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European Journal of Pharmacology 426 (2001) 35–38

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Neuroprotection afforded by NAAG and NAALADase inhibition requires glial cells and metabotropic glutamate receptor activation

Ajit G. Thomas [1], Jennifer L. Olkowski [1], Barbara S. Slusher *

Guilford Pharmaceuticals Inc., 6611 Tributary Street, Baltimore, MD 21224, USA

Received 10 April 2001; received in revised form 3 July 2001; accepted 6 July 2001

Abstract

N-acetylated-α-linked-acidic-dipeptidase (NAALADase or glutamate carboxypeptidase II) cleaves the neuropeptide *N*-acetyl-aspartyl-glutamate (NAAG) to glutamate and *N*-acetyl-aspartate (NAA). Previously, NAAG and 2-(phosphonomethyl)-pentanedioic acid (2-PMPA), a potent and selective NAALADase inhibitor, were found to be neuroprotective in neuronal/glial co-cultures and in animals following transient middle cerebral artery occlusion. In this report, we examined the involvement of glial cells and metabotropic glutamate (mGlu) receptors in neuroprotection mediated by NAAG and 2-PMPA in an in vitro model of metabolic inhibition. Neuroprotection of neuronal/glial co-cultures by both NAAG and 2-PMPA, against metabolic inhibition, was significantly higher than neuroprotection in the absence of glia. Similarly, (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG IV), a selective group II mGlu receptor agonist, was less neuroprotective in the absence of glia. Selective group II mGlu receptor antagonists and (*S*)-α-methyl-4-carboxyphenylglycine (MCPG), a non-selective mGlu receptor antagonist, reduced the protection afforded by both NAAG and 2-PMPA when using neuronal/glial co-cultures. In contrast, groups I and III mGlu receptor antagonists did not affect NAAG or 2-PMPA neuroprotection. These results underscore the critical involvement of glia and group II mGlu receptors in NAAG and 2-PMPA-mediated neuroprotection.

Keywords: NAALADase (*N*-acetylated-α-linked-acidic-dipeptidase); NAAG (*N*-acetyl-aspartyl-glutamate); mGlu receptor; Ischemia; Neuroprotection; 2-PMPA (2-phosphonomethyl)-pentanedioic acid

1. Introduction

N-acetylated-α-linked-acidic-dipeptidase (NAALADase or glutamate carboxypeptidase II; EC 3.4.17.21, Barrett et al., 1998) is a neuropeptidase that hydrolyzes the abundant brain peptide *N*-acetyl-aspartyl-glutamate (NAAG) to *N*-acetyl-aspartate (NAA) and glutamate (Robinson et al., 1987). 2-(Phosphonomethyl)-pentanedioic acid (2-PMPA), a potent and selective NAALADase inhibitor (Jackson et al., 1996), provides neuroprotection in both in vitro and in vivo models of cerebral ischemia (Slusher et al., 1999). Coincident with NAALADase inhibition, at neuroprotective doses, 2-PMPA increases NAAG and decreases extracellular glutamate levels in vivo (Slusher et al., 1999). NAAG, like NAALADase inhibition, provides significant protection in an in vitro model of metabolic inhibition (Thomas et al., 2000) and in in vivo models of toxicity (Orlando et al., 1997) and cerebral ischemia (Lu et al., 2000).

NAAG acts as an agonist at the group II $mGlu_3$ receptor (Wroblewska et al., 1993, 1997), a receptor found on both neuronal (Wroblewska et al., 1993) and glial cells (Santi et al., 1995). In this report, we show that neuroprotection by NAAG and NAALADase inhibition are mediated by glial, rather than neuronal cells, in a tissue culture model of metabolic inhibition. In addition, we provide evidence to support the involvement of group II metabotropic glutamate (mGlu) receptors in their neuroprotection.

2. Materials and methods

All procedures reported here have been carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and put forth by the National Institutes of Health of the United States.

* Corresponding author. Tel.: +1-410-631-6802; fax: +1-410-631-6804.

E-mail address: slusherb@guilfordpharm.com (B.S. Slusher).

[1] Both authors contributed equally.

0014-2999/01/$ - see front matter
PII: S0014-2999(01)01198-0

Table 1
Neuroprotection by 2-PMPA against metabolic inhibition in both neuronal/glial co-cultures and neuronal cultures.
Cultures were exposed to 2-deoxyglucose (2-DG, 10 mM) and potassium cyanide (KCN, 5 mM) as described in Methods.

	EC_{50} in neuronal/glial co-cultures	EC_{50} in neuronal cultures
2-PMPA	0.77 ± 0.1 nM	7 ± 2.4 μM
+ Group I antagonist, AIDA	1.30 ± 0.3 nM	–
+ Group II antagonist, EGLU	265 ± 118 nM	–
+ Group III antagonist, MSOP	2 ± 1 nM	–
+ Nonselective antagonist, MCPG	250 ± 37 nM	–

EC_{50} values are the average ± S.E.M. of four individual determinations. Antagonists were used at 100 μM.

2.1. Materials

NAAG was obtained from either Tocris-Cookson (Ballwin, MO) or Bachem (Torrance, CA), and (*S*)-α-methyl-4-carboxyphenylglycine (MCPG), (2*S*)-α-ethylglutamic acid (EGLU), (*RS*)-1-aminoindan-1,5-dicarboxylic acid (AIDA), (*RS*)-α-methylserine-*O*-phosphate (MSOP) and (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG IV) from Tocris-Cookson. 2-PMPA was synthesized by SRI International (Menlo Park, CA). All other drugs and chemicals were obtained from Sigma (St. Louis, MO). Regular and phosphate-free HEPES-buffered saline solution (HBSS) was custom-made by Paragon Biotech Inc. (Baltimore, MD). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from GIBCO (Rockville, MD). The serum used in making up the tissue culture medium was obtained from HyClone Laboratories (Logan, UT) and timed pregnant rats were obtained from Harlan Sprague Dawley (Indianapolis, IN).

2.2. Methods

Neuronal/glial co-cultures were prepared from E17 rats (Vornov, 1995). In order to obtain mainly neuronal cultures, 4-day-old cultures were treated with 5-fluoro-2′-deoxyuridine (FUDR, 40 μM) for 4 days (Dawson et al., 1993). On days 18–20, cultures were exposed to 2-deoxyglucose (10 mM) and potassium cyanide (KCN, 5 mM) in HBSS for 20 min at 37 °C. When using 2-PMPA, phosphate-free HBSS was used. Cultures were allowed to recover in DMEM for 24 h at 37 °C, 95% humidity and 5% CO_2. Unless otherwise stated, drugs, at pH 7.4, were present during the metabolic inhibition and throughout recovery (24 h). Cellular injury was quantified by measuring the lactate dehydrogenase (LDH) released from cultures after recovery. Metabolic inhibition, in the presence of buffer alone, caused complete morphological destruction of cultured neurons (100% LDH release). Data were normalized as a percent of this maximal injury.

3. Results

3.1. 2-PMPA and NAAG-mediated neuroprotection requires glial cells

Neuronal/glial co-cultures were protected by 2-PMPA against metabolic inhibition (EC_{50} = 0.77 nM ± 0.1, Table 1). In neuronal cultures, the ability of 2-PMPA to protect against insult was reduced 1000-fold (EC_{50} = 7.0 ± 2.4 μM, Table 1). NAAG also provided robust neuroprotection in the neuronal/glial co-culture system (83 ± 2.9% protection at 100 μM, Table 2). In neuronal cultures, NAAG-mediated neuroprotection was also substantially reduced (16 ± 0.5% at 100 μM, Table 2).

3.2. 2-PMPA and NAAG-mediated neuroprotection requires mGlu receptor activation

NAAG is known to be an agonist at the group II $mGlu_3$ receptor (Wroblewska et al., 1997, 1993), and this receptor is found in both glial (Santi et al., 1995) and neuronal cells (Wroblewska et al., 1993). We first examined the effect of a selective group II mGlu receptor agonist on metabolic inhibition in the presence and absence of glia. In the presence of glia, the EC_{50} of DCG IV was 35 ± 11.7 nM (Table 3). The EC_{50} shifted to 881 ± 380 nM (Table 3) in the absence of glia. We also examined the effect of several mGlu receptor antagonists on NAAG and 2-PMPA-mediated neuroprotection. In the presence of MCPG (100 μM), a non-selective group II mGlu receptor antagonist, the neuroprotective effects of both 2-PMPA and NAAG were

Table 2
Effect of mGlu receptor antagonists on NAAG-mediated neuroprotection in neuronal/glial co-cultures.
Cultures were exposed to 2-deoxyglucose (2-DG, 10 mM) and potassium cyanide (KCN, 5 mM) as described in Methods.

	% Inhibition of LDH release in neuronal/glial co-cultures	% Inhibition of LDH release in neuronal cultures
NAAG	83 ± 2.9	16 ± 0.5
+ Group I antagonist, AIDA	82 ± 1.4	–
+ Group II antagonist, EGLU	16 ± 2.5	–
+ Group III antagonist, MSOP	80 ± 2.7	–
+ Nonselective antagonist, MCPG	0 ± 14.5	–

Percent inhibition of LDH release is the average ± S.E.M. of four individual determinations. NAAG and the antagonists were used at 100 μM.

Table 3
Effect of DCG IV (100 μM) against simulated ischemia in both neuronal/glial co-cultures and neuronal cultures
Cultures were exposed to 2-deoxyglucose (2-DG, 10 mM) and potassium cyanide (KCN, 5 mM) as described in Methods.

	EC_{50} in neuronal/glial co-cultures	EC_{50} in neuronal cultures
DCG-IV	35 ± 11.7 nM	881 ± 380 nM

EC_{50} values are the average ± S.E.M. of four individual determinations.

reduced. The EC_{50} of 2-PMPA shifted 300-fold from 0.77 ± 0.1 to 250 ± 37 nM in the presence of MCPG (Table 1). NAAG-mediated protection against metabolic inhibition was also reversed by MCPG (Table 2). EGLU, a group II mGlu receptor antagonist, significantly reduced the protective effects of both 2-PMPA ($EC_{50} = 0.77$ nM to $EC_{50} = 265$ nM, Table 1) and NAAG (83% protection to 16%, Table 2). In contrast, AIDA, a group I mGlu receptor antagonist, and MSOP, a group III mGlu receptor antagonist did not affect 2-PMPA (Table 1) or NAAG neuroprotection (Table 2).

4. Discussion

NAAG is an abundant neuropeptide found in millimolar concentrations in the brain (Coyle et al., 1991, 1997). NAAG is released from neurons after depolarization by a calcium-dependent process (Tsai et al., 1988), and is both an agonist at group II $mGlu_3$ receptors (Wroblewska et al., 1993, 1997) and a mixed agonist/antagonist at NMDA receptors (Puttfarcken et al., 1993). NAALADase, a membrane bound metalloprotease, is responsible for hydrolyzing NAAG to *N*-acetyl-aspartate and glutamate (Slusher et al., 1990). Using the potent and selective inhibitor 2-PMPA (Jackson et al., 1996), we have shown previously that NAALADase inhibition is neuroprotective in both in vitro and in vivo models of ischemia via increases in extracellular levels of NAAG and decreases in extracellular levels of glutamate (Slusher et al., 1999). A blockade of NAALADase could have neuroprotective effects by decreasing the liberation of glutamate from NAAG, thus preventing toxic glutamate accumulation. In addition, NAALADase inhibition could have its neuroprotective effects by increasing NAAG, and thus influencing mGlu receptors or NMDA receptors. In this study, we examined the involvement of glial cells and mGlu receptor in the neuroprotection mediated by both NAAG and 2-PMPA against metabolic inhibition.

2-PMPA neuroprotection was found to be very potent in neuronal/glial co-cultures ($EC_{50} = 0.77 \pm 0.1$ nM). NAAG was also found to be neuroprotective ($83 \pm 2.9\%$ protection at 100 μM). In cultures treated with FUDR, to decrease the amount of glial cells (Dawson et al., 1993), there was a significant decrease in neuroprotection with both 2-PMPA (Table 1) and NAAG (Table 2). Neuroprotection was largely glial mediated. The 2-PMPA data are consistent with reports that NAALADase resides primarily on glial cells (Berger et al., 1999; Cassidy and Neale, 1993).

As has been described previously for NAAG, we observed a U-shaped dose–response curve for NAAG-mediated neuroprotection (Bruno et al., 1998). At higher concentrations of NAAG, we observed toxicity, presumably due to NMDA agonism (Thomas et al., 2000). Since NAAG acts at the group II $mGlu_3$ receptor, we looked at the effects of another group II mGlu receptor agonist, DCG IV, on metabolic inhibition in the presence and absence of glia. In the absence of glia, the EC_{50} shifted from 35 ± 11.7 to 881 ± 380 nM (Table 3). We also looked at the effects of the non-selective receptor antagonist MCPG on the neuroprotective abilities of 2-PMPA and NAAG in co-cultures. Protection of neuronal/glial co-cultures by 2-PMPA was significantly reduced in the presence of MCPG (Table 1). Likewise, when different concentrations of MCPG were used in the presence of NAAG, neuroprotection was gradually obliterated in a dose-dependent manner (Thomas et al., 2000). MCPG by itself had no effect on metabolic inhibition (Thomas et al., 2000). Protection was reversed by EGLU, a group II mGlu receptor antagonist, but not by AIDA or MSOP, selective groups I and III mGlu receptor antagonists, respectively (Table 2). These results are consistent with the hypothesis that NAAG's protective effects are primarily mediated through group II mGlu receptors on glial cells.

In summary, neuroprotection by NAAG or NAALADase inhibition in neuronal/glial co-cultures, against metabolic stress, appears to be dependent on the presence of glial cells. Secondly, neuroprotection was reversed by non-selective and group II mGlu receptor antagonists and it was unaffected by groups I and III mGlu receptor antagonists. Taken together, these results suggest a critical involvement of glial mGlu receptors in NAAG and 2-PMPA-mediated neuroprotection.

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