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Biochemical Pharmacology 61 (2001) 417–425

Biochemical Pharmacology

Extracellular adenosine-induced apoptosis in mouse neuroblastoma cells Studies on involvement of adenosine receptors and adenosine uptake

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Received 12 January 2000; accepted 18 July 2000

Abstract

The induction of apoptosis by adenosine was studied in the mouse neuroblastoma cell line N1E-115. Apoptosis was characterized by fluorescence and electron microscopy, fluorescence-activated cell sorter (FACS) analysis, and caspase activity assays. A sixteen-hour exposure to 100 μM of adenosine led to chromatin condensation and caspase activation. However, selective agonists for all four adenosine receptors were ineffective. Caspase activation could be blocked partially by an inhibitor of the nucleoside transporter, dipyridamole, and completely by uridine, a competing substrate for adenosine transport. 2′-Deoxycoformycin, an inhibitor of adenosine deaminase, enhanced caspase activation by adenosine but had no effect by itself. Caspase activation could be blocked by 5′-amino-5′-deoxyadenosine, which inhibits the phosphorylation of adenosine by adenosine kinase. These results indicate that adenosine receptors are not involved in adenosine-induced apoptosis in N1E-115 cells, but that uptake of adenosine and its subsequent phosphorylation is required.

Keywords: Apoptosis; Adenosine; Adenosine receptors; Nucleoside transporters

1. Introduction

The process of programmed cell death has been suggested to play an important role in certain neurodegenerative diseases such as Alzheimer's disease [1]. Cell death by apoptosis progresses through a series of well-regulated morphological and biochemical phases, including chromatin condensation [2] and caspase activation [3]. The stimuli which may lead to the activation of pathways resulting in cell death in neurodegenerative diseases are largely unknown; however, it has been shown that damaged cells release large amounts of ATP and adenosine [4,5], which could play a role in neuronal cell death [6,7]. Therefore, we started investigations on the role of adenosine in neurodegenerative diseases.

There are several sources of adenosine *in vivo*. It is produced intracellularly from adenine, AMP, or *S*-adenosyl-L-homocysteine, and can also be generated extracellularly as a breakdown product of extracellular ATP. Under normal conditions, extracellular adenosine concentration in the brain is in the nanomolar range [8]. After cell damage, however, the concentration rises rapidly; local concentrations in the micromolar range can be reached [4]. This is expected to affect the functioning of neighbouring cells, because adenosine has neuromodulatory properties [9,10]. It has been implicated in induction of apoptosis in several cell types such as astrocytes [6], rat microglia cells [11], and sympathetic neurons [7]. However, it was found that it also protected damaged neuronal cells against cell death [12]. These opposite effects of adenosine may be related to the

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Abbreviations: NECA, 5′-(*N*-ethylcarboxamido)adenosine; AMDA, 5′-amino-5′-deoxyadenosine; CPA, N^6-cyclopentyladenosine; NBI, nitrobenzylthioinosine; 2-Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide; AMV–RT, Avian myeloblastosis virus–reverse transcriptase; PCR, polymerase chain reaction; DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; and EM, electron microscopy.

0006-2952/01/$ – see front matter
PII: S0006-2952(00)00573-6

fact that there are several adenosine receptors on the cell surface, which could produce opposite survival signals inside the cell. In addition, adenosine can be transported into the cell by several carriers, potentially leading to other cellular effects. Extracellular adenosine acts mainly through the activation of the adenosine receptors of which four subtypes are known: A_1, A_{2A}, A_{2B}, and A_3 [13]. These receptors are members of the G protein-coupled receptor superfamily. A_1 [14] and A_3 [15] adenosine receptors are coupled via inhibitory G proteins to the inactivation of adenylate cyclase, while the A_2 [14,16] receptors mediate, via stimulatory G proteins, the activation of this enzyme.

Alternatively, adenosine may be rapidly transported into the cell by nucleoside transporters. Two major types of nucleoside transport have been distinguished based on their mechanism of action: sodium-dependent, active transport [17] and passive transport by means of facilitated diffusion. The latter is further subdivided into two classes, based upon sensitivity towards the transport inhibitor NBI [18]: the *es* transporter is susceptible to inhibition by NBI, while the *ei* transporter is insensitive to NBI. Once inside the cell, adenosine can be converted to AMP via adenosine kinase (AK) or to inosine via adenosine deaminase (ADA).

We have investigated the effect of extracellular adenosine in a mouse neuroblastoma cell line, N1E-115 [19], in an attempt to gain more insight into the mechanism of adenosine-induced apoptosis in neuronal tissue. We report that adenosine can induce apoptosis in N1E-115 cells, which is a receptor-independent process but one dependent upon cellular uptake of adenosine.

2. Materials and methods

2.1. Materials

Adenosine, uridine, dipyridamole, 2′-deoxyadenosine, NECA, and AMDA were purchased from Sigma. 2′-Deoxycoformycin (pentostatin) was a gift from Parke Davis, and CPA and 2-Cl-IB-MECA were from RBI. zVAD-fmk was from BACHEM and Nonidet P-40 from Roche Molecular Biochemicals. All primers were from Eurogentec; DNase, AMV–reverse transcriptase, and AMV–RT buffer were from Promega; Taq DNA polymerase and PCR reaction buffer were from Pharmacia; the caspase substrate DEVD-AMC was from Calbiochem. Propidium iodide was from Molecular Probes. The radioligands [^{3}H]NBI and [^{3}H]D-PCPX were from NEN Life Science Products.

2.2. Cell culture

Murine neuroblastoma cells, N1E-115 [19], were a gift from the Department of Molecular Carcinogenesis, LUMC, Leiden University. The cells were cultured in 75-cm^2 flasks (Greiner) in Dulbecco's modified Eagle's medium (GIBCO) containing 4.5 g/L of glucose, 2% (v/v) fetal bovine serum (GIBCO), and penicillin (50 U/mL)/streptomycin (50 μg/mL) (GIBCO). Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°, and subcultured every 4 days. They were used for 10–15 passages. The cells were grown for 3–4 days in 6-well plates (Greiner) before incubation with adenosine or other compounds.

2.3. Isolation of mRNA and RT–PCR

Total mRNA was isolated from 1*10^6 cells. Cells were washed twice with 1 mL PBS and resuspended in 250 μL Tris buffer pH = 7.8 containing 150 mM NaCl, 10 mM Tris, 1 mM EDTA. After addition of 20 μL of a 10% (v/v) Nonidet P-40 solution, the mixture was kept on ice for 60 sec followed by centrifugation for 1 min at 15,000 *g*. To the supernatant was added 250 μL RNA extraction buffer containing 350 mM NaCl, 20 mM Tris, 20 mM EDTA, and 1% (w/v) SDS, pH = 7.8. After mixing, 500 μL phenol/chloroform/isoamyl alcohol (25:24:1) was added followed by 1 min of vortexing. After centrifugation for 5 min at 15,000 *g*, the aqueous phase was isolated and 1 mL of 96% (v/v) ethanol was added. The mixture was kept at −20° overnight and centrifuged for 30 min at 15,000 *g* at 4°. The RNA pellet was washed with 70% (v/v) ethanol and dried on air. The RNA was dissolved in 20 μL TE containing 10 mM Tris and 1 mM EDTA, pH = 7.4. The concentration of RNA was measured spectrophotometrically at 260 nm.

Ten micrograms of the isolated mRNA was treated with DNase in 1*DNase buffer at 37° for 30 min. cDNA was synthesized using AMV–RV and an oligo dT primer according to the Promega protocol that was enclosed with the AMV–RT. PCRs were performed in a 25 μL reaction mixture containing 0.1 μL Taq DNA polymerase, 1* PCR reaction buffer, 0.2 mM of each dNTP, and 0.2 μL BSA (10 mg/mL) using specific primers for each receptor and transporter subtype. PCR reactions were carried out at 94° (30 sec), 58° (60 sec), and 72° (60 sec) for 33 cycles with the Perkin Elmer 2400 PCR system using the primers shown in Table 1. The PCR products were separated on a 1% (w/v) agarose gel (Sigma) in 44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA.

2.4. Radioligand-binding studies

N1E-115 cells were harvested and collected in ligand-binding (LB) buffer containing 140 mM NaCl, 5 mM KCl, 5 mM glucose, and 20 mM Tris, pH = 7.4 at 25°. Ligand-binding studies were performed on whole cells in a final volume of 400 μL containing 200 μL cell suspension (25,000 cells/200 μL) in LB buffer, 100 μL competing ligand in LB buffer, and 100 μL radioligand in LB buffer. The radioactive ligand [^{3}H]NBI (2 nM) was used for the *es* transporter and [^{3}H]DPCPX (0.4 nM) to label adenosine A_1 receptors. The samples were incubated at 24°. Incubations were carried out for 30 min with agitation and were terminated by vacuum filtration over glass fiber filters (GF/B, Whatman). The filter was rapidly washed with ice-cold buffer

using an M-24R cell harvester (Brandel). Non-selective binding of [^{3}H]NBI was determined by addition of 10 μM dipyridamole [20]. Non-selective binding of [^{3}H]DPCPX was determined in the presence of 10 μM NECA. [^{3}H]Radioactivity was measured in an LKB 1214 RackBeta liquid scintillation counter (Wallace) with 3 mL Emulsifier-Safe scintillation cocktail (Packard).

2.5. Hoechst staining

N1E-115 cells were harvested and centrifuged at 800 *g* for 3 min. The cells were fixed in formaldehyde 3.7% (w/v) for 15 min followed by addition of 2 μg/μL of Hoechst 33258 dye in PBS. After 15 min, the cells were centrifuged at 800 *g* for 3 min, washed with PBS, resuspended in 50% (v/v) glycerol/PBS, and fixed on a coverslip. Nuclear condensation was determined by optical imaging of the fluorescent staining at 380 nm.

2.6. Electron microscopy

N1E-115 cells were grown in 6-cm ∅ dishes for three days. Thereafter, 100 μM adenosine was added to the cells. Part of the cells was floating after treatment with adenosine; they were prepared for EM separately as described below. Sixteen hours later, the attached cells were fixed in Petri dishes in a fixative containing 0.1% glutaraldehyde in 0.14 M cacodylate buffer (pH = 7.4) for 30 min at room temperature. The samples were washed twice in PBS and post-fixed in 1% osmium tetroxide in Millonig phosphate buffer (pH = 7.3) for 60 min at 4° [21]. Thereafter, the cells were dehydrated in a graded series of ethanol. After incubation with epoxy resin LX-112 (Ladd Research Industries), Beem capsules filled with epoxy resin were placed on top of the cell culture and polymerized for 72 hr at 60°. After 16 hr, the floating cells in the adenosine-treated cultures were pelleted in 2% Bactro-agar at 60°, cut into 1-mm^3 blocks, dehydrated in graded series of ethanol, and embedded in epoxy resin LX-112 and polymerized. Ultrathin sections (70 nm) were cut on an ultramicrotome (Reichert OM U3), collected on copper grids, stained with uranyl acetate and lead hydroxide, and examined in a Philips EM 410 transmission electron microscope.

2.7. FACS analysis

The percentages of apoptotic, necrotic, and viable cells were determined by flow cytometric analysis (FACScalibur, Becton Dickinson) using Alexa™488-labeled annexin V and propidium iodide. Viable cells are negative for both stains, apoptotic cells only bind annexin V, and necrotic cells are stained by annexin V as well as propidium iodide [22]. N1E-115 cells were harvested and centrifuged at 500 *g* for 5 min in Dulbecco's modified Eagle's medium. The cell pellet was resuspended in 200 μL binding buffer containing 10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$ (pH = 7.4), supplemented with Alexa™488-labeled annexin V. The cells were left on ice for 15 min in the dark. Propidium iodide (10 μM) was added to the cells 1 min prior to FACS analysis.

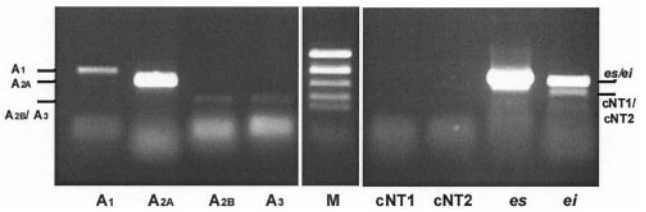

Fig. 1. RT–PCR analysis of adenosine receptors and transporters in N1E-115 cells. The positions of the fragments are indicated. Length of the different PCR products: A_1: 783 bp; A_{2A}: 619 bp; A_{2B}: 337 bp; A_3: 359 bp; cNT1: 468 bp; cNT2: 570 bp; *es*: 740 bp; *ei*: 677 bp. The marker has fragments of 1444, 946, 721, 471, and 357 bp, respectively. Equal amounts of cDNA were used for the PCR.

2.8. Caspase assay

The activity of caspase-3-like proteases was measured as described previously [23]. N1E-115 cells were harvested and centrifuged in Dulbecco's modified Eagle's medium. The pellet was resuspended in 50 μL lysis buffer containing 10 mM HEPES, 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, and 5 mM EGTA, pH = 7.0. After 3 cycles of freeze/thawing, the samples were centrifuged for 10 min at 15000 *g*. Protein concentrations were determined by measuring absorption at 590 nm in 96-well plates using Bradford reagent (Bio-Rad) [24]. A 10-μg sample of protein in a total volume of 20 μL was added to 80 μL of reaction buffer containing 100 mM HEPES pH = 7.25, 10% (w/v) sucrose, 0.1% (v/v) Nonidet P-40, 10 mM dithiothreitol, and 25 μM DEVD-AMC in a white 96-well plate (Costar). The fluorescence of the cleaved substrate was measured every 2 min for 45 min at 37° in a bioassay reader (Perkin Elmer) at 360-nm excitation and 446-nm emission. Calibration curves were constructed using free AMC.

2.9. Statistics

The statistical significance was tested with Student's *t*-test. The level of significance was set at $P < 0.05$.

Results

3.1. Identification of adenosine receptors and transporters in N1E-115 cells

In order to assess the effect of adenosine on N1E-115 cells, we first determined whether the adenosine receptors and transporters were present in this cell line. Two methods were used: RT–PCR and ligand binding.

Using RT–PCR (Fig. 1), we found that the mRNAs of

Table 1
Primers used for RT–PCR

Receptor/ Transporter	Forward (5′-3′)	Reverse (5′-3′)	Length (bp)
A_1	CGAGGTGCTCATTGCCTTGG	GGCATAGACGATGGGGTTCA	783
A_{2A}	TGGGCTCCTCGGTGTACATC	CTCCATCTGCTTCAGCTGTC	619
A_{2B}	GCTGTCCTCTGGGTCCTTGC	ATCATAGCCAGTGACTTGGC	337
A_3	CTGCGGGTCAAGCTGACA	GCTGTCTTGAACTCCCGTCC	359
es	CCATCATGAGTGGCCAGGGC	CCCGAAGCACATGCAGAGGC	740
ei	ACCTGGTCGGGATCAGCTTC	CAGCTTTGGTCTCCAGCTCC	677
cNT1	GGAAGCTTCTGCAGGGAGCAC	CCTGGTTCTGTTCTGATGAC	468
cNT2	TCTGCTCATCCGTCCCTACC	CCAGAGAGACGTTTGTTCTTG	570

Sequence of the different primers used for RT–PCR. Column 4 shows the fragment size of the PCR products.

both the A_1 and the A_{2A} receptor were present in the cells at relatively high concentrations, with the A_{2A} signal being the strongest. The mRNAs for the A_{2B} and A_3 receptors were present as well, albeit at a lower concentration. Furthermore, the cells contained mRNA for the *es* and *ei* transporter. These nucleoside transporter mRNAs were also present at high levels. The cNT1 and cNT2 primers for the sodium-dependent transporter mRNA, however, did not yield a PCR product (Table 1, Fig. 1), which suggests that the mRNAs are not present in N1E-115 cells. Thus, the RT–PCR showed the presence of the mRNAs for all four adenosine receptors and of the *es* and *ei* nucleoside transporters. Negative controls were carried out, but showed no PCR products (not shown).

In addition, we performed ligand-binding studies. Only for the A_1 receptor and the transporter (*es*) did we observe detectable specific binding. In Fig. 2, the results are shown for [^{3}H]NBI binding to the *es* transport protein. Binding studies for the A_{2A} and A_3 receptor were performed but no significant binding was found.

3.2. Adenosine-induced apoptosis

N1E-115 cells were incubated with adenosine to study induction of apoptosis and analyzed by fluorescence microscopy (after Hoechst staining) and EM. Both morphological methods showed the induction of condensed nuclei, indicating the presence of apoptotic cells after treatment with adenosine (Fig. 3, B, C, and F). Some 10–20% of the cells detached during the incubation period. We collected floating and attached cells separately and analyzed them by EM. Apoptotic nuclei were only present in the floating cells (Fig. 3F); the attached cells were not different from the controls (Fig. 3, D and E).

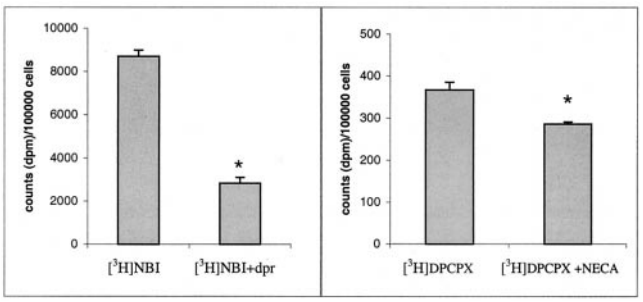

Fig. 2. Identification of the *es* transporter in N1E-115 cells by radioligand-binding studies. Dipyridamole (dpr, 10 μM) was used to determine non-specific binding for [^{3}H]NBI. Shown are the means ± SEM of six experiments. * indicates $P < 0.01$ compared to total binding.

The extent of cell death was determined by flow cytometry. Cells were stained with Alexa™488-labeled annexin V, which stains phosphatidyl serines, and with propidium iodide, which stains the nucleus of death cells exclusively. Cells that stained positive for annexin V and propidium iodide were counted as necrotic, cells stained only for annexin V were counted as apoptotic, and cells that were negative for both stains were counted as viable. Both apoptosis and necrosis were induced by adenosine (Fig. 4, B and C). The extent of apoptosis after treatment with adenosine remained stable during the 24 hr of the experiment, but the number of necrotic cells increased with time. To examine whether this necrosis was secondary necrosis, as a consequence of apoptosis, the caspase inhibitor zVAD-fmk was added to the cells (Fig. 4D). zVAD-fmk decreased the extent of necrosis, indicating that the necrosis observed was indeed due to initial apoptosis.

To confirm the induction of apoptosis by adenosine, we measured caspase-3-like activity in the cells. Cells were cultured for three days and subsequently exposed to adenosine for various time periods (Fig. 5). The lowest adenosine concentration, 30 μM, had no effect, but at 100 μM an 8-fold increase in caspase activity was found. After stimulation with adenosine, the caspase activity reached a peak after 16 hr. For all other experiments, therefore, an incubation time of 16 hr was used unless mentioned otherwise.

3.3. The effect of adenosine receptor agonists

Next we determined whether the apoptotic effect of adenosine as measured by caspase activation involved a receptor-mediated process. Various adenosine receptor agonists were used: NECA, a non-selective agonist, activates all four receptors, while CPA, 2-carboxyethyl-amino-5′-*N*-ethylcarboxamidoadenosine (CGS21680), and 2-Cl-IB-

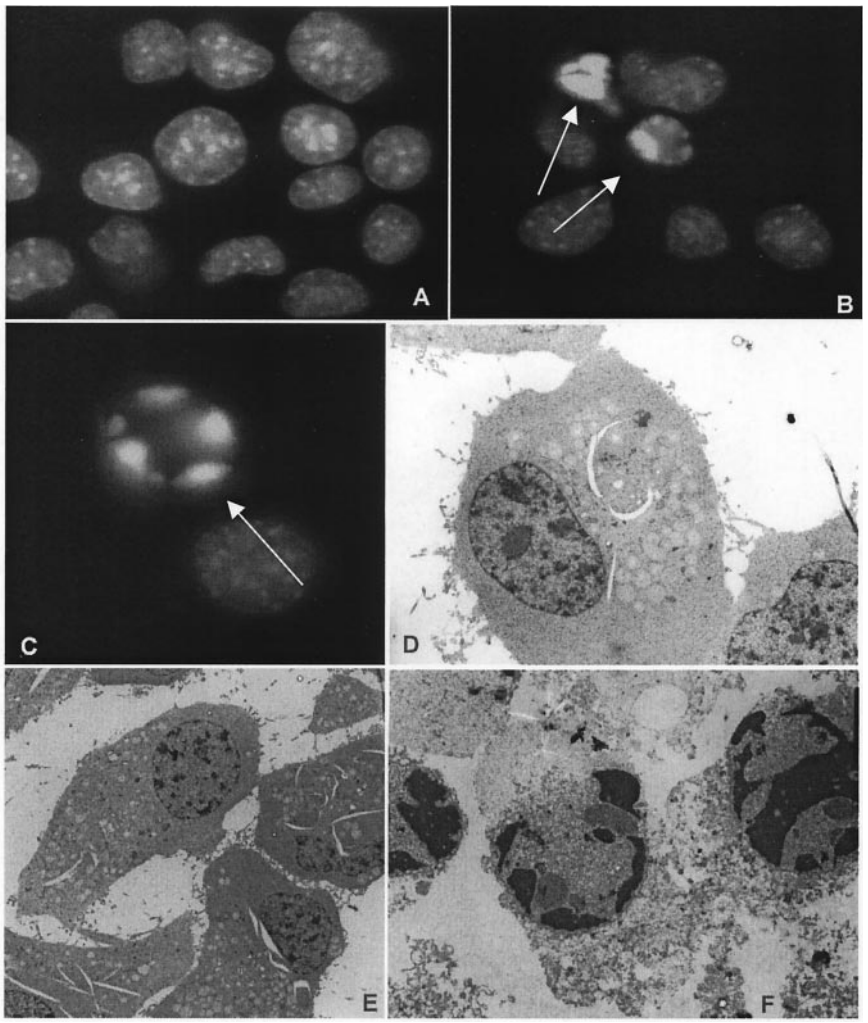

Fig. 3. Morphological effects of adenosine on N1E-115 cells. (a) Nuclei of untreated cells stained with Hoechst 33258 (magnification 400×). (b and c) Magnification 400× and 800×, respectively: nuclei of cells treated with adenosine (100 μM) for 18 hr; the arrow indicates apoptotic nuclei. (d–f) Electron microscopic pictures of N1E-115 cells without (d) (magnification 3000×) and with 100 μM adenosine (e and f) for 16 hr. (e) The attached cells (magnification 1500×); (f) the floating cells (magnification 3000×).

MECA are selective agonists for the A_1, A_{2A}, and A_3 receptors, respectively. A selective agonist for the A_{2B} receptor is not available. The ligands were used in concentrations that ensure full receptor occupancy [25–27]. None of the agonists had any effect on caspase activity (Fig. 6), indicating that adenosine receptors are not involved in adenosine-induced apoptosis in N1E-115 cells.

3.4. Effect of adenosine uptake inhibitors

In order to study the possible involvement of nucleoside transporters, we tested the effect of an *es* transport inhibitor, dipyridamole, on adenosine-induced caspase activity. Dipyridamole blocked induction of caspase activity at 100 μM adenosine by 60% (Fig. 7).

Another inhibitor of adenosine uptake is uridine, which is a competing substrate for the nucleoside transport protein. The activation of caspases by 100 μM adenosine was completely prevented by uridine (Table 2). Uridine itself had no effect on caspase activity at 50 μM. Taken together, the data suggest that transport of adenosine into the cell is necessary to induce caspase activity.

Finally, we tested 2′-deoxyadenosine, an adenosine analogue which can be transported by the nucleoside transporters [28], but which has a very low affinity for the adenosine receptors (affinity is about 1000-fold lower than that of adenosine) [29]. It also activated caspases in N1E-115 cells (Fig. 8).

3.5. The role of adenosine metabolites in adenosine-induced apoptosis

In the cell, adenosine can be converted to AMP by adenosine kinase, or to inosine by adenosine deaminase. To study the involvement of these enzymes, we used selective inhibitors. Inhibition of adenosine kinase by AMDA re-

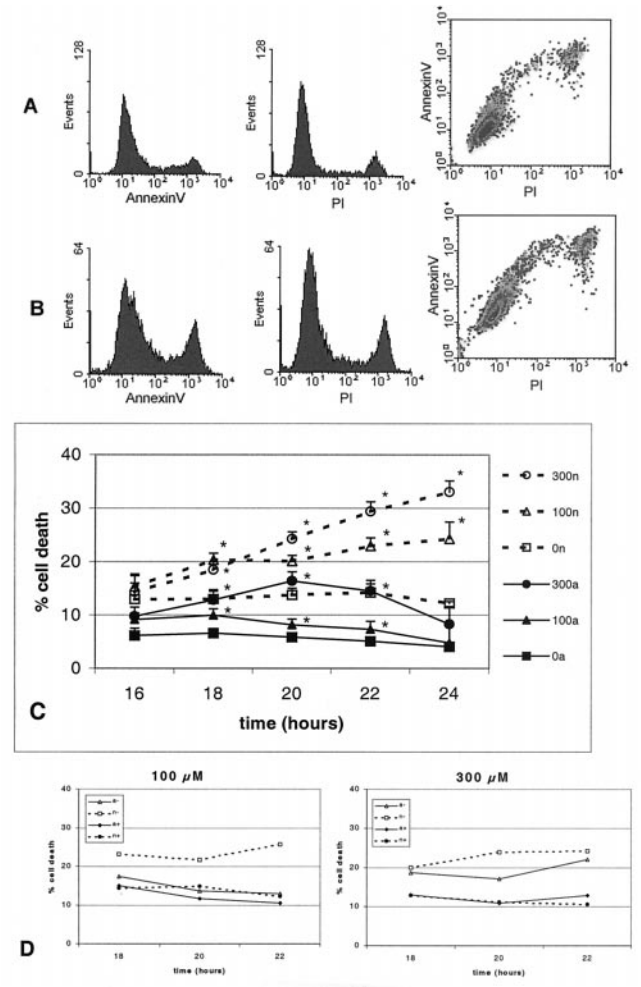

Fig. 4. Fluorescence-activated cell sorter (FACS) analysis of adenosine-treated and untreated cells. (a) Annexin V and propidium iodide (PI) staining of control cells. (b) Annexin V and propidium iodide (PI) staining of cells treated with 100 μM adenosine. (c) N1E-115 cells were treated with 100 or 300 μM adenosine. Mean values ± SEM of three independent experiments are shown. * $P < 0.05$ compared to control. (d) N1E-115 cells were treated with 100 or 300 μM adenosine alone or together with 1 μM zVAD-fmk and were harvested after 18, 20, or 22 hr. a: apoptotic; n: necrotic; −: no zVAD-fmk added to the cells; +: zVAD-fmk added to the cells 30 min before addition of adenosine. Data of one representative experiment are shown.

duced the adenosine-induced caspase activation by 60% (Fig. 9A). 2′-Deoxycoformycin, an inhibitor of adenosine deaminase, itself had no apoptotic effect (Fig. 9B). However, it did cause adenosine to become apoptotic at concentrations where adenosine itself had no effect, e.g. at 30 μM.

4. Discussion

The N1E-115 cell line was chosen to study a possible role of adenosine in neurodegenerative diseases. Our results show that adenosine caused apoptosis in this cell line. Therefore, we investigated the mechanisms involved, in particular the role of adenosine receptors and adenosine

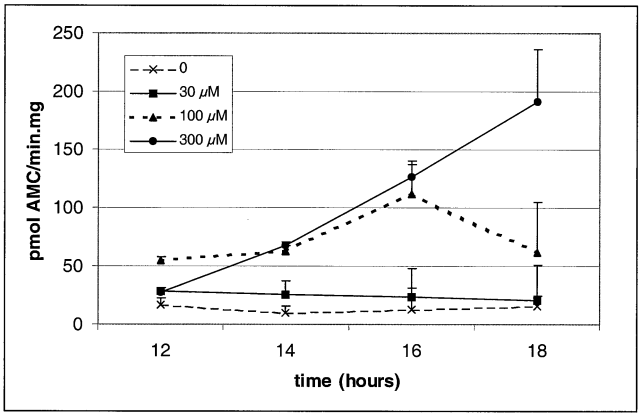

Fig. 5. Adenosine-induced caspase activity in N1E-115 cells. Cells were incubated with adenosine for different time periods. Cells were harvested after the indicated time points and caspase activity of a 10-μg protein sample was measured. Mean values ± SEM of four independent experiments are shown.

transporters. We first assessed which of the adenosine receptors and nucleoside transporters were present in N1E-115 cells. RT–PCR indicated that A_1 and A_{2A} receptor mRNA was present. The other two receptors, A_{2B} and A_3, were also detected by RT–PCR but were present in a lower amount. The presence of the A_1 and A_2 receptors in N1E-115 cells had already been suggested by Murphy and Byczko [30] who determined the effects of the adenosine receptor agonists $R(-)N^6$-(2-phenylisopropyl)adenosine (R-PIA), NECA, and CPA on the cellular cyclic adenosine monophosphate (cAMP) concentration. They also demonstrated that adenosine could enter N1E-115 cells, but the

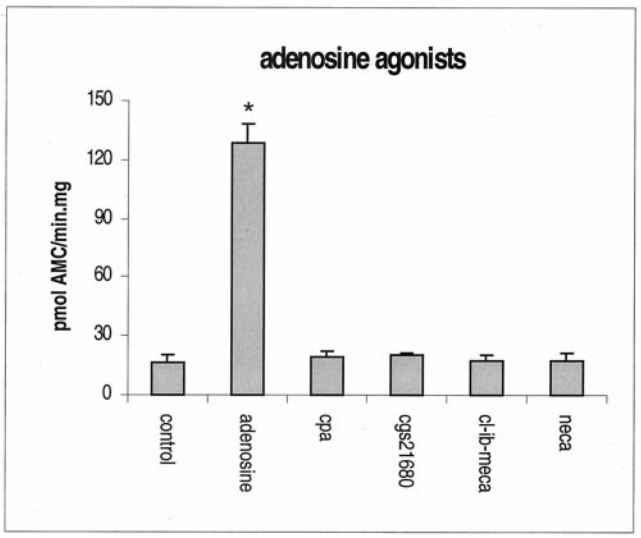

Fig. 6. The effect of adenosine and adenosine receptor agonists in N1E-115 cells. Cells were incubated with 100 μM adenosine, 100 nM CPA (A_1 agonist), 1.5 μM 2-carboxyethyl-amino-5′-*N*-ethylcarboxamidoadenosine (GS21680) (A_{2A} agonist), or 50 nM 2-Cl-IB-MECA (A_3 agonist). Cells were harvested after 16-hr incubation with adenosine and caspase activity was measured. Mean values ± SEM of three independent experiments are shown. * $P < 0.01$ compared to control.

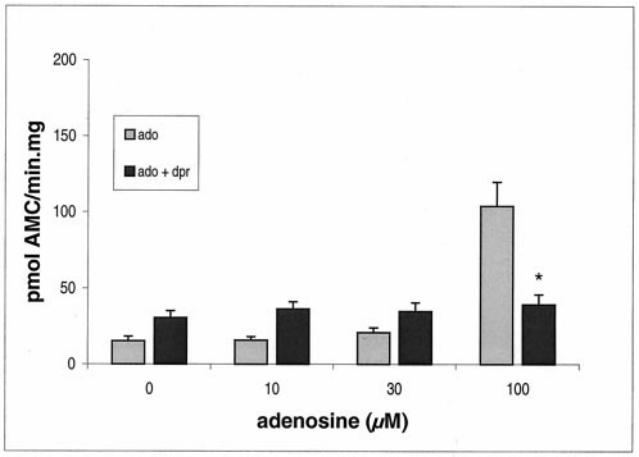

Fig. 7. The effect of a nucleoside transport inhibitor on adenosine-induced apoptosis in N1E-115 cells. Cells were incubated with 10 μM dipyridamole (dpr) and increasing concentrations of adenosine. Cells were harvested after 16-hr incubation with adenosine and caspase activity was measured. Mean values ± SEM of three independent experiments are shown. * $P <$ 0.05 compared to corresponding incubations without dipyridamole.

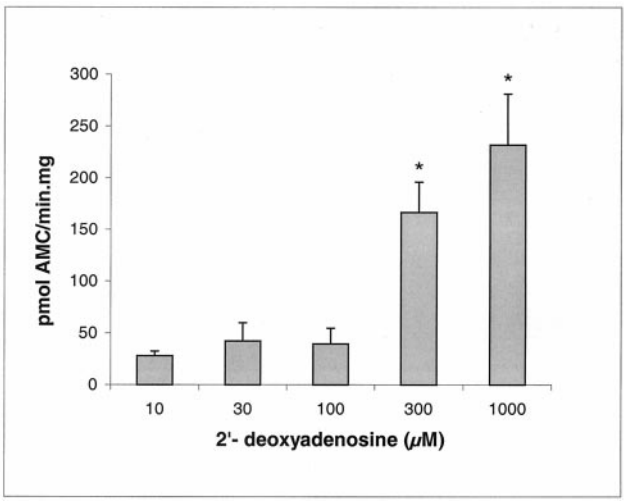

Fig. 8. The effect of 2′-deoxyadenosine on N1E-115 cells. 2′-Deoxyadenosine was added to N1E-115 cells at increasing concentrations. Cells were harvested after 16-hr incubation with adenosine and caspase activity was measured. Mean values ± SEM of three independent experiments are shown. * $P <$ 0.01 compared to control.

transporters involved were not identified [31]. Our results showed the presence of the nucleoside transporters *es* and *ei* on N1E-115 cells; the cNT1 and cNT2 transporters could not be detected. Ligand-binding studies confirmed the presence of adenosine A_1 receptors and *es* transporters only. The ligand-binding experiments did not show the presence of the A_{2A}, possibly due to the fact that the A_{2A} receptor is translationally regulated [32]. It is possible that in N1E-115 cells the mRNA is present but is not translated into significantly high levels of the protein to allow detection.

Adenosine induced apoptosis in the N1E-115 cells: both caspase activation and apoptotic nuclei were observed after treatment with adenosine. Only detached cells that floated in the culture medium upon adenosine exposure contained apoptotic nuclei as shown by EM, most likely because cells lose their ability to attach when they become apoptotic [33,34]. The FACS data confirmed the increase in cell death observed by microscopy and caspase activation. Cell death ("secondary necrosis") could be blocked by zVAD-fmk, indicating that the cells died after apoptosis. To test the

Table 2
Effect of uridine and dipyridamole on adenosine-induced apoptosis

Additions	Control (pmol AMC/min/mg)	+Adenosine (pmol AMC/min/mg)
	15 ± 3	104 ± 16*
uridine 50 μM	12 ± 2	18 ± 2**
dipyridamole 10 μM	31 ± 5	39 ± 7**

N1E-115 cells were incubated with adenosine with or without uridine or dipyridamole. Cells were harvested after 16 hr and caspase activity was measured. Shown are the means of three independent experiments ± SEM.

* $P <$ 0.01 compared to control.

** $P <$ 0.01 compared to corresponding incubations without uridine or dipyridamole.

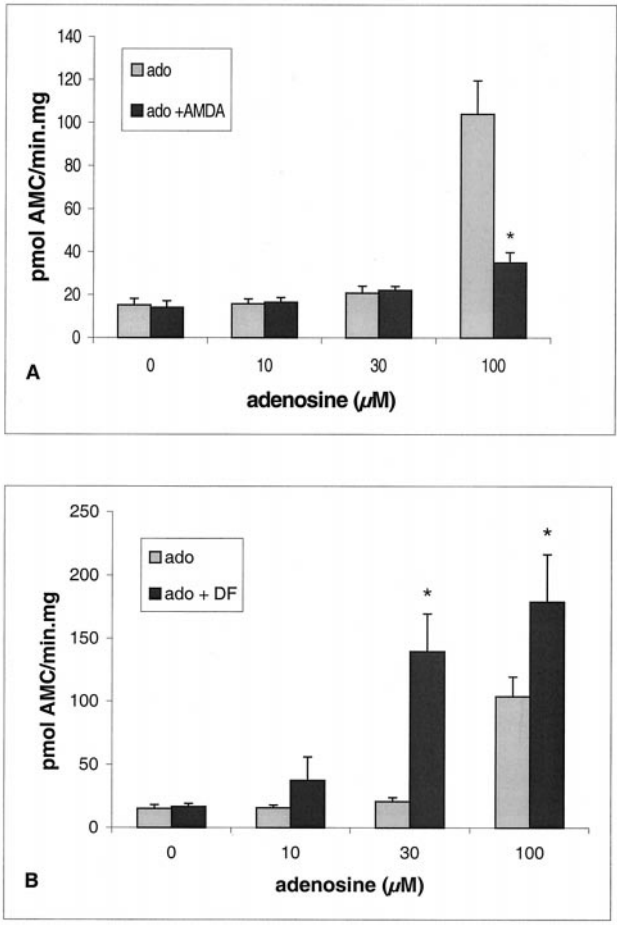

Fig. 9. Effect of inhibition of adenosine kinase and adenosine deaminase on caspase activity. AMDA (20 μM, a) or 100 nM 2′-deoxycoformycin (DF, b) was added to N1E-115 cells alone or together with increasing concentrations of adenosine. Cells were harvested after 16-hr incubation with adenosine and caspase activity was measured. Means of three independent experiments ± SEM are shown. Statistical significance ($P <$ 0.01) is represented by * for comparison without AMDA or 2′-deoxycoformycin.

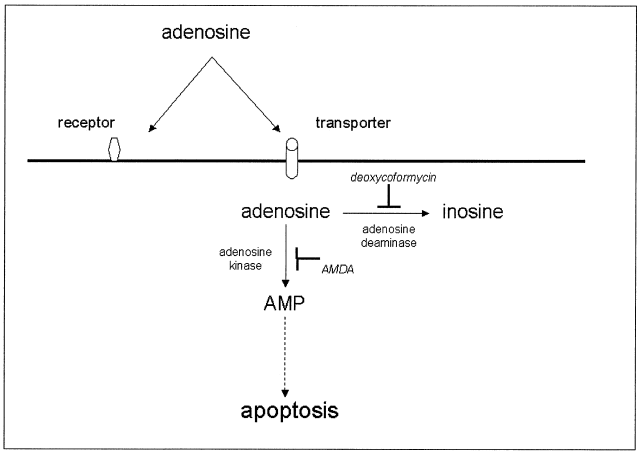

Fig. 10. Scheme of the possible routes for adenosine-induced apoptosis in N1E-115 cells.

involvement of adenosine receptors in induction of apoptosis, selective adenosine agonists were tested for activation of caspase activity in N1E-115 cells. None of the agonists used in this experiment activated the caspases. Even if all the adenosine receptors were activated by the non-selective adenosine receptor agonist NECA, no activation of caspases was observed. Abbracchio *et al*. [35] and Kohno *et al*. [36], however, suggested the involvement of the A_3 receptor in adenosine-induced apoptosis using 2-Cl-IB-MECA (in rat astrocytes and the human promyelocytic leukemia cell line HL-60 cells, respectively). Both authors used high agonist concentrations (10 μM), rendering a selective effect questionable [25].

2′-Deoxyadenosine is transported by the nucleoside transporters into the cell [28]; an intracellular effect unrelated to the adenosine receptors is apparently responsible for the induction of apoptosis by 2′-deoxyadenosine. Thus, it was argued that the effect of adenosine might be subsequent to uptake of adenosine by the cell, similar to reports by Tanaka *et al*. [37] in the human leukemia cell line HL-60 and by Wakade *et al*. [7] in chick embryonic sympathetic neurones. The *es* transporter-specific inhibitor dipyridamole could largely prevent the induction of caspase activation by adenosine, and uridine almost completely blocked activation of the caspases, confirming that it is necessary for adenosine to enter the cell before it can activate caspases. 2′-Deoxyadenosine, which has a very low affinity for the adenosine receptors [29], activated caspases as did adenosine.

In further investigations, two conversion routes of adenosine were blocked. Caspase activation was partly prevented when the phosphorylation of adenosine was blocked by AMDA. Conversely, when the breakdown of adenosine into inosine was inhibited by 2′-deoxycoformycin, an even higher caspase activation by adenosine was found. It is likely that in this case more AMP is formed, because adenosine can no longer be converted to inosine. The importance of the phosphorylation of adenosine has also been suggested by Wakade *et al*. [7], using chick embryonic sympathetic neurons.

The results in this study indicate that adenosine receptors do not play a role in adenosine-induced apoptosis in N1E-115 cells, but that uptake of adenosine is required for apoptosis. The conversion of adenosine to AMP (Fig. 10) most likely plays a role, but the further pathway leading to adenosine-induced apoptosis in N1E-115 cells needs to be identified. Whether these same effects occur *in vivo* has yet to be established.

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