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A_{2A} adenosine receptor ligands and proinflammatory cytokines induce PC 12 cell death through apoptosis

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Abstract

A_{2A} adenosine receptor-mediated signaling affects a variety of important processes in the central nervous system both in physiological and pathological conditions, and has been indicated as possible novel therapeutic target in several nervous system diseases. In the present work, cell death induction was investigated after neuronal PC 12 cell treatment with proinflammatory cytokines and adenosine receptor ligands. Interleukin-1-beta (IL-1-beta, 500 U/mL), tumor necrosis factor-alpha (TNF-alpha, 1000 U/mL) and the non selective adenosine receptor agonist, 5′-*N*-ethylcarboxamidoadenosine (NECA), caused a significant reduction of cell viability with a maximal effect within 3–48 hr. Moreover, an addictive effect was detected when the cells were simultaneously treated with Interleukin-1-beta and NECA for 3 hr. To investigate the adenosine receptor subtypes involved in PC 12 cell death, the effects of several adenosine receptor agonists/antagonists were evaluated. The endogenous nucleoside, adenosine, and the selective A_{2A} adenosine receptor agonist, 2-(carboxyethylphenylethylamino)adenosine-5′-carboxamide (CGS21680) reduced PC 12 cell viability. This effect was counteracted by the selective A_{2A} adenosine receptor antagonist, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3e]-1,2,4-triazolo[1,5c]pyrimidine (SCH58261), but not by selective A_{2B} adenosine receptor antagonist *N*-(4-acethylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]acetamide (MRS1706), suggesting the specific involvement of A_{2A} adenosine receptor subtype in adenosine-mediated cytotoxicity. Moreover, the selective A_1 adenosine receptor agonist, N^6-cyclohexyladenosine (CHA), did not induce any significant effect on cell viability. By ELISA immunoassay cell death detection and transmission electron microscopy (TEM) we demonstrated that A_{2A} adenosine receptor ligands and cytokines induced cell death through an apoptotic pathway. In conclusion, our results showed that A_{2A} adenosine receptors are involved in the control of PC 12 cell survival/death and may contribute to modulate cellular activity in response to tissue damage associated with inflammatory mediator production.

Keywords: A_{2A} adenosine receptors; A_{2A} adenosine receptor ligands; Proinflammatory cytokines; Rat PC 12 cells; Cell viability; Apoptosis

1. Introduction

The nucleoside adenosine (ADO) is an endogenous neuromodulator involved in the regulation of many functions within the central nervous system, whose effects are mediated by at least four distinct receptors: A_1, A_{2A}, A_{2B} and A_3 [1,2]. A_{2A} ARs, contribute to the regulation of dopamine-dependent behaviors [3], and appear to be involved in neurodegenerative processes (see below). Some lines of evidence suggest a regulatory connection between adenosine receptors and proinflammatory cytokines [4,5]. Cytokines are potent multifunctional pleiotropic proteins which are constitutively expressed at low levels in healthy adult brain and increase in response to injury or infection

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Abbreviations: GPCRs, G protein-coupled receptors; A_{2A} AR, A_{2A} adenosine receptors; NECA, 5′-*N*-ethylcarboxamidoadenosine; CGS21680, 2-(carboxyethylphenylethylamino)adenosine-5′-carboxamide; CHA, N^6-cyclohexyladenosine; ADO, adenosine; PC 12, rat pheochromocytoma cells; Tris, tris(hydroxy-methyl)aminomethane; TNF-alpha, tumor necrosis factor-alpha; IL-1-beta, interleukin-1-beta; SCH58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3e]-1,2,4-triazolo[1,5c]pyrimidine; MRS1706, *N*-(4-acethylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]acetamide; [^{3}H] DPCPX, 2-chloro-N^6-cyclopentyladenosine; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2-*H*-tetrazolium, inner salt; TEM, transmission electron microscopy.

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[6–9]. These proteins, interacting with specific receptors on the plasma membrane, activate different intracellular phosphorylative cascades involved in cell differentiation, cell survival and apoptosis. Moreover, modulating the activity of transcription factors they may regulate several genes, including those encoding for AR. It has been demonstrated that proinflammatory cytokine, TNF-alpha, up-regulates the expression and function of A_{2A} AR in human monocytic THP-1 cell lines [4]. In neuronal cell lines, we demonstrated that IL-1-beta and TNF-alpha cause an A_{2A} AR up-expression [5]. The physiopathological significance of this A_{2A} AR up-regulation is not yet clear: this may represent a cell defense mechanism towards cytokine-induced cell damage or may play a synergistic effect on cell death. Adenosine modulates proliferation, survival and apoptosis of many different cell types [10] by a mechanism which involves either the entry of the nucleoside inside the cells [11] or the activation of transmembrane AR [12,13]. Concerning the A_{2A} AR effects, it has been demonstrated that: (1) selective A_{2A} receptor agonists/antagonists regulate striatal glutamate release [14–16]; (2) A_{2A} receptor antagonists have a neuroprotective role in a rat model of Huntington's disease [17] and in different cerebral models of ischemia/hypoxia and excytotoxic damage [18–21]; (3) mice lacking A_{2A} AR are less vulnerable to ischemia and neuronal damage [22]. These observations support the idea that blockade of A_{2A} AR may have neuroprotective effects in neurodegenerative diseases [23–25], although the direct effects of A_{2A} AR agonists on neuronal cell survival/death have not yet been investigated.

The aim of the present study was to investigate the effect of AR ligands and proinflammatory cytokines in the control of neuronal cell death. In this study, PC 12 cells have been chosen as cellular model, for three main reasons: (1) they express A_{2A} AR subtype at high levels; (2) they have been widely used in "*in vitro*" ischemic studies and to investigate both the effects of growth factors and the regulation of neurotransmitter release; (3) they have been used to study the mechanisms of neuronal death.

2. Materials and methods

2.1. Materials

Adenosine, NECA, CGS21680, CHA and Trypan blue were from RBI/Sigma. Cell culture media and fetal calf serum (FCS) were from Bio-Whittaker and Boehringer/Roche, respectively. SCH58261 was a gift from Schering-Plough. MRS1706 was from Tocris. [^{3}H] DPCPX was from Perkin-Elmer, Life Science. All other chemicals were supplied by standard commercial sources.

2.2. Cell culture and treatments

PC 12 cells were maintained in RPMI 1640 medium supplemented with 5% FCS, 10% horse serum, 2 mM L-glutamine and 100 U/mL penicillin/streptomycin in a humidified atmosphere (5% CO_2) at 37°.

Cytokines and NECA treatment: cells were treated for 3 or 48 hr with IL-1-beta (500 U/mL) or TNF-alpha (1000 U/mL) in the absence or in the presence of the AR agonist, NECA (1 μM).

Adenosine receptor ligands treatment: samples of cells were treated with adenosine (ADO) or AR agonists, NECA (20 nM–1 μM), CGS21680 (20 nM–1 μM) or CHA (100 nM). The specific involvement of A_{2A}/A_{2B} AR subtypes was evaluated incubating the cells with the agonist NECA in the presence of the A_{2A}/A_{2B} AR selective antagonists, SCH58261 (1 μM) and MRS1706 (10 nM), respectively.

After treatments, cells were harvested for cell viability, apoptosis and electron microscope studies.

2.3. Cell viability

The number of living cells was determined by evaluating the mitochondrial dehydrogenase activity using [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2-*H*-tetrazolium, inner salt (MTS) that is converted into formazan product in living cells (Cell Titer 96® AQueous one solution assay, Promega). Cells were plated in 96-multiwell plates; 100 μL of complete medium were added to each well with different concentrations of adenosine receptor agonists/antagonists and/or proinflammatory cytokines. The cells were then incubated for different time intervals. At the end of incubation period, 20 μL of MTS solution were added to each well. The plates were incubated for 2 hr at 37° and the optical density of each well was read on a spectrophotometer at 490 nm. For each experiments three individual wells of each drug concentration were prepared. Each experiments was repeated three times. Cell viability was also measured using Trypan blue esclusion assay [26].

2.4. Measurement of apoptosis

Cells were plated in 96-multiwell plates and treated with proinflammatory cytokines and/or AR agonists/antagonists for 48 hr. At the end of incubation time, apoptosis was measured with a photometric enzyme immunoassay (cell death detection ELISA, Boehringer/Roche) for the quantitative detection of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes/10^5 cells ± SEM). Results were from three different experiments performed in triplicate.

2.5. Electron microscope observation

To evaluate the morphological features of PC 12 cell death, control cells or cells treated with cytokines and/or AR ligands were processed for TEM [5]. Briefly, control

and treated cells were pelletted by centrifugation and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 hr at 4°. After rinsing in cacodylate buffer, specimens were postfixed in 1% cacodylate-buffered osmium tetroxide for 2 hr at room temperature, dehydrated in a graded series of ethanol, briefly transferred to propylene oxide and embedded in Epon-Araldite. Ultrathin sections (60–80 nm thick) were cut with a diamond knife, placed on formvar-carbon coated copper grids (200 mesh), stained with uranyl acetate and lead citrate and observed with a Jeol 100 SX TEM.

2.6. A_1 AR radioligand binding assay

PC 12 cells were homogenized in ice-cold hypotonic buffer (5 mM Tris–HCl, pH 7.4, containing 2 mM EDTA) and centrifuged at 48,000 *g* for 15 min. The membrane pellet was resuspended in 50 mM Tris–HCl buffer pH 7.4, frozen in liquid nitrogen at a protein concentration of 1–3 mg/mL and stored at −80°. [^{3}H] DPCPX binding to PC 12 cell membranes was tested as previously reported [27]. Nonspecific [^{3}H] DPCPX binding was defined in the presence of 100 μM R-PIA. Protein concentrations were determined with a protein assay kit from Biorad, using BSA as the standard.

2.7. Data analysis

For data analysis and graphic presentations we used the nonlinear multipurpose curve-fitting program Graph-Pad Prism (GraphPad).

All data are presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA. Significance refers to results where $P < 0.05$ was obtained.

3. Results

The effects on cell viability of short- and long-time PC 12 cell exposure to proinflammatory cytokines and/or to AR ligands were evaluated. The conversion of MTS to a formazan product by mitochondrial dehydrogenases was used as an index of mitochondrial viability.

As shown in Fig. 1, 3 hr cell incubation with proinflammatory cytokines, IL-1-beta (500 U/mL) and TNF-alpha (1000 U/mL), induced a significant reduction of PC 12 cell viability (86.5 ± 1.5% and 75.2 ± 5.2% vs. control 100%, respectively).

A weak reduction of PC 12 cell viability was also observed when the cells were incubated for 3 hr with the AR agonist, NECA, at micromolar concentration (90.25 ± 1.75%; $P = 0.0366$ vs. control), suggesting an adenosine receptor-mediated cytotoxic effect.

To evaluate a possible additive effect between adenosine receptor ligand and cytokines in short-time cellular response, cells were simultaneously exposed to the two

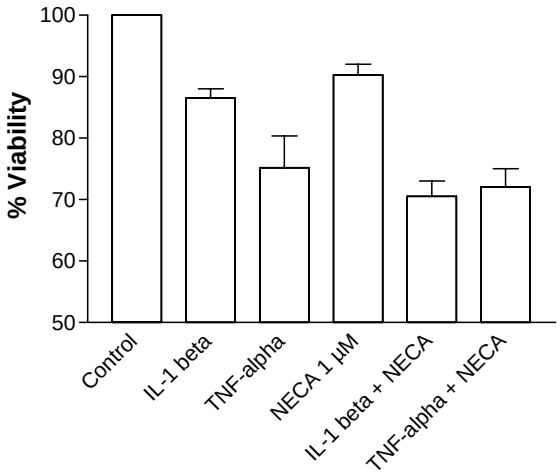

Fig. 1. Short-time effect of proinflammatory cytokines and/or adenosine receptor agonist, NECA, on PC 12 cell viability. PC 12 cells were treated with 500 U/mL IL-1-beta or 1000 U/mL TNF-alpha in the absence or in the presence of 1 μM NECA for 3 hr, as described in Section 2. After incubation, cell viability was determined using MTS conversion assay. Data were normalized to untreated cells (control) as 100% and shown as mean ± SEM for 3 wells per group vs. vehicle-treated cells. Each experiments were performed three times with similar results.

agents. Cell incubation with NECA and IL-1-beta induced a significant increase in cell death (70.5 ± 2.5% cell viability vs. 86.5 ± 1.5 with IL-1-beta alone; $P = 0.031$) (Fig. 1). On the contrary no increase in TNF-alpha-mediated cell death was detected after simultaneous incubation with TNF-alpha and NECA (72 ± 3.0% cell viability vs. 75.2 ± 5.2 with TNF-alpha alone; $P > 0.05$).

The reported experimental conditions were carried out even for longer periods of time: cell viability was evaluated after 48 hr cell incubation. Long-time cytokine cell exposure did not significantly affected the percentage of cell death ($P > 0.05$) (Fig. 2), suggesting that cytokine-mediated cytotoxicity occurred within few hours and remained constant for prolonged period of time. The maximum effect on cell death was obtained with TNF-alpha (74.8 ± 3.0% of cell viability) rather than with IL-1-beta (81.0 ± 6.2% of cell viability).

On the contrary, 48 hr cell exposure to micromolar NECA concentration induced a significant increase in agonist-mediated effect (cell viability of 77 ± 2.0% vs. 90.2 ± 1.75% after 3 hr incubation; $P = 0.038$; Fig. 2) suggesting that the cellular response mediated by activation of adenosine receptor needed a longer time.

Incubation with NECA and IL-1-beta for 48 hr did not increase the percentage of cell death if compared with the two agents singularly; similar results were obtained when the treatment was carried out adding IL-1-beta and NECA simultaneously (Fig. 2) or when the cells were pretreated for 3 hr with cytokines and then, incubated with NECA for 48 hr (data not shown).

To dissect the implication of specific AR subtypes in the regulation of PC 12 cell death/survival, the effect on

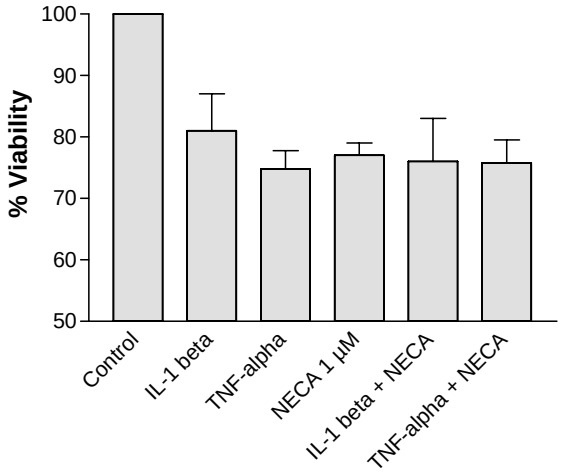

Fig. 2. Long-time effect of proinflammatory cytokines and/or adenosine receptor agonist, NECA, on PC 12 cell viability. PC 12 cells were treated with 500 U/mL IL-1-beta or 1000 U/mL TNF-alpha for 48 hr in the absence or in the presence of 1 μM NECA. After incubation, cell viability was determined using MTS conversion assay. Data were normalized to untreated cells (control) as 100% and shown as mean ± SEM for 3 wells per group vs. vehicle-treated cells. Each experiments were performed three times with similar results.

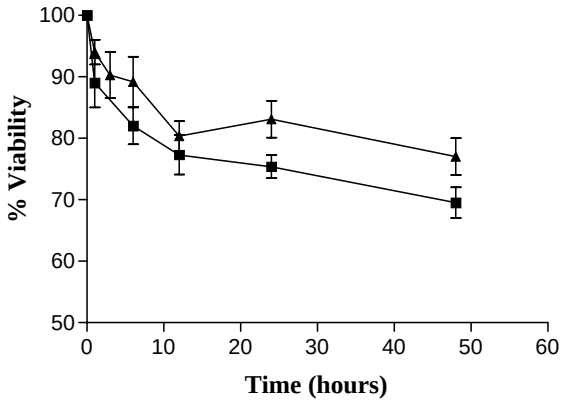

Fig. 3. Effect of NECA on PC 12 cell viability: time course. PC 12 cells were treated with the agonist NECA, 20 nM (■) or 1 μM (▲) for different time intervals (1–48 hr), as described in Section 2. After incubation, cell viability was determined using MTS conversion assay. Data were normalized to untreated cells (control) as 100% and shown as mean ± SEM for 3 wells per group vs. vehicle-treated cells. Each experiments were performed three times with similar results.

cell viability of different AR agonists/antagonists was evaluated.

The presence of A_{2A} AR has been confirmed in our laboratory as previously demonstrated [5]. The expression of A_1 AR in PC 12 cells was assessed by radioligand binding assay using the selective A_1 AR radioligand [^{3}H] DPCPX. At 3 nM radioligand concentration, a specific [^{3}H] DPCPX binding was detected (85 fmol/mg of proteins) demonstrating that A_1 AR subtype was expressed at low level in this cell line.

The nonselective AR agonist, NECA (20 nM and 1 μM), reduced cell viability in a time-dependent manner with a $t_{1/2}$ of 4.51–4.53 hr and reached a maximum effect after 48 hr (Fig. 3). The effect of the nucleoside, ADO (30 μM) and of the selective A_1 and A_{2A} AR agonists, CHA (100 nM) and CGS21680 (1 μM), was also evaluated. ADO and CGS21680 induced a significant reduction of cell viability

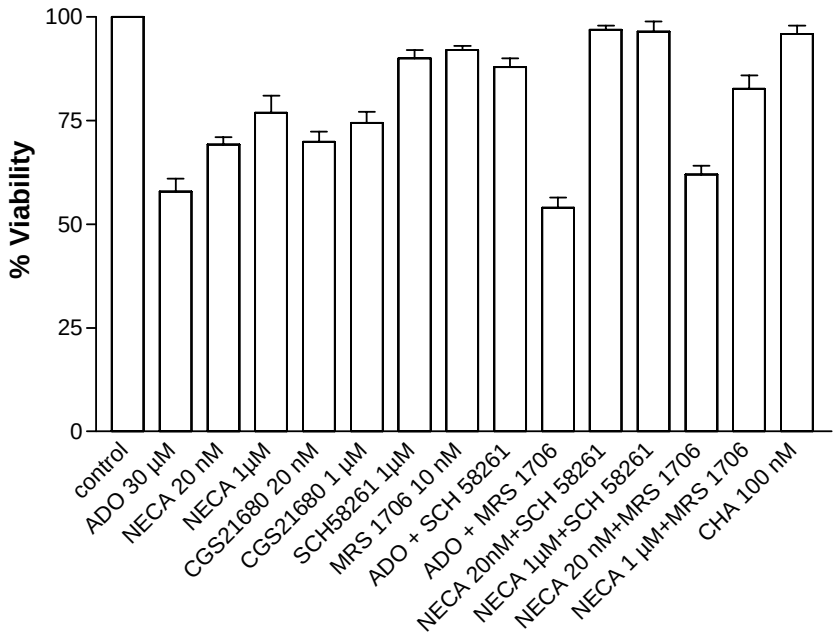

Fig. 4. Effect of adenosine receptor agonists/antagonists on PC 12 cell viability. PC 12 cells were treated with the endogenous nucleoside, ADO (30 μM), or with the synthetic agonists NECA (20 nM–1 μM), CGS21680 (20 nM–1 μM), or CHA (100 nM) for 48 hr. A sample of the cells were pretreated with the antagonist SCH58261 (1 μM) or MRS1706 (10 nM) before treatment with the agonist ADO or NECA for 48 hr. Then, cell viability was determined using MTS conversion assay. Data were normalized to untreated cells (control) as 100% and shown as mean ± SEM for 3 wells per group vs. vehicle-treated cells. Each experiments were performed three times with similar results.

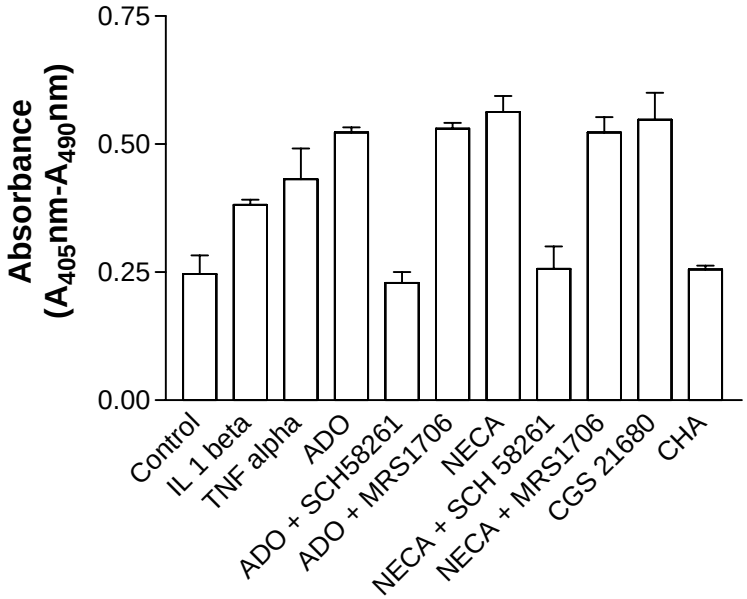

Fig. 5. Proapoptotic effects of cytokines and adenosine-receptor ligands. PC 12 cells were treated with proinflammatory cytokines or with the endogenous nucleoside, ADO (30 μM), or with the synthetic agonists NECA (1 μM) or CGS21680 (1 μM) or CHA (100 nM) for 48 hr. A sample of the cells were pretreated with the antagonist SCH58261 (1 μM) or MRS1706 (10 nM) before treatment with the agonist ADO or NECA for 48 hr. The quantitative determination of mono- and oligonucleosomes were determined using an ELISA kit as described in Section 2 and expressed as absorbance/10^5 cells ± SEM.

whereas CHA did not show any significant effect (Fig. 4). The effects of selective A_{2A} and A_{2B} AR antagonists, SCH58261 and MRS1706, were assessed. Incubation of the cells with the antagonists alone did not induce a significant effect on PC 12 cell viability. SCH58261 prevented the cytotoxic effects mediated by ADO increasing cell viability to 88 ± 2.1%. The antagonist was also able to counteract the effects mediated by both nanomolar and micromolar NECA concentration (97 ± 1% and 96.5 ± 2.5% of cell viability, respectively). On the contrary, the A_{2B} AR antagonist, MRS1706 (10 nM), did not affect the cytotoxicity mediated by either ADO and NECA. These results suggest that, in PC 12 cells, the adenosine-mediated cell death mainly occurred through the activation of the A_{2A} AR subtype whereas neither A_{2B} or A_1 ARs are involved. By using Trypan blue exclusion assay we observed that cytokines and AR ligands affected cell viability with results similar to the previous method (data not shown). The viability of control untreated cells was about 97%.

To evaluate whether PC 12 cell death occurred through an apoptotic program, we quantified the cytoplasmic histone-associated DNA fragments by cell death detection

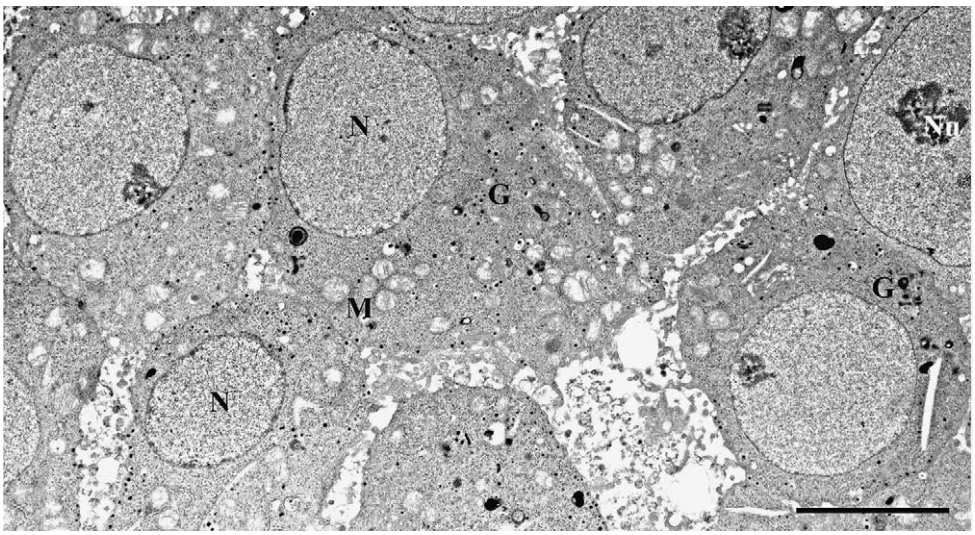

Fig. 6. TEM micrograph of untreated PC 12 cells. The nucleus (N) contains finely dispersed chromatin and a prominent nucleolus (Nu). In the cytoplasm a large amount of free ribosomes, some scattered or clustered mitochondria (M) and electron-dense granules (G) of different size are visible. Scale bar = 5 μm.

ELISA assay. Cells were incubated with proinflammatory cytokines or with AR agonists/antagonists for 48 hr and their effects on apoptosis were evaluated (Fig. 5). Both IL-1-beta and TNF-alpha induced apoptosis of PC 12 cells with respect to untreated control cells. Similar effects were obtained after cell incubation with ADO or AR agonists, NECA and CGS21680. No significant apoptotic effect was obtained after incubation of the cells with the selective A_1 AR agonist, CHA. SCH58261 (1 μM) significantly reduced the proapoptotic effect of ADO and NECA whereas, the selective A_{2B} AR antagonist, MRS1706, had not significant effect on agonist-mediated apoptosis. Both antagonists had no effect on PC 12 cell apoptosis when tested alone. These results confirmed that A_{2A} AR are primarily involved in adenosine-mediated cell death by an apoptotic mechanism.

Finally, to investigate the morphological features of cellular death (necrosis and/or apoptosis) induced by

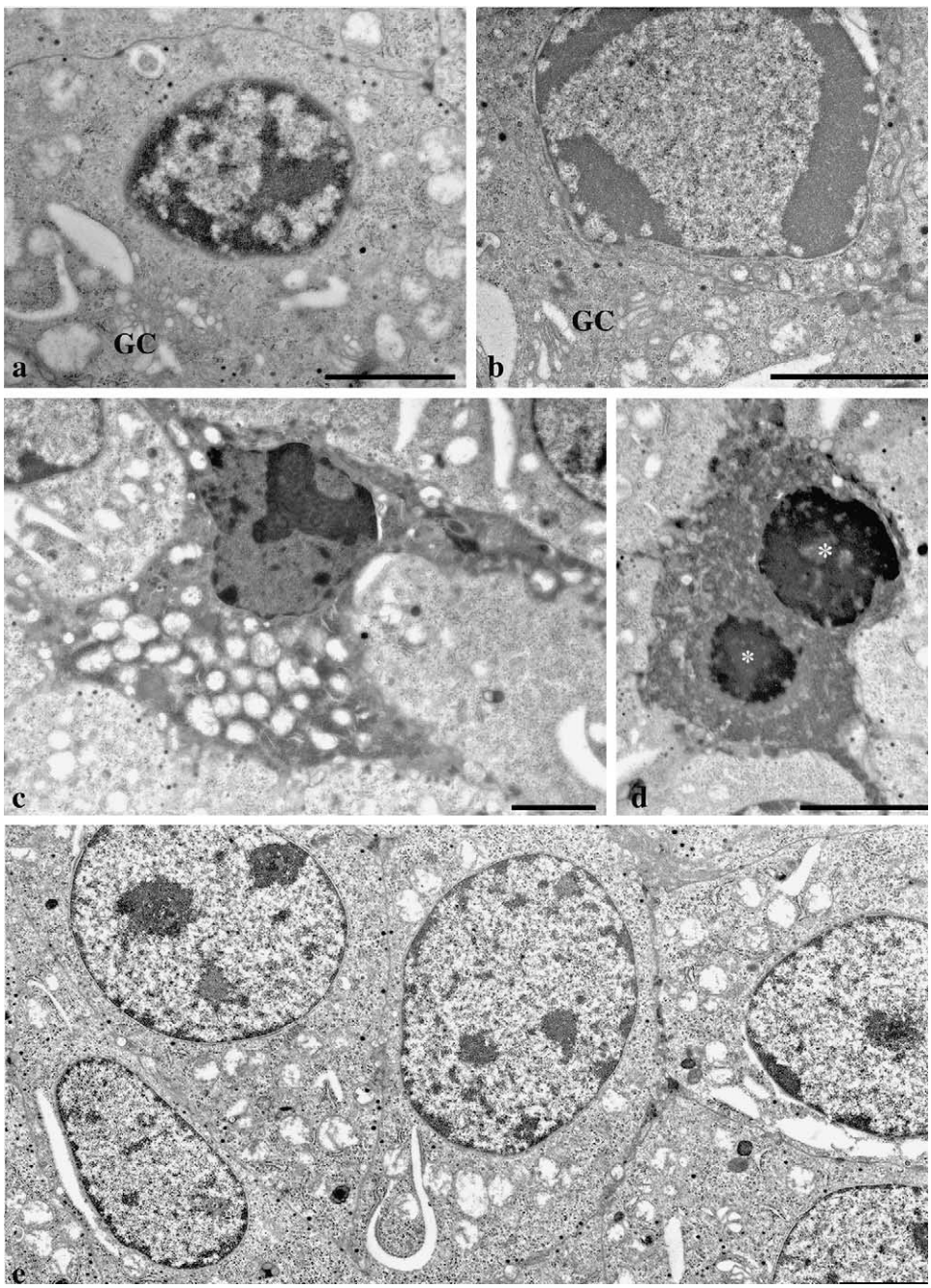

Fig. 7. TEM micrographs of PC 12 cells after NECA treatment (48 hr incubation) in the absence (a–d) or in the presence (e) of 1 μM SCH58261. Parts (a) and (b) show early steps of apoptosis. The chromatin begins to condense and to compact at the nuclear periphery and exhibits a granular appearance. Some mitochondria (M), Golgi complexes (GC) and electron-dense granules are visible in the cytoplasm. Parts (c) and (d) show advanced stages of apoptosis. Note the condensed cytoplasm with swollen mitochondria (c) and an apoptotic body (d), with two micronuclei (∗) and remnants of the degraded cytoplasm, representing the final stage of the apoptotic process. Part (e) shows unaffected PC 12 cells. Scale bars = 2.5 μm.

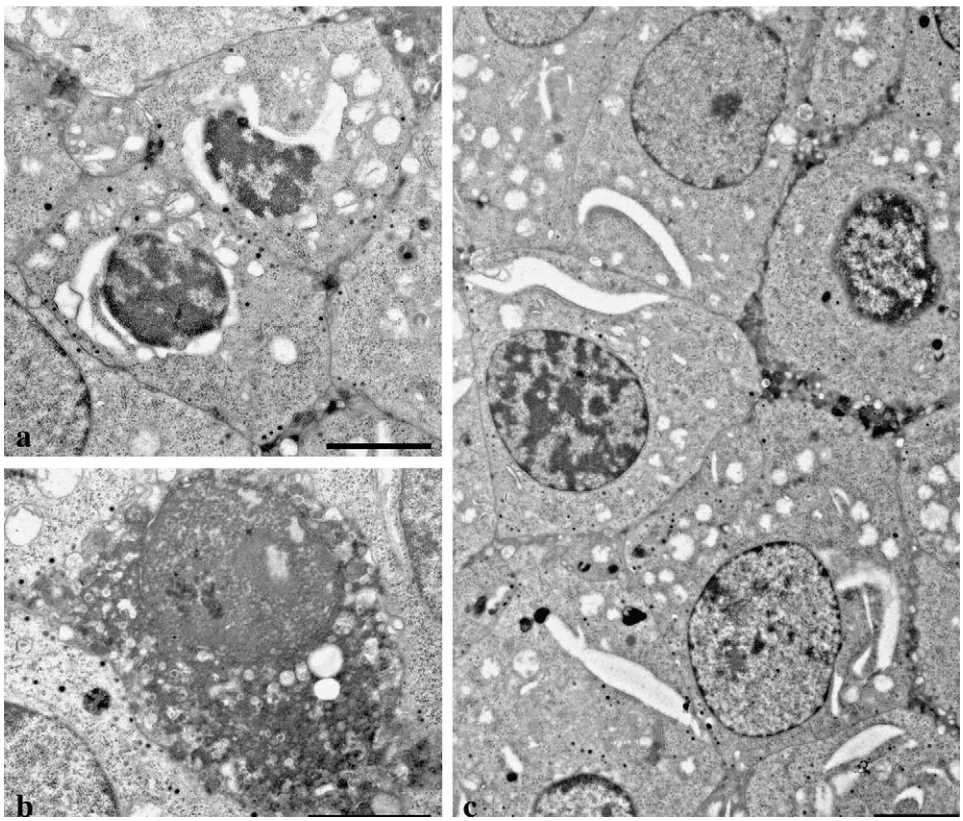

Fig. 8. TEM micrographs of PC 12 cells after 48 hr treatment with TNF-alpha (a and b) or IL-1-beta (c). Parts (a) and (b) show initial and final stages of the apoptotic process, respectively. In part (c) only some nuclei exhibit condensed chromatin characteristic of early stages of apoptosis. Scale bars = 2.5 μm.

A_{2A} AR ligands and cytokines, PC 12 cells were analyzed by TEM. Untreated PC 12 cells exhibited their typical ultrastructure characterized by a well-preserved plasma membrane, a nucleus with finely granular and uniformly dispersed chromatin, a cytoplasm containing randomly distributed organelles and electron-dense granules (Fig. 6). When cells were exposed to NECA for 48 hr, unequivocal signs of apoptosis were detected. The morphological changes observed in these cells included a progressive margination and condensation of the chromatin abutting on the inner nuclear envelope (Fig. 7a and b), nuclear fragmentation, cytoplasm shrinkage. Sometimes, the formation of membrane-bound cell fragments (apoptotic bodies) of different size in which nuclear material and cell organelles were randomly distributed (Fig. 7c and d) was observed. At early stage of apoptosis, mitochondria appeared to maintain their integrity while, at later stages, they showed swelling and crystolysis. When cells were incubated with NECA in the presence of selective A_{2A} AR antagonist, SCH58261, no alterations in the morphology of the nucleus and cytoplasmic organelles were observed (Fig. 7e), confirming the data obtained through viability assays.

Detectable signs of apoptosis were also observed in PC 12 cell treated with TNF-alpha, as evidenced by margination and condensation of the chromatin, associated with hypertrophy and crystolysis of mitochondria (Fig. 8a) and the final shrinkage of the vacuolized cytoplasm (Fig. 8b). On the contrary, IL-1-beta induced only a weak effect on cell death, due to the fact that the majority of the cells are morphologically unaltered (Fig. 8c).

4. Discussion

The main finding arising from the present study is that proinflammatory cytokines and/or A_{2A} AR ligands induce PC 12 cell death through an apoptotic mechanism.

In a previous work, we have demonstrated that proinflammatory cytokines, IL-1-beta and TNF-alpha, released at high levels following brain damage, are involved in the heterologous regulation of A_{2A} AR in PC 12 cells, which mainly express this AR subtype [5]. In particular, we have demonstrated that cytokines induce an up-regulation of the A_{2A} AR gene and protein expression levels without affecting the A_{2A} AR-mediated cAMP intracellular pathway. In the present work, we investigated the effect of cytokines and A_{2A} AR ligand treatment on PC 12 cell death control. As demonstrated by cell death detection ELISA assay and confirmed by TEM, IL-1-beta and TNF-alpha, were able to induce different degree of apoptotic cell death. The low degree of cell death induced by cytokines in our experimental model was in accordance with the data previously reported in these neuronal cell lines [28,29]. The effects

induced by cytokines were detected after 3 hr of cell treatment supporting the hypothesis that cytokines may play an important role in the brain response to acute hypoxic-ischemic injury. In fact, in different experimental models of cerebral damage, such as chemical hypoxia and glucose deprivation [30], the expression of both cytokine mRNA and corresponding proteins occurred within 30 min and 2 hr.

Three hours cell incubation with IL-1-beta in the presence of the AR agonist, NECA, resulted in an increase of cell death; following a longer time (48 hr exposure) this additive effect could not be reproduced probably because (at this time) cytokine itself induced a maximal effect.

In the attempt to identify the specific AR involved, different AR agonists/antagonists were tested. PC 12 cells have been widely used to study A_{2A} AR subtypes which are the mainly expressed receptors in this cell line. Whereas PC 12 cells do not express A_3 AR subtype, the presence of A_1 AR subtype in these cells is still a matter of debate [31]. By radioligand binding studies, we demonstrated that PC 12 cells express A_1 AR at low levels. Then, the role of A_1, A_{2A} and A_{2B} AR in PC 12 cell death was investigated.

ADO induced a significant reduction of cell viability supporting a cytotoxic effect of the nucleoside in this cell line. The nonselective AR agonist, NECA, induced PC 12 cell death in a time dependent manner with a peak at 48 hr: the maximum effect was obtained with nanomolar agonist concentrations and no further increase was observed with micromolar concentrations, probably because the receptor undergo desensitization when it is strongly activated [32]. The finding that SCH58261 but not MRS1706 significantly prevented the effects induced by the agonist NECA and by ADO demonstrated the A_{2A} AR specific involvement in cell death. This is in line with some data suggesting that A_{2A} AR activation could participate in neuronal damage generation [14–16] in the tissues that specifically express this receptor subtype.

Our data suggest that adenosine and cytokines, which are released at high concentrations during cerebral damage, participate in cell apoptosis and showed an additive effect in the regulation of short-time cellular responses. Several studies indicate that adenosine can modulate the proliferation, survival and apoptosis of many different cell types, ranging from epithelial, endothelial and smooth muscle cells to cells of the immune and neuronal lineage [10]. Of the four adenosine G protein coupled receptors identified so far, the A_{2A} and A_3 receptors have been specifically implicated in cell death modulation [33,34]. Some intriguing findings indicate that A_{2A} play a specific role in immune and neuronal cell death suggesting a possible therapeutic use of A_{2A} AR selective agonists in different pathologies. Among these are included adenosine deaminase-immunodeficiency syndrome (a pathology characterized by accumulation of adenosine to toxic levels) [33] and tumors, where the induction of apoptosis *via* activation of specific receptors may be desirable. In parallel, evidence for neuroprotection by A_{2A} AR antagonists was first obtained by Gao and Phillis [35] and later confirmed in different models of ischaemia and excitotoxic damage [18,23,36–38].

Although only a single antagonist compound was tested in the present study, the protective effect exerted by SCH58261 can be likely ascribed to the blockage of the A_{2A} AR subtype. On the other hand, SCH58261 is a specific and selective A_{2A} AR ligand with an affinity in the nanomolar range (K_i 2.3 nM) and a high selectivity vs. A_1, A_{2B} and A_3 receptors [39,40]. A_{2A}-mediated cytotoxicity was also confirmed by the pro-apoptotic effects of the selective A_{2A} AR agonist, CGS21680. Furthermore, our data suggest that adenosine-mediated cytotoxicity primarily involves a receptor-regulated mechanism; the finding that SCH58261 did not completely prevented ADO-mediated effects could suggest that the nucleoside regulated cell death also by an intracellular pathway. Further studies are required to investigate the role of intracellular toxicity of ADO.

Taken together, our results suggest that, althought A_{2A} AR ligands reduced pro-inflammatory cytokine production [41–43], they may potentiate the pro-inflammatory effects of those compounds. The molecular mechanisms by which A_{2A} AR stimulation triggers cell death have not yet been carefully explored. In PC 12 cells, as well as in other different cell lines, it has been demonstrated that A_{2A} receptor activation induce cAMP accumulation and a parallel transduction signaling pathway *via* PKC [44]. A_{2A} AR-mediated cAMP signaling pathway is not affected by PC 12 cell treatment with proinflammatory cytokines suggesting that probably cAMP is not the primary effector for A_{2A} AR toxicity. Therefore, PKC pathway may be involved in A_{2A} AR-mediated toxicity: in human melanoma cells [13], A_{2A} AR-mediated cell death involves mitogen activated protein kinases and PKC signaling pathway. To date, works in our laboratory are in progress to investigate the intracellular pathways involved in the A_{2A} AR-mediated cytotoxicity in PC 12 cells and the effects of proinflammatory cytokines on A_{2A} AR-mediated PKC signaling. These results might clarify the pathophysiological significance of A_{2A} AR up-regulation induced by cytokines during cerebral damage.

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