Changes in synaptosomal ectonucleotidase activities in two rat models of temporal lobe epilepsy

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Abstract

Adenosine has been proposed as an endogenous anticonvulsant which can play an important role in seizure initiation, propagation and arrest. Besides the release of adenosine per se, the ectonucleotidase pathway is an important metabolic source of extracellular adenosine. Here we evaluated ATP diphosphohydrolase and 5′-nucleotidase activities in synaptosomes from hippocampus and cerebral cortex at different periods after induction of status epilepticus (SE) by intraperitoneal administration of pilocarpine or kainate. Ectonucleotidase activities from synaptosomes of hippocampus and cerebral cortex of rats were significantly increased at 48–52 h, 7–9 days and 45–50 days after induction of SE by pilocarpine. In relation to kainate model, both hippocampal enzymes were enhanced at 7–9 days and 45–50 days, but only 5′-nucleotidase remained elevated at 100–110 days after the treatment. In cerebral cortex, an increase in ATP diphosphohydrolase was observed at 48–52 h, 7–9 days and 45–50 days after induction of SE by kainate. However, 5′-nucleotidase activity only presented significant changes at 45–50 and 100–110 days. Our results suggest that SE can induce late and prolonged changes in ectonucleotidases activities. The regulation of the ectonucleotidase pathway may play a modulatory role during the evolution of behavioral and pathophysiological changes related to temporal lobe epilepsy. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Adenosine is an endogenous nucleoside that possesses anticonvulsant (Cavalheiro et al., 1987; Kostopoulos et al., 1989; During and Spencer, 1992) and neuroprotective properties (Turski et al., 1985; MacGregor et al., 1997). This action probably involves the inhibition of seizure initiation and propagation ‘in vivo’ and ‘in vitro’ (Kostopoulos et al., 1989; Whitcomb et al., 1990), an effect which has been attributed by the activation of A1 receptors (Brundege and Dunwiddie,
In addition, adenosine has well established presynaptic inhibitory actions due to reduction in neurotransmitters release, including glutamate, dopamine, serotonin and acetylcholine (Ribeiro, 1995; Brundege and Dunwiddie, 1997). The ability of endogenous adenosine to diffuse locally and exert modulatory effects on independent pathways in hippocampus might suggest that this nucleoside could contribute to the phenomena of synaptic plasticity (Mendonça and Ribeiro, 1997).

Besides the release of adenosine as such, biochemical studies have established that a potential source of adenosine is its formation in the extracellular space from adenine nucleotides (Dunwiddie et al., 1997). ATP is a chemical mediator of fast excitatory transmission and its action occurs through P2-purinoceptors (Abbracchio and Burnstock, 1998; Di Iorio et al., 1998). It has been showed that, on high-frequency (100 Hz) burst stimulation, the release of ATP is enhanced (Cunha et al., 1996), and marked increases in adenosine levels can occur under various conditions (e.g. hypoxia, ischemia, seizures) (Brundege and Dunwiddie, 1997; Braun et al., 1998). Once adenine nucleotides reach the extracellular space, they are subsequently converted to adenosine through the action of ecto-enzymes (Zimmermann, 1996; Bonan et al., 1998). 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) (Sarkis and Saltó, 1991; Battastini et al., 1995). Ecto-ATP diphosphohydrolase is an enzyme able to hydrolyze adenine triphosphonucleoside (ATP) to its equivalent monophosphonucleoside (AMP) and inorganic phosphate. Then, AMP can produce adenosine by the action of an ecto-5'-nucleotidase (Zimmermann, 1996). The recently cloned ecto-apyrase is a noncovalent tetrameric protein (Wang et al., 1998) expressed in primary neurons and astrocytes (Wang et al., 1997), presenting a wide distribution in cerebral cortex, hippocampus, cerebellum, glial and endothelial cells (Wang and Guidotti, 1998).

Human temporal lobe epilepsy (TLE) is associated with complex partial seizures that can produce secondarily generalized seizures and motor convulsions (Engel et al., 1997; Mather et al., 1997). The pilocarpine and kainic acid models are useful animal models to investigate the development and neuropathology related with TLE. These models induce a prolonged status epilepticus (SE), which causes neuronal loss, mossy fiber sprouting in hippocampus and spontaneous recurrent seizures (Ben-Ari, 1985; Cavalheiro et al., 1991; Cronin et al., 1992; Mello et al., 1993). Here we investigated the ATP diphosphohydrolase and 5'-nucleotidase activities at different times after induction of SE by pilocarpine and kainate models. The results can provide new information about biochemical events during the temporal evolution of long-lasting changes induced by both studied models.

2. Material and methods

2.1. Pilocarpine model

Female Wistar rats (age, 70–90 days; weight, 200–240 g) from our breeding stock were housed five to a cage, with water and food ad libitum. The animal house was kept on a 12-h light, 12-h dark cycle (lights on at 07:00 h) at a temperature of 23 ± 1°C. The pilocarpine model has been previously described (Cavalheiro et al., 1991; Mello et al., 1993). A total of 75 animals was injected with pilocarpine and monitored behaviorally for at least 6 h. Total mortality was 40%, and 4% of the rats did not develop SE. Only animals (42 rats) that evolved to SE characterized by generalized motor seizures were used. In summary, 30 min after s.c. pretreatment with scopolamine methylnitrate 1 mg kg⁻¹ (to minimize peripheral cholinergic effects), a single dose of pilocarpine (350 mg kg⁻¹, dissolved in saline) was injected i.p. After a pilocarpine injection, the animal became hypoactive; generalized convulsions and limbic SE usually occur 40–80 min after the injection. After a silent period of 14 ± 3.0 days (ranging from 4–44 days), all animals developed spontaneous seizures (2–15 per month), characterizing the chronic period. There is no evidence of spontaneous remission for at least 6 months (Cavalheiro et al., 1991; Mello et al., 1993).
2.2. Kainate model

In this model, female Wistar rats (age, 70–90 days; weight, 200–250 g) were maintained in the same conditions reported above. The kainate model has been described previously (Lothman et al., 1981; Ben-Ari, 1985; Cronin et al., 1992). A total of 60 animals were injected with kainate and monitored behaviorally for at least 6 h. Total mortality was 21%, and 9% of the animals did not develop SE. Only animals that reached SE characterized by generalized motor seizures were used (42 rats) in the experiments. In summary, animals received a single dose of kainate i.p. (10 mg kg\(^{-1}\), dissolved in saline). During the first 20–30 min, they had ‘staring’ spells. During the next 30 min animals had head nodding and several wet-dog shakes. One hour after kainic acid treatment, they had recurrent limbic motor seizures, evolving to a full motor limbic SE. This model did not consistently induce spontaneous recurrent seizures (i.e. often < 50% of the treated rats were observed to have seizures) (Cronin et al., 1992).

2.3. Animal preparation and subcellular fractionation

Animals were sacrificed by decapitation at different times after SE initiation (24–28 h; 48–52 h; 7–9 days, 45–50 days, 100–110 days). When chronic animals were tested, subcellular fractionation was prepared during the interictal period. After removal, brains were placed in ice-cold isolation medium (320 mM sucrose, 5 mM Hepes, pH 7.5, and 0.1 mM EDTA) and were cut longitudinally. Total hippocampi (100–110 mg of tissue) and total cerebral cortex (450–500 mg of tissue) of both hemispheres were immediately dissected on ice. In pilot experiments, we have determined that the vehicle (saline) or scopolamine injection did not alter the enzyme activities at the different times tested. Therefore, a group of naive rats were used as control and the subcellular fractionation and enzyme assays were carried out simultaneously with the kainate or pilocarpine treated-groups at the different times studied. The total hippocampi and cerebral cortex were gently homogenized in 5 and 10 vol., respectively, of ice-cold isolation medium with a motor-driven Teflon-glass homogenizer. The synaptosomes were isolated as described previously (Nagy and Delgado-Escueta, 1984). 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 15,000 \( \times \) g for 20 min with the same ice-cold medium to remove the contaminating Percoll and the synaptosome pellet was resuspended to a final protein concentration of approximately 0.5 mg ml\(^{-1}\). The material was prepared fresh daily and maintained at 0–4°C throughout preparation.

2.4. Enzyme assays

The reaction medium used to assay the ATP diphosphohydrolase activity was essentially as described previously (Battastini et al., 1991) 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 \( \mu \)l. The synaptosome preparation (10–20 \( \mu \)g protein) was added to the reaction mixture and preincubated for 10 min 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 \( \mu \)l of 10% trichloroacetic acid; 100-\( \mu \)l samples were taken for the assay of released inorganic phosphate (Pi) (Chan et al., 1986). The reaction medium used to assay the 5’-nucleotidase activity contained 10 mM MgCl\(_2\), 0.1 M Tris–HCl, pH 7.0 and 0.15 M sucrose in a final volume of 200\( \mu \)l (Heymann et al., 1984). The synaptosome preparation (10–20 \( \mu \)g protein) was preincubated for 10 min 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 \( \mu \)l 10% trichloroacetic acid. The samples were chilled on ice for 10 min and 100-\( \mu \)l samples were taken for the assay of released inorganic phosphate (Pi) as described by Chan et al. (1986). 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 (Bradford, 1976), using bovine serum albumin as standard.

2.5. Statistical analysis

The data obtained for the enzyme activities are presented as mean ± S.D. of a number of animals studied in each condition. The statistical analysis used for in vivo experiments was one-way ANOVA, followed by Duncan’s multiple range test. The in vitro effect of convulsant drugs on ectonucleotidase activities was evaluated by Student’s t-test. P < 0.05 was considered to represent a significant difference in both statistical analyses used.

3. Results

3.1. Ectonucleotidase activities after SE induced by pilocarpine

The effect of temporal evolution of pilocarpine model was evaluated on ecto-ATP diphosphohydrolase and 5'-nucleotidase activities in synaptosomes from hippocampus and cerebral cortex of rats. As showed in Fig. 1A, there was a 78% increase in ATP hydrolysis in hippocampal synaptosomes of rats at 48–52 h after the induction of SE. This increase reached the maximum value (86% increase) at 7–9 days after the SE when compared to the respective control group. The ATPase activity remained high and then gradually decreased (36% increase in relation to control group) at 45–50 days after pilocarpine-induced SE. The results related to ADP hydrolysis by ATP diphosphohydrolase showed a significant 50% increase at 48–52 h after the pilocarpine-induced SE. This increase attained a peak (187%) at 7–9 days after the induction of the SE and gradually decreased (37% increase in relation to control group) at 45–50 days after the event (Fig. 1A).

The data for about 5'-nucleotidase showed a very similar profile when compared to ATP diphosphohydrolase. It is possible to observe a significant increase of 5'-nucleotidase activity at 48–52 h (64%), 7–9 days (134%) and 45–50 days (99%) after the induction of SE (Fig. 1A). Both ectonucleotidas activities did not change at 24–28 h and 100–110 days after SE induced by pilocarpine in hippocampal synaptosomes of rats.

Since these ectonucleotidas are widely expressed in rat brain (Wang and Guidotti, 1998), we also evaluate possible changes in these enzyme activities in synaptosomes from cerebral cortex after induction of SE by pilocarpine. In relation to ATP hydrolysis, a significant increase (34%) was observed only in the group tested at 48–52 h after SE (Fig. 1B). However, under the same conditions, ADPase activity was significantly increased at 48–52 h and 7–9 days after the SE (44 and 49%, respectively). The 5'-nucleotidase activity showed a significant peak of activity (104% increase) at 7–9 days and gradually decreased (62% increase) at 45–50 days after SE, recovering the normal activity at 100–110 days after the event (Fig. 1B). In order to avoid the influence of age in animals tested at 100–110 days after SE, we analyzed these enzyme activities in both hippocampus and cerebral cortex, in naive animals with the same age (180–200 days). There was no significant difference in the ectonucleotidase activities when compared to younger control rats (age, 90–100 days) (data not shown).

3.2. Ectonucleotidase activities after SE induced by kainate

There were no significant changes in the enzyme activities studied in hippocampal synaptosomes at 24–28 and 48–52 h after induction of SE by kainate (Fig. 2A). However, a significant increase in ATP (53%), ADP (114%) and AMP (31%) hydrolysis was observed at 7–9 days after SE. These effects remained significant at 45–50 days after SE (46, 80 and 131% for ATP, ADP and AMP, respectively) in hippocampal synaptosomes. However, the ATP and ADP hydrolysis promoted by ATP diphosphohydrolase gradually decreased, recovering the normal activity at 100–
110 days after event (Fig. 2A). With respect to 5'-nucleotidase at the same period, it is still possible to observe a significant increase (60%) in AMP hydrolysis.

The same temporal analysis was performed in synaptosomes from cerebral cortex of adult rats after SE induced by kainate. A significant increase of ATP diphosphohydrolase (23 and 20% for ATP and ADP, respectively) was observed at 48–52 h after SE. However, under the same condition, there was no significant effect on 5'-nucleotidase (Fig. 2B). Fig. 2B also shows an increase

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![Fig. 1](image.png)

**Fig. 1.** Effects of pilocarpine model on ATP (■), ADP (●) and AMP (▲) hydrolysis in synaptosomes from hippocampus (A) and cerebral cortex (B) at different times after the induction of status epilepticus in adult rats. The control activities in hippocampal synaptosomes were 120.9±9.1, 42.4±7.7 and 15.8±3 nmol Pi min⁻¹ mg⁻¹ protein for ATP, ADP and AMP hydrolysis, respectively. The control activities in synaptosomes from cerebral cortex were 142.8±12.3, 47±5.5 and 15.3±2.6 nmol Pi min⁻¹ mg⁻¹ protein for ATP, ADP and AMP hydrolysis, respectively. Bars represent means ± S.D. of at least eight animals. *ATP hydrolysis in treated-group significantly different from control ATP hydrolysis. (a) ADP hydrolysis in treated-group significantly different from control ADP hydrolysis. (b) AMP hydrolysis in treated-group significantly different from control AMP hydrolysis (*P < 0.05, Duncan’s Test).
of ATP (28%) and ADP (43%) hydrolysis 7–9 days after the event. In the group tested at 45–50 days after SE, an increase in ectonucleotidase activities (42, 46 and 94% for ATP, ADP and AMP, respectively) was also observed, but only 5'-nucleotidase activity remaining increased (41%) at 100–110 days after the event in synaptosomes from cerebral cortex of rats.

3.3. Effects in vitro of convulsant drugs on ectonucleotidase activities

In order to discard the influence of the residual drugs used to induce SE on the ectonucleotidase activities during early periods, we analyzed the effect in vitro of these drugs on ATP diphosphohydrolase and 5'-nucleotidase. The drugs and con-
centrations tested were 5 mM kainate, 10 mM pilocarpine, 0.5 mM scopolamine or 10 mM pilocarpine plus 0.5 mM scopolamine in both synaptosomal fractions analyzed. The results revealed no statistically significant alterations in vitro for ectonucleotidase activities in the presence of these drugs in synaptosomes from hippocampus or cerebral cortex of rats, suggesting that the increased nucleotide hydrolysis is induced by the models and not by the drug administration (data not shown).

4. Discussion

This work was carried out in order to investigate possible changes in ectonucleotidases activities at different times after spontaneous recovering of status epilepticus induced by pilocarpine and kainate. The co-localization of the genes for human ecto-apyrase/CD39 (10q 23.1-24.1) and for the susceptibility to partial epilepsy (10q 22-24) suggests that a mutation of the ecto-apyrase gene might exert a significant role in the epilepsy (Maliszewski et al., 1994; Ottman et al., 1995; Wang et al., 1997). Our results have shown a substantial increase in ectonucleotidases activities in hippocampal synaptosomes between 48 h and 50 days after SE induced by pilocarpine. In cerebral cortex, we observe an increase in ATP diphosphohydrolase activity 48-52 h after SE, but only ADP hydrolysis remained increased at 7–9 days after the event. This result indicates the participation of an ATP diphosphohydrolase, since ADP is considered a substrate marker for this enzyme activity (Battastini et al., 1991; Sarkis and Saltó, 1991). However, it is important to note that the apparent temporal dissociation observed between both substrates (ATP and ADP) can be due to the simultaneous presence of two different enzymes involved in the nucleotide hydrolysis, an ATP diphosphohydrolase and an ecto-ATPase (Kegel et al., 1997). These enzymes have been identified in molecular terms and differ in their preference for the substrates. (Zimmermann, 1996). Both enzymes are expressed as ecto-enzymes in the brain, but their specific localization and interactions are still unknown.

In the kainic acid model, the results have shown a novel feature, that is the activation of 5′-nucleotidase 100–110 days after SE. It is interesting to observe that the transient alterations and different time course for ectonucleotidases activities observed between the models could be due to differences in the behavioral course, including the time for the first spontaneous seizure (1–3 weeks in kainate model; 45–100 days in pilocarpine model), the number of animals that present chronic epilepsy (lower in the kainate treatment, often < 50% of the treated rats), as well as pharmacological and histopathological findings specific for each model (Cronin et al., 1992; Cavalheiro, 1995).

The cellular pattern of expression of 5′-nucleotidase can vary substantially both with developing and on lesioning of nervous tissue (Zimmermann, 1996). Whereas 5′-nucleotidase in the adult nervous system has a predominantly glial association, it is transiently present also at synapses that are related to the regenerative sprouting responses and synaptogenesis in this system (Schoen et al., 1999). Thus, it appears that 5′-nucleotidase is expressed on reactive glial as well as neural cells (Zimmermann, 1996). The procedure used in our experiments for the isolation of synaptosomes allows the preparation of a synaptosomal-enriched fraction, but not immunopurified, presenting a glial and myelin contamination in the range of 0.3% (Nagy and Delgado-Escueta, 1984). Therefore, it is not possible to exclude glial contamination in our preparation. However, it is important to consider that, in all treatments studied, we prepared synaptosomal fractions from control group under the same conditions used for the treated group. Thus, independently of the localization of ecto-5′-nucleotidase, this enzyme activity was significantly increased after kainate or pilocarpine treatments.

Changes in other synaptosomal enzymes involved in ATP hydrolysis have been observed after the induction of SE. Alterations in synaptosomal ecto-ATPase in rat brain during prolonged SE induced by lithium and pilocarpine have been observed (Nagy et al., 1997). Nagy et al. (1990) also demonstrated that synaptosomal ecto-ATPase activity is decreased in the anterior region of...
hippocampus (epileptogenic zone), but it is increased in the posterior zone (non-epileptogenic zone), containing CA3 and granule cells of dentate gyrus. A significant decrease in \( \text{Na}^+, \text{K}^+ \)-ATPase activity was observed in the acute and silent period of pilocarpine model, but an increase of this activity was observed during the chronic period (Fernandes et al., 1996). Similar results were observed in the hippocampus of rats treated with kainate (Anderson et al., 1994). Alterations in \( \text{Na}^+, \text{K}^+ \)-ATPase activity could promote a control of excitability, produced by release of \( \text{Ca}^{2+} \) and glutamate during seizures (Fernandes et al., 1996).

Although our findings did not show a correlation between the extent of changes in ectonucleotidases activities and the extent of sprouting, we also cannot completely discard this possibility. Recently, Schoen et al. (1999) has shown that 5′-nucleotidase is present in synapses of aberrant mossy fibers in the inner molecular layer of the dentate gyrus of rats after seizures induced by systemic kainate treatment or electrical kindling. The expression of 5′-nucleotidase on synaptic membranes of sprouting mossy fibers could be an adaptive response of the epileptic hippocampus. Therefore, it is concluded that in both hippocampus from normal and epileptic rats, 5′-nucleotidase is associated with axons capable of a plastic sprouting response (Schoen et al., 1999). The glycoprotein 5′-nucleotidase may help stabilize a newly formed, sprouted synapse, due to other non-enzymatic functions. 5′-Nucleotidase is involved in signal transduction as differentiation antigen CD73. It may also serve cell recognition since it is attached to cell membrane by a glycosyl phosphatidylinositol anchor, carries the HNK-1 epitope during periods of synaptic maturation and binds to proteins of the extracellular matrix (Schoen et al., 1999). The involvement of the ATP diphosphohydrolase in the synaptic plasticity could be related to cell adhesion. Adhesion is a phenomenon often coupled to particular ATP diphosphohydrolases — either directly via intrinsic ecto-ATP diphosphohydrolase activity exhibited by some known adhesion molecules, e.g. NCAM, or indirectly as shown by homotypic adhesion triggered by monoclonal antibodies against CD39, recently shown to be an ecto-ATP diphosphohydrolase (Dzhandzhugazyan et al., 1998).

Immunohistochemical studies are in progress in our laboratory in order to observe the distribution of ectonucleotidases activities after induction of SE induced by kainate and pilocarpine models.

Our findings lead us to the hypothesis that an increase in ectonucleotidases activities could modulate the seizure activity in a time window (48 h–110 days) after SE, contributing to production of extracellular adenosine, a known endogenous neuromodulator (During and Spencer, 1992; Brundege and Dunwiddie, 1997). If ATP is released in large amounts and for a long time, it may promote a dramatic increase in intracellular calcium levels mediated by \( \text{P}_{2X} \) receptors, that could represent a significant damage, as that induced by excess of glutamate (Edwards et al., 1992). If all members of the ectonucleotidase pathway work at an elevated rate, an efficient removal of extracellular ATP and enhanced adenosine production could occur in this condition. Then, adenosine could modulate the release of a variety of neurotransmitters, including glutamate, acetylcholine, noradrenaline and dopamine (Brundege and Dunwiddie, 1997; Di Iorio et al., 1998). In summary, after SE, an important adaptive plasticity of ectonucleotidase pathway could occur in order to decrease ATP levels, an excitatory neurotransmitter, and to increase adenosine levels, a neuroprotective compound. Recently, we have reported the participation of these ectonucleotidases in specific biochemical events related to memory acquisition and consolidation, suggesting an important role of this pathway in synaptic plasticity during physiologic events (Bonan et al., 1998).

In summary, the results reported here show significant alterations in the synaptosomal ectonucleotidase activities at different times after SE induced by two different animal models of epilepsy — pilocarpine and kainate models. Accurate knowledge of the changes in these enzyme activities at different periods after SE could be used to design a novel class of drugs for epilepsy treatment.
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