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Modulation of Apoptosis by Adenosine in the Central Nervous System: a Possible Role for the A₃ Receptor:

Pathophysiological Significance and Therapeutic Implications for Neurodegenerative Disorders^a

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INTRODUCTION

A great body of evidence has been accumulating in the last 20 years supporting a role for adenosine as a neurotransmitter and neuromodulator in the central nervous system.¹ In brain, adenosine acts as a potent depressant of excitatory neurotransmission and is colocalized (either as adenosine *per se* or as its precursor molecule, adenosine triphosphate (ATP)) with “classic” excitatory transmitters in many presynaptic terminals, whence it is released during physiological neurotransmission. It is now well established that the multiple effects of this nucleoside are mediated by activation of specific cell surface receptors, which, based on biochemical, pharmacological and molecular cloning studies, have been classified into four subtypes, denoted as A₁, A_{2A}, A_{2B} and A₃.² All the adenosine receptors are members of the guanine nucleotide-binding protein (G protein)-coupled receptor family and possess seven transmembrane helical regions.³ The functional roles of some of the adenosine receptor subtypes (*e.g.*, the A₁ and A_{2A} receptors) are relatively well established (see below), whereas the role(s) of the A_{2B} and the recently cloned A₃ receptors are still largely unknown.

ROLE OF ADENOSINE RECEPTOR SUBTYPES IN ADENOSINE MODULATION OF NEUROTRANSMISSION

The potent depressant, sedative and anticonvulsant actions of adenosine are mainly mediated by the A₁ receptor subtype, located to both pre- and postsynapses. The A₁ receptor seems to be connected through G_i/G_o-proteins to a variety of transduction mechanisms, including

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inhibition of adenylyl cyclase activity, modulation of phospholipase C-dependent intracellular Ca^{2+} regulation and modulation of K^+ , Ca^{2+} and Cl^- channels.^{2,3} In brain presynaptic terminals, a direct inhibition of membrane Ca^{2+} conductance seems to represent a main mechanism responsible for A_1 receptor-mediated inhibition of neurotransmitter release.^{4,5}

The A_2 receptors have been defined on the basis of their ability to stimulate adenylyl cyclase activity. Thus, they probably interact with G_5 proteins, although stimulation of phosphoinositide-dependent phospholipase C activity has been reported as well.⁶ Differently from the A_1 subtype, which can be detected in all brain areas (although to discrete levels of expression), the A_{2A} receptor subtype is particularly abundant and almost exclusively located to dopamine-rich brain areas, such as corpus striatum and caudatus putamen. This receptor has been involved in regulation of motor functions.² More recently, the presence of A_{2A} -like receptors in brain areas other than basal ganglia has been reported.⁷ These receptors have been proposed to play an entirely different role and to be responsible for stimulation of neurotransmitter release. The A_{2B} receptor has low affinity for adenosine and, differently from the A_1 and A_{2A} subtypes, which are activated by nM nucleoside concentrations, but similarly to the A_3 subtype (see below), requires micromolar concentrations of adenosine to be activated. The A_{2B} receptor can be found in low density in almost all cell types, including brain astrocytes. Recent data indicate that this receptor participates in the regulation of the interleukin 6 gene in astrocytoma cells,⁸ therefore suggesting a role for this receptor subtype in neuropathological conditions characterized by increased brain levels of this cytokine (*e.g.*, Alzheimer's disease and brain ischemia).

Very little is known about the A_3 receptor, initially identified by molecular cloning and whose pharmacological and molecular characterization has revealed unexpected findings for an adenosine receptor.⁹ The A_3 receptor has a unique structure-activity relationship profile, tissue distribution and effector coupling, being coupled to both inhibition of adenylyl cyclase activity and stimulation of phosphoinositide-dependent phospholipase C.^{10,11} Although there may be species differences,^{10,12} actions at this receptor are relatively insensitive to xanthine derivatives,⁹ which behave as antagonists at the A_1 and A_2 subtypes.¹³ Moreover, activation of the A_3 receptor requires high (*i.e.*, pathological) concentrations of adenosine; the K_i value of adenosine at this receptor is approximately 1 μM , versus 10 and 30 nM at rat A_1 and A_{2A} receptors, respectively.⁹ Thus, the pathophysiological actions of the A_3 receptor may be very different from those of the other adenosine receptors. Insights into the possible role of this receptor in brain have come from recent *in vivo* studies employing the first really selective A_3 receptor agonist N^6 -(3-iodobenzyl)-5'-(*N*-methyluronamide)adenosine (IB-MECA).¹⁴ In mice, administration of IB-MECA resulted in potent locomotor depression, with >50% reduction in activity. This behavioral effect was not counteracted by xanthine antagonists, therefore confirming the involvement of the A_3 receptor subtype.¹⁵ More recently, this receptor has also been implicated in the development of ischemic brain damage (see following section) which makes it a novel target for therapeutic interventions in central nervous system diseases characterized by neurodegenerative events.

ADENOSINE AND BRAIN ISCHEMIA: A DIFFERENTIAL ROLE FOR THE DIFFERENT ADENOSINE RECEPTOR SUBTYPES?

Treatment of cerebral ischemia with drugs acting at adenosine receptors was proposed over 15 years ago.¹⁶ Experimental studies rapidly validated the original concept, showing that, as also expected from the well documented depressant actions of adenosine, the administration of adenosine analogues to experimental animals either before or after the ischemic insult results in substantial improvement of survival, reduction of brain injury and amelioration of

associated neurological deficits.^{1,16,17} With the only exception of A₃-selective agents (see below), all adenosine analogues, especially those selective for the A₁ receptor, were shown to be neuroprotective (elegantly reviewed in Ref. 1). Consistently, administration of adenosine receptor blockers either immediately before or during the ischemic insult has been shown to worsen the ischemic outcome.¹ Conversely, subchronic treatment with xanthine adenosine receptor blockers before induction of ischemia resulted in protection against the subsequent ischemic insult, maybe through an upregulation of neuroprotective A₁ receptors.^{1,17}

We now have substantial information on the mechanisms at the basis of adenosine neuroprotective actions. Following trauma and ischemia, brain concentrations of adenosine are strongly and transiently increased from resting values of 50–300 nM to 10–50 μM. Notably, during ischemia, there is a dramatic increase of extracellular adenosine in the affected tissue, mainly as a result of intracellular catabolism of ATP and subsequent transport out of the cells by several specific carrier-mediated membrane transporters.¹ There is evidence that during ischemia/hypoxia and trauma, not only decreased energy supply but also enhanced energy demand by the supraphysiological activation of excitatory glutamatergic neurotransmission leads to increased adenosine release, as a result of massively increased release of neurotransmitters from brain terminals.¹⁸ Under these conditions, adenosine contributes to limiting the deleterious consequences of sustained activation of glutamate receptors by both reducing further release of neurotoxic mediators via presynaptic A₁ receptors, and by counteracting the postsynaptic actions of excitatory transmitters, including the excessive entry of Ca²⁺ ions, which has been associated to the development of programmed cell death.

The role of the adenosine A₃ receptor in ischemia has been recently investigated by von Lubitz and co-workers. These authors showed that the *in vivo* chronic administration of IB-MECA dramatically improved the histopathological and neurological outcome and preserved short-term memory following cerebral ischemia in gerbils.¹⁹ Chronic IB-MECA was also protective in chemically induced (*N*-methyl-D-aspartate or pentamethylenetetrazole) seizures.²⁰ Significant improvement of seizure latency, neurological impairment and survival was observed. At present, it is unknown whether the protective effects of chronically administered IB-MECA are due to effects on blood flow, neurons and/or other brain cells (see below).

A regimen-dependent inversion of the experimental result was seen after the acute administration of the A₃ selective agonist in the stroke model.¹⁹ Enhanced mortality and extensive brain damage were observed after a single IB-MECA administration immediately prior to induction of ischemia, suggesting that the A₃ receptor may participate to the development of ischemic damage.¹⁹ Such regimen-dependent opposite effects raised the hypothesis that, differently from the “neuroprotective” A₁ receptor, the brain A₃ receptor may mediate induction of cell death, and that its desensitization following chronic exposure to agonists (as may occur as a result of the chronic administration protocol), may play a role in the observed attenuation of ischemic brain damage.

ADENOSINE REGULATES ASTROGLIAL CELL DEATH: A ROLE FOR THE A₃ RECEPTOR?

As underlined above, the exact contribution of different brain cells to the *in vivo* effects of adenosine A₃ receptor agonists is not yet established. In brain, adenosine receptors are not only expressed by neuronal cells, but are also abundantly present on endothelial and glial cells, which may therefore significantly contribute to the detected effects.

In recent years, our laboratory has been particularly interested at characterizing the functional roles of adenosine receptors on astrocytes, a cell type which is now recognized to play important roles in both central nervous system development and in brain repair following trauma and ischemia (for review, see Refs. 21, 22). Not only do astroglial cells provide a metabolic support to neurons, but they also exert a number of highly specific functions. In developing brain, during specification of cortical areas, radial glia offer substratum and guide to migrating neurons and at the end of neurogenesis transforms into type 1 astrocytes. In adult brain, astrocytes express neurotransmitter receptors and uptake systems and directly participate in neurotransmission. Moreover, astroglial cells are known to respond to various types of injury by rapid and vigorous astrogliosis, a reaction characterized by both increased astroglial cell proliferation and astrocytic hypertrophy, as shown by “stellation” and increased expression of glial fibrillary acidic protein (GFAP), the astrocyte-specific intermediate filament protein. Although there is still debate about whether reactive astrogliosis is beneficial or detrimental to neuronal repair mechanisms, it is known that activated astrocytes are needed for axonal growth and guidance.²³

On these bases and in keeping with the well documented protective actions of adenosine analogues, we undertook a study aimed at investigating the effects of the adenosine A₁/A₃ nonselective agonist 2-chloroadenosine (2-CA) on rat brain primary astrocytes. Choice of this agonist was simply due to the fact that it is relatively hydrolysis-resistant, and therefore seemed to us particularly suited for long-term “trophic” studies. Surprisingly, exposure of cultures to 2-CA resulted, 48–72 hr later, in a marked reduction of astrocytic cell number, as shown by a dramatic decrease of GFAP-positive cells.²⁴ 2-CA-induced decrease of astrocytic cell number was not counteracted by either xanthine derivatives or blockers of the adenosine transport system, which suggested the involvement of a xanthine-insensitive extracellular receptor. In an attempt to elucidate the molecular mechanisms responsible for 2-CA-induced reduction of astrocytes, we preincubated cultures with bromodeoxyuridine (BrdU) and then double-labeled cells with both an anti-GFAP antibody (to detect astrocytes) and an anti-BrdU antibody (to quantify the percentage of astrocytes undergoing DNA synthesis). We were surprised to find out that, despite the marked reduction of cell number, 2-CA significantly increased the percentage of cells showing anti-BrdU immunoreactivity in nuclei.²⁴ Other examples had been previously reported in the literature where cell death was shown to be accompanied by a concomitant “paradoxical” increase of DNA synthesis. This phenomenon has been well characterized for secretory epithelial prostate cells that undergo extensive apoptosis following castration. Colombel & co-workers demonstrated that prostatic cells undergoing apoptotic death incorporate BrdU into nuclear DNA prior to DNA fragmentation.²⁵ Based on these data, they concluded that quiescent epithelial cells undergo apoptosis as a result of two sequential events initiated by testosterone depletion. The first event is an active reentry of these cells into the cell cycle. The second event is the apoptotic destruction resulting from the inability of differentiated cells to successfully complete this cycle (“abortive mitosis”).²⁵ Moreover, a number of data has been accumulating in recent years suggesting that two strikingly different cellular programs as cell proliferation and cell death by apoptosis indeed share initial identical patterns of protooncogene expression,^{26–29} so that the same primary stimulus activates a common pathway that can lead to either event; the timing of a secondary growth stimulus determines the final outcome.³⁰

On this background, we decided to verify whether astroglial cell death induced by 2-CA was apoptosis. Flow cytometric analysis of propidium iodide-stained nuclei showed the appearance of an hypodiploid DNA peak in cultures exposed to 2-CA that was not present in control cultures, suggesting induction of apoptosis, which was also confirmed by light and transmission electron microscopy analysis of cells.¹¹ An example of 2-CA-induced apoptosis is shown in Figure 1. This study represented the first demonstration in favor of a

novel action for adenosine (induction of cell death by apoptosis) in the central nervous system.

More recently, we extended this study to the investigation of the receptor subtype responsible for adenosine-induced apoptosis. A possible role for the A₃ receptor was indirectly suggested by the demonstration that 2-CA effects were not reversed by xanthine antagonists.²⁴ We therefore tested the effects of the selective A₃ receptor agonist IB-MECA and its more recent 2-chloro derivative, 2-C1-IB-MECA.³² Exposure of rat astrocytes to high (μM) concentrations of either agonist resulted in development of apoptosis, as shown by both morphological and flow cytometric criteria.³³ On both rat astrocytes and human astrocytoma cells (ADF cells),³⁴ at concentrations 2–3 orders of magnitude lower (10–100 nM), these same agonists induced a marked reorganization of the cytoskeleton, with appearance of stress fibers and numerous cell protrusions. These morphological changes were accompanied by a significant reduction of the number of spontaneously detached apoptotic cells in the culture medium.³³

The opposing actions induced on astroglial cells by nM and μM A₃ agonist concentrations may be, at least in part, the bases of the strikingly different ischemic outcome observed *in vivo* after either acute or chronic administration of IB-MECA.¹⁹ We speculate that a robust and acute activation of these receptors during ischemia as a consequence of massive release of adenosine (see the previous section) may contribute to the development of ischemic damage. It could be hypothesized that A₃ receptor-mediated apoptosis of astroglial cells may result in a reduced survival rate of neuronal cells (of course, this does not rule out that possible direct effects of adenosine A₃ receptors located on other cell types may contribute to ischemia-induced damage as well). In the von Lubitz *et al.* study, a marked cerebroprotection associated to increased survival rate was demonstrated if ischemia was induced after a subchronic treatment with low doses of IB-MECA.¹⁹ Desensitization of central A₃ receptors as a consequence of prolonged agonist exposure was hypothesized to reduce the putative deleterious contribution of this receptor to ischemic damage. Our data on cells of the astroglial lineage show that, under certain experimental conditions, A₃ agonists can indeed activate cell protection mechanisms (e.g., changes of the cytoskeletal machinery making cells more resistant to subsequent insults). However, since these beneficial effects are induced at nM agonist concentrations, molecular mechanisms other than agonist-induced receptor desensitization may be involved. A subthreshold stimulation of the A₃ receptor prior to the induction of ischemia may result in activation of protective mechanisms that make the brain less sensitive to a subsequent ischemic insult (“ischemic tolerance”). In this respect, it was previously shown that, in gerbils, mild ischemic treatments (e.g., a 2-min carotid occlusion) induce tolerance to a subsequent, and what would be lethal, ischemic stress.³⁵ Alternatively, the differential effects induced by different A₃ agonist concentrations may be related to either expression of two distinct astroglial A₃ receptor subtypes (endowed with different affinities and mediating cell protection and cell death, respectively) or to coupling of the same receptor to different transduction pathways, simply depending upon the degree of receptor activation. Studies are currently in progress in our laboratory aimed at elucidating this important point.

CHARACTERISTICS OF ADENOSINE-INDUCED APOPTOSIS

Adenosine and adenosine analogues have been previously demonstrated to induce apoptosis of cell types other than astroglial cells (summarized in Table 1).

In lymphoid cells, the apoptotic effect of 2-chloro-2'-deoxyadenosine (2-CdA, cladribine, which is utilized as an anticancer agent) has been shown to require intracellular phosphorylation to 2-CdATP (cladribine-5'-triphosphate) which directly competes with

dATP for DNA polymerase binding, being incorporated during DNA synthesis and repair to arrest chain extension.³⁶ DNA strand breakage is the immediate consequence of 2-CdA exposure and is more likely the primary lesion for apoptosis in these cells.^{37–39} Relief from deoxyadenylate stress imbalances was obtained by stably transfecting cells with the antiapoptotic molecule bcl-2.⁴⁰

In chick embryonic sympathetic neurons, adenosine (1–100 μ M) inhibited neurite outgrowth and killed about 80% of cells; also in this case, adenosine toxicity required the nucleoside to enter the cells and to be phosphorylated, as suggested by prevention of toxicity by both inhibitors of adenosine membrane transporter and by inhibitors of adenosine kinase.⁴¹

In human thymocytes, adenosine analogues were demonstrated to induce cell death via at least two independent pathways, one requiring the activation of an “atypical” extracellular receptor subtype linked to stimulation of adenylyl cyclase and Ca^{2+} increases,⁴² the other one being independent of extracellular receptors for adenosine.⁴³ For example, the effects of 2-CdA were prevented by addition of the nucleoside transport inhibitor dipyridamole, suggesting that 2-CdA has to be taken up by cells in order to trigger cell death.⁴³ On the contrary, but similar to astrocytes,³¹ the lethal effects of 2-CA were unaffected by dipyridamole, suggesting an extracellular site of action for the nucleoside. This conclusion is in agreement with the recent demonstration that both IB-MECA and CI-IB-MECA can induce apoptosis of HL-60 human promyelocytic leukemia cells.⁴⁴ This effect, similar to 2-CA-induced astroglial cell death, was not counteracted by xanthine blockers, further confirming an atypical extracellular site of action for these adenosine derivatives.⁴⁴

In conclusion, it seems that adenosine may utilize different pathways to modulate apoptosis in target cells. Adenosine can either induce cell death by activating a specific extracellular receptor (likely the A_3 subtype) linked to some as yet unidentified signaling pathway, or, alternatively, can directly enter cells to exert its toxicity. Some cells apparently express only one of these two pathways leading to cell death; in some other cells, both pathways are operative and the choice of either one of the two may simply depend on specific pathophysiological conditions.

PATHOPHYSIOLOGICAL IMPLICATIONS

The demonstration that adenosine can regulate the viability of various cell types has important pathophysiological implications.

Adenosine-induced apoptosis of thymocytes may play a critical role in the intrathymic cell selection process which functions to prevent autoimmunity and to remove thymocytes expressing functionally inactive T cell receptors. It may be hypothesized that a dysregulation of adenosinergic mechanisms in this crucial phase of T cell development may lead to serious immune system dysfunctions, as also documented by the adenosine-deaminase immunodeficiency syndrome.^{45,46} Moreover, confirmation of an active role of the adenosine A_3 receptor in regulating apoptosis in lymphoid cells⁴⁴ may lead to the development of novel anticancer agents of potential value in the treatment of leukemias and other tumors.

Adenosine-regulated apoptosis of nervous system cells may have important pathophysiological implications as well. Purine-induced cell death could play a role in naturally occurring apoptosis during brain development.⁴⁷ Additionally, this mechanism could play a role in the remodeling of the brain circuitries following trauma and ischemia, by favoring the elimination of irreversibly damaged cells in order to spare energy and space for recovering ones.²² Moreover, in contrast to necrosis, where cellular contents (including neurotoxic mediators) are massively released in the extracellular space, cell death by apoptosis occurs with preservation of cell membrane integrity; cellular contents are safely

sealed until phagocytosis intervenes and death occurs with no inflammation.⁴⁸ It might be speculated that adenosine-induced apoptosis in infarcted brain may function as a signal to limit the spreading of cell death following ischemic stroke.

Further possible pathophysiological implications come from a recent revaluation of the significance of apoptosis in the central nervous system. For many years apoptosis has been regarded simply as a physiological form of cell death.⁴⁹ It was believed that physiologically appropriate death is due to apoptosis, and that pathological mechanisms involve necrosis.^{49–51} However, recent evidence suggests that in a number of nervous system diseases characterized by neurodegenerative events, pathological cell death may also occur by apoptosis. Apoptotic cell death has been demonstrated in ischemia (but see also above), status epilepticus, HIV-1 encephalopathy and neurodegenerative diseases such as amyotrophic lateral sclerosis, Parkinson's and Alzheimer's disease.⁵² On these bases, the pharmacological modulation of apoptosis would open new avenues to the therapy of invalidating diseases which are also leading causes of death. A direct confirmation of the involvement of the adenosine A₃ receptor subtype in induction of cell death in the brain may therefore lead to the discovery of a novel class of potent modulators of apoptosis of potential use in neurological disorders characterized by neurodegenerative events.

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FIGURE 1.

Induction of apoptosis by 2-CA in rat primary astrocytic cultures: light microscopy evidence. Cultures were prepared as described in Ref. 31 and grown on glass coverslips in the absence (control not shown) or presence of the adenosine analogue (10^{-4} M). After 72 hr, cells were fixed, incubated with the Hoechst 33258 chromatin dye for 20 min to label nuclei, washed, immunostained with an anti-GFAP antibody as previously described to identify astroglial cells,³¹ and analyzed under a fluorescence microscope. Immunofluorescence micrographs show a GFAP staining of cultures (A) and corresponding Hoechst staining (B). Exposure to the adenosine analogue results in condensation of chromatin and appearance of apoptotic bodies (indicated by *arrows* in B), which are not normally detected in corresponding control cultures.

TABLE I

Induction of Apoptosis by Adenosine or Adenosine Analogues^a

Cell Type	Apoptotic Stimulus	Mechanism	Reference No.	Comments
Lymphoid cells	2-CdA	receptor-independent; arrest of DNA chain extension	38–40	
HL-60 cells	IB-MECA and 2-Cl-IB-MECA	A ₃ receptor-mediated; intracellular mechanism unknown; [Ca ²⁺] _i increases involved	44	potential value in the treatment of leukemias and other tumors; possible role in intrathymic T cell selection
Human thymocytes	2-CA	A ₂ receptor? cAMP and [Ca ²⁺] _i increases involved	42	
Human thymocytes	2-CdA	receptor-independent; increase of apoptotic p53 protein involved	43	
Chick sympathetic neurons	adenosine	receptor-independent; depletion of pyrimidine nucleotides and consequent inhibition of DNA synthesis	41	possible role in development and in brain remodeling following trauma and ischemia
Rat brain astrocytes	2-CA; IB-MECA and 2-Cl-IB-MECA	A ₃ receptor-mediated; abortive mitosis? effect on cytoskeleton?	31, 33	

^aSee text for further details.