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Effect of the L- or D-aspartate on ecto-5′nucleotidase activity and on cellular viability in cultured neurons: participation of the adenosine A_{2A} receptors

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Summary. Glutamate increases the extracellular adenosine levels, an important endogenous neuromodulator. The neurotoxicity induced by glutamate increases the ecto-5′-nucleotidase activity in neurons, which produces adenosine from AMP. L- and D-aspartate (Asp) mimic most of the actions of glutamate in the N-methyl-D-aspartate (NMDA) receptors. In the present study, both amino acids stimulated the ecto-5′-nucleotidase activity in cerebellar granule cells. MK-801 and AP-5 prevented the L- and D-Asp-evoked activation of ecto-5′-nucleotidase. Both NMDA receptor antagonists prevented completely the damage induced by L-Asp, but partially the D-Asp-induced damage. The antagonist of adenosine A_{2A} receptors (ZM 241385) prevented totally the L- Asp-induced cellular death, but partially the neurotoxicity induced by D-Asp and the antagonist of adenosine A_1 receptors (CPT) had no effect. The results indicated a different involvement of NMDA receptors on the L- or D-Asp-evoked activation of ecto-5′-nucleotidase and on cellular damage. The adenosine formed from ecto-5′-nucleotidase stimulation preferentially acted on adenosine A_{2A} receptor which is probably co-operating with the neurotoxicity induced by amino acids.

Keywords: L- and D-aspartate – Neurotoxicity – Ecto-5′-nucleotidase – Adenosine receptors

Introduction

Excitatory neurotransmission in the mammalian CNS is mediated by excitatory amino acids (EAAs), such as L-, D-aspartate (L-, D-Asp) and principally glutamate (Glu). Rapid uptake of EAAs by high-affinity EAAs transporters is believed to be the principal mechanism of the removal of EAAs from synaptic cleft. Nevertheless, in the neuropathological conditions these transporters have an important role on EAAs release by reversal of transporter activity, triggering neuronal death (Rossi et al., 2000).

Excitotoxicity mediated by Glu and L-Asp has been described in many instances to excessive activation of the N-methyl-D-aspartate (NMDA) receptors-channel complex (Rothman and Olney, 1995). The EAAs, L-and D-Asp, are putative NMDA receptors agonists mimicking most of the actions of Glu on NMDA receptors by sharing the same binding site (Olverman et al., 1988). The activation of NMDA receptors promotes the increase of extracellular adenosine levels (Hoehn and White, 1990), an important endogenous neuromodulator. The extracellular adenosine can be released via a bi-directional transporter and/or formed from adenine nucleotides released that are degraded by an extracellular chain of ecto-nucleotidases. The nucleotides ATP and ADP are hydrolyzed through ecto-ATP diphosphohydrolase activity resulting in the formation of AMP, which products adenosine by ecto-5′-nucleotidase activity. Ecto-5′-nucleotidase activity is a pivotal step of the extracellular adenosine production from enzymatic chain (Zimmermann and Braun, 1999). The activation of adenosine A_1 receptors inhibits neuronal firing, decreases calcium uptake (Palmer and Stiles, 1995) and inhibits release of excitatory neurotransmitters such as Glu and Asp (Poli et al., 1991), contributing to the neuroprotection. The activation of adenosine A_{2A} receptors enhances synaptic transmission and the glutamate release (O'Regan et al., 1992), which could contribute to the neurotoxicity. In cultured neurons (Boeck et al., 2000) as well as hippocampal slices (Bruno et al., 2002) have been demonstrated that Glu stimulates extracellular

nucleotides hydrolysis via ectonucleotidases. On the other hand, it is unclear whether the adenosine produced acts on adenosine A_1 or A_{2A} receptors since the affinity of adenosine for these subtypes of receptors is similar (reviewed in Cunha, 2005). The stimulation of ecto-nucleotidase activities induced by Glu in neurons was mimicked by NMDA (Boeck et al., 2000). Since L- and D-Asp are agonists to NMDA receptor, in the present study we investigated if neurotoxic dose of L- and D-Asp mimic the stimulation of ecto-5′-nucleotidase activity via NMDA receptors activation. Moreover, we tested the potential neurotoxic effects of L- and D-Asp and if the NMDA receptor antagonists and the adenosine A_1 or A_{2A} receptors antagonist could be able to affect the L- and D-Asp-induced cellular damage in cerebellar granule cells.

Materials and methods

Materials

L-aspartic acid and D-aspartic acid were obtained from Sigma (Sto Louis, MO, USA). N-cyclopentyladenosine (CPA), 8-cyclopentyl-1,3-dimethylxanthine (CPT) was obtained from RBI-Research Biochemical International (Natick, MA, USA). Dizocilpine (MK-801), (2-amino-5-phosphonopentanoic acid (A-P5) and (4-(2[7-amino-2-(2-furyl{1,2,4}-triazolo{2,3-a{1,3,5}triazian-5-yl-aminoethyl)phenol (ZM 241385) were obtained from Tocris Cookson (Bristol, UK). Propidium Iodite (PI) was purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA, USA). Cell culture solutions were purchased from Sigma or Gibco BRL (Life Technologies, Scotland, UK).

Cerebellar granule cell culture

Primary cultures of cerebellar granule cells were prepared from 8-day-old Wistar rats (Boeck et al., 2000). Briefly, freshly dissected cerebella were incubated with 0.025% trypsin solution for 15 min at 37 °C and disrupted mechanically with a fire-polished Pasteur pipette in the presence of 0.08 mg/ml DNase and 0.05% trypsin inhibitor. Cells were seeded at a density of $3.0 \times 10^5/\text{cm}^2$ in a 24-well multiwell dish (Nunc) coated with 10 μg/ml poly-D-lysine in Eagle's basal medium (BME) supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil), 50 μg/ml gentamicin and 25 mM KCl. The growth of non-neuronal cells was inhibited by an addition of 20 μM cytosine arabinofuranoside 18–20 h after seeding and the medium was maintained without any change during the culture period. Due to medium evaporation, water and 0.1% glucose were added to a final volume of 100 μL at 7 days in vitro (DIV). These cultures contain > 90% granule cells, 4–6% GABAergic neurons, and a small number of glial (2–3%) and endothelial cells (<1%) (Kingsbury et al., 1985).

Determination of ecto-5′-nucleotidase activity

After 8 DIV, the nutrient medium was carefully removed from dishes, and the cell monolayer was gently washed once with pre-warmed sodium containing HEPES-buffered saline (120 mM NaCl, 5 mM KCl, 2 mM CaCl2 and 10 mM glucose) (Boeck et al., 2000). Cells were pre-incubated for 30 min in the presence or absence of 1 mM L- or D-Asp. Neurotoxicity doses were used, because our aim was to analyze the possible correlation between the activity of this enzyme and at the same time the neurotoxicity induced by these ligands. As specified, 1 μM dizocilpine (MK801, non-competitive NMDA receptor antagonist) or 100 μM (±)-2-amino-5-phosphonopentanoic acid (AP-5, competitive NMDA receptor antagonist) were present (Tremblay et al., 2000). Incubation of cerebellar granule cells started by the addition of 1 mM AMP (final concentration) and incubated for 10 min at 37 °C. The reaction was stopped by the removal of aliquots, which were then mixed with 5% trichloroacetic acid (TCA, final concentration). After a centrifugation of 5 min at 12 000 × *g* at 4 °C, the aliquots were taken for assay of released inorganic phosphate (Pi) (Chan et al., 1986). Non-enzymatic Pi released from nucleotide into assay medium without cells and Pi released from cells incubated without nucleotide was subtracted from the total Pi released during incubation, giving values for enzymatic activity. Enzymatic activity was expressed as nmol Pi/mg protein/min. We have observed, as previously described, that at the end of the experiment the survival and morphology of cells is maintained (Boeck et al., 2000). Protein was determined using bovine serum albumin as standard (Bradford, 1976).

L- and D-Asp neurotoxicity and cellular damage

At 8 DIV, the nutrient medium was carefully removed from dishes, and the cell monolayer was gently washed twice with pre-warmed HEPES-buffered saline. Cells were incubated with or without 1 mM L- or D-Asp in the presence or absence of NMDA receptor antagonists (1 μM MK-801 or 100 μM AP-5) for 30 min in order to verify the possible involvement of NMDA receptors activation in the neurotoxicity induced by EAAs. To verify the effect of adenosine formed from endogenous AMP hydrolysis induced by L- or D-Asp, the cells were incubated with 100 nM CPT, adenosine A_1 receptors antagonist, (Manzoni et al., 1994) or 50 nM ZM 241385, adenosine A_{2A} receptor antagonist, (Dall'Igna et al., 2003) in the presence of erytro-9-[2-hydroxy-3-nonyl]adenine (10 μM EHNA, inhibitor of adenosine deaminase) and nitrobenzylthioinosine (10 μM NBTI, blocker of adenosine transporters) for 10 min before L- or D-Asp incubation. After the neurotoxicity assay period, cells were carefully washed twice with pre-warmed HEPES-buffered saline and previously the saved sister culture medium (culture conditioned medium) was replaced. Cell viability was monitored before and after each experiment by the inverted phase-contrast microscopy. Cells death was assessed 24 h post-neurotoxicity assay period using uptake of the fluorescent exclusion dye propidium iodide (PI), which is a polar compound that only enters into the dead or dying cells with damage membranes. Cells were incubated with 5 μg/ml PI for 30 min and then imaged on a standard inverted microscope (Nikon Eclipse TE 300) using a rhodamine filter set (excitation 540 nm; emission 617 nm).

The PI uptake was quantified by a densitometric analysis using Scion Image software (Scion Corporation). The quantification of cell death, which corresponds to PI uptake into cells damaged, was expressed as the percentage of dead cells from control cells (untreated cells).

Statistics

Statistical analysis among groups was performed from independent cultures ($n = 3$–4) and the data were evaluated by a one-way analysis of variance (ANOVA) followed by Duncan's post-hoc test. Differences were considered significant at <95% confidence.

Results

Since either Glu or NMDA increase the AMP hydrolysis, we investigated whether the Glu analogs and ligands for NMDA, L- and D-Asp, have the same effect on ecto-5′-nucleotidase activity. Cells were pre-incubated with either 1 mM L- or D-Asp for 30 min and the hydrolysis of

AMP was measured. Figure 1 shows the stimulatory effect of both L- and D-Asp on ecto-5′-nucleotidase activity from 28.5 ± 2 to 40.1 ± 6 nmol Pi/mg protein/min ($40.9 \pm 15\%$, $n = 3$–4) (Fig. 1A); or from 25.5 ± 2 to 39.8 ± 3 nmol Pi/mg protein/min ($57.8 \pm 17\%$, $n =$ 3–4) (Fig. 1B), respectively. At the end of the incubation with L- or D-Asp we could not observe any cellular morphologic alterations monitored by phase microscopy and the amount of Pi released by cells during incubation period with L- or D-Asp alone (without AMP) was not significantly different of control cells; consequently this effect was not due to cell disruption (data not shown). To determine whether the increase of AMP hydrolysis evoked by L- and D-Asp was mediated by NMDA receptors activation, MK-801 or AP-5 (NMDA receptor antagonists) were present during the pre-incubation with L- or D-Asp. Both antagonists prevented the stimulatory effect induced by L-Asp and D-Asp on AMP hydrolysis. Neither

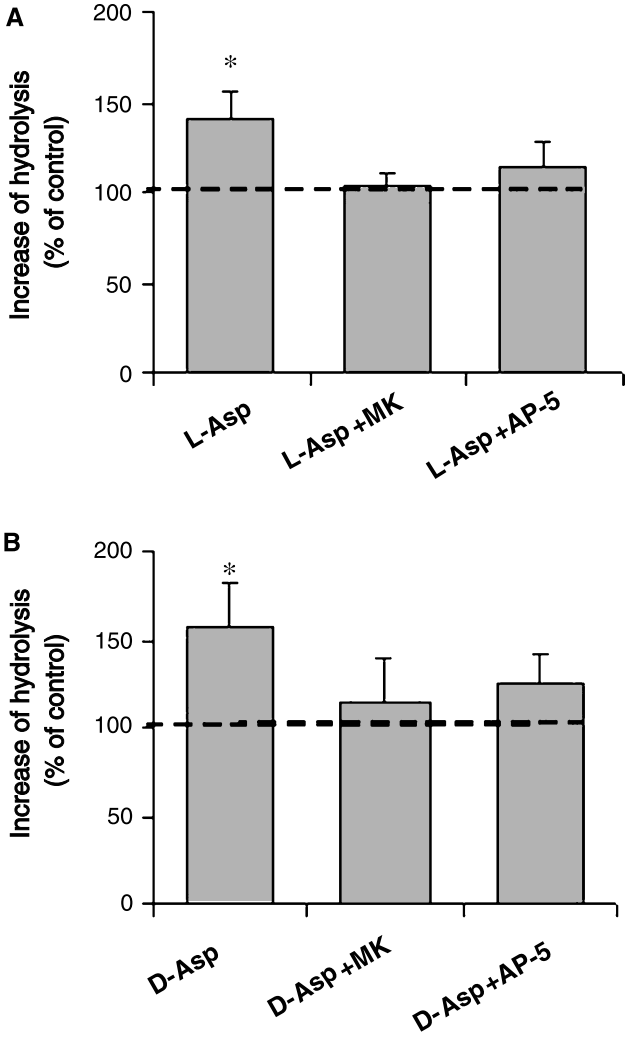

Fig. 1. Stimulation of the ecto-5′-nucleotidase activity induced by L-Asp or D-asp (1 mM) in the presence or absence of antagonists of NMDA receptors (1 μM MK801 or 100 μM AP-5) in cerebellar granule cells. **A** L-Asp, **B** D-Asp. *Dashed line*, control group. Values are mean ± SD of independent cultures ($n = 3$–4). $^{*}p < 0.05$ from all other groups

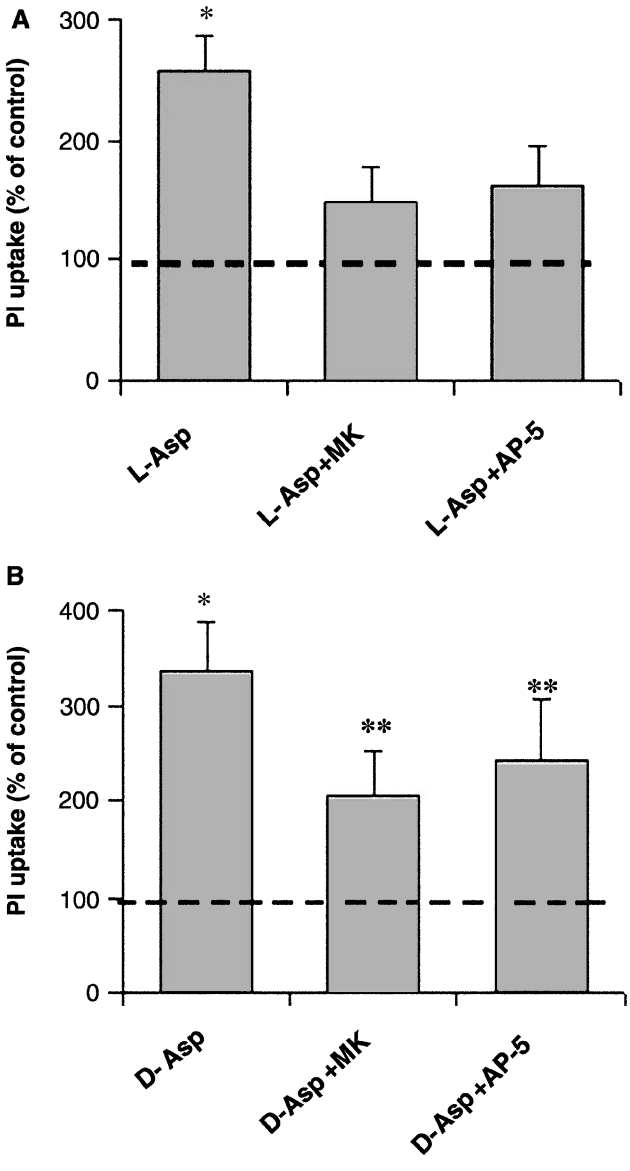

Fig. 2. Effect of NMDA receptors antagonist on neurotoxicity evoked by L-Asp (**A**) or D-Asp (**B**). Cells were treated with or without L- or D-Asp (1 mM) in the presence or absence of antagonists of NMDA receptors (1 μM MK-801 or 100 μM AP-5). Images were captured and then analyzed using Scion Image software. The area where Pi fluorescence was detectable above background was determined by densitometric analysis. Values of the quantification of L- or D-Asp-induced cellular damage are expressed as mean ± S.E.M. of independent cultures ($n = 3$). MK-801 or AP-5 applied alone had no effect on PI uptake. *Dashed line*, control group (0.59 ± 0.07 of PI uptake). $^{*}p < 0.05$ vs respective control, L- or D-Asp + MK-801 and L- or D-Asp + AP-5 groups; $^{**}p < 0.05$ vs control and D-Asp groups

MK801 nor AP-5 alone has any effect on AMP hydrolysis. In order to investigate the involvement of NMDA receptors on the neurotoxicity induced by EAAs, cells were incubated with L- or D-Asp in the presence or absence of MK-801 or AP-5 during the neurotoxicity assay period. Following 24 h the cellular damage was assayed by PI uptake (Fig. 2). L- Asp and D-Asp induced cells to damage [$156.8 \pm 29.7\%$ ($n = 3$) and $236.4 \pm 53.1\%$ ($n = 3$) of PI uptake from control cells, respectively] in a similar way to that observed with Glu (data not shown). Both NMDA receptor antagonists prevented completely the neuronal death induced by L-Asp ($47.4 \pm 29.8\%$ for MK-801 and $61.1 \pm 34.6\%$ for AP-5), but partially the D-Asp-induced cellular death ($106.6 \pm 48.3\%$ for MK-801 and $143.3 \pm 62.7\%$ for AP-5).

Considering that the increase of the ecto-5′-nucleotidase activity induced by neurotoxic dose of L-and D-Asp leads to enhance in the extracellular adenosine levels, cells were exposed to adenosine A_1 or A_{2A} receptor antagonists to evaluate their effect on EAAs neurotoxicity. Since cerebellar granule cells possess the bidirectional nucleoside transporter sensitive to NBTI (Sweeney, 1996) this experiment was carried out in presence of NBTI in order to exclude the participation of adenosine released by this mechanism and /or adenosine uptake.

EHNA was also included in the incubation medium to avoid adenosine degradation. Cells were incubated with L- or D-Asp plus NBTI and EHNA in the presence or absence of CPT or ZM 241385 for 30 min. Following 24 h the cellular damage was assayed by PI uptake (Fig. 3). The cellular damage induced by L- Asp and D-Asp was $137.7 \pm 38.1\%$, $n = 4$ and $133.9 \pm 37.1\%$, $n = 4$ of PI uptake from control cells, respectively. ZM 241385 (adenosine A_{2A} receptor antagonist) prevented completely the neurotoxicity induced by L-Asp ($18.7 \pm 14.9\%$), but partially the neuronal damage induced by D-Asp ($45.0 \pm 13.2\%$) and CPT (adenosine A_1 receptor antagonist) had no effect on the cellular death induced by these EAAs ($119.4 \pm 40.7\%$ for L-asp and $107.1 \pm 48.1\%$ for D-Asp). The neuronal death observed in cells incubated with L- or D-Asp alone was the same observed in the group incubated with these EAAs in presence of NBTI and EHNA. In addition, NBTI and EHNA had no effect when applied alone (data not shown).

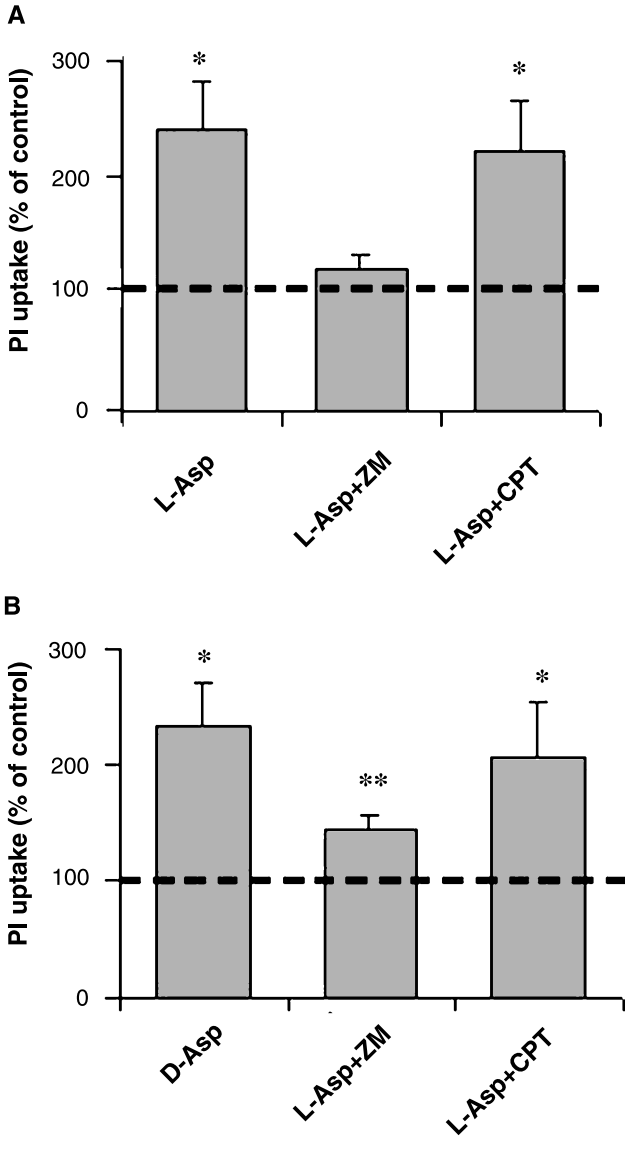

Fig. 3. Effect of selective adenosine A_1 and A_{2A} receptors antagonist on neurotoxicity evoked by EAAs. Cells were treated with or without 1 mM L- (**A**) or D-Asp (**B**) (plus 10 μM NBTI and 10 μM EHNA) in the presence or absence of CPT (100 nM) or ZM 241385 (50 nM). Images were captured and then analyzed using Scion Image software. The area where Pi fluorescence was detectable above background was determined by densitometric analysis. Values of the quantification of L- or D-Asp-induced cellular damage are expressed as mean ± S.E.M. of independent cultures ($n = 4$). CPA or ZM applied alone had no effect on PI uptake. *Dashed line*, control group (0.8 ± 0.2 of PI uptake). $^{*}p < 0.05$ vs respective control and L- or D-Asp + ZM groups; $^{**}p < 0.05$ vs control, D-Asp and D-Asp + CPT groups

Discussion

The present report demonstrates that L- and D-Asp were able to stimulate the ecto-5′-nucleotidase activity and led cerebellar granule cells to damage. The stimulation evoked by L-Asp or D-Asp was mediated by NMDA receptors activation, since the non-competitive (MK-801) and the competitive antagonist (AP-5) NMDA receptor antagonists were able to prevent their stimulation upon ecto-5′-nucleotidase. We also observed the protective

effect evoked by NMDA receptor antagonists against cellular damage induced by L-Asp, but with partial effect upon D-Asp-induced neurotoxicity. Altogether these results described herein could suggest a different participation of NMDA receptors on the L-Asp or D-Asp actions in cerebellar granule cells. Therefore, the effect of L-asp and D-asp on AMP hydrolysis was due to activation of NMDA receptors, but the neurotoxicity induced by D-Asp probably involves activation of other subtype of Glu receptors. It has been demonstrated that D-Asp, but not L-Asp, reduces the amplitude of miniature excitatory postsynaptic current in cultured hippocampal neurons by competing with L-Glu in binding to the agonist binding site on the AMPA receptors (Gong et al., 2005). Thus, our results corroborate with the studies that indicate L-Asp as agonist to NMDA receptors, but the NMDA receptor activation by D-Asp depends on the parameter analyzed. Since the activation of the AMP hydrolysis induced by high dose of EAAs enhances the extracellular adenosine levels and this adenosine could be acting on adenosine A_1 or A_{2A} receptors, we investigated the effect of the antagonist for both sub-types of adenosine receptors on EAAsinduced neurotoxicity. This experimental design was carried out in presence of NBTI, a blocker of bi-directional adenosine transporter, in order to avoid adenosine release and/or adenosine uptake by this bi-directional carrier. Our results demonstrated that adenosine A_{2A} receptor antagonists (ZM 241385) prevented totally the L-Asp-induced cellular death, but partially the neurotoxicity induced by D-Asp and adenosine A_1 receptor antagonist (CPT) had no effect. Therefore, these data suggest that the adenosine produced by the stimulation of ecto-5′-nucleotidase induced by L- or D-Asp promoted activation of adenosine A_{2A} receptors, which are contributing with a neurotoxicity induced by these EAAs. Since the neurotoxicity induced by D-Asp was partially blocked by NMDA and adenosine A_{2A} receptors antagonists, we could suppose that D-Asp is also acting through other mechanisms in order to promote the cellular damage. Our observations are in agreement with previous studies showing that adenosine released by bi-directional nucleoside carrier leads to preferential adenosine A_1 receptors activation, whilst formed from ectonucleotidases pathway leads to favored adenosine A_{2A} receptors activation (Cunha et al., 1996; Cunha, 2005; Ribeiro, 1999). It is known that adenosine A_{2A} receptors activation is involved on the EAAs release (O'Regan et al., 1992), which promotes neuronal damage and it could explain in part the neuroprotective properties of adenosine A_{2A} receptor antagonists. However, this hypothesis does not satisfactorily explain the neuroprotection observed by ZM 241385 in the present report, since the neurons were incubated with high concentration of EAAs. Additionally, it has been shown that the efficacy of some neuroprotective drugs can not be simply ascribed to their ability to inhibit glutamate release (Calabresi et al., 2000). Thus, the cellular mechanisms involved on the neuroprotection displayed by adenosine A_{2A} receptors antagonists remain to be elucidated.

Recently we demonstrated that chemical preconditioning, which prevented cellular death induced by Glu, abolished the activation of ecto-5′-nucleotidase induced by Glu and at the same time promoted the desensitization of adenosine A_{2A} receptors, suggesting strongly the view that these two events are tightly interconnected (Boeck et al., 2005). Furthermore, it has been shown that noxious stimuli cause a parallel increase of the expression of ecto-5′-nucleotidase and of adenosine A_{2A} receptors (Napieralski et al., 2003). Therefore, we suggest that adenosine formed from ecto-nucleotidases stimulation by EAAs is acting on adenosine A_{2A} receptor, co-operating with the EAAs-induced neurotoxicity.

Conclusion

The present study indicated a different involvement of NMDA receptors on the L-Asp or D-Asp actions in cerebellar granule cells. The neurotoxicity and the stimulation of the ecto-5′-nucleotidase activity displayed by L- Asp was due to NMDA receptors activation, but the contribution of this receptor on the neurotoxicity induced by D-Asp was just partial, probably the activation of other subtype of Glu receptors are involved in this effect. Besides the adenosine formed from ecto-5′-nucleotidases stimulation acted preferentially on adenosine A_{2A} receptor and this effect co-operated with the neurotoxicity induced by EAAs.

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