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Neuroprotective effects of nicotinamide and 1-methylnicotinamide in acute excitotoxicity *in vitro*

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Abstract

Nicotinamide (NAM), an important cofactor in many metabolic pathways, exhibits at high doses neuroprotective abilities of an unclear mechanism. In the present study we evaluated the unknown protective capability of its immediate metabolite 1-methylnicotinamide (MNA) in comparison to NAM in primary cultures of rat cerebellar granule cells (CGC) submitted to acute excitotoxicity. Neurotoxicity was evaluated with propidium iodide staining 24 h after 30 min exposure to glutamate (GLU) and NMDA. NAM and MNA reduced NMDA toxicity only at 25 mM concentration, while neurotoxicity of 0.5 mM GLU was slightly diminished only by 25 mM NAM. Both compounds at 25 mM reduced GLU-induced ^{45}Ca uptake and dose-dependently inhibited NMDA-induced ^{45}Ca accumulation. Neither NAM nor MNA interfered with GLU-evoked intracellular calcium transients evaluated with calcium orange fluorescent probe or inhibited [^{3}H]MK-801 binding to rat cortical membranes. NAM and MNA failed to change GLU-evoked decrease in mitochondrial membrane potential monitored using the fluorescent dye rhodamine 123. Analysis with a hydroperoxide-sensitive fluorescent probe demonstrated significant reduction by 20 and 25 mM MNA, but not NAM, of oxidative stress in cultures after 1 h treatment with GLU. CGC accumulated radiolabelled NAM and MNA in a time and concentration dependent manner, NAM being transported more rapidly. These findings demonstrate that weak neuroprotective ability of MNA in excitotoxicity, accompanied by incomplete stabilization of calcium imbalance and lessening of oxidative stress, is not connected with direct inhibition of NMDA receptors. The exact mechanisms of these effects require further investigation.

Key words: *calcium, cerebellar granule cells, glutamate, NMDA, mitochondrial potential, neuroprotection, oxidative stress.*

Introduction

Among several vitamins and exogenous cofactors vital for cell metabolism, nicotinamide (NAM), the amide form of niacin (vitamin B3) plays an important role. It is an essential precursor of NAD^+ required for cellular energy metabolism [24]. The other repeatedly suggested role of nicotinamide may be its involvement in neurotoxicity directly evoked by the product of its methylation, 1-methylnicotinamide (MNA). Epidemiological data point to a correlation between niacin supply in the diet and risk of Parkinson's disease [44,45]. The activity of nicotinamide N-methyl

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transferase (NNMT, EC2.1.1.1) in the brain has been demonstrated [17,33]. Moreover, the results of some studies suggest that its product MNA may be neurotoxic [16,17,32]. In contrast to these speculations recent studies demonstrated neuroprotective ability of NAM under various experimental conditions. NAM was neuroprotective in *in vitro* models of cytotoxicity, and *in vivo* in brain ischaemia, provided very high concentrations or doses are applied [3,8,9,14,23,30,42]. The suggested mechanisms of NAM-induced neuroprotection are complex and remain uncertain (for a review see [26]).

As mentioned above, the hypothetical neurotoxicity of NAM has been explained by a direct damaging effect of MNA [17]. However, our recent results demonstrated absence of such a toxic effect and even neuroprotective activity of this compound in homocysteine toxicity in a model of cerebellar granule cells (CGC) in primary culture [41]. Other studies showed in the periphery anti-inflammatory and anti-thrombotic activity of MNA mediated by prostacyclin [4,6,19].

It has been generally accepted that glutamate-induced excitotoxicity is mainly mediated by activation of the NMDA receptors, neuronal calcium overload, oxidative stress and impairment of mitochondrial functions [1,7,36]. It has been suggested that stabilization of the mitochondrial potential is an important mechanism of NAM-evoked neuroprotection [26]. Still, it is not clear if MNA shares the neuroprotective properties of its precursor NAM also in the classical model of excitotoxicity induced by glutamate or by NMDA, which is an agonist of the principal calcium-permeable ionotropic glutamate receptors.

The aim of the present study was to evaluate the neuroprotective activity of MNA in comparison with NAM using primary cultures of rat CGC and an acute model of GLU- and NMDA-evoked excitotoxicity. To elucidate their potential mechanisms we also studied the effects of NAM and MNA on excitotoxicity-evoked calcium imbalance, free radical formation and disturbances in mitochondrial potential.

Material and Methods

Reagents

L-glutamate, N-methyl-D-aspartate (NMDA), propidium iodide (PI) dye and materials for cell culture were purchased from Sigma Chemical Company (St. Louis, MO, USA). Nicotinamide and 1-methylnicotinamide, as well as [^{3}H]methylnicotinamide (250 µCi/mmol), were obtained from the Institute of Applied Radiation Chemistry, Technical University of Lodz, Poland (courtesy of Professors Jerzy Gebicki and Jan Adamus). [^{3}H]nicotinamide (250 µCi/mmol) was purchased from Hartmann Analytical GmbH, Braunschweig, Germany. $^{45}CaCl_2$ (30 µCi/mg Ca) was produced by Polatom, Swierk, Poland. The fluorescent probes calcium orange-AM, rhodamine 123, and DCFH2-DA were purchased from Molecular Probes, OR, USA. Other chemicals were of analytical grade.

The animals

Primary cultures of granule neurons were prepared from the cerebella of 7-day-old rats bred in the Animal Colony of the Medical Research Centre, Polish Academy of Sciences in Warsaw. The rat pups were treated in accordance with Polish and European Community regulations concerning experiments on animals, and the procedure was approved by the First Local Ethical Committee in Warsaw.

Neuronal cultures

Granule neurons were prepared by the method of Schousboe et al. [38], and cultivated in modified BME medium containing 25 mM KCl, exactly as described previously in detail [48-50]. The cells were used for experiments on the 7th day *in vitro* (DIV).

Neurotoxicity

Acute excitotoxicity of L-glutamate or NMDA was induced by replacing the BME growth medium with Locke 5 buffer containing: 154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 5.6 mM glucose and 10 mM HEPES pH 7.35, with aliquots of freshly prepared L-glutamate of NMDA alone or with NAM or MNA as required. After 30 min, the Locke medium was replaced with the original growth medium and the cultures continued for 24 h. To evaluate neurotoxicity, the cells were fixed with 80% methanol, stained with 0.5 µg/ml PI, and viable and dead cells were counted using a Zeiss Axiovert fluorescence microscope by an investigator unaware of the exact experimental conditions, as described previously [48,49]. Results were expressed as the percentage of live cells. The number of repetitions (identically treated wells) within each trial was n=6. The experiments were repeated at least 3 times with different cultures, obtaining the

same qualitative results, and the representative data have been shown.

^{45}Ca uptake

^{45}Ca uptake was determined in magnesium-free low potassium Locke 5 buffer supplemented with 5 µM glycine. After preincubation for 10 min at 37°C, and additional 5 min incubation with glycine, the co-agonist of the NMDA receptor complex, and with NAM or MNA, $^{45}CaCl_2$ (1 µCi/well) was added together with 0.5 mM L-glutamate or NMDA for 10 min. Then the medium was removed and the cells were washed 3 times with ice cold glucose- and calcium-free medium containing 2 mM EGTA. Then the cells were dissolved in ice cold 0.5 M NaOH, and radioactivity in neurons was measured using the Wallac 1409 liquid scintillation counter.

Fluorescent methods

For loading with calcium orange-AM, which is a calcium-sensitive fluorescent probe, the cells were incubated with 20 µM of a dye for 15 min. After washing the cells with Locke buffer the basal fluorescence reflecting the steady-state level of intracellular calcium concentration ($[Ca^{2+}]_i$) was monitored for 90 sec and recorded at each 30 sec, using the confocal microscope Zeiss LSM 510 with filter pairs of excitation and emission wavelength of 530 nm and 560 nm, respectively. The control (basal) fluorescence level is presented in graphs as 100%. Then 0.5 mM GLU was added and the fluorescence was recorded for 90 sec at each 30 sec using LSM 510 computer program (version 3.2).

Changes in mitochondrial membrane potential were monitored using the fluorescent dye rhodamine 123 (Rh123, Molecular Probes), a cell permeable cationic dye, which preferentially partitions into mitochondria. Depolarization of the membrane results in the loss of Rh123 from the mitochondria and an increase in intracellular fluorescence. Rh123 was added to cultures to attain a final concentration of 10 µM for 30 min at 37°C. After that, cells were washed from the culture medium with Locke buffer and treated with tested substances. Changes in the fluorescence in cells were monitored using a microplate reader (Fluoroscan, LabScan, Finland) at 490 nm excitation and 530 emission wavelengths.

The fluorescent probe DCFH2-DA, which is oxidized to fluorescent 2'7'-dihydrofluorescein (DCF) by hydroxyperoxides, was used to measure relative levels of cellular peroxides. CGC were incubated with 50 µM dye for 30 min at 37°C and then washed with the Locke buffer. Next tested substances were given to appropriate wells with 1×10^6 cells, and changes in fluorescence level were measured at different time points using a microplate reader (Fluoroscan, LabScan, Finland) set at excitation wavelength of 485 nm and emission of 530 nm.

[^{3}H]MK-801 binding

Preparation of the fraction enriched with crude synaptic membranes from the rat cerebral cortex used for measurements of [^{3}H]MK-801 binding was done by differential centrifugation and extensive washing exactly as described previously [37,43]. The final pellets were suspended in 0.5 ml of Tris-HEPES buffer and stored at –70°C. Before the experiment frozen membranes were defrosted and washed three more times in Tris-HEPES buffer to remove endogenous glutamate. For the MK-801 binding assay, the tubes containing brain membranes (100 µg of protein), radio-labelled non-competitive antagonist of NMDA receptors [^{3}H]MK-801 (final concentration 4 nM) 100 µM NMDA, 10 µM glycine, different concentrations of NAM and MNA and Tris-HEPES, pH 7.4 (final volume 500 µl) were incubated for 1 hour at 30°C. Incubation was terminated by rapid filtration using Whatman GF/B filters and a Brandel MPR-24 Cell Harvester, and radioactivity was measured using a liquid scintillation counter (Wallac 1409). Non-specific binding of [^{3}H]MK-801 was determined in the presence of unlabelled 10 µM MK-801.

Radiolabelled nicotinamide and methylnicotinamide uptake

Radiolabelled nicotinamide and methylnicotinamide uptake in cultured cerebellar granule cells was assessed after 7 days in culture at a cell density of 4×10^6 per wall. Cells were washed with uptake buffer (4 mM Tris-HCL, 6.25 mM HEPES, 120 mM NaCl, 5 mM KCL, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.6 mM D-glucose, final pH 7.4) and incubated with 1 µCi [^{14}C]nicotinamide or [^{14}C]methylnicotinamide in the presence of different concentrations of unlabelled nicotinamide and methylnicotinamide. Incubation was performed at 37°C at three time points. Uptake was terminated by aspirating the uptake buffer and washing with ice

cold Krebs buffer. Radioactivity was measured using a liquid scintillation counter.

Statistics

The results are presented as means ± standard deviation (SD). For comparisons between groups, one-way analysis of variance (ANOVA) followed by Dunnett's test was used, with differences considered significant at $P<0.05$.

Results

Experiments focused on neuroprotective effects of NAM and MNA were performed using the model of acute exposure of the cells to agonists of glutamate receptors. As shown in Fig. 1A, application of 0.5 mM GLU or NMDA for 30 min resulted in a significant drop in the number of living cells evaluated 24 h later. Co-application of NAM or MNA in concentrations up to 10 mM did not inhibit acute GLU and NMDA-evoked neurotoxicity (results not shown). In our experiments significant reduction of the neurotoxic effect of NMDA was achieved only at 25 mM NAM and MNA. In the case of GLU-induced toxicity, significant neuroprotection was evoked by 25 mM NAM, but not MNA (Fig. 1A). It has been generally accepted that excessive calcium influx to neurons accompanying excitotoxic insult may trigger oxidative stress and impairment of the mitochondrial functions leading to excitotoxic neuronal damage. In subsequent experiments we attempted to relate the observed pattern of NAM and MNA-evoked neuroprotection after the excitotoxic challenge to possible modification by NAM and MNA of these pathogenic processes. Data presented in Fig. 1B demonstrate that both tested substances administered at 25 mM concentrations significantly inhibited ^{45}Ca accumulation in CGC evoked by 0.5 mM NMDA or GLU.

These initial findings suggested possible interference of NAM and MNA with activity of the NMDA receptor. To test this hypothesis we further studied effects of NAM or MNA at concentrations from 1 to 25 mM on NMDA-evoked ^{45}Ca uptake. As presented in Fig. 2, neither tested substance had a significant effect on ^{45}Ca uptake in the control cells untreated with this agonist, but significantly reduced NMDA-evoked ^{45}Ca accumulation in a concentration-dependent manner.

Data presented in Fig. 3A, B demonstrate that inhibition by NAM and MNA of NMDA-evoked calcium uptake to neurons is not accompanied by adequate interference of these compounds with glutamate-induced increase in the intracellular calcium concentration. Calcium transients were evaluated by the rise in calcium orange fluorescence

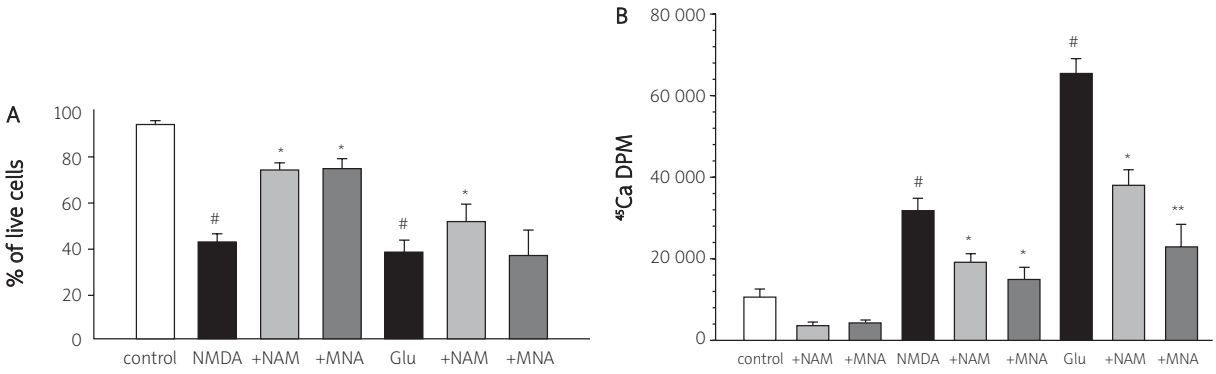

Fig. 1. Effects of nicotinamide and methylnicotinamide on acute excitotoxicity vs. ^{45}Ca uptake in neurons. **A.** Excitotoxic insult was induced by 30 min incubation of cerebellar granule cells with 0.5 mM NMDA or glutamate. Nicotinamide (NAM) or 1-methylnicotinamide (MNA) was applied in 25 mM concentrations for 30 min together with excitotoxins. The number of live cells (as percentage) was evaluated after 24 h. **B.** ^{45}Ca uptake by neurons, evoked by 10-min incubation with 0.5 mM NMDA or glutamate and 0.5 μM glycine (in DPM per well containing 4×10^6 cells), was measured in the presence of 25 mM NAM and MNA. Results are means ± SD (n=6). # effects of excitotoxins significantly different from the control; * means significantly different from the effects of corresponding excitotoxins ($p<0.05$)

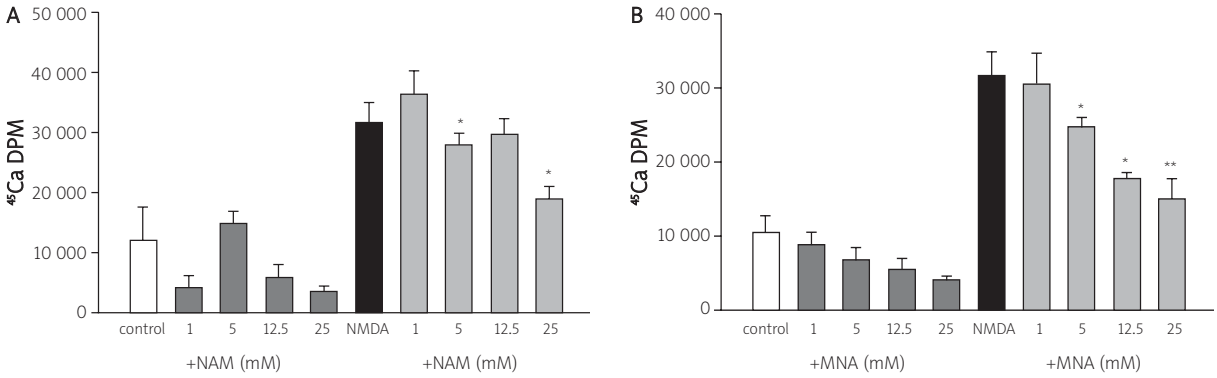

Fig. 2. Concentration-dependent effects of nicotinamide (A) and 1-methylnicotinamide (B) on NMDA-evoked ^{45}Ca uptake in cultured cerebellar granule cells. Cells were incubated for 10 min in the presence of ^{45}Ca, 0.5 mM NMDA, 5 µM glycine, nicotinamide (NAM) or 1-methylnicotinamide (MNA) as indicated. Results expressed as ^{45}Ca accumulated in DMP per well containing 4 × 10^6 cells are means ± SD (n=6). * means significantly different from the effect of 0.5 mM NMDA ($p<0.05$), ** ($p<0.01$).

in the cells. Application of 0.5 mM GLU induced a rapid increase in fluorescence, which was not modified by NAM in the concentration range from 15 mM (not shown) to 25 mM (Fig. 3A). A tendency for slight decrease in the GLU-induced calcium signal, not reaching statistical significance, was observed only with 25 mM MNA (Fig. 3B).

To evaluate possible interference of NAM and MNA with the activity of NMDA receptors, we studied effects of these compounds on [^{3}H]MK-801 binding to the membranous fraction isolated from the rat brain cortex in the presence of NMDA and glycine, e.g. under conditions promoting maximal opening of the NMDA channel. As presented in

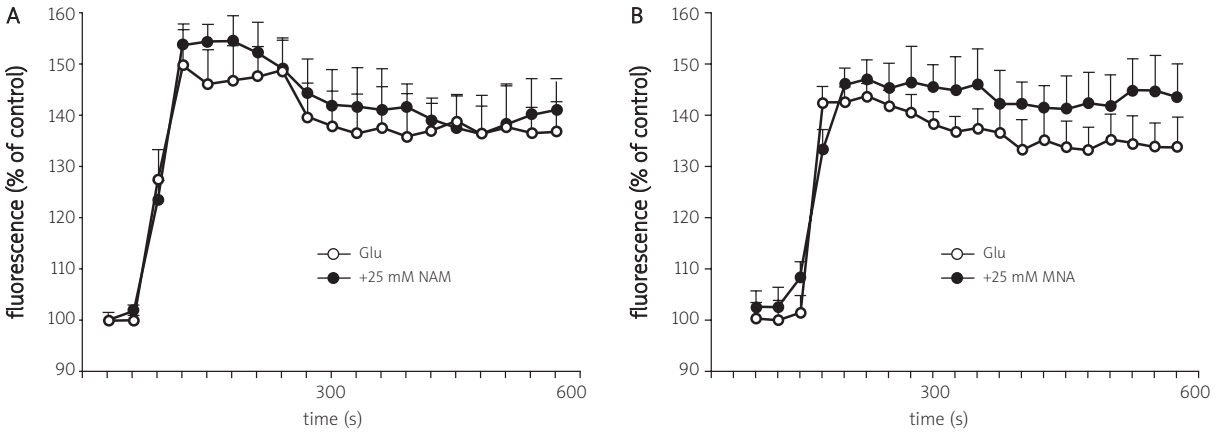

Fig. 3. Effects of 25 mM nicotinamide **(A)** and 1-methylnicotinamide **(B)** on calcium transients in cultured cerebellar granule cells, visualized with calcium orange fluorescent probe. Three initial laser-scanner confocal images of cultures, control or preincubated with 25 mM nicotinamide (NAM) or 1-methylnicotinamide (MNA) were taken at 0, 30 and 60 sec to establish the control (basal) fluorescence level, presented in graphs as 100%. Then 0.5 mM glutamate (GLU) was applied. The results are presented as percent changes in the intensity of fluorescence compared with the basal level. Results are means ± SD (n=6). Differences between control and NAM or MNA-treated cultures did not reach statistical significance ($p<0.05$)

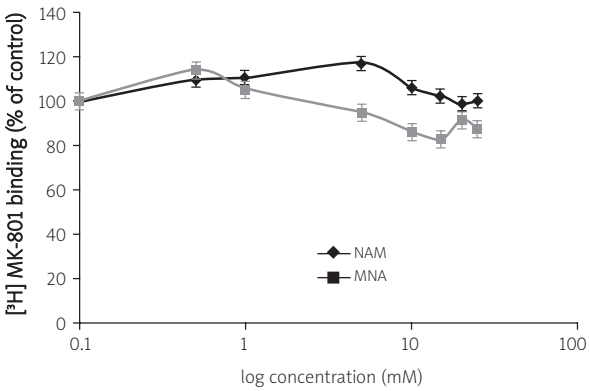

Fig. 4. Effects of nicotinamide (NAM) and 1-methylnicotinamide (MNA) on [^{3}H]MK-801 binding to rat brain membranes. [^{3}H]MK-801 was used at 4 nM, NMDA at 10 µM with 10 µM glycine. NAM and MNA were used in seven concentrations from 0.5 mM to 25 mM as indicated. Non-specific binding was determined in the presence of 10 µM unlabelled MK-801. The data are expressed as percentages of the control (means ± SD, n=6)

Fig. 4, neither compound tested significantly changed the binding. Thus, our results do not support the hypothesis that NMDA receptors may be direct targets for NAM and MNA-evoked inhibition leading to neuroprotection.

Measurements of changes in the fluorescence of Rh123 allowed evaluation of the drop in potential of mitochondrial membranes in cultured neurons submitted to excitotoxic insult and effects of NAM and MNA. As shown in Fig. 5A, application of GLU induced a very significant and prolonged increase in Rh123 fluorescence, reflecting its efflux from the depolarized mitochondria to the cytosol. Neither NAM nor MNA significantly modified this effect irrespective of time of incubation up to 60 min (Fig. 5B).

To visualize accumulation of free radicals in cultured neurons, in this study we used the fluorescent probe DCFH2-DA. Data presented in Fig. 6A, B demonstrate that incubation of the cells in the presence of 0.5 mM GLU resulted in the intracellular accumulation and rise in fluorescence of its oxidized product DCF, indicating production of free radical species. This effect was significantly inhibited by 20 and 25 mM MNA, pointing to antiradical activity of this compound (Fig. 6B), whereas NAM had no effect.

The results of this study do not present a consistent picture of MNA-induced neuroprotection, clearly identifying its cell surface (receptor) or intracellular targets. This raises the question whether CGC in culture are able to accumulate MNA. Therefore in the final experimental approach we evaluated ability of CGC in culture to accumulate MNA, in comparison to NAM, in relation to time and concentration of these substances. As presented in Fig. 7A, B, CGC accumulated both tested compounds. The accumulation of NAM was slightly more rapid, reaching within less than 15 min of incubation with 25 mM NAM a plateau at the level of 3-3.5 nmol/4 × 10^6 cells. The cells during 60 min of incubation with 25 mM MNA accumulated with a linear relation to time about 2.5 nmol per 4 × 10^6 cells. Irrespective on these differences, these results indicate that MNA may be accumulated in CGC and therefore its interference with intracellular targets is possible.

Discussion

In the present study we verified a working hypothesis that MNA has neuroprotective potential in acute excitotoxicity. Using primary cultures of rat cerebellar granule cells we compared protective effects of NAM and MNA in relation to their possible interference with the activity of NMDA receptors, stabilization of calcium homeostasis, protection of mitochondrial integrity and antiradical activity. We also studied uptake of MNA and NAM in our model neuronal cultures. The results of this study demonstrate that both NAM and MNA applied exclusively at 25 mM concentration induce modest neuroprotection, but based on the obtained data it is difficult to propose the relevant mechanism of this effect. Our results indicate that although both substances inhibit NMDA- and GLU-evoked ^{45}Ca uptake, they do not lessen GLU-induced calcium transients and do not interfere directly with activity of the NMDA receptors/channels. Although we demonstrated accumulation of NAM and MNA in cerebellar granule cells, there was no significant effect of these substances on GLU-induced decrease in the mitochondrial membrane potential, and only limited antiradical activity of MNA was noted. Provisionally we tend to explain these phenomena as a reflection of the pleiotropic effects of MNA on neurons, resulting in indirect reduction of their excitability.

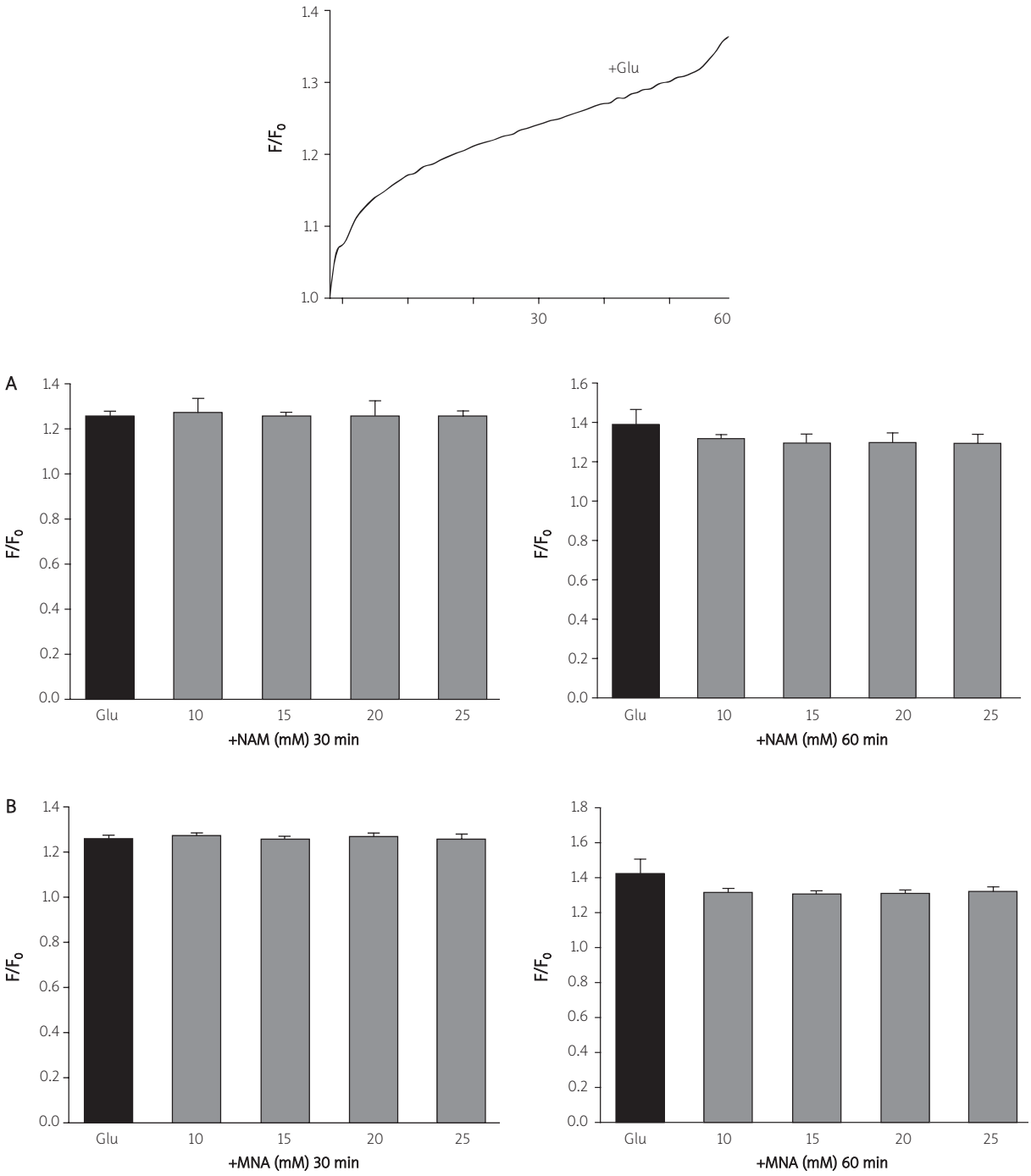

Fig. 5. Effects of nicotinamide (NAM) and 1-methylnicotinamide (MNA) on glutamate-evoked changes in mitochondrial membrane potential in cultured cerebellar granule cells, visualized with rhodamine 123 fluorescence. Upper panel: effect of 0.5 mM glutamate. Lower panels – concentration-dependent effects of NAM (A) and MNA (B). The data are expressed as changes in the intensity of fluorescence compared with the basal level (F/Fo) (means ± SD, n=6). Differences between control and NAM or MNA-treated samples did not reach statistical significance ($p<0.05$)

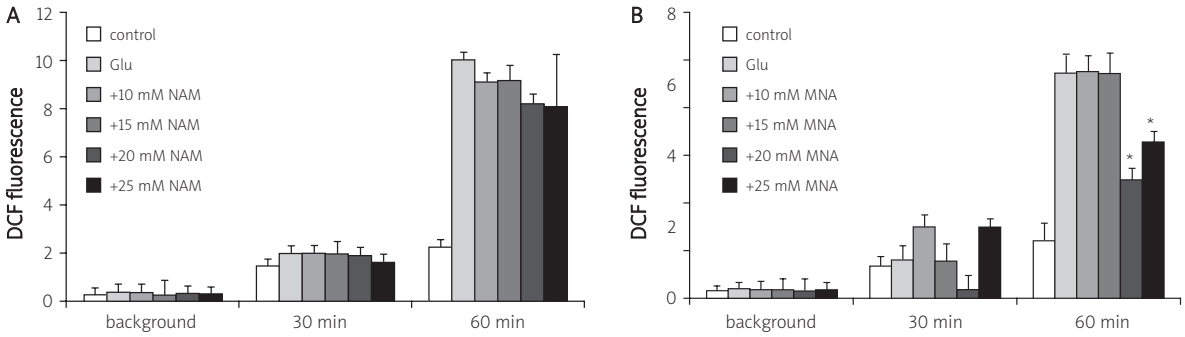

Fig. 6. Effects of nicotinamide (NAM – panel **A**) and 1-methylnicotinamide (MNA – panel **B**) on glutamate-evoked rise in the level of cellular peroxides in cultured cerebellar granule cells, visualized with 2'7'-dichydrofluorescein (DCF) fluorescence. The data are expressed in arbitrary units of fluorescence intensity (means ± SD, n=6). * means significantly different from the effect of 0.5 mM glutamate ($p<0.05$)

Since this study was focused on neuroprotective potential of NAM and its methylated metabolite in excitotoxicity, we used as a model the primary cultures of cerebellar granule cells (CGC). These neurons after 7 days of culture *in vitro* have been widely used for neurotoxicological studies on excitotoxicity. It is known that after this period in culture they reach maximal cell density and have matured glutamate receptors [18,28,38]. Since CGC undergo apoptosis after 8-9 days *in vitro*, they are particularly suitable for studying models of acute neurotoxicity. However, one should keep in mind that the results of studies using this *in vitro* model may differ from responses of mature neurons *in situ* in the brain of adult animals. These possible differences may result from distinct reactivity and inherent unstable viability of relatively pure cultures of developing cerebellar granule neurons. Other mechanisms, particularly enhanced oxidative stress, may predominate in the aged brain [42]. Using a protocol of acute neurotoxicity, CGC in culture were exposed to 0.5 mM concentrations of GLU or NMDA for 30 min in the presence of NAM and MNA and neuronal viability was evaluated 24 h later [1,49,50]. Under these experimental conditions we observed relatively weak neuroprotective

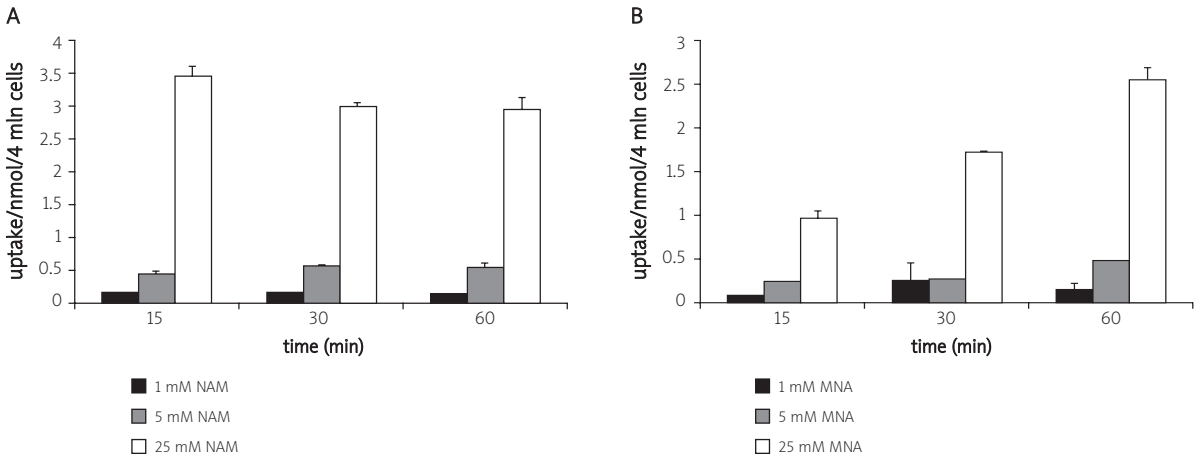

Fig. 7. Time course and effect of concentration on [^{14}C]nicotinamide and [^{14}C]methylnicotinamide uptake in cultured cerebellar granule cells. Uptake of labelled compounds was measured at 37°C for 15 to 60 min as indicated. Data represent means ± SD (n=6)

potential of both compounds tested, when applied at 25 mM concentration.

Neuroprotective abilities of NAM have been observed *in vivo* in various models of brain ischaemia [14,23,30] and also *in vitro* in cultured neurons submitted to excitotoxic insults [39]. It has been generally accepted that excitotoxicity plays an important role in the mechanism of hypoxic and ischaemic brain damage [21,34,36]; thus consistent results of *in vivo* and *in vitro* experiments should be expected. Nevertheless, available data utilizing different experimental *in vitro* models provide conflicting data concerning NAM-induced neuroprotection. Kaneda et al. [22] noticed that chronic preincubation of neurons in rat retinal cultures with 20 and 200 μM nicotinamide, but not co-application with glutamate, reduces its acute excitotoxic action. Also Lin et al. [25] could not find any statistically significant neuroprotective effect of NAM applied in the concentration range from 0.5 to 5 mM. In turn Shen et al. [39] demonstrated that NAM at higher millimolar concentrations reduces injury of cultured cortical neurons evoked by acute exposure to oxygen and glucose deprivation, which induces endogenous excitotoxicity. Our findings confirm that only 25 mM NAM is capable of reducing acute GLU- and NMDA-evoked excitotoxic neuronal damage. To sum up, the results of our present study indicate, in agreement with Shen et al. [39], that neuroprotective activity of NAM *in vitro* in the model of acute excitotoxicity requires high millimolar concentrations of this compound. Interestingly, MNA applied also at 25 mM concentration induced neuroprotection in NMDA-evoked acute neurotoxicity, but had no effect on excitotoxicity caused by GLU. Our results from previous *in vivo* studies demonstrated neuroprotective effects of MNA applied at much lower doses than NAM in a rat model of neonatal asphyxia [40]. Also *in vitro* experiments demonstrated that preincubation with MNA applied at lower concentrations than NAM produces neuroprotection in primary cultures of rat cerebellar granule cells submitted to homocysteine neurotoxicity [41]. In order to find an explanation for this inconsistency, we continued mechanistic studies concerning effects of both substances tested.

It has been generally accepted that the influx of extracellular calcium to neurons via NMDA receptors/channels mediates glutamate-evoked excitotoxicity (for a review see [36]). Previous studies concerning effects of NAM on intracellular calcium concentration in neurons challenged with excitotoxicity provided conflicting data, whereas to our knowledge the effects of MNA on calcium homeostasis have not been studied yet. Frantseva et al. [15], using organotypic cultures of rat hippocampal slices submitted to hypoxia/hypoglycaemia, did not find any significant effect of 25 mM NAM on the intracellular calcium transient measured with fluo-3 fluorescent probe, although they detected a reduction of mitochondrial calcium accumulation. In turn Shen et al. [39] demonstrated inhibition by 20 mM NAM of the intracellular calcium transient evoked by oxygen and glucose deprivation in primary cortical cultures. Also the results of our studies characterizing effects of NAM and MNA on calcium homeostasis in neurons treated with NMDA and GLU are not coherent. On the one hand we observed inhibition by 25 mM NAM and MNA of GLU-evoked ^{45}Ca uptake and concentration-dependent at the millimolar range inhibition by these compounds of NMDA-induced accumulation of ^{45}Ca, suggesting possible interference of these compounds with activity of NMDA receptors. However, this was not followed by significant suppression by NAM or MNA of the increase in intracellular calcium concentration evoked by GLU. On the other hand, we did not find any significant effect of NAM and MNA on [^{3}H]MK-801 binding, which argues against their direct inhibition of NMDA receptors.

Our provisional interpretation of these findings is based on differences between characteristics of calcium homeostasis used in our study. Accumulation of radioactive calcium in neurons treated with excitatory amino acid receptor agonists is a commonly used method of evaluating calcium load and characterizing the activity of NMDA receptors in living neurons [27,49]. In turn, intracellular calcium transients (increases in intracellular calcium concentrations evoked by glutamatergic agonists) result from different mechanisms of calcium homeostasis including calcium influx, release for intracellular stores and calcium buffering (for a review see [36]). Finally, [^{3}H]MK-801 binding to isolated fractions or the rat cortical membrane reflects activation of the NMDA channels independently of the plasma membrane potential [2,37]. Thus inhibition of GLU and NMDA-evoked ^{45}Ca uptake by NAM and MNA without interference with intracellular calcium transient and [^{3}H]MK-801 binding might indicate that these compounds do not interfere directly with the activity

of NMDA channels, and are unable to inhibit all the significant pathways of GLU-induced calcium influx to the cytosol, including mobilization from the intracellular stores. However, it seems that both NAM and MNA may selectively inhibit activation of NMDA receptors in living neurons. This phenomenon is known to be strongly dependent on plasma membrane potential and might be inhibited by compounds stabilizing this potential and reducing neuronal excitability [36]. In fact, a weak affinity of NAM to the GABA-benzodiazepine receptor complex has been repeatedly demonstrated [10,29]. It is not known if NAM shares these properties with its methylated product, MNA. Further studies are required to elucidate the mechanisms of these effects.

In agreement with the lack of significant effects of NAM and MNA on GLU-evoked calcium transients in neurons, we did not observe any protection by these compounds of a drop in the mitochondrial membrane potential in neurons treated with GLU, which was measured with the fluorescent probe Rho123. Stabilization by NAM of the mitochondrial membrane potential was described as the principal mechanism of NAM-evoked neuroprotection and protection of cerebral endothelial cells [8,9].

The results of our experiments using the fluorescent probe DCF sensitive to radical oxygen species demonstrate that GLU-induced excitotoxicity is accompanied by oxidative stress and free radical formation. This is in agreement with many previous studies [11,12,35,36]. Although there are controversies concerning the sequence of events in excitotoxic calcium-mediated neuronal damage, no doubt free radical formation together with mitochondrial calcium overload are major triggers of mitochondrial dysfunction, which in turn participates in production of free radicals and aggravates calcium imbalance. Previous studies demonstrated that NAM is a free radical scavenger in nervous tissue [15,39]. Unexpectedly, in our study NAM had no such effect, whereas 20 and 25 mM MNA significantly reduced free radical formation induced by prolonged 60 min incubation with GLU. At present we have no explanation for this effect.

Based on the results of this study it was difficult to ascribe the pharmacological profile of exogenous MNA to particular subcellular structures such as plasma membrane receptors (e.g. GABA-benzodiazepine complex) or to unidentified intracellular sites. Therefore we addressed the question whether this compound may be accumulated by cultured cerebellar granule cells. Our data demonstrate that CGC accumulate both NAM and MNA at a comparable rate, although NAM uptake was more rapid. It is known that MNA is transported in the renal and hepatic cells by organic cation transporters involving a proton exchange mechanism [31,47]. In human neurons the cation transporter hOCT2 has been identified as being involved in the electrogenic uptake of choline, MNA, tetraethylammonium, MPP+ and related compounds [5]. Consistent with that, MNA interferes with transport of choline in the brain [13]. However, it is difficult to judge whether the same transporter is instrumental in the MNA uptake in rat CGC cultures, as human and rat cation transporters have been shown to differ in their properties [20]. Still, these data indicate that MNA may be accumulated inside neurons and therefore neuronal plasma membrane is not an exclusive site of its action in neurons.

Taken together, this study demonstrates that MNA as well as NAM, applied at high millimolar concentrations to primary cultures of rat CGC, provide neuroprotection in acute excitotoxicity. This effect is combined with inhibition of NMDA receptor activation in cultured neurons, without significant effects on NMDA receptor/channel activity in isolated brain membranes. These data may be explained by the stabilizing effect of NAM and MNA on neuronal potential, e.g. via the GABA-benzodiazepine receptor complex. Our results demonstrate that both compounds studied are accumulated in CGC in comparable rates, so their interference with intracellular sites cannot be excluded. However, in our study MNA as well as NAM did not alter GLU-induced intracellular calcium transients or drop in the mitochondrial membrane potential. Moreover, only MNA, and not NAM, exhibited modest antiradical activity in GLU-induced oxidative stress.

In conclusion, 1-methylnicotinamide like nicotinamide has neuroprotective ability. The exact mechanism of this phenomenon is unclear. We propose that it might be at least partially connected with GABA/benzodiazepine receptor-mediated stabilization of the plasma membrane potential, resulting in the inhibition of voltage-dependent activation of NMDA receptors in neurons. Moreover, both these substances are transported to neurons and therefore intracellular location of their

neuroprotective actions including antiradical activity is also possible.

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