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Region-specific neuroprotective effect of ZM 241385 towards glutamate uptake inhibition in cultured neurons

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ABSTRACT

Active uptake by neurons and glial cells is the main mechanism for maintaining extracellular glutamate at low, non-toxic concentrations. Adenosine A_{2A} receptors regulate extracellular glutamate levels by acting on both the release and the uptake of glutamate. The aim of this study was to evaluate whether the inhibition of the effects of glutamate uptake blockers by adenosine A_{2A} receptor antagonists resulted in neuroprotection. In cortical and striatal neuronal cultures, the application of L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC, a transportable competitive inhibitor of glutamate uptake), induced a dose-dependent increase in lactate dehydrogenase (LDH) levels, an index of cytotoxicity. Such an effect of PDC was significantly reduced by pre-treatment with the adenosine A_{2A} receptor antagonist ZM 241385 (50 nM) in striatal, but not cortical, cultures. The protective effects of ZM 241385 were specifically due to a counteraction of PDC effects, since ZM 241385 was totally ineffective in preventing the cytotoxicity induced by direct application of glutamate to cultures. These results indicate that adenosine A_{2A} receptor antagonists prevent the toxic effects induced by a transportable competitive inhibitor of glutamate uptake, that such an effect specifically occurs in the striatum and that it does not depend on a direct blockade of glutamate-induced toxicity.

1. Introduction

Glutamate is the main excitatory transmitter in the central nervous system (CNS). While a physiological glutamatergic transmission ensures numerous CNS processes, such as learning and memory, excessive glutamate concentrations may result in neuronal toxicity (excitotoxicity) and eventual neuronal cell death (Choi, 1988, 1992). Glutamate homeostasis is accomplished by the activity of glutamate transporters (excitatory amino-acid transporters, EAATs) expressed by neurons and glial cells (see Danbolt, 2001, for review), which represent the primary mechanism for maintaining low, non-toxic extracellular concentrations of glutamate. Indeed, an impairment of the glutamate transporter system is thought to play a pathogenetic role in conditions such as cerebral ischemia (Seki et al., 1999; Rossi et al., 2000) and chronic neurodegenerative diseases (Harris et al., 1995; Lin et al., 1998; Behrens et al., 2002).

Adenosine A_{2A} receptors are being regarded as promising targets for the development of neuroprotective strategies, in particular for those neurodegenerative diseases in which excitotoxicity plays a critical pathogenetic role. Adenosine A_{2A} receptor antagonists blocked high-K^+- (Corsi et al., 2000; Pintor et al., 2001), ischemia- (Melani et al., 2006; Marcoli et al., 2003) or quinolinic acid (QA)-stimulated glutamate outflow (Popoli et al., 2002, 2003), an effect most probably due to the blockade of adenosine A_{2A} receptors on pre-synaptic glutamatergic terminals (Hettinger et al., 2001). In addition, A_{2A} receptors have been reported to negatively modulate glutamate transport (Nishizaki et al., 2002). In agreement, in a striatal microdialysis study, the selective adenosine A_{2A} receptor antagonists SCH 58261 and ZM 241385 prevented the raise in extracellular glutamate induced by glutamate uptake inhibitors (Pintor et al., 2004). Whether the inhibition of the effects of glutamate uptake blockers by A_{2A} receptor antagonists may result in neuroprotection has never been investigated.

The aim of the present work was to evaluate the influence of the selective adenosine A_{2A} receptor antagonist ZM 241385 on the cytotoxicity induced by glutamate uptake inhibition in primary neuronal cultures. To better mimic reverse transport occurring in some disease states such as ischemia (Nafia et al., 2008 and references therein; Rossi et al., 2000), L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC), a transportable competitive inhibitor that also induces glutamate release through heteroexchange (Volterra et al., 1996; Koch et al., 1999) was used. Two brain areas characterized by a very different expression of adenosine A_{2A} receptors (namely the cortex and the striatum) were compared.

2. Materials and methods

2.1. Western blotting

Since adenosine A_{2A} receptors regulate the glial glutamate transporter-1 (GLT-1) (Nishizaki et al., 2002; Pintor et al., 2004), and since a certain

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degree of neuronal GLT-1 expression has been reported in hippocampal neurons (Mennerick et al., 1998), we wanted to verify first the content of GLT-1 in our primary cortical and striatal neurons (see below) by Western blot analysis. In the same samples we verified also the presence of excitatory amino-acid carrier-1 (EAAC 1). Proteins extracted from cortex of C57BL/6 mice were used as positive control for GLT-1. After 15 days of culture, cells were lysed in Ripa buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml PMSF, leupeptin, aprotinin, pepastatin A and sodium orthovanadate) for 30 min on ice. The lysates were centrifuged at 12,000 rcf for 20 min at 4 °C and the pellets were discarded. Protein analysis was conducted by the Biorad Protein Assay (Bio-Rad Laboratories, Milan, Italy) using BSA as standard. Fifty micrograms of protein was separated onto 8% acrylamide gels and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories). Membranes were blocked with 5% non-fat dry milk in TBST buffer (0.1 M Tris base, 0.15 M NaCl, 0.05% Tween 20, pH 7.4) and then incubated with anti-GLT-1 antibody (1:2000) or with anti-EAAC 1 antibody (1:500) overnight at 4 °C. Following incubation with HRP-linked anti-rabbit antibody (1:5000), visualization of the bound antibodies was performed with the Enhanced ChemiLuminescence (ECL) system (Pierce, S.I.A.L., Rome, Italy). The blots were exposed to X-ray film and then scanned. Anti-glutamate transporter GLT-1 (AB1783), anti-EAAC 1 (MAB 1587) and HRP-linked anti-rabbit antibodies were purchased from Chemicon International (Milan, Italy). Supersignal West Pico Chemiluminescent substrate was purchased from Pierce (S.I.A.L., Rome, Italy).

2.2. Cytotoxicity in primary neuronal cultures

To evaluate the potential neuroprotective effects of ZM 241385 towards PDC-induced toxicity, primary neuronal cultures were used. Neuronal cultures were preferred because of their higher vulnerability to uptake blockers with respect to mixed astrocytic/neuronal or astrocyte-enriched cell cultures (Blitzblau et al., 1996).

Animal care and use followed the directives of the Council of the European Communities (86/609/EEC). Brain areas were isolated from 17–18 day old embryos. Pregnant rats were ether anaesthetized, decapitated and the foetuses collected and rapidly decapitated. After removal of the meninges, the cortices and the striata were dissected, transferred to Hank's balanced salt solution (HBSS) and mechanically fragmented. The tissue fragments were transferred to 0.025% trypsin solution and incubated for 15 min at 37 °C. Striatal and cortical cells were then washed in HBSS and resuspended in Neurobasal medium supplemented with 0.5 mM L-glutamine, 2% B-27 supplement, 5 U/ml penicillin and 5 μg/ml streptomycin (referred as complete medium). Aliquots of $2–3 \times 10^4$ cells were placed in 24-well culture plates coated with poly-L-lysine (5 μg/ml) and maintained at 37 °C in humidified air with 5% CO_2.

Every 4 days, 0.5 ml of medium was removed and replaced by the same volume of fresh complete medium. Under these experimental conditions, cultures consist predominantly of neurons as previously described (Brewer et al., 1993; Brewer, 1995). In particular, in our cultures the percentage of contaminating astrocytes is normally ≤10%. Assays were done on 15 day old cultures. At the time of the experiment, culture medium was removed and substituted by an appropriate volume of Neurobasal medium supplemented with penicillin (5 U/ml) and streptomycin (5 μg/ml). Cultured cells were then exposed to PDC (12.5–200 μM) or glutamate (12.5–100 μM) for 1 h, preincubated or not for 15 min in ZM 241385 (50 nM), MK-801 (10 μM) or control medium. All drug treatments were performed at 37 °C in humidified air with 5% CO_2. Following exposure to the drugs, culture medium was removed and replaced with fresh complete medium. Cultures were then returned to the incubator and cellular damage was evaluated, 24 h later, by measuring the amount of lactate dehydrogenase (LDH) released into the medium using a cytotoxicity detection kit (Roche Diagnostic, Indianapolis, IN). The reaction was run at room temperature with light protection for 30 min.

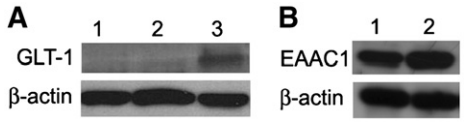

Fig. 1. GLT-1 is not expressed in neuron-enriched cortical and striatal cultures. The figure shows a representative Western blotting analysis. Panel A: GLT1 is clearly revealed in the positive control (cortex of C57BL/6 mice, lane 3), while no expression is seen in cortical and striatal cultures (lanes 1 and 2). Beta-actin was used as a loading control. Panel B: the presence of EAAC 1 in cortical and striatal cultures (lanes 1 and 2) is confirmed. Beta-actin was used as a loading control.

Results are expressed as percentage of control, which was considered as 100%, and represent means ± SEM values of 3–4 independent experiments, assayed in triplicate.

2.3. Drugs

L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC) and 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino] ethyl)phenol (ZM 241385) were purchased from Tocris Cookson (Bristol, UK). MK-801 was purchased from RBI (Natick, MA, USA).

L-glutamic acid was from Sigma-Aldrich (Milan, Italy).

3. Results

3.1. Western blotting

As shown in Fig. 1A, it is not possible to reveal GLT-1 presence either in cortical (lane 1) or in striatal (lane 2) primary cultures, where, on the contrary, EAAC 1 is clearly expressed (Fig. 1B). GLT-1 can be detected only in the cortex of C57BL/6 mice used as positive control (Fig. 1A, lane 3).

3.2. Influence of ZM 241385 on PDC-induced striatal and cortical cell injury

One hour incubation of striatal cultures with PDC (12.5–200 μM, $N = 4–12$/dose) induced a significant, dose-dependent increase in LDH release with respect to basal levels (Fig. 2A). PDC-induced toxicity was mainly mediated by the NMDA receptors, since it was prevented by MK-801 (MK-801 10 μM + PDC 100 μM: LDH release 88 ± 3.8% of basal, $N = 3$, Fig. 2A). Exposure to ZM 241385 (50 nM, $N = 4–9$/group) 15 min before and then together with PDC significantly reduced LDH release with respect to PDC alone (Fig. 2B). When applied alone, neither MK-801 nor ZM 241385 influenced basal LDH release (not shown).

PDC ($N = 4–9$/dose) induced a concentration-dependent cytotoxicity also in cortical cultures, as shown in Fig. 2C, an effect prevented, also in this case, by MK-801 (MK-801 10 μM + PDC 100 μM: LDH release 98.7 ± 5.9% of basal, $N = 3$, Fig. 2C). Unlike in the striatal cultures, ZM 241385 (50 nM) did not influence LDH release vs. PDC alone (Fig. 2D). In a limited number of experiments ZM 241385 was tested at 100 nM and it was ineffective as well in reducing PDC toxicity in cortical neurons ($N = 3$, data not shown). These data indicate that the protective effects of ZM 241385 towards PDC are region-specific.

3.3. Influence of ZM 241385 on glutamate-induced neuronal cell injury

To verify whether ZM 241385 effects depended upon a specific influence on glutamate transport, this antagonist was tested towards the toxicity induced by direct application of glutamate.

One hour incubation of striatal and cortical cells with glutamate (12.5–100 μM, $N = 6–9$/dose) induced a significant, dose-dependent increase in LDH release with respect to basal levels (Fig. 3A and C). Glutamate-induced toxicity depended on NMDA receptor activation, since it was fully prevented by MK 801 both in striatal and cortical cultures (MK 801 10 μM + Glu 100 μM: LDH release 101.5 ± 6.7% of