

Calpain inhibitor I retards seizure offset in the hippocampus of freely moving rats

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Abstract

The implications of increased calpain-mediated proteolysis during epileptic seizures are still unclear, and in this study we investigate the effect of the continuous perfusion of calpain inhibitor I on picrotoxin-induced seizures in chronic freely moving rats. Continuous intrahippocampal microperfusion of 500 μ M calpain inhibitor I had no effect on basal EEG, but doubled ($P < 0.05$) average seizure duration, and increased more than five-fold ($P < 0.01$) the total seizure time and three-fold ($P < 0.01$) the seizure offset time compared to picrotoxin alone, in each individual rat. However, seizure type and onset time were not modified by calpain inhibitor I. These results indicate that a calpain-mediated mechanism may be responsible for seizure offset, probably through AMPA glutamate receptors internalization and further degradation. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

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Calpain is a calcium-dependent neural protease which has been shown to play an important role in synaptic plasticity, and to be necessary for long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus [1,12]. These effects are probably related to neural pathology involving excitatory amino acid induction of hippocampal damage [19]. Calpain activation has been attributed to the increase of intracellular calcium through *N*-methyl-D-aspartate (NMDA) receptor activity, and can produce rapid modifications in the properties of the subunits of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) glutamate receptors [4,10]. The NMDA receptor-induced alterations are reportedly involved both in kindling [14] and in the hyperexcitability preceding seizure activity [5].

Calpain inhibitor I (*N*-acetyl-leu-leu-norleucinal, CPI-I) has been shown to cross the cell membrane and to prevent the formation of long-term potentiation both in vitro [8] and

after kainate-induced seizures [9]. Recent evidence has demonstrated that seizure activity induced by systemic injection of kainic acid in rats is accompanied by calpain activation in several brain structures [10]. However, its action on conscious, freely moving animals, and its effects on seizure models which do not directly activate glutamate receptors, remain unclear. Enhancing synaptic activity by blockade of GABAergic inhibition with picrotoxin does not affect spinal density [7] and does not seem to facilitate LTP [6]. Also, it has been suggested that a receptor regulation system, somehow related with rapid calpain-mediated proteolysis [20], may compensate for the alterations in inhibitory synaptic inputs. On this basis, we have investigated the effect of the intrahippocampal perfusion of calpain inhibitor I on picrotoxin-induced seizures in chronic freely moving rats.

Picrotoxin microdialysis in the hippocampus of chronic freely moving rats, has proved to be a good model for inducing controlled and reproducible seizures in repeated experiments in the same animal [17]. Using this method,

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it is possible to induce a short number of seizures, minimizing the effects of repeated seizures elicited in other seizure models. This method has been useful in studying the effects of receptor antagonist hippocampal microperfusion on seizures [18].

Male Sprague–Dawley rats, initially weighing 250–300 g were used. They were housed in groups of three under controlled environmental conditions (ambient temperature $21 \pm 1^\circ\text{C}$, 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 with 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 our chronic animal protocols were designed to reduce the number of animals used [17]. Animal care followed Spanish legislation on Protection of Animals Used in Experimental and Other Scientific Purposes, in agreement with the European Union regulations. The rats were anaesthetized with pentobarbital (40 mg/kg, injected intraperitoneally) and placed in a stereotaxic instrument (D. Kopf, Tujunga, CA). Under aseptic conditions, two 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 [15] 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 MD 1-95L I, 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.

The experiments were carried out on conscious, freely moving rats 10 days after surgery. From the fourth day the animals were placed for three hours 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, UK), and also with a Minihuit electroencephalograph (Alvar Electronic, Paris, France).

During an experimental session recording time was distributed as follows. (a) A 15 mm reference EEG was recorded before every probe introduction. (b) A 120 mm 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 mm post picrotoxin 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. 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 seizure was defined as the threshold seizure. All animals with rapidly recurrent seizures were treated with diazepam (4 mg/kg) to avoid unnecessary distress and brain damage after the 1 h period of recording. Seizure types and rest periods between experimental sessions were described previously in detail [17].

We used a CMA/120 system for freely moving animals (CMA/Microdialysis AB, Stockholm, Sweden) and CMA/12 micro-dialysis 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 [17].

For the control experiments, Ringer's solution was perfused at a constant flow rate of 2 $\mu\text{l}/\text{min}$ during 105 min. Picrotoxin (Sigma Chemical Co., St Louis, MO) dissolved in Ringer's solution was perfused at the same rate during the following 5 min. After picrotoxin administration, the perfusion of Ringer's solution continued for a further 1 h. Calpain inhibitor I (Boehringer Mannheim, Germany) was dissolved in Ringer's solution at a concentration of 500 μM and perfused continuously throughout the experiment following the same protocol for Ringer's solution in the control experiments. Threshold control experiments were performed in all animals a week after calpain inhibitor I administration to ensure that no permanent modification had been induced in the time, duration or number of seizures.

EEG records were analyzed using the Medilog 9200 software, version 7.2. Wakefulness, somnolence, and sleep EEG activity (sleep spindles and slow wave sleep) was 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 CPI I administration. 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. Then, the brain was 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. 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.

Threshold seizures were absence-like seizures in 80% of the animals. The total number of seizures was 1.8 ± 1.2 , mean seizure duration was 22.5 ± 6.9 s and the total time in seizures was 42.5 ± 28.2 s. (Fig. 1). In no case were seizures present more than 35 min after picrotoxin administration (onset time 13.3 ± 3.9 mm, offset time 15.3 ± 7.6 mm). In most of the animals, fast picrotoxin activity disappeared completely 35–40 mm after picrotoxin perfusion and was substituted by sleep EEG activity. Continuous perfusion of calpain inhibitor I had no effect on basal EEG patterns prior to picrotoxin perfusion. However, it increased significantly the total number of seizures (5 ± 1.7 , $P < 0.01$), total time in seizures (236.7 ± 89.4 s, $P < 0.01$), and the duration of seizures (47.1 ± 16.6 s, $P < 0.05$) when the same picrotoxin dose was administered (Fig. 1). In 60% of the rats, seizure activity remained one h after picrotoxin (onset time 10.9 ± 4.1 mm, offset time 51 ± 2.7 mm, $P < 0.01$, Fig. 1), and 40% developed spike and wave status epilepticus associated with absence-like behavior. Those animals were treated with intraperitoneal diazepam if seizures persisted 1 h after picrotoxin administration. Despite this significant increase in seizure duration, only one rat developed motor seizures.

This study reports on the previously uninvestigated effect of calpain inhibitor I on picrotoxin-induced seizure activity in conscious, freely moving rats. We found that calpain inhibition potentiates picrotoxin-induced paroxysmal activity, increasing seizure frequency and duration, but not necessarily to a more severe seizure type as happens when the picrotoxin dose is increased [17]. These results suggest a calpain-mediated mechanism which may be a part of a seizure offset system at the cell or the network level, and offer a clear alternative to the previous interpretation of the in vitro experiments. A study of the increase in products generated by calpain-mediated proteolysis [2,3] after kainate administration and electrical stimulation demonstrated a close relationship among calpain activation and seizures. However, there is no direct evidence of the physiological and pathological significance of the increased calpain activity.

Calpain has been demonstrated to be involved in NMDA receptor-mediated changes in synaptic efficacy [1,16]. NMDA receptor activation produces an increase in the intracellular calcium concentration which activates calpain, resulting in complex modifications of AMPA receptor properties. However, as several authors have pointed out [20], the regulation of glutamate receptor activation may depend on the previous physiological state of the neuron, compensating for alterations in the synaptic input with 'homeodynamic' receptor regulation. Furthermore, LTD has been shown to be prevented by calcium-dependent protease inhi-

bitors [11]. Standley et al. [20], indicate that calpain activation produces a partial proteolysis of the AMPA receptors which may be a signal for receptor internalization and further degradation leading to depression if receptors are not protected by phosphorylation. These authors hypothesize that lower intracellular calcium concentrations activate phosphatases and calpain, leading to receptor degradation, whereas higher calcium concentrations activate receptor phosphorylation protecting receptors from calpain-mediated proteolysis. Thus, the same calpain-activated mechanism may be responsible, at the cell level, for both up and down-regulation of glutamate AMPA receptors. This could explain how calcium-dependent protease inhibition prevents the establishment of LTP after kainate treatment [10], where massive calcium entry, sprouting, and slow synaptic reorganization are involved, but also may block LTD and seizure arrest after acute picrotoxin seizures by

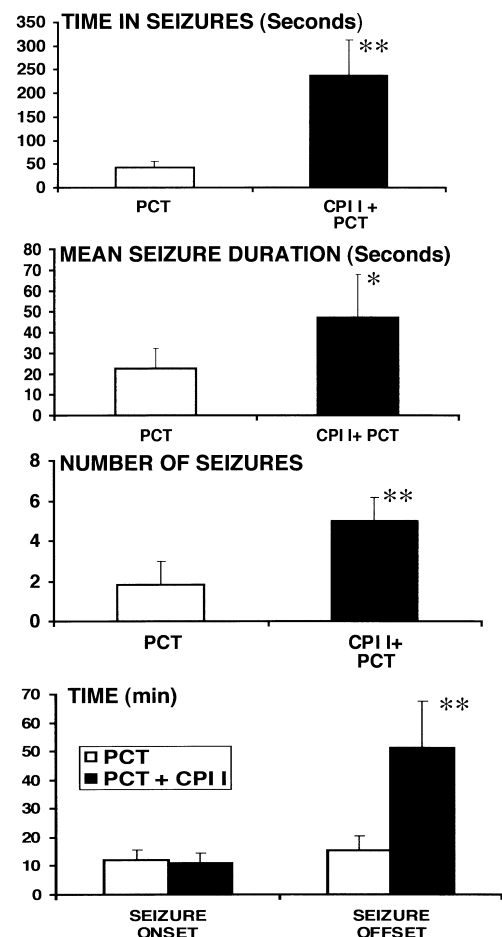


Fig. 1. Effect of calpain inhibitor I continuous microperfusion (500 μ M) on picrotoxin-induced seizures. PCT: Picrotoxin; CPI I: Calpain inhibitor I. Data are mean \pm SE ($n = 10$) of the values obtained by the analysis of the electroencephalographic recordings. For rats which developed spike and wave status epilepticus, 'time in seizures' refers to seizures presented prior to the establishment of status and 'seizure offset' corresponds to the time of diazepam administration (60 mm. after picrotoxin). * $P < 0.05$, ** $P < 0.01$ by Student's paired *t*-test.

preventing fast receptor inactivation as a response to diminished inhibition.

However there is also a plausible explanation at the network level: calpain inhibition may prevent the potentiation of glutamatergic feedback circuits which, during neuronal hyperexcitation, may enhance GABAergic inhibition [13], thus compensating the picrotoxin action. This may also explain the difference in our results when comparing those observed in hippocampal slices and cultures, where some of the feedback circuits may have been lost. Calpain activation following seizure onset exhibits a specific spatio-temporal pattern, with activation in restricted interneurons preceding widespread activation in pyramidal neurons [3]. These authors found that one of the early events elicited by seizure activity is a modification of the AMPA receptors located in subsets of interneurons in stratum oriens and in the hilus of the dentate gyrus. Eight hours after kainate seizures, calpain activity has disappears from interneurons but can be found in pyramidal cell bodies in the hippocampus. This initial activity in selected interneurons has been interpreted as a trigger for neuronal pathology, but in the light of the in vivo action of calpain inhibition, may also be interpreted as a feedback system which activates inhibitory circuits, in an attempt to compensate for hyperexcitability.

We found that in intact animals, when seizures are produced by the blockade of GABAergic inhibition, a calpain-mediated mechanism may be responsible for seizure offset, probably through AMPA glutamate receptors internalization and further degradation. Our results suggest a plausible calpain-mediated mechanism for seizure arrest following alterations of GABAergic inhibition. Calpain inhibition may also interfere with the glutamatergic feedback circuits responsible for re-activating GABA-mediated inhibition. Our results differ somehow from previous in vitro experiments, but it has been pointed out that most in vitro paradigms do not work in vivo preparations [1]. Further in vivo research is needed to explain some of the biochemical changes that lead to status epilepticus, and the in vivo effect of calpain inhibitor I may provide a new model to study these changes in chronic freely moving rats.

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