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Apoptosis induced by extracellular ATP in the mouse neuroblastoma cell line N1E-115: studies on involvement of P2 receptors and adenosine

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Abstract

Adenosine triphosphate (ATP) can be released in large amounts from (damaged) cells, leading to locally high concentrations. In this study, we investigated the effect of such high concentrations of ATP on neuroblastoma cells. ATP ($\geq$30 μM) induced apoptosis in the mouse neuroblastoma cell line N1E-115. Activation of the ATP receptor $P2X_7$ is one of the routes via which ATP has been shown to induce apoptosis. Although the $P2X_7$ receptor was present in N1E-115 cells, both at the protein and mRNA level, studies with the $P2X_7$ receptor agonist benzoyl–benzoyl ATP showed that this receptor was not involved in ATP-induced apoptosis. It has been shown previously that adenosine induces apoptosis in N1E-115 cells after transport inside the cell. In this study, both dipyridamole, a nucleoside transport protein blocker, and uridine, a substrate for this transporter, were able to block ATP-induced apoptosis. This indicated that ATP had to be broken down to adenosine to induce apoptosis. The ecto-nucleotidase inhibitors 6-*N*,*N*-diethyl-β-dibromomethylene-D-adenosine-5′-triphosphate (ARL67156) and α,β-methylene adenosine 5′-diphosphate (AOPCP) commonly used to slow breakdown of ATP did not inhibit ATP breakdown appreciably, while the ATP antagonist PPADS inhibited the breakdown of AMP to adenosine; PPADS was also the only compound capable of inhibiting ATP-induced apoptosis. We conclude that the main route of ATP-induced apoptosis in N1E-115 cells was via breakdown to adenosine.

Keywords: Apoptosis; ATP; Ecto-nucleotidases; Adenosine; P2 receptors

1. Introduction

Neuronal cell death, especially by apoptosis (''programmed cell death''), plays an important role in certain neurodegenerative diseases [1]. One of the compounds that could be important in this process is extracellular ATP. Extracellular ATP has profound physiological and pathological effects in several biological processes [2,3]. Under normal conditions ATP functions as a neurotransmitter and neuromodulator [4]. It is released in high amounts but the extracellular concentration is kept low by ecto-nucleotidases [5], which rapidly hydrolyze ATP to ADP, AMP and adenosine. However, ATP can also be released locally from damaged cells in amounts large enough to play a role in neuronal cell death [6].

ATP can, in principle, activate cells via P2 receptors of which there are two classes, P2X and P2Y [7,8]. P2X receptors are ligand-gated ion channels of which seven have been cloned and characterized so far. The P2Y receptors are G protein-coupled receptors; so far five of these receptors have been identified and accepted as members of the P2 receptor family [9].

One P2 receptor is especially interesting when studying apoptosis: the $P2X_7$ receptor. It differs from the other P2X receptors because it converts to a pore when ATP is continuously present [10]. This pore is permeable to small

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Abbreviations: ATP, adenosine triphosphate; FACS, fluorescence activated cell sorter; DEVD-AMC, *N*-acetyl-Asp–Glu–Val–Asp-7-amino-4-methylcoumarin; PPADS, pyridoxal phosphate 6-azo phenyl 2′,4′-disulfonic acid; AOPCP, α,β-methylene adenosine 5′-diphophate.

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molecules and ions. The $P2X_7$ receptor has been implicated in induction of apoptosis in several cell lines such as mesangial, microglial and dendritic cells [11–14].

Activation of the $P2X_7$ receptor is not the only way in which ATP can cause apoptosis. As mentioned above, extracellular ATP is rapidly broken down after its release to ADP, AMP and adenosine by ecto-nucleotidases. Recently, we showed that one of these products, namely adenosine, induces apoptosis in N1E-115 cells after intracellular uptake [15]. In the present study, we investigated whether ATP induces apoptosis in N1E-115 cells via the purine P_2 receptors or indirectly, after its breakdown to adenosine. The results suggest that ATP induces apoptosis mainly through breakdown to adenosine. Although the $P2X_7$ receptor is present in these cells, it does not seem to be involved in ATP-induced apoptosis.

2. Materials and methods

2.1. Materials

Adenosine 5′-triphosphate was purchased from Boeringer Mannheim. The $P2X_7$ antibody, oxidized ATP, 2′,3′-*O*-(4-benzoyl–benzoyl) ATP, α,β-methylene adenosine 5′-diphosphate, ADP, AMP, uridine, dipyridamole, pyridoxal phosphate 6-azo phenyl 2′,4′-disulfonic acid (PPADS), 2-methylthioADP, cordycepin-5′-triphosphate, 5′-adenylylimido diphosphate, UTP, 2-methylthioATP, α,β-methylene ATP, β,γ-methylene ATP, ARL67156 and 5′ iodotubercidin were purchased from Sigma; 2′-deoxycoformycin (pentostatin) was a gift from Parke Davis; adenosine-5′-*O*-[3-thiotriphosphate] was from Roche; zVAD-fmk was from BACHEM; the caspase substrate *N*-acetyl-Asp–Glu–Val–Asp-7-amino-4-methylcoumarin (DEVD-AMC) was from Calbiochem; Protifar was from Nutricia; propidium iodide was from Molecular Probes; Suramin was from Bayer.

2.2. Cell culture

Murine neuroblastoma cells, N1E-115 [16] were a gift of 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 glucose, 2% (v/v) fetal bovine serum (GIBCO) and penicillin (50 unit/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 the compounds.

2.3. FACS analysis

N1E-115 cells (1×10^5) were incubated with 30 or 100 μM ATP for 24 hr, after which the cells were collected in tubes and centrifuged for 5 min at 800 *g*. The cell pellet was resuspended in 300 μL phosphate buffered saline (PBS)/EDTA (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 2H_2O$, 1.8 mM KH_2PO_4) and 700 μL 96% aqueous ethanol was added. Cells were kept at −20° for at least 30 min and at most 1 week. Before the cells were measured on the flow cytometer, they were centrifuged for 5 min at 800 *g* and resuspended in PBS containing 7.5 mM propidium iodide and 50 μM RNase A. After incubation at room temperature in the dark for 1 hr, the cells were measured on the flow cytometer (FACScalibur, Becton Dickenson, San Jose, CA, USA).

2.3.1. Hoechst staining

N1E-115 cells (1×10^5) were harvested and centrifuged at 800 *g* for 5 min. The cells were fixed in 3.7% (w/v) formaldehyde with 2 μg/μL Hoechst 33258 dye for 30 min. After 30 min the cells were centrifuged at 800 *g* for 5 min, washed with PBS, resuspended in PBS and fixed on a cover slip. Nuclear condensation was determined by optical imaging of the fluorescence staining at 380 nm.

2.4. Caspase assay

The activity of caspase 3-like proteases was measured as described before [15]. N1E-115 cells (1×10^5) 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 15,000 *g*. Protein concentrations were determined using Bradford reagent (Bio-Rad) [17] and measuring absorption at 590 nm in 96-well plates. 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-P40, 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.

2.5. Western blot

N1E-115 cells (1×10^5) were scraped in ice-cold PBS, pH = 7.4. After centrifuging for 5 min at 800 *g*, the pellet was resuspended in lysis buffer containing kinase and protease inhibitors (50 mM Tris pH 8.0, 150 mM NaCl, 1% Nonidet-P40, 1 μM DTT, 0.005 μg/mL leupeptin, 0.01 μg/mL aprotinin, 10 μM vanadate, 50 μM NaF, 5 μM PMSF). Twenty-five microgram of protein were separated on a 7.5% polyacrylamide gel. Proteins were transferred to a polyvinylidene difluoride Immunobilon-P membrane (Millipore, Bedford, MA) using a Bio-Rad mini

protean II blotting apparatus (Bio-Rad, Hercules, CA). For immunostaining, primary antibodies (in 1% (w/v) Protifar in TBS-T (0.5 M NaCl, 200 mM Tris pH 7.4, 0.5% Tween-20)) were added for 1 hr at RT. The secondary antibodies in 1% (w/v) Protifar in TBS-T were added for 1 hr at RT. Finally, the membrane was incubated with ECL reagent for 1 min.

2.6. Immunostaining

N1E-115 cells were grown on coverslips for 3 days, after which they were washed twice with PBS and fixed for 10 min in 3.7% (w/v) formaldehyde. After fixation the cells were washed twice with 1 mg/mL $NaBH_4$ for 10 min and blocked in TBP (PBS containing 0.1% triton and 0.5% BSA) for 10 min. The cells were incubated with the $P2X_7$ antibody in TBP for 1 hr at room temperature after which the cells were washed with TBP and the second antibody, goat anti-rabbit labeled with Alexa 568, was added to the cells. This was incubated at room temperature for 1 hr in the dark. The staining of the cells was visualized using a video intensified fluorescence microscope.

2.7. Isolation of mRNA and RT-PCR

Total mRNA was isolated from 1×10^6 N1E-115 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-P40 solution the mixture was kept on ice for 60 s followed by centrifugation for 1 min at 15,000 *g*. To the supernatant 250 μL RNA extraction buffer was added 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% aqueous ethanol was added. The mixture was kept at −20° over night 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 (10 mM Tris, 1 mM EDTA) containing 10 mM Tris and 1 mM EDTA, pH = 7.4. The concentration of RNA was measured spectrophotometrically at 260 nm.

Ten microgram of the isolated mRNA was treated with DNase in DNase buffer at 37° for 30 min. cDNA was synthesized using AMV reverse transcriptase and an oligo dT primer according to the Promega protocol that was enclosed with the AMV-RT. Polymerase chain reactions (PCRs) were performed in a 25 μL reaction mixture containing 0.1 μL Taq DNA polymerase, PCR reaction buffer, 0.2 mM dNTP each 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 s), 58° (60 s) and 72° (60 s) for 33 cycles with the Perkin-Elmer 2400 PCR system using the following primers: forward 5′-CTTGCTGCAGCTGGAACGAT-3′ and reverse 5′-TTTTCGGCGCTCCTCAAGAG-3′. 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.

2.8. HPLC analysis of ATP, ADP and AMP

For the measurement of ATP breakdown 100,000 cells were grown in P90 dishes with 10 mL Dulbecco's modified Eagle's medium (GIBCO) containing 2% (v/v) fetal bovine serum (GIBCO) for 2 days. After 2 days, 100 μM ATP, in the presence or absence of 100 μM PPADS, ARL67156, or AOPCP, was added to the medium. PPADS, ARL67156, or AOPCP was added 15 min prior to ATP. After 0, 30, 60, 120, 180, 240, 300, and 360 min 500 μL acetonitrile was added to 500 μL medium and the mixture was centrifuged for 1 min at 15,000 *g*. The supernatant was filtered and analyzed by reverse-phase high-pressure liquid chromotography. Of the sample 50 μL was injected on to an Adsorbosphere HS C18 column (Aldrich). The gradient of the mobile phase changed from 10 mM KH_2PO_4, 50 mM tetrabutylammonium bromide (TBAB) pH = 5.0 with 1% acetonitrile (A) to 10 mM KH_2PO_4, 50 mM TBAB pH = 5.0 with 50% acetonitrile (B). The elution cycle consisted of a linear gradient with a flow of 0.750 mL/min starting with 100% A leading to 100% B in 17 min, 5 min at 100% B, in 3 min from 100% B to 100% A and 5 min at 100% A before reinjection. Detection was at 254 nm.

2.9. Statistics

The statistical significance was tested with students *t*-test. The level of significance was set at $P < 0.053$.

3. Results

3.1. ATP-induced apoptosis

To study induction of apoptosis by ATP, N1E-115 cells were incubated with ATP, harvested after 22 hr, and the nuclei were stained with Hoechst 33258, to check for condensation of DNA. Cells treated with both 30 and 100 μM ATP showed condensed nuclei typical for apoptotic cells (Fig. 1). The extent of cell death was determined by flow cytometry. Cells were treated with ATP, fixed after 24 hr of incubation and the DNA was stained with propidium iodide. The first peak is the G1/G0 phase (Fig. 2). Because the DNA of the apoptotic cells is fragmented the sub-G1/G0 phase (M1) is counted as apoptotic. To examine whether this sub-G1/G0 phase really represents apoptosis the caspase inhibitor zVAD-fmk was added to the cells. ATP increased the sub-G1/G0 phase at 100 μM and to a lesser extent at 30 μM (Table 1). zVAD-fmk prevented this, indicating that the cells treated with ATP were indeed apoptotic.

To confirm the induction of apoptosis by ATP we measured caspase 3-like activity in the cells. Cells were

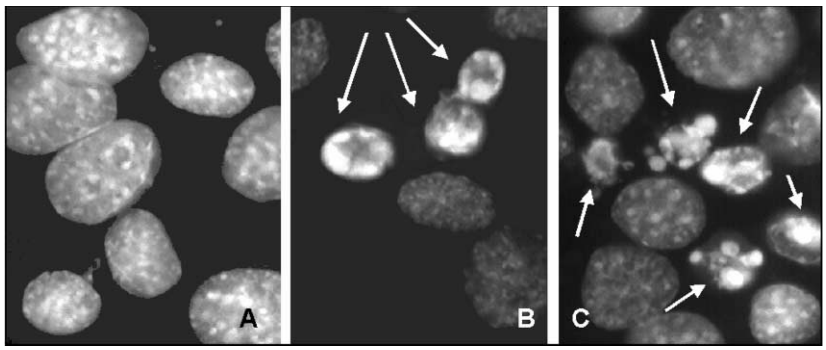

Fig. 1. Morphological effects of ATP on N1E-115 cells. Cells were harvested after 22 hr incubation with ATP and the nuclei were stained with Hoechst 33258 (magnification 400×). (A) Untreated cells; (B) 30 μM ATP; and (C) 100 μM ATP. The arrows indicate apoptotic nuclei.

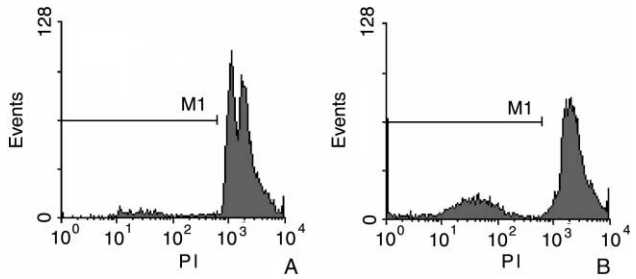

Fig. 2. FACS analysis of N1E-115 cells treated with ATP. Cells were harvested after 24 hr of incubation; DNA content was measured using propidium iodide (PI). (A) Control cells; (B) cells treated with 100 μM ATP. M1 is the sub-G1/G0 population. The *x*-axis shows fluorescence intensity of PI. Mean values of three independent experiments are shown.

cultured for 3 days and subsequently exposed to various concentrations of ATP for 18 hr (Fig. 3). ATP caused an increase in caspase activity, starting at 30 μM. These results show that incubation of N1E-115 cells with ATP leads to apoptosis at ATP concentrations of 30 μM and higher.

3.2. Involvement of the $P2X_7$ receptor

The $P2X_7$ receptor is regarded as a potentially important factor in ATP-induced apoptosis [11–14]. Therefore, we examined whether the $P2X_7$ receptor was involved in the apoptosis induced by ATP in N1E-115 cells. First we confirmed the presence of the $P2X_7$ receptor in N1E-115 cells at the mRNA level with RT-PCR, and at the protein level with immunostaining and Western blot (Fig. 4). To study whether this receptor was involved in ATP-induced apoptosis an agonist for the $P2X_7$ receptor, benzoyl–benzoyl ATP (bzATP) [6] was used. bzATP did not increase caspase activity in N1E-115 cells at a concentration of 100 μM after 18 hr (Fig. 5). The lack of effect of bzATP indicates that the $P2X_7$ receptor does not mediate apoptosis in N1E-115 cells. The $P2X_7$ antagonist oxidized ATP [18] increased caspase activity by itself at 10 μM; therefore, it could not be determined whether the compound could block ATP-induced apoptosis (Fig. 5).

Table 1
Sub-G0/G1 contents of N1E-115 ells treated with ATP with or without the caspase inhibitor zVAD-fmk

	% Sub-G0/G1 ± SEM
Control	17 ± 2
30 μM ATP	20 ± 2*
100 μM ATP	36 ± 5*
100 μM zVAD-fmk	14 ± 2
100 μM ATP + 100 μM zVAD-fmk	18 ± 3#

N1E-115 cells were incubated with 30 or 100 μM ATP in the presence or absence of zVAD-fmk for 24 hr. The sub-G0/G1 population was measured on the FACS using propidium iodide staining. Data are mean values of three independent experiments ± SEM. *$P < 0.05$ compared to control; #$P < 0.05$ compared to 100 μM ATP without zVAD-fmk.

3.3. The effect of ATP agonists and antagonists

Because the $P2X_7$ receptor did not seem to be involved, the role of other P2 receptors was tested. The ATP analogues used were divided into three groups, because there are no truly selective ligands for the different P2 receptors. Adenosine-5′-*O*-[3-thiotriphosphate] (ATPγS), 2-methylthioADP (MeSADP), cordycepin-5′-triphosphate (3′-dATP), and 5′-adenylylimido diphosphate (AMP.PNP) were used as non-selective agonists. UTP and 2-methylthioATP (MeSATP) mainly activate P2Y receptors, while α,β-methylene ATP (AMP.CPP), β,γ-methylene ATP

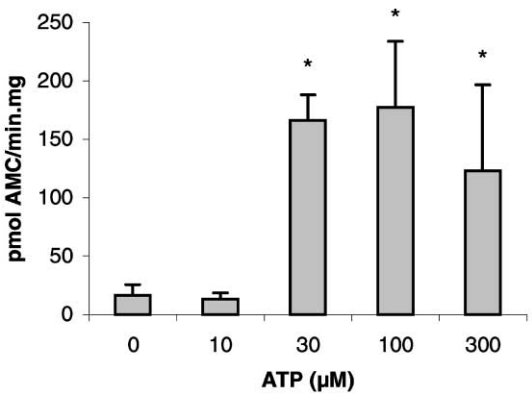

Fig. 3. Caspase activity induced by ATP in N1E-115 cells. Cells were treated with various concentrations of ATP. Caspase activity was measured after 18 hr. Mean values ± SEM of three independent experiments are shown.

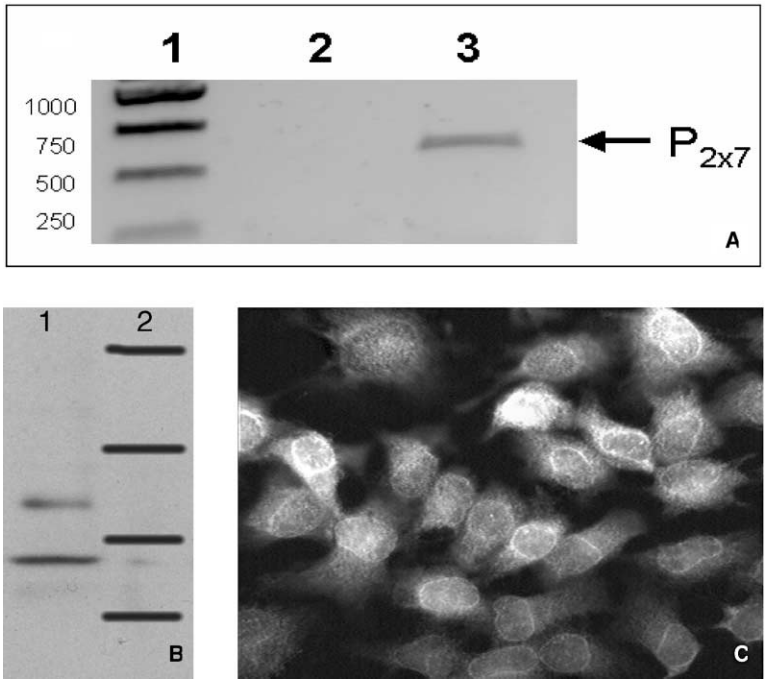

Fig. 4. Presence of the $P2X_7$ receptor in N1E-115 cells. (A) RT-PCR of $P2X_7$ receptor mRNA; lane 1: marker, lane 2: negative control (H_2O with primers); lane 3: N1E-115 cells; (B) Western blot using a $P2X_7$ antibody, lane 1: N1E-115 cells, lane 2: marker (175, 83, 62 and 47.5 kDa); (C) immunostaining of N1E-115 cells with $P2X_7$ antibody.

(AMP.PCP) have a higher affinity for the P2X receptors [9]. All compounds were used at a concentration of 100 μM. Although some of the agonists induced a slight increase in caspase activity, none of them were as active as ATP itself (Fig. 6).

We also tested the effect of two ATP receptor antagonists, suramin and PPADS [19,20]. Both are non-selective antagonists, which are also capable of inhibiting ecto-nucleotidases [21]. Further, two ecto-nucleotidase inhibitors were tested, ARL67156 [22] and AOPCP [23]. AOPCP inhibits only the ecto-5′ nucleotidases, which are responsible for the breakdown of AMP to adenosine. PPADS was the only compound that was able to block ATP-induced caspase activity (Table 2): it almost completely blocked the ATP-induced increase in caspase activity. A slight, but not significant inhibition by ARL67156 and AOPCP was observed (Table 2).

In order to assess whether the inhibitors used were indeed capable of decreasing the hydrolysis of ATP, we determined the breakdown of ATP with HPLC. ATP breakdown in medium without cells was also measured (data not shown). Although breakdown could be seen, it was not as fast as that in medium with cells ($t_{1/2}$: 4 hr vs. 30 min, respectively). ATP itself was completely broken down by N1E-115 cells in 60–120 min. None of the inhibitors could inhibit this process completely (Fig. 7). ARL67156 only

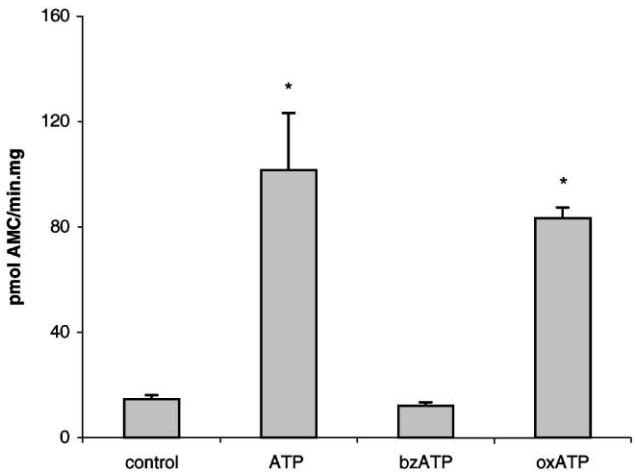

Fig. 5. Involvement of the $P2X_7$ receptor in ATP-induced caspase activity. Cells were treated with 100 μM ATP, 100 μM bzATP ($P2X_7$ agonist) or 10 μM oxATP ($P2X_7$ antagonist). Caspase activity was measured after 18 hr. Mean values ± SEM of three independent experiments are shown. $^*P < 0.05$ compared to control.

Table 2
Caspase activity of N1E-115 cells treated with ATP and various antagonists and ecto-nucleotidase inhibitors

	Control	30 μM ATP	100 μM ATP
–	26 ± 5	132 ± 24	144 ± 30
PPADS	21 ± 3	23 ± 3*	29 ± 2*
Suramin	56 ± 17	152 ± 39	182 ± 89
AOPCP	16 ± 1	117 ± 37	102 ± 24
ARL67156	21 ± 5	106 ± 25	98 ± 13

N1E-115 cells were incubated with different concentrations of ATP, with or without 100 μM of the ATP antagonists PPADS or suramin, the 5′ nucleotidase inhibitor AOPCP or the ecto-nucleotidase inhibitor ARL67156. Caspase activity was measured after 18 hr. Mean values ± SEM of three independent experiments are shown. $^*P < 0.05$ compared to corresponding concentrations without antagonist/inhibitor.

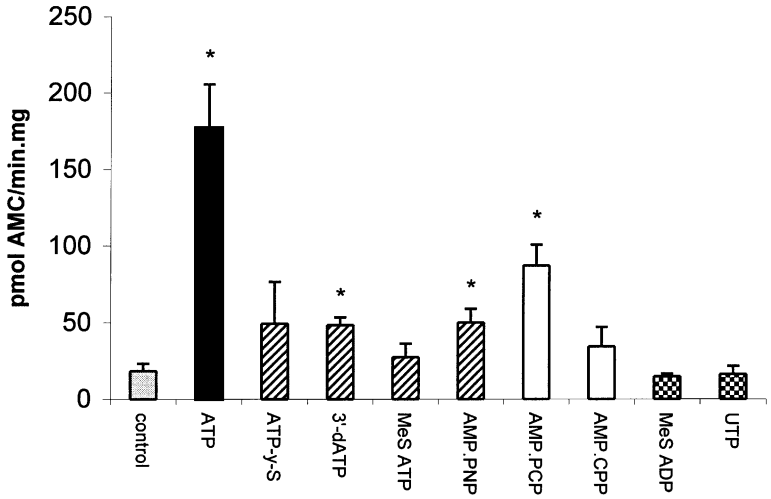

Fig. 6. Effect of P_2 receptor agonists on caspase activity. N1E-115 cells were treated with 100 μM ATP (■) or 100 μM of ATP agonist: (▨) non-selective agonists, (□) P2X receptor agonists, (▩) P2Y receptor agonists. Caspase activity was measured after 18 hr. Mean values ± SEM of three independent experiments are shown. $^*P < 0.05$ compared to control.

slowed down the breakdown of ATP and ADP, while the 5′ nucleotidase inhibitor AOPCP was not able to inhibit breakdown in our system. The P2 antagonist PPADS did not decrease the conversion of ATP to ADP and AMP but, surprisingly, inhibited the breakdown of AMP quite strongly (Fig. 7C).

3.4. The effect of ATP metabolites, adenosine-uptake inhibitors and adenosine kinase inhibitors

The effect of ADP and AMP on N1E-115 cells was examined to find out whether the effect of ATP on N1E-115 cells was caused by a metabolite of ATP rather than by ATP itself. Both ADP and AMP induced caspase activity (Fig. 8). We had shown previously that adenosine causes apoptosis in these cells [15], therefore, the inhibitors that were able to block adenosine-induced apoptosis were tested to investigate the involvement of adenosine in ATP-induced apoptosis. Dipyridamole is an inhibitor of the nucleoside transporter and blocks the transport of adenosine into the cell. We showed previously that this inhibitor was able to block adenosine-induced apoptosis in N1E-115 cells. Uridine, which is a competitive substrate for uptake via the nucleoside transporter, was also tested. Both compounds inhibited the ATP-induced caspase activity (Fig. 9). Secondly, the adenosine kinase inhibitor 5′ iodotubercidin (5′ IT) was used. It was previously shown that adenosine kinase inhibitors were able to block adenosine-induced apoptosis [15]. 5′ IT inhibited apoptosis induced by 30 μM, but not 100 μM ATP (Fig. 9).

4. Discussion

Both the nuclear fragmentation and the FACS data showed that ATP induces apoptosis in N1E-115 cells. This was confirmed by the activation of caspases by ATP in these cells. Normally, ATP is very rapidly broken down to adenosine; we have already shown that adenosine can cause apoptosis in this cell line [15]. We now show that ATP-induced apoptosis was decreased by inhibition of adenosine uptake using the nucleoside transport blocker dipyridamole. Altogether, these results suggested that ATP induces apoptosis in N1E-115 cells via extracellular breakdown to adenosine. AMP is an important mediator in adenosine-induced apoptosis [15,24].

To confirm this suggestion ATP breakdown was measured by HPLC. The breakdown of ATP by cells is expected to be caused mainly by ecto-nucleotidases. In our study, the conversion of ATP was followed over a period of 5 hr; the inhibitors ARL67156 and AOPCP were little effective in slowing down the breakdown of ATP and other metabolites. Other studies on the ecto-nucleotidase inhibitors, suramin, PPADS and ARL67156 [25–27], confirm that total inhibition of ATP breakdown could not be achieved with any of the compounds. The ecto-nucleotidase inhibitor ARL67156, which caused only an insignificant decrease in caspase activity, only slightly inhibited ATP breakdown. The same effect could be seen for the 5′ nucleotidase inhibitor AOPCP.

The experiments with the two P2 receptor antagonists PPADS and suramin showed intriguing results. Although PPADS was able to inhibit ATP-induced apoptosis, suramin had no effect. Because PPADS is also capable of inhibiting ecto-nucleotidase activity [21,26–28], it was tested with HPLC to establish whether PPADS inhibited ATP breakdown. PPADS neither inhibited the conversion of ATP to ADP, nor from ADP to AMP. However, it did inhibit the breakdown of AMP, rendering it a potent ecto-5′ nucleotidase inhibitor. This also is consistent with the concept that adenosine is an important mediator in ATP-induced apoptosis.

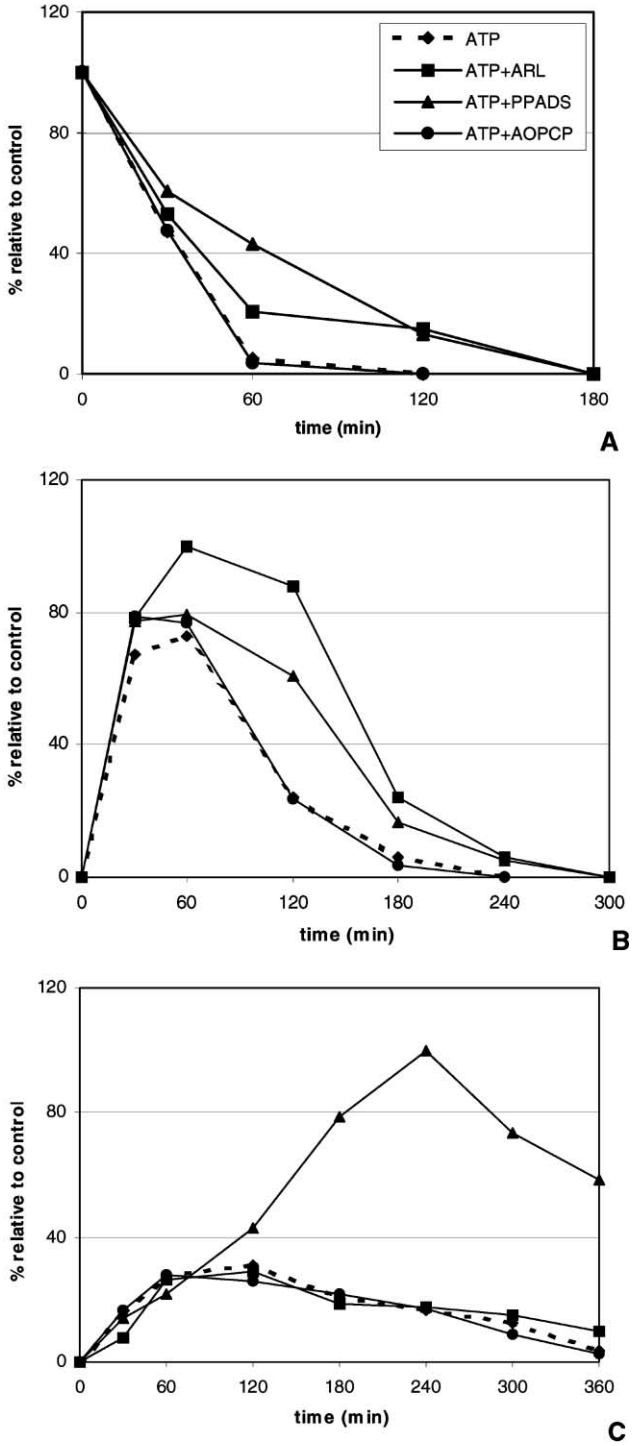

Fig. 7. Effect of the ecto-nucleotidase inhibitor ARL67156, the ATP receptor antagonist PPADS, and the 5′ nucleotidase inhibitor AOPCP on ATP breakdown by N1E-115 cells. The nucleotides were measured by HPLC. (A) Breakdown of ATP; (B) breakdown of ADP; (C) breakdown of AMP. ATP without inhibitors was used as the control. ATP (◆); ATP + ARL (■); ATP + PPADS (▲); ATP + AOPCP (●).

It was shown previously that blocking the intracellular conversion from adenosine to AMP with the adenosine kinase inhibitor 5′ IT gave a decrease in apoptosis induced by adenosine [15]. This could also been seen in the experiments with 30 μM ATP. However, at 100 μM ATP,

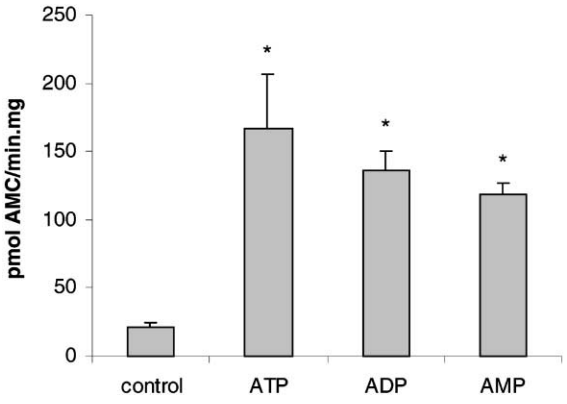

Fig. 8. Effect of metabolites of ATP on caspase activity. N1E-115 cells were treated with 100 μM ATP, ADP or AMP. Caspase activity was measured after 18 hr. Mean values ± SEM of three independent experiments are shown. $^{*}P < 0.05$ compared to control.

5′ IT did not have an effect. 5′ IT was also unable to inhibit adenosine-induced apoptosis at higher concentrations (data not shown). This might be due to the possible involvement of other routes.

It is conceivable that ATP induces apoptosis both via breakdown to adenosine and activation of one of its own receptors. The $P2X_7$ receptor, in particular, has been implicated in induction of apoptosis in several cell lines [11–14]. Our results showed that, although the $P2X_7$ receptor is present on N1E-115 cells, both at mRNA and protein level, it was not involved in ATP-induced apoptosis in these cells. The $P2X_7$ receptor antagonist oxATP caused apoptosis by itself. The molecular mechanism for this is not clear on the basis of these results.

None of the other agonists used in this study showed any substantial increase in caspase activity. The interpretation of findings with P2 receptor agonists and antagonists is

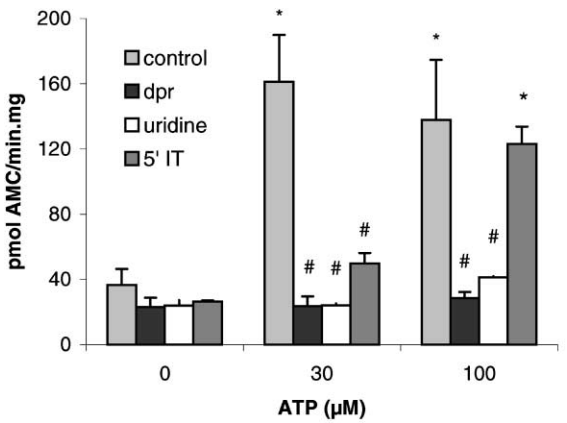

Fig. 9. Effect of nucleoside transport inhibition and adenosine kinase inhibition on ATP-induced apoptosis. N1E-115 cells were incubated with 30 and 100 μM ATP together with 10 μM of an inhibitor of the nucleoside transporter, dipyridamole (dpr), 50 μM of a competitive substitute for adenosine uptake, uridine, or 20 nM of the adenosine kinase inhibitor 5′-iodotubercidin (5′ IT). Caspase activity was measured after 18 hr. Mean values ± SEM of three independent experiments are shown. $^{*}P < 0.05$ compared to values in the absence of ATP. $^{\#}P < 0.05$ compared to corresponding concentrations without inhibitor.

cumbersome, though, since most of them are non-selective and have other pharmacological effects as well [9,28]. Overall, our results lead to the conclusion that activation of the P_2 receptors is not a major route for ATP-induced apoptosis in N1E-115 cells. Although P2 receptors might be involved, the main route for ATP-induced apoptosis in N1E-115 cells is via breakdown to adenosine.

Adenosine was shown to induce apoptosis at a concentration of 100 μM and higher [15]. Here we showed that 30 μM of ATP already brought about an increase in caspase activity, although we postulate that it needs to be broken down to adenosine before exerting its effect. It has been suggested that the slow continuous release of adenosine in human epidermoid carcinoma cells (A431) is more toxic for a cell than a high one-off concentration of adenosine [29]. Our results might also be explained if exposure to a lower concentration of adenosine over a longer period is more harmful to the cell than a high concentration administered in a single dose.

Altogether, these data show that extracellular ATP is a potent inducer of apoptosis. This makes ATP an important compound to consider when examining apoptosis in neurodegenerative diseases.

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