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Effect of nitric oxide donors on extracellular ATP, ADP, and AMP catabolism in rat hippocampal synaptosomes

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ABSTRACT: Extracellular adenine nucleotides acting as signaling molecules are inactivated by hydrolysis catalyzed by ectonucleotidases. Adenosine triphosphate (ATP) diphosphohydrolase (apyrase, EC 3.6.1.5) and 5′-nucleotidase (EC 3.1.3.5) are involved in an enzymatic chain for the hydrolysis of ATP to adenosine in the synaptic cleft. In this study, we investigated the *in vitro* effect of nitric oxide (NO) donors on extracellular ATP, adenosine diphosphate (ADP), and adenosine monophosphate (AMP) catabolism in hippocampal synaptosomes of rats. We evaluated the effect of the incubation time on ATP, ADP, and AMP hydrolysis in the absence and in the presence of 1 mM sodium nitroprusside (SNP). The inhibitory effect of SNP increased with the incubation time and the maximal inhibition was observed after 180 min for both enzyme activities. The inhibition observed attained a maximum at 1 mM SNP for ATP, ADP, and AMP hydrolysis, with the enzyme activities being markedly reduced at this concentration of SNP. However, other NO donors tested, such as S-nitroso-N-acetyl-penicillamine and isosorbide dinitrate, did not affect the enzyme activities. The effect of the NO donor, SNP, on extracellular ATP and ADP catabolism was increased by the addition of the thiol glutathione but this effect was not observed on extracellular AMP catabolism. The results suggest that the increased production of NO could have a modulatory role on the ectonucleotidase activities. © 2001 Elsevier Science Inc.

KEY WORDS: ATPase, ADPase, Apyrase, NO, Ectonucleotidases, Sodium nitroprusside.

INTRODUCTION

The role of adenosine triphosphate (ATP) and adenosine in the modulation of synaptic transmission has been extensively studied in the central and peripheral nervous systems. ATP may function as a transient cellular signal or as a fast excitatory neurotransmitter at synapses between neurons, together with other neurotransmitters [1,13]. The participation of extracellular ATP on cell signaling systems seems to be mediated by two subclasses of P_2-purinoreceptors, P_{2x} and P_{2y} [30]. Furthermore, ATP may be used as a substrate for ecto-protein kinases, being involved in the extracellular phosphorylation of proteins [14–16].

Extracellular ATP and adenosine concentration could be modulated by a chain of ectonucleotidases [43]. Previous studies from our laboratory [4,31] have shown that the neurotransmitter, ATP, is hydrolyzed to adenosine monophosphate (AMP) in synaptosomes of the central and peripheral nervous system by the action of an ATP diphosphohydrolase (apyrase, ATPDase,EC 3.6.1.5). The AMP produced is then hydrolyzed to adenosine by the action of a 5′-nucleotidase (EC 3.1.3.5). Adenosine is an important and ubiquitous neuromodulator in the mammalian central nervous system and acts through the activation of P_1-purinoreceptors [42]. These two enzymes (apyrase and 5′-nucleotidase) have a dual function in controlling the availability of cell ligands (ATP, ADP, AMP, adenosine) for nucleotide or nucleoside receptors and the duration and extent of receptor activation [43]. Recently, it has been shown that ecto-apyrase is expressed in primary neurons and astrocyte cultures [38] and is widely distributed in cerebral cortex, hippocampus, cerebellum, and glial and endothelial cells [39]. Furthermore, Kegel et al. [20] have shown that an ecto-ATPase is co-expressed with an ecto-ATP diphosphohydrolase in rat brain.

Nitric oxide (NO) is a widespread and multifunctional biological messenger molecule which is synthesised from L-arginine in the nervous system by three NO synthase (NOS) isoforms (neuronal, endothelial and immunologic) [41]. NO may play a role in physiological neuronal functions such as long-term potentiation (LTP) as a retrograde messenger [34,25] and in the regulation of gene expression [41]. Furthermore, it can act as a potent vasodilator and an inhibitor of platelet aggregation [19,37] and, as has been reported, in the S-nitrosylation of proteins [2,29,36]. Conversely, NO may play a role not only in physiological functions but in a variety of disorders, because it impairs DNA replication, promotes oxidative damage and energy failure [19], and is involved in N-methyl-D-aspartate (NMDA) receptor-mediated neurotoxicity [11,37,40].

Because NO may have an important role in events related to synaptic plasticity, changes in ectonucleotidase activities have been shown to be altered in these situations [5,32], the objective of the present study was to investigate the *in vitro* effect of NO

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donors on the extracellular ATP, ADP, and AMP catabolism in synaptosomes from the hippocampus of adult rats.

MATERIALS AND METHODS

Materials

Nucleotides (ATP, ADP, AMP), Hepes, Trizma Base, EDTA, sodium nitroprusside (SNP), isosorbide dinitrate, glutathione (GSH), S-nitroso-N-acetyl-penicillamine (SNAP), and Percoll were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sucrose, ferricyanide, and ferrocyanide were obtained from Merck (Darmsdadt, Germany). Percoll was routinely filtered through millipore AP 15 pre-filters to remove aggregated and incompletely coated particles. All other reagents were of analytical grade.

Subcellular Fractionation

Adult male Wistar rats (age, 70–90 days; weight, 240–280 g), were housed under controlled conditions in a 12-h light/dark cycle at a constant temperature (23±2°C) and with free access to food and water. The animals were sacrificed by decapitation, and the brain was rapidly excised. After dissection, the hippocampus was gently homogenized in 5 vol. of an ice-cold medium consisting of 320 mM sucrose, 0.1 mM EDTA, and 5 mM Hepes, pH 7.5, with a motor driven Teflon-glass homogenizer. The synaptosomes were isolated as described by Nagy and Delgado-Escueta [27]. Briefly, 0.5 ml of crude mitochondrial fraction was mixed with 4 ml of an 8.5% Percoll solution and layered onto an isoosmotic Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosomal fractions were washed at 15,000 *g* for 20 min with the same ice-cold medium to remove contaminating Percoll. The synaptosomal pellet was resuspended to a final protein concentration of approximately 0.5 mg/ml. The material was prepared fresh daily and maintained at 0–4°C throughout preparation.

Enzyme Assays

The reaction medium used to assay ATP and ADP hydrolysis was essentially as described by Battastini et al. [3] and contained 5 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris-HCl buffer, pH 8.0 in a final vol. of 200 μl. The reaction medium used to assay extracellular AMP hydrolysis was essentially as described by Heymann et al. [18] and contained 10 mM $MgCl_2$, 0.1 M Tris-HCl, pH 7.5, and 0.15 mM sucrose in a final volume of 200 μl.

A preliminary set of experiments was performed to choose the adequate preincubation time and the concentration of NO donors. The enzyme preparation (10–20 μg protein) was added to the reaction mixture and preincubated for different times (0–180 min) at 37°C in the presence of 1 mM SNP. Controls were made without SNP. The effect of SNP was tested in the 0.01–2 mM range at 37°C with 180 min of preincubation. Under the same conditions we tested 1 mM SNAP, 1 mM isosorbide dinitrate, 1 mM ferricyanide, or ferrocyanide and 1 mM SNP plus 0.01-0.1 mM GSH as described for each experiment. All reactions were started by the addition of substrate (ATP, ADP, or AMP) to a final concentration of 1 mM and stopped by the addition of 200 μl 10% trichloroacetic acid. The samples were chilled on ice for 10 min and 100-μl samples were taken to assay released inorganic phosphate (Pi) using malachite green as the colorimetric reagent [9].

For all enzyme assays, incubation time (20 min) and protein concentrations were chosen in order to assure linearity of the reaction. Controls with the addition of enzymatic preparations after the addition of trichloroacetic acid were used to correct for non-enzymatic hydrolysis of the substrates. All samples were run in duplicate or triplicate and processed in the dark inside a sealed tube. Specific activity is expressed as nmol of Pi released per min per mg of protein at 37°C.

Protein Determination

Protein was assayed by the Coomassie Blue method according to Bradford [7] using bovine serum albumin as standard.

Statistical Analysis

The data obtained for the enzyme activities are presented as mean ± SEM and the number of experiments indicated. Data were analyzed statistically by one-way analysis of variance followed by the Duncan multiple range test, with the level of significance set at $p < 0.05$.

RESULTS

The ATP, ADP, and AMP catabolism by hippocampal synaptosomes exposed to different times of preincubation and to different SNP concentrations are shown in Figs. 1A,B. We observed a decrease in both activities in a time and concentration-dependent manner.

The effect of 1 mM SNP was significantly different after 120 min of preincubation for ADP hydrolysis and after 180 min of preincubation for both ATP and ADP hydrolysis (Fig. 1A). We also evaluated the activity of 5′-nucleotidase. AMP hydrolysis was also significantly decreased by the presence of 1 mM SNP after 180 min of preincubation (Fig. 1A). Thus, the complete ectonucleotidase pathway (ATP diphosphohydrolase plus 5′-nucleotidase) was affected by 1 mM SNP after 180 min of preincubation.

After 3 h of preincubation of the synaptosomal fraction with 0.01 mM SNP, we observed an inhibitory effect on extracellular ATP, ADP, and AMP catabolism (Fig. 1B). The nucleotide catabolism showed a decrease of 31% (ATP), 33% (ADP), and 24% (AMP), respectively in the presence of 1 mM SNP. This inhibition was statistically significant ($p < 0.05$) when compared to the control (no SNP addition). This parallelism in the inhibition of the ATP–ADP hydrolysis reinforces previous studies from our laboratory which demonstrated one enzyme (ATP diphosphohydrolase) hydrolyzing both substrates in the central nervous system.

In order to confirm if NO was indeed responsible for the inhibitory effect of SNP on extracellular ATP, ADP, and AMP catabolism we determined the effect of other NO donors (SNAP and isosorbide dinitrate). Based on the results obtained with SNP, we used 1 mM as the standard concentration to evaluate the effect of SNAP and isosorbide dinitrate. SNAP and isosorbide dinitrate, both at 1 mM, did not induce significant changes in ATP, ADP, and AMP catabolism (data not shown).

To evaluate the participation of ferricyanide and ferrocyanide (which are produced from SNP after NO release) in the inhibition produced by SNP, the effect of these molecules upon ATP, ADP, and AMP catabolism was tested. Ferricyanide and ferrocyanide did not cause significant changes in ATPase, ADPase, and AMPase activities when compared to the control (data not shown).

The possible participation of a mechanism of S-nitrosylation by NO as the first step in the inactivation of the enzymes involved in extracellular ATP, ADP, and AMP hydrolysis was investigated. GSH, an endogenous thiol, was used as a NO scavenger, *in vitro*. GSH, at 0.1 mM, increased the inhibition observed for ATP hydrolysis in the presence of 1mM SNP. Similar results were obtained for ADP hydrolysis in the presence of 0.01 and 0.1 mM GSH (Fig. 2). Incubation with GSH alone had no effect on both activities (results not shown). The inhibition of AMP hydrolysis

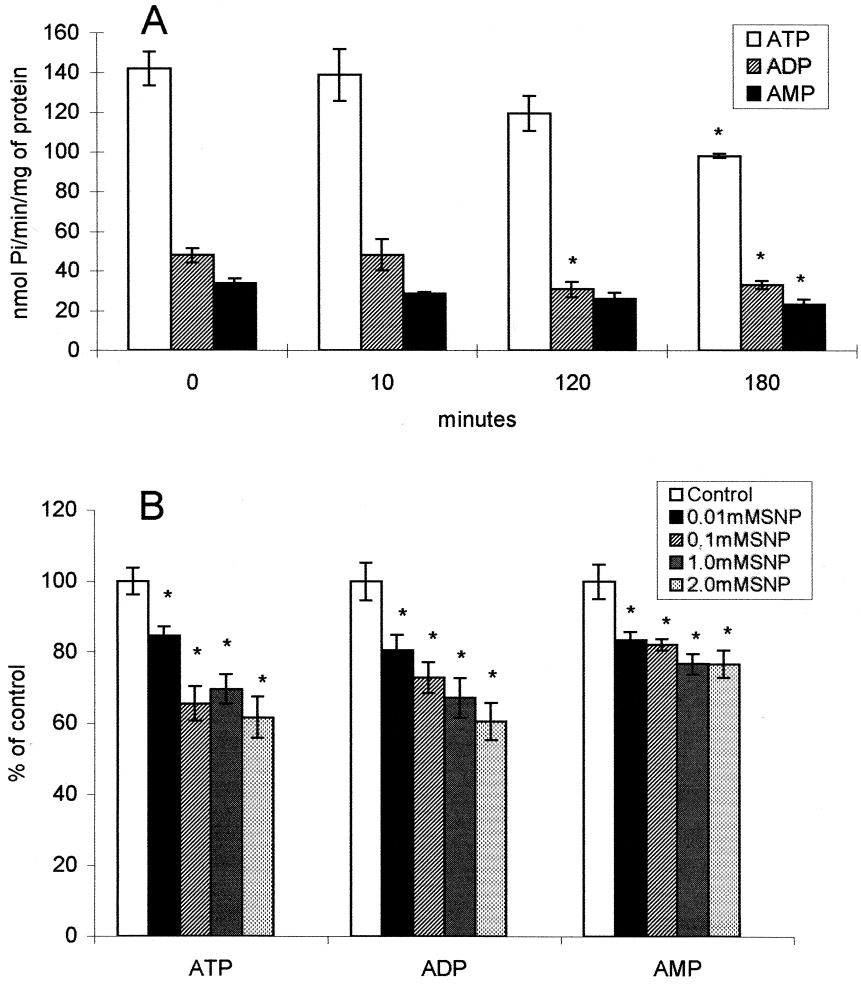

FIG. 1. *In vitro* effect of sodium nitroprusside (SNP) on extracellular adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) catabolism. (A) Synaptosomes were preincubated with 1 mM SNP during the indicated periods of time. Zero time represents the control (B) Synaptosomes were preincubated for 3 h with 0.01–2 mM SNP. The nucleotides were incubated in a final concentration of 1 mM. Bars represent the means ± SEM of at least three experiments. *Significantly different from control ($p < 0.05$, Duncan test).

promoted by SNP was not increased by the presence of GSH (Fig. 2).

DISCUSSION

In the present study we investigated the effect of NO donors on the enzyme activities involved in the cleavage of ATP to adenosine (ATP diphosphohydrolase and 5′-nucleotidase) in the synaptosomal fraction from the hippocampus of adult rats. This enzymatic chain of ectonucleotidases participates in a pathway that results in the control of the rate, amount and timing of ATP disappearance and adenosine formation in the synaptic cleft [4,44]. Adenosine has been shown to modulate synaptic plasticity [12] by activating inhibitory A_1 and facilitatory A_2 adenosine receptors, which modulate synaptic transmission [10]. Recently our group [5,6] has shown a decrease in ectonucleotidase activities in processes related to memory formation and, probably, to synaptic plasticity. Other conditions, such as epilepsy and neurite outgrowth, are also able to alter the ectonucleotidase activities [6,33]. Furthermore, it has been shown that the administration of NO donors (hydroxylamine and S-nitrosocysteine) to hippocampal slices facilitates the induction of LTP [24]. The results presented here show the occurrence of a significant inhibition of extracellular ATP, ADP, and AMP catabolism in the presence of 1 mM SNP after 3 h of preincubation, which could be related to an increase in ATP concentration and a decrease in adenosine production in the synaptic cleft. Based on these considerations, it is possible to speculate on the action of NO as one of the mechanisms involved in the inhibition of the ectonucleotidase pathway observed immediately posttraining [5].

Congeners of nitrogen monoxide (NO) are neuroprotective and/or neurodestructive structures [22]. The effects of these compounds are characterized by alternative redox states of NO, such as nitric oxide ($NO^{\bullet}$), nitrosyl (NO^{+}), and nitroxyl (NO^{-}) [2,22]. SNP, SNAP, and isosorbide dinitrate are known NO^{+} donors, but SNP can readily generate $NO^{\bullet}$ in a reductive activation with thiol ($NO^{+} + e^{-} \rightarrow NO^{\bullet}$). $NO^{\bullet}$, generated from NO donors or synthesized endogenously after NMDA receptor activation, can lead

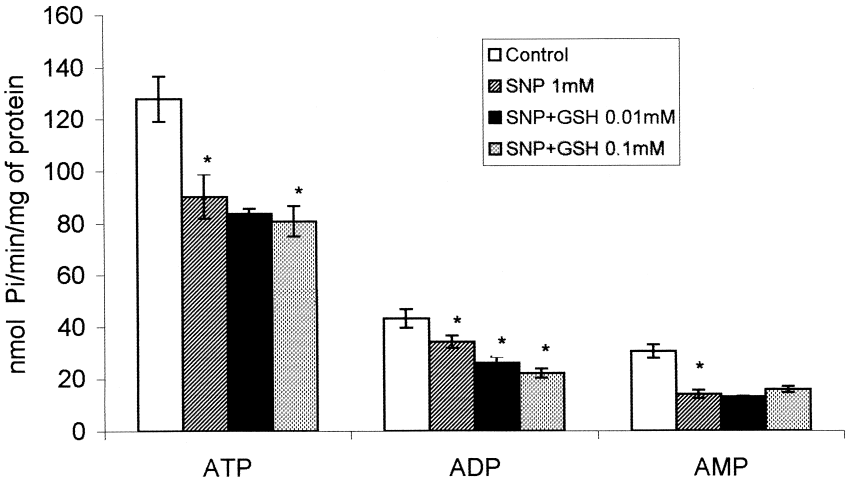

FIG. 2. Effect of glutathione on sodium nitroprusside (SNP)-induced inhibition of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) hydrolysis in hippocampal synaptosomes of rats. All experiments were performed using a preincubation time of 3 h and in the presence of 1 mM SNP. Control represents preincubation in the presence of SNP. The glutathione (GSH) preparation without SNP did not affect the enzyme activity. The nucleotides were incubated in a final concentration of 1 mM. Bars represent the means ± SEM of at least three experiments. *Significantly different from control ($p < 0.05$, Duncan test).

to neurotoxicity [10], while NO^+ can provide neuroprotection [23]. The $NO^\bullet$ mediated neurotoxicity is engendered at least in part by the reaction with superoxide anion ($O_2^{\bullet -}$), apparently leading to the formation of peroxynitrite ($ONOO^-$). In contrast to $NO^\bullet$, NO^+ groups do not react with superoxide anion [24]. These characteristics probably explain the lack of significant changes observed in the presence of other NO donors (SNAP and isosorbide dinitrate) when compared with the inhibitory effect of SNP on ATP, ADP, and AMP hydrolysis. As ferrocyanide and ferricyanide moieties did not affect either of the enzyme activities evaluated, ruling out the possibility of a molecular effect of these moieties, our results point to the effect of NO *per se*.

S-nitrosylation results from the transfer of a NO group to cysteine sulfhydryls on proteins. This occurrence is becoming increasingly recognized as a ubiquitous regulatory reaction comparable to phosphorylation [8]. S-nitrosylation was reported to affect the activity of a large number of membrane bound, cytosolic, and nuclear protein [2,8,23,26]. Biological activity can be controlled by protein S-nitrosylation [8,23]. S-nitrosylation may play an important role in many processes ranging from signal transduction, DNA repair, host defense, and blood pressure control to ion channel regulation and neurotransmission [8]. Moreover, under physiological conditions, S-nitrosylation is a favorable reaction that stabilizes NO in a uniquely bioactive form [36]. The inhibition of ATP/ADP hydrolysis (ATP diphosphohydrolase) and AMP hydrolysis (5′-nucleotidase) in the presence of SNP may result from interaction with NO, or with the congeners NO^+ and $NO^\bullet$.

The result showing that the effect of SNP was potentiated in the presence of thiol glutathione (Fig. 2) is an apparent discrepancy, but it has been recently reported that glutathione reacts *in vivo* to form S-nitrosoglutathione [23]. 5′-nucleotidase has a well-documented paradoxical effect with verapamil using $FeCl_2$/ascorbate as a free radical-generating system [21]. We may suggest that the RSNO, a nitrosothiol-generated compound, could act as an ATP diphosphohydrolase inhibitor, probably by S-nitrosylation. This effect was described previously for an ecto-enzyme involved in nucleotide hydrolysis (ecto- 5′-nucleotidase) from *Opossum* kidney cells [35] and in the present work, we observed a similar effect for ATP/ADP hydrolysis in synaptosomes from central nervous system. Conversely, we did not observe any change in the ecto-5′-nucleotidase inhibition promoted by SNP in the presence of GSH. Considering that the ATP/ADP hydrolysis is inhibited by SNP (a NO donor) and that the ATP diphosphohydrolase is one of the two enzymes involved in the degradation of the neurotransmitter ATP to adenosine, the observed inhibition by NO could promote an increase in ATP levels in the synaptic cleft. This fact could explain the neurotoxicity of ATP and may be one of the mechanisms involved in the brain damage. It is important to note that NO donors may also promote the release of purines from rat hippocampal slices [17]. Some effects of NO donors on ecto-nucleotidases are mediated through cyclic guanosine monophosphate [28] and we have no results to discharge this possibility.

In conclusion, in the present study, we suggest that NO species inhibit the enzymes involved in the catabolism of extracellular adenine nucleotides probably by S-nitrosylation. This raises the interesting possibility that NO may act as a modulator of the ATP metabolizing enzyme chain in processes related to synaptic plasticity.

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