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Activation of mGlu1 but not mGlu5 metabotropic glutamate receptors contributes to postischemic neuronal injury in vitro and in vivo

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Abstract

In order to investigate the involvement of mGlu1 and mGlu5 metabotropic glutamate receptors in the development of postischemic neuronal death, we examined the effects of selective agonists and antagonists in models of cerebral ischemia in vitro and in vivo. In murine cortical cell cultures and rat organotypic hippocampal slices exposed to oxygen and glucose deprivation (OGD), the mGlu1 antagonists 1-aminoindan-1,5-dicarboxylic acid (AIDA; 300 μM), (*S*)-(+)-2-(3′-carboxybicyclo[1.1.1]pentyl)-glycine (CBPG; 300 μM), 7-hydroxyiminocyclopropan[*b*]chromen-1*a*-carboxylic acid ethyl ester (CPCCOEt; 10–30 μM) and (+)-2-methyl-4-carboxyphenylglycine (LY367385; 30–100 μM) reduced neuronal loss when added to the medium during OGD and the subsequent 24-h recovery period. On the contrary, the potent and selective mGlu5 antagonist methyl-6-(phenylethynyl)-pyridine (MPEP; 0.1–1 μM) did not exhibit neuroprotection in any of these in vitro models. Incubation with the nonselective mGlu1 and mGlu5 agonist 3,5-dihydroxyphenylglycine (3,5-DHPG; 300 μM) but not with the mGlu5 agonist (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG; 1 mM) enhanced the severity of OGD-induced neuronal damage. In gerbils subjected to global ischemia, intracerebroventricular administration of AIDA (100 nmol two times) or CBPG (300 nmol, two times) afforded consistent protection against CA1 pyramidal cell death, whereas MPEP (10 pmol i.c.v two times and 10 mg/kg i.p two times) failed to reduce postischemic hippocampal damage. Our results suggest that activation of mGlu1 but not mGlu5 receptor contributes to postischemic neuronal injury. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Metabotropic glutamate receptors; MPEP; AIDA; Neuroprotection; Oxygen–glucose deprivation; Global ischemia

1. Introduction

Recent evidence suggests that metabotropic glutamate (mGlu) receptors contribute to the neurotoxic effects of glutamate. In particular, Group I mGlu receptors coupled to phosphoinositide hydrolysis (i.e., mGlu1 and mGlu5) have been extensively investigated for their possible role in neurodegeneration (Bordi and Ugolini, 1999; Nicoletti et al., 1999). Receptors of the mGlu1 and mGlu5 subtypes are known to be associated with a number of potentially detrimental events such as the increase in cytosolic free Ca^{2+} and activation of protein kinase C, the potentiation of *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor responses, and the enhancement of glutamate release. The results of neurotoxicity studies have shown that Group I mGlu receptor antagonists are consistently neuroprotective, whereas the use of agonists has generated conflicting results. Discrepancies may arise from the poor selectivity of available mGlu receptor ligands. It has also been proposed that a number of alternative factors, including the subunit composition of NMDA receptors, the exposure time to drugs or toxic insults and the function of astrocytes may affect the responses to mGlu receptor agonists (for a review, see Nicoletti et al., 1999).

We have shown that 1-aminoindan-1,5-dicarboxylic acid (AIDA) and (*S*)-(+)-2-(3′-carboxybicyclo[1.1.1]pentyl)-glycine (CBPG), two Group I mGlu receptor antagonists

Abbreviations: (1*S*,3*R*)-ACPD, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid; AIDA, 1-aminoindan-1,5-dicarboxylic acid; BHK, baby hamster kidney; CBPG, (*S*)-(+)-2-(3′-carboxybicyclo[1.1.1]pentyl)-glycine; CHPG, (*RS*)-2-chloro-5-hydroxyphenylglycine; CPCCOEt, 7-hydroxyiminocyclopropan[*b*]chromen-1*a*-carboxylic acid ethyl ester; 3,5-DHPG, 3,5-dihydroxyphenylglycine; DIV, days in vitro; IP, inositol phosphate; LDH, lactate dehydrogenase; LY367385, (+)-2-methyl-4-carboxyphenylglycine; mGlu, metabotropic glutamate; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; NMDA, *N*-methyl-D-aspartate; OGD, oxygen–glucose deprivation; PI, propidium iodide.

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displaying preferential activity on mGlu1 over mGlu5 receptors (Mannaioni et al., 1999; Moroni et al., 1997), reduce neuronal damage in in vitro and in vivo models of cerebral ischemia (Pellegrini-Giampietro et al., 1999a,b). A number of similar studies in other experimental models also point to mGlu1 receptors as a potential target for neuroprotection (Bruno et al., 1999; Mukhin et al., 1996; Strasser et al., 1998). Although mGlu5 receptors have been implicated in the potentiation of ionotropic glutamate receptor responses (Alagarsamy et al., 1999; Attucci et al., 2001; Doherty et al., 1997; Ugolini et al., 1999) and antagonism of mGlu5 receptors has been demonstrated to be neuroprotective against NMDA-induced excitotoxicity (Bruno et al., 2000; Movsesyan et al., 2001), the contribution of mGlu5 receptor blockade to neuroprotection in postischemic neuronal injury is still unclear. In the present study, we used the potent and selective mGlu5 noncompetitive antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) (Gasparini et al., 1999), in comparison with available selective mGlu1 antagonists, in order to investigate whether mGlu5 receptors contribute to neuronal injury following oxygen and glucose deprivation (OGD) in vitro and global ischemia in vivo.

2. Materials and methods

2.1. Materials

AIDA and CBPG were synthesized as described (Pellicciari et al., 1995, 1996). (1*S*,3*R*)-1-Aminocyclopentane-1, 3-dicarboxylic acid [(1*S*,3*R*)-ACPD], (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG), 7-hydroxyiminocyclopropan[*b*]chromen-1*a*-carboxylic acid ethyl ester (CPCCOEt), 3,5-dihydroxyphenylglycine (3,5-DHPG), (+)-2-methyl-4-carboxyphenylglycine (LY367385) and MPEP were purchased from Tocris Cookson (Bristol, UK). Tissue culture reagents were obtained from GIBCO-BRL (San Giuliano Milanese, MI, Italy) and ICN Pharmaceuticals (Opera, MI, Italy). lactate dehydrogenase (LDH) activity was quantitated using the Cytotoxicity Detection Kit (LDH) from Boehringer Mannheim Italia (Monza, MI, Italy). Propidium iodide (PI) was purchased from Molecular Probes (Leiden, the Netherlands).

2.2. Determination of agonist-induced phospholipase C activity in BHK transfected cells

Baby hamster kidney (BHK) cells stably transfected with mGlu1a or mGlu5a receptors were provided by Dr. C. Thomsen (Novo Nordisk, Malov, Denmark) and cultured in Dulbecco's modified Eagle's medium supplemented with 5% dialyzed fetal bovine serum, 2 mM glutamine, 0.05 mg/ml gentamicin and 0.1 mg/ml neomycin in a humidified atmosphere (95% air/5% CO_2) at 37 °C. In addition, the incubation medium of the transfected cells was supplemented with G-418 and methotrexate. Cells were maintained in a humidified atmosphere (95% air/5% CO_2) at 37 °C and subcultured (every second day) using 0.05% trypsin/EDTA. Measurements of pospholipase C-catalyzed [^{3}H]inositol phosphate (IP) formation in transfected cells expressing mGlu receptors were performed as previously described in detail (Moroni et al., 1997).

2.3. OGD in murine cortical cell cultures

Cultures of mixed cortical cells containing both neuronal and glial elements were prepared from fetal (E14–15) mouse brain, used at 14 days in vitro (DIV) and exposed to OGD as previously described in detail (Pellegrini-Giampietro et al., 1999a,b). The culture medium was replaced by thorough exchange with a glucose-free balanced salt solution, which had previously been saturated with 95% N_2/5% CO_2 and heated to 37 °C. Multiwells were then sealed into an airtight incubation chamber equipped with inlet and outlet valves and 95% N_2/5% CO_2 was blown through the chamber for 10 min to ensure maximal removal of oxygen. The chamber was then sealed and placed into the incubator at 37 °C for 60 min. OGD was terminated by removing the cultures from the chamber, replacing the exposure solution with oxygenated medium and returning the multiwells to the incubator under normoxic conditions. The extent of neuronal death was assessed 24 h later. In this system, 60-min OGD induced a neuronal damage that was approximately 75% of the maximal degree of neuronal injury achieved by exposing the cultures for 24 h to 1 mM glutamate (see Pellegrini-Giampietro et al., 1999a).

2.4. OGD in rat organotypic hippocampal cultures

Organotypic hippocampal slice cultures were prepared from 8-day-old rats, used at 14 DIV and exposed to OGD as previously reported in detail (Pellegrini-Giampietro et al., 1999a,b). Briefly: the slices were preincubated for 30 min in serum-free medium and then subjected to OGD by exposing them to a serum-free medium devoid of glucose and previously saturated with 95% N_2/5% CO_2. Following 30 min incubation at 37 °C in the airtight anoxic chamber, the cultures were transferred to oxygenated serum-free medium containing 5 mg/ml glucose and 5 μg/ml PI and returned to the incubator under normoxic conditions until neuronal injury was evaluated 24 h later. In this system, 30-min OGD induced a CA1 pyramidal cell damage that was approximately 75% of the maximal damage achieved by exposing the slices for 24 h to 10 mM glutamate (see Pellegrini-Giampietro et al., 1999a).

2.5. Assessment of neuronal injury in vitro

Cell damage in mixed cortical cultures was quantitatively evaluated by measuring the amount of LDH released from injured cells into the extracellular fluid 24 h following exposure to OGD or glutamate, as previously described

(Pellegrini-Giampietro et al., 1999a,b). Background LDH release was determined in control cultures not exposed to OGD and was subtracted from all experimental values. The resulting value correlated linearly with the degree of cell loss estimated by observation of cultures under phase-contrast microscopy or under bright-field optics following of 5 min incubation with 0.4% trypan blue, which stains debris and nonviable cells. Cell injury was assessed in organotypic hippocampal cultures using PI, a polar dye which enters the cells only if the membrane is damaged and becomes fluorescent upon binding to DNA. PI fluorescence was viewed using an Intracellular Imaging (Cincinnati, OH, USA) imaging system, consisting of a Xenon-arc lamp, a Nikon TMS-F inverted microscope equipped with a Groony fluorescence optics module for epi-illumination, a low power objective (four times), and a rhodamine filter set. In order to quantify cell death, the CA1 hippocampal subfield was identified and encompassed by a frame using the drawing function in the image software and the intensity of PI fluorescence (i.e., the mean of the fluorescence intensity values of each pixel in the area defined by the frame) was collected (see, for details, Pellegrini-Giampietro et al., 1999a,b). Background PI fluorescence was determined in control cultures not exposed to OGD and was subtracted to all experimental values. There was a linear correlation between CA1 PI fluorescence intensity and the number of injured CA1 pyramidal cells as detected by morphological criteria (see Pellegrini-Giampietro et al., 1999a).

2.6. Transient global ischemia and assessment of CA1 pyramidal cell injury in gerbils

Mongolian gerbils (*Meriones unguiculatus*, Morini, Reggio Emilia, Italy) weighing 60–80 g were anesthetized with chloral hydrate (300 mg/kg i.p) and positioned in a stereotaxic frame for the implantation of a polyethylene cannula (inner diameter: 0.28 mm, outer diameter: 0.61 mm) into their right cerebral ventricle using the following stereotaxic coordinates: 0.5 mm posterior from bregma, 0.5 mm lateral, 2.2 mm below the skull surface. After 24-h recovery, all (both control and treated) animals were subjected to transient global ischemia as previously described (Pellegrini-Giampietro et al., 1994). Briefly, gerbils were anesthetized with a mixture of 2% halothane, 75% nitrogen and 20% oxygen. Through a ventral midline neck incision, both common carotid arteries were isolated and occluded for 5 min using micro-arterial clamps. At the end of the occlusion period, the clamps were released allowing restoration of carotid blood flow and the incision was sutured. Halothane administration was discontinued immediately after carotid occlusion and the animals, which remained unresponsive for approximately 20 min, were considered exposed to forebrain ischemia. Sham-operated controls were anesthetized and subjected to the neck incision but their carotids were not occluded. Sham-operated and ischemic gerbils were treated intracerebroventricularly with either vehicle or mGlu receptor antagonists dissolved in 5 μl of saline immediately after the carotid occlusion and 60 min postischemia. In a separate set of experiments, MPEP was administered intraperitoneally at 10 mg/kg immediately after the occlusion and 3 h postischemia, according to Rao et al. (2000). Body temperature was monitored and maintained at 37 ± 0.5 °C with a rectal thermistor and heating pad until the animals had fully recovered from anesthesia. The animals were then placed in a warm room and the rectal temperature was periodically recorded. Seven days after the ischemic insult, the gerbils were sacrificed by decapitation, their brains rapidly removed and frozen in dry ice. Coronal sections (20 μm) were cut in a cryostat and stained with toluidine blue. At least four microscopic sections for each animal were analyzed by a researcher who was blind to the experimental treatment. Hippocampal injury was assessed quantitatively by counting the number of CA1 pyramidal cells appearing histologically normal as previously described in detail (Pellegrini-Giampietro et al., 1994). The experimental protocol was carried out according to the Italian guidelines for animal care (DL 116/92) in application of the European Communities Council Directive (86/609/EEC) and was formally approved by the Animal Care Committee of the Department of Pharmacology of the University of Florence.

3. Results

3.1. Effects of Group I mGlu receptor antagonists on agonist-induced phosphoinositide hydrolysis in BHK cells transfected with mGlu1a or mGlu5a receptors

We have previously shown that AIDA is a competitive antagonist displaying preferential activity on mGlu1 (IC_{50} = 215 μM) over mGlu5 (IC_{50} > 1 mM) receptors (Moroni et al., 1997) and that CBGP is a slightly more potent mGlu1 antagonist (IC_{50} = 65 μM) that exhibits partial agonist activity on mGlu5 receptors (Mannaioni et al., 1999). Accordingly, relatively large concentrations of AIDA (300 μM) inhibited the formation of $[^3H]$IP induced by 100 μM (1S,3R)-ACPD in BHK cells transfected with mGlu1a but not with mGlu5a receptors, whereas CBPG (300 μM) reduced the phosphoinositide response in cells expressing both receptor subtypes (Fig. 1) (see also Attucci et al., 2001). The noncompetitive antagonist CPCCOEt (Litschig et al., 1999) displayed a significant degree of selectivity for mGlu1 over mGlu5 receptors at 10 μM (not shown) and 30 μM (Fig. 1) when tested against the formation of $[^3H]$IP evoked by 100 μM (1*S*,3*R*)-ACPD. Its potency as an antagonist was, however, quite similar in cells transfected with mGlu1a (IC_{50} = 10 μM) and mGlu5a (IC_{50} = 50 μM) receptors, confirming that at concentrations higher than 30 μM CPCCOEt also blocks the phosphoinositide response evoked by stimulation of mGlu5 receptors (Attucci et al., 2001; Casabona et al., 1997). Fig. 1 shows that MPEP was a

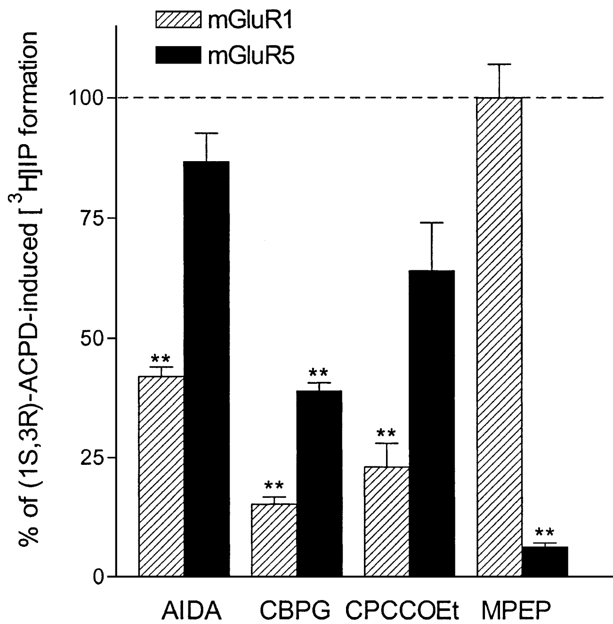

Fig. 1. Inhibition of agonist-evoked [^{3}H]IP formation in BHK cells expressing mGlu1 or mGlu5 receptors: effects of 300 μM AIDA, 300 μM CBGP, 30 μM CPCCOEt and 1 μM MPEP. Each bar represents the formation of [^{3}H]IP formation calculated as a percent of maximal response induced by 100 μM (1*S*,3*R*)-ACPD and is the mean ± S.E.M. of at least three experiments conducted in triplicate. ** $P<.01$ vs. maximal response (ANOVA + Tukey's *w* test).

potent and selective mGlu5 receptor antagonist, with an IC_{50} of 35 nM, and it had no significant effect in BHK expressing mGlu1a receptors up to 10 μM.

3.2. Neuroprotective effect of mGlu1 but not mGlu5 receptor antagonists against OGD in vitro

In murine cortical cell cultures, 60-min OGD led to the degeneration of approximately 75% of the neurons, whereas exposure to 1 mM glutamate lesioned virtually all neuronal cells with no damage of the underlying glial cell layer. In this study, we compared the neuroprotective effects of a number of mGlu1 receptor antagonists with those of the potent and selective mGlu5 receptor antagonist MPEP. In confirmation of our previous results (Pellegrini-Giampietro et al., 1999a,b), the addition of AIDA (300 μM) and CBPG (300 μM) to the incubation medium during the 60-min OGD challenge and the subsequent 24-h recovery period significantly attenuated OGD neurotoxicity by 83% and 69%, respectively (Table 1). Similarly, CPCCOEt (10–30 μM) and the competitive mGlu1 receptor antagonist LY367385 (30–100 μM) displayed a significant neuroprotection in this model, as revealed by the reduction of the OGD-induced efflux of LDH into the medium (Table 1). Fig. 2A shows that LY367385, in accordance with its reported IC_{50} of 8.8 μM (Clark et al., 1997), was more potent than AIDA in producing its neuroprotective effect. On the contrary, when the selective mGlu5 antagonist MPEP was added to the cultures at 0.1 or 1 μM, no protective effect could be observed (Table 1 and Fig. 2A). At higher concentrations, which are known to reduce NMDA receptor-mediated responses (Movsesyan et al., 2001; O'Leary et al., 2000), MPEP exhibited a slight attenuation of OGD-induced neuronal death (Fig. 2A). Incubation of cortical cultures with the nonselective Group I mGlu agonist 3,5-DHPG (300 μM) but not with the selective mGlu5 receptor agonist CHPG (1 mM) significantly enhance the release of LDH evoked by OGD (Table 1), further suggesting a role for mGlu1 receptors in mediating OGD neurotoxicity in this model.

The neuroprotective effects of MPEP were also investigated in rat organotypic hippocampal slice cultures, another in vitro model that has been widely used to study postischemic neuronal injury. Exposure of hippocampal slices to OGD for 30 min led to a significant increase in PI fluorescence in the hippocampal CA1 subregion 24 h later. The results obtained with mGlu1 receptor antagonists in organotypic slices (Table 1 and Fig. 2B) were very similar to those observed in cortical cells, except for the fact that CPCCOEt (1–300 μM) failed to reduce CA1 pyramidal cell death following OGD. In accordance with the results obtained in mixed cortical cultures, no changes in CA1 pyramidal cell death were observed when organotypic hippocampal slices exposed to OGD were treated with MPEP or CHPG (Table 1 and Fig. 2B).

Table 1
Effects of Group I mGlu receptor agents on neuronal injury induced by OGD in murine cortical cell and rat organotypic hippocampal cultures

	% of OGD-induced neuronal death			
	Cortical cells		Hippocampal slices	
	LDH release (units/l)	%	CA1 PI fluorescence	%
OGD	312 ± 10	100	81 ± 6	100
OGD + 300 μM AIDA	53 ± 7 **	17	25 ± 3 **	31
OGD + 300 μM CBGP	96 ± 7 **	31	32 ± 3 **	40
OGD + 10 μM CPCCOEt	174 ± 12 *	56	79 ± 3	98
OGD + 30 μM CPCCOEt	103 ± 7 **	33	77 ± 2	96
OGD + 30 μM LY367385	154 ± 22 *	49	52 ± 4	65
OGD + 100 μM LY367385	46 ± 25 **	14	37 ± 5 *	47
OGD + 0.1 μM MPEP	311 ± 25	100	80 ± 2	100
OGD + 1 μM MPEP	257 ± 25	83	76 ± 3	95
OGD + 300 μM 3,5-DHPG	448 ± 15 **	144	138 ± 5 **	172
OGD + 1 mM CHPG	335 ± 22	107	75 ± 15	93

OGD was applied for 60 (mixed cortical cells) or 30 min (organotypic hippocampal cultures), and 24 h later neuronal damage was assessed by measuring LDH release or the intensity of PI fluorescence, respectively. Drugs were added to the incubation medium during the OGD insult and the subsequent 24-h recovery period. Data are expressed as percent of OGD-induced neuronal injury. In both models, OGD-induced neuronal death was significantly reduced by the addition of 300 μM AIDA, 300 μM CBPG or 30 and 100 μM LY367385. CPCCOEt (10 and 30 μM) was neuroprotective only in cortical cell cultures exposed to OGD. No protection was observed when MPEP was added in both models. A total of 300 μM 3,5-DHPG but not 1 mM CHPG enhanced the severity of OGD-induced neuronal damage. Values represent the mean ± S.E.M. of at least five experiments.

* $P<.05$ vs. OGD.

** $P<.01$ vs. OGD (ANOVA + Tukey's *w* test).

3.3. Neuroprotective effects of mGlu1 but not mGlu5 receptor antagonists against global ischemia in vivo

We then examined the effects of mGlu1 and mGlu5 receptor antagonists on CA1 pyramidal cell death following global ischemia in the gerbil. In corroboration of our previous results (Pellegrini-Giampietro et al., 1999a,b), both AIDA (100–300 nmol two times) and CBPG (100 nmol two times) were neuroprotective when administered intracerebroventricularly immediately after and 60 min after the carotid occlusion (Table 2). The mGlu5 receptor antagonist MPEP, however, was unable to attenuate CA1 pyramidal cell death in this model, neither when administered intracerebroventricularly (at 10 pmol two times) as AIDA or CBPG, nor when administered intraperitoneally (at 10 mg/kg two times) using the paradigm described by Rao et al. (2000) (Table 2).

Table 2
Effects of Group I mGlu receptor agents on CA1 pyramidal cell death following transient global ischemia in gerbils

	% of normal CA1 pyramidal cells	*n*
Vehicle	7 ± 2	15
100 nmol AIDA icv	72 ± 6 **	7
300 nmol AIDA icv	85 ± 8 **	7
30 nmol CBPG icv	21 ± 5	6
100 nmol CBPG icv	62 ± 8 **	8
10 pmol MPEP icv	15 ± 7	7
10 mg/kg MPEP ip	18 ± 10	6

Gerbils were subjected to 5 min bilateral carotid occlusion, and 7 days later CA1 pyramidal cell death was assessed by morphological criteria. AIDA, CBGP and MPEP were administered intracerebroventricularly at the indicated dose at time 0 (carotid occlusion) and 60 min postischemia. When given intraperitoneally, MPEP was administered at time 0 and 3 h postischemia. Data are expressed as percent of normal pyramidal cells counted in the CA1 subfield. The number of CA1 pyramidal cells appearing histologically normal in a section of sham-operated animals was 220 ± 20. Administration of 100–300 nmol AIDA and 100 nmol CBGP but not MPEP induced significant neuroprotection.

** $P < .01$ vs. vehicle (ANOVA + Tukey's *w* test).

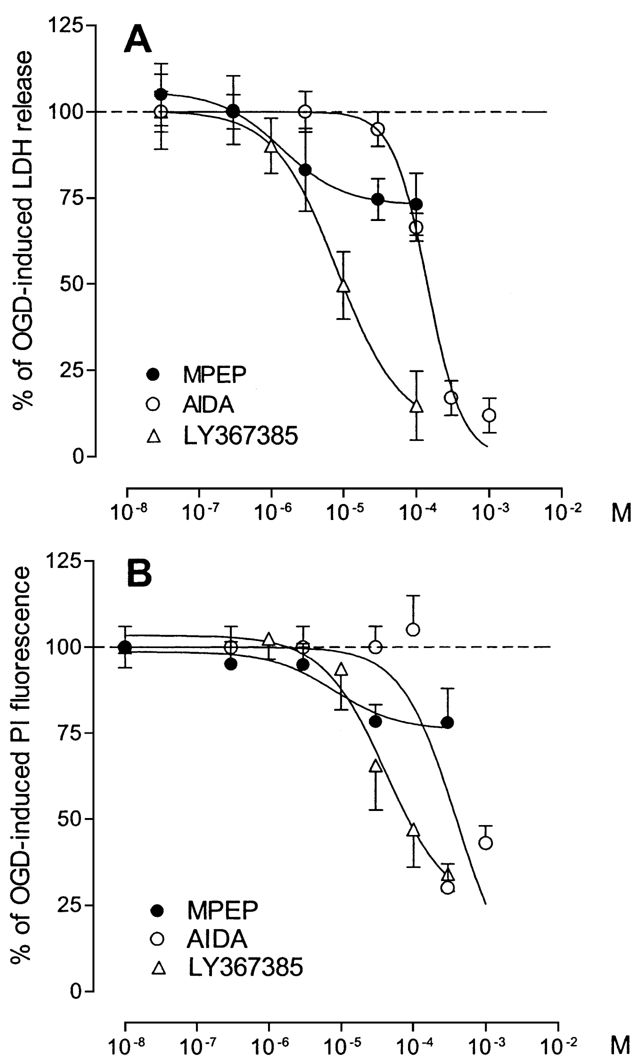

Fig. 2. AIDA and LY367385 but not MPEP protect against OGD neurotoxicity in murine cortical cell (A) and rat organotypic hippocampal (B) cultures. Neuronal death was reduced by the addition of increasing concentrations of AIDA and LY367385 to the incubation medium during the OGD insult and the subsequent 24-h recovery period. In both models, MPEP displayed a slight neuroprotective effect only at concentrations >30 μM. Data are expressed as percent of OGD-induced neuronal damage. Each point represents the mean ± S.E.M. of at least five experiments.

4. Discussion

Our results show that the presence in the incubation medium of AIDA, CBPG, CPCCOEt and LY367385, four antagonists displaying different degrees of selectivity and potency for mGlu1 receptors (Clark et al., 1997; Litschig et al., 1999; Mannaioni et al., 1999; Moroni et al., 1997), significantly attenuated neuronal death in mixed neorcortical cell and organotypic hippocampal cultures exposed to OGD. On the contrary, the highly selective noncompetitive mGlu5 receptor antagonist MPEP (Gasparini et al., 1999) was not neuroprotective in these models. Neuronal injury produced by OGD was potentiated by the nonselective Group I agonist 3,5-DHPG but was not modified by the selective mGlu5 receptor agonist CHPG. In a similar manner, mGlu1 receptor antagonists but not MPEP reduced the delayed degeneration of CA1 pyramidal cells following transient bilateral carotid in the gerbil. Taken together, these results suggest that mGlu1 but not mGlu5 receptors are involved in the mechanisms that contribute to postischemic neuronal death.

We have previously demonstrated that AIDA and CBPG reduce neuronal death in vitro and in vivo models of cerebral ischemia (Pellegrini-Giampietro et al., 1999a,b). In the present study, we have extended our observations by using two relatively new mGlu1 receptor antagonists: CPCCOEt (Litschig et al., 1999) and LY367385 (Clark et al., 1997). Whereas LY367385 proved to be equally neuro-

ptrotective both in mixed cortical cells and organotypic hippocampal slices exposed to OGD, the noncompetitive antagonist CPCCOEt significantly reduced neuronal death only in the cortical cell model. It is possible that esterases present in the medium may cleave CPCCOEt, and thus prevent accumulation of adequate and predictable concentrations of the intact compound in the slices. The IC_{50} value for the inhibition of agonist-evoked phosphoinositide hydrolysis observed for CPCCOEt in BHK cells transfected with mGlu1 receptors correlated well with the concentrations (10 and 30 μM) that exhibited neuroprotection against OGD in cortical cells. We did not use higher concentrations in hippocampal slices because at >30 μM CPCCOEt exhibited also a reduction in the formation of IPs in cells transfected with mGlu5 receptors (see also Attucci et al., 2001).

In order to elucidate whether mGlu5 receptors are involved in determining neuronal death after ischemia, we used the potent and selective noncompetitive antagonist MPEP. However, at concentrations (0.1–1 μM) that selectively abolished the phosphoinositide breakdown evoked by mGlu5 but not mGlu1 receptor stimulation, MPEP failed to attenuate neuronal death induced by OGD in cortical cells and hippocampal slices. MPEP showed a slight degree of neuroprotection at relatively higher concentrations (>30 μM), which have been shown to interact with NMDA receptors (Movsesyan et al., 2001; O'Leary et al., 2000). Similarly, MPEP did not reduce CA1 pyramidal cell death in the global ischemia gerbil model, neither when administered intracerebroventricularly like the neuroprotective mGlu1 receptor antagonists AIDA and CBPG (see also Pellegrini-Giampietro et al., 1999a), or intraperitoneally using the same MPEP administration protocol that reduced neuronal injury in a recent similar study (Rao et al., 2000). At present, the reasons for the discrepancy between our study and that of Rao and colleagues are not obvious, and may be ascribable to differences in the strain of animals or in the ischemic protocol.

The results of this study are in line with previous observations demonstrating that pharmacological or antisense oligonucleotide blockade of mGlu1 receptors is neuroprotective against excitotoxic insults (Battaglia et al., 2001; Bruno et al., 1999; Strasser et al., 1998), posttraumatic injury (Agrawal et al., 1998; Faden et al., 2001; Lyeth et al., 2001; Mukhin et al., 1996) and in vitro and in vivo postischemic neuronal death (Allen et al., 2000, 2001; Bruno et al., 1999; Pellegrini-Giampietro et al., 1999a,b). Antagonism of mGlu5 receptors with MPEP has been shown to protect neurons against NMDA- or β-amyloid-induced neurotoxicity (Bruno et al., 2000; Battaglia et al., 2001), whereas the use of antisense oligonucleotides directed against mGlu5 receptors was ineffective against posttraumatic neuronal injury (Mukhin et al., 1996). In a number of studies, the efficacy of higher doses of MPEP against excitotoxicity (O'Leary et al., 2000), posttraumatic injury (Movsesyan et al., 2001) or transient focal ischemia (Bao et al., 2001) has been ascribed to its action on NMDA receptors. Therefore, whereas experimental data show compelling evidence for the involvement of mGlu1 receptors in neurodegeneration, mGlu5 receptors do not appear to represent a potentially important target for neuroprotection. Rather, they are likely to play a fundamental role in a number of other CNS disorders, particularly in pain and anxiety (Spooren et al., 2001).

Antagonism of mGlu1 receptor may attenuate postischemic neuronal injury through multiple mechanisms. Antagonists may prevent Ca^{2+} overload triggered by mGlu1 receptor-induced activation of phospholipase C and inositol 1,4,5-trisphosphate receptors (Aramori and Nakanishi, 1992) or by the facilitatory coupling between ryanodine receptors and L-type Ca^{2+} channels (Chavis et al., 1996). Antagonism of mGlu1 receptors may also exacerbate neuronal injury by blocking the facilitatory action of mGlu1 receptor agonists upon NMDA receptors (Aniksztejn et al., 1992; Mannaioni et al., 1996) or through an enhanced release of arachidonic acid (Allen et al., 2001). Conversely, the mechanisms underlying a differential role for mGlu1 and mGlu5 receptors in postischemic neuronal death are presently unknown. The two receptors are highly homologous and they are both coupled to G_q proteins and to the activation of the phosphoinositide hydrolysis. However, functional differences for mGlu1 and mGlu5 have been detected when they are expressed in the same cells. For example, stimulation of mGlu1 and mGlu5 receptors in transfected cells leads to diverse response patterns of intracellular Ca^{2+} increase (Kawabata et al., 1996), while in the CA1 hippocampal area the roles of mGlu1 and mGlu5 receptors in regulating the excitability of pyramidal cells are highly segregated (Mannaioni et al., 2001). Moreover, because mGlu5 receptors are expressed in principal neurons and mGlu1 receptors are preferentially localized on GABAergic interneurons in mixed cultures of cortical cells (Battaglia et al., 2001) and in the hippocampal CA1 area (Baude et al., 1993), it has been proposed that the selective blockade of mGlu1 receptors leads to a distinct mechanism of neuroprotection that operates via the release of GABA in these regions (Battaglia et al., 2001; Pellegrini-Giampietro et al., 1999b). Recently, it has been hypothesized that the differential expression of the NMDA receptor subunit NMDAR-2C in neurons or mGlu5-expressing astrocytes may have an influence on neurotoxic/neuroprotective effects mediated by group I mGlu receptor agonists and antagonists (Nicoletti et al., 1999).

In conclusion, we have demonstrated that inhibition of mGlu1 receptors is neuroprotective in three different models of cerebral ischemia, whereas the selective blockade of mGlu5 receptor is ineffective. These results point out the differential role of the two Group I mGlu receptors subtypes, suggesting that mGlu1 rather than mGlu5 receptors are implicated in the pathways leading to postischemic neuronal injury. Therefore, mGlu1 receptors antagonists should be regarded as promising targets for the development of innovative drugs against cerebral ischemia.

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