevation of $[Ca^{2+}]_i$ was decreased by 32% in the presence of NMDA antagonists, MK-801 and CPP (3-[2-carboxypiperazine-4-yl]propyl-1-phosphonic acid), in Mg^{2+} free buffer. Kainate receptor-activated glutamate release was also decreased (30%) by MK-801 or CPP. These results show that certain extent of elevations of intracellular Ca^{2+} and extracellular glutamate by kainate is due to coactivation of NMDA receptors.

Key words : N-methyl-D-aspartate, Kainate, Ca^{2+} influx, Glutamate release, Cerebellar granule neuron

INTRODUCTION

Excitatory amino acid (EAA) receptors are now generally accepted as the main transmitters mediating synaptic excitation in the mammalian CNS (Watkins and Evans, 1981; Monaghan *et al.*, 1989; Collingridge and Lester, 1989). They are involved in many physiological phenomena ranging from the processing of sensory information to cognitive processes such as learning and memory. The presynaptic release of the neurotransmitter, glutamate, activates several subtypes of EAA receptors that can be distinguished by the selective agonists N-methyl-D-aspartate (NMDA), kainate (KA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA).

It is well known that the NMDA receptors are blocked in a voltage-dependent manner, thus producing a region of negative-slope conductance in media which contained Mg^{2+} at physiological concentration (Mayer and Westbrook, 1984). Thus NMDA receptor-mediated responses are likely to be highly voltage dependent at membrane potentials close to the resting state. The blocking effect of Mg^{2+} is seen as a high frequency flicker, as Mg^{2+} rapidly enter, block, and then dissociate from NMDA receptor channels when observed at the single-channel level (Nowak *et al.*, 1984; Ascher and Nowak, 1988). The blocking action only occurs when the membrane potential is held at a negative voltage. Membrane voltage above -40 mV releases the Mg^{2+} from NMDA receptor, then allowing other ions to flow through the receptor channel. When NMDA receptor is opened, it allows not only the monovalent cations such as Na^+, but also divalent cation such as Ca^{2+} (10 times higher than monovalent) to influx through channel (Mayer and Westbrook, 1987). The physiological significance of glutamate receptor channels is determined by their Ca^{2+} permeability. NMDA receptor channels are permeable to Ca^{2+} while kainate receptor channels are permeable to Na^+ though Ca^{2+} are only 0.15 times as permeate as Na^+ (Mayer and Westbrook, 1987). However, the influx of Na^+ elevates membrane potential, opens voltage sensitive Ca^{2+} channel and then removes Mg^{2+} from NMDA re-

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ceptors which results in rise of intracellular Ca^{2+}.

The Ca^{2+} influx elicited in cerebellar granule cells by kainate includes at least two components. One is directly through kainate receptors and the other is related to the ability of kainate to release endogenous glutamate, then released glutamates activate NMDA receptors. In our experiment, to define the effect of the latter component kainate-induced intracellular Ca^{2+} elevation and extracellular glutamate release were measured with application of NMDA receptor antagonists.

MATRIALS AND METHODS

Materials

CPP (3-(2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid) and MK-801 were purchased from RBI (Natick, MA), and Fura-2 AM from Molecular Probes (Eugene, OR). N-methyl-D-aspartate (NMDA), kainate and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cerebellar granule cell culture

Cerebellar granule cells were cultured as described (McCaslin and Morgan, 1987; McCaslin and Ho, 1994) with slight modifications. Briefly, 8-day-old rat pups (Sprague-Dawley, Harlan, Indianapolis, IN) were decapitated, and the heads were partially sterilized by dipping them in 95% ethanol. The cerebellum was dissected from the tissue and placed in culture medium which lacks serum and bicarbonate. Dissociated cells were collected at a density of about 2×10^6 cells/ml. Growth medium (5 ml/60 mm dish, 300 mOsm) was 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), 6% bovine calf serum (Hyclone, Logan, UT) and 6% fetal bovine serum (JRH Bioscience, Lenexa, KS). After 2 days incubation (37°C; 10% CO_2), growth medium was aspirated from the cultures and new growth medium (5 ml/dish, 300 mOsm) containing 25 mM KCl was added with 5 μM cytosine arabinoside to prevent proliferation of non-neuronal cells (McCaslin and Ho, 1994). The cells were then incubated for 14 days at 37°C under 10% CO_2-90% filtered room air.

Measurement of intracellular Ca^{2+} levels

Calcium influx was determined by ratio fluorometry as described by others (Grynkiewicz, 1985; Cai and McCaslin, 1992). The cells were grown on glass cover slides loaded with 10 μM fura-2 AM (dissolved in DMSO) for 1 hr at 37°C in physiological saline HEPES (PSH) buffer solution containing the following mM concentrations: 135 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 40 bicarbonate, 10 glucose and 5 HEPES (pH 7.4, 300 mOsm) and washed with PSH buffer. Cell culture slides were cut with diamond pencil and mounted into stirred spectrophotometer cuvettes containing 2.5 ml PSH buffer (without bicarbonate). Fluorescence was measured with a FLUOROLOG-2 spectrophotometer (SPEX Ind. Inc., Edison, NJ) by exciting cells at 340 and 380 nm and measuring light emission at 505 nm for 10 min. Baseline $[Ca^{2+}]_i$ was measured for 60 sec before the addition of various experimental compounds. Calcium concentrations were calculated according to the method of Grynkiewicz *et al.*, (1985) using a K_D of 224 nM by using TM 3000 software (SPEX). Rmin and Rmax were determined by addition of EGTA (final concentration, 20 mM) and ionomycin (final concentration, 10 μM), respectively.

Glutamate measurement

After cells were grown for 10-14 days, growth medium was washed from the cells, and they were placed in PSH buffer. After a 30 min equilibriation period in PSH buffer (37°C; 10% CO_2), cells were washed and reincubated in the presence of various compounds at 37°C for 1 h in CO_2 incubator. The amount of glutamate secreted into the buffer was separated and quantified by HPLC as described below (Ellison *et al.*, 1987). A small amount of buffer (500 μl) was collected from the culture dish, and the glutamate concentration was quantified by HPLC (BAS 200, Bioanalytical Systems, IN) with an EC detector after precolumn derivatization of sample aliquots (50 μl) with 20 μl of o-phthaldialdehyde/2-mercaptoethanol reagent for 2 min. The C18, 5 mm, 4.6×150 mm, reverse-phase column (Rainin, CA) was eluted with mobile phase (pH 5.2) containing 0.1 M sodium phosphate buffer with 37% (v/v) HPLC-grade methanol at a rate of 1.0 ml/min.

RESULTS

The representative curves showing changes in $[Ca^{2+}]_i$ in response to application of EAAs with NMDA antagonist are shown in Fig. 1. NMDA (100 μM) induced large increase over baseline $[Ca^{2+}]_i$ which were markedly suppressed by an NMDA antagonist, MK-801 (10 μM) in Mg^{2+} free buffer. To exclude the possibility of blocking effect of MK-801 (non-competitive type) on kainate receptor, another NMDA receptor antagonist, CPP (competitive type) was tested on kainate (100 μM)-induced elevation of $[Ca^{2+}]_i$. (Fig. 2). Either MK-801 or CPP (each 10 μM) surely suppressed NMDA receptor response, and these NMDA receptor antagonists also decreased kainate-induced elevation of $[Ca^{2+}]_i$ (32.4 and 33.9%, respectively).

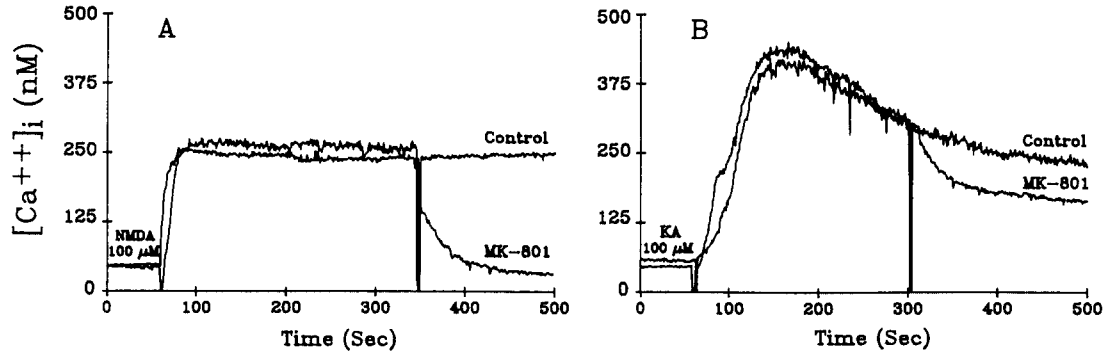

Fig. 1. NMDA and kainate induced elevations of $[Ca^{2+}]_i$ in cerebellar granule cells in Mg^{2+} free buffer. Fura-2 loaded cells were stimulated with either NMDA or kainate (100 μM each at A, B respectively) at the first break in the curve and buffer (control) or MK-801 (10 μM) was added at the second break in the curve.

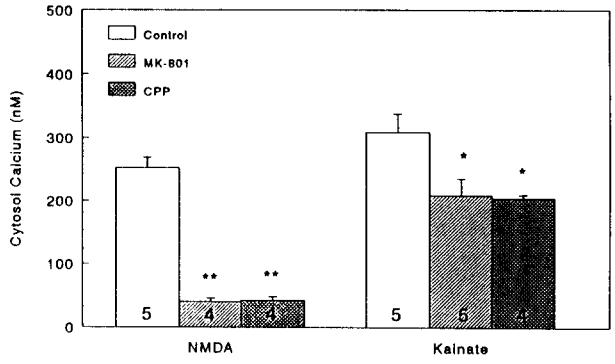

Fig. 2. NMDA receptor antagonists decreased kainate-as well as NMDA-induced elevations of $[Ca^{2+}]_i$ in Mg^{2+} free buffer. Experiments were performed as described in Fig. 1. Values present mean±SEM of nM. The numbers for each group are shown in the columns. Data were analyzed by an ANOVA followed by the Duncan's test. *p<0.05, **p<0.01 from each control group

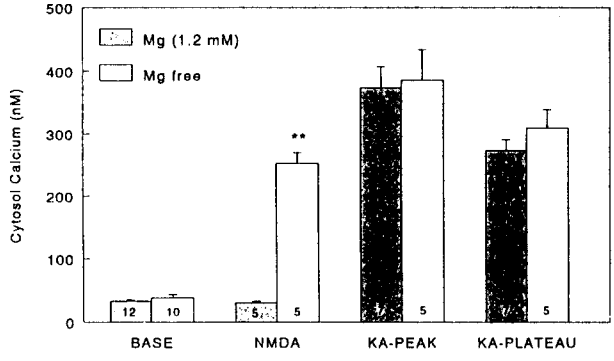

Fig. 3. Comparison of excitatory amino acid induced elevations of $[Ca^{2+}]_i$ with Mg^{2+} and without Mg^{2+} in the experimental buffer. Fura-2 loaded cells were stimulated with either NMDA or KA (each 100 μM). The patterns of $[Ca^{2+}]_i$ curve are identical to the curves of control in Fig. 1. Peak values of KA-induced elevations of $[Ca^{2+}]_i$ were measured at the maximal elevations (around 165 sec) of Ca^{2+}. Values present mean±SEM of nM. The numbers for each group are shown in the columns. Data were analyzed by an ANOVA followed by the Duncan's test. **p<0.01 from Mg^{2+} contained group

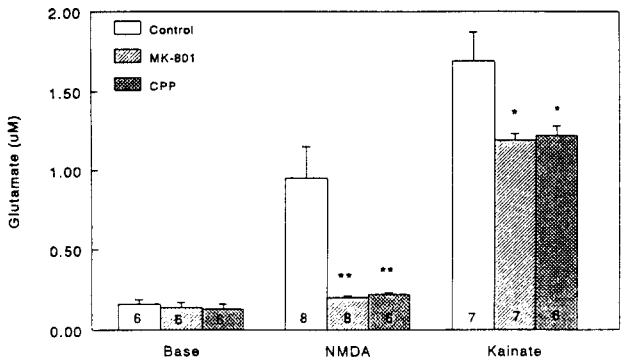

Fig. 4. Effects of NMDA antagonists on NMDA-and kainate-induced glutamate release for 1 h incubation. Cerebellar granule cells were grown for 14 days in vitro and then washed with PSH buffer as described in the text. After a 30 min equilibriation period in PSH buffer, cells were washed again and incubated with NMDA (100 μM) or kainate (50 μM) in the NMDA antagonist (MK-801 or CPP, each 10 μM) containing buffer for 1 h. Values present the mean±SEM of μM in 3 ml of extracellular buffer. The numbers for each group are shown in the columns. Data were analyzed by an ANOVA followed by the Duncan's test. *p<0.05, **p<0.01 from each control (cells treated with agonist alone) group

To test hypothesis that kainate receptor activation induces membrane depolarization which then results in removal of Mg^{2+} from NMDA receptors allowing Ca^{2+} influx, NMDA and kainate induced $[Ca^{2+}]_i$ were compared in physiological concentration of Mg^{2+} (1.2 mM) and Mg^{2+} free buffer (Fig. 3). The physiological concentration of Mg^{2+} strongly blocked NMDA-induced elevation of $[Ca^{2+}]_i$, however, same concentration of Mg^{2+} did not affect kainate-induced elevation of $[Ca^{2+}]_i$.

NMDA and kainate gradually elevated extracellular glutamate in accordance with the exposure time up to 1 h but not significantly elevated in short time (up to 10 min) in our previous report (Oh *et al.*, 1995). The incubation of cells for 1 h with NMDA or kainate resulted in elevation of extracellular glu-

tamate in Mg^{2+} free buffer. NMDA antagonists, both MK-801 and CPP, inhibited NMDA-induced glutamate release to almost basal level (0.16 ± 0.03 μM) and also decreased (30%) kainate-induced glutamate release significantly (Fig. 4).

DISCUSSION

Several studies have linked that the activation of kainate receptors affects NMDA receptor function via elevation of membrane potential resulted in removal of Mg^{2+} following Ca^{2+} influx. Therefore, we believed that part of Ca^{2+} influx by kainate receptor activation is secondary to NMDA receptor opening. However, in our results (Fig. 1,2), kainate-induced Ca^{2+} elevation was significantly decreased by NMDA receptor antagonists, MK-801 and CPP, in Mg^{2+} free buffer. These results suggest that voltage dependent Mg^{2+} removal would not be a major factor involving in indirect activation of NMDA receptors by kainate.

The voltage dependence of NMDA responses under physiological conditions makes it suitable for the integrative functions of synaptic transmission, both in the temporal and spatial regions. Such integrative functions could occur in release of mixed agonist, such as glutamate, from presynaptic neuron onto both NMDA receptors and kainate receptors on the postsynaptic neuron. This could result in a compound KA/NMDA receptor-activated Ca^{2+} influx where the relative functional contributions of the two receptor types would depend not only on the proportions of the two receptor types present postsynaptically, but also on the current membrane potential of the postsynaptic neuron (Forsythe and Westbrook, 1988). The activation of kainate receptor can induce glutamate release; Ca^{2+} dependent release and/or reversal of glutamate uptake. The released glutamate can activate NMDA receptors as well as kainate receptors. The threshold for activation of NMDA receptors by glutamate is approximately 50 nM in single channel recording (Mayer *et al.*, 1989), thus activation of NMDA receptors is possible with released low concentration of glutamate. Indeed, if the postsynaptic membrane is relatively hyperpolarized, then the glutamate would open predominantly kainate receptor-associated channels, due to the blockade of the NMDA receptor-associated channels by Mg^{2+} ions. The kainate receptor-mediated depolarization could ultimately be sufficient to relieve the Mg^{2+} blockade of the NMDA ionopore, thus allowing a further depolarization of the postsynaptic membrane due to the action of the glutamate at NMDA receptors. Also in our result (Fig. 3), NMDA receptor but not kainate receptor function was suppressed under physiological concentration of Mg^{2+} since NMDA did not elevate $[Ca^{2+}]_i$ in Mg^{2+} buffer while kainate-induced $[Ca^{2+}]_i$ elevation was not blocked in Mg^{2+} free buffer. This would be an indirect support for the independent activation of the NMDA receptors by the released glutamate. The lack of responsiveness to NMDA in the presence of Mg^{2+} was not due to a shortage of glycine since glycine addition in Mg^{2+} buffer produced little response (data not shown). The actual extracellular Mg^{2+} concentration in the normally zero Mg^{2+} was found to be about 0.2 μM (Jahr and Stevens, 1990). However, this concentration of Mg^{2+} did not affect NMDA- or kainate-induced $[Ca^{2+}]_i$ levels in preliminary experiment.

There is a possibility that intracellular Mg^{2+} could be removed by membrane voltage elevation with kainate receptor activation. In the resting membrane voltage, the intracellular Mg^{2+} dissociation constant (8 mM) is close to the extracellular Mg^{2+} dissociation constant calculated from single channel experiments (Ascher and Nowak, 1988). This suggests that Mg^{2+} from either side of the membrane may be blocking at the same site. We can not exclude another possibility that chimeric NMDA/KA receptor could be activated by either NMDA or kainate and inhibited by either antagonist, so the NMDA and kainate binding sites could not be physically separated and exhibited functional interaction (Henley *et al.*, 1992). Therefore, it could be presumed when MK-801 blocks to the NMDA-gated channel, it also blocks subsequent responses to kainate. Indeed, NMDA antagonist AP-5, which reduced (80%) the current evoked by NMDA, also antagonized the response to the same concentration of kainate by 10 to 20%. In addition, non-NMDA antagonist (DNQX, 100 μM) antagonized the response to 50 μM kainate by more than 95%, also antagonized the response to 33 μM NMDA by approximately 90% in the Xenopus oocyte expression system (Brackley and Usherwood, 1993). However, MK-801 had no effect on the response to kainate application alone and MK-801 partially blocked kainate response only in the presence of NMDA in the oocyte system and it is not clear whether chimeric NMDA/KA receptor occur in the rat CNS *in vivo*.

Analogous to blocking effect (32%) of NMDA receptor antagonists to kainate-induced $[Ca^{2+}]_i$, both MK-801 and CPP decreased (30%) kainate-induced glutamate release. The incubation of cells for 1 h with NMDA or kainate resulted in elevation of extracellular glutamate, and kainate shows higher potency than NMDA in terms of extracellular glutamate accumulation.

In conclusion, the present observations provide evidence that the activation of kainate receptors can activate NMDA receptors by liberating glutamate which results in additive elevations of intracellular Ca^{2+} and extracellular glutamate in a Mg^{2+}-independent manner. These results suggest that addition of physiological

concentration of Mg^{2+} in experimental buffer is not enough to block NMDA receptor functions under kainate receptor activation.

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