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Acute Caffeine Treatment Increases Extracellular Nucleotide Hydrolysis from Rat Striatal and Hippocampal Synaptosomes

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The psychostimulant caffeine promotes behavioral effects such as hyperlocomotion, anxiety, and disruption of sleep by blockade of adenosine receptors. The availability of extracellular adenosine depends on its release by transporters or by the extracellular ATP catabolism performed by the ecto-nucleotidase pathway. This study verified the effect of caffeine on NTPDase 1 (ATP diphosphohydrolase) and 5′-nucleotidase of synaptosomes from hippocampus and striatum of rats. Caffeine and theophylline tested *in vitro* were unable to modify nucleotide hydrolysis. Caffeine chronically administered in the drinking water at 0.3 g/L or 1 g/L for 14 days failed to affect nucleotide hydrolysis. However, acute administration of caffeine (30 mg/kg, ip) produced an enhancement of ATP (50%) and ADP (32%) hydrolysis in synaptosomes of hippocampus and striatum, respectively. This activation of ATP and ADP hydrolysis after acute treatment suggests a compensatory effect to increase adenosine levels and counteract the antagonist action of caffeine.

KEY WORDS: Adenosine; caffeine; ecto-nucleotidases; NTPDases; 5′-nucleotidase; theophylline.

INTRODUCTION

Caffeine is a psychoactive substance present in several beverages and food. It has been considered that the caffeine is a potential model drug of abuse (1,2). The biochemical mechanism that underlies the actions of caffeine in relevant concentrations for human consumption is blockade of adenosine A_1 and A_{2A} receptors (1). Adenosine A_1 receptors modulate synaptic activity by inhibiting the release of several neurotransmitters and are highly distributed in the central nervous system (CNS), with particularly high concentrations in the hippocampus, cortex, cerebellum, and thalamus (3,4). Adenosine A_{2A} receptors are particularly expressed to the striatum, where they interact with dopamine D_2 receptors (3,4). Blockade of A_1 and A_{2A} receptors is of particular interest because one of the actions of adenosine in the CNS is related with the regulation of release of several neurotransmitters. The interaction of the A_1 and A_{2A} receptors with the dopaminergic receptors D_1 and D_2, respectively, is a potential therapeutic target in disorders such as Parkinson's disease and schizophrenia (5,6)

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The effects of caffeine and theophylline on adenosine receptors have been widely studied in different species and tissues, where changes in expression and binding of adenosine A_1 and A_2 receptors have been associated with administration of these substances (7,8). The intake of caffeine and its metabolite, theophylline, has been suggested to modulate adenosine A_1 receptors, promoting an upregulation in rat brain that is not always dependent on mRNA changes (9). However, in neonatal life and pregnancy, a downregulation of these receptors has been shown (8). Svenningsson et al. (10) showed a downregulation in adenosine A_{2A} receptors levels and their mRNA in rostral parts of striatum, but an increased expression of adenosine A_1 receptors mRNA in the lateral amygdala after chronic and acute treatment with caffeine, respectively.

The physiological activation of adenosine receptors is performed by extracellular adenosine derived from the bidirectional transporter system or by the extracellular catabolism of adenine nucleotides (3). The different sources of adenosine have been related to selective activation of the adenosine receptors, because it has been hypothesized that the adenosine released as such mainly activates inhibitory A_1 receptors, whereas A_{2A} receptors would be mainly activated by adenosine provided from the catabolism of nucleotides (11). In recent years, studies have demonstrated that members of several families of ecto-nucleotidases can contribute to the extracellular hydrolysis of nucleotides (for review, see 12). Nucleoside 5′-tri–and diphosphates can be hydrolyzed by members of the E-NTPDase family (ectonucleoside triphosphate diphosphohydrolase family), whereas nucleosides 5′-monophosphates are mainly subjected to hydrolysis by ecto-5′-nucleotidase (12,13), producing the respective nucleoside. These enzymes promote the complete ATP hydrolysis to adenosine and may play an important role in physiological and pathological conditions (14), controlling the activation and the availability of ligands to nucleotide and nucleoside receptors (12).

There are few studies on the xanthine effects on the ATPase and ecto-nucleotidases. Theophylline caused increase of Ca^{2+},Mg^{2+}-ATPase activity from crude synaptosomal membranes in hippocampal region (15). Theophylline and caffeine, in concentrations of 1 mM, inhibited Na^{+},K^{+}-ATPase (EC 3.6.1.38) activity in rat pancreatic islets (16). Studies showed a significant inhibition of rabbit renal 5′-nucleotidase (EC 3.1.3.5), cyclic nucleotide phosphodiesterase (EC 3.1.4.17), and adenosine deaminase (EC 3.5.4.4) by theophylline, but only at millimolar concentration (17). Furthermore, a significant inhibition of a 5′-nucleotidase from rat brain by different xanthine derivatives has been observed (18–20).

Considering that this pathway has been postulated to be the main source of adenosine at the synaptic level (21), we investigated ATP, ADP, and AMP hydrolysis in hippocampal and striatal synaptosomes obtained from rats submitted to chronic and acute treatment with caffeine. In addition, we verified the influence of caffeine and theophylline on this pathway *in vitro* using synaptosomes from hippocampus and striatum of rats.

EXPERIMENTAL PROCEDURE

Animals and Treatments. A total of 70 male Wistar rats (205 ± 37 g, age 60–80 days) from our breeding stock were used in the study. Animals were housed in cages with food *ad libitum* under a 12-h light/12-h dark cycle (light on at 07:00 AM) at a temperature of 25 ± 1°C. In the acute caffeine treatment, animals received a single intraperitoneal injection of 30 mg/kg of caffeine (1 mL/kg, dissolved in 0.9% NaCl solution) at 60 min before the sacrifice. Controls received a single injection of saline. In the chronic caffeine treatment, the drinking solutions with 0.3 g/L or 1 g/L of caffeine were exchanged every third day to fresh solution. Control animals received ordinary tap water. The daily intake was measured in all groups.

Procedures for the care and use of animals were adopted according to the regulations published by the Brazilian Society for Neuroscience and Behavior (SBNeC).

Synaptosomes Preparation. Animals were sacrificed by decapitation, and the brain structures were removed to an ice-cold medium solution (320 mM sucrose, 5 mM HEPES, pH 7.5, and 0.1 mM EDTA). Structures were gently homogenized in 5 volumes of ice-cold medium solution with a motor-driven Teflon glass homogenizer. The synaptosomes were isolated as described previously by Nagy and Delgado-Escueta (22). Briefly, 0.5 ml of the 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 then washed twice at 15,000 × *g* for 20 min with the same ice-cold medium to remove the contaminating Percoll. The synaptosome pellet was resuspended to a final protein concentration of approximately 0.5 mg/ml. To ascertain that the extracellular nucleotides hydrolysis was provided by intact synaptosomes, a continuous monitoring was made by determination of LDH activity. 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 previously (23) and contained 5.0 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 volume of 200 μl. The reaction medium used to assay 5′-nucleotidase activity contained 10 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.5, and 0.15 M sucrose in a final volume of 200 μl (24). For *in vitro* assays, caffeine or theophylline, at the concentrations of 10, 100, 500, and 1000 μM, were added to the reaction mixture. The synaptosomal fractions (10–20 μg protein) were added to the reaction mixture, preincubated for 10 min, and incubated for 20 min at 37°C. The reaction was initiated by the addition of 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 samples were taken for the assay of released inorganic phosphate (Pi) (25). Incubation times and protein concentration were chosen to ensure the linearity of the reaction. Controls with the addition of the enzyme preparation after addition of trichloroacetic acid were used to correct nonenzymatic hydrolysis of the substrates. All samples were run in triplicate. Protein was measured by the Coomassie blue method (26), using bovine serum albumin as standard.

Statistical Analysis. The data are expressed as mean ± SD and were analyzed by Student's t test or by one-way ANOVA, followed by the Duncan test as post hoc test, considering a level of significance of 5%.

RESULTS

Effect of Acute Caffeine Treatment on Ecto-Nucleotidase Activities. As shown in Fig. 1A, acute treatment with 30 mg/kg caffeine induced a significant increase of ATP hydrolysis in synaptosomes from hippocampus of rats (50% $P < .05$), but no significant changes in ADP and AMP hydrolysis in the same condition. In synaptosomes from striatum, acute caffeine treatment produced a significant increase in ADP hydrolysis (32%; $P < .05$), but ATP and AMP hydrolysis were not altered by acute treatment with caffeine (Fig. 1B).

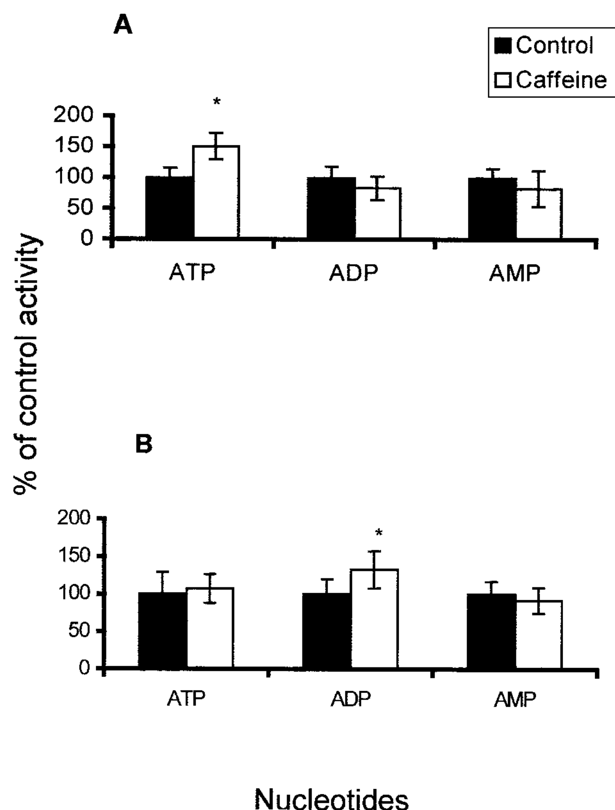

Fig. 1. Effect of acute caffeine treatment (30 mg/kg, ip) on ATP, ADP, and AMP hydrolysis from hippocampal **(A)** and striatal **(B)** synaptosomes. Bars represent mean ± SD. n per group was 5–7 animals. In synaptosomes from hippocampus, control activities were 114.29 ± 18.26, 55.93 ± 10.14, and 34.30 ± 5.03 nmol min^{-1} mg^{-1} of protein for ATP, ADP, and AMP hydrolysis, respectively. In synaptosomes from striatum, control activities were 144.43 ± 41.64, 49.40 ± 9.83, and 25.12 ± 4.34 nM Pi min^{-1}/mg^{-1} of protein for ATP, ADP, and AMP hydrolysis, respectively. *Significantly different from the respective control group (Student's t test, $P < .05$).

Effect of Chronic Caffeine Treatment on Ecto-nucleotidase Activities. The animals were submitted to chronic treatment with caffeine at 0.3 g/L and 1 g/L in their drinking water for 14 days. Caffeine consumption was estimated from the loss of water from the drinking bottles. Daily water intake in all groups of rats (control and caffeine-treated with 0.3 g/L or 1 g/L) was not significantly different (Table I). There were no differences in the ATP, ADP, and AMP hydrolysis in synaptosomes from the hippocampus (Fig. 2A) and striatum (Fig. 2B) of rats treated for 14 days in both caffeine concentrations used.

Effect of Caffeine and Theophylline on Nucleotide Hydrolysis in Vitro. Caffeine and theophylline, at the concentrations tested (10, 100, 500, and 1000 μM), were unable to modify ATP, ADP, and AMP hydrolysis when compared to the control (no caffeine or theophylline added) in synaptosomes from hippocampus and striatum of rats (data not shown).

DISCUSSION

The aim of this study was to verify the effect of acute and chronic treatment with caffeine on the ecto-nucleotidase pathway in synaptosomes from hippocampus and striatum of rats. Furthermore, *in vitro* studies have been conducted to provide more elements about the direct interaction of caffeine and theophylline and the enzymes involved in nucleotide hydrolysis. The ecto-5′-nucleotidase (EC 3.1.3.5) and NTPDase (EC 3.6.1.5) activities from hippocampus and striatum of adult rats did not demonstrate significant changes in the presence of theophylline and caffeine in the concentrations tested.

Table I. Caffeine Consumption in Chronic Treatment

Groups (14 days)	Fluid consumption (ml/day/kg)	Dose of caffeine (mg/day/kg)
Control	149.5 ± 38.2	0
0.3 mg/ml	159.9 ± 25.2	48 ± 7.6
1 mg/ml	164.4 ± 26.8	164.4 ± 26.8

Note: Values are expressed as means ± SD.

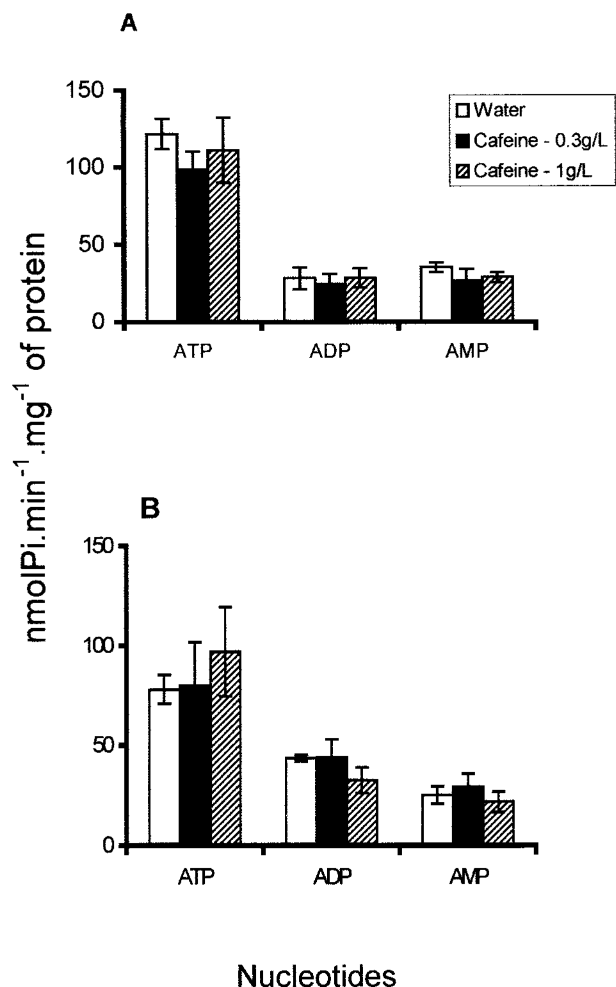

Fig. 2. Effect of chronic caffeine treatment (0.3 or 1 g/L in the drinking water, during 14 days) on the ATP, ADP, and AMP hydrolysis in hippocampal **(A)** and striatal **(B)** synaptosomes of rats. Bars represent mean ± SD. n per group was 5–7 animals.

Studies about the differences in acute and chronic exposure to adenosine ligands showed that the effects of acute administration of a particular ligand can be opposite to its chronic effects, which could be due to differences between the periods of treatment, caffeine doses, administration methods, animal gender and age (1,8,27). When we tested the effect of acute treatment of caffeine (30 mg/kg, ip) on the ecto-nucleotidase pathway, we observed an increase in ATP and ADP hydrolysis in synaptosomes from hippocampus and striatum, respectively. These results suggest that caffeine, at acute doses, may play a modulatory role on the ecto-nucleotidase pathway, indirectly modulating the availability of adenine nucleotides and, consequently, adenosine levels in the synaptic cleft. The hippocampus presents a high density of A_1 receptors, and the antagonist actions of caffeine promote a decrease in the inhibitory tonus exerted by adenosine on these receptors (4). Thus it is possible to suggest that caffeine could impair the neuromodulatory actions of adenosine, leading to an increase of neurotransmitter release, including ATP, because it is coreleased with several neurotransmitters (28). Consequently, an increase of ATP and ADP hydrolysis by the ecto-nucleotidase pathway may be a compensatory mechanism to increase adenosine levels and counteract the antagonist action of caffeine.

Furthermore, there is a different expression of adenosine receptors in hippocampus and striatum. The different effects induced by caffeine on nucleotide hydrolysis in these structures could indicate a functional compartmentalization of ecto-nucleotidases. It has been shown that an ecto-ATPase (NTPDase 2) is co-expressed with an ecto-ATP diphosphohydrolase (NT-PDase 1) in the rat brain (29). Furthermore, studies have demonstrated that the ecto-apyrase and ecto-AT-Pase may be differently distributed with specialized and different functions in different regions of the same tissue (30). The differences observed in ATP and ADP hydrolysis in synaptosomes from hippocampus and striatum suggest that the two related extracellular nucleotide-hydrolyzing enzymes may be involved in the effects observed. The increase in ATP hydrolysis in synaptosomes from hippocampus might involve an ecto-ATPase (NTPDase 2), whereas the increase in ADP hydrolysis in synaptosomes from striatum may be related to an ecto-apyrase.

It has been shown that systemic administration of caffeine can preferentially increase extracellular levels of dopamine and glutamate using *in vivo* microdialysis in rats (31). Studies have observed that glutamate can stimulate ADP and AMP hydrolysis in cultured neuronal cells and increase ATP hydrolysis in hippocampal slices (32,33). It is possible to suggest that the acute administration of caffeine can contribute to an increase in glutamate levels, which could exert a modulatory effect on ecto-nucleotidase activities. In this condition, the ecto-nucleotidase pathway could be responding to the glutamate increase in relation to balance of extracellular ATP, ADP, and adenosine levels in hippocampus and striatum of rats. In contrast, the absence of these effects in chronic treatment could be due to tolerance development and the reestablishment of the inhibitory tonus exerted by adenosine on the neurotransmitters release, such as glutamate.

Chronic treatment with caffeine is mainly associated with an upregulation of adenosine A_1 receptor (27). However, other studies pointed out a downregu-

lation or absence of effect on the A_1 receptor after long-term caffeine exposure (8,34). Léon et al. (8) showed a downregulation of A_1 receptors after chronic caffeine intake (1 g/L for 14 days) and deduced that the antagonism of A_1 receptors by caffeine can increase extracellular adenosine levels and promote downregulation by an excess of the agonist. This is supported by studies that demonstrated that A_1 receptors antagonists promote increase of extracellular adenosine levels (35). However, there are few studies exploring the effects of caffeine on ecto-nucleotidase activities (1,21). In our investigation, the administration of 0.3 g/L (equivalent dose of normal human consumption) and 1 g/L caffeine in the drinking water during 14 days did not promote any effect on nucleotide hydrolysis in synaptosomal fractions. Animals submitted to long-term caffeine treatment develop tolerance, which is possibly related to upregulation of A_1 receptors (8,36). Svenningsson et al. (10) showed that oral administration of caffeine (0.3 g/L for 14 days) leads to development of tolerance to the stimulatory effect of a challenge with caffeine. These results suggests that the modulation of nucleotide hydrolysis is not relevant for the mechanisms of tolerance development to caffeine, because there are no significant changes in nucleotide hydrolysis after long-term intake of caffeine.

In conclusion, we have shown that acute administration of caffeine promoted an enhancement of ATP and ADP hydrolysis in synaptosomes of hippocampus and striatum, respectively. In addition, chronic caffeine exposure was unable to modify NTPDase and 5′-nucleotidase activities. Thus, it seems that high acute concentrations of caffeine can modulate the ecto-nucleotidase pathway, which could produce an increase in adenosine levels to counteract the antagonist actions of caffeine.

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