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Research report

Changes in nucleotide hydrolysis in rat blood serum induced by pentylenetetrazol-kindling

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Abstract

There is growing pharmacological evidence from several animal models of seizure disorders that adenosine possesses endogenous anticonvulsant activity. Apart from being released from cells, adenosine can be produced by the degradation of adenine nucleotides by ectoenzymes or soluble nucleotidases. These enzymes constitute an important mechanism in synaptic modulation, as they hydrolyze ATP, an excitatory neurotransmitter, to adenosine, a neuroprotective compound. We recently demonstrated an increase in ectoenzyme activity in rat brain synaptosomes after pentylenetetrazol-kindling in rats resistant to kindling, suggesting a role for ectonucleotidases in the seizure control. The present work investigates the effect of seizures induced by pentylenetetrazol kindling on the enzymes that could be playing a role in ATP, ADP and AMP hydrolysis to adenosine in rat blood serum. Animals received injections of PTZ (30 mg/kg, i.p., dissolved in 0.9% saline) once every 48 h, totaling 10 stimulations and the controls animals were injected with saline. The hydrolysis of ATP, ADP and AMP were significantly increased (42, 40, and 45%, respectively), while phosphodiesterase activity was unchanged. These results suggest once more that an increase in the ATP diphosphohydrolase and 5′-nucleotidase activities and, possibly, in adenosine levels, could represent an important compensatory mechanism in the development of chronic epilepsy. Moreover, the fact that this increase can also be measured in serum could mean that these enzymes might be useful as plasma markers of seizures in epilepsy.

Theme: Disorders of the nervous system

Topic: Epilepsy: basis mechanisms

Keywords: Adenosine; Epilepsy; PTZ kindling; ATP diphosphohydrolase; 5′-Nucleotidase; Rat

1. Introduction

Chemical kindling is widely used as an experimental model of epilepsy and epileptogenesis. This phenomenon is characterized by progressive intensification of seizures activity after repeated administration of subconvulsant doses of different CNS stimulants, including pentylenetetrazol [26,32]. Pentylenetetrazol (PTZ) is a commonly used convulsant, apparently acting via $GABA_A$ receptor complex [28] as well as by altering potassium permeability of the cell membrane via a voltage-dependent mechanism [23]. Studies have shown that the mechanisms involved in PTZ kindling may include a decrease in central GABAergic function [11]. The enhanced seizure susceptibility induced by kindling is probably attributable to plastic changes in the synaptic efficacy [9].

ATP is currently recognized as an excitatory neurotransmitter in the central nervous system [13]. Extracellular ATP exists normally at low concentrations, but in pathological situations, ATP can be released in large quantities from platelets and endothelial and vascular tissues [5,2]. However, ATP released in the synaptic cleft can be hydrolyzed to adenosine, a neuroprotective and neuromodulatory agent, and the enzymes catalyzing this

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conversion are thought to have a role in modulating and controlling excitatory synaptic transmission [37].

Adenosine is an important regulator of neuronal excitability in the central nervous system [22]. Activation of presynaptic adenosine A_1 receptors reduces neurotransmitter release [18], depressing the neuronal activity in the central nervous system [31]. Several studies have shown that the administration of adenosine and its derivatives protect from experimentally-induced seizures in vivo [14,15]. Some studies indicate that the anticonvulsant response of adenosine is predominantly mediated by changes in A_1 receptors [24], which are increased after PTZ-kindling [27].

Moreover, experimental seizures raise the cerebral levels of adenosine [36,3], and many pathological situations are associated with a rapid increase in circulating plasma adenosine concentration in man [30]. Synaptic adenosine levels increase during periods of increased metabolic demand, such as those that exist during seizures [7]. Furthermore, an increase in hippocampal adenosine release before and during seizures has been demonstrated after administration of proconvulsants, including pentylenetetrazol [19].

Adenosine is mainly produced from the hydrolysis of adenine nucleotides by an extracellular chain of ectonucleotidases [15,40]. This chain of enzymes includes ecto-ATPases (NTPDase2, CD39L1, EC 3.6.1.3), ATP diphosphohydrolase (apyrase, EC 3.6.1.5), ecto-5′-nucleotidase (EC 3.1.3.5) and 5′-nucleotide phosphodiesterase (PDEase, PC-1, EC 3.1.4.1). We have demonstrated in central and peripheral nervous system, that ATP is hydrolyzed to adenosine by the conjugated action of an ATP diphosphohydrolase (NTPDase1, CD39, ecto-apyrase, EC 3.6.1.5) and a 5′-nucleotidase (lymphocyte surface protein, CD73, EC 3.1.3.5) [1,34].

ATP diphosphohydrolases are enzymes that hydrolyze ATP and ADP almost equally well. Ecto-5′-nucleotidase plays an important role in the formation of adenosine from extracellular AMP [40,12]. The 5′-nucleotide phosphodiesterases or PDEases, are members of the PDNP family capable of hydrolyzing ATP and ADP as well as UDP-galactose, NAD, DNA and RNA [33]. *p*-Nitrophenyl-5′-thymidine-monophosphate (*p*-Nph-5′-TMP) has been used as an artificial substrate marker to 5′-nucleotide phosphodiesterase generating *p*-nitrophenol. Additionally, soluble nucleotidases are also involved in the breakdown of ATP to adenosine [37]. The stimulation of vascular endothelial cells induces a concomitant release of endogenous ATP and soluble enzymes that degrade both ATP and AMP [37].

There are evidences to suggest a participation of nucleotidases in the modulatory processes occurring in chronic epilepsy [35,40]. Besides, the co-localization of the genes for human ATP diphosphohydrolase/CD39 (10q 23.1-24.1) and for the susceptibility to partial epilepsy (10q 22-24) suggests that a mutation of the apyrase gene might exert a significant role in the epilepsy [25,29]. We have recently demonstrated that acute seizures induced by pentylenetetrazol elicit a significant increase in ATP diphosphohydrolase and 5′-nucleotidase activities in rat blood serum lasting up to 48 h after the seizure [8]. Moreover, these same changes were observed in synaptosomes of rats after status epilepticus induced in different animal models of epilepsy [4].

Therefore, we decided to investigate if the same pattern of change holds true for plasmatic enzyme activities, by measuring the effect of PTZ-kindling on the enzymes that could be acting in ATP, ADP and AMP hydrolysis in rat blood serum.

2. Material and methods

2.1. Pentylenetetrazol (PTZ) kindling

Adult female Wistar rats, weighing 150–250 g, were used. Animals were housed in cages with food and water available ad libitum and were maintained under a 12-h light/dark cycle (light on at 07:00 h) at a room temperature of 25 °C. The rats submitted to kindling model and controls were divided in two groups of five animals. One group received injections of PTZ (35 mg/kg, i.p., dissolved in 0.9% saline) once every 48 h, totaling 10 stimulations. After each PTZ injection the animals were observed for 30 min and seizures were classified according to the stages proposed by Racine [32]; stage 0, no response; 1, facial clonus; 2, head nodding; 3, forelimb clonus; 4, rearing with bilateral forelimb clonus; 5, rearing and falling with bilateral forelimb clonus. Meanwhile, control animals were injected with saline solution (10 injections as in the treated group). All the injections were performed during the day, between 11:00 and 14:00 h. Animals were killed by decapitation 48 h after the last injection, a time at which the increase in nucleotidase activity seen after acute seizures was previously found to have disappeared, with levels of the enzymes returning to normal. Therefore, effects seen 48 h after kindling would likely be due to chronic changes in the brain induced by this process. Only animals showing at least three consecutive stage 5 seizures were included in the study.

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

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 and the serum was maintained in ice at 4 °C throughout the experiments.

2.3. Measurement of ATP and ADP hydrolysis

ATP and ADP hydrolysis was determined using a modification of the method described by Yegutkin [39]. The reaction mixture containing ADP or ATP (3 mM) as a substrate, in 112.5 mM Tris–HCl, pH 8.0, was incubated 40 min 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% trichloroacetic acid (TCA). Incubation times and protein concentration were chosen to ensure the linearity of the reaction (results not shown). The absorbance was measured at 630 nm using a Spectrophotometer Genesis 20. Inorganic phosphate (P_i) released was determined as previously described by Chan et al. [10]. Controls to correct for non-enzymatic hydrolysis were performed by adding the serum after the reaction was stopped with trichloroacetic acid. All samples were assayed in triplicate. Enzyme activities were generally expressed as nmol P_i released per min per mg of protein.

2.4. Measurement of AMP hydrolysis

The reaction mixture containing AMP (3 mM) as a substrate in 100 mM Tris–HCl, pH 7.5, was incubated 40 min with 1.0–1.5 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 trichloroacetic acid. All samples were assayed in triplicate. Enzyme activities were generally expressed as nmol P_i released per min per mg of protein. All other procedures were the same as for ATP and ADP hydrolysis, as described above.

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

The reaction mixture containing *p*-Nph-5′-TMP (0.5 mM) as a substrate in 100 mM Tris–HCl, pH 8.9, was incubated 5 min 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). *p*-Nitrophenyl-5′-thymidine-monophosphate (*p*-Nph-5′-TMP) hydrolysis was determined essentially as described by Sakura et al. [33]. 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 nmol of *p*-nitrophenol released per min per mg of protein.

2.6. 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, Brasil) [38]. The absorbance was measured at 340 nm.

2.7. Protein determination

Protein was determined by the Coomassie blue method, according to Bradford [6] using bovine serum albumin as standard.

2.8. Statistical analysis

The data obtained are expressed as means±standard deviation of at least five 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

As expected, PTZ-kindling produced a progressive increase in the seizure susceptibility of the treated animals. Enzyme assays demonstrated an increase of approximately 41% in ATP hydrolysis in kindled rats (0.82±0.1 nmol P_i/min/mg, $P<0.05$) when compared to control rats (0.58±0.04 nmol P_i/min/mg) (Fig. 1). The results obtained with ADP hydrolysis showed a similar profile, with an increase of 42% in treated rats (0.78±0.08 nmol P_i/min/mg, $P<0.05$) in relation to controls animals (0.55±0.03 nmol P_i/min/mg) (Fig. 1). We also observed an increase of 45% in AMP hydrolysis (0.33±0.08 and 0.48±0.06 nmol P_i/min/mg in control and treated rats, respectively, $P<0.05$), indicating a probable increase in 5′-nucleotidase activity (Fig. 1).

Considering that the enzyme phosphodiesterase is also expressed in blood serum [33] and can also act in ATP and ADP hydrolysis, we also measured phosphodiesterase activity through our experimental model. Our results confirmed that there was a phosphodiesterase activity in rat blood serum, but no significant difference was observed in kindled rats when compared to control rats (Fig. 2). Therefore, the increase in ATP and ADP hydrolysis can not to be related to a phosphodiesterase activity.

In order to analyze if kindling was causing significant cellular disruption which could theoretically cause an increase in nucleotidases present in serum, we measured the activity of the cytosolic enzyme lactate dehydrogenase (LDH), a marker for tissue damage, in rat blood serum. However, there were no significant changes in the LDH activity observed in PTZ-treated rats (601.4±24 U/l) and control rats (610.2±19 U/l), indicating that the increases in nucleotide hydrolysis are not likely to be due to cellular breakdown.

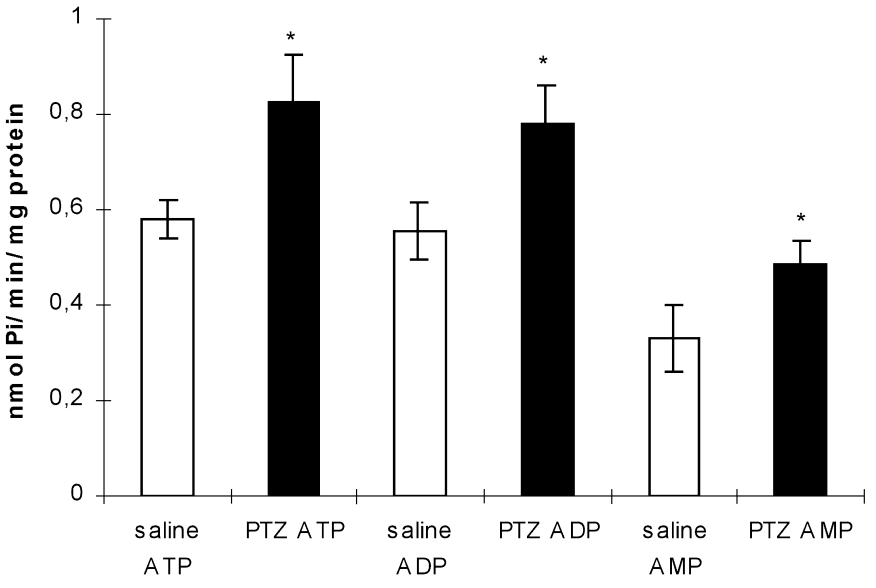

Fig. 1. Effects of PTZ-kindling on ATP, ADP and AMP hydrolysis from rat blood serum. Bars represent means±S.D. of at least five animals. * PTZ-treated group significantly different from control group ($P<0.05$, Student's *t*-test).

4. Discussion

Our results show that PTZ-kindling induces an increase in ATP, ADP and AMP hydrolysis lasting for at least 48 h in the rat brain. Since these changes were not found 48 h after acute seizures induced by the same convulsant [8], it is reasonable to consider that the changes observed were probably due to the chronic changes in synaptic excitability induced by kindling. In addition, we recently demonstrated that these changes in nucleotides hydrolysis are induced by seizures and not by the convulsant itself [8].

These results are in agreement with the evidence that adenosine can play a role in modulation of brain activity in epilepsy, by reducing neurotransmitter release [18] and depressing the neuronal activity in the central nervous system [31]. Experimental evidence suggests that others enzymes systems are also altered by PTZ kindling [17]. We have recently demonstrated an increase in ATP diphosphohydrolase and 5′-nucleotidase activities in synaptosomes from the hippocampus and cerebral cortex in pilocarpine and kainic acid models of epilepsy in rats, both of which are characterized by chronic spontaneous seizures [4].

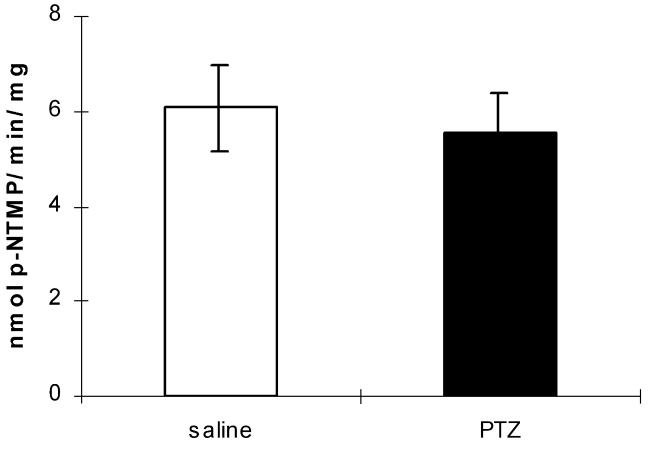

Fig. 2. Effects of PTZ-kindling on phosphodiesterase activity from rat blood. Bars represent means±S.D. of at least four animals.

When measured in serum, the enzymes ATP diphosphohydrolase and 5′-nucleotidase were shown to be increased as soon as 30 min after acute seizures induced by a convulsive dose of PTZ [8]. However, the new data presented herein show that the chronic administration of the convulsant, can induce changes in these enzymes which last longer than those occurring after a single acute seizure. Furthermore, the changes observed in ATP and ADP hydrolysis after chronic treatment with PTZ, can be attributed to an ATP diphosphohydrolase because the enzyme phosphodiesterase, that could be acting on the hydrolysis of these nucleotides in rat blood serum, was not altered in this situation. The present work, also demonstrated an activation in 5′-nucleotidase activity after PTZ-kindling, acting in association with an ATP diphosphohydrolase to complete hydrolysis of ATP to adenosine.

It has been demonstrated that soluble nucleotidases are released in the bloodstream upon electrical stimulation [21] and under conditions of shear stress from vascular endothelial cells [39]. Under conditions of stimulation these cells release not only soluble enzymes, but also high concentration of endogenous ATP [39]. Moreover, large quantities of ATP can be released in pathological conditions from platelets and vascular smooth muscle cells [19]. Since this massive release of ATP could cause

significant death [20] and cellular damage [16], the concomitant release of enzymes appears to be an important protective mechanism.

It is tempting to view the changes observed in plasmatic enzymes as being due to altered processes in the rat brain, but hemodynamic changes, changes in body temperature and the sheer muscular stress of a tonic–clonic seizure could conceivably give rise to processes resulting in altered enzyme activity, especially acutely. On the other hand, the fact that the enzymes were found to be increased 48 h after the last seizure, a time at which enzyme activity was shown to have returned to normal after a single acute seizure [8], makes it somewhat more likely that their origin is indeed the chronic brain alterations produced by kindling.

Additionally, the diagnosis of seizures in clinical practice frequently presents as a problem, as most seizures are not witnessed by a physician and many neurological and systemic conditions such as migraine and syncope, can sometimes be confused with seizures. Moreover, some patients can present psychogenic events known as 'pseudo-seizures' which can be virtually identical to real epileptic events. Therefore, potential serum markers of both acute seizures and chronic epilepsy could be of great diagnostic importance in clinical practice. However, the search for consistent ones has been largely unsuccessful up to. Much more work has to be performed, of course, to say if the measurement of nucleotidases in plasma could eventually be useful in this kind of situation.

In summary, our results show an increase of the enzymatic activities measured in the serum of rats submitted to PTZ-kindling, complementing the data already showing shorter-lasting increases after acute seizures [8]. These results suggest the involvement of nucleotidases in modulatory processes occurring in response to epilepsy. Nevertheless, more studies need to be carried out to determine the exact origin of the rise in enzyme activities both in acute seizures and in chronic epilepsy.

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