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Brain Research 675 (1995) 38–44

BRAIN RESEARCH

Research report

Glycine site NMDA receptor antagonists provide protection against ischemia-induced neuronal damage in hippocampal slice cultures

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Accepted 13 December 1994

Abstract

Ischemia-induced neuronal injury can be reduced by glutamate antagonists acting at the *N*-methyl-D-aspartate (NMDA) receptor. 7-Chlorokynurenic acid and the recently synthesized compound Acea 1021 block NMDA receptors by acting at the strychnine-insensitive glycine site. The anti-ischemic properties of these compounds were tested by evaluating their ability to reduce CA1 neuronal damage in hippocampal slice cultures deprived of oxygen and glucose. Acea 1021 and 7-chlorokynurenic acid significantly reduced CA1 injury produced by oxygen and glucose deprivation in a dose-dependent manner. The neuroprotective effect of these compounds was reversed by the addition of glycine. The phencyclidine site NMDA antagonist MK-801 also provided significant protection to CA1 neurons against the same insult, and this protection was not affected by the addition of glycine. These results indicate that Acea 1021 and 7-chlorokynurenic acid can provide protection to CA1 neurons against ischemia-induced injury by a glycine-sensitive mechanism.

Keywords: Glutamate; Glycine; NMDA receptor; Glycine site; Ischemia; Organotypic hippocampal cultures; Acea 1021; 7-Chlorokynurenic acid; MK-801

1. Introduction

Glutamate antagonists have been shown to be effective in reducing ischemia-induced neuronal injury in a variety of experimental models [1,9,13,15,27,35,36]. Activation of the *N*-methyl-D-aspartate (NMDA) sub-class of glutamate receptors, and associated calcium influx has been shown to play a role in the pathogenesis of neuronal injury following ischemia [1,15,33]. A number of different NMDA antagonists have been synthesized and tested in experimental models of ischemia, and have been considered for clinical studies as cerebral protective agents. Activation of NMDA receptors requires glycine as a co-factor and a group of NMDA antagonists acts by blocking the glycine site on the receptor complex [7,20,22,23,32,39,45]. The recently synthesized quinoxalinedione derivative, Acea 1021, is a specific glycine site NMDA antagonist which crosses the blood–brain barrier and does not produce phencyclidine (PCP)-like side effects in animals [2,7,42,44]. Phencyclidine-like side effects are commonly seen in animals and in humans when many of the currently available NMDA antagonists are administered [2,5,17]. Balster et al. [2] showed that the PCP-like side effects common to many NMDA antagonists, did not occur in monkeys or in rats given Acea 1021. Behavioral side effects including agitation and psychosis may be seriously limit the use of NMDA antagonists currently undergoing clinical evaluation [5,17]. A clinical trial employing the non-competitive NMDA antagonist CGS 19755 in stroke patients found that some patients experienced marked temporary behavioral side effects, which limited the dose that could be used [17]. It is therefore important to establish the usefulness of glycine site antagonists as neuroprotective agents, which do not have these effects.

We previously described an in vitro system, using organotypic hippocampal slice cultures to evaluate compounds for protective effects on CA1 neurons against artificial ischemia (glucose and oxygen deprivation). The use of hippocampal slice cultures eliminates some of the confounding physiologic variables such as temperature, which can complicate the results of in

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0006-8993/95/$09.50
SSDI 0006-8993(95)00039-9

vivo models [4,27]. We were able to demonstrate that the NMDA receptor antagonist dizocilpine (MK-801) reduced neuronal injury produced by oxygen and glucose deprivation in hippocampal slice cultures [27]. The objectives of this study were to examine the ability of NMDA antagonists which act at the glycine site, to protect CA1 pyramidal neurons from damage resulting from exposure to glucose and oxygen deprivation. The glycine site antagonists Acea 1021 and 7-chlorokynurenic acid were tested for neuroprotective effects using cultured slices of rat hippocampus, exposed to a carefully controlled reproducible 'ischemic' insult. We also examined reversal of the neuroprotective effect of Acea 1021 and 7-chlorokynurenic acid, by adding exogenous glycine. The neuroprotective effect of MK-801, a non-competetive NMDA antagonist which does not act at the glycine site, was also studied in the presence of added glycine.

2. Materials and methods

2.1. Preparation of compounds

Acea 1021 (Aeca Pharmacueticals, Irvine Ca.) and 7-chlorokynurenic acid (Research Biochemicals Incorporated Natick, MA) were dissolved in dimethylsulfoxide (DMSO) (Sigma Chemical, St. Louis, MO). The stock concentration of both compounds in DMSO was 10 mM. The stock solutions were diluted and delivered to the cultures as described, giving a final concentration of 1% DMSO in the medium. The concentration of DMSO used for controls was 1%. For the dose response curves, Acea-1021 and 7-chlorokynurenic were diluted progressively in DMSO to achieve final concentrations of 100.0, 10.0, 1.0, 0.10 and 0.01 μM. Glycine (Sigma Chemical, St. Louis, MO) was dissolved in glucose free HBSS and added to the cultures before and after exposure to the insult, to achieve final concentrations of 1.0, 0.1, 0.01, 0.001 mM. The control cultures and cultures with no glycine received an identical dilution of glucose free HBSS. MK-801 (Research Biochemicals Incorporated Natick, Ma.) was prepared as a 10 mM solution in glucose free HBSS and added to the cultures as a 1:100 dilution (10 μM and 100 μM final concentrations).

2.2. Culture preparation

Organotypic cultures of the rat hippocampus were prepared according to the methods of Gahwiler et al. [10] and Stoppini et al. [37]. Hippocampal slices were prepared from 4 to 7 day old neonatal rats (Sprague–Dawley, B and K Universal, Inc., Fremont, CA) by removing the brain, dissecting the hippocampal formation and making transverse slices (4–5 μM) using a McIlwain tissue slicer (Brinkman Instruments, Westbury, NY). Slices were placed in Gey's Balanced Salt Solution (Gibco, Grand Island, NY) supplemented with glucose (Sigma Chemical, St. Louis, MO) to a final concentration of 6.5 mg/ml for 1 h at 4°C. Individual slices were transferred onto 25 mm sterile transparent Anocell interface membranes (VWR Scientific, Seattle, WA). The membranes were placed in 6 well culture trays with 1.5 cc growth medium consisting of 50% minimal essential medium (MEM), (Gibco, Grand Island, NY) supplemented with 10 mM Hepes (Sigma Chemical, St. Louis, MO), sodium bicarbonate, 25% Hank's Balanced Salt Solution (HBSS) (Gibco, Grand Island, NY), 25% horse serum (Gibco, Grand Island, NY) and glucose to a final concentration of 6.5 mg/ml. The cultures were grown at 36.5°C in an atmosphere with 90 to 100% humidity and 5% CO_2. The culture medium was changed every 3 days. No antibiotics were used. All cultures were grown for 12 to 14 days in vitro before experimentation. The cultures were selected for use were examined under light microscopy, and only

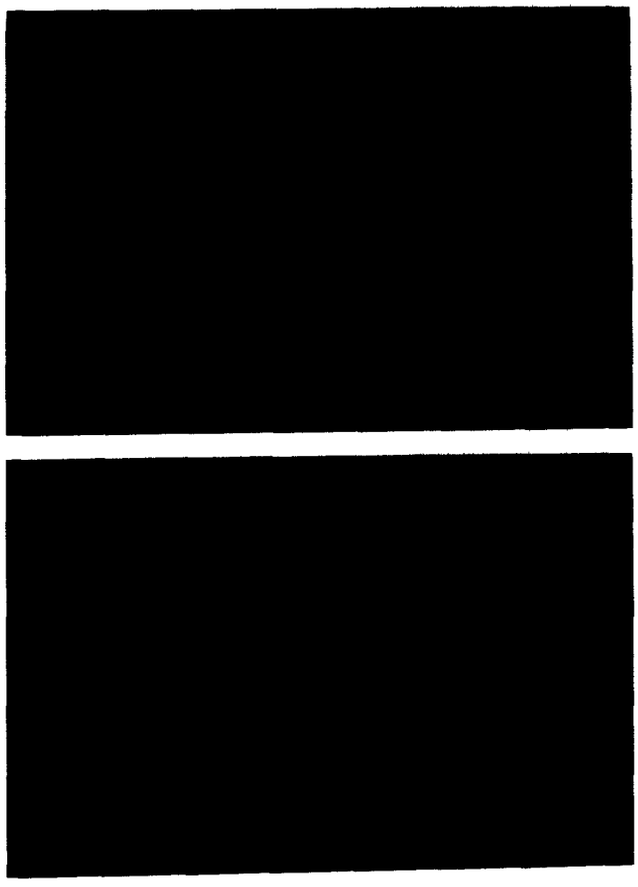

Fig. 1. Upper panel: illustration of the propidium iodide fluorescence in a hippocampal culture exposed to 35 min of oxygen and glucose. Lower panel: illustration of the marked reduction in propidium iodide fluorescence in a hippocampal culture exposed to 35 min of oxygen and glucose deprivation in the presence of 100 μM Acea 1021. DG, dentate gyrus.

cultures with a complete anatomy were used. Cultures were randomized into groups for experimentation.

2.3. Induction of artificial ischemia

Artificial ischemia was induced by a slight modification of the method previously described by Newell et al. [27]. After triple rinsing, the cultures were placed in 1.5 cc of HBSS with no glucose. The HBSS had been brought to an equimolar concentration to the culture medium with sucrose. Drug or vehicle were added 10–15 min before exposure to anoxia/hypoglycemia. The temperature of each individual culture well was confirmed to be at 36.5°C with an electronic thermometer, and was carefully controlled to be equivalent in all cultures, to insure that the results were independent of changes in temperature.

The cultures were transferred into an anaerobic chamber (Forma Scientific) which was pre-equilibrated to 36.5°C and had an atmosphere of 0% oxygen, 10% hydrogen and 5% CO_2. The hydrogen was present for interaction with a palladium catalyst which maintained the oxygen concentration at 0%. An oxygen meter was present at all times to confirm anoxic conditions. Following equilibration in the medium (for 10 min), the culture tray was placed in the anaerobic chamber at 36.5°C for 35 min.

Upon removal of the tray from the anaerobic chamber, membranes were transferred to pre-warmed growth medium containing drug or vehicle in the same concentrations used for pretreatment. The growth medium also contained propidium iodide (Sigma Chemical, St. Louis, MO) at a final concentration of 0.5 μg/ml. Propidium iodide, is a fluorescent dye that is non toxic to neurons. Propidium iodide is excluded from healthy cells with intact plasma membranes but enters damaged cells, binds to nucleic acids, and displays bright fluorescence [24] (see Fig. 1). The cultures were transferred back to a CO_2 incubator at 36.5°C for 48 h before they were evaluated for cell death.

2.4. Assessment of cell death

Imaging of the relative amount of fluorescence of propidium iodide in the CA1 subfield of the cultures was used as an index of the percentage of cell death [28,29]. The first imaging of the cultures, following the insult, was done after 48 h of incubation with propidium iodide. The cultures were examined using a Nikon Diaphot inverted fluorescent microscope and fluorescent images were obtained using a Dage 72 CCD camera (Michigan City, IN), with the auto gain disabled. The images were digitized and saved, using Optimas image analysis software (Bio-Scan, Inc., Edmonds, WA).

Forty eight hours after the initial 'ischemic' insult, the remaining neurons were killed by exposing the cultures to 3 h of anoxia. The fluorescent intensity associated with 100% damage to CA1 was then determined and compared to the fluorescent intensity fol-

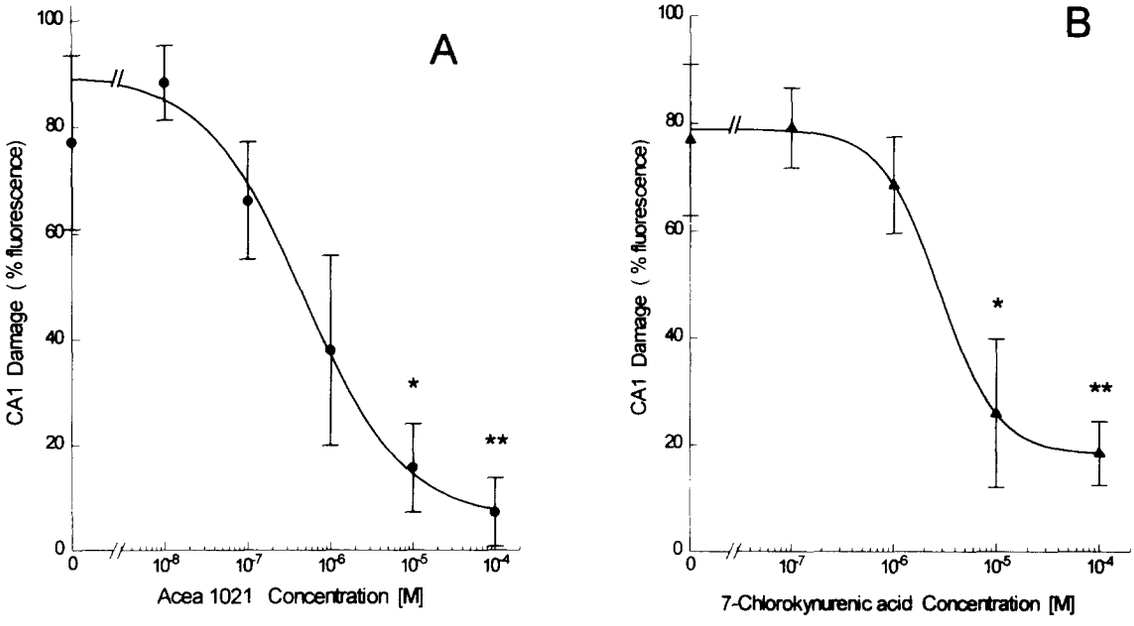

Fig. 2. A: dose-response curve showing the effect of increasing concentration of Acea 1021 on reducing propidium iodide fluorescence in CA1 caused by oxygen and glucose deprivation for 35 min. $n = 8$ for each data point. B: dose-response curve showing the effect of increasing concentration of 7-chlorokynurenic acid on reducing propidium iodide fluorescence in CA1 caused by oxygen and glucose deprivation for 35 min. $n = 8$ for each data point. ** $P < 0.01$, * $P < 0.05$ compared to control using Mann–Whitney test with Bonferroni correction for multiple comparisons.

lowing the original ischemic insult. The integrated gray values (fluorescent intensity) from the CA1 subregion following the 'ischemic' exposure were expressed as a percentage of the value obtained from that identical region following 3 h of anoxia. The values were averaged for each group and compared. All values were expressed as mean ± S.E.M. Statistical analysis of the results was performed using the Mann–Whitney test with Bonferroni correction for multiple comparisons. Significant differences were considered at the P value < 0.05.

3. Results

Exposure of the hippocampal slice cultures to artificial ischemia (oxygen and glucose deprivation) for 35 min at 36.5°C produced marked propidium iodide fluorescence in the neurons in the cultures, most prominently in CA1. The presence of 100 μM Acea 1021 or 100 μM 7-chlorokynuerenic acid, caused a marked reduction the amount of fluorescence detected in the CA1 region of the cultures (Fig. 1). Both compounds exhibited a dose-dependent reduction in neuronal damage and were effective in the micromolar range (Fig. 2). The addition of 0.1 mM and 1.0 mM glycine reversed the neuroprotective effect of 100 μM Acea 1021, and 1.0 mM glycine reversed the neuroprotective effect of 100 μM 7-chlorokynurenic acid. Glycine alone (1.0 mM) however, was not neurotoxic (Figs. 3 and 4).

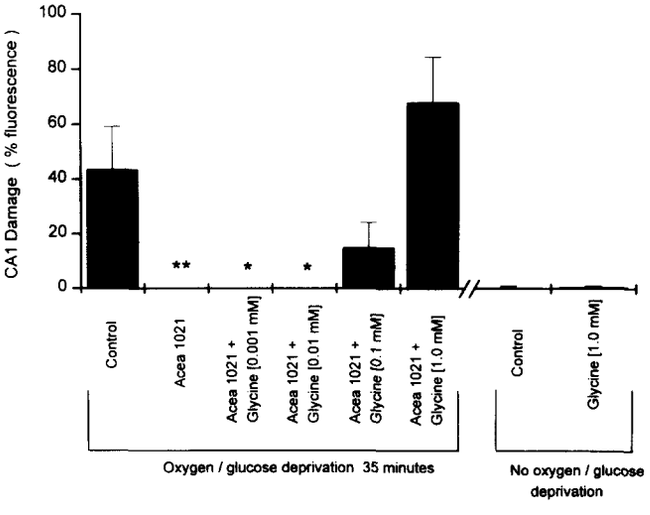

Fig. 3. Illustration of the neuronal protection provided by Acea 1021 (100 μM) and the reversal of the protection by the addition of exogenous glycine at concentrations of 0.1 mM and 1.0 mM, but no effect at lower concentrations (0.001–0.01 mM). Glycine alone, did not cause significant ($P = 0.14$ one-tailed Mann–Whitney test) CA1 damage at a 1.0 mM concentration when compared to control. $n = 8$ for each data point except for first control $n = 7$. ** $P < 0.01$, * $P < 0.05$ using Mann–Whitney test with Bonferroni correction for multiple comparisons.

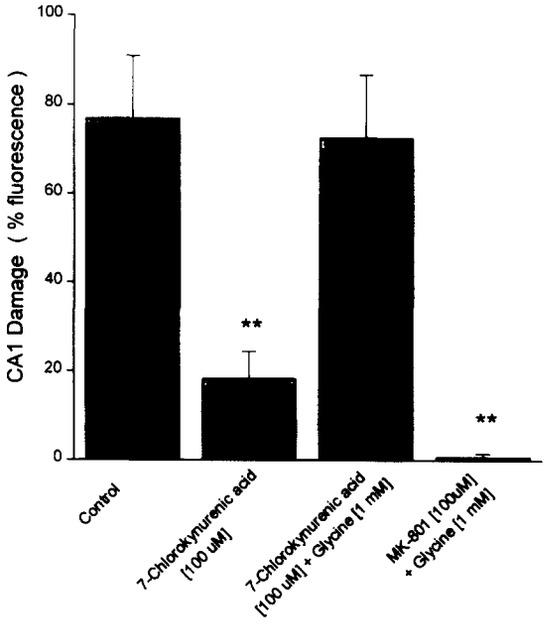

Fig. 4. Illustration of the neuronal protective effect of 7-chlorokynurenic acid (100 μM) when compared with the control cultures, and the reversal of the neuronal protective effect by the addition of 1.0 mM glycine. The same concentration of glycine had no effect on the protection provided by MK-801 (100 μM). $n = 8$ for each data point. ** $P < 0.01$, * $P < 0.05$ using Mann–Whitney test with Bonferroni correction for multiple comparisons.

In contrast, 100 μM MK-801 provided significant neuronal protection against oxygen and glucose deprivation in the presence of the same concentration of glycine (1.0 mM), that reversed the protective effects of Acea 1021 and 7-chlorokynurenic acid (Figs. 3 and 4). A lower concentration (10 μM) of MK-801 was equally protective against the same insult with 1 mM glycine present (data not shown).

4. Discussion

In this study, our results demonstrate that the glycine site NMDA receptor antagonists Acea 1021 and 7-chlorokynuerenic acid, protect CA1 neurons in hippocampal slice cultures against ischemia-induced injury. These compounds act in a dose-dependent manner and are effective in the micromolar range.

NMDA receptor antagonists have been shown to provide neuronal protection in a variety of in vivo and in vitro experimental models of ischemia, including dissociated cell cultures, focal ischemia models, and transient forebrain ischemia models [13–16,31,33,34]. The value of NMDA antagonists in providing protection to CA1 neurons against transient forebrain ischemia has been questioned however. It has been suggested that a small reduction in body temperature

induced by MK-801 rather than its action on the NMDA receptor confers its protective effect on CA1 neurons observed in the transient forebrain ischemia model [4]. Hippocampal slice cultures provide a valuable in vitro model system to evaluate the protective effect of various compounds against ischemic damage to CA1 neurons under well-controlled experimental conditions, and in the absence of physiologic variables such as temperature, blood pressure, and microcirculatory effects which can be problematic when using in vivo ischemia models [4,12,27,41]. Alterations of these physiological effects therefore cannot account for the protective effects of Acea 1021 and 7-chlorokynurenic acid against CA1 damage observed in these experiments. The presence of preserved synaptic circuitry [11,25,26], expression of major surface receptors [11,19,38], and preserved neuronal glial interactions in hippocampal slice cultures, creates a neural tissue-like environment which distinguishes this preparation from dissociated cell culture models [8,16].

We employed a previously described computerized quantitative image analysis system to determine the severity of cell damage following the insult [21,28,29]. This method of analysis quantitates the fluorescent intensity of PI in the CA1 subfield of the hippocampal slice cultures and uses this measure as an estimate of cell damage. Previous comparisons of PI fluoresence to histological staining in hippocampal cultures, by us [28] and by others [40,41], indicates that most of the fluorescence is due to neuronal cell death. Glia are resistant to damage from glucose and oxygen deprivation at the severity used in these experiments [16], but will demonstrate PI fluorescence with increased exposure to this insult (6–8 h). The usefulness of PI as an indicator of neuronal cell death has been confirmed by comparing PI fluorescence to histological and electrophysiologic correlates of cell death in dissociated cell cultures and histological correlates in organotypic hippocampal cultures [24,28,40,41].

Our results indicate that the primary mechanism by which Acea 1021 and 7-chlorokynurenic acid exert their effect is mediated through the glycine site of the NMDA receptor. The recently developed experimental compound Acea 1021, has been shown to be a potent glycine site selective NMDA antagonist [7]. 7-Chlorokynuerenic acid also is a potent glycine site antagonist [22] but both compounds have some effect at non-NMDA receptors at higher concentrations [7,22]. The complete reversal of the protective effect afforded by these compounds by adding exogenous glycine, however, strongly suggests that the primary protective effect is glycine mediated, presumably through NMDA receptors. As expected, the potent non-competitive NMDA antagonist MK-801 was also highly effective in providing neuronal protection to CA1 neurons. Unlike the glycine site antagonists, however, protection provided by MK-801, which acts primarily at the PCP site on the NMDA receptor [5,30], was not prevented by the addition of 1 mM glycine.

In addition to the lack of neurobehavioral effects, glyine site NMDA antagonists may offer advantages over other types of NMDA antagonists. Unlike competitive NMDA antagonists, glycine site antagonists should be resistant to displacement by high levels of glutamate seen during ischemia [3]. Although our results show that high levels of glycine can overcome the protective effect of these competitive glycine site antagonists, previous studies indicate that glutamate reaches higher levels than glycine during anoxia and ischemia, and glycine may not reach the levels required to overcome the inhibition [3,6,18].

7-Chlorokynurenic acid and Acea 1021 have recently been shown to be effective in reducing ischemia-induced neuronal injury induced in in vivo ischemia models [42,43]. Wood et al. [43] showed that intraventricular injections of 7-chlorokynurenic acid reduced ischemia-induced CA1 damage, and also learning deficits in rats subjected to transient forebrain ischemia. The clinical usefulness of this compound is limited, however, due to poor blood–brain barrier penetration. Warner et al. [42] found that intraperitoneal administration of Acea 1021 significantly reduced infarct size following middle cerebral artery occlusion in rats. 7-Chlorokyneurinic acid has also been shown to protect dissociated cortical neurons from damage due to oxygen and glucose deprivation [15]. Our results, using the hippocampal slice culture model, are consistant with the findings of these other investigators and demonstrate that Acea 1021 provides effective neuroprotection against oxygen and glucose deprivation, independent of physiologic effects. The apparent lack of behavioral side effects associated with Acea 1021 and its ability to cross the blood–brain barrier suggest that it may prove to be a clinically useful compound for providing neuroprotection in patients suffering cerebral ischemic insults.

Acknowledgements

We thank Vera Papermaster for preparing the hippocampal cultures. We also thank Paul Schwartz and Janet Clardy for preparing the illustrations and also Lois Downey for assistance with the statistical analysis. We thank Dr. Eckhard Weber and Dr. M.J. Whitehouse for kindly providing the Acea 1021 used, and also for providing valuable advice regarding the manuscript. This work was supported through a grant from a National Stroke Association (D.W.N.), and also through Clinician Investigator Development Award 1K08 NS 015969 01 (D.W.N.), and NIH Grants 1 P50 NS 30305-01 (D.W.N., A.T.M.), and NS28650 (A.T.M.).

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