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ORIGINAL ARTICLE

The role of ATP and adenosine in the brain under normoxic and ischemic conditions

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Abstract By taking advantage of some recently synthesized compounds that are able to block ecto-ATPase activity, we demonstrated that adenosine triphosphate (ATP) in the hippocampus exerts an inhibitory action independent of its degradation to adenosine. In addition, tonic activation of P2 receptors contributes to the normally recorded excitatory neurotransmission. The role of P2 receptors becomes critical during ischemia when extracellular ATP concentrations increase. Under such conditions, P2 antagonism is protective. Although ATP exerts a detrimental role under ischemia, it also exerts a trophic role in terms of cell division and differentiation. We recently reported that ATP is spontaneously released from human mesenchymal stem cells (hMSCs) in culture. Moreover, it decreases hMSC proliferation rate at early stages of culture. Increased hMSC differentiation could account for an ATP-induced decrease in cell proliferation. ATP as a homeostatic regulator might exert a different effect on cell trophism according to the rate of its efflux and receptor expression during the cell life cycle. During ischemia, adenosine formed by intracellular ATP escapes from cells through the equilibrative transporter. The protective role of adenosine A_1 receptors during ischemia is well accepted. However, the use of selective A_1 agonists is hampered by unwanted peripheral effects, thus attention has been focused on A_{2A} and A_3 receptors. The protective effects of A_{2A} antagonists in brain ischemia may be largely due to reduced glutamate outflow from neurones and glial cells. Reduced activation of p38 mitogen-activated protein kinases that are involved in neuronal death through transcriptional mechanisms may also contribute to protection by A_{2A} antagonism. Evidence that A_3 receptor antagonism may be protective after ischemia is also reported.

Keywords Adenosine · Adenosine A_{2A} receptors · ATP · Ecto-ATPase inhibitors · Ischemia · P2 purinergic receptors

Abbreviations

ATPγS	Adenosine-5′-o-(3-thio)triphosphate
α,β-meATP	Alpha,beta-methylene ATP
ARL 67156	6-N,N-diethyl-D-β,γ-dibromomethylene ATP
BBG	Brilliant blue G
BGO 136	1-Hydroxynaphthalene-3,6-disulfonate
CGS 15943	9-Chloro-2-(2-furanyl)-[1,2,4] triazolo[1,5-c] quinazolin-5-amine
CGS 21680	2-p-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride
Cl-IB-MECA	1-[2-Chloro-6[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-ß-D-ribofuranuronamide
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
fEPSP	field extracellular postsynaptic potential
GIRK	Kir 3.2 and 3.4 channels: potassium inward rectifiers
hMSCs	Human mesenchymal stem cells
LTP	Long-term potentiation
LTD	Long-term depression
MAPKs	Mitogen-activated protein kinases
MRS 1191	3-Ethyl 5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(6)-dihydropyridine-3,5-dicarboxylate

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MRS 2179	2′-Deoxy-N^6-methyladenosine 3′,5′-bisphosphate
NTPDase	Nucleoside triphosphate diphosphohydrolase
OGD	Oxygen/glucose deprivation
PPADS	Pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid
PS	Population spike
PV4	Hexapotassium dihydrogen monotitanoundecatungstocobaltate(II) tridecahydrate, $K_6H_2[TiW_{11}CoO_{40}]\cdot 13H_2O$
RB2	Reactive blue 2
SCH 58261	5-Amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine
ZM 241385	4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a] [1,3,5]triazin-5-yl-amino]ethyl)phenol

Historical overview

The physiological roles of adenosine triphosphate (ATP) and its metabolite adenosine have been historically linked to cell metabolism since ATP is an ubiquitous intracellular energy source in a number of enzymatic processes. However, over the years both metabolites have emerged as very versatile molecules of biological systems, being implicated in a variety of cell processes, from platelet aggregation to neurotransmission. The term 'purinergic signalling' was first introduced in the scientific literature by Burnstock [1]. An implicit concept for sustaining the hypothesis of purinergic neurotransmission proposed in the 1970s was the existence of purinergic receptors. The first evidence in this direction suggested the existence of two different subfamilies of such 'purinoceptors', identified as P1 and P2 receptors, selective for adenosine and ATP respectively [2]. Four different subtypes of P1-G-protein-coupled receptors: A_1, A_{2A}, A_{2B} and A_3 are known at present [3], whereas P2 purinoceptors belong to two major families: P2X ligand-gated ion channel receptors and P2Y G-protein-coupled receptors [4]. Cloning experiments supported this classification and helped to subdivide P2 receptors into seven P2X and eight P2Y subtypes [5] (plus the recently deorphanized GPR17 receptor [6]).

Roles of ATP in neurotransmission under normoxic conditions

The first studies on the role of extracellular ATP in hippocampal neurotransmission indicated prominent inhibitory action of this purinergic nucleotide on synaptic activity [7, 8]. However, several lines of evidence led to the hypothesis that ATP-mediated inhibition of hippocampal neurotransmission was probably mediated by adenosine acting on A_1 receptors. In fact, this response was theophylline-sensitive [7–9] and absent in A_1 knockout mice [10]. This assumption was supported by the fact that, in the hippocampus, extracellular ATP is rapidly converted into adenosine by ecto-ATPases and ecto-nucleotidases [11] and that adenosine acting on A_1 receptors exerts a well-described inhibitory role on CA1 excitatory neurotransmission [3]. In recent years, a more detailed observation of ATP-evoked effects, supported by the synthesis of new pharmacological tools, has helped to clarify the effective role of purinergic nucleotides in the hippocampus. In particular, it emerged that ATP mediates inhibitory effects by P2 receptor activation [12–14].

In a recent paper, we contributed to elucidating the role of ATP on CA1 hippocampal neurotransmission, and we reinforced the concept that ATP-mediated effects are not necessarily linked to adenosine formation in this brain region [15]. ATP is hydrolysed by ecto-NTPDase, enzymes located on the cell surface in the CNS that limit ATP, ADP and AMP spatio-temporal activity [11, 16]. There are three different known NTPDases: NTPDase1 hydrolyzes ATP and ADP equally well, NTPDase2 has a high preference for ATP, NTPDase3 is a functional intermediate, preferably hydrolyzing ATP [17]. In our study, we took advantage of some recently synthesized compounds: ARL 67156, which, at micromolar concentrations, inhibits rat NTPDase1 and 3 transiently transfected in Chinese hamster ovary cells, showing negligible activity on NTPDase2 [18]; BGO 136, a new inhibitor described as a selective NTPDase1 and 2 blocker with *Ki* values in the high micromolar range [19]; and the recently synthesized PV4, which strongly inhibits rat NTPDase1, 2 and 3 with *Ki* values in the nanomolar range [20]. By using these inhibitors that are able to block NTPDase activity without interfering with P2 receptor activation, we demonstrated that ATP exerts an inhibitory action, independent of its degradation to adenosine. As shown in Fig. 1, the application of ATP during a NTPDase activity blockade still elicits a decrease in evoked synaptic responses which is even more pronounced than that evoked by ATP alone.

An excitatory effect of exogenous ATP on hippocampal neurotransmission has also been reported by different authors. This effect, which was observed after drug removal, was described to persist in in vitro preparations for a relatively prolonged period, up to 1 h [21–25]. This 'long-lasting' potentiation of synaptic responses was compared to electrically evoked LTP, firstly described in the hippocampus by Abrams and Kandel [26] and called 'ATP-induced LTP'. In agreement, we observed a potentiation of the synaptic responses after drug removal when slices were superfused in the presence of the metabolically stable ATP-analogue ATPγS (Fig. 2) [15]. In addition to this 'long-lasting' excitatory effect evoked by the exogenous application of P2 agonists, we also demonstrated an excitatory tone exerted by endogenous ATP.

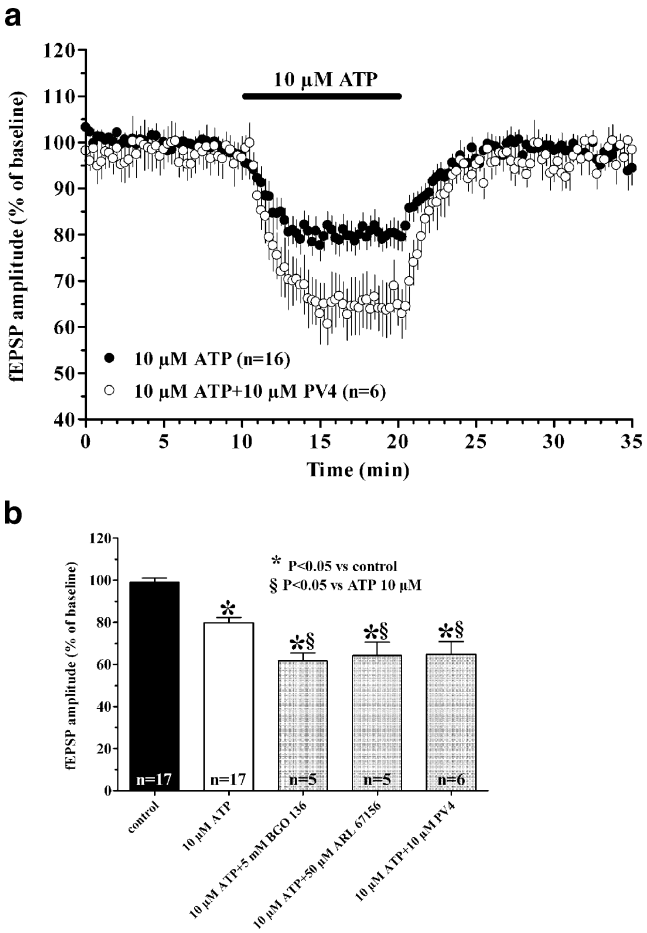

Fig. 1a, b The inhibitory effect induced by ATP on fEPSP amplitude is potentiated in the presence of different NTPDase inhibitors. **a** Time-course of fEPSP amplitude before, during and after the application of ATP in the absence or in the presence of the NTPDase1,2,3 inhibitor PV4. Each *point* in the graph represents the mean ± SE of fEPSP value measured as percent of baseline, pre-drug level. **b** *Columns* in the graph summarize the average amplitude (mean ± SE) of evoked fEPSP recorded from CA1 hippocampal region in control conditions, 5 min after superfusion of ATP alone and 5 min after ATP superfusion in the presence of different ecto-ATPases inhibitors. Note that the inhibitory effect of ATP on fEPSP amplitude is potentiated by BGO 136, PV4 and ARL 67156. *$P<0.05$ one-way ANOVA, Newman-Keuls multiple comparison post-hoc test versus pre-drug value. §$P<0.05$, one-way ANOVA, Newman-Keuls multiple comparison post-hoc test versus 10 µM ATP treated slices. (Modified from [15])

Slices superfused with P2 antagonists show a small but significant reduction in synaptic transmission (Fig. 3). These data demonstrate that tonic activation of P2 receptors contributes to glutamatergic excitatory neurotransmission in the hippocampus, an observation that is in line with previous work [27].

Roles of ATP in ischemic conditions

The role of ATP may become critical during pathological conditions such as ischemia, when extracellular ATP concentrations increase. An enhanced outflow of radioactive ATP from hippocampal slices during in vitro ischemic-like insults was first reported by Juranyi and co-workers [28], and the first demonstration that ATP outflow increases in vivo during the induction of focal ischemia in the rat was reported by Melani and colleagues [29]. Thus, at the ischemic site, levels of extracellular nucleotides may remain elevated for long periods of time after injury. Evidence supports the idea that, under such pathological conditions, released ATP may exert an excitotoxic role by acting on its receptors, thus enhancing Ca^{2+} inward currents and altering synaptic activity and cellular plasticity. Non-selective antagonists of P2 receptors, suramin and PPADS, and the selective antagonists, BBG and MRS 2179 of $P2X_7$ and $P2Y_1$ receptors respectively, prevent the irreversible failure of neurotransmission induced by a prolonged period of OGD in hippocampal slices [15]. Moreover, they protect from development of anoxic depolarization (AD), which is a rapid and regenerative wave of depolarization that propagates in tissue and represents an unequivocal sign of sufferance [15]. Antagonists of P2 receptors have been proved protective against cell death induced by either ATP itself, hypoglycemia or glutamate exposure in primary cultures of brain-derived neurones [30–33]. Intrastriatal ATP injection in rats induced, 24 h later, a clearly lesioned area [34]. The ATP-induced damage was concentration-dependent, mimicked by ATPγS and α,β meATP (but not by ADP or adenosine) and blocked by RB2, a non-specific P2 antagonist [34]. Suramin, another non-specific P2 receptor antagonist, administered 30 min before occlusion of the middle cerebral artery, resulted in a significant decrease in infarct and oedema volume 6 h after brain injury [35]. In agreement, it was demonstrated that RB2 [36] and PPADS [37] improve neurological deficit and

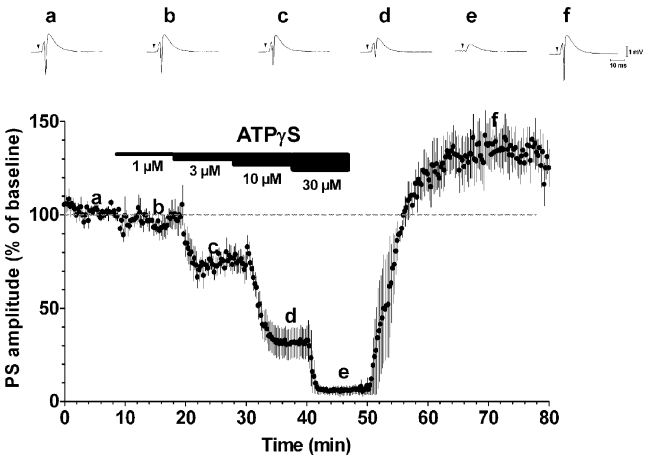

Fig. 2 Inhibitory and excitatory effects of the stable ATP analogue ATPγS. Averaged time-course ($n=4$) of PS amplitude before, during and after the application of different concentrations of ATPγS. PS amplitude (mean ± SE) is measured as percent of baseline level. *Upper panels* represent single traces recorded in a typical experiment before, during and after ATPγS application at different concentrations

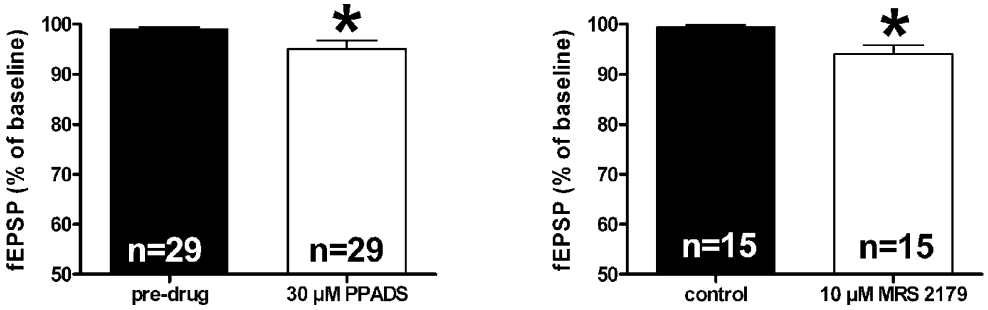

Fig. 3 Excitatory effects of endogenous ATP. *Bars* in the graphs represent the average of fEPSP amplitude in the presence of P2 antagonists: PPADS (30 μM) and MRS 2179 (10 μM). *$P<0.05$, paired Student's *t*-test. (Modified from [15])

reduce the damage induced in rats in a model of focal ischemia in vivo. Moreover, RB2 induces the expression of $P2X_7$ receptors on reactive microglia in the remote ipsi and contralateral cingulate and medial frontal cortex and striatum. Although a pro-apoptotic role has been attributed to the $P2X_7$ receptor, results suggest that microglial cells expressing the $P2X_7$ receptor can be implicated in tissue damage as well as in the defence and repairing processes in the remote ipsi and contralateral undamaged areas [36].

In considering the effect of ATP during ischemia, it must be taken into account that ATP itself is involved in control of cerebrovascular regulation [38] and that the metabolite of ATP, ADP, that interacts with $P2Y_1$/$P2Y_{12}$ receptors, is a potent platelet aggregator. However, clinical studies that checked the antiplatelet therapy of the selective $P2Y_{12}$ receptor antagonist clopidogrel reported a response variability [39, 40].

Although evidence suggests that ATP exerts a detrimental role under ischemia, there are reports that it may exert a trophic role in terms of cell division and differentiation in both differentiated and undifferentiated cells [41–43], including adult neural stem cells [44]. Interesting data correlate these trophic effects with the occurrence of spontaneous Ca^{2+} waves that propagate among adjacent cells in a self-renewing manner. Kawano et al. [45] reported that ATP-induced initiation and propagation of intracellular Ca^{2+} waves in human mesenchymal stem cells (hMSCs) promote activation of transcription factors (e.g. NFAT) that are involved in cell differentiation. In the same study, they demonstrated that ATP-induced Ca^{2+} waves disappear in the fully differentiated adipogenic phenotype. We recently reported that ATP is spontaneously released from hMSCs during the early stages of culture (P0-P5). Moreover, we reported that ATP decreases proliferation rate (Fig. 4) and modulates specific ionic current hMSCs [46]. A high extracellular ATP concentration at earlier cell culture passages suggests an important role of ATP in regulating cell differentiation. Increased hMSC differentiation may account for an ATP-induced decrease in cell proliferation.

Therefore, it can be envisaged that ATP, as an autocrine/paracrine homeostatic regulator, exerts different effects on

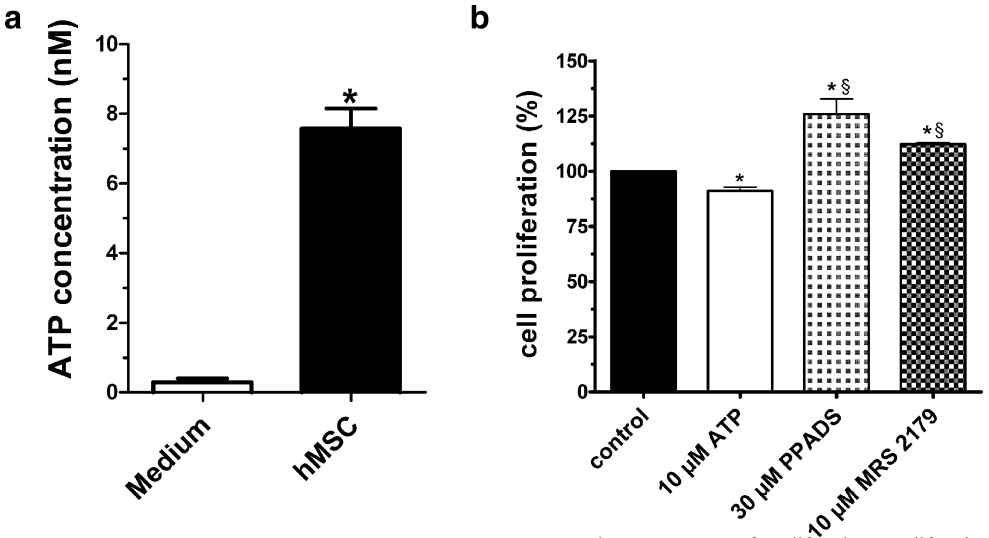

Fig. 4a, b Human mesenchymal stem cells in culture spontaneously release ATP that modulates cell proliferation. **a** Extracellular concentrations of ATP were measured in the medium containing hMSCs and in control medium not containing cells. Data are expressed as mean ± SE, $n=11$, unpaired Student's *t*-test: *$P<0.0001$ vs medium alone. **b** Effect on hMSC proliferation after daily application of ATP (10 μM) and P2 antagonists, PPADS (30 μM) and MRS 2179 (10 μM). Data are expressed as percentage of proliferation. Proliferation of untreated cells was assumed as 100%. The cell number was determined after 5 days of culture by a culture counter. Each *column bar* represents the mean ± SE of $n=4$ for each experimental condition. Paired Student's *t*-test: *$P<0.05$ vs respective control; one-way ANOVA, Newman-Keuls post-test: §$P<0.05$ vs 10 μM ATP-treated cells. (Modified from [46])

cell trophism according to its extracellular concentrations, distinct cell populations involved, differential expression and recruitment of P2 receptors and of more or less sustained stimulation of the same receptor. Balancing these effects may be relevant in the post-ischemic brain, when a neuroregenerative process could promote tissue repair [47]. On this basis, determination of ATP extracellular concentrations at various times after ischemia induction may help to identify which types of receptors may be stimulated under ischemia and explain the role of detrimental versus trophic ATP during ischemia. ATP present in the extracellular space is rapidly metabolised by membrane-bound NTPDase. Extracellular ATP concentrations, evaluated in the brain to date, have been underestimated, since it has not been possible to selectively inhibit ecto-NTPDase. In fact, the selective inhibitor ARL 67156, tentatively used thus far to inhibit ecto-NTPDase, interferes with the ATP assay method [29].

Role of adenosine in cerebral transmission under normoxic conditions

Adenosine exerts an important tonic modulation of synaptic transmission in the brain. This tonic inhibition of synaptic transmission is evoked by stimulation of A_1 receptors, as demonstrated in several brain regions, such as the hippocampus, striatum and olfactory cortex [48, 49]. The inhibitory effect of adenosine A_1 receptor stimulation has a pre- and postsynaptic component. Activation of the presynaptic A_1 receptors reduces Ca^{2+} influx through the preferential inhibition of N-type and, probably, Q-type channels [50, 51]. Inhibition of presynaptic calcium currents decreases transmitter release [52], and adenosine, by stimulation of A_1 receptors, has been found to inhibit the release of virtually all classical neurotransmitters: glutamate, acetylcholine, dopamine, noradrenaline and serotonin (see in [53]). In particular, powerful suppression of glutamate release from presynaptic terminals has been described in the hippocampus [54, 55], where adenosine A_1 receptor activation reduces the number of quanta released (but not the size of individual quanta nor postsynaptic glutamate receptor sensitivity) in the Schaffer collateral-commissural pathway [56]. The postsynaptic effect of A_1 receptors consists of direct hyperpolarisation of neurones via activation of GIRK channels (Kir 3.2 and 3.4 channels: potassium inward rectifiers) [57, 58]. Endogenous adenosine exerts tonic inhibition of excitatory neurotransmission. The selective A_1 antagonist, DPCPX, causes a 15% increase in synaptic potential amplitude in in vitro brain slices [59]. This is an expected result in a brain region where the adenosine concentration at a receptor level was calculated around 200 nM [59, 60] and A_1 receptors, whose affinity for adenosine is in the low nanomolar range, are highly expressed. These data are confirmed by the fact that, in slices taken from homozygous A_1 receptor knockout mice, no evidence was found for an endogenous inhibitory action by adenosine in the Schaffer collateral pathway in the CA1 region of the hippocampus or at the mossy fibre synapses in the CA3 region [61].

Opposite effects from A_1-mediated synaptic inhibition are elicited by A_{2A} receptor activation, which has been shown to mediate excitatory actions in synaptic function [62–64]. In the hippocampus in vitro, A_{2A} receptor stimulation results in a Ca^{2+}-dependent release of acetylcholine [65, 66]. Furthermore, the application of CGS 21680, a selective A_{2A} receptor agonist, decreases the ability of A_1 receptor agonists to inhibit excitatory neurotransmission [9, 67]. This effect suggests that A_{2A} receptor stimulation increases synaptic transmission through A_1 receptor desensitisation [68, 69]. However, there is also evidence that A_{2A} receptors increase excitatory amino acid release. In fact, the selective stimulation of adenosine A_{2A} receptors augments the amount of glutamate released in the hippocampus and striatum of young rats [70–72].

In spite of the excitatory role in neurotransmission brought about by A_{2A} receptors, the net effect of adenosine is an inhibitory tonus on neurotransmission, in accordance with observations suggesting that activation of A_{2A} receptors requires protracted stimulation to induce evident effects on synaptic transmission [59]. It is worth noticing that the role of A_{2A} receptors in the striatum is recently gaining interest in light of their heterodimerisation with D_2 dopamine receptors. The association between A_{2A} and D_2 receptors results in an antagonistic interaction that provides a rationale for evaluating A_{2A}-selective antagonists in Parkinson's disease, supported by epidemiological evidence indicating an inverse relationship between caffeine consumption and the risk of developing this pathology [73, 74]. It was suggested that A_{2A} antagonists not only provide symptomatic relief but also decelerate dopaminergic neurone degeneration in patients.

Discrepancies about the role of adenosine A_3 receptors in the brain are present in the literature. Activation of this receptor subtype has been associated with both excitatory and inhibitory effects, even in the same brain region. An excitatory role of A_3 receptors has been supported by evidence indicating that, in the rat hippocampus, their activation attenuates LTD and allows induction of LTP elicited by a subliminal weak-burst protocol [75]. In addition, in the same brain area, A_3 receptor activation through a selective adenosine A_3 agonist has been shown to antagonize the adenosine A_1 receptor-mediated inhibition of excitatory neurotransmission [8]. Moreover, A_3 receptor stimulation always attenuates the inhibition of hippocampal slice neurotransmission, in a PKC-dependent manner, caused by presynaptic metabotropic glutamate receptors [76]. Whole-cell patch clamp recordings in CA3 hippocampal pyramidal neurones demonstrate that A_3 receptor activation results in a significant potentiation of high threshold hippocampal Ca^{2+}

currents by a PKA-dependent mechanism [77]. Finally, facilitation of the onset of epileptiform discharge has been observed in the presence of the selective A_3 receptor agonist Cl-IB-MECA [78], and a reduction in such epileptic activity was observed when A_3 receptors, activated by endogenously released adenosine during seizures, were blocked by the selective antagonist MRS 1191 [79].

Contrary to previous results, an inhibitory action has been attributed to A_3 receptors by Brand and colleagues [80], who demonstrated that, in rat cortical neurones, the selective activation of this adenosine receptor subtype is involved in inhibition of excitatory neurotransmission, suggesting a synergistic action with the inhibitory effect of adenosine brought about by A_1 receptor activation. Despite results obtained by A_3 receptor stimulation, evidence that a selective block of A_3 receptors does not affect neurotransmission in the CA1 region of the hippocampus under normoxic conditions indicates that endogenous adenosine at physiological concentrations does not exert tonic activation of A_3 receptors [8, 81]. This is in line with evidence that A_3 receptor activation requires micromolar levels of extracellular adenosine, which can be reached only during pathological conditions of impairment in energy supply (for example during hypoxia or ischemia [82–85]).

Role of adenosine in ischemic conditions

Extracellular adenosine concentrations increase dramatically during ischemia [82, 86, 87]. During ischemia, following the imbalance between ATP degradation and resynthesis, the intracellular concentration of adenosine increases. Therefore, increased adenosine concentrations in the extracellular space during ischemia are likely due to the equilibrative transporter that carries adenosine out of cells. Adenosine formation can also take place at the extracellular level, through the hydrolysis of extracellular ATP operated by NTPDases and ecto-5′-nucleotidase (e5′-NTs) (see Fig. 5). Hence, these enzymes have a dual function in modulating purinergic neurotransmission: (1) they rapidly interrupt ATP-mediated signalling by degrading extracellular nucleotides and (2) they give rise to extracellular adenosine, which activates P1 receptors. However, recent evidence in vitro suggests that released ATP does not substantially contribute to the adenosine concentration in the extracellular milieu during ischemia [88].

Adenosine-potentiating agents, which elevate endogenous adenosine levels by either inhibiting its metabolism by adenosine deaminase or kinase [89, 90] or preventing its transport [91, 92], offer protection against ischemic neuronal damage in different in vivo ischemia models. Moreover, adenosine infusion into the ischemic striatum during transient focal ischemia proves to significantly ameliorate the neurological outcome and reduce infarct volume [93].

A temporal correlation exists between adenosine outflow and synaptic potential inhibition in rat hippocampal slices during ischemia-like conditions [84, 88]. Synaptic inhibition during ischemia is greatly dependent on adenosine, which, by stimulating A_1 receptors, exerts a protective role by reducing the Ca^{2+} influx, thus counteracting the presynaptic release of excitatory neurotransmitters [55, 94] and, in particular, glutamate, which exerts an excitotoxic role during ischemia mainly by overstimulation of NMDA receptors [95]. By directly increasing the K^+ and Cl^- ion conductan-

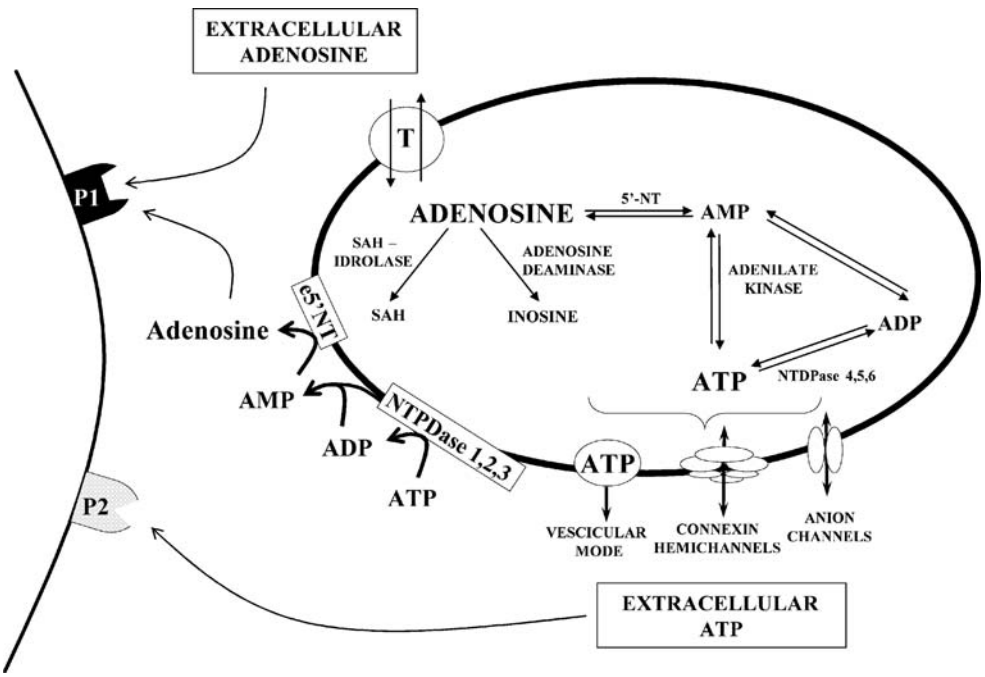

Fig. 5 Schematic drawing of intracellular and extracellular adenosine formation. In the extracellular space, adenosine and ATP act on own purinergic receptor subtypes: P1 and P2 receptors, respectively. *ADP* adenosine diphosphate, *AMP* adenosine monophosphate, *ATP* adenosine triphosphate, *e5′-NT* ecto-5′-nucleotidase, *5′-NT* 5′-nucleotidase, *NTDPase* ecto-nucleoside triphosphate diphosphohydrolases, *P1* adenosine receptor, *P2* ATP receptor, *SAH* S-adenosylhomocysteine, *T* bidirectional nucleoside transporter. (Modified from [142])

ces, adenosine stabilises the neuronal membrane potentials, thus reducing neuronal excitability [96]. Consequent reductions in cellular metabolism and energy consumption [97] and moderate lowering of the body/brain temperature [98] are protective in ischemia.

A_1 receptor agonists are shown to attenuate ischemic or excitotoxic neuronal damage in both in vitro and in vivo models of cerebral ischemia (for review, see [99, 100]). In accordance, adenosine A_1 antagonists given acutely exacerbate the damage induced by ischemia in different animal models of ischemia. An unselective A_1 receptor antagonist, theophylline, increased mortality [101, 102]. Unlike acute treatment, chronic administration of A_1 agonists worsened survival and increased neuronal loss [103], a phenomenon thought to depend on A_1 receptor desensitization.

Although data converge in demonstrating a neuroprotective effect of adenosine through A_1 receptors during ischemia, the use of selective A_1 agonists is hampered by unwanted peripheral effects, e.g. sedation, bradycardia, hypotension [104]. Von Lubitz and co-workers [105] have reported that post-ischemic administration of the A_1 receptor agonist adenosine amine congener (ADAC), which induces fewer undesirable effects, increases survival in gerbils. Moreover, we may consider that administration of agents that elevate the local concentration of adenosine at the injury site, by inhibiting its metabolism to inosine or rephosphorylation to AMP or reuptake, may have the advantage of restricting the effect of such inhibitors to areas of injury-induced adenosine release [106].

More recently, the role of A_{2A} receptors in ischemic neuroprotection has been studied. Gao and Phillis [107] demonstrated for the first time that the non-selective A_{2A} receptor antagonist CGS 15943 reduces cerebral ischemic injury in the gerbil following global forebrain ischemia. Subsequently, many reports have confirmed the neuroprotective role of A_{2A} receptor antagonists in different models of ischemia. The selective A_{2A} receptor antagonist SCH 58261 reduced ischemic brain damage in neonatal [108] and adult [83, 109] rat models of focal cerebral ischaemia. The same antagonist, subchronically administered, was protective against both brain damage and neurological deficit in the adult rat model of focal cerebral ischemia [110, 111]. Studies in A_{2A} receptor knockout mice supported the neuroprotective role of A_{2A} receptor antagonists on ischemic brain damage [112]. The beneficial effects of A_{2A} antagonists in stroke were mainly attributed to reduced glutamate outflow [83, 110, 113]. Prolonged application of the A_{2A} selective agonist CGS 21680 significantly reduced synaptic depression brought about by OGD in the hippocampus [59] and the same agonist promotes glutamate release under normoxic and ischemic conditions [70, 71, 114, 115]. In addition to neurones, adenosine A_{2A} receptors are located on microglia [116, 117] and astrocytes [118]. On glial cells, A_{2A} receptors mediate inhibition of the glutamate uptake transporter, GLT-1, and stimulate glutamate outflow [119]. A_{2A} antagonists prevent the increase in glutamate levels induced by glutamate uptake inhibitors [120]. Therefore, the protective effects of A_{2A} antagonists in brain ischemia may be largely due to reduced glutamate outflow from neurones and glial cells.

Further support of a major role for glia in the neuroprotective effect of A_{2A} antagonism in ischemia comes from the observation that subchronic administration of the A_{2A} receptor antagonist SCH 58261 reduces p38 mitogen-activated protein kinase (MAPK) activation in striatal and cortical microglia 24 h after permanent focal ischemia [111]. Evidence indicates that p38 MAPK is activated in glia up to 24 h after ischemia [121, 122] and is involved, through transcriptional mechanisms, in neuronal death [123, 124]. Since SCH 58261 reduces glutamate outflow in the first hours after ischemia [83], reduced p38 MAPK activation may be due to a direct effect on glial A_{2A} receptors or may be secondary to a reduction in the excitotoxic cascade that primes p38 activation [125]. The importance of A_{2A} receptors under ischemia is highlighted by the observation that A_{2A} receptor expression increases on neurones and microglia after ischemia [126]. Selective inactivation of A_{2A} receptors on bone-marrow-derived cells (wild-type mice transplanted with A_{2A} receptor knockout bone marrow cells) attenuates infarct volumes and ischemia-induced expression of several proinflammatory cytokines in the brain [127]. Therefore, protective effects of A_{2A} antagonists may be attributed to inhibition of inflammation product production.

In several studies, A_{2A} receptor agonists have been found to be protective in the global ischemia model in the gerbil [128, 129]. Jones and co-workers [130] show that peripheral administration of the A_{2A} receptor agonist CGS 21680 protects the hippocampus against kainate-induced excitotoxicity. However, the direct injection of CGS 21680 into the hippocampus failed to afford protection, while the A_{2A} antagonist ZM 241385, when injected directly into the hippocampus, reduced kainate-induced neuronal damage [131]. These data suggest that the neuroprotective properties of A_{2A} agonists are mainly due to peripherally mediated effects. Major mechanisms that may account for A_{2A}-mediated protection include inhibition of platelet aggregation and vasodilation [89] and anti-inflammatory actions. A_{2A} receptors on neutrophils may account for inhibition of adhesion to endothelial cells and ensuing production of free radicals [132, 133].

On this basis, adenosine and its receptors are considered targets for therapeutic implementation in the treatment of stroke. At the moment, a possible adenosynergic therapeutic strategy after ischaemia that is worth consideration is that of increasing adenosine concentrations at the ischemic sites by inhibitors of adenosine metabolism or reuptake in association with adenosine A_{2A} antagonists. When considering the

possible use of adenosine kinase inhibitors, it should be taken into account that adenosine represents only a small percentage of nucleotide content [134], therefore inhibition of its rephosphorylation to ATP by adenosine kinase inhibitors does not weigh upon the ATP content.

The few studies present in the literature concerning the role of A_3 receptors in the pathophysiology of cerebral ischemia are rather contradictory. We have demonstrated that selective antagonism of A_3 receptors facilitates the recovery of synaptic activity induced by ischemic preconditioning in rat hippocampal slices [81]. A harmful role of A_3 receptors during in vitro OGD was confirmed by our observation that blocking the A_3 adenosine receptor consistently abolishes or delays the occurrence of anoxic depolarization (AD) and significantly protects from the irreversible disruption of excitatory neurotransmission caused by a severe ischemic episode [135]. These results are in agreement with the observation that acute administration of a selective adenosine A_3 agonist exacerbates the damage elicited by global ischemia in the gerbil [136]. On the contrary, it was demonstrated that chronic pre-ischemic administration of an A_3 agonist protects against ischemic neuronal damage [136]. This effect may be attributed to desensitisation of A_3 receptors. In fact, both human and rat A_3 receptors are desensitised within a few minutes after agonist exposure [137, 138].

Contrary to the above information, Hentschel and colleagues [139] demonstrated that under hypoxic conditions, selective activation of A_3 adenosine receptors brings about an inhibition of excitatory neurotransmission on cortical neurones, indicating that A_3 receptors may sustain the neuroprotective action of adenosine induced by A_1 receptors. Consistent with these reports, mice lacking A_3 adenosine receptors show increased neurodegeneration in response to repeated episodes of moderate hypoxia [140] or an increase in cerebral infarction after transient ligation of the middle cerebral artery [141]. These opposite results regarding an excitatory or inhibitory role of A_3 receptors on synaptic activity under hypoxia/ischemia may be reconciled by our recent data [135], suggesting that in a first phase of ischemia, A_3 receptors play a protective synergistic role with A_1 receptors. Severe ischemia would transform the A_3 receptor-mediated effects from protective to injurious.

Taken together, these data suggest that the outcome of A_3 receptor stimulation on synaptic transmission during hypoxic/ischemic phenomena depends on the intensity and duration of stimulation.

Concluding remarks

Purinergic signalling, e.g. adenosine and ATP, by activating specific membrane receptors (P1 and P2 respectively), is strictly correlated in orchestrating brain cell functions either under physiological normoxic or ischemic circumstances. However, the respective contribution of each single element is hard to discern from the total outcome, due to the rapid and ubiquitous enzymatic interconversion of these molecules.

In the brain, both compounds are likely to play a role in neuronal activity, and the inhibitory action of endogenous adenosine in excitatory neurotransmission (mainly due to A_1 receptor activation) is well documented. Conversely our data, in line with previous observations in the literature, suggest that tonic activation of P2 receptors contributes to excitatory transmission in the hippocampus. Such an effect is modest, but it is unmasked by the application of P2 purinergic antagonists that reduce evoked synaptic responses.

An inhibitory effect elicited by exogenous ATP application has been frequently described and is mostly due to enzymatic degradation to adenosine and the subsequent activation of A_1 receptors. However, our evidence demonstrates that ATP itself may inhibit hippocampal synaptic transmission since an even more pronounced effect is observed when enzymatic ATP degradation is blocked. Moreover, in our studies in the hippocampus, a potentiation of neurotransmission by the ATP analogue, ATPγS, develops a few minutes after the inhibitory effect has been washed out, and it persists for 45 min to 1 h. Within the brain, ATP is involved in the synaptic plasticity phenomenon and may be an important mediator of long-lasting effects in synaptic plasticity.

Either adenosine or ATP seems to become a particularly important signalling molecule under pathological conditions, such as ischemia, when the extracellular concentration of either compound drastically rises. It is well known that adenosine exerts important neuroprotective effects during brain ischemic insults by activating adenosine A_1 receptors, which profoundly inhibit synaptic transmission and in particular the release of glutamate, known to contribute to ischemic damage. Under severe ischemia, a prolonged stimulation of both A_{2A} and A_3 receptors may be deleterious. The role of endogenously released ATP during cerebral ischemia is mainly deleterious, as found in our work and in the literature, since the application of P2 antagonists always results in a reduction in ischemic damage. The mechanisms underlying these effects are still unknown, but they can be correlated to the tonic excitatory action of endogenous ATP found in many brain regions (for example in the hippocampus and prefrontal cortex).

Purinergic signalling has an ancient phylogenetic origin. Expression of P2 receptors on cell membrane and responses to extracellular ATP are found in primitive prokaryotic species up to evolved animals and plants, suggesting an important and highly conserved role of extracellular purine nucleotides during evolution. Similarly, ontogenetic development in several species seems to involve purinergic signalling, especially during its first stages. Our observation that hMSCs,

an undifferentiated line of cells able to originate a number of different cell lineages (adipocytes, chondrocytes, osteoblasts, neurones), spontaneously release ATP into the extracellular space and express functional purinergic P2 receptors, which modulate different kinds of membrane currents, and that ATP, during the early stages of culture, inhibits proliferation indicates an autocrine/paracrine mechanism of action for extracellular ATP in modulating cell functions of undifferentiated stem cells at early developmental stages. Such effects may be important in brain neurogenesis during development and in responses to neurodegenerative stimuli.

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