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Agmatine protects against cell damage induced by NMDA and glutamate in cultured hippocampal neurons

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Abstract

Agmatine is a polyamine and has been considered as a novel neurotransmitter or neuromodulator in the central nervous system. In the present study, the neuroprotective effect of agmatine against cell damage caused by *N*-methyl-D-aspartate (NMDA) and glutamate was investigated in cultured rat hippocampal neurons. Lactate dehydrogenase (LDH) activity assay, β -tubulin III immunocytochemical staining and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay were conducted to detect cell damage. Exposure of 12-day neuronal cultures of rat hippocampus to NMDA or glutamate for 1 h caused a concentration-dependent neurotoxicity, as indicated by the significant increase in released LDH activities. Addition of 100 μ M agmatine into media ablated the neurotoxicity induced by NMDA or glutamate, an effect also produced by the specific NMDA receptor antagonist dizocilpine hydrogen maleate (MK801). Arcaine, an analog of agmatine with similar structure as agmatine, fully prevented the NMDA- or glutamate-induced neuronal damage. Spermine and putrescine, the endogenous polyamine and metabolic products of agmatine without the guanidine moiety of agmatine, failed to show this effect, indicating a structural relevance for this neuroprotection. Immunocytochemical staining and TUNEL assay confirmed the findings in the LDH measurement. That is, agmatine and MK801 markedly attenuated NMDA-induced neuronal death and significantly reduced TUNEL-positive cell numbers induced by exposure of cultured hippocampal neurons to NMDA. Taken together, these results demonstrate that agmatine can protect cultured hippocampal neurons from NMDA- or glutamate-induced excitotoxicity, through a possible blockade of the NMDA receptor channels or a potential anti-apoptotic property.

Keywords

Agmatine; Hippocampus; Glutamate; NMDA; Apoptosis; Lactate dehydrogenase

1. Introduction

Agmatine is an endogenous polyamine derived from enzymatic decarboxylation of L-arginine (Tabor and Tabor, 1984). In the past decade, accumulating evidence indicated agmatine's several levels of pharmacological and physiological importance. Agmatine is present in the brain and other tissues of mammals (Lortie et al., 1996; Li et al., 1994). In neuronal tissues, agmatine is present in axon terminals associated with synaptic vesicles

(Reis et al., 1998) and can be taken up into synaptosomes via a Na⁺-independent system (Sastre et al., 1997). Agmatine has been reported to act as a ligand of the imidazoline receptor (Li et al., 1994). It inhibits all isoforms of nitric oxide synthase (NOS; Galea et al., 1996) and blocks nicotinic receptor (Loring, 1990), voltage-gated Ca²⁺ channels and N-methyl-D-aspartate (NMDA) receptor channels (Yang and Reis, 1999). All these functional characteristics suggest that agmatine may play a role as a neurotransmitter or neuromodulator in the brain.

Agmatine has previously been shown to exert its neuroprotective action by reducing the size of ischemic infarctions or the loss of cerebellar neurons after focal or global ischemia in vivo (Gilad et al., 1996b; Kim et al., 2004). Its neuroprotective effects also include attenuating the extent of neuronal loss following excitotoxic spinal cord injury (Fairbanks et al., 2000) and preventing neurotoxicity produced by glutamate in cultured cerebellar granule cells (Olmos et al., 1999). Our previous study demonstrated that agmatine can protect the cultured rat cortex neurons and PC12 cells from cell death after exposing to NMDA and glutamate (Zhu et al., 2003). However, more compelling evidence is needed for elucidating its neuroprotective role on different brain neurons.

The hippocampus plays a vital role in learning and memory (Eichenbaum and Otto, 1992). It also influences autonomic and vegetative functions (Jacobson and Sapolsky, 1991). Many studies have demonstrated that the hippocampus is one of the most vulnerable brain regions as regards to various neurological insults such as hypoxia-ischemia, seizure and prolonged stress. These insults produce excessive synaptic-glutamate accumulation, which triggers a series of intracellular biochemical changes and finally leads to degenerative events in the neuron, including cytoskeletal degradation, the collapse of mitochondrial potentials and protein misfolding. These glutamate-induced cell damages have been shown to contribute to pathological consequences in different kinds of disorders such as major depression, seizure and Alzheimer's diseases (Portera-Cailliau et al., 1995). Also, glutamate is a major excitatory neurotransmitter in the brain and a potent excitotoxin. Exposure of neuronal cultures to micromolar concentrations of glutamate has been reported to lead to neurotoxicity (Choi, 1988) and induce neuronal apoptosis (Kure et al., 1991). Given the abundance of agmatine in the hippocampus (Feng et al., 1997; Reis et al., 1998), studying the effects of agmatine on hippocampal neurons will provide important information regarding the potential role of this proposed neurotransmitter in this area.

In the present investigation using primary cultured hippocampal neurons of rats, we examined the neuroprotective effect of agmatine against neuronal damage induced by NMDA and glutamate. We observed that agmatine prevents hippocampal neurons from excitatory cell damage, an effect dependent on its capacity to block NMDA receptor channels, as well as its potential anti-apoptotic effects. Our data suggest that as an important polyamine in the hippocampus, agmatine may play a role in the physiology of hippocampal neurons. Further elucidation of the effects of agmatine on hippocampal neurons may lead to novel therapeutic strategies for the diseases related to the hippocampus.

2. Results

2.1. Neuroprotective effects of agmatine on NMDA- or glutamate-induced cell damage in cultured hippocampal neurons

As previously reported (Brewer et al., 1993), neurobasal medium supplemented with B-27 represents an optimized medium for sustaining the long-term survival of hippocampal neurons with little glial growth. The double immunocytochemical staining performed in 12-day hippocampal cultures with β T-III (to display putative neurons) and GFAP (for astrocytes) in the present study showed a nearly pure neuronal population with very little

GFAP staining (less than 0.5%, data not shown), which is consistent with the report by Brewer et al. (1993).

We examined the neuroprotective effects of agmatine on NMDA-induced neuronal damage. Twelve-day cultured hippocampal neurons were exposed to 10, 100, and 200 μ M NMDA or 1, 10, and 100 μ M glutamate in serum-free buffer for 1 h followed by continued incubation in normal growth medium for an additional 23 h. LDH assays showed that NMDA or glutamate caused a concentration-dependent neurotoxicity, as indexed by a significant increase in LDH activity after exposure to 200 μ M NMDA (by 35%; $F_{7,52} = 4.37$, $P < 0.05$) or 10 and 100 μ M glutamate (by 78 and 98%, respectively; $F_{7,35} = 6.82$, $P < 0.01$), respectively (Fig. 1). Concurrent exposure of these neurons to 200 μ M NMDA or 100 μ M glutamate with different concentrations of agmatine showed that 100 μ M agmatine fully prevented increased LDH activities produced by NMDA or glutamate (Fig. 1). Agmatine (100 μ M) alone did not cause a significant change in LDH activity.

The neuroprotective effects of agmatine were further studied using immunocytochemical stainings with β T-III (Fig. 3) and TUNEL assay (Fig. 4) in cultured hippocampal neurons treated in the same way as those described in the experiment for LDH measurement. Control cultures exhibited β T-III-positive cells with homogeneous and compact morphology (Fig. 3A) or sparse TUNEL-labeled cells (Fig. 4A). Exposure of cultures to 200 μ M NMDA resulted in a significant loss of β T-III-positive neurons (Fig. 3B), with the disappearance of neuritis, disrupted membranes, distorted somata, and condensed nuclei. The degree of cell death estimated by microscopic examination of immunostained cells on coverslips was consistent with the results obtained with LDH assay (data not shown). Also this exposure resulted in a large number of TUNEL-positive cells (Fig. 4B). The neuronal loss and increased TUNEL-positive cells were prevented by the addition of 100 μ M agmatine (Figs. 3C and 4C) to the cultures. 100 μ M agmatine by itself did not markedly affect the morphology of β T-III-positive neurons and the numbers of TUNEL-positive cells, compared to the control cultures (data not shown).

2.2. Comparison of effects of agmatine, the analog of agmatine and MK801 on NMDA- or glutamate-induced neuronal damage

In these experiments, cultured hippocampal neurons were exposed to 200 μ M NMDA or 100 μ M glutamate in combination with either 100 μ M agmatine, 10 μ M MK801, 100 μ M arcaine, spermine or putrescine by the same paradigm as described above. As illustrated in Fig. 2, 100 μ M agmatine prevented the increase in LDH activity produced by 200 μ M NMDA or 100 μ M glutamate. In like manner, 10 μ M MK801 fully abolished the NMDA- or glutamate-induced LDH increase ($F_{6,41} = 7.09$, $P < 0.01$), as did 100 μ M arcaine, an agmatine analog with similar structures. However, spermine, an endogenous polyamine, and putrescine, a metabolic product of agmatine, both without the guanidino group, failed to prevent the increased LDH activity ($F_{6,41} = 9.14$, $P < 0.01$), after co-incubation with NMDA or glutamate.

Similar effects between agmatine and MK801 were also evident in the studies of immunocytochemical staining and TUNEL assay. Co-incubation of NMDA with MK801 or agmatine prevented neuronal death (Figs. 3C and D) and significantly reduced TUNEL-labeled cells (Figs. 4C and D). This protective effect was substantial: the ratios of TUNEL-positive to TUNEL-negative cell numbers counted in the cultures that were co-incubated with NMDA and agmatine or MK801 cultures were 1.67 ± 0.65 and 0.65 ± 0.14 , respectively, which were significantly lower than those of NMDA-treated cultures (7.10 ± 0.70) ($F_{2,16} = 32.26$, $P < 0.01$) (Fig. 4E).

3. Discussion

Since the last decade, a large body of in vitro and in vivo experimental evidence has demonstrated the neuroprotective effects of agmatine. This neuroprotective effect has been hypothesized to be related to its ability to block heteromeric NMDA receptor channels (Yang and Reis, 1999; Olmos et al., 1999). In the present study, 100 μ M agmatine completely abolished the increased LDH levels and prevented neuronal death caused by NMDA or glutamate. This effect was shared by MK801, a non-competitive NMDA receptor antagonist, and arcaine, a synthetic analog of agmatine with two terminal guanidino groups required to block the NMDA receptor channels, but not by spermine and putrescine, two endogenous polyamines with no guanidino group. Our results extend previous observations and confirm that one mechanism of agmatine's neuroprotective effects is through its ability to block NMDA receptor channels.

While many studies support the neuroprotective role of agmatine, exposure of rat cerebellar granule neurons to 200–800 μ M agmatine was found to result in cell death (Abe et al., 2003). The discrepancy between that study and ours may be in part due to different experimental conditions. It has been shown that agmatine exerted neurotoxicity in medium with high K^+ (up to 27.5 mM) but not low K^+ (under 10 mM) because high concentrations of K^+ in the medium may trigger exocytosis and lead to release of glutamate from neurons. The K^+ concentration in our exposure medium is less than 6 mM. Also, the highest concentration of agmatine used in the current study is 100 μ M, a concentration that has been demonstrated to block NMDA receptor channels in rat hippocampal neurons (Yang and Reis, 1999). Nevertheless, our experiments and those of Abe et al. (2003) do suggest that whether agmatine exerts neuroprotection or neurotoxicity partly depends on the experimental conditions, including the concentrations of K^+ and agmatine applied.

The ability of NMDA to induce apoptosis in cultured hippocampal neurons has been well documented (Diebaili et al., 2002; Vincent et al., 2002; Kajta et al., 2004; Prehn, 1996; Lee et al., 2003). Therefore, the NMDA induced neuronal death observed in the present study might also be due to apoptosis. Our TUNEL assay shows that exposure of cultured hippocampal neurons to NMDA significantly increased TUNEL-positive cell numbers, which were prevented by co-incubation with agmatine or MK801, suggesting that agmatine may exert its neuroprotective effect through its anti-apoptotic property. However, more study is needed to categorically define the anti-apoptotic characteristic of agmatine.

In conclusion, it is interesting to note that LDH measurements, immunocytochemical staining and TUNEL assay correlated well to demonstrate that agmatine fully protects hippocampal neurons against neurotoxicity induced by exposure to NMDA or glutamate. As glutamate-induced hippocampal dysfunction is implicated in seizure, Alzheimer's disease and major depression, the neuroprotection of agmatine against NMDA/glutamate induced excitotoxic insults has broad implications for potential new therapeutic strategies for these diseases.

4. Experimental procedures

4.1. Chemicals and reagents

Agmatine sulfate salt, *N*-methyl-D-aspartic acid (NMDA), L-glutamate acid monosodium salt, dizocilpine hydrogen maleate (MK801), arcaine sulfate salt, spermine dehydrochloride and putrescine dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO).

4.2. Culture of hippocampal neurons

Hippocampi were dissected out from the brains of 18-day fetuses of Long Evans rats and placed in Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (Gibco BRL, Grand Island, NY) containing 1 mM sodium pyruvate and 10 mM HEPES. Then the hippocampal tissues were dissociated in HBSS solution containing 0.125% trypsin solution and 0.1 mg/ml deoxyribonuclease for 15 min at 37 °C. Subsequently, tissues were triturated by repeated passage through a constricted Pasteur pipette. The dispersed tissues were allowed to settle for 3 min. The supernatant was transferred to a fresh tube and centrifuged at 2000 rpm for 90 s. The pellet was resuspended in a neuron-defined culture medium, serum-free Neurobasal medium (Gibco BRL, Grand Island, NY), supplemented with B-27, 0.5 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin and 25 μM glutamate. Trypan blue-excluding cells were counted, and cells were then plated onto 6-well plates coated with poly-D-lysine (100 $\mu\text{g}/\text{ml}$; BD Biosciences, Bedford, MA) at $2.5\text{--}3 \times 10^5$ per well. Cell cultures were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Half of the medium was replaced with fresh medium without glutamate every 3–4 days. For immunocytochemical staining and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay, a 13-mm glass coverslip coated with poly-D-lysine (100 $\mu\text{g}/\text{ml}$) was placed in the middle of each well in 12-well plates which were not coated. 1×10^5 cells were plated onto each well. Cells adhered to and grew on the coverslip. The medium was changed as previously done for the 6-well plates.

4.3. Drugs exposures

The experiments to investigate the effect of agmatine on NMDA- or glutamate-induced cell damage were conducted using 12-day primary hippocampal cultures. The neuronal cultures were washed twice with Mg^{2+} -free, HEPES-buffered saline (HBS, 146 mM NaCl, 10 mM HEPES, 2 mM CaCl_2 , 5 mM KCl, 10 mM D-glucose, pH 7.4) and incubated in the same solution in the absence (control) or presence of different concentrations of NMDA or glutamate, alone or in combination with agmatine (1–100 μM), MK801 (10 μM), arcaine (100 μM), spermine (100 μM) or putrescine (100 μM), for 1 h. The choice of 100 μM concentration for these polyamines is based on previously published data that 100 μM agmatine blocked NMDA receptor channels, but same concentration of putrescine or spermine failed to do so (Yang and Reis, 1999). However, the real concentrations of these polyamines in the hippocampus are different: agmatine 4.7 nmol/g wet wt (Feng et al., 1997), putrescine 7.1 nmol/g wet wt and spermine 213 nmol/g wet wt (Seiler and Schmidt-Glenewinkel, 1975). Therefore, the concentrations used in our in vitro experiment serve only for pharmacological comparisons. After two washes with fresh HBS solution, cells were subsequently re-incubated in culture media lacking any drugs for an additional period of 23 h. At the end of exposure, cultured neurons were harvested and LDH activity in the medium and cells measured. Cultured neurons used for immunocytochemical staining or TUNEL assay were treated the same way. The coverslips, where neurons were growing, were washed twice with Dulbecco's phosphate-saline (PBS) and fixed in 4% paraformaldehyde. Immunocytochemical staining or TUNEL assay was performed immediately on the fixed cultures.

4.4. Lactate dehydrogenase (LDH) assay

The release of LDH, a widely used index of cellular injury (Koh and Choi, 1987), was measured using a Cytotoxicity Detection kit (LDH) (Roche Diagnostics Corporation, Indianapolis, IN) according to manufacturer's instructions. Briefly, culture medium was collected and immediately centrifuged to remove any cells and debris. The cells were collected into microfuge tubes by adding 1 ml PBS, and cell homology was prepared by ultra-sonication for 25 s twice and centrifuging at 14,000 rpm for 4 min to remove cellular

debris. LDH activities in the medium and cell homology were measured based on the protocol from the kit using a Microplate Reader (Bio-Rad, Hercules, CA). Results are expressed as percentage of LDH released into the medium compared to total LDH (medium + cells).

4.5. Immunocytochemical staining for β -tubulin III (β T-III)

Immunocytochemical staining was performed using a monoclonal antibody to β T-III (Chemicon, Temecula, CA). After fixing with 4% paraformaldehyde, coverslips bearing neuronal cultures were preincubated in 5% bovine serum in PBS supplemented with 0.2% Triton-X 100 for 1 h at room temperature, followed by incubation in primary antibody (1:500 dilution, in PBS containing 0.2% Triton-X 100) overnight at 4 °C. The following day, binding of β T-III antibody was detected with a biotinylated secondary antibody using ABC kit (Vector Laboratories, Burlingame, CA) according to the instructions of the manufacturer. 3,3-Diaminobenzidine tetra-hydrochloride (DAB) was used as the substrate. Staining for β T-III was then visualized and analyzed microscopically. To verify the concurrent presence of glial cells in the culture, double immunocytochemical staining for β T-III and glial fibrillary acidic protein (GFAP) was performed on some coverslips bearing neuronal cultures using a protocol of Multiple Antigen Labeling (Vector Laboratories, Burlingame, CA).

4.6. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay

DNA damage in dying cells was identified using ApopTag Peroxidase In situ Apoptosis Detection kits (Chemicon, Temecula, CA). Briefly, coverslips bearing neuronal cultures were incubated in an equilibration buffer (30 min) followed by addition of terminal deoxynucleotidyl transferase (TdT) and digoxigenin-dUTP reaction buffer (60 min) at 37 °C. Coverslips were then washed in stop/wash buffer for 10 min at 37 °C, followed by incubation in anti-digoxigenin antibody conjugated to peroxidase for 30 min at room temperature. The digoxigenin-dUTP-peroxidase complex was visualized by reacting with DAB to generate a brown reaction product. Negative controls were performed by substituting distilled water for TdT in the working solution. TUNEL assay was then analyzed microscopically. Using a 10 $\times$ 10 reticule and 20 $\times$ objective, the total numbers of TdT-positive cells were counted in five randomly chosen fields on each treated and control coverslip. The results are expressed as ratio of TdT-positive to TdT-negative cell numbers in 5–6 coverslips.

4.7. Statistics

Data are presented as means $\pm$ SEM values and analyzed by analysis of variance (ANOVA) using single-factor ANOVA (SuperANOVA program, Abacus concepts, Berkeley, CA). In the presence of significant *F* values, individual comparisons between means were made using the Student–Newman–Keuls test. For analyzing of the quantitative results of TUNEL labeling, original data which were expressed as ratio of TUNEL-positive to TUNEL-negative cells in coverslips were first converted to arcsin values using Excel program. Then these arcsin values were analyzed using one-way ANOVA as described above.

Abbreviations

ANOVA	analysis of variance
DAB	3,3-diaminobenzidine tetrahydrochloride
LDH	lactate dehydrogenase

βT-III	β-tubulin III
GFAP	glial fibrillary acidic protein
MK801	dizocilpine hydrogen maleate
NMDA	<i>N</i> -methyl-D-aspartate
TUNEL	terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling

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Fig. 1.

The release of LDH activity in primary culture of rat hippocampal neurons illustrating the neurotoxic effects of NMDA or glutamate and neuroprotective effects of agmatine. Data are shown as mean $\pm$ SEM of 6–10 measurements. * $P < 0.05$, ** $P < 0.01$, compared to controls (0). † $P < 0.05$, compared to the group treated with 200 μ M NMDA. ‡ $P < 0.01$, compared to the group treated with 100 μ M glutamate (Glu).

Fig. 2.

The comparative neuroprotective effects of agmatine, MK801, arcaine, spermine, and putrescine against cell damage caused by NMDA or glutamate. Data are shown as mean $\pm$ SEM of 6 measurements. * $P < 0.05$, ** $P < 0.01$, compared to the controls (0). † $P < 0.05$, compared to the group treated with 200 μ M NMDA; ‡ $P < 0.01$, compared to the group treated with 100 μ M glutamate (Glu).

Fig. 3.

Agmatine reduces cell death in NMDA-treated rat hippocampal cultures. Hippocampal neurons (12 days) were exposed to vehicle (A), 200 μ M NMDA either alone (B) or in combination with 100 μ M agmatine (C), or 10 μ M MK801 (D) for 1 h. The neuronal cultures were immunocytochemically stained with antibody to β -tubulin III. Calibration bar: 100 μ m for all figures.

Fig. 4. TUNEL assay in cultured rat hippocampal neurons (12 days) exposed to vehicle (A), 200 μ M NMDA for 1 h either alone (B) or in combination with 100 μ M agmatine (C), or 10 μ M MK801 (D), followed by continue incubation in fresh growing media for additional 23 h. TUNEL assay shows the presence of DNA fragmentation in the nuclei of cultured hippocampal neurons as indicated by dark spots. Calibration bar: 100 μ m for all figures. Above TUNEL assays were quantitatively analyzed (E). Data were expressed as ratio of TUNEL-positive to TUNEL-negative cells in 5–6 coverslips from 4 experiments. * $P < 0.01$ versus NMDA exposure.