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ScienceDirect
Pharmacology, Biochemistry and Behavior 84 (2006) 511–516
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
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Anticonvulsant effect of the calcineurin inhibitor ascomycin on seizures induced by picrotoxin microperfusion in the rat hippocampus

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Received 18 January 2006; received in revised form 13 June 2006; accepted 21 June 2006
Available online 26 July 2006

Abstract

The potential in vivo anticonvulsant effect of calcineurin (protein phosphatase 2B) inhibitor ascomycin against seizures induced by intrahippocampal microdialysis of picrotoxin was examined in the present study. After establishing individual picrotoxin seizure thresholds, ascomycin was continually microperfused into the rat hippocampus through microdialysis probes at concentrations 10, 50 and 100 μM. No behavioral or electroencephalographic effects were observed during microperfusion of ascomycin alone. Low concentrations (10 μM) of ascomycin did not prevent picrotoxin seizures, however, 50 and 100 μM ascomycin showed antiepileptic effect, completely suppressing seizures in 41.7% and 75% of the animals studied respectively. Mean seizure duration and mean number of seizures were significantly reduced ($P<0.01$) by microperfusion of 100 μM ascomycin. Calcineurin activity might be involved in the biochemical changes leading to picrotoxin-induced epileptic seizures. The present findings provide additional in vivo evidence of the involvement of phosphorylation/dephosphorylation mechanisms in the development of epileptic seizures, suggesting that calcineurin modulation may be a possible strategy in the search for new anticonvulsant drugs.

Keywords: Ascomycin; Calcineurin; Hippocampus; Microdialysis; Picrotoxin; Seizures

1. Introduction

Calcineurin (protein phosphatase 2B) is a calcium/calmodulin-dependent phosphatase highly enriched in neural tissue (Pallen and Wang, 1985). Calcineurin-mediated dephosphorylation is an important modulatory factor in several cellular processes, including development of learning and memory (Riedel, 1999), regulation of neuronal plasticity (Groth et al., 2003) and induction of apoptosis (Springer et al., 2000). Additionally, calcineurin may regulate the activity of the $GABA_A$ receptor (Amico et al., 1998; Huang and Dillon, 1998) and the NMDA (Tong et al., 1995; Shi et al., 2000) and AMPA (Beattie et al., 2000; Lin et al., 2000) glutamate receptors. Within neurons, calcineurin is predominantly found at the post-synaptic densities and cell soma (Groth et al., 2003), but the particular targets of calcineurin dephosphorylation following synaptic activity depend upon the subcellular localization of the different pools of calcineurin in relation to the Ca^{2+} signal.

Much research has focused in the role of intracellular Ca^{2+} concentration mediated by *N*-methyl-D-aspartate receptor (NMDAR) receptor activation in epilepsy. In several animal models, prolonged activation of the NMDAR-Ca^{2+} transduction pathway induces long-lasting plasticity changes in hippocampal neurons causing increased excitability leading to the occurrence of recurrent epileptiform discharges (Dingledine et al., 1990; DeLorenzo et al., 1998, 2005). Alterations in calcium-regulated systems and loss of calcium homeostasis have also been implicated in many pathological conditions, such as ischemia (Choi, 1988; Parsons et al., 1997, 1999), traumatic brain injury (Rzigalinski et al., 1998), status epilepticus (Pal et al., 1999; Parsons et al., 2000) and hypoxia-induced seizures (Sanchez et al., 2005). Specifically, influx of calcium through the NMDA subtype of glutamate receptor is suspected to be important for physiological changes occurring after status epilepticus (Rice and De-Lorenzo, 1998; Pal et al., 1999). NMDA-linked increases in intracellular calcium affect a number of calcium-

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0091-3057/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2006.06.015

controlled cellular mechanisms and enzymes including calcineurin (Montoro et al., 1993; Lieberman and Mody, 1994). Furthermore, calcineurin inhibitors such as FK 506 and cyclosporin have been proposed as neuroprotectants in neurodegenerative disorders associated with acute brain ischemia (Sharkey et al., 2000; Sanchez et al., 2005).

However, the role of calcineurin in the development and propagation of epileptic seizures is still controversial. Moia et al. (1994) showed that the concentration of calcineurin in the brain of rats, as detected immunohistochemically, increases after completion of electrical kindling, and two calcineurin inhibitors, FK 506 and cyclosporin A, inhibit progression of electrical kindling in rats. Kurz et al. (2001) have reported a significant increase in calcineurin activity in the rat pilocarpine model of status epilepticus, occurring through a NMDA-dependent mechanism. Sanchez et al. (2005) have shown that GABAergic synaptic transmission is downregulated by calcineurin after seizures in the developing rat brain. However, Suzuki et al. (2001) have reported a facilitation in pentylenetetrazol-induced chemical kindling in rats treated with FK 506, and a decreased expression of calcineurin mRNA in the mouse hippocampus after kainic acid-induced seizures has been reported (Solá et al., 1998). Recently, Misonou et al. (2004) have shown that in the kainate model of continuous seizures in the rat, a calcineurin-dependent loss of voltage-dependent Kv2.1 potassium channel clustering is observed, suggesting an important link between calcium influx leading to calcineurin activation and the intrinsic excitability of pyramidal neurons.

The aim of this study is to improve the knowledge about the possible role of calcineurin in epileptic seizures induced in vivo by picrotoxin microdialysis in the hippocampus of freely moving rats. For this purpose we have used a whole-animal model (Sierra-Paredes and Sierra-Marcuño, 1996) in which partial seizures can be elicited repeatedly on different days without changes in threshold or seizure patterns. Picrotoxin seizure thresholds remain constant in the same animal in repeated experiments for time periods as long as six months (Sierra-Paredes and Sierra-Marcuño, 1996), thus providing a good model to study possible modifications in neuronal excitability.

Ascomycin is a FK 506 analog which inhibits calcineurin phosphatase in the nanomolar range (Meadows et al., 1993). We used ascomycin for inhibiting calcineurin activity because of good inhibitory action and solubility in an aqueous medium for an adequate perfusion through microdialysis probes. Picrotoxin and ascomycin were dialyzed through the probe to avoid possible dynamic effects imposed by the blood–brain barrier on some systemic administrated drugs (Aguilar-Veiga et al., 1991). Our method is completely reversible, thus permitting the independent study in the same animal of the effect of different ascomycin concentrations.

2. Experimental procedure

2.1. Animals and surgical procedure

Eight male Sprague–Dawley rats, initially weighing 250–300 g were used. They were housed in groups of three under controlled environmental conditions (ambient temperature 21 ±1 °C, humidity 50–60%, 12:12 h light/dark cycle) with free access to food and water except during testing. Rats were obtained from the animalary of the University of Santiago. All experiments were performed in a laboratory under controlled environmental conditions and at the same time in the morning in order to avoid circadian variations. All efforts were made to minimize animal suffering, and the animal protocols were designed to reduce the number of animals used (Sierra-Paredes and Sierra-Marcuño, 1996). Animal care complied with Spanish legislation on Protection of Animals Used in Experimental and Other Scientific Purposes, and with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The rats were anaesthetized with pentobarbital (Sigma St Louis, MO, USA; 40 mg/kg, injected intraperitoneally) and placed in a stereotaxic instrument (D. Kopf, Tujunga, CA, USA). Under aseptic conditions, 2 stainless steel microscrews to be used as electrodes for EEG recording were positioned in the skull above the frontal and occipital areas of each hemisphere; one screw, used as a reference electrode, was anchored in the mid-line, 7–9 mm rostrally to the coronal suture. The intracerebral guide for the microdialysis probe (CMA/12, CMA/Microdialysis AB, Stockholm, Sweden) was sterilized with 70% ethanol, rinsed in sterile saline and was implanted vertically into the ventral hippocampus. Stereotaxis coordinates derived from the atlas of Paxinos and Watson (1997) were 5 mm posterior, 4.8 mm lateral and 4 mm ventral for the tip of the cannula relative to bregma and dural surface. Wires from the microscrews were soldered to a miniature plug (Cannon MD1-9SL1, USA) and fixed firmly to the skull with dental cement. After surgery, the rats were placed in individual cages and received intramuscular amoxicillin therapy (10 mg/kg every day) for 4–5 days.

2.2. Microdialysis and EEG recording

The experiments were carried out on conscious, freely moving rats 10 days after surgery. From the fourth day the animals were placed for 3 h daily in the experimental unit for habituation and EEG control of wakefulness and sleep activity. Bipolar cortical EEGs were recorded on magnetic tapes using a Holter-EEG system (Oxford-Medilog 9200, Oxford, U.K.), and also with a Minihuit electroencephalograph (Alvar Electronic, Paris, France).

During an experimental session recording time was distributed as follows:

a) A 15 min reference EEG was recorded before every probe introduction.
b) A 120 min basal control EEG. This long control period was chosen to let the animal recover from possible local modifications induced by the tip of the probe.
c) A 60 min post-picrotoxin (Sigma) microperfusion control.

All habituation and experimental sessions were recorded on videotape using a standard camera in order to relate behaviorally observed seizures with the EEG recordings. In our experiments, seizure threshold was defined as the lowest picrotoxin

concentration which produced a specific EEG pattern and/or behavioral seizures in a particular rat after 5 min perfusion through the rat hippocampus. Only one picrotoxin dose was perfused in each experimental session. The lowest picrotoxin concentration used was 100 μM, and the dose was slowly increased (+25 μM each step) in each animal in successive experimental sessions at 3–4 day intervals until an EEG-behavioral seizure was induced. This picrotoxin concentration was the threshold dose. Seizure types and rest periods between experimental sessions were described previously in detail (Sierra-Paredes and Sierra-Marcuño, 1996).

We used a CMA/120 system for freely moving animals (CMA/Microdialysis AB, Stockholm, Sweden) and CMA/12 microdialysis probes with 4 mm of membrane length. The probe was connected via polyethylene tubing to a syringe selector (CMA/111), and to 1 ml syringes mounted on a micro-injection pump (CMA/100). Before starting each experiment, the probe was perfused with ethanol and distilled water. After checking the integrity of the probe under light microscopy, it was perfused with a sterile Ringer's solution (NaCl 147 mM, KCl 4.0 mM, $CaCl_2$ 2.4 mM) for 10 min, and then introduced into the rat hippocampus through the chronically implanted intracerebral guide. Between re-use, the probe was maintained in distilled water, and before every introduction it was sterilized and the integrity of the dialysis membrane was checked. A detailed description of the whole-animal model and the method to induce seizures is presented elsewhere (Sierra-Paredes and Sierra-Marcuño, 1996).

For the control experiments, Ringer's solution was perfused at a constant flow rate of 2 μl/min during 2 h. Then, picrotoxin dissolved in Ringer's solution was perfused at the same rate during 5 min. After picrotoxin administration, the perfusion of Ringer's solution continued for one more hour (Table 1).

Ascomycin (Sigma) was dissolved in Ringer and perfused continuously throughout the experiment in all the animals on different days at 10, 50 and 100 μM concentrations in a random order, following the same protocol for Ringer's solution and picrotoxin administration in the control experiments (Table 1). Each dose was administered once in each animal with resting periods between experiments of at least one week during a total period of 2–3 months.

Threshold control experiments were performed on all animals to ensure that no permanent modification had been induced in the duration or number of seizures using the same picrotoxin dose. After finishing ascomycin administration, frequent 3 h EEG controls (2–3 times a week,) with simultaneous video recording were performed in all animals without probe introduction, in order to monitor possible long-term effects of ascomycin and the picrotoxin/ascomycin combination.

At the end of the experiments rats were anaesthetized with Nembutal and killed by decapitation. A probe was introduced and perfused with Sudan black to localize easily the position of the probe. The brain was then removed and placed in 4% phosphate buffered formaldehyde solution. A week later 50 μm coronal sections were cut and stained with cresyl violet, and the position of the probe was checked under light microscopy.

Table 1
Experimental protocol for the microdialysis of picrotoxin and ascomycin

Time (min)				
0	15	135	140	200
Picrotoxin experiments	Probe insertion Ringer	Picrotoxin	Ringer	End
Ascomycin experiments	Probe insertion Ascomycin	Picrotoxin EEG /video	Ascomycin	End

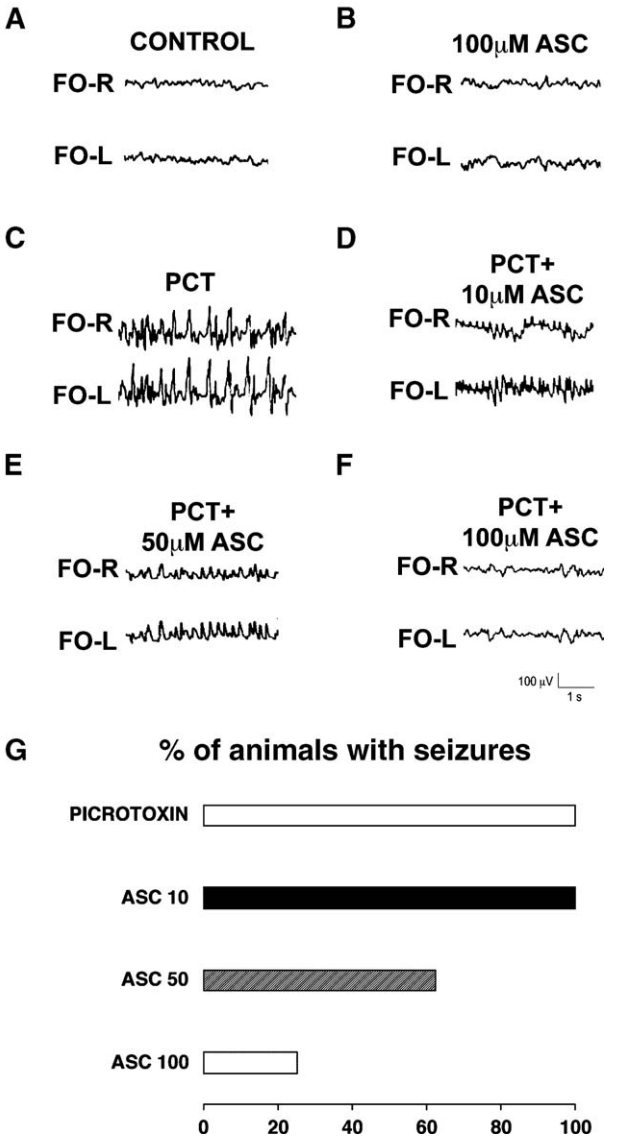

Fig. 1. A: Control EEG recording from a rat. B: EEG recording during ascomycin microperfusion (100 μM). C: EEG recording after intrahippocampal microperfusion of picrotoxin (seizure induced with a threshold dose). D: EEG recording from a rat treated with a picrotoxin threshold dose and 10 μM ascomycin E: EEG recording from a rat treated with a picrotoxin threshold dose and 50 μM ascomycin. F: EEG recording from a rat treated with a picrotoxin threshold dose and 100 μM ascomycin. G: Percentage of animals showing seizures after picrotoxin and ascomycin administration. EEG records are from the same animal using the same picrotoxin dose on different days. FOR: Fronto-occipital right. FOL: Fronto-occipital left.

2.3. Data analysis

EEG records were analyzed using the Medilog 9200 software, version 7.2. Wakefulness, somnolence, and sleep EEG activity (sleep spindles and slow wave sleep) were measured as a percentage of total time in the control record. Spike and wave discharge duration, seizure duration, and seizure onset and offset times were evaluated after picrotoxin and ascomycin administration. Statistical significance of the difference in duration, total time of seizures and seizure onset and offset times was determined by Student's paired *t*-test. All results with $P<0.01$ were considered significant.

3. Results

Our results on individual seizure thresholds and seizure types were consistent with those described previously (Sierra-Paredes and Sierra-Marcuño, 1996). Seizure threshold among animals varied between 100 and 250 μM picrotoxin, although it remained unchanged in repeated between-day experiments within individual rats. The lowest seizure threshold induced three types of individual seizures: I) arrest behavior (absence-like); II) arrest behavior followed by facial clonus, alimentary automatisms and clonus of the contralateral forepaw; III) rearing with alimentary automatisms, weak facial clonus and forelimb clonus. Latency for the lowest seizure threshold as measured from the end of the 5 min picrotoxin microperfusion to the start of electro-behavioral seizures showed a range from 14 to 36 min after the end of picrotoxin perfusion. However, latency did not show significant variation in repeated experiments with individual rats.

Ascomycin microperfusion in the hippocampus of freely moving rats (10, 50 and 100 μM) did not change the electroencephalographic activity and there was no difference in comparison with Ringer solution perfused controls (Fig. 1). No changes in the sleep/wakefulness cycle were observed during ascomycin microperfusion. No behavioral or electroencephalographic chronic effects (up to four months after the last ascomycin administration) were observed in rats receiving picrotoxin alone or ascomycin and picrotoxin, except for two animals which presented seizures during ascomycin 100 μM administration (Fig. 1), when a total of three spontaneous seizures were observed in the subsequent inspection of the animals.

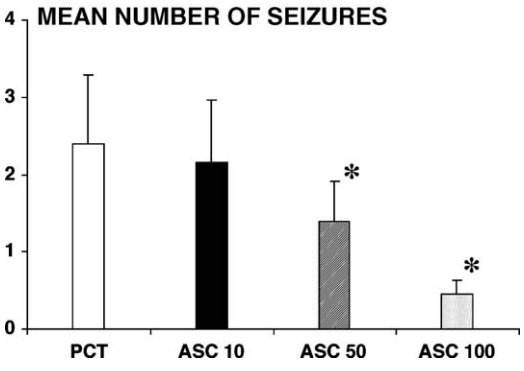

Fig. 2. Mean number of seizures after picrotoxin administration (threshold dose) and 10, 50 and 100 μM ascomycin. Data are mean±S.D. ($n=8$) *$P<0.01$ by Student's paired *t*-test.

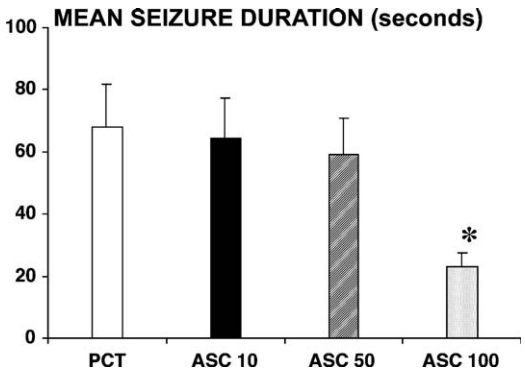

Fig. 3. Mean seizure duration (s) after picrotoxin administration (threshold dose) and 10, 50 and 100 μM ascomycin. Data are mean±S.D. ($n=8$) *$P<0.01$ by Student's paired *t*-test.

10 μM ascomycin did not show anticonvulsant effect (Fig. 1). When ascomycin 10 μM was perfused, no significant reduction in the mean number of seizures (from 2.38±0.52 to 2.16±0.63; $P>0.05$; Fig. 2) or seizure duration (from 68± 17.5 s to 64.3 ±14.7; $P>0.05$; Fig. 3) were observed. However, 50 μM ascomycin completely prevented picrotoxin-induced seizures in the 41.7% of the rats (Fig. 1), and significantly reduced the mean number of seizures (1.400±3, $P<0.01$; Fig. 2) No significant differences were found in mean seizure duration (59±14.1 s, $P<0.01$; Fig. 3) with this dose. 100 μM ascomycin completely suppressed seizures in 75% of the animals (Fig. 1), and significantly reduced seizure duration and severity in non-protected rats, mean number of seizures (0.46± 0.12; $P<0.01$; Fig. 2) and mean seizure duration (23±6.9 s; $P<0.01$; Fig. 3) were also significantly decreased by 100 μM ascomycin. Ascomycin treatment did not modify seizure latency at any of the doses studied.

4. Discussion

This study reports on the previously uninvestigated in vivo effect of ascomycin on picrotoxin-induced seizures. We have found that ascomycin shows anticonvulsant effect against picrotoxin seizures when perfused into the rat hippocampus at 50 and 100 μM concentrations. No effects were observed with a 10 μM dose. Previous studies have been performed in order to determine the pro- or anticonvulsant effect of several calcineurin inhibitors (Moia et al., 1994; Suzuki et al., 2001; Sanchez et al., 2005), however, they have been limited by the complications of systemic administration such as blood–brain barrier transport and brain tissue distribution. The present study is the first to use direct application to the hippocampus of awake rats, thus allowing the maximum inhibitor concentration at the seizure focus rather than distributed over the large brain areas. Continuous microperfusion permits also to keep a steady extracellular ascomycin concentration over the microdialysis period, minimizing the effect of individual differences in absorption and clearance of the enzyme inhibitor. However, the

possibility of chemical interaction among ascomycin and picrotoxin will have to be excluded in further research.

Furthermore, the previously investigated kindling (Moia et al., 1994), kainate (Solá et al., 1998; Misonou et al., 2004) and hypoxia (Sanchez et al., 2005) models of epilepsy induce neuronal damage in the hippocampus such as neuronal loss followed by reorganization of the circuitry during the epileptogenic phase (Scharfman, 2002) which could affect significantly calcineurin enzymatic activity through a secondary mechanism.

Calcineurin enzymatic activity has been related to epileptic seizures in several animal models. Kurz et al. (2001) demonstrated a significant increase in calcineurin activity in cortical and hippocampal homogenates during status epilepticus induced by pilocarpine. However, results obtained from status epilepticus are difficult to extrapolate to single acute seizures. Calcineurin is a calcium/calmodulin-stimulated enzyme (Klee et al., 1998) and would be stimulated by increased intracellular calcium concentrations. An increase in intracellular free calcium has been found during and after status epilepticus (Pal et al., 1999). The increased intracellular calcium associated with status epilepticus could be responsible for activating calcineurin above its normal physiological level, because status epilepticus induces a loss of function of the endoplasmic reticulum Mg^{2+}/Ca^{2+} ATPase (Parsons et al., 2000). This enzyme sequesters calcium ions into the microsomes of the smooth endoplasmic reticulum, providing a high affinity mechanism for regulating intracellular calcium concentration (Carafoli, 1987; Miller, 1991). After status epilepticus, ATPase-mediated uptake of calcium into the microsomes is less efficient (Parsons et al., 2000), which could potentially result in higher than normal resting calcium concentrations inside the cell, affecting the status epilepticus-induced increase in calcineurin dephosphorylation.

We have previously reported that reduced PKA-mediated phosphorylation protects against picrotoxin-induced seizures (Vázquez-López et al., 2005). The results presented here show that restoring phosphorylation by inhibiting calcineurin phosphatase action also has an anticonvulsant effect. We can only speculate that, during acute picrotoxin seizures, PKA-mediated phosphorylation mechanisms might be involved in facilitating excitatory neurotransmission, while calcineurin-mediated dephosphorylation may affect mainly to $GABA_A$ receptors.

In our model, neuronal mechanisms which regulate calcineurin dephosphorylation under normal conditions could be highly altered as a result of picrotoxin-induced impairment in GABAergic activity. One important calcineurin-mediated mechanism is modulation of the $GABA_A$ receptor. $GABA_A$ receptors are the primary receptors responsible for the fast inhibitory response in neuronal tissue (Macdonald and Olsen, 1994), and play a major role in preventing the neuronal hyperexcitability associated with epilepsy. Several recent studies have demonstrated an inhibitory modulation of $GABA_A$ receptor function by calcineurin (Chen and Wong, 1995; Amico et al., 1998; Lu et al., 2000). Calcineurin activity might be involved in the biochemical changes leading to picrotoxin-induced epileptic seizures, because picrotoxin binding to $GABA_A$ receptors results in a net disinhibition of cellular excitability and may lead to increased dephosphorylation. Thus, calcineurin inhibition might favor GABAA receptor activation antagonizing the effect of picrotoxin.

In two animals which had seizures under strong calcineurin inhibition (100 μM), we observed late spontaneous seizures. This suggests that eventhough calcineurin inhibition shows an acute antiepileptic effect, some calcineurin activity is required in order to prevent long-term changes leading to epileptogenesis, in accordance to the data shown by Suzuki et al. (2001). Thus, a possible explanation of previously contradictory results may lie in the different calcineurin-related biochemical responses related to inhibitory and excitatory activity. Calcineurin inhibitors might acutely potentiate GABAergic transmission but, when excessive excitatory activity induces massive Ca^{2+} entry through NMDA or Ca^{2+}-permeable AMPA receptors (Sanchez et al., 2005), some calcineurin activity may be needed to prevent the activation of long-term molecular cellular mechanisms (Lieberman and Mody, 1994) leading to the sustained recurrent excitatory activity which induces late spontaneous seizures.

This study provides additional evidence of the involvement of phosphorylation/dephosphorylation mechanisms in the development of acute epileptic seizures. Our results show in vivo that calcineurin participates in the mechanisms of picrotoxin-induced epileptic seizures in the rat hippocampus, and suggest that calcineurin modulation may be a possible strategy in the search for new anticonvulsant drugs.

Further research in chronic experimental models of epilepsy will be required to determine the effect of long-term calcineurin inhibition and the relationships among calcineurin and aminoacid neurotransmitter receptors during epileptic seizures.

Acknowledgements

The authors gratefully acknowledge the work of the laboratory of Neuroanatomy (Director: Prof. J.L. Labandeira) who performed the histological study and Prof. J.L. Otero-Cepeda (Department of Statistics) for statistical analysis.

This work was supported by grant XUGA PGIDIT03PXIB20803PR from the Consellería de Educación e Ordenación Universitaria, Xunta de Galicia, Galicia, Spain.

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