ELSEVIER

European Journal of Pharmacology 275 (1995) 311–314

ejp

Short communication

N-Methyl-D-aspartate neurotoxicity in hippocampal slices: protection by aniracetam

Marina Pizzi *, Ornella Consolandi, Maurizio Memo, PierFranco Spano

Division of Pharmacology and Experimental Therapeutics, Department of Biomedical Sciences and Biotechnologies, School of Medicine, University of Brescia, Brescia 25123, Italy

Received 17 January 1995; accepted 20 January 1995

Abstract

Aniracetam, a drug known to elicit cognition enhancing properties in both animals and humans, was found to counteract the neurotoxicity induced by excitatory amino acids in primary cultures of cerebellar neurons. We report here that aniracetam prevents the neurotoxic effect induced by *N*-methyl-D-aspartate (NMDA) in rat hippocampal slices. Time-course experiments showed that the aniracetam-induced neuroprotection does not require preincubation of the slices with the drug. Maximal effective concentration of aniracetam was 10 μM. Since the NMDA-mediated cell death in hippocampal slices is considered a valuable experimental model of ischemia, these results suggest a possible novel therapeutic application for aniracetam.

Keywords: NMDA (*N*-methyl-D-aspartate); Aniracetam; Hippocampus

1. Introduction

Glutamate is the major excitatory neurotransmitter in the brain. It participates in modulating many neurological functions, including cognition, memory, movement and sensation (Gasic and Hollmann, 1992) and is crucial for the developmental plasticity of synaptic connections. However, in various pathological conditions, including stroke and various neurodegenerative disorders, excessive activation of glutamate receptors may mediate neuronal injury and death. Extensive studies on the neurotoxic effects of excitatory amino acids have revealed that stimulation of the ionotropic *N*-methyl-D-aspartate (NMDA) receptors is the primary event triggering the process of rapid neuronal damage (Choi, 1987).

It has been shown that glutamate added for brief periods of time in the absence of magnesium, to facilitate the NMDA receptor activation, induces the death of rat cerebellar granule cells grown in culture (Favaron et al., 1988). This, as well as other models of neurons in culture, have been extensively used to develop a variety of neuroprotective drugs including compounds modulating NMDA receptor function (Choi et al., 1988) or acting at other cellular sites (Favaron et al., 1988; Miyamoto et al., 1989; Pizzi et al., 1991).

We originally found that in the primary culture of rat cerebellar granule cells, glutamate-induced neurotoxicity can be concentration-dependently counteracted by aniracetam (Pizzi et al., 1993), a pyrrolidinone derivative endowed with cognition-enhancing activity both in animals and in man (Lee and Benfield, 1994).

Since cultured cerebellar granule cells present intrinsic limitations, we evaluated in this study aniracetam-mediated neuroprotection using slices from rat hippocampus. This preparation offers multiple advantages which make it more predictive for neuropathological diseases. Among others, it contains a heterogeneous cell population including the most vulnerable brain neurons to excitotoxic injury.

2. Materials and methods

2.1. Experimental technique

The experiments were carried out on transverse slices of 8-day-old rat hippocampus, cut at a thickness

* Corresponding author. Department of Biomedical Sciences & Biotechnologies, School of Medicine, University of Brescia, Via Valsabbina, 19, 25123 Brescia, Italy. Tel. 39.30.3715283, fax 39.30.3701157.

Elsevier Science B.V.
SSDI 0014-2999(95)00047-X

of 0.5 mm according to the method of Gathwaite and Garthwaite (1989) with minor modifications. Briefly, slices were preincubated at 37°C for 30 min in a Krebs solution containing glucose 11 mM and equilibrated with 95% O_2, 5% CO_2 (pH 7.4). Then, 30 μM NMDA (Sigma) was added and incubation was carried out for 30 min. At the end of this period, slices were washed and further incubated in fresh buffer, with or without aniracetam (Hoffmann-La Roche) for 90 min in order to allow irreversibly damaged neurons to become visibly necrotic while giving reversibly damaged cells time to recover (Hajios et al., 1986). Aniracetam, when used, was added to slices during and after the NMDA exposure. In the time-course study, aniracetam was present in the reaction mixture from the preincubation time. Slices were fixed in a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C and then transferred to fresh phosphate buffer solution overnight. Finally, slices were dehydrated in a series of ethanol, infiltrated with a gradient of acetone-resin and embedded in epoxy resin. Semithin (1 μm) sections were cut in the plane of the slices stained with methylene blue and azur II and examined by light microscopy. To perform a quantitation of cell loss, adjacent cells were counted in cell layer fields taken from CA1, CA3 and the dorsal blade of dentate gyrus in each slice. The considered fields measured 1.5×10^4 μm^2. Living neurons appeared homogeneous and compact with a blue cytoplasm and a brighter nucleus while lesioned cells were oedematous, contained white vacuoles and dark shrinking nucleus. The percentage of cell survival was calculated by the ratio between living cells and total cell number.

2.2. Statistics

In each separate experiment, several slices from different brains were subjected to each condition. Points represent the means ± S.E.M. of at least three experiments run in quadruplicate. Statistical significance of the differences was evaluated by Wilcoxon's rank sum test.

3. Results

In agreement with previous findings (Gathwaite and Garthwaite, 1989; Nadler et al., 1981; Steiner et al., 1984), application of 30 μM NMDA for 30 min to hippocampal slices induced a specific cell injury. Virtually, all pyramidal neurons of CA1, CA3 and granule cells of dentate gyrus became acutely necrotic (Fig. 1d,e,f). They exhibited highly swollen cytoplasms containing large vacuoles, nuclear shrinkage and focal clumping of chromatin. Addition of 200 μM aniracetam to hippocampal slices maximally prevented the NMDA-mediated damage. A complete neuroprotection was observed in CA1, CA3 as well as in the dentate gyrus (Fig. 1g,h,i). Aniracetam per se, at 200 μM concentration did not modify the viability of hippocampal neurons.

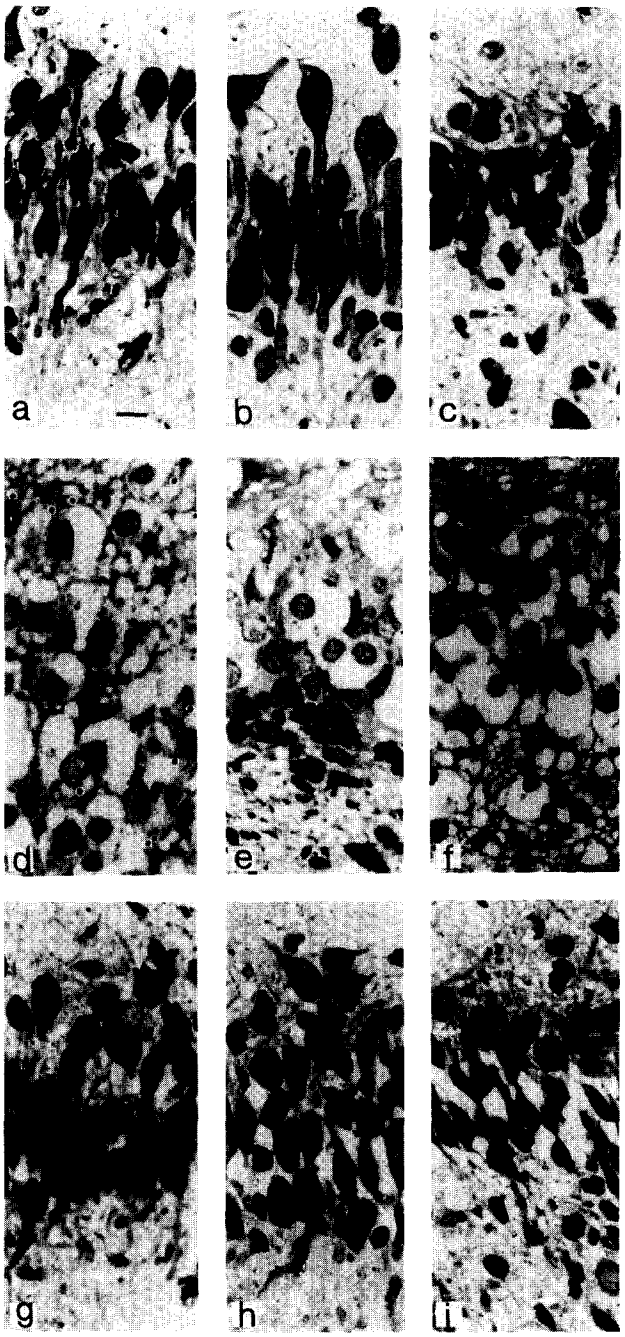

Fig. 1. Excitotoxic effect of NMDA in rat hippocampal slices: prevention by aniracetam. a–c : controls; d–f: 30 μM NMDA; g–i: 30 μM NMDA plus 200 μM aniracetam. The regions illustrated are CA1 (a,d,g), CA3 (b,e,h) and dentate gyrus (c,f,i). Scale bar = 10 μm.

The neuroprotective effect elicited by aniracetam was evaluated in a time-course study by varying the

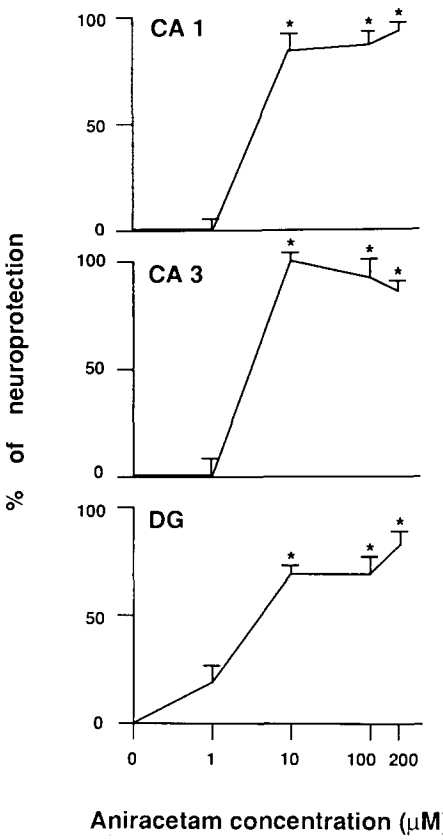

Fig. 2. Effect of different concentrations of aniracetam on NMDA (30 μM)-induced cell loss in CA1 (upper panel), CA3 (middle panel) and dentate gyrus (lower panel). Aniracetam was added to the incubation mixture during and after the NMDA exposure. Points represent the means ± S.E.M. of three experiments run in quadruplicate. * $P < 0.01$ vs corresponding NMDA alone.

time at which aniracetam was added to the slices, according to the following protocols: before, during and after NMDA exposure; during and after NMDA exposure; and after NMDA exposure. Maximal efficacy was obtained when aniracetam was added before and/or during NMDA exposure in addition to the recovery period. No protection was produced when aniracetam was added to the slices at the end of the NMDA pulse (data not shown).

By taking the previous results into consideration, we investigated the neuroprotective effect of different concentrations of aniracetam (Fig. 2). A significant neuroprotection against NMDA toxicity was found at concentrations of aniracetam higher than 1 μM. Maximal neuroprotection was obtained with as low as 10 μM concentration. Concentration dependency, if present, appears to be characterized by a very deep curve within a range of concentrations of one order of magnitude. The theoretically calculated EC_{50} values were 3 μM, 2.5 μM, and 5 μM in CA1, CA3, and dentate gyrus respectively.

4. Discussion

The present study shows that aniracetam significantly counteracts the NMDA-mediated toxicity in hippocampal slices from 8-day-old rats. This finding extends previous work demonstrating the neuroprotective effects of aniracetam in primary cultures of rat cerebellar granule cells exposed to different excitatory amino acid receptor agonists, such as glutamate, kainate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (Pizzi et al., 1993). Aniracetam efficacy is now confirmed on a heterogeneous population of neurons which have been differentiated in vivo.

The time-course study of the effect of aniracetam revealed that the presence of the drug is not essential during preincubation and recovery periods, while it is crucial during the exposure to NMDA. Although previous ligand binding studies failed to demonstrate a close interaction between aniracetam and NMDA receptor moiety (Martin and Haefely, 1993), these results suggest that aniracetam should directly modulate the early NMDA receptor-mediated processes to produce cell survival. Indeed, experiments performed in rat cerebellar granule cells revealed that aniracetam can also prevent the NMDA-mediated intracellular calcium rise (Pizzi et al., in preparation) by acting distally to the NMDA receptor complex.

The effect of aniracetam was tested at different concentrations. The range of neuroprotective concentrations in brain slices appears superimposable on that described in primary culture of neurons (Pizzi et al., 1993) and is compatible with that presumably present in the rat brain after either oral or parenteral administration of aniracetam (Mayersohn et al., 1993).

In primary culture of cerebellar granule cells we also demonstrated a neuroprotective effect elicited by glutamate metabotropic receptor (mGlu receptor) stimulation (Pizzi et al., 1993). Particularly, we reported that aniracetam potentiated mGlu receptor transmission in terms of both signal transduction and neuroprotection. Since mGlu receptor-mediated neuroprotection was also confirmed in hippocampal slices (Pizzi et al., in preparation) the possible interaction between aniracetam and mGlu receptor in hippocampus will be a matter for further investigation.

In summary, the present data confirm the efficacy of aniracetam in preventing NMDA-mediated cell injury. The extension of the aniracetam effect to the ex vivo hippocampal neurons makes it now imperative to explore the activity of this compound also in in vivo models of excitotoxicity.

Acknowledgements

The authors wish to thank MariaPia Molinari Tosatti and Fabrizio Bonardi for their assistance in setting up

histochemical techniques and Amanda Wright for assistance in the revision.

References

Choi, D.W., 1987, Ionic dependence of glutamate neurotoxicity, J. Neurosci. 7, 369.

Choi, D., J. Koh and S. Peters, 1988, Pharmacology of glutamate neurotoxicity in cortical cell culture, J. Neurosci. 8, 185.

Favaron, M., H. Manev, H. Alho, M. Bertolino, B. Ferret and A. Guidotti, 1988, Gangliosides prevent glutamate and kainate neurotoxicity in primary culture of neonatal rat cerebellum and cortex, Proc. Natl. Acad. Sci. USA. 85, 7351.

Gasic, G.P. and M. Hollmann, 1992, Molecular neurobiology of glutamate receptors, Annu. Rev. Physiol. 54, 507.

Gathwaite, G. and J. Garthwaite, 1989, Differential dependence on Ca^{2+} of *N*-methyl-D-aspartate and quisqualate neurotoxicity in young rat hippocampal slices, Neurosci. Lett. 97, 316.

Lee, R.C. and P. Benfield, 1994, Aniracetam. An overview of its pharmacodynamic and pharmacokinetic properties, and a review of its therapeutic potential in senile cognitive disorders, Drugs Aging 4(3), 257.

Martin J.R. and W.E. Haefely, 1993, Pharmacology of aniracetam a novel pyrrolidinone derivative with cognition enhancing activity, Drug Invest. 5 (Suppl. 1), 4.

Mayersohn, M., G. Roncari and G. Wendt, 1993, Disposition pharmacokinetics and metabolism of aniracetam in animals, Drug Invest. 5 (Suppl. 1), 73.

Miyamoto, M., T.H. Murphy, R.L. Schnaar and J.T. Coyle, 1989, Antioxidants protect against glutamate-induced cytotoxicity in a neuronal cell line, J. Pharmacol. Exp. Ther. 250, 1132.

Nadler, J.V., D.A. Evenson and G.J. Cuthbertson, 1981, Comparative toxicity of kainic acid and other amino acids towards rat hippocampal neurons, Neuroscience 6, 2505.

Pizzi, M., M. Ribola, A. Valerio, M. Memo and P.F. Spano, 1991, Various Ca^{2+} entry blockers prevent glutamate-induced neurotoxicity, Eur. J. Pharmacol. 109, 169.

Pizzi, M., C. Fallacara, V. Arrighi, M. Memo and P.F. Spano, 1993, Attenuation of excitatory amino acid toxicity by metabotropic glutamate receptor agonists and aniracetam in primary culture of cerebellar granule cells, J. Neurochem. 61, 683.

Steiner, H.X., G.J. McBean, C. Kohler, P.J. Roberts and R. Schwarcz, 1984, Ibotenate-induced neuronal degeneration in immature rat brain, Brain Res. 307, 117.