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Differential role of mGlu1 and mGlu5 receptors in rat hippocampal slice models of ischemic tolerance

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Abstract

Activation of glutamate receptors has been proposed as a key factor in the induction of ischemic tolerance. We used organotypic rat hippocampal slices exposed to 30 min oxygen–glucose deprivation (OGD) to evaluate postischemic pyramidal cell death in the CA1 subregion. In this model, 10 min exposure to OGD 24 h before the exposure to toxic OGD was not lethal and reduced the subsequent OGD neurotoxicity by ~53% (ischemic preconditioning). Similarly, a 30 min exposure to the group I mGlu receptor agonist DHPG (10 μM) significantly reduced OGD neurotoxicity 24 h later (pharmacological preconditioning). Ischemic tolerance did not develop when either the selective mGlu1 antagonists LY367385 and 3-MATIDA or the AMPA/KA antagonist CNQX were present in the incubation medium during exposure to sublethal OGD. Neither the NMDA antagonist MK801 nor the mGlu5 antagonist MPEP affected the preconditioning process. On the other hand, pharmacological preconditioning was prevented not only by LY367385 or CNQX, but also by MPEP. In preconditioned slices, the toxic responses to AMPA or NMDA were reduced. The neurotoxicty of 100 μM DHPG in slices simultaneously exposed to a mild (20 min) OGD was differentially altered in the two preconditioning paradigms. After ischemic preconditioning, DHPG neurotoxicity was reduced in a manner that was sensitive to LY367385 but not to MPEP, whereas after pharmacological preconditioning it was enhanced in a manner that was sensitive to MPEP but not to LY367385. Our results show that mGlu1 and mGlu5 receptors are differentially involved in the induction and expression of ischemic tolerance following two diverse preconditioning stimuli.

Introduction

Exposure of brain slices or isolated neurons in culture to a short sublethal period of oxygen and glucose deprivation (OGD) has been shown to reduce cell death induced by a subsequent more prolonged and otherwise lethal hypoxic or ischemic insult (Schurr *et al*., 1986; Grabb & Choi, 1999). The molecular mechanisms leading to an increased neuronal resistance to ischemic injury (a phenomenon known as ischemic preconditioning or ischemic tolerance) are still not clearly understood and a number of possible induction pathways, including activation of glutamate receptors and changes in the concentration of intracellular free Ca^{2+}, have been proposed (Dirnagl *et al*., 2003; Bickler & Fahlman, 2004; Gidday, 2006). Recently, it has been demonstrated that brief activation of group I metabotropic glutamate (mGlu) receptors (consisting of mGlu1 and mGlu5 receptor subtypes) with the selective agonist (S)-3,5-dihydroxyphenylglycine (DHPG) reduces NMDA-mediated Ca^{2+} inflow and the resulting cell death, suggesting that activation of these mGlu subtypes may contribute to the induction of tolerance towards excitotoxic events (Blaabjerg *et al*., 2003; Baskys *et al*., 2005).

Both mGlu1 and mGlu5 receptors are present in the hippocampus: mGlu5 is the predominant receptor expressed on CA1 pyramidal cells (Baude *et al*., 1993; Romano *et al*., 1995) whereas the mGlu1 subtype in CA1 has been detected mostly in GABAergic interneurons of the stratum oriens (Shigemoto *et al*., 1992; Berthele *et al*., 1998; Ferraguti *et al*., 1998). Activation of mGlu1 receptors has been shown to regulate CA1 pyramidal cell excitability by modulating GABAergic transmission (Gereau & Conn, 1995; Fitzsimonds & Dichter, 1996) and/or by directly acting on principal neurons (Mannaioni *et al*., 2001). Previous studies from our laboratory have shown that mGlu1 but not mGlu5 receptor antagonists significantly reduce postischemic neuronal damage in both *in vitro* and *in vivo* models of cerebral ischemia (Pellegrini-Giampietro *et al*., 1999a,b; Cozzi *et al*., 2002; Meli *et al*., 2002), suggesting that activation of this particular receptor subtype significantly contributes to the events leading to postischemic neurodegeneration. Along this line, we also showed that DHPG exacerbated neuronal injury in mixed cortical cultures and organotypic hippocampal slices exposed to OGD (Pellegrini-Giampietro *et al*., 1999a).

With the aim of investigating the role of group I mGlu receptors in both the induction and the expression of ischemic tolerance, we used organotypic hippocampal slices exposed to either ischemic or pharmacological preconditioning paradigms, and a number of glutamate receptor agonists and antagonists. In particular, we examined whether activation of mGlu1 and/or mGlu5 receptors was necessary to initiate the process leading to ischemic tolerance and whether the expression of ischemic tolerance was associated with changes in glutamate receptor-mediated neurotoxicity.

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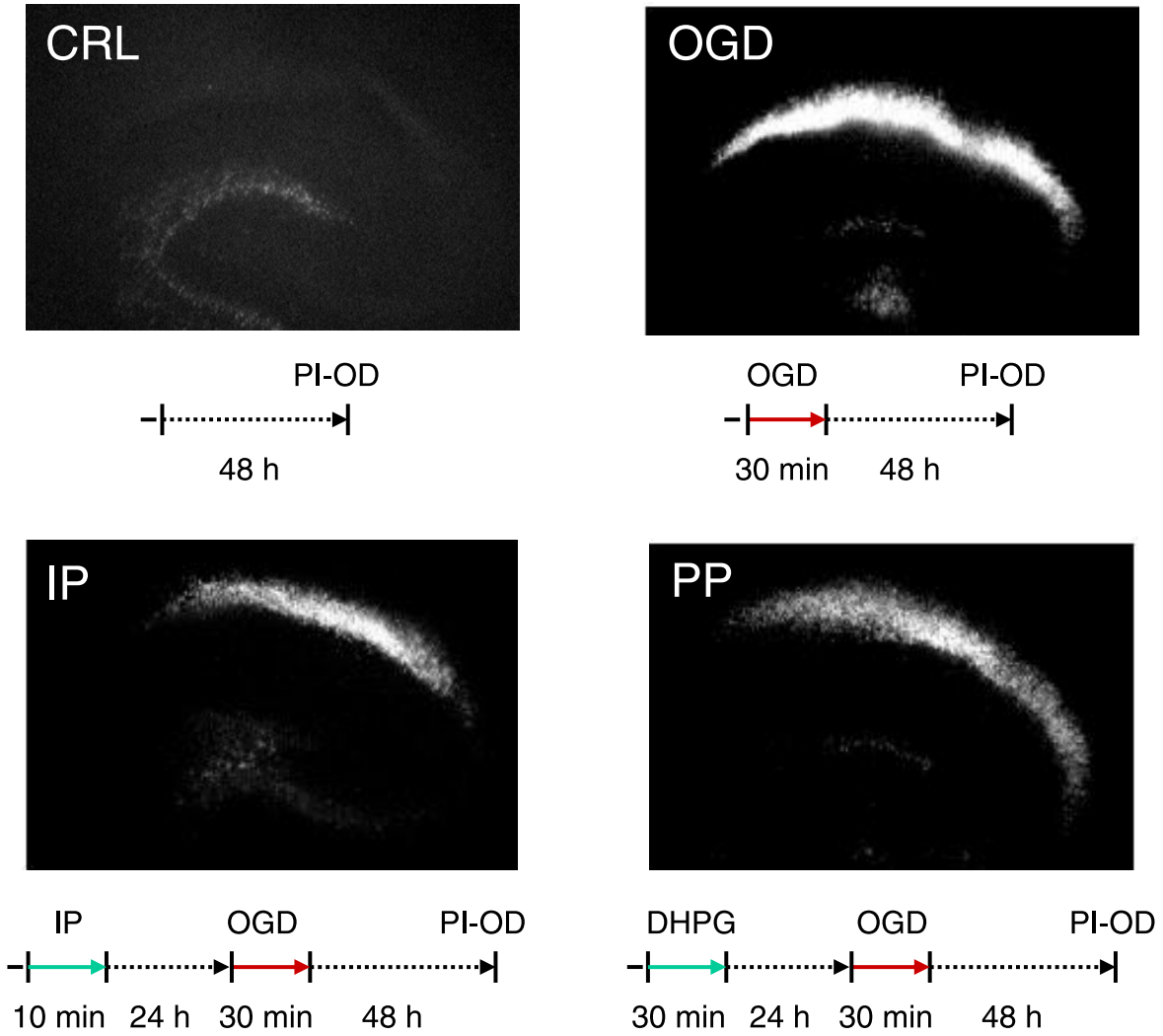

FIG. 1. Ischemic and pharmacological preconditioning in organotypic hippocampal slices. Cultured slices were exposed to 30 min OGD and 48 h later incubated with PI for fluorescence detection of its optical density (OD). (CRL) Hippocampal slice under normoxic and drug-free conditions (background PI fluorescence). (OGD) Hippocampal slice exposed to 30 min OGD, displaying more intense PI labelling in the CA1 region than in the CA3 and dentate gyrus. (IP) Hippocampal slice exposed to ischemic preconditioning (IP) 24 h prior to OGD, displaying reduced CA1 PI labelling as compared to OGD alone. (PP) Hippocampal slice exposed to pharmacological preconditioning (PP: DHPG 10 μM, 30 min) 24 h prior to OGD, displaying a similar reduction in CA1 PI labelling.

Materials and methods

Materials

The glutamate receptor agents DHPG, (S)-(+)-alpha-amino-4-carboxy-2-methylbenzene-acetic acid (LY367385), 3-methyl-aminothiophene dicarboxylic acid (3-MATIDA), 2-methyl-6-(phenyl-ethynyl)-pyridine (MPEP), (+)MK801 maleate (MK801) and 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX) 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). Propidium iodide (PI) was purchased from Molecular Probes (Leiden, the Netherlands). If not otherwise declared, all cell culture media were purchased from MP Biomedicals (Irvine, CA, USA).

Preparation of organotypic cultures

Animal use was approved by the Ethical Committee for Animal Care, Department of Pharmacology, University of Florence. All experiments conformed to Italian and international guidelines on the ethical use of animals. The number of animals was kept to the minimum level necessary for this study. Organotypic slice cultures of the hippocampus were prepared as previously described in detail (Pellegrini-Giampietro *et al.*, 1999a). Briefly: neonatal pups (8–10 days old) of Wistar rats were decapitated and the hippocampi were dissected free from the forebrain and transversely sliced (400 μm) on a McIlwain tissue chopper. Slices were placed in Hanks's balanced salt solution (supplemented with 5 mg/mL glucose and 3.75 μg/mL amphotericin B) at 4 °C. The slices were then transferred onto 30-mm-diameter membrane inserts (Millicell-CM; Millipore, Italy) and placed into six-well culture trays with 1.2 mL of slice culture medium per well. The slice culture medium consisted of: Eagle's minimal essential medium, 50%; Hanks's balanced salt solution, 25%; heat-inactivated horse serum, 25%; L-glutamine, 2 mM; glucose, 5 mg/mL; and amphotericine B, 3.75 μg/mL. The cultures were maintained at 37 °C in an atmosphere of humidified air and 5% CO_2. The slice culture medium was changed twice a week. Slices were kept in culture for 12–14 days before experiments. At the end of this culture period and prior to the

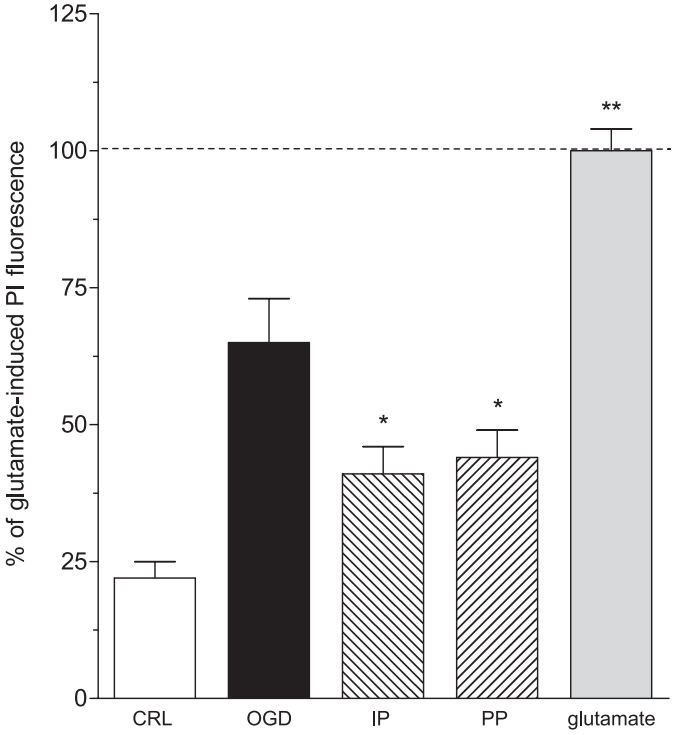

FIG. 2. Ischemic (IP) and pharmacological (PP) preconditioning attenuated CA1 damage produced by OGD in organotypic hippocampal slices. OGD was applied for 30 min and 48 h later CA1 damage was assessed by measuring the intensity of PI fluorescence. Data are expressed as percentages of maximal neuronal death, as measured following the application of 10 mM glutamate for 48 h. CA1 damage was significantly reduced when slices were pre-exposed to a brief (10 min) OGD (IP) or to 10 μM DHPG for 30 min (PP) 24 h before the toxic challenge of 30 min OGD. Bars represent the mean ± SEM of $n \geq 6$ experiments run in quadruplicate. *$P < 0.05$ and **$P < 0.01$ vs. OGD.

TABLE 1. Effects of glutamate receptor antagonists on the induction of ischemic preconditioning in rat organotypic hippocampal slice cultures

	CA1 PI fluorescence	(%)
Basal	54 ± 11	(22)
30 min OGD	145 ± 20	(62)
Glutamate (10 mM)	232 ± 10**	(100)
Ischemic preconditioning (IP)	97 ± 20*	(41)
IP + 100 μM LY367385	169 ± 22	(73)
IP + 10 μM MPEP	75 ± 16*	(31)
IP + 100 μM MK801	87 ± 20*	(36)
IP + 10 μM CNQX	144 ± 4	(60)

Data are presented as means ± SEM, with percentages of glutamate-induced neuronal injury in parentheses. OGD was applied for 30 min and, 48 h later, neuronal damage was assessed by measuring the intensity of PI fluorescence. Maximal damage was achieved by exposing the slices to 10 mM glutamate for 48 h. Ischemic preconditioning (IP) was performed by exposing the slices to a sublethal period (10 min) of OGD 24 h before the toxic challenge of 30 min OGD. Drugs were added to the incubation medium 15 min prior to and during IP, but not during the subsequent recovery period. Data are expressed as CA1 fluorescence 48 h after the exposure to glutamate or OGD. OGD-induced neuronal death was significantly reduced by IP. LY367385 (100 μM) and CNQX (10 μM) but not MPEP (10 μM) nor MK801 (100 μM) prevented IP. Values represent the mean ± SEM of $n \geq 6$ experiments. *$P < 0.05$ and **$P < 0.01$ vs. 30 min OGD.

experiments all slices were screened for viability by incubating them for 30 min with PI (5 μg/mL); slices displaying signs of neurodegeneration were discarded from the study.

TABLE 2. Effects of glutamate receptor antagonists on the induction of pharmacological preconditioning with DHPG in rat organotypic hippocampal slice cultures

	CA1 PI fluorescence	(%)
Basal	56 ± 11	(23)
30 min OGD	162 ± 22	(71)
Glutamate (10 mM)	241 ± 1**	(100)
DHPG 10 μM (PP)	107 ± 13*	(44)
PP + 100 μM LY367385	180 ± 7	(74)
PP + 10 μM MPEP	161 ± 11	(67)
PP + 100 μM MK801	66 ± 15*	(28)
PP + 10 μM CNQX	194 ± 13	(80)

Data are presented as means ± SEM, with percentages of glutamate-induced neuronal injury in parentheses. OGD was applied for 30 min and, 48 h later, neuronal damage was assessed by measuring the intensity of PI fluorescence. Maximal damage was achieved by exposing the slices to 10 mM glutamate for 48 h. Pharmacological preconditioning (PP) was performed by exposing the slices to 10 μM DHPG for 30 min 24 h before the toxic challenge of 30 min OGD. Drugs were added to the incubation medium 15 min prior and during PP, but not during the subsequent recovery period. Data are expressed as CA1 fluorescence 48 h after the exposure to glutamate or OGD. OGD-induced neuronal death was significantly reduced by PP. LY367385 (100 μM), MPEP (10 μM) and CNQX (10 μM), but not MK801 (100 μM) prevented PP. Values represent the mean ± SEM of $n \geq 6$ experiments. *$P < 0.05$ and **$P < 0.01$ vs. 30 min OGD.

OGD in rat hippocampal slices

Organotypic hippocampal slice cultures were exposed to OGD as previously reported in detail (Pellegrini-Giampietro *et al*., 1999a). Briefly, the slices were subjected to OGD by exposing them to a serum- and glucose-free medium saturated with 95% N_2 and 5% CO_2 at 37 °C in a gassed incubator equipped with an oxygen gas controller (BioSpherix, New York, USA). Following 30 min of OGD the cultures were transferred to oxygenated serum-free medium (Eagle's minimal essential medium, 75%; Hank's balanced salt solution, 25%; L-glutamine, 2 mM; and amphotericin B, 3.75 μg/mL) containing 5 mg/mL glucose and returned to the incubator under normoxic conditions until neuronal injury was evaluated 48 h later. The potential toxicity of amphotericin B was tested in a series of experiments carried out in the absence and in the presence of the drug; these produced virtually identical results.

Assessment of neuronal injury

Cell injury was assessed using the fluorescent dye PI, a highly polar compound which is normally excluded from cells with an intact membrane. When the membrane is damaged, PI enters the cells and upon binding to exposed DNA becomes highly fluorescent. PI (5 μg/mL) was added to the medium at the end of the 48-h post-OGD recovery period. Thirty minutes later, fluorescence was viewed using an inverted fluorescence microscope (Olympus IX-50; Solent Scientific, Segensworth, UK) equipped with a xenon-arc lamp, a low-power objective (4×) and a rhodamine filter. Images were digitized using a video image obtained by a CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) controlled by software (InCyt Im1^{TM}; Intracellular Imaging Inc., Cincinnati, OH, USA) and subsequently analysed using the Image-Pro Plus morphometric analysis software (Media Cybernetics, Silver Spring, MD, USA). In order to quantify cell death, the CA1 hippocampal subfield was identified and encompassed in a frame using the drawing function in the image software (ImageJ; NIH, Bethesda,

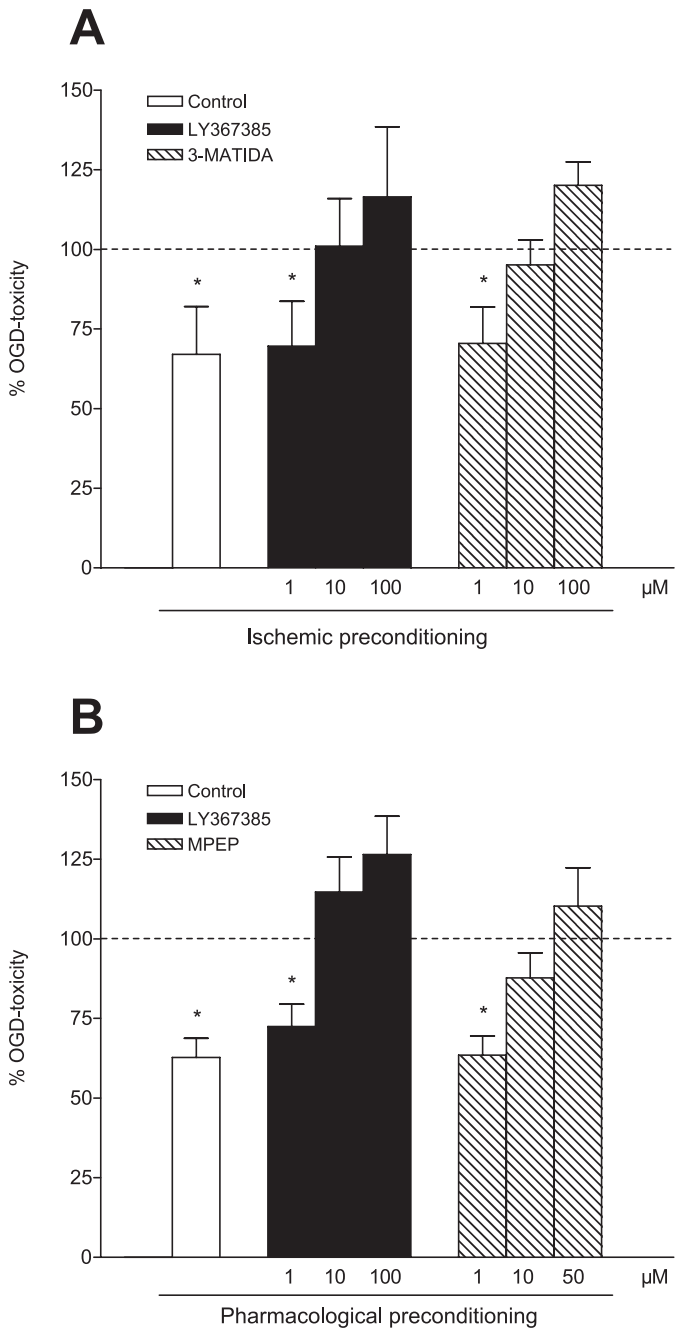

FIG. 3. Effects of group I mGlu receptor antagonists on the induction of (A) ischemic and (B) pharmacological preconditioning (IP and PP, respectively) in rat organotypic hippocampal slices. Experiments were carried out as indicated in the legends to Tables 1 and 2. Data are expressed as percentages of OGD-induced CA1 toxicity, as determined by the intensity of PI fluorescence in this region. OGD toxicity was significantly reduced by (A) IP and (B) PP. IP was significantly prevented by the mGlu1 receptor antagonists LY367385 (10–100 µM) and 3-MATIDA (10–100 µM), whereas PP was significantly prevented by LY367385 (10–100 µM) and by the mGlu5 receptor antagonist MPEP (10–50 µM). Bars represent the mean ± SEM of $n \geq 4$ experiments run in quadruplicate. *$P < 0.05$ vs. OGD.

MD, USA) and the optical density of PI fluorescence was detected. There was a linear correlation between CA1 PI fluorescence and the number of injured CA1 pyramidal cells as detected by morphological criteria (see Pellegrini-Giampietro *et al.*, 1999a).

Experimental design

Typical examples of slice cultures and time settings for preconditioning experiments are given in Fig. 1. Paradigms for control and OGD experiments are shown in the upper row and preconditioning protocols are shown in the lower row. For ischemic preconditioning experiments, slices were pre-exposed to a sublethal period of OGD (10 min) and then, 24 h later, to 30 min OGD (Fig. 1, IP). For pharmacological preconditioning experiments with DHPG (Fig. 1, PP), slices were pre-exposed to the group I mGlu receptor agonist DHPG (10 µM) for 30 min and then, 24 h later, to 30 min OGD.

Statistical analysis

Data are given as mean ± SEM of n experiments. Statistical significance of differences was analysed using ANOVA followed by Tukey's w-test. A P-value of 0.05 was considered significant. All statistical calculations were performed using GraphPad Prism 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Ischemic and pharmacological preconditioning

In accord with previous observations (Pellegrini-Giampietro *et al.*, 1999a,b; Meli *et al.*, 2002), exposure of hippocampal slices to OGD for 30 min caused an increase in PI fluorescence in the CA1 subregion that was ~65% of the fluorescence intensity obtained by exposing the slices to 10 mM glutamate for 48 h (100% neuronal damage). Figures 1 and 2 and Table 1 show that exposure to a sublethal period of OGD (10 min) reduced the toxic effects of a subsequent (24 h later) 30 min exposure to OGD; this ischemic preconditioning paradigm significantly reduced PI fluorescence by 53 ± 5%. Interestingly, a similar significant reduction (by 49 ± 5%) in CA1 damage induced by 30 min OGD was observed by pre-exposing the preparation to DHPG (10 µM) for 30 min 24 h before the toxic challenge (pharmacological preconditioning; Figs 1 and 2, Table 2). No reduction was observed when pre-exposure of slices to the same paradigm (10 µM DHPG for 30 min) was carried out 2 h before exposure to 30 min OGD. Larger DHPG concentrations (100 µM) did not induce pharmacological preconditioning but rather exacerbated the subsequent OGD-induced cell death (data not shown).

Effects of glutamate receptor antagonists on the induction of ischemic or pharmacological preconditioning

To evaluate whether activation of ionotropic or group I metabotropic glutamate receptors is involved in the development of ischemic or pharmacological preconditioning, a number of selective antagonists were used. Tables 1 and 2 show that the AMPA/kainate receptor antagonist CNQX (10 µM) prevented the development of tolerance to OGD when present in the incubation medium during pre-exposure of slices to both sublethal OGD (ischemic preconditioning) and to 10 µM DHPG (pharmacological preconditioning). In contrast, the NMDA antagonist MK801 (100 µM) did not modify the induction of tolerance to OGD. Ischemic preconditioning was also prevented by the selective mGlu1 receptor antagonists LY367385 (10–100 µM) and 3-MATIDA (10–100 µM) (Fig. 3A, Table 1) but not by the mGlu5 receptor antagonist MPEP (10 µM, Table 1). Interestingly, the tolerance to OGD induced by DHPG could be completely prevented by either LY367385 (10–100 µM) or MPEP (10–50 µM Fig. 3B, Table 2), suggesting that the mechanisms and processes of tolerance induction are not identical in the two experimental paradigms used.

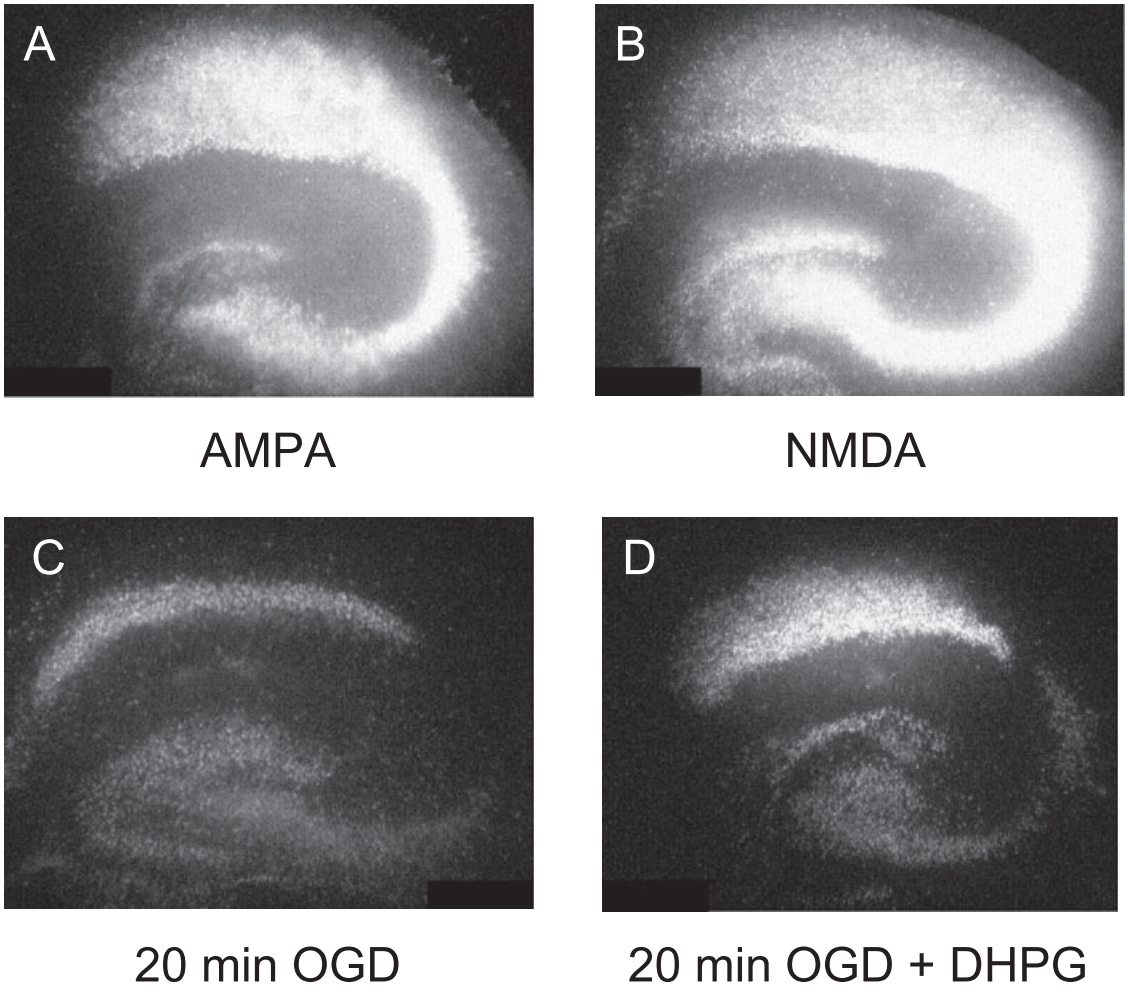

FIG. 4. Excitotoxic injury in organotypic hippocampal slices. Slices were exposed to (A) 100 μM AMPA for 60 min, (B) 300 μM NMDA for 60 min, (C) mild (20 min) OGD or (D) 100 μM DHPG + mild OGD, and 48 h later incubated with PI and observed under fluorescence optics. AMPA and NMDA produced striking and nonselective injury in CA1, CA3 and in the dentate gyrus, whereas mild OGD induced a modest and selective CA1 damage that was clearly enhanced by the addition of DHPG.

Preconditioning and glutamate receptor-mediated neuronal death

In order to evaluate the mechanisms leading to tolerance towards the OGD insult, we investigated the effects of ischemic or pharmacological preconditioning on the subsequent neurotoxicity induced by ionotropic and group I metabotropic glutamate receptor agonists. Exposure of slices to AMPA (100 μM) or NMDA (300 μM) for 60 min produced a pattern of neurotoxicity, as observed 48 h later, that was slightly more intense and less selective for CA1 than that induced by 30 min OGD (Fig. 4). When we tested whether a 24-h pre-exposure to preconditioning stimuli could affect AMPA or NMDA toxicity, we observed that both the ischemic and the pharmacological preconditioning paradigms reduced the neurotoxicity caused by 3–30 μM AMPA or 10–100 μM NMDA but were unable to attenuate the injury induced by the highest concentrations of ionotropic glutamate receptor agonists (Fig. 5A and B).

We have previously shown that 100 μM DHPG is not toxic by itself but significantly aggravates CA1 injury when added to hippocampal slices exposed to OGD (Pellegrini-Giampietro *et al*., 1999a). In order to evaluate whether ischemic or pharmacological preconditioning could affect mGlu1 and mGlu5 receptor sensitivities, we exposed preconditioned slices to a protocol in which the agonist DHPG (100 μM) enhanced the modest CA1 damage produced by a brief period (20 min) of OGD (Figs 4, 6 and 7). In slices pre-exposed to ischemic preconditioning (Fig. 6), the DHPG-triggered increase in mild OGD neurotoxicity was markedly reduced (from 182 ± 24% vs. mild OGD alone, to 120 ± 11%), in a manner that was completely prevented by the mGlu1 receptor antagonist LY367385 (100 μM) but not by the mGlu5 receptor antagonist MPEP (10 μM). In contrast, in slices pre-exposed to pharmacological preconditioning (Fig. 7), the increase in CA1 neuronal death induced by DHPG plus mild OGD was remarkably increased (from 165 ± 13% vs. mild OGD alone, to 358 ± 68%). In this case, the selective mGlu5 antagonist MPEP (10 μM), but not the mGlu1 antagonist LY367385 (100 μM), prevented the increased neurotoxic response to DHPG in mild OGD.

Discussion

In models of cerebral preconditioning, stimuli that are harmful or toxic to the brain can induce neuroprotection when administered for brief periods or at low doses. In accord with results obtained in other laboratories (for reviews see: Dirnagl *et al*., 2003; Gidday, 2006), we observed that a brief period (10 min) of sublethal OGD in organotypic hippocampal slices increased neuronal survival after

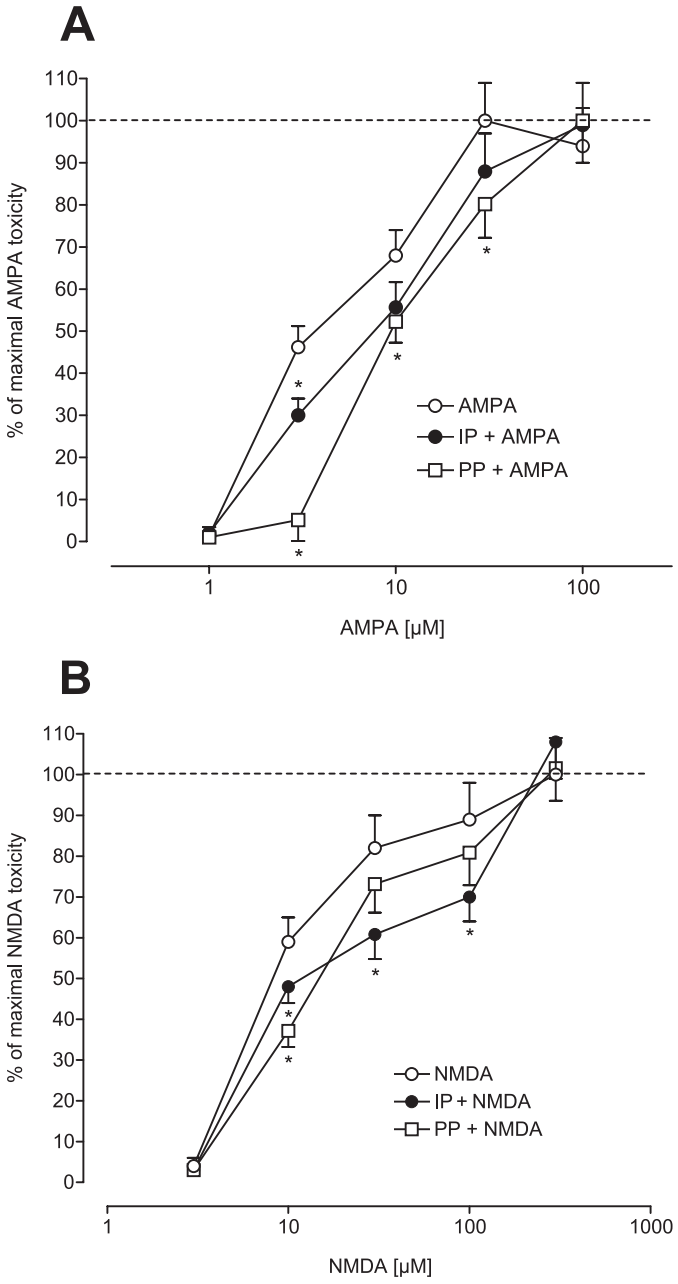

FIG. 5. Ischemic (IP) and pharmacological (PP) preconditioning reduced the toxicity induced by ionotropic glutamate receptor agonists in rat organotypic hippocampal slices. Slices were exposed to (A) 1–100 μM AMPA or (B) 3–300 μM NMDA for 60 min and 48 h later CA1 damage was assessed by measuring the intensity of PI fluorescence. Data are expressed as percentages of maximal (A) AMPA or (B) NMDA toxicity. The toxicity evoked by (A) 3–30 μM AMPA or (B) 10–100 μM NMDA was reduced when slices were pre-exposed 24 h earlier to a brief (10 min) OGD (IP) or to 10 μM DHPG for 30 min (PP). Values represent the mean ± SEM of $n \geq 3$ experiments run in quadruplicate. *$P < 0.05$ vs. correspondent AMPA or NMDA concentration.

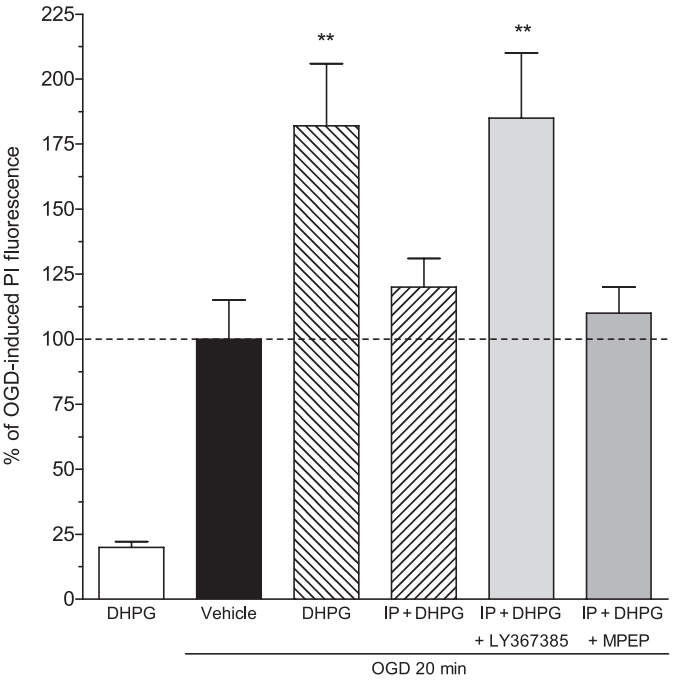

FIG. 6. Ischemic preconditioning (IP) reduced DHPG toxicity in rat organotypic hippocampal slices. Slices were exposed to 100 μM DHPG, OGD or DHPG + OGD for 20 min and 48 h later CA1 damage was assessed by measuring the intensity of PI fluorescence. Data are expressed as percentages of 20 min OGD-induced neuronal death. The toxicity evoked by DHPG was significantly reduced when slices were pre-exposed 24 h earlier to a brief (10 min) OGD (IP). The mGlu1 receptor antagonist LY367385 (100 μM), but not the mGlu5 receptor antagonist MPEP (10 μM), was able to prevent the reduction induced by IP. Bars represent the mean ± SEM of $n \geq 4$ experiments run in quadruplicate. **$P < 0.01$ vs. OGD 20 min alone.

a subsequent, more prolonged (30 min), and otherwise lethal OGD insult. In other experiments, we observed that DHPG (10 μM), a group I mGlu receptor agonist, applied to the slices for 30 min 24 h before the lethal OGD challenge, significantly attenuated CA1 injury, whereas a higher concentration (100 μM) of DHPG exacerbated the subsequent OGD-induced neuronal death. These observations suggest that activation of group I mGlu receptors is sufficient to trigger mechanisms culminating in an increased resistance to ischemic insults (ischemic tolerance). It has been previously reported that it is possible to increase neuronal resistance to ischemic insults by pre-exposing slices to glutamate (Schurr *et al.*, 2001), NMDA (Raval *et al.*, 2003), adenosine A1 receptor agonists (Lange-Asschenfeldt *et al.*, 2004) or a period of moderate increase in intracellular Ca^{2+} concentrations (Bickler & Fahlman, 2004). Because group I mGlu receptors share with these other approaches the ability to increase intracellular Ca^{2+} levels, the latter might represent a common pathway able to lead to an increased neuronal resistance to ischemic insults. Another possible mechanism that may contribute to the group I mGlu receptor-mediated development of ischemic tolerance is the rapid increase in excitability that has been reported in hippocampal pyramidal cells exposed to relatively low doses (10 μM) of DHPG (Young *et al.*, 2004).

In cultured hippocampal slices, selective antagonists of NMDA, AMPA or mGlu1 receptors significantly reduce CA1 injury when added to the incubation medium during and after a 30-min OGD insult (Pellegrini-Giampietro *et al.*, 1999a,b). In the present study, we evaluated the effects of glutamate receptor antagonists when added to the medium during two different preconditioning procedures that were performed 24 h before the lethal exposure to OGD. Interestingly, AMPA or mGlu1 antagonists prevented the development of ischemic tolerance but, to our surprise, even elevated concentrations of MK801 (10–100 μM) failed to do so. In contrast, other laboratories have shown that NMDA receptor antagonists (including 10 μM MK801) prevent the induction of ischemic tolerance (Raval *et al.*, 2003). It has also been shown that activation of group I mGlu receptors produces

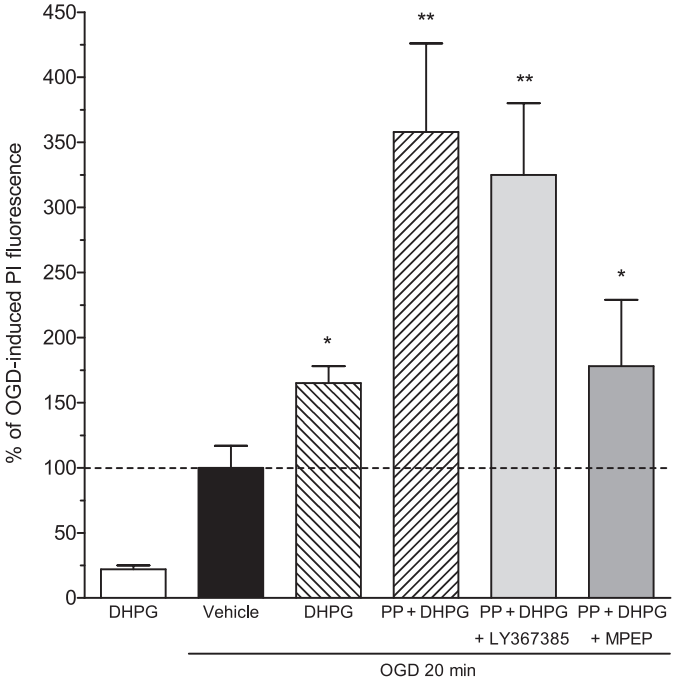

FIG. 7. Pharmacological preconditioning (PP) enhanced DHPG toxicity in rat organotypic hippocampal slices. Slices were exposed to 100 μM DHPG, OGD or DHPG + OGD for 20 min and 48 h later CA1 damage was assessed by measuring the intensity of PI fluorescence. Data are expressed as percentages of 20 min OGD-induced neuronal death. The toxicity evoked by DHPG was significantly exacerbated when slices were pre-exposed 24 h earlier to 10 μM DHPG for 30 min (PP). The mGlu5 receptor antagonist MPEP (10 μM), but not the mGlu1 receptor antagonist LY367385 (100 μM), was able to prevent the potentiation of DHPG neurotoxicity induced by PP. Bars represent the mean ± SEM of $n \geq 4$ experiments run in quadruplicate. *$P < 0.05$ and **$P < 0.01$ vs. OGD 20 min alone.

rapid protection against NMDA-mediated neuronal death (Arnett *et al*., 2004). The different time periods used for the preconditioning procedures (10 vs. 15 min) or the different intervals used before the exposure to the toxic stimuli may explain these apparent discrepancies.

The use of selective mGlu1 and mGlu5 receptor antagonists allowed us to differentiate the processes activated by either ischemic or pharmacological preconditioning. Both MPEP, a selective mGlu5 antagonist (Gasparini *et al*., 1999), and LY367385, a selective competitive mGlu1 antagonist (Kingston *et al*., 1999), significantly reduced the preconditioning effects induced by DHPG (10 μM), suggesting that activation of both mGlu1 and mGlu5 receptors was necessary to induce ischemic tolerance in our model of pharmacological preconditioning. On the other hand, the preconditioning effect activated by a brief (10 min) OGD exposure was reduced by LY367385 and by another mGlu1 receptor antagonists, 3-MATIDA (Moroni *et al*., 2002), but not by the mGlu5 receptor antagonist MPEP, indicating that under these conditions mGlu1 receptors contribute more substantially than mGlu5 receptors to the induction of ischemic tolerance. It is interesting to note that, under selected conditions, mGlu1 but not mGlu5 receptors appear to be involved in the mechanisms leading to postischemic neurodegeneration (Pellegrini-Giampietro, 2003).

In an attempt to understand the underlying mechanisms in the development of ischemic tolerance, we also examined whether ischemic or pharmacological preconditioning could alter the excitotoxic effects of glutamate receptor agonists. We used a range of concentrations of AMPA and NMDA to induce increasing excitotoxic responses and we evaluated their effects in control slices and in slices exposed 24 h earlier to either the ischemic or the pharmacological preconditioning protocol. Our results indicate that the excitotoxic potential of AMPA and NMDA receptor agonists was reduced in preconditioned slices; this is in accord with reports showing that cortical cultures preconditioned with KCN (McLaughlin *et al*., 2003) or organotypic hippocampal slices preconditioned with DHPG (Blaabjerg *et al*., 2003) are protected against NMDA excitotoxicity. The reduced NMDA response after preconditioning stimuli has been associated with caspase 3 activation and increased production of neuroprotective proteins such as HSP 70 (McLaughlin *et al*., 2003) or with Rab5b-stimulated endocytosis of NMDA receptors (Baskys *et al*., 2005). Similarly, ischemic preconditioning confers neuroprotection against global ischemia *in vivo* by attenuating the postischemic modifications in subunit composition that lead to the formation of Ca^{2+}-permeable AMPA receptors (Tanaka *et al*., 2002).

In a similar manner, ischemic preconditioning significantly reduced the excitotoxic potential of the group I mGlu receptor agonist DHPG, as evaluated by applying the compound (which was not toxic by itself) to slices simultaneously exposed to a mild (20 min) OGD. As predicted (Gasparini *et al*., 1999; Pellegrini-Giampietro *et al*., 1999a), DHPG significantly exacerbated CA1 injury in control slices while, in slices preconditioned 24 h earlier with a brief (10 min) OGD, DHPG excitotoxicity was diminished. Because group I mGlu receptors are known to desensitize depending on their level of phosphorylation (Dale *et al*., 2002), it is possible that ischemic preconditioning may trigger phosphorylation mechanisms leading to receptor desensitization and hence to ischemic tolerance. Again, the results of the experiments performed using mGlu1- and mGlu5-selective antagonists suggest that a loss of mGlu1 rather than mGlu5 receptor function may be responsible for the increased tolerance to OGD.

In contrast, a brief exposure of group I mGlu receptors to their selective agonist DHPG (10 μM), as observed in our pharmacological preconditioning model, may sensitize mGlu1 and/or mGlu5 receptors, thereby activating transient neurotoxic mechanisms. Indeed, in pharmacologically preconditioned slices, DHPG excitotoxicity in mild OGD was more than doubled. The enhanced excitotoxic DHPG response was blocked not by LY367385 but by MPEP, indicating that it was mGlu1-independent and presumably mediated via mGlu5 receptor activation. Thus, pharmacological preconditioning with 10 μM DHPG may activate processes that sensitize mGlu5 receptors; the subsequent addition of 100 μM DHPG during mild OGD appears to unmask their excitotoxic potential. Receptor sensitization may be a result of the recruitment of mGlu5 receptors previously uncoupled to their effector G-proteins (receptor reserve). Further studies are necessary to investigate which intracellular signalling cascades are involved in mGlu1 or mGlu5 receptor-dependent induction of ischemic tolerance.

In conclusion, our results show that group I mGlu receptors are differentially involved in the mechanisms leading to ischemic tolerance following either ischemic or pharmacological preconditioning. The decreased mGlu1 receptor-induced excitotoxicity in ischemically preconditioned slices suggests that changes in mGlu1 receptor function following a brief exposure to OGD may be responsible for the development of ischemic tolerance. The results in slices preconditioned by a brief pre-exposure to DHPG suggest that under these circumstances mGlu5 receptors may become sensitized to subsequently produce an enhanced toxic response to the receptor agonist.

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Abbreviations

3-MATIDA, 3-methyl-aminothiophene dicarboxylic acid; CNQX, 6-cyano-7-nitro-quinoxaline-2,3-dione; DHPG, (S)-3,5-dihydroxyphenylglycine; LY367385, (S)-(+)-alpha-amino-4-carboxy-2-methylbenzene-acetic acid; mGlu, metabotropic glutamate; MK801, (+)MK801 maleate; MPEP, 2-methyl-6-(phenyl-ethynyl)-pyridine; OGD, oxygen and glucose deprivation; PI, propidium iodide.

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