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NMDA receptor antagonists MK-801 and memantine induce tolerance to oxygen and glucose deprivation in primary cultures of rat cerebellar granule cells

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Preconditioning is an experimental strategy for reducing ischemic brain damage. There are reports that brief exposure of neurons to NMDA-receptor antagonists may be an adequate preconditioning stressor. We studied effects of preconditioning of the cerebellar granule cells (CGC) in primary culture by 30-minute exposure to NMDA receptor antagonists 0.5 μM MK-801 or 5 μM memantine. CGC were challenged with oxygen and glucose deprivation (OGD) or excitotoxic glutamate and cell viability was tested 24 h later using calcein/ethidium homodimer-1 staining. We studied glutamate-induced increases in ^{45}Ca uptake and in the intracellular Ca^{2+} level assessed with the fluorescent probe fluo-3. The number of living cells in OGD-treated cultures decreased by 42%. Preconditioning with MK-801 or memantine 24 h earlier reduced cell death to 8% and 30% and 48 h earlier to 27% and 33%, respectively. Pretreatment with MK-801 followed by the standard MK-801 wash out was slightly cytoprotective in a glutamate excitotoxicity test performed immediately; the protection increased significantly 24 h after preconditioning. In both cases the extensive wash out of MK-801 after preconditioning resulted in loss of cytoprotection. The increase in the intracellular Ca^{2+} level evoked by glutamate was decreased 24 h after preconditioning and even halved in the neuronal cultures 48 h after preconditioning with MK-801 and memantine. Glutamate-induced ^{45}Ca uptake in these cells was decreased by 18%, irrespective of the time laps after preconditioning. These results demonstrate that preconditioning of CGC with NMDA receptor antagonists induces prolonged tolerance to OGD, which is accompanied by the reduction of glutamate-evoked calcium fluxes. The causal relationship between these effects may be suggested.

Key words: calcium transients, glutamate, neuroprotection, OGD, preconditioning

INTRODUCTION

Cerebral ischemia is one of the leading causes of death or permanent disability in developed countries (Doyle et al. 2008). It is known that the mechanisms leading to irreversible ischemic brain damage include, *inter alia*, energy deficit, over-stimulation of excitatory amino acid receptors (especially of the NMDA receptor), a disorder of calcium homeostasis in neurons, damage of mitochondria and oxidative stress (Szydlowska and Tymianski 2010). They lead to necrotic or apoptotic cell death, or to autophagy (Balduini et al. 2012). The promising results of studies on the pharmacological neuroprotection in cerebral ischemia, including the use of NMDA receptor antagonists, for various reasons have not resulted in advances in the treatment of stroke (Cheng et al. 2004, Gladstone et al. 2002). There is therefore a need for new effective therapeutic approaches in brain ischemia.

One of the novel potential treatments of stroke is preconditioning of the brain, consisting of sublethal exposure to various stressors, which leads to the induction of brain tolerance to ischemia (Kirino 2002). Studies concerning brain preconditioning are at the stage of pre-clinical tests. In recent years considerable progress was made in understanding the mechanisms of acquisition of tolerance to ischemia by the brain and other organs, and in the knowledge of the characteris-

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tics of this phenomenon (Dirnagl et al. 2009, Kitagawa 2012). The discovery of the effectiveness of ischemic brain postconditioning (i.e. the acquisition of tolerance after suffering an episode of brain ischemia) (Zhao 2009) and the finding that the tolerance of the brain can be obtained by pharmacological methods (Dirnagl et al. 2009), increased the likelihood of the practical use of induced brain tolerance to ischemia.

It has been repeatedly demonstrated that moderate stimulation of the NMDA receptors in neurons induces their tolerance (Kato et al. 1992, Tauskela et al. 2001, Jiang et al. 2003). Paradoxically, Tremblay and colleagues (2000) demonstrated that transient exposure of the cultured cortical neurons to NMDA receptor antagonists induces long-term tolerance to various death signals. We have shown previously that tolerance to the toxic effects of glutamate by a brief treatment with NMDA receptor antagonists can be induced also in primary cultures of rat cerebellar granule cells (CGC) (Kuszczyk et al. 2010). Still, it is unknown if preconditioning with NMDA receptor antagonists induces tolerance to brain ischemia. In the original study of Tremblay et al. (2000) it was shown that exposure of cortical neurons to MK-801 induces their tolerance to oxygen and glucose deprivation (OGD), which is a commonly used *in vitro* model of ischemia-like conditions. These authors also demonstrated that such a treatment does not interfere with NMDA-induced calcium transients in neurons tested 48 h later. However, it is not known whether these phenomena may be reproduced using other types of neuronal cultures such as CGC, with the NMDA receptor antagonists other than MK-801, and how the results would depend on time lapse after preconditioning.

Thus, in the present study we revisited the subject of inducing tolerance to OGD in CGC neurons by preconditioning with NMDA receptor antagonists. For preconditioning we used two uncompetitive NMDA receptor antagonists of high and low affinity to the channel, MK-801 and memantine, respectively. Then, 24 and 48 hours later we tested the tolerance of CGC to OGD and effects of preconditioning on glutamate-evoked changes in the intracellular Ca^{2+} level and ^{45}Ca uptake.

METHODS

Glutamate, fetal bovine serum and other components of cell culturing media were obtained from Sigma-Aldrich sp. z o.o., (Poznań, Poland). (+)-5 Methyl-10,11-dihydro-5H-dibenzo[a,d]cycloheptan-5,10-imine hydrogen maleate (MK-801, disocilpine) and memantine were purchased from RBA (Natic, MA, USA). The Live/Dead® Viability/Cytotoxicity Kit for mammalian cells was purchased from Molecular Probes Inc., Paisley, UK. ^{45}Ca was obtained from the Radioisotope Research Development Centre (Świerk, Poland). Fluo-3/AM was produced by Molecular Probes Inc. (Eugene, OR, USA). All other chemicals were of analytical grade.

For preparing neuronal primary cultures, 7 day-old pups of the Wistar rats were used. The procedure, performed in accordance with domestic and international regulations concerning experiments on animals, was approved by the Forth Local Ethical Committee in Warsaw. All efforts were made to reduce the number of animals used and to minimize their suffering. Primary cultures of CGC were prepared and cultured in basal Eagle medium (BME) supplemented with 10% fetal calf serum, 25 mM KCl, 4 mM glutamine, streptomycin (50 μg/ml) and penicillin (50 U/ml), as has been described in details previously (Kuszczyk et al. 2010, Ziemińska et al. 2012). For preconditioning with the NMDA receptor antagonists, doses of 0.5 μM MK-801 or 5 μM memantine were administered 24 or 48 h prior to OGD for 30 minutes at 37°C in Locke 25 incubation buffer containing 134 mM NaCl, 25 mM KCl, 4 mM $NaHCO_3$, 5 mM HEPES, pH 7.4, 2.3 mM $CaCl_2$ and 5 mM glucose (Kuszczyk et al. 2010). Control cultures were treated with Locke 25 buffer without the NMDA receptor antagonists. After preconditioning the cells were washed twice with Locke 25 buffer and cultured in the original growth medium. In the separate set of experiments this standard washout procedure after preconditioning was compared with the washout expanded to 5 washes with the Mg^{2+}-free Locke 25 buffer supplemented with 1 mM $MgCl_2$ and 15 μM NMDA as described by McKay and coauthors (2013), which was followed by one final wash with the Mg^{2+}-free Locke 25 buffer without these additives.

The OGD treatment of CGC cultured *in vitro* for 7 days was performed as described previously (Ziemińska et al. 2012) with only minor modifications. The cultures were incubated for 90 minutes at 37°C in the OGD buffer (116 mM NaCl, 25 mM KCl, 26.2 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1 mM NaH_2PO4 (pH 7.4) without glucose and in an atmosphere containing 5% CO_2 and 95% N_2 using the C-chamber connected with Proox Model 110 and ProCO_2 Model 120 gas control-

lers (BioSpherix, Lacona, NY, USA). In addition, as positive control, a group of previously untreated cultures was challenged with OGD in the presence of 0.5 μM MK-801 or 5 μM memantine. After OGD, the cells were cultured for 24 h under standard conditions in the original growth medium. In selected experiments performed in order to evaluate indirectly completeness of the washout of MK-801 after preconditioning, 10 min or 24 h after the standard or expanded washout of that antagonist the cells were challenged for 30 min with 250 μM glutamate in the Locke 25 buffer which was followed by 24 h culturing under standard conditions. The viability of neurons was assessed by an observer blind to the experimental groups using calcein/ethidium homodimer-1 staining and the multidetection microplate reader FLUOstar Omega (GBM Labtech GmbH, Offenburg, Germany) for measuring the fluorescence (Kuszczyk et al. 2010). The proportion of live cells was expressed as a percentage of the total cell number.

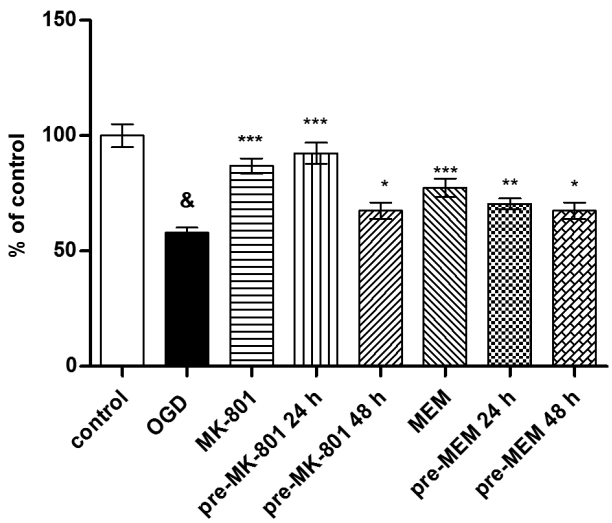

Fig. 1. Neuroprotection of rat cerebellar granule cells against oxygen-glucose depravation (OGD) provided by 0.5 μM MK-801 or 5 μM memantine (MEM) applied during the insult or by preconditioning (pre) with these antagonists performed 24 or 48 h before OGD. After preconditioning the cells were washed twice with Locke 25 buffer and cultured in the original growth medium. Survival of the neurons was examined 24 h after OGD using calcein/ethidium homodimer-1 staining. Results are the means ± SEM (n=6); results significantly different from the control – $^{\&}P<0.001$; results significantly different from the OGD group – $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$.

For evaluating glutamate-induced changes in Ca^{2+} homeostasis, CGC were submitted to a replacement of the growth medium by the Locke 5 buffer. To measure changes in the intracellular Ca^{2+} concentration, the CGC were incubated for 30 minutes in a Locke 5 buffer containing the calcium-sensitive fluorescent probe 16 μM fluo-3/AM. Then, after washing with Locke 5 buffer, changes of fluorescence, basal and evoked by 500 μM glutamate, were recorded every 5 minutes, over a period of 30 minutes, using a multidetection microplate reader FLUOstar Omega at 485 nm excitation and 538 nm emission wavelengths. The results are presented as percent changes in the intensity of fluorescence compared to the basal level. In ^{45}Ca uptake experiments performed as described before (Antkiewicz-Michaluk et al. 2006) the cells were preincubated for 10 minutes at 37°C, then $^{45}CaCl_2$ (1 μCi/well) together with 500 μM glutamate was added and the cells were incubated for 10 min. After medium removal and 3 washes with ice-cold glucose and $CaCl_2$-free medium with 2 mM EGTA, the cells were solubilised in ice-cold 0.5 M NaOH, and the radioactivity was measured by liquid scintillation spectroscopy using Wallac 1409 counter (Wallac, Turku, Finland).

The results are presented in figures as mean ± SEM (n=6). One-way ANOVA followed by Dunnett's test or Wilcoxon matched pairs test were used for comparisons between groups. Significance was taken at $P<0.05$.

RESULTS

As presented in Figure 1, OGD lasting 90 minutes induced a 42% decrease in the number of surviving neurons, which is highly significant (P=0.03). When 0.5 μM MK-801 or 5 μM memantine were present in the incubation medium during OGD, this cytotoxic effect of OGD was significantly reduced to 13% and 23%, with $P<0.0001$ and 0.002, respectively. A very similar level of cytoprotection was achieved after preconditioning of CGC with 0.5 μM MK-801 or 5 μM memantine 24 h before OGD. Here, pretreatment with MK-801 and memantine reduced neuronal loss to 8% and 30%, respectively (P=0.007 and 0.0043). Extending the time interval between preconditioning and OGD to 48 h lessened neuroprotection. However, under these conditions OGD-evoked cytotoxicity in the cultures preconditioned with MK-801 and memantine of 27%

and 33%, respectively (Fig. 1), was still significantly lower than in control (P=0.043 and 0.036).

In the additional control experiments we extended the MK-801 wash out procedure to five washes with the ionic medium containing 1 mM Mg^{2+} and 15 μM NMDA, using the technique adapted from McKay and colleagues (2013). Exposure of cultures for 30 min to 250 μM glutamate followed 24 h later by assessment of viability was used to evaluate resistance of cells to excitotoxicity. The excitotoxicity test performed immediately after the MK-801 wash out demonstrated that cell viability [presented here as mean ± SEM (n=7–8)] significantly decreased from 90.4±0.73% in cultures submitted to wash out without other treatments to 57.5±1.85% *(*P<0.001) in cultures challenged with glutamate which were not preconditioned. In cultures preconditioned with MK-801 and then submitted to the standard wash out procedure viability also significantly decreased to 64.9±1.90% (P<0.001). This decrease was significantly reduced comparing to cultures which were not preconditioned (P=0.02), demonstrating a slight cytoprotection in cells submitted to the standard wash out procedure. In cultures extensively washed after MK-801 with Mg^{2+} and NMDA neuronal viability was reduced to 56.9±1.72%, and this result did not differ statistically from cultures that were not preconditioned (P=0.72). The cultures were also submitted to excitotoxicity test 24 h after preconditioning with MK-801 which was followed by the standard or extended wash out procedures. The results demonstrated that viability of cells in cultures which were not preconditioned with MK-801 decreased after treatment with glutamate to 54.4±2.11%, whereas in cultures preconditioned with MK-801 and then submitted to the standard wash out procedure we detected cell viability of 75.4±2.56% which was significantly higher than in cultures not preconditioned and in cells preconditioned immediately before the excitotoxic stimulus (P<0.01). However in cultures which were extensively washed out after MK-801 with Mg^{2+} and NMDA viability of cells was reduced to the level of 51.2±1.71% that did not differ significantly from the corresponding control cultures which were not preconditioned (P=0.25).

In order to relate tolerance induced by preconditioning CGC with the NMDA receptor antagonists to possible changes in generation of calcium signaling in the preconditioned neurons, we evaluated effects of preconditioning on glutamate-induced increase in the intracellular Ca^{2+} level in neurons. As presented in

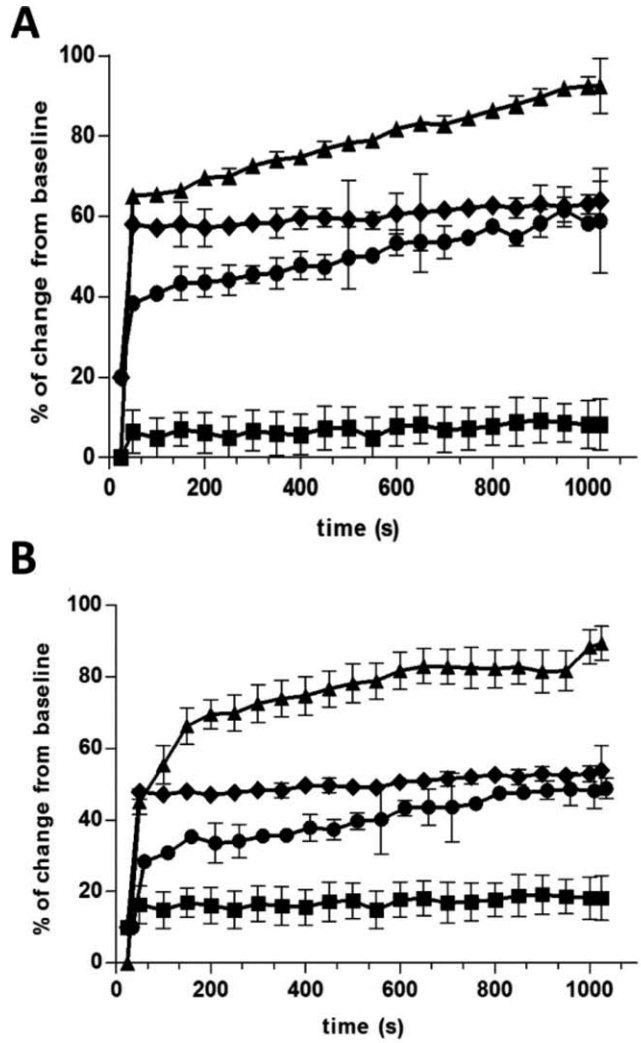

Fig. 2. Glutamate-induced increase in the intracellular Ca^{2+} concentration in primary cultures of rat cerebellar granule cells: modulation by preconditioning with 0.5 μM MK-801 or 5 μM memantine performed 24 h (A) and 48 h (B) before the test. After preconditioning the cells were washed twice with Locke 25 buffer and cultured in the original growth medium. After 25-second long measurements of the basal fluorescence of the fluo-3 loaded neurons, 500 μM glutamate was administered. Changes in the intracellular Ca^{2+} concentration were evaluated by measurement of fluo-3 fluorescence and expressed in percent of the basal level. Squares – control cells without glutamate; triangles – control cells with glutamate; circles – cells preconditioned with MK-801; diamonds – cells preconditioned with memantine. Results are means ± SEM (n=6). All the results representing effects of preconditioning with MK-801 and memantine taken during steady state phase are significantly different from the control cells treated with glutamate at the corresponding experimental time points (Wilcoxon matched pairs test, P<0.05).

Figure 2, application of 500 μM glutamate resulted in a huge rise in fluo-3 fluorescence reflecting the increase in Ca^{2+} concentration in CGC. This effect was significantly reduced (P<0.0001 for both tested substances) by 16% and 14% in cultures preconditioned 24 h before the test with 0.5 μM MK-801 or 5 μM memantine, respectively (Fig. 2A). As presented in Figure 2B, the increase in the intracellular Ca^{2+} level induced by glutamate in CGC preconditioned 48 h before test was halved in comparison to control (P<0.0001).

Reduced glutamate-induced calcium transients in CGC which were observed 24 h and 48 h after preconditioning with MK-801 and memantine (Fig. 2A,B), might have resulted from the decreased influx of extracellular Ca^{2+} to neurons stimulated with glutamate. In order to check this hypothetical explanation, we evaluated the effects of preconditioning on glutamate-induced uptake of ^{45}Ca in CGC. As shown in Figure 3, application of 500 μM glutamate resulted in a huge 40% rise in the accumulation of the radioactive calcium in CGC (P<0.0001). Pretreatment of CGC with 0.5 μM MK-801 reduced the glutamate-induced ^{45}Ca uptake in CGC by 17% 24 h after preconditioning (P=0.0087) and by 16% 48 h after that treatment (P=0.0043). Uptake of ^{45}Ca in neurons measured 48 h after preconditioning with memantine was inhibited to a similar extent as after preconditioning with MK-801 (P=0.026). However, 24 h after preconditioning that difference lost its statistical significance (P=0.053).

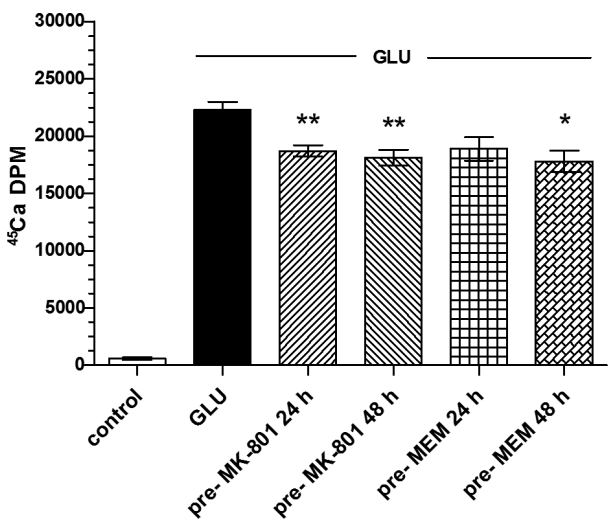

Fig. 3. Glutamate-induced increase in ^{45}Ca uptake in primary cultures of rat cerebellar granule cells: modulation by preconditioning with 0.5 μM MK-801 or 5 μM memantine (MEM) performed 24 or 48 h before the test. Neuronal cultures, untreated or preconditioned (pre) 24 or 48 h earlier with 0.5 μM MK-801 or 5 μM memantine (MEM), then washed twice with Locke 25 buffer and cultured in the original growth medium, were incubated for 10 minutes with 500 μM glutamate and $^{45}CaCl_2$ (1 μCi/well). In groups marked MK-801 or MEM the antagonists were present during the incubation. Then radioactivity accumulated was measured and presented in DPM/well. Results are the means ± SEM (n=6); results significantly different from the GLU group – **P<0.01; *P<0.05.

DISCUSSION

The results of this study demonstrated that a 30-minute exposure of CGC in the primary cultures to NMDA receptor antagonists 0.5 μM MK-801 or 5 μM memantine performed 24 h or 48 h before OGD results in cytoprotection, which is accompanied by the reduction of glutamate-evoked rise in the intracellular Ca^{2+} level and by a slight inhibition of glutamate-evoked ^{45}Ca uptake. We claim that these findings may be attributed to preconditioning with NMDA receptor antagonists which induces tolerance of CGC to OGD, and that the decrease in calcium signaling in glutamatergic receptors may participate in its mechanism. A model of cerebellar granule cells in the primary culture that was used in this study had previously been successfully used by us and by other authors for testing the OGD-induced damage to neurons (Kalda et al. 1998, Scorziello et al. 2001, Ziemińska et al. 2012). The experimental model of OGD that is regarded as the *in vitro* equivalent of stroke has been widely used in studies of responses to hypoxia and ischemia, not only in nerve cells (see above) but also in astrocytes and microglia (Fan et al. 2013, Ziomka-Nałęcz et al. 2013). The concentrations of MK-801 and memantine used for inducing tolerance were chosen based on our previous experience (Kuszczyk et al. 2010).

We interpret the aforementioned effects of brief exposure of neurons to NMDA receptor antagonists as manifestations of the phenomenon of preconditioning inducing tolerance of neurons to OGD. The alternative explanation is that these effects might result from the retention of the antagonists in cultures, their physical presence and direct action during the tests i.e. 24 or even 48 h after their application. This might concern MK-801 but not memantine which is a low-affinity antagonist of the NMDA receptor (Möbius et al. 2004). Due to repeated washing of the cells after the

exposure upon preconditioning and further exchange of the media before OGD, retention of memantine in pharmacological concentrations is highly unlikely. In turn MK-801-evoked inhibition of NMDA receptors has a reputation of being irreversible in terms of electrophysiological experiments taking the scale of minutes to a few hours (Li et al. 2002). Nevertheless, we speculate that during one or two days of incubation this high affinity antagonist may ultimately defuse from the binding sites inside NMDA receptor channels and may be metabolized. Most importantly, Tremblay and coworkers (2000) previously demonstrated that neuroprotection generated 48 h after brief pretreatment of cortical neurons with MK -801 may be blocked by the protein synthesis inhibitor cycloheximide, and hereby this phenomenon meets the criteria of tolerance induced by delayed preconditioning (Barone et al. 1998).

Based on the above arguments we claim that preconditioning of the neuronal cultures with both NMDA receptor antagonists induces tolerance to OGD and excitotoxicity. Still in the present study we examined to what extent possible presence of residual MK-801 after its washing may also participate in neuroprotective effects observed after preconditioning with MK-801. The recent study of McKay and others (2013) confirmed that MK-801 is very difficult to wash out and described protocols of repeated washing of neurons which were pretreated with MK-801 with the medium containing a low concentration of NMDA and magnesium that significantly accelerate recovery of NMDA receptors from MK-801 blockade. Our present results comparing the effects of standard and extended techniques of the MK-801 wash out after preconditioning revealed a statistically significant difference in the susceptibility of cultures to a glutamate excitotoxicity test. In cultures challenged with glutamate immediately after preconditioning with MK-801 followed by a standard wash out a significant but relatively slight cytoprotection was noticed. This protective effect was much more pronounced when the excitotoxic stimulus was applied 24 h after preconditioning with MK-801 followed by a standard wash out procedure. However application of an extended wash out with a magnesium and NMDA containing medium in both cases resulted in a complete elimination of neuroprotection. Collectively this data indicate that in our experiments traces of MK-801 remain in cultures after preconditioning followed by a standard wash out procedure. Consequently, in the excitotoxicity test applied a few minutes after wash out these traces of MK-801 provided relatively slight but statistically significant cytoprotection, which was not noticed after more effective extended wash out of MK-801 with magnesium and NMDA-containing medium. Our finding that cytoprotection induced by exposure of CGC to MK-801 followed by a standard wash out is much more pronounced after 24 h is consistent with known features of delayed preconditioning (Dirnagl et al. 2009) and suggests that the induced tolerance plays a key role in the mechanism of this phenomenon. Lack of this effect in cultures extensively washed out after MK-801 preconditioning may indicate that a prolonged presence of residual MK-801 resulting in persistent partial suppression of the activity of NMDA receptors during 24 h incubation of cultures after preconditioning is the key component of the induction of the neuronal tolerance by MK-801. Subsequent studies are necessary to test the latter hypothetical explanation.

Already Tremblay and coauthors (2000) demonstrated that preconditioning of the rat primary cortical cultures with brief exposure to NMDA receptor antagonist MK-801 induces tolerance to OGD applied 48 h later. Our present results brought new data to these pioneering findings. We show that such a tolerance to OGD may be induced not only in the cortical neuronal cultures but also in CGC, which suggests the universal nature of this effect. Beyond the already known effect of MK-801, we have also detected a similar but slightly weaker effect of memantine, a drug that is used in humans in the treatment of Alzheimer's disease symptoms (Danysz and Persons 2012). The previous study of Tremblay et al. (2000) has not detected reduction by preconditioning with MK-801 of NMDA-induced increase in the intracellular Ca^{2+} level in cortical neurons. In contrast, we have shown here a lessening of intracellular calcium transients evoked by glutamate in CGC 24 h and also 48 h after preconditioning with both MK-801 and memantine. Our present findings that preconditioning with the NMDA receptor antagonists 24 h and 48 h later mildly reduces the glutamate-induced ^{45}Ca uptake are only partially consistent with the results obtained by Aizenman and others (2000) demonstrating alterations in the properties of NMDA receptors in primary neuronal cultures 24 h, but not 48 h after preconditioning with sublethal chemical ischemia.

The convergence of the occurrence of neuroprotection and inhibition of glutamate-induced calcium signaling in CGC after preconditioning with NMDA receptor antagonists that was observed in the present study, may suggest a causal relationship between these phenomena. The role of disorders of calcium homeostasis and signaling in the cells in the mechanisms of ischemia-induced neuronal damage is commonly known (for review see Szydlowska and Tymianski 2010). Still, further studies are needed to characterize the mechanism of this phenomenon and to make clear how to refer the highly pronounced inhibition of the glutamate-evoked intracellular Ca^{2+} transients to only a slight reduction of glutamate-induced ^{45}Ca uptake. Perhaps preconditioning with NMDA receptor antagonists mainly modifies a phenomenon of calcium-induced calcium release (CICR) from the intracellular stores that follows the NMDA receptor-mediated Ca^{2+} influx to neurons and potentiates generation of calcium signaling in neurons (for review see Verkhratsky and Toescu 2003). Provisionally, we suggest that changes in the generation of calcium signaling in glutamate receptors which we observed may contribute to the mechanism of tolerance to OGD gained by preconditioning with NMDA receptor antagonists and that this event is primary to inhibition of PKC inactivation and suppression of calpain activation, which were previously described by Tremblay and colleagues (2000).

In our previous study the tolerance of CGC to glutamate-induced excitotoxicity which was induced by preconditioning with the NMDA receptor antagonists was very substantial and long lasting (Kuszczyk et al. 2010). Our present work reveals that in CGC preconditioning to OGD with the NMDA receptor antagonists, although significant, was of lower efficiency and shorter duration. Similar differences between our previous results (Kuszczyk et al. 2010) and our data concern cytoprotection provided by NMDA receptor antagonists present during the excitotoxic challenge or OGD. These distinctions may be due to the more complex mechanisms of cytotoxicity induced by OGD, that apart from the obvious component of excitotoxicity implicates pronounced fluxes of monovalent cations and significant energy disturbances leading to pronounced necrotic component of neuronal death (Strasser and Fischer 1995, Beck et al. 2003, Jones et al. 2004). Still, our present results demonstrating the ability to induce tolerance to OGD in neurons preconditioned with NMDA receptor antagonists point to the possible use of such a strategy in the prevention of the ischemia-induced brain damage, especially since OGD has been considered as the *in vitro* model of ischemia (for reviews see Noraberg et al. 2005, Daviaud et al. 2013). Our recent results confirm this possibility, at least for the rat model of perinatal asphyxia (Makarewicz et al. 2014).

CONCLUSION

The results of this study showed that the 30-minute pretreatment of the primary cultures of rat cerebellar granule cells to the NMDA receptor antagonists MK-801 and memantine 24 or 48 h before OGD results in cytoprotection, which is accompanied by the inhibition of glutamate-induced increase in intracellular calcium levels. We suggest that this phenomenon is a reflection of preconditioning and induced tolerance, and that inhibition of calcium signaling in the glutamate-sensitive receptors may be involved in its mechanism. These data indicate that also under *in vivo* conditions preconditioning with NMDA receptor antagonists might induce brain tolerance to ischemia.

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