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Antiepileptic Drugs Prevent Changes Induced by Pilocarpine Model of Epilepsy in Brain Ecto-Nucleotidases

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Abstract Ecto-nucleotidases, one of the main mechanisms involved in the control of adenosine levels in the synaptic cleft, have shown increased activities after the pilocarpine model of epilepsy. Here we have investigated the effect of the antiepileptic drugs (AEDs) on ecto-nucleotidase activities from hippocampal and cerebral cortical synaptosomes of rats at seven days after the induction of the pilocarpine model. Expression of these enzymes were investigated as well. Our results have demonstrated that phenytoin (50 mg/kg) and carbamazepine (30 mg/kg) were able to prevent the increase in ecto-nucleotidase activities elicited by pilocarpine in brain synaptosomes. However, sodium valproate (at 100 mg/kg) was only able to avoid the increase on ATP and ADP hydrolysis in hippocampal synaptosomes. Increase on ATP hydrolysis in hippocampal synaptosomes was also prevented by sodium valproate at 286 mg/kg, which corresponds to ED50 for

pilocarpine model. NTPDase1, NTPDase2, NTPDase3, and ecto-5′-nucleotidase expressions were not affected by pilocarpine in cerebral cortex. However, expressions of NTPDase2, NTPDase3, and ecto-5′-nucleotidase were increased by pilocarpine in hippocampus. Our results have indicated that previous treatment with AEDs was able to prevent the increase in hippocampal ecto-nucleotidases of pilocarpine-treated rats. These findings have shown that anticonvulsant drugs can modulate plastic events related to the increase of nucleotidase expression and activities in pilocarpine-treated rats.

Keywords Epilepsy · Adenosine · NTPDases · Ecto-5′-nucleotidase · Antiepileptic drugs

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Introduction

Epilepsy is one of the most frequent disorders of the CNS, affecting nearly 1.5% of the world population [1]. Among the several kinds of epileptic disorders, human temporal lobe epilepsy occurs mainly in adult individuals and has high incidence, morbidity and clinical importance, producing secondarily generalized seizures and motor convulsions [2]. Several animal models of epilepsy, including pilocarpine model, have been useful to study this disorder. Pilocarpine model of epilepsy induces a prolonged *status epilepticus* (SE), neuronal loss, mossy fiber sprouting in hippocampus and spontaneous recurrent seizures [3, 4]. This model presents three distinct periods: an acute phase which begins after the exposure to pilocarpine and is characterized by behavior changes such as facial automatisms and motor limbic seizures [5]; a silent or latent

period, when animals begin to present an apparent normal behavior [6], molecular changes occur and may be important to the development of epileptogenesis [7]; and a chronic phase characterized by spontaneous recurrent seizures [6].

Classical antiepileptic drugs (AEDs), such as phenytoin (PHT), sodium valproate (VPA), and carbamazepine (CBZ) have been extensively used for the treatment of epilepsy. The main mechanism of action of these drugs is related to the blockade of voltage-gated Na^+ channels [8]. There is evidence that conventional AEDs have some influence on the purinergic transmission in CNS [9]. Previous studies have shown that adenosine receptor agonists can enhance the anticonvulsant action of classical AEDs, such as CBZ, PHT, and VPA [9, 10]. Furthermore, PHT blocked the adenosine uptake promoted by nucleoside transporter system [11] and CBZ acts as an adenosine A_1 receptor antagonist [12–14].

Adenosine is a well-known neuromodulator [15], which exerts its effects through G protein-coupled receptors named A_1, A_{2A}, A_{2B}, and A_3. Adenosine is able to act as an endogenous anticonvulsant [16, 17] by activation of adenosine A_1 receptors promoting inhibition on neurotransmitter release [18]. The ability of endogenous adenosine to diffuse locally and exert modulatory effects could contribute to the phenomena of synaptic plasticity. Extracellular adenosine can be provided by the nucleoside bidirectional transport or produced through sequential catabolism of ATP by ecto-nucleotidases, such as NTPDases (nucleoside triphosphate diphosphohydrolases) and ecto-5′-nucleotidase [19]. Ecto-nucleotidases are membrane-bound enzymes with catalytic site located in the extracellular medium. NTPDase1, 3, and 8 slightly prefer ATP over ADP by a ratio of 1, 3, and 2, respectively. Whereas, NTPDase2 prefers triphosphonucleosides [20]. Ecto-5′-nucleotidase hydrolyses monophosphate nucleosides, e.g. AMP, and is directly involved in adenosine production in the synaptic cleft [21]. These enzymes control the rate, amount, and timing of nucleotide degradation and formation, promoting the regulation of nucleotide-mediated signaling in purinoceptors [22, 23].

It is known that several enzymes have their expression altered by pilocarpine treatment [24–26]. Previous studies from our laboratory have shown increased ecto-nucleotidase activities in synaptosomes from cerebral cortex and hippocampus of rats submitted to pilocarpine, kainic acid models of epilepsy [27], and pentylenetetrazole (PTZ) kindling [28]. The more significant increase of ATP, ADP, and AMP hydrolysis was observed at 7–9 days after the induction of the pilocarpine model presenting an activation of ecto-nucleotidase activities in the range of 86–187% in hippocampal synaptosomes of pilocarpine-treated rats [27]. Despite several studies about this issue, the role of ecto-nucleotidases in epilepsy, as well as, their interactions with therapeutic agents used in the management of this disorder need to be clarified. Therefore, the aim of this study was to investigate the effect of PHT, CBZ, and VPA on the ecto-nucleotidase expression and activities from cerebral cortex and hippocampus of adult rats submitted to pilocarpine model of epilepsy.

Experimental procedure

Animals

In all experiments, male Wistar rats (age, 70–90 days; weight, 200–280 g) from our breeding stock were used and housed five to a 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 $23 \pm 1°C$. Procedures for the care and use of animals were adopted according to the regulations of Colégio Brasileiro de Experimentação Animal (COBEA), based on the Guide for the Care and Use of Laboratory Animals (National Research Council).

Chemicals

Sodium valproate, carbamazepine, 5,5–diphenylhydantoin sodium salt, Nucleotides (ATP, ADP, and AMP), Percoll, Trizma base, Malachite Green Base, Coomassie Brilliant Blue G, EDTA, HEPES, pilocarpine hydrochloride and methylscopolamine nitrate were purchased from Sigma, St. Louis, MO, U.S.A. All other reagents were of analytical grade.

In vitro experiments

Different concentrations of PHT (1–1,000 μM), VPA, and CBZ (both 10–1,000 μM) were tested on nucleotidase activities in synaptosomes from hippocampus and cerebral cortex of naive rats. PHT and VPA were diluted in saline solution (0.9%). CBZ was diluted in ethanol (final concentration: 2%) and control, containing the vehicle, was used to exclude its effect on the enzyme activities. Anticonvulsant solutions were added to reaction medium in the preincubation phase of the enzyme assays.

In vivo experiments

Sub-effective doses of the anticonvulsants were tested on ecto-nucleotidase activities. All latency times (time between anticonvulsant administration and pilocarpine treatment) and doses of the drugs tested were chosen according to literature [29, 30]. VPA was diluted in 0.9% saline solution. CBZ and PHT were diluted in 3% Tween 80. PHT (50 mg/kg), VPA (100 mg/kg and 286 mg/kg), and CBZ (30 mg/kg) were injected i.p. (1 ml/kg) in naive rats. After the latency period of the drugs (PHT: 120 min; VPA: 30 min; CBZ: 45 min), the animals were sacrificed by decapitation and the synaptosomal fractions were isolated in order to perform the enzyme assays. After the injection of AED as described above, a group of animals was submitted to pilocarpine model as described below. Controls receiving 3% Tween 80 were also tested to evaluate if the vehicle had any effect on the enzyme activities of rats submitted to saline (controls) or pilocarpine treatments.

Pilocarpine model

The pilocarpine model has been previously described [3, 4]. The animals were injected with pilocarpine and monitored behaviorally for at least 6 h. Only animals that evolved to SE characterized by generalized motor seizures were used. In summary, thirty minutes after subcutaneous pretreatment with scopolamine methylnitrate 1 mg/kg (to minimize peripheral cholinergic effects), a single dose of pilocarpine (350 mg/kg, dissolved in saline) was injected intraperitoneally. After pilocarpine injection, the animal became hypoactive; generalized convulsions, and limbic SE usually occur 40–80 min after injection. The mortality reached 44% in rats that received only the pilocarpine administration. The mortalities for AED administration followed pilocarpine treatment were 50%, 58%, 33%, and 41% for PHT, CBZ, VPA (100 mg/kg and 286 mg/kg) groups, respectively. All animals treated or not with the previous anticonvulsant injection presented the behavior described above. However, VPA-treated animals had a delay in the beginning of pilocarpine-induced behavior changes (approximately 10 min and 30 min for 100 mg/kg and 286 mg/kg VPA, respectively). We have also observed that 3 animals presented only hypoactivity, salivation, and piloerection when pretreated with ED50 of VPA (286 mg/kg) and four animals had a typical SE induced by pilocarpine. Therefore, we divided these individuals in two groups: more or less-convulsive rats. All animals were used

7 days after the administration of pilocarpine in order to study the silent period of the model.

Animal preparation and subcellular fraction

Animals were sacrificed by decapitation and the brains were removed and placed into 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 (450–600 mg of tissue) of both hemispheres were immediately dissected on ice. In pilot experiments, we have determined that scopolamine injections did not alter the enzyme activities (data not shown). Therefore, the control group received only saline injections and the subcellular fractionation and enzyme assays were carried out simultaneously with the anticonvulsant or pilocarpine treated-groups. The total hippocampi and cerebral cortex were gently homogenized in 5 and 10 volumes, respectively, of ice-cold isolation medium with a motor-driven Teflon-glass homogenizer. The synaptosomes were isolated as described previously [31]. Briefly, 0.5 ml of 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% and 16%). The synaptosomes that banded at 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosomal fractions were washed twice at $15000 \times g$ 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. The material was prepared fresh daily and maintained at 0–4°C throughout preparation.

Enzyme assays

The reaction medium used to assay the ATP and ADP hydrolysis in hippocampal and cerebral cortical synaptosomes was essentially as described previously [32] 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 preparation (10–20 μg 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 μl 10% trichloroacetic acid. The released inorganic phosphate (Pi) was measured as previously described [33].

The reaction medium used to assay the ecto-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 μl [34]. The synaptosomal preparation (10–20 μg 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 μl 10% trichloroacetic acid; the released inorganic phosphate (Pi) was measured as described previously [33]. In all 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. Specific activity was expressed as nanomole of Pi released per minute per milligram of protein. All samples were run in triplicate.

Protein determination

Protein was determined by the Coomassie Blue method [35] using bovine serum albumin as standard in all enzyme assays.

Statistical analysis

The data obtained are represented as mean ± S.D. Statistical analysis were performed by one-way analysis of variance (ANOVA), followed by a Duncan multiple range test, considering $P < 0.05$ as significant. We have used this test for comparing three different treatments (saline, pilocarpine, and anticonvulsant drugs plus pilocarpine).

Analysis of gene expression by semi-quantitative RT-PCR

The analysis of the expression of NTPDases1, NTP-Dase2, NTPDase3, and ecto-5′-nucleotidase was carried out by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Seven days after treatment with pilocarpine and AEDs, the hippocampus and cerebral cortex of rats were isolated for total RNA extraction with Trizol reagent (Invitrogen) in accordance with the manufacture instructions. The cDNA species were synthesized with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) from 3 μg of total RNA and oligo (dT) primer in accordance with the suppliers. RT reactions were performed for 50 min at 42°C. cDNA (0.1 μl) was used as a template for PCR with specific primers for

NTPDase1, NTPDase2, NTPDase3, NTPDase8, and ecto-5′-nucleotidase (CD73). β-actin-PCR was performed as a control for cDNA synthesis. PCR reactions were performed (total volume of 25 μl) using a concentration of 0.4 μM of each primer indicated below and 200 μM and 1 U Taq polymerase (Invitrogen) in the supplied reaction buffer.

Conditions for all PCRs were as follow: Initial 1 min denaturation step at 94°C, 1 min at 94°C, 1 min annealing step (NTPDase1, NTPDase3, and ecto-5′-nucleotidase: 65°C; NTPDase 2: 66°C; β-actin: 58.5°C), 1 min extension step at 72°C for 35 cycles and a final 10 min extension at 72°C. The amplification products were: NTPDase1—543 bp; NTPDase2—331 bp; NTP-Dase3—267 bp; ecto-5′-nucleotidase—405 bp; β-actin—210 bp. Primers for NTPDase8 (394 bp) were also used in this study. PCR products were submitted to electrophoresis using a 1% agarose gel. Bands intensities were analyzed by Kodak 1D v.3.5.4 software. The following set of primers were used: for NTPDase1: 5′-GAT CAT CAC TGG GCA GGA GGA AGG-3′ and 5′-AAG ACA CCG TTG AAG GCA CAC TGG-3′; for NTPDase2: 5′-GCT GGG TGG GCC GGT GGA TAC G-3′ and 5′-ATT GAA GGC CCG GGG ACG CTG AC-3′; for NTPDase3: 5′-CGG GAT CCT TGC TGT GCG TGG CAT TTC TT-3′ and 5′-TCT AGA GGT GCT CTG GCA GGA ATC AGT-3′; for ecto-5′-nucleotidase (CD73): 5′-CCC GGG GGC CAC TAG CAC CTC A-3′ and 5′-GCC TGG ACC ACG GGA ACC TT-3′; for β-actin: 5′-TAT GCC AAC ACA GTG CTG TCT GG-3′ and 5′-TAC TCC TGC TTC CTG ATC CAC AT-3′; for NTPDase8: 5′-CCA CAC TGT CAC TGG CTT CCT TG-3′ and 5′-ACG AGG ATG TAT AGG CCT GAG G-3′.

Results

We have evaluated the effect in vitro of AEDs on ATP, ADP, and AMP hydrolysis from synaptosomes of cerebral cortex and hippocampus of naive rats. These experiments were performed in order to elucidate whether these drugs have any direct effect on the enzyme activities that could influence the in vivo experiments. The results have shown that PHT (1–1000 μM), CBZ, and VPA (both 10–1000 μM) have not altered the nucleotidase activities in synaptosomes from hippocampus and cerebral cortex of rats (data not shown). Furthermore, in vivo treatment with PHT, VPA (both doses tested), and CBZ did not promote any effect on the ecto-nucleotidase activities from

synaptosomes of hippocampus and cerebral cortex of naive rats (Fig. 1).

Previous studies from our laboratory have shown increased ecto-nucleotidase activities in female Wistar rats submitted to the pilocarpine model of epilepsy [27]. In order to verify whether male Wistar rats are subjected to similar biochemical changes, we have studied the influence of the pilocarpine model of epilepsy on ATP, ADP, and AMP hydrolysis from hippocampal and cerebral cortical synaptosomes. In synaptosomes of hippocampus from male rats, the enzyme activities were increased in 55%, 98%, and 101% for ATP, ADP, and AMP hydrolysis, respectively, at 7–9 days after pilocarpine administration (Fig. 2). The pilocarpine model has also enhanced the ecto-nucleotidase activities by 33% (ATP hydrolysis), 41% (ADP hydrolysis), and 70% (AMP hydrolysis) in cerebral cortical synaptosomes from male rats (Fig. 3).

We have evaluated if AEDs have any effect on the ecto-nucleotidase activities from male rats submitted
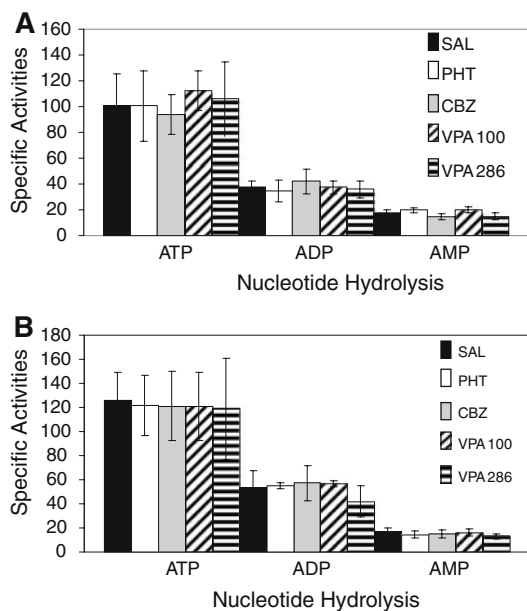

Fig. 1 Effect of PHT (50 mg/kg), CBZ (30 mg/kg) and VPA (100 and 286 mg/kg) ATP, ADP, and AMP hydrolysis from synaptosomes of hippocampus (**A**) and cerebral cortex (**B**). The control activities (SAL) in hippocampal synaptosomes were 100.7 ($\pm$24), 37.9 ($\pm$10.7), and 17.8 ($\pm$4.8) nmol Pi min^{-1} mg^{-1} of protein for ATP, ADP, and AMP, respectively. In synaptosomes of cerebral cortex the control activities (SAL) were 113.8 ($\pm$18.4), 47.6 ($\pm$7.9), and 16.8 ($\pm$3.8) nmol Pi min^{-1} mg^{-1} of protein for ATP, ADP, and AMP, respectively. The data represent a mean $\pm$ SD (n = 5 animals) and were expressed in nmol Pi per minute per mg protein. Statistical analysis were performed by one-way analysis of variance (ANOVA), followed by a Duncan multiple range test, considering $P < 0.05$ as significant (*)
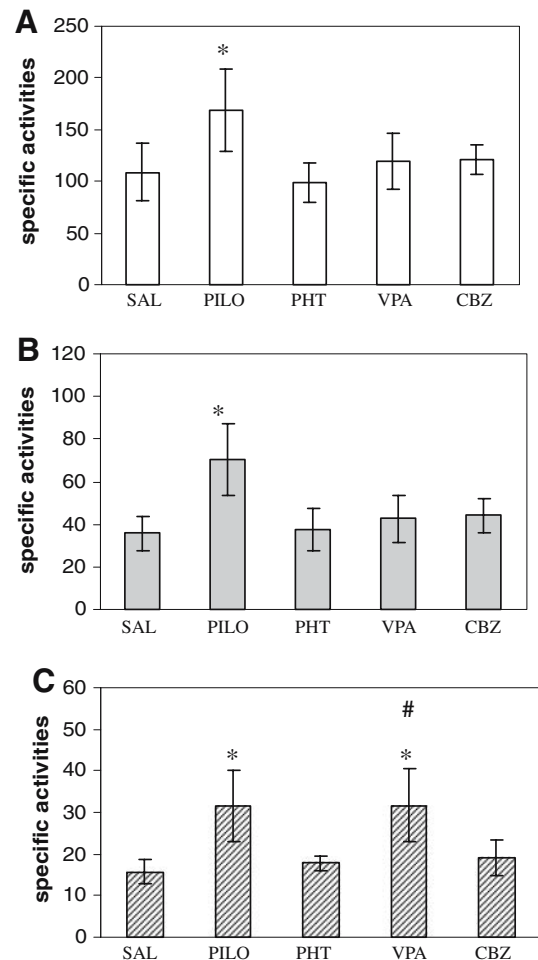
Fig. 2 Influence of PHT (50 mg/kg), VPA (100 mg/kg), and CBZ (30 mg/kg) on ATP (**A**), ADP (**B**), and AMP (**C**) hydrolysis from hippocampal synaptosomes of rats with epilepsy. Control values (SAL) were 109.0 ($\pm$27.6), 35.6 ($\pm$8.3), and 15.6 ($\pm$2.8) nmol Pi min^{-1} mg^{-1} of protein for ATP, ADP, and AMP, respectively. The data represent a mean $\pm$ SD (n = 8 animals) and were expressed in nmol Pi per minute per mg protein. All groups were compared to the control (SAL). *$P < 0.05$, using one-way ANOVA followed by Duncan multiple range test. #Represents $P > 0.05$ when compared to pilocarpine group (PILO)

to the pilocarpine model. Our results have shown that CBZ and PHT can prevent the pilocarpine-induced increase on ATP, ADP, and AMP hydrolysis in synaptosomes from hippocampus (Fig.2) and cerebral cortex (Fig. 3). VPA (at 100 mg/kg) has prevented only ATP and ADP hydrolysis in synaptosomes from hippocampus of pilocarpine-treated rats (Fig. 2A, B). In cerebral cortex, increased ATP, ADP, and AMP hydrolysis induced by pilocarpine were not prevented by VPA administration (100 mg/kg) (Fig. 3). We have also verified the effects promoted by VPA in the dose related to ED50 for pilocarpine-induced seizures
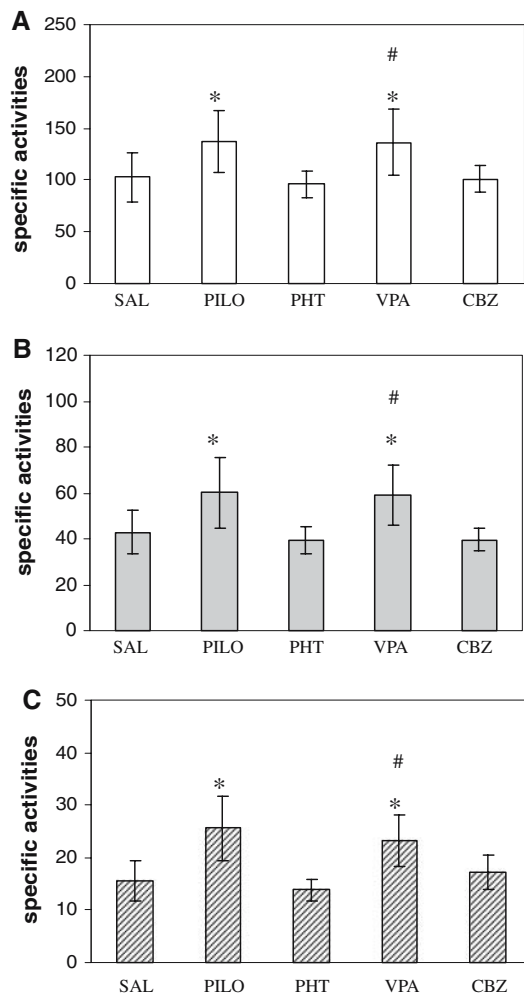
Fig. 3 Influence of PHT (50 mg/kg), VPA (100 mg/kg), and CBZ (30 mg/kg) on ATP (**A**), ADP (**B**), and AMP (**C**) hydrolysis from cerebral cortical synaptosomes of rats with epilepsy. Control values (SAL) were 102.8 ($\pm$24.0), 42.7.6 ($\pm$9.3), and 15.5 ($\pm$3.9) nmol Pi min^{-1} mg^{-1} of protein for ATP, ADP and AMP, respectively. The data represent a mean $\pm$ SD (n = 8 animals) and were expressed in nmol Pi min^{-1} mg^{-1} protein. All groups were compared to the control (SAL). *P < 0.05, using one-way ANOVA followed by Duncan multiple range test. #Represents P > 0.05 when compared to pilocarpine group (PILO)

(286 mg/kg, i.p.). There were no significant differences in nucleotide hydrolysis between animals with more or less intense seizures after pretreatment with 286 mg/kg VPA. Our results have also shown that the VPA dose related to ED50 did not prevent the changes observed on ATP (138.75 $\pm$ 12.23 nmol Pi min^{-1} mg^{-1} protein), ADP (86.35 $\pm$ 7.66 nmol Pi min^{-1} mg^{-1} protein), and AMP hydrolysis (25.10 $\pm$ 1.4 nmol Pi min^{-1} mg^{-1} protein) after the induction of the pilocarpine model in synaptosomes from cerebral cortex when compared to control values (92.39 $\pm$ 20.57; 44.61 $\pm$ 10.86;

14.36 $\pm$ 3.3 nmol Pi min^{-1} mg^{-1} protein for ATP, ADP, and AMP hydrolysis, respectively). In synaptosomes from hippocampus, we have observed that VPA dose related to ED50 prevented the changes on ATP hydrolysis (127.1 $\pm$ 16.7 nmol Pi min^{-1} mg^{-1} protein) observed in the pilocarpine group (178.3 $\pm$ 32.2 nmol Pi min^{-1} mg^{-1} protein). However, in relation to ADP hydrolysis, it has been observed a trend toward the prevention induced by VPA (60.6 $\pm$ 10 nmol Pi min^{-1} mg^{-1} protein) on changes promoted by pilocarpine (80.8 $\pm$ 9.5 nmol Pi min^{-1} mg^{-1} protein). Changes induced by pilocarpine on AMP hydrolysis (42.47 $\pm$ 9.1 nmol Pi min^{-1} mg^{-1} protein) were not prevented by VPA at 286 mg/kg (33.4 $\pm$ 2.3 nmol Pi min^{-1} mg^{-1} protein).

The relative expression of enzymes has also been analyzed by semi-quantitative RT-PCR. NTPDase8 was not expressed in hippocampus and cerebral cortex of rats, which was in accordance to previous studies [36]. The relative expression of NTPDase1, NTP-Dase2, NTPDase3, and ecto-5′-nucleotidase were not altered by the treatment with pilocarpine in cerebral cortex (data not shown). However, in hippocampus of rats submitted to pilocarpine, there was an increase in relative expression of NTPDase2 (28%), NTPDase3 (28%), and ecto-5′-nucleotidase (54%) (Fig. 4). NTP-Dase1 expression was not altered after pilocarpine treatment in hippocampus. In order to verify whether or not AEDs have any influence on enzyme expressions, we have also analyzed the effect of the prior treatment with CBZ (30 mg/kg), PHT (50 mg/kg), and VPA (100 mg/kg) in the relative expression of pilocarpine-treated rats (Fig. 5). Previous treatments with PHT and VPA were able to prevent the increase in relative expression of NTPDase2 from hippocampus of pilocarpine-treated rats (Fig. 5A). Furthermore, CBZ treatment was able to prevent the increase in relative expression of NTPDase3 (Fig. 5B) and ecto-5′-nucle-otidase (Fig. 5C).

Discussion

A study from our laboratory has shown increased brain ecto-nucleotidases activities in the pilocarpine model of epilepsy, mainly during 7–9 days after the convulsant administration [27]. Unlike adult rats, synaptosomal ecto-nucleotidases from young rats were not increased 7–9 days after pilocarpine treatment [37]. In addition, other significant difference is that pilocarpine-treated young rats did not present recurrent spontaneous seizures, a feature of the chronic period

Fig. 4 Representative semi-quantitative RT-PCR mRNA for NTPDase1, 2, 3, and ecto-5′-nucleotidase from hippocampus of saline-treated (**A**) and pilocarpine-treated (**B**) rats. The expression was evaluated by ecto-nucleotidases to *β*-actin mRNA ratio. Three independent experiments were performed with entirely consistent results
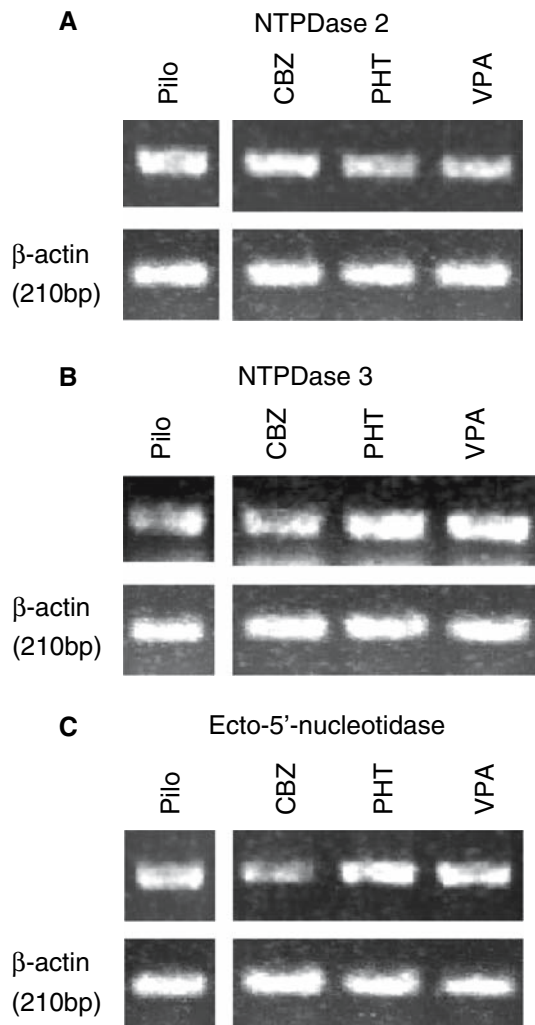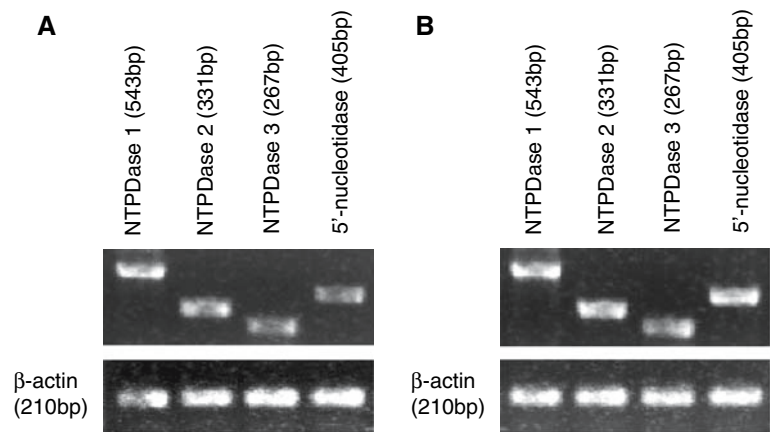
Fig. 5 Representative semi-quantitative RT-PCR mRNA for NTPDase 2 (**A**), NTPDase 3 (**B**), and ecto-5′-nucleotidase (**C**) from hippocampus of pilocarpine-treated rats or pilocarpine-treated rats submitted to previous therapy with PHT (50 mg/kg), CBZ (30 mg/kg), and VPA (100 mg/kg). The expression was evaluated by ecto-nucleotidases to *β*-actin mRNA ratio. Three independent experiments were performed with entirely consistent results

of pilocarpine model in adult rats [38]. Therefore, the importance of the silent period has been stressed by many groups because there is a great deal of evidence that in this phase several molecular changes occur and may contribute to the process of epileptogenesis [6, 7]. Studies on the silent period seem to be more critical since there is a good chance that molecular mechanisms could block plastic changes and could also suppress the development of epilepsy [39].

The interaction between AEDs and the pilocarpine model of epilepsy has been extensively studied. According to literature, CBZ (up to 50 mg/kg) and PHT (up to 200 mg/kg) were totally ineffective against pilocarpine-induced SE. In contrast, VPA inhibited pilocarpine-induced convulsions with an ED50 of 286 mg/kg [29]. Also, there is evidence that the chronic treatment with CBZ (40 mg/kg, 3 times daily during 56 days) was not effective against pilocarpine-induced epileptogenesis [40]. Our objective was to elucidate whether or not AEDs were able to modulate the changes induced by pilocarpine model on ecto-nucleotidase activities. Therefore, sub-effective doses of PHT, VPA, and CBZ were chosen since ATP, ADP, and AMP hydrolysis were not affected by these treatments (Fig. 1). Our results have shown that PHT and CBZ can prevent the increase in ecto-nucleotidase activities from synaptosomes of epileptic rats eventhough neither avoided the motor limbic seizures, a typical feature of SE induced by pilocarpine.

There is some evidence that AEDs can interfere in purinergic transmission in the CNS [9]. In fact, PHT can act as an inhibitor of the adenosine reuptake, but this effect is observed in high and non-therapeutic doses [11, 41]. Therefore, our results have suggested that PHT also could control adenosine levels by preventing the increase on ecto-nucleotidase activities promoted by pilocarpine.

It has been shown that CBZ can inhibit the in vitro activity of ecto-ATPase from synaptosomal plasma membranes of rat brain [42]. However, we have observed that CBZ treatment has not been able to change ATP, ADP, and AMP hydrolysis from brain synaptosomes of naive rats. In addition, several studies have shown that CBZ can act as an antagonist of adenosine A_1 receptors and induce up-regulation of these receptors in the brain [13, 14, 43]. It has been reported that the anticonvulsant action of adenosine is mediated by activation of adenosine A_1 receptors [19, 44], and the levels of these receptors are enhanced in the cerebral cortex and hippocampus of epileptic rats [45]. However, studies have shown a lower density of hippocampal adenosine A_1 receptors in electric-kindled or kainate-injected rats [46, 47]. In spite of the neuroprotective role of adenosine, under certain circumstances, this nucleoside could have an opposite effect contributing to neuronal damage and death [15]. Thus, the increase in the adenosine production induced by pilocarpine could be an important biochemical change as consequence of adaptive plasticity of the model, rather than a neuroprotective mechanism. In this study, the preventive effect of CBZ on the increased nucleotidase activities induced by pilocarpine reinforces the idea that these changes in the enzyme activities are a consequence of the plasticity promoted by the model.

We also have investigated the effect of VPA (100 mg/kg) on the nucleotidases from synaptosomes of hippocampus and cerebral cortex of rats submitted to pilocarpine model. This drug was able to prevent the increase of ATP and ADP hydrolysis of hippocampal synaptosomes, but not the increase in ecto-5′-nucleotidase. Similar effects occurred when animals were pretreated with 286 mg/kg of VPA in hippocampal synaptosomes of rats submitted to pilocarpine, except for ADP hydrolysis, which presented a trend toward to prevention of the pilocarpine-induced effects. ATP, ADP, and AMP hydrolysis from synaptosomes of cerebral cortex did not return to control levels after VPA administration in both doses tested. The mechanism of action of VPA is similar to PHT and CBZ, involving the blockade of voltage-gated Na^+ channels [48]. Pilocarpine administration induces hippocampal mossy fibers sprouting and a marked cellular death in CA1, CA3 regions, and dentate gyrus [4] and the initial firing of epileptiform discharges occurs in hippocampal region diffusing to cerebral cortex and amygdala [5]. This fact indicates there are differences between hippocampus and cerebral cortex in relation to the development of the model and, consequently, the susceptibility of ecto-nucleotidases to VPA in different

brain regions. Furthermore, doses of VPA tested could not be sufficient to prevent the increase on ecto-nucleotidase activities from cerebral cortical synaptosomes and ecto-5′-nucleotidase from hippocampal synaptosomes of pilocarpine-treated rats. Previous studies have tested the effect of conventional AEDs on the recurrent spontaneous seizures in the pilocarpine model of epilepsy, which VPA was effective at a dose of 600 mg/kg [49]. However, our study has been carried out in the silent phase of pilocarpine model of epilepsy, a critical phase for the development of spontaneous recurrent seizures characteristic of the chronic period.

The kinetic effect observed could be a consequence of transcriptional control and/or post-translational mechanisms. It is known that expression of several enzymes can be altered by this model [24–26]. Therefore, we have studied the relative expression of NTPDases1, NTPDase2, NTPDase3, and ecto-5′-nucleotidase from cerebral cortex and hippocampus of pilocarpine-treated rats. No alteration in relative expression of all enzymes in cerebral cortex was observed, whereas NTPDase2, NTPDase3, and ecto-5′-nucleotidase expressions were increased in hippocampus of pilocarpine-treated rats. The major hippocampal susceptibility to pilocarpine [5] could explain the increased relative expression observed in this structure. The influence of AEDs at transcriptional level of hippocampal NTPDase 2, NTPDase3, and ecto-5′-nucleotidase was also demonstrated. NTPDase3 and ecto-5′-nucleotidase transcription was regulated by CBZ and NTPDase2 by PHT and VPA. It has been demonstrated that AEDs could act direct or indirectly regulating gene expression. For example, PHT was able to promote keratinocyte growth factor receptor expression by more than 150% [50], whereas CBZ and VPA were inhibitors of histone deacetylases (HDAC), which is an important regulator of gene expression [51].

The other plausible explanation for the changes in the enzyme activities after pilocarpine treatment may involve post-translational events. According to analysis performed in NetPhosk, a kinase-specific prediction of protein phosphorylation sites tool (http://www.cbs.dtu.dk/), NTPDases1, NTPDase2, NTPDase3, and ecto-5′-nucleotidase sequences present possible PKC phosphorylation sites. In fact, the expression of different isoforms of protein kinase C (PKC) is upregulated by pilocarpine treatment [52]. This fact leads us to the hypothesis that phosphorylation may exert a modulation on these enzyme activities in cerebral cortex and hippocampus of rats. Based on the different expression profiles observed in these

brain structures, it is possible to suggest that increased relative expression of hippocampal NTPDase2, NTPDase3, and ecto-5′-nucleotidase may act in synergy with post-translational events, such as phosphorylation, in order to up-regulate ecto-nucleotidase activities.

In summary, the findings reported here have shown that anticonvulsant drugs can modulate plastic events related to the increase of nucleotidase expression and activities in pilocarpine-treated rats. Our results also contribute to a better understanding about the pharmacology of classical AEDs and their interaction with purinergic neurotransmission.

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