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Short communication

Adenosine and ADP prevent apoptosis in cultured rat cerebellar granule cells

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Abstract

Cerebellar granule cells (CGCs) explanted in vitro undergo death via apoptosis when the concentration of potassium is shifted from 25 mM to 5 mM. We report that adenosine and ADP, which act as neurotransmitters and neuromodulators in the brain, exert in cultured cerebellar granule cells a specific and marked antiapoptotic action with half-maximal effect in the 10–100 μM range. The action of adenosine is partly inhibited by the A1AR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and is mimicked by the A1AR agonist 2-chloro-*N*6-cyclopentyladenosine (CCPA), while ADP effect, that is completely blocked by the P2x, P2y receptors noncompetitive antagonist suramine, is restored in the presence of the selective P2x purinoceptors agonist β,γ-methylene-L-ATP. These findings demonstrate that adenosine and ADP markedly inhibit the program of cell death in cerebellar granule cells and suggest that such an action is mediated via interaction with, respectively, A1 and P2x receptors.

Keywords: Cerebellar granule cell; Apoptosis; Purinoceptor; Adenosine; ADP

We have previously reported that lowering extracellular K^+ from 25 mM to 5 mM triggers apoptosis in cultured rat cerebellar granule cells [6] via a partial closure of L-type Ca^{2+} channels with consequent drop of intracellular concentrations of this cation [8]. Pharmacological treatments that counteract the Ca_i^{2+} drop block apoptosis, while antagonists of L-type Ca^{2+} channels, such as nifedipine, cause apoptosis even in 25 mM K^+ [8]. It has been reported that cyclic ADP ribose (cADPR) releases Ca^{2+} in various cell types and its production is achieved by CD38, a type II transmembrane glycoprotein with properties of bifunctional ectoenzyme which is present also in cerebellar granule cells (CGCs) [5].

In view of the crucial role played by Ca_i^{2+} homeostasis in these neurons and of the action of cADPR in inducing release of this cation from intracellular stores, we tested the possibility that this cyclic nucleotide and its precursor βNAD could counteract the apoptotic pathway. Since only βNAD, but not cADPR, showed a significant and reproducible neuroprotective action (see also Fig. 1), a systematic search for the active component of this compound responsible of the antiapoptotic action was undertaken and led to identify adenosine and ADP as the constituents fully endowed with the antiapoptotic activity.

Since nucleotides and nucleosides have been shown or postulated to play also the function of trophic substances [16], besides a neurotransmitter and neuromodulatory role, the marked and specific antiapoptotic action exerted on CGCs first reported in this paper could have relevance to all conditions whereby a control of the program of cell death could play a crucial role. Among these are the events controlling the extent and site(s) of cell death during development, when approximately 50% of neurons are doomed to die during formation of neural networks and several neurodegenerative disorders affecting the adult brain.

Cultures enriched in granule neurons were obtained from dissociated cerebella of eight-day-old Wistar rats (Charles River Breeding Laboratories), according to the procedure previously described [13]. Cells were plated in basal medium Eagle (BME; GIBCO) supplemented with fetal bovine serum (FBS) on four-well dishes (NUNC) previously coated with poly-L-lysine (10 μg/ml). Cellular density was 0.5×10^6 cells per well. After 18–22 h, 1-β-arabinofuranosylcytosine (10 μM) was added to the culture medium to prevent replication of non-neuronal cells.

In order to induce apoptosis, after 6–7 DIV, the culture medium containing 25 mM KCl was replaced with an identical medium devoid of serum and containing 5 mM KCl [6] plus or minus (controls) the substance tested for its possible antiapoptotic action. The antagonists were added

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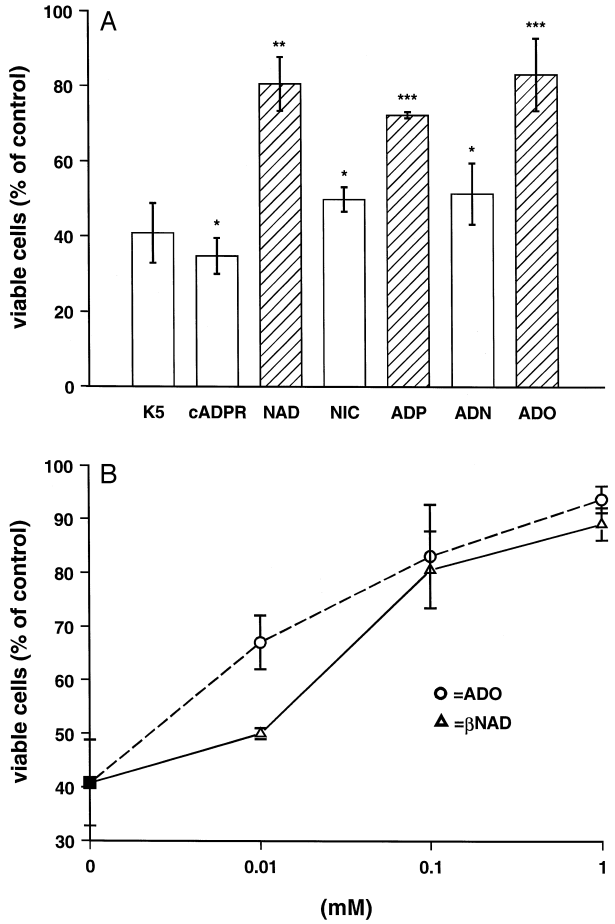

Fig. 1. (a) Survival of neurons in 5 mM KCl after treatment with various agents. Induction of apoptosis was performed switching 6 DIV-cultured cerebellar granule cells from culture medium containing 10% FBS and 25 mM KCl to serum-free medium with 5 mM KCl and no additives (K5) or with 10 μM cyclic ADPR (cADPR), 100 μM βNAD (NAD), 100 μM nicotinamide (NIC), 100 μM ADP, 100 μM adenine (ADN), or 100 μM adenosine (ADO). Forty-eight hours later, viable cells were counted and compared to controls in serum-free medium containing 25 mM KCl. (b) Concentration dependence of the antiapoptotic action of βNAD and adenosine (ADO). Cells treated as above were tested with different concentrations of βNAD and adenosine. Forty-eight hours later, viable cells were counted and compared to controls. The maximum antiapoptotic activity is achieved at 1 mM, whereas half-maximal effect occurs in the 10–100 μM range. Each bar represents mean ± S.D. Statistically significant differences were estimated with Duncan's multiple range test. * D = n.s. compared to K5; ** D = s. compared to K5; *** D = n.s. compared to 100 μM βNAD. The experiments were repeated four times in duplicate with different cell preparations ($n = 4$).

15 min before addition of corresponding agonists. Controls underwent the same procedure, but their medium contained 25 mM KCl.

After 24 or 48 h of incubation in 5 mM KCl and 25 mM KCl medium, viable cells were determined by counting intact nuclei at contrast phase microscope as previously described [23] or were processed for TUNEL (terminal UTP end labeling) in order to detect DNA degradation typical of apoptosis. To this aim, the cells were fixed for 10 min in 4% paraformaldehyde, rinsed in Tris–EDTA 0.5 μg/ml (TE), briefly processed in TdT buffer (25 mM Tris–HCl, 200 mM sodium cacodylate, 5 mM cobalt chloride) and subsequently incubated with the labeling mix (20 U TdT-Boehringer, 0.5 nmol digoxigenin-11-ddUTP-Boehringer in 100 μl TdT buffer) for 120 min at 37°C. After the TdT reaction, the cells were rinsed in Tris-buffered saline (TBS), incubated in TBS containing 0.3% Triton X-100 and 2% bovine serum albumin for 60 min with anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (Boehringer) diluted 1/250 in TBS-Triton. After washing in TBS, the colour reaction was visualised by incubating the cells in colour buffer (100 mM Tris–HCl, 100 mM sodium chloride, 50 mM magnesium chloride, pH 9.5) containing 4-nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer). The reaction was monitored under the microscope and stopped in TE. The labeled cells withstand intensive cells washings providing evidence that ddUTP is covalently incorporated by the TdT and that DNA breaks appearing in apoptotic cells really function as primer for the in situ reaction.

Data analysis was performed with Stat-100 software (Biosoft). Each test was repeated four or five times in duplicate (see the figure legends) and collected results were processed with One-way analysis of variance (ANOVA) for repeated measurements. If the effect was significant ($p = 0.05$) Duncan's multiple range test post-hoc analysis was used for multiple comparison of means ($p < 0.05$ was considered to indicate a significant difference). Further, D = s. will be used for a significant Duncan's test result, while D = n.s. for a not significant one, compared to controls when not specified.

Adenosine, ADP, βNAD, and DPCPX were from Sigma. Suramine, CCPA, DPMA, CSC, N^6-benzyl-NECA, Reactive-blue 2 and β,γ-methylene-L-ATP were from RBI.

In a preliminary series of experiments, 6–7 DIV-cultured cerebellar granule cells were switched in 5 mM KCl (K5) serum-free medium in the absence or in the presence of cADPR and of its precursor βNAD. After 24 h, the extent of cell death via apoptosis was monitored. As can be seen in Fig. 1a, while cADPR at all concentrations tested (1 μM, 10 μM, 100 μM) is not able to block the death process (D = n.s.); on the contrary, 100 μM βNAD enhances survival from 40.8% ± 8 (mean value ± S.D.) of the controls in 5 mM KCl to 88.9% ± 3 (D = s.). In order to assess which constituent of βNAD was endowed with the anti-apoptotic action, we separately tested nicotinamide, adenine, adenosine, and ADP (Fig. 1a). We found that neither nicotinamide nor adenine has a significant effect (D = n.s.), whereas adenosine and ADP exhibit an effect overimpossible to that of βNAD. Concentration-dependent experiments reported in Fig. 1b indicate that the maximum protective activity is achieved at 1 mM both for βNAD and adenosine and that half-maximal effect occurs in the 10–100 μM range. The slightly lower activity of βNAD at all concentrations tested is probably attributable

to the fact that in order to be active, it must be first degraded to adenosine by nucleotidase present in the cytoplasmic membrane. The experimental test based on the evaluation of the extent of cell death does not allow to calculate in a more precise fashion the concentration providing half-maximal protection.

Parallel experiments performed with the TUNEL procedure (Fig. 2) confirm and extend previous findings that the 25 mM to 5 mM KCl shift causes death via apoptosis characterized by DNA laddering due to fragmentation into internucleosomal particles [6]. We found that only a fraction of all neurons that, at any given time, have undergone apoptotic death are labeled with this technique, suggesting that only for a limited time window neurons undergoing apoptosis are available for nick end labeling. As shown in Fig. 2c,d, in the presence of βNAD and adenosine, labeled neurons are barely detectable, and are comparable to controls kept in 25 mM KCl.

Moreover, morphological examinations at phase contrast microscope (not shown) indicate that cultures main-

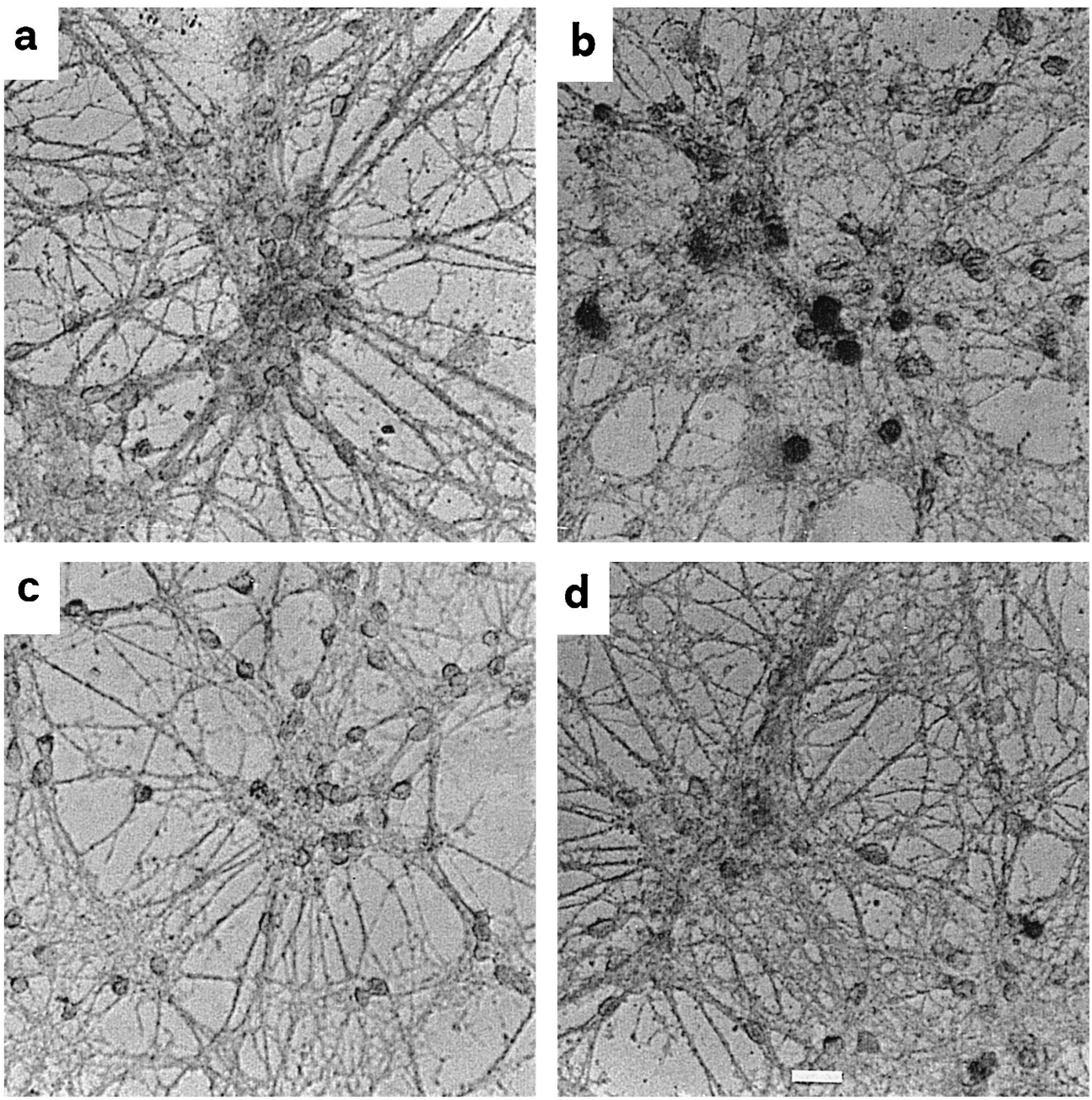

Fig. 2. Prevention of low KCl-induced apoptosis by βNAD and adenosine revealed by TUNEL staining. Six DIV neurons were shifted in low KCl (5 mM) serum-free medium, in the absence (b) or in the presence of 100 μM βNAD (c) or 100 μM adenosine (d). Forty-eight hours later, cultures were processed with TUNEL technique to detect dead cells and photographed using an inverted light microscope. (a) Control in 25 mM serum-free medium. Experiments were repeated three times with similar results, thus, pictures are representative fields. Scale bar (shown in d): 10 μm.

tained for 48 h in 5 mM KCl medium supplemented with βNAD, adenosine, and ADP are populated by a number of healthy neurons comparable to that detectable in 25 mM KCl, with well-defined cell bodies and a developed network of neurites, while detached cells, shrunken bodies, and fragmented neurites are rare in all fields.

Attempts were therefore performed to assess the type of adenosine receptor responsible of this antiapoptotic activity, considering that previous studies had shown that A1 adenosine receptors are predominantly expressed in cerebellum and particularly in CGCs [20,25] and involved in the neuroprotective activity of purines [14] and that ADP induces Ca^{2+} influx via P2x receptors [12,22]. To this aim, the A1AR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) [3,17], the A1AR agonist 2-chloro-*N*6-cyclopentyladenosine (CCPA) [11,17] and the noncompetitive P2x, P2y receptors antagonist suramine [4] were used. As it can be seen in Fig. 3, in the presence of 10 μM CCPA, neurons survive (80.0% ± 6.0; D = s. compared to 5 mM KCl) to an extent overimpossible to that occurring with ADP and slightly lower than with adenosine (86.0% ± 6.0; D = n.s. compared to 100 μM adenosine). On the other hand, 200 μM DPCPX only in part reverses adenosine neuroprotection (68.0% ± 6.0; D = s. compared with 100 μM adenosine), while suramine completely inhibits the protection of ADP (35.0% ± 3.0; D = n.s. compared to 5 mM KCl). Controls in 25 mM KCl and in 5 mM KCl media with DPCPX or suramine alone show that these substances have no toxic effect at the concentrations employed in these studies (not shown). In order to assess the possible role of the other types of adenosine receptors and which of the two P2 receptors subtypes recognized by suramine is responsible of ADP protection, we used the A2AR agonist N^6-2-(3,5-dimethoxyphenyl)-2-2-methylphenyl-ethyl adenosine (DPMA), the $A2_aAR$ antagonist 1,3,7-trimethyl-8-(3-chlorostyryl) xanthine (CSC) alternatively in association with DPMA and adenosine, the selective A3AR agonist N^6-benzyl-5'-*N*-ethylcarboxamidocadenosine (N^6-benzyl-NECA), the P2y receptor antagonist Reactive-blue 2 and the P2x receptor agonist β,γ-methylene-L-ATP [12,17]. Ten micromolars of DPMA (37.4% ± 6.2; D = n.s.), 10 μM and 100 μM N^6-benzyl-NECA (47.6% ± 6.4; D = n.s.) did not influence the viability of the neurons, while 100 μM DPMA had a toxic action. On the other hand, 100 μM adenosine was not antagonized by the addition of 200 μM CSC (72.0% ± 3.3; D = n.s. compared to 100 μM adenosine), therefore, ruling out the participation of A2 and A3 receptors in the neuroprotective activity. As far as suramine inhibition of ADP action is concerned, we observed that Reactive-blue 2 exerts a toxic effect on granule cultures at all tested concentrations (20, 100, 200 μM) whereas β,γ-methylene-L-ATP elicits a protective activity already at 10 μM (63.0% ± 4.2; D = n.s. compared to 10 μM ADP) reaching ADP protection level at 100 μM (75.0% ± 0.0; D = n.s. compared to 100 μM ADP) (Fig. 3).

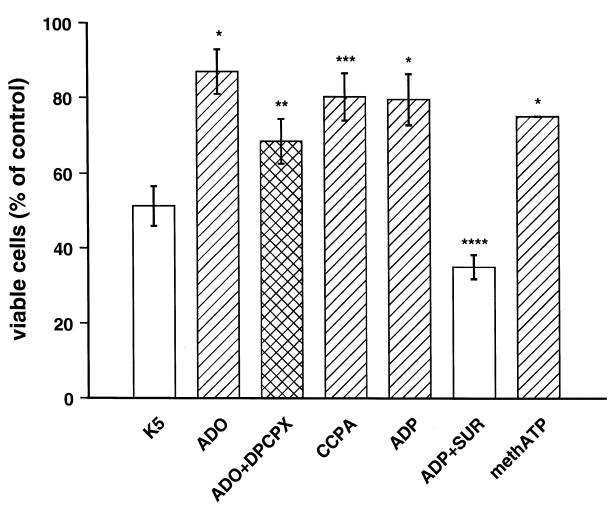

Fig. 3. Survival of neurons after 24 h of treatment in low K^+ without additives (K5) or with adenosine (ADO, 100 μM) alone or with the A1AR antagonist DPCPX (200 μM), with the A1AR agonist CCPA (10 μM),with 100 μM ADP alone or with the noncompetitive P2x, P2y antagonist suramine (SUR, 100 μM), with the selective P2x receptor agonist β,γ-methylene-L-ATP (methATP, 100 μM). * D = s. compared to K5; ** D = s. compared to 100 μM ADO; *** D = n.s. compared to 100 μM ADO; **** D = s. compared to 100 μM ADP. Values are means of five experiments repeated in duplicate (n = 5).

The findings reported in this paper demonstrate that adenosine and ADP inhibit the proapoptotic stimulus of low extracellular KCl in a concentration-dependent fashion with half-maximal effect at 10–100 μM and a plateau at 1.0 mM. It has been reported [15] that adenosine acts as an endogenous activator of the cellular antioxidant system and that reactive oxygen species (ROS) are produced during apoptosis. Our data suggest that the antiapoptotic action of adenosine and ADP could be achieved via inhibition of ROS production during early stages of apoptosis and that this effect is mediated by the interaction of these ligands with their receptors. Another action of adenosine and ADP could consist in restoring ATP levels which are generally impaired during apoptosis in several cell systems [19]. The question then arises as to the possible biological significance of this effect and of the mechanism through which it is reached. The observations that CCPA can mimic adenosine properties, DPCPX partially antagonizes its antiapoptotic action, and that β,γ-methylene-L-ATP inhibits the death of neurons to the same extent as ADP, suggest the involvement of both A1AR and P2x purinoceptors. Future studies should clarify this problem and elucidate whether the antiapoptotic action is achieved by restoration of Ca^{2+} homeostasis imbalanced by the Ca_i^{2+} drop caused by potassium withdrawal from culture medium [8] or, alternatively, via activation of alternate pathway(s) downstream the Ca_i^{2+} drop.

As for the possible functional significance, it is worth mentioning that adenosine is released in vitro by CGCs [21], that low concentrations of this nucleoside are present in vivo in the extracellular space and that several physio-

logical or pathological stimuli may cause its rise via a bidirectional facilitated diffusion [9] or via extracellular degradation of ATP [24]. Furthermore, electrical and chemical depolarization [2,7] or ischemia-hypoxic conditions [3,10] evoke adenosine release in the extracellular space where it can exert its action both at pre- and post-synaptic sites by inhibiting the release of excitatory neurotransmitters [1,18] and by decreasing EPSP (excitatory post-synaptic potential) via A1ARs [26].

Thus, adenosine and its derivatives may exert their action not only as neurotransmitters and neuromodulators, but also by stabilizing neuronal functions via different routes consisting in provoking vasodilation to supply oxygen and nutrients [21], inhibiting excitotoxicity by neurotransmitters and preventing neurons from delayed death via apoptosis as reported in this paper.

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