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Altered ATP Hydrolysis Induced by Pentylenetetrazol Kindling in Rat Brain Synaptosomes

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The ectonucleotidase pathway is an important metabolic source of extracellular adenosine. Adenosine has potent anticonvulsant effects on various models of epilepsy. One of these models is pentylenetetrazol (PTZ) kindling, in which repeated administration of subconvulsive doses of this drug induces progressive intensification of seizure activity. In this study, we examine the effect of a single convulsive injection (60 mg/kg, i.p.) or 10 successive (35 mg/kg, i.p.) injections of PTZ on synaptosomal ectonucleotidases. Our results have shown that no changes in ectonucleotidase activities were seen at 0, 1, and 24 h or at 5 days after a single convulsive PTZ injection. However, after PTZ-kindling, rats which were more resistant to seizure development presented an increase in ATP hydrolysis in synaptosomes from hippocampus and cerebral cortex (44% and 28%, respectively). These results suggest that changes in nucleotide hydrolysis may represent an important mechanism in the modulation of chronic epileptic activity in this model.

KEY WORDS: Ectonucleotidases; adenosine; epilepsy; kindling; pentylenetetrazol.

INTRODUCTION

Kindling is widely used as an experimental model of epilepsy and epileptogenesis. This model refers to a phenomenon whereby repeated administration of subconvulsant electrical or chemical stimuli gradually raises the susceptibility of the animal until the same stimuli eventually become convulsive (1,2,3). One of the ways to induce kindling is by repeated systemic administration of subconvulsant doses of pentylenetetrazol (PTZ), which induce progressive intensification of seizure activity in response to the same dose (3). The enhanced seizure susceptibility induced by kindling is a long-lasting, possibly permanent, alteration in the neuronal excitability, probably attributable to plastic changes in the synaptic efficacy (4).

Several studies have shown that adenosine, an ubiquitous neuromodulator, has potent anticonvulsant effects on various models of epilepsy, including PTZ-kindling (5–7). Adenosine agonists administered either centrally or peripherally reduce seizure activity in a dose-dependent manner in electrically kindled rats (8). An increased affinity for adenosine A_1 receptors has been observed in the hippocampus of kindled rats, suggesting that these receptors might play a role in the anticonvulsant effects of adenosine analogues (9). Furthermore, drugs capable of reducing adenosine effects, such as methylxanthines, increase the duration of seizures and facilitate the development of status epilepticus (10).

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Changes in adenosine-mediated neuromodulation involve not only adenosine release as such, but also the extracellular catabolism of ATP by ectonucleotidases, which constitute a highly sophisticated pathway designed to control the rate, amount and timing of adenosine formation (11,12). We have demonstrated that ATP is hydrolyzed to adenosine in the synaptic cleft by the conjugated action of an ATP diphosphohydrolase (apyrase, ATPDase, EC 3.6.1.5) and a 5′-nucleotidase (EC 3.1.3.5) (13,14). Furthermore, Kegel et al. (15) have shown that an ecto-ATPase (EC 3.6.1.3) is co-expressed with an ecto-ATP diphosphohydrolase in rat brain. These enzymes had previously been identified in biochemical terms and were shown to hydrolyze a variety of purine and pyrimidine nucleoside di-and triphosphates (12,16). However, ecto-ATPase and ecto-ATP diphosphohydrolase differ in their preference for nucleoside 5′-diphosphates. Ecto-ATPase has a very high preference for ATP over ADP whereas the related ecto-ATP diphosphohydrolase (ecto-apyrase) hydrolyzes both substrates equally as well (12). Subsequently, an ecto-5′-nucleotidase participates, together with ecto-ATPase and ecto-ATP diphosphohydrolase, in the complete hydrolysis of ATP to adenosine in the synaptic cleft (16,17). Ecto-5′-nucleotidase is an enzyme able to hydrolyze monophosphonucleosides (AMP) to its equivalent nucleoside (adenosine) and inorganic phosphate (16).

Considering that adenosine seems to play a role in the modulation of seizure behavior induced by kindling, we decided to study if changes in ectonucleotidases can be involved in this process. For this purpose, we examine the effect of single or chronic injections of PTZ on synaptosomal ectonucleotidases in the hippocampus and cerebral cortex of rats.

EXPERIMENTAL PROCEDURE

Treatments. Female Wistar rats (age, 60–80 days; weight, 150–200 g) from our breeding stock were housed five per cage, with water and food *ad libitum.* The animal house was kept on a 12 h light-dark cycle (lights on at 7:00 am) at a temperature of 23 ± 1 °C. Procedures for the care and use of animals were adopted according to the regulations published by the Brazilian Society for Neuroscience and Behavior.

The kindling procedure consisted of i.p. injections of PTZ (35 mg/kg, dissolved in 0.9% saline) once every 48 h, totaling 10 stimulations. After each PTZ injection the animals were observed for 30 minutes and seizures were classified according to the stages proposed by Racine (2): 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. The control group was injected with saline (10 injections with the same periodicity and volume as the treated group) and was also observed for 30 minutes (18). All the injections were performed during daytime, at times varying from 11:00 am to 3:00 p.m. After the stimulation period animals were divided into two groups according to the mean of the 3 highest kindling stages attained in the last five injections. The kindled animals were divided in group I (GI), with mean kindling stage lower than 3 in all animals, and group II (GII), with mean stage higher than 3 for all animals. Animals were killed by decapitation 5 days after the last injection, the brain structures were removed and subcellular fractionation and enzyme assays were carried out.

In the acute seizure model, animals received a single convulsive injection of PTZ (60 mg/kg, i.p., dissolved in 0.9% saline). Only animals showing tonic-clonic seizures within 4 min after the injection were included in the study. Treated groups were killed by decapitation either immediately, 1 h, 24 h or 5 days after the injection. Control group was injected with saline and the subcellular fractionation and enzyme assays were carried out simultaneously with treated-groups at the different times studied.

Subcellular Fractionation. After removal, brains were placed into ice-cold isolation medium (320 mM sucrose, 5 mM HEPES, pH 7.5, and 0.1 mM EDTA) and hippocampi and cerebral cortices were immediately dissected on ice. The hippocampi and cerebral cortices were gently homogenized in 5 and 10 volumes, respectively, of ice-cold isolation medium with a motor-driven Teflon-glass homogenizer. Synaptosomes were isolated as described previously (19). Briefly, 0.5 ml of the crude mitochondrial fraction was mixed with 4.0 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 twice at 15000 *g* for 20 min with the same ice-cold medium to remove the contaminating Percoll and 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 previously (17) 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 synaptosomal fraction (10–20 µg protein) was added to the reaction mixture and preincubated for 10 minutes at 37°C. The reaction was initiated by the addition of ATP or ADP to a final concentration of 1.0 mM and stopped by the addition of 200 µl 10% trichloroacetic acid. The samples were chilled on ice for 10 minutes and 100 µl samples were taken for the assay of released inorganic phosphate (Pi) (20).

The reaction medium used to assay the 5′-nucleotidase activity contained 10 mM $MgCl_2$, 0.15 M sucrose and 0.1 M TRIS-HCl, pH 7.0 in a final volume of 200 µl (21). The synaptosomal fraction (10–20 µg protein) was preincubated for 10 minutes at 37°C. The reaction was initiated by the addition of AMP to a final concentration of 1.0 mM and stopped by the addition of 200 µl of 10% trichloroacetic acid; 100 µl samples were taken for the assay of released inorganic phosphate (Pi) (20). In both enzyme assays, incubation times and protein concentration were chosen in order to ensure the linearity of the reactions. 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 duplicate. Protein was measured by the Coomassie Blue method (22), using bovine serum albumin as standard.

Statistical Analysis. The data obtained for the enzyme activities are presented as mean ± SD of a number of animals studied in each condition. The statistical analysis used for kindling experiments was one-way ANOVA, followed by Duncan multiple range test. In the acute treatment, results were compared using two-way ANOVA and one-way ANOVA, followed by Duncan multiple range test. A $p < 0.05$ was considered to represent a significant difference in statistical analysis used.

RESULTS

As expected, PTZ-kindling produced a progressive increase in the seizure susceptibility of the treated rats. After the 10th stimulation, animals were divided into two groups: group I (GI, n = 6), in which the mean kindling stage was 3 or less, with an overall average of 2.35 and group II (GII n = 6), which the mean stage was greater than 3 and averaged 4.2. Enzyme assays in these animals showed a significant increase in ATP hydrolysis (44%) among the less severely kindled animals (GI) in synaptosomes from hippocampus, when compared to the saline group ($p < 0.05$) (Table I). The kindling-resistant group (GI) also presented a significant increase (28%) on ATP hydrolysis in synaptosomes from cerebral cortex (Table II). On the other hand, the kindling-susceptible group (GII), which presented more severe seizures, did not show significant changes in ATP hydrolysis in both fractions analyzed (hippocampus and cerebral cortex), when compared to controls (Tables I and II). There was no significant difference in ADP and AMP hydrolysis among the three groups (Tables I and II).

In order to examine if the altered ATP hydrolysis was due to the chronic, long-lasting changes induced by kindling or to a residual effect of the drug, we investigated the enzymes activities at different times after a single acute seizure induced by PTZ (60 mg/kg, i.p.). Following the single PTZ injection, all animals included in the study presented generalized tonic-clonic seizures. However, our results did not show significant differences in ATP, ADP and AMP hydrolysis in all times tested (immediately, 1h, 24h and 5 days) after the single injection of PTZ in synaptosomes of hippocampus and cerebral cortex (data not shown).

TABLE I. Effect of PTZ Kindling on Ectonucleotidase Activities in Synaptosomes from Hippocampus of Rats

Group	*n*	ATP	ADP	AMP
Saline	6	101.7 ± 15.8	37.2 ± 9.8	18.4 ± 3.1
G I	6	146.6 ± 8.03[a]	40.9 ± 10.8	17.1 ± 1.8
G II	6	120.4 ± 20.1	41.4 ± 11.1	20.4 ± 3.9

Data are expressed as mean ± S.D. Saline = saline-injected group. GI = kindling-resistant group (mean kindling stage < 3, average = 2.35). GII = kindling-sensitive group (mean kindling stage >3, average = 4.2).
[a] Significantly different from the saline group ($p < 0.05$) by one-way ANOVA, followed by Duncan multiple range test.

TABLE II. Effect of PTZ Kindling on Ectonucleotidase Activities in Synaptosomes from Cerebral Cortex of Rats

Group	*n*	ATP	ADP	AMP
Saline	6	120.9 ± 14.1	40.1 ± 7.9	17.8 ± 4.1
G I	6	154.7 ± 14.2[a]	44.1 ± 9.3	15.9 ± 3.8
G II	6	119.7 ± 25.7	47.6 ± 11.1	13.3 ± 2.7

Data are expressed as mean ± S.D. Saline = saline-injected group. GI = kindling-resistant group (mean kindling stage < 3, average = 2.35). GII= kindling-sensitive group (mean kindling stage >3, average = 4.2).
[a] Significantly different from the saline group ($p < 0.05$) by one-way ANOVA, followed by Duncan multiple range test.

DISCUSSION

The present study demonstrates that the ATPase activity promoted by ectonucleotidases is significant increased in synaptosomes from hippocampus and cerebral cortex of rats more resistant to kindling, when compared to controls. It is important to note that different effects for ATP and ADP hydrolysis can be due to the simultaneous presence of two different enzymes involved in the nucleotide hydrolysis, an ATP diphosphohydrolase and an ecto-ATPase (15). These results suggest the participation of an ecto-ATPase, since ATP is considered a substrate marker for this enzyme activity (12,16).

Changes in synaptosomal enzymes involved in ATP hydrolysis have been observed in other animal models of epilepsy. For example, alterations in synaptosomal ecto-ATPase in rat brain are observed during prolonged *status epilepticus* induced by lithium and pilocarpine (23). Furthermore, Fernandes et al. (24) have showed a significant decrease in Na^+, K^+-ATPase activity in acute *status epilepticus* and in the silent period of the pilocarpine model of epilepsy. However, a significant increase in this activity was observed during the chronic period, characterized by recurrent spontaneous seizures (24). Similar results were observed in hippocampus of rats treated with kainate (25). Alterations in Na^+, K^+-ATPase activity could promote a control of the excitability produced by the release of Ca^{2+} and glutamate during seizures (25).

If ATP is released in large amounts and for a long time, it may promote an dramatic increase in intracellular calcium levels mediated by $P_{2\times}$ receptors, which could cause significant damage similar to that induced by excessive glutamate release (26). Our findings lead us to the hypothesis that an increase in ATP hydrolysis by an ecto-ATPase, can produce high levels of ADP. Then ADP, by stoichiometric effect, is hydrolyzed by conjugate action of an ATP diphosphohydrolase and a 5′-nucleotidase, producing an increase in adenosine levels. Thus, the activation of ecto-ATPase, the first enzyme in the pathway of ATP hydrolysis to adenosine, can contribute to an increase in the concentration of the last metabolite, the neuromodulator adenosine (5,27). In summary, adaptive plasticity in chronic epilepsy could involve a decrease in the levels of ATP, an excitatory neurotransmitter, and a simultaneous increase in the concentration of adenosine, a neuroprotective compound. This balance can be achieved through a delicate regulation of the amount of ATP released and of the rate of ATP hydrolysis by ectoenzymes. The measurements of the ectonucleotidase activities require one to add ATP, ADP or AMP, in saturating concentrations, in order to initiate the reaction and later determine the release of inorganic phosphate, which prevent the measure of original levels of nucleotides in experimental and control conditions. However, our experiments are able to demonstrate possible alterations in ectonucleotidase activities in a condition that triggers mechanisms able to modulate these enzymes. In the future, the measurements of nucleotide and nucleoside levels can constitute an important contribution to the knowledge of the role of purines in epilepsy.

It has been proposed that adenosine and adenosine analogs possess anticonvulsant properties, an effect which has been attributed by the activation of A_1 receptors (28). Single and repeated pentylenetetrazol-induced convulsions are associated with significant increases of A_1 adenosine receptors in the cortex, hippocampus and cerebellum (29). It has been suggested that the increase in A_1 receptor number, along with the rise in nucleotidase activities and cerebral adenosine levels, might contribute to prevent cellular excitation and to maintain the neuronal homeostasis (30).

Therefore, our results suggest that an increase in ATP hydrolysis and, consequently, in adenosine levels could represent an important compensatory mechanism in the development of chronic epilepsy, explaining why these changes are associated with less severe seizures in PTZ-kindled rats. These alterations seems to be related to the chronic, long-lasting synaptic plasticity induced by kindling, since such changes are not seen in acute seizures, which are insufficient to activate these mechanisms. When these results are considered together, they support the hypothesis that changes in nucleotide hydrolysis may represent an important mechanism in the modulation of epileptogenesis.

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