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Increase of nucleotidase activities in rat blood serum after a single convulsive injection of pentylenetetrazol

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Abstract

Adenosine has been shown to be a major regulator in convulsive disorders exerting its anticonvulsant effects on various seizure models. The ectonucleotidase pathway is an important metabolic source of extracellular adenosine. In this study, we evaluated ATP, ADP and AMP hydrolysis in rat serum after a single convulsive injection of pentylenetetrazol (PTZ). The animals were sacrificed at 5 and 30 min, 1, 5, 12, 24 and 48 h after an intraperitoneal injection of PTZ (60 mg/kg). ATP, ADP and AMP hydrolysis by rat blood serum were significantly increased (40–50%) until 24 h after PTZ injection. There were no significant differences in the nucleotide hydrolysis when the in vitro effect of different concentrations of PTZ was analyzed. Changes in nucleotide hydrolysis observed after acute administration of PTZ could not be attributed to phosphodiesterase activity since PTZ-treated rats did not demonstrate significant differences in the hydrolysis of the substrate marker of this enzyme when compared with control rats. These results suggest that the stimulation of the nucleotidase pathway may play an important role in attenuating seizure activity.

Keywords: Adenosine; Nucleotidases; Epilepsy; Seizures; ATP diphosphohydrolase; 5′-Nucleotidase

1. Introduction

Adenosine is an endogenous neuromodulator that possess anticonvulsant and neuroprotective properties (Kostopoulos, 1988). Synaptic adenosine levels increase during periods of increased metabolic demand, such as those that exist during seizures (During and Spencer, 1992; Berman et al., 2000). Adenosine and its analogues depress neuronal activity in the central nervous system by decreasing membrane excitability and/or neurotransmitter release (Phillips and Wu, 1981). This depressant effect has been attributed to the activation of A_1 adenosine receptors (Dragunow, 1988). Moreover, it was previously demonstrated that adenosine and adenosine A_1 receptor agonists significantly protect against seizures induced by acute pentylenetetrazol (PTZ) administration (Malhotra and Gupta, 1997).

PTZ is a commonly used proconvulsant, acting via the $GABA_A$ receptor complex (Olsen, 1981) and by altering the potassium permeability of the cell membrane via a voltage-dependent mechanism (Madeja et al., 1996). PTZ-induced generalized convulsions are associated with a significant increase in A_1 adenosine receptors (Pagonopoulou et al., 1993) and these changes precede or coincide with an increase in PTZ-seizure latency (Angelatou et al., 1990).

Biochemical studies have established that adenine nucleotides are thought to be an important potential source of extracellular adenosine (Dunwiddie et al., 1997; Cunha, 2001). Once released, these adenine nucleotides are metabolized and rapidly converted to adenosine through the action of ecto-enzymes (Zimmer-

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mann, 1996; Bonan et al., 1998). It has been demonstrated that in the central nervous system, the neurotransmitter ATP is hydrolyzed to adenosine by the conjugated action of ecto-nucleotidases, which includes an ATP diphosphohydrolase and a 5′-nucleotidase (Battasttini et al., 1991; Bonan et al., 1998). Furthermore, the 5′-nucleotide phosphodiesterases (PDEase, NPPase, EC 3.1.4.1) are enzymes that releases mononucleoside-5′-monophosphate from the 3′-OH terminal of the nucleotides. *p*-Nitrophenyl-5′-thymidine-monophosphate (*p*-Nph-5′-TMP) has been used as an artificial substrate for 5′-nucleotide phosphodiesterase, generating *p*-nitrophenol. These enzymes are known to hydrolyze not only ATP and ADP but also UDP-galactose, NAD, DNA and RNA (Sakura et al., 1998).

Although it is well established that the breakdown of ATP is mediated by the membrane-bound ectonucleotidases, recent studies indicate that soluble nucleotidases, probably released from sympathetic nerves, were also involved in ATP breakdown to adenosine (Todorov et al., 1997; Yegutkin et al., 2000). The release of specific nucleotidases may represent a novel mechanism for terminating the actions of the neurotransmitter ATP. Considering that soluble nucleotidases can act together with ectonucleotidases to produce adenosine and that this last structure plays an important role in the modulation of seizures, we investigated the effect of acute seizures induced by PTZ on the soluble enzymes that could be acting in ATP, ADP and AMP hydrolysis to adenosine in rat blood serum.

2. Material and methods

2.1. Pentylenetetrazol treatment

Female Wistar rats, 60–90 days old and weighing 150–250 g, were used. Animals received a singe convulsive injection of PTZ (60 mg/kg, i.p. dissolved in 0.9% saline). Control animals were injected with the same volume of saline. The animals were sacrificed by decapitation either 5 and 30 min 1, 5, 12, 24 or 48 h after the injection of PTZ or saline.

2.2. Isolation of blood serum fraction

Blood was drawn after the decapitation of female Wistar rats (approximately 60 days old). Blood samples were centrifuged in plastic tubes for 5 min at 5000 × *g* at 20 °C before freezing serum at 0 °C. Serum was used immediately in experiments.

2.3. Measurement of ATP and ADP hydrolysis

ATP and ADP hydrolysis was determined using a modification of the method described by Yegutkin (1997). The reaction mixture containing ADP or ATP as a substrate, in 112.5 mM Tris–HCl, pH 8.0, was incubated with 1.0–1.5 mg serum protein at 37 °C in a final volume of 0.2 ml. The reaction was stopped by the addition of 0.2 ml 10% TCA. Incubation times and protein concentration were chosen to ensure the linearity of the reaction (results not shown) and absorbance was measured at 630 nm. Inorganic phosphate (Pi) released was determined as previously described by Chan et al. (1986). Controls to correct for non-enzymatic hydrolysis were performed by adding the serum after the reaction was stopped with TCA. All samples were assayed in triplicate. Enzyme activities were generally expressed as nanomoles of Pi released per min per milligram of protein.

2.4. Experiments performed in vitro

ATP and ADP hydrolysis was determined using a modification of the method described by Yegutkin (1997). To evaluate the effect of the drug on the nucleotide hydrolysis, the in vitro experiments were performed using different concentrations of PTZ (in the range of 0.05–20 mM) in presence of ADP or ATP as a substrate, 112.5 mM Tris–HCl, pH 8.0 in rat blood serum. The control sample was performed using saline solution instead of PTZ. All the others procedures were the same described above in Section 2.3.

2.5. Measurement of AMP hydrolysis

The reaction mixture containing AMP as a substrate in 100 mM Tris–HCl, pH 7.5, was incubated with 1.0 mg to 1.5 mg protein serum at 37 °C in a final volume of 0.2 ml. Controls to correct for non-enzymatic hydrolysis were performed by adding the serum after the reaction was stopped with TCA. All samples were assayed in triplicate. Enzyme activities were generally expressed as nanomoles of Pi released per min per milligram of protein. All others procedures were the same as for ATP and ADP hydrolysis, as described above.

2.6. Measurement of p-Nph-5′-TMP hydrolysis

p-Nitrophenil-5′-thymidine-monophosphate (*p*-Nph-5′-TMP) hydrolysis was determined essentially as described by Sakura et al. (1998). The reaction mixture containing *p*-Nph-5′-TMP as a substrate in 100 mM Tris–HCl, pH 8.9, was incubated with 1.0–1.5 mg serum protein at 37 °C for 8 min in a final volume of 0.2 ml. The reaction was stopped by the addition of 0.2 ml NaOH 0.2 N. Incubation times and protein concentrations were chosen to ensure the linearity of the reaction (results not shown). The amount of *p*-nitrophenol was measured at 400 nm. Controls to correct for

non-enzymatic hydrolysis were performed by adding the serum after the reaction was stopped with NaOH. All samples were assayed in triplicate. Enzyme activities were generally expressed as nanomoles (nmol) of *p*-nitrophenol released per min per milligram of protein.

2.7. Lactate dehydrogenase (LDH) activity

The LDH activity was determined in the serum of PTZ-treated animals and control animals using a commercial kit (Kinetic Method Labtest Diagnóstica, MG-Brazil). The absorbance was measured at 340 nm.

2.8. Protein determination

Protein was determined by the Coomassie Blue method, according to Bradford (1976) using bovine serum albumin as standard.

2.9. Statistical analysis

The data obtained are expressed as mean ± S.D. of at least four animals. The results were analyzed statistically by Student's *t*-test. A P value of less than 0.05 was considered to represent a significant difference.

3. Results

In order to evaluate the effect of acute seizures induced by PTZ on the ATPase and ADPase activities, ATP and ADP hydrolysis was measured in the blood serum of rats sacrificed at different times after the PTZ injection. There was a significant increase of 51 ± 0.028% ($n = 4$) in ATP hydrolysis (Fig. 1A) and 50 ± 0.022% ($n = 4$) in ADP hydrolysis (Fig. 1B) at 5 min after the PTZ-treatment. At 30 min after PTZ-treatment, ATP and ADP hydrolysis increased by 50 ± 0.018% ($n = 4$) and 56 ± 0.024% ($n = 4$), respectively, in relation to controls (Fig. 1A and B). Serum ATPase and ADPase activities of rats killed 1 h after the PTZ injection, significantly increased 44 ± 0.025% ($n = 4$) and 45 ± 0.017% ($n = 4$), respectively, in relation to control animals. In addition, 5 h after the PTZ injection, the enzyme activity was increased by 54 ± 0.027% ($n = 4$) for ATP (Fig. 1A) and by 56 ± 0.011% ($n = 4$) for ADP (Fig. 1B) when compared with control animals. This increase reached maximum activation (69 ± 0.018%; $n = 4$ and 70 ± 0.023%; $n = 4$ for ATP and ADP hydrolysis, respectively) at 12 h after the treatment with PTZ, but the specific activity values of the enzyme decreased in relation to the other times tested. This decrease in enzyme activity was also observed 24 h after PTZ injection, but the ATP and ADP hydrolysis remained significantly increased 55 ± 0.020% ($n = 4$) and 54 ± 0.031% ($n = 4$), respectively, in relation to the control (Fig. 1A and B). At 48 h after PTZ-treatment, no significant difference was observed in ATP and ADP hydrolysis when compared with controls (Fig. 1A and B). Considering that the observed changes in ATPase and ADPase activities could be attributed to the effect of the drug itself rather than to the acute seizures, experiments to evaluate the effect of the drug on the nucleotide hydrolysis were realized in vitro using crescent concentrations of PTZ (in the range of 0.05–20 mM). Results did not reveal any statistically significant alterations in the ATP and ADP hydrolysis, in vitro, in the presence of PTZ, suggesting strongly that the increased nucleotides hydrolysis is induced by seizures and not by the drug itself (Fig. 2). It is important to note the parallel effect on ATP and ADP hydrolysis.

The results regarding the 5′-nucleotidase showed a similar profile when compared with ATP and ADP hydrolysis. Significant increase in 5′-nucleotidase activity were observed, when compared with the respective control group, at 5 min (33 ± 0.0046%; $n = 4$), 30 min (48 ± 0.0048%; $n = 4$), 60 min (50 ± 0.010%; $n = 4$), 12 h (45 ± 0.058%; $n = 4$) and 24 h (44 ± 0.032%; $n = 4$) after the acute seizures induced by PTZ injection (Fig. 3). However, at 48 h after PTZ-treatment, no significant difference was observed in AMP hydrolysis when compared with the control group (Fig. 3). Then, the AMP hydrolysis in this model changes exactly as the ATP–ADP hydrolysis.

Considering that a phosphodiesterase enzyme is expressed in blood serum (Sakura et al., 1998) and also can act in ATP and ADP hydrolysis, we evaluated the activity of this enzyme in the serum of rats treated with PTZ or saline using *p*-Nph-5′-TMP, an artificial substrate (marker for the phosphodiesterase activity).

Fig. 4 demonstrates that there is a phosphodiesterase activity in the rat blood serum, but that this activity is not significantly changed in PTZ-treated rats when compared with control rats at any time tested.

In order to investigate the role of the PTZ treatment in cellular disruption we measured the activity of the cytosolic enzyme, LDH, a marker of tissue damage, in rat blood serum. There were no significant differences in LDH activity in the serum of PTZ-treated rats when compared with control rats (data not shown). These results indicate that the increase in nucleotide hydrolysis can not be attributed to cellular breakdown.

4. Discussion

Previous studies have shown an increase in adenosine release and metabolism during pre-seizure and seizure activity induced by the administration of the proconvulsants, such as PTZ (Berman et al., 2000). Adenosine and the adenosine A_1 receptor agonist demonstrate

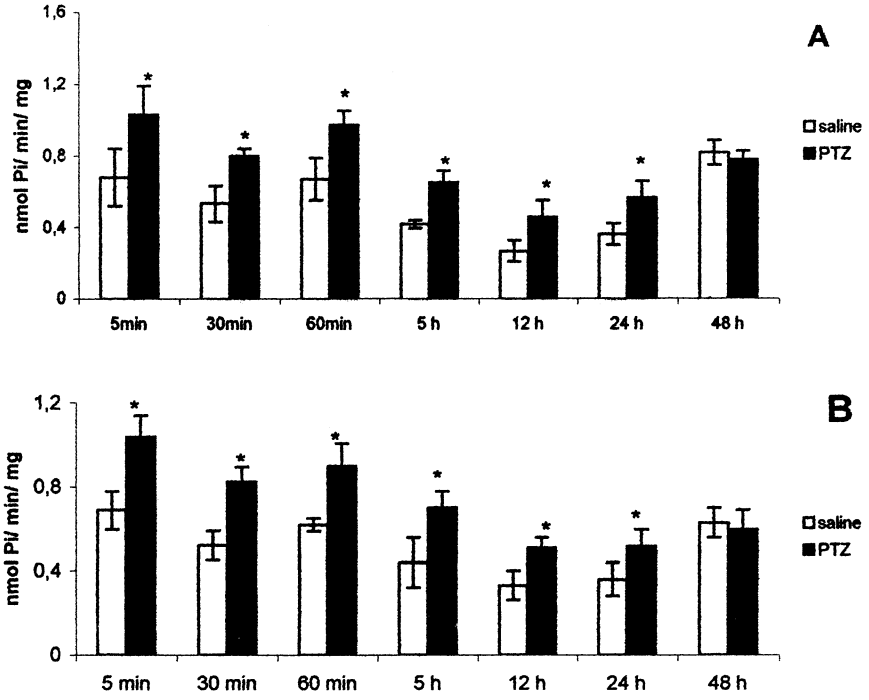

Fig. 1. Effects of seizures induced by acute administration of PTZ (60 mg/kg, i.p.) on ATP (A) and ADP (B) hydrolysis in rat blood serum at different times after the induction of seizures. Bars represent mean ± S.D. of at least four animals. *PTZ-treated group significantly different from control group ($P < 0.05$, Student's t-test).

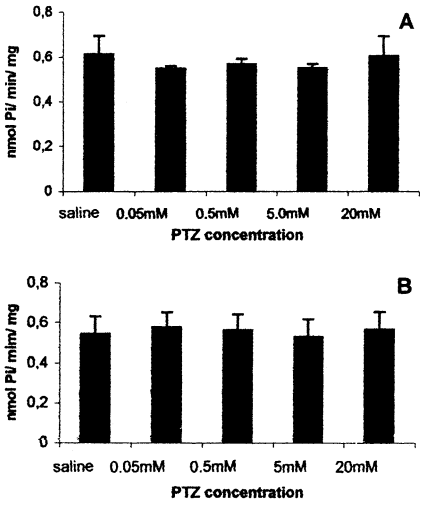

Fig. 2. Effect of increasing concentrations of PTZ (0.05–20 mM) on ATP (A) and ADP (2) hydrolysis in rat blood serum, in vitro. Bars represent mean ± S.D. of at least four experiments.

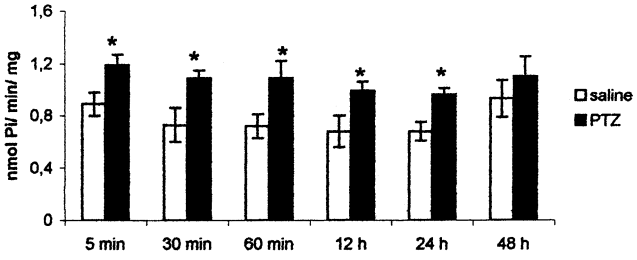

Fig. 3. Effects of seizures induced by acute administration of PTZ (60 mg/kg, i.p.) on 5′-nucleotidase activity from rat blood serum at different times after the induction of seizures. Bars represent mean ± S.D. of at least four animals. *AMP hydrolysis in treated-group significantly different from control group ($P < 0.05$, Student's t-test).

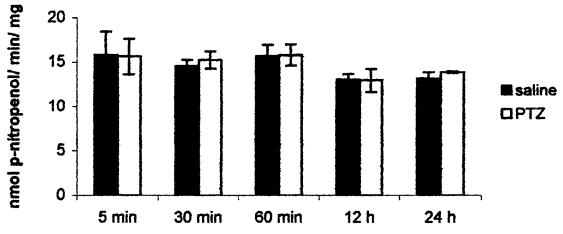

Fig. 4. Effects of seizures induced by acute administration of PTZ (60 mg/kg, i.p.) on phosphodiesterase activity in rat blood serum at different times after the induction of seizures. Bars represent mean ± S.D. of at least four animals.

significant protection against acute PTZ-induced seizures (Malhotra and Gupta, 1997). Moreover, reduction of extracellular adenosine formation by an injection of an ecto-5′-nucleotidase inhibitor, produce generalized seizures (Zhang et al., 1992). In addition, single and repeated PTZ-induced convulsions are associated with significant increase in A_1 adenosine receptors in different brain areas (Angelatou et al., 1990) and this upregulation of A_1 adenosine receptors was observed within 1 h following seizures (Pagonopoulou and Angelatou, 1998), 24 h after seizures (Pagonopoulou et al., 1993) and remained for at least 14 days after the

PTZ-induced seizures (Angelatou et al., 1990). Additionally, stroke and transient ischemic attacks are associated with a rapid increase in circulating plasma adenosine concentration in man, detectable in the peripheral vein up to 15 days after the acute event (Pasini et al., 2000).

Taken together, the protector effect of adenosine, the effect of A_1 adenosine receptor agonist and the convulsant action of an ecto-5′-nucleotidase inhibitor, further support the role of endogenous adenosine as a neuroprotective and anticonvulsant structure. Thus, it is important to consider the pathway involved in the extracellular adenosine production.

Recently, we demonstrated an increase in ATP diphosphohydrolase and 5′-nucleotidase activities in synaptosomes of the hippocampus and cerebral cortex of rats at different periods after status epilepticus induced in two different animal models of epilepsy (Bonan et al., 2000). Therefore we investigated the effect of acute seizures induced by PTZ on the enzymes that could be acting in ATP, ADP and AMP hydrolysis to adenosine in rat blood serum, at different times after the induction of seizures.

The present study demonstrates a significant increase in ATP, ADP (Fig. 1A and B) and AMP (Fig. 3) hydrolysis by serum of rats and these results suggest an effect promoted by the seizures, since that in vitro there were not significant changes an ATP and ADP hydrolysis in the presence of PTZ (Fig. 2). However, the phosphodiesterase activity (evaluated using a substrate marker for this enzyme), which could also be acting in ATP and ADP hydrolysis in rat blood serum, did not present any statistically significant increase when compared treated animals with control animals (Fig. 4). As we had a parallel increase in ATP–ADP hydrolysis and an increase in AMP hydrolysis but we did not have changes in the substrate marker for the phosphodiesterase, these results provide strong evidence for the involvement of an ATP diphosphohydrolase and a 5′-nucleotidase in adenosine production in rat blood serum, suggesting a role for these enzymes in the modulation of seizures induced by the acute administration of PTZ.

Furthermore, it was demonstrated that experimental seizures raise cerebral levels of adenosine within seconds following the onset of seizures (Winn et al., 1979). In this study, we observed an increase in nucleotide hydrolysis within 5 min of the administration of PTZ. Moreover, soon after the treatment with PTZ, the numeric values of ATPase and ADPase activities were higher in relation to the other times tested, although the ATP and ADP hydrolysis remained significantly increased in relation to the control (Fig. 1A and B). These increased values of enzyme activity soon after PTZ-injection, may be attributed to the effect of stress caused by injection and manipulation of animals, since soluble nucleotidases are released during shear stress (Yegutkin et al., 2000).

Since our results were obtained in rat blood serum, it is important consider that upon electrical stimulation, the sympathetic nerve releases not only ATP, but also soluble enzymes that can act in conjunction with membrane ectonucleotidases in the breakdown of ATP to adenosine (Todorov et al., 1997). Furthermore, CD39, the first human gene reported to encode a protein with ecto-ATP diphosphohydrolase activity (Maliszewski et al., 1994) is expressed in macrophages, suggesting that this protein is present in the circulation (Mulero et al., 1999).

The levels of adenosine are increased in hypoxia (Lloyd et al., 1993) and the plasma adenosine also increased during brain ischaemia in humans (Pasini et al., 2000). Furthermore, acute exposure to hypoxia causes a decrease in the enzyme activities of adenosine kinase and adenosine deaminase accompanied by an increase in 5′-nucleotidase activity (Kobayash et al., 2000). These results are important because adenosine is an endogenous activator of the cellular antioxidant defense system (Maggirwar et al., 1994). Then, the hypoxia that occurs during seizure activity could contribute to increase in nucleotide hydrolysis and, possibly increasing adenosine levels. Moreover, it is possible to consider the increase in 5′-nucleotidase activity due to activation of the enzyme via phosphorylation by protein kinase C. However, these hypothesis must be confirmed in futures experiments.

In summary, the results reported here show that acute seizures induced by PTZ elicit a significant increase in ATP diphosphohydrolase and 5′-nucleotidase activities, probably contributing to the increase in the levels of adenosine and consequently in the modulation of seizure activity.

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