

Role of cAMP-Dependent Protein Kinase on Acute Picrotoxin-Induced Seizures

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cAMP-dependent protein kinase (PKA) is a major modulator of synaptic transmission likely to be involved in molecular and cellular events leading to epileptogenesis, but little is known about how it affects the onset of acute epileptic seizures. In this study, we determined PKA enzymatic activity in the rat hippocampus during picrotoxin-induced seizures, using H-9 dihydrochloride, a PKA inhibitor, to investigate the *in vivo* effects of this enzyme on seizures induced by picrotoxin microdialysis in the rat hippocampus. No significant modifications were found in PKA activity during seizures as compared to control rats, but H-9 dihydrochloride microperfusion (100 μ M) prevented picrotoxin seizures in 50% of the animals and significantly reduced the mean number of seizures and mean seizure duration. These results suggest that acute picrotoxin-induced seizures occur without an increase in hippocampal PKA activity, but reduced PKA-mediated phosphorylation protects against picrotoxin seizures, probably by increasing the inhibitory potential of GABA_A receptors. The possibility of other targets for H-9 dihydrochloride, such as PKC, PKG or CAMKII, however, cannot be ruled out.

KEY WORDS: Hippocampus; microdialysis; picrotoxin; protein kinase A; seizures.

INTRODUCTION

The neurochemical events underlying the hyperexcitability of neurons and neuronal networks, which may lead to epileptic seizures, are still unclear. There is a considerable volume of scientific data indicating that the pathogenesis of epileptic seizures may result from alterations of the synaptic function and several intrinsic properties of neurons. Although development of circuitry with recurrent excitatory synapses is emerging as a common theme in many experimental models of epilepsy, it seems probable that the intrinsic properties of the neurons

within a network will have a powerful influence on its excitability (1,2). In order to investigate the molecular basis for such modifications, a large number of animal models of epilepsy have been developed (3).

Much research has focused on the intracellular effects of glutamate and GABA neurotransmission. In several animal models, prolonged activation of the *N*-methyl-D-aspartate receptor (NMDAR) induces long-lasting plasticity changes in hippocampal neurons causing increased excitability leading to the occurrence of recurrent epileptiform discharges (4–8). NMDAR-linked increases in intracellular calcium affect a number of calcium-controlled cellular mechanisms and enzymes including several protein kinases and phosphatases (9–12). A substantial body of data also supports the involvement of GABA_A receptors in the initiation and spread of seizures. Administration of GABA_A antagonists such as bicuculline or picrotoxin (3,13) produces convulsions, while agonists are usually anticonvulsant (14).

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It was proposed that increased excitability in neurons underlying epileptic seizures would be maintained by abnormalities in protein phosphorylation systems (15,16). Regulation of NMDA and AMPA (17,18) receptors by cAMP-dependent protein kinase (PKA) is essential for synaptic transmission. Ca^{2+} influx through NMDA receptors is closely related with protein kinase activity. PKA phosphorylation directly controls the synaptic incorporation of AMPA receptors in the rat hippocampus (19) contributing to diverse mechanisms underlying synaptic plasticity.

GABAergic inhibitory synaptic transmission (20) and GABA_A receptors (21) are also regulated by phosphorylation. Biochemical approaches demonstrated that GABA_A receptors can be phosphorylated directly and consequently can be functionally modulated by PKA (22–24). However, in contrast to the familiar plasticity of glutamate receptors, fewer studies implicate this process in altering the efficacy of GABAergic synaptic transmission (21), and most research into the role of PKA in epileptic seizures has focused exclusively on seizure-prone genetic models in mice and rats.

The aim of this study is to improve our knowledge of the possible role of PKA in epileptic seizures induced *in vivo* by picrotoxin administered both through intraperitoneal injection and microdialysis in the hippocampus of freely moving rats. For this latter purpose, a whole-animal model (13) was used 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 (13), thus providing a good model to study possible modifications in neuronal excitability. We used H-9 dihydrochloride (*N*-(2-aminoethyl)-5-isoquinolinesulfonamide dihydrochloride) as a PKA inhibitor (25) because of its good inhibitory action and solubility in an aqueous medium for an adequate perfusion through microdialysis probes. Enzyme inhibitors were dialyzed through the probe to avoid possible dynamic effects imposed by the blood–brain barrier on some systemic administered drugs (26). Our method is completely reversible, thus allowing independent study, in the same animal, of picrotoxin seizure thresholds and the effect of the PKA inhibitor.

EXPERIMENTAL PROCEDURE

Drugs and Reagents. ProFluor™ PKA assay kit was purchased from Promega (Madison, WI, USA). H-9 dihydrochloride

was obtained from TOCRIS (Bristol, UK). All other drugs and reagents were purchased from Sigma/RBI (St Louis, MO, USA).

Animals and Surgical Procedure. 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 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 our chronic animal protocols were designed to reduce the number of animals used (13). Animal care complied with Spanish legislation on Protection of Animals Used in Experimental and Other Scientific Purposes, and 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, USA). 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 (27) 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.

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, UK), 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 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 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 (13).

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 microinjection 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, CaCl₂ 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 (13).

For the control experiments, Ringer's solution was perfused at a constant flow rate of 2 ml/min during 2 h. 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.

H-9 dihydrochloride was dissolved in Ringer at a 100 μ M concentration and perfused continuously throughout the experiment in all the animals on different days, following the same protocol for Ringer's solution and picrotoxin administration in the control experiments. H-9 dihydrochloride is a cell-permeable inhibitor of protein kinase A ($K_i = 1.9 \mu$ M) (25). Threshold control experiments were performed on all animals a week after the first threshold seizure to ensure that no permanent modification had been induced in the duration or number of seizures.

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 μ m coronal sections were cut and stained with cresyl violet, and the position of the probe was checked under light microscopy.

PKA Activity Assay. For the determination of PKA activity, two groups of eight rats were used. Rats from the control group were killed by decapitation 15 min after receiving an intraperitoneal saline injection. Rats from the seizure group were intraperitoneally injected with 4 mg/kg of picrotoxin, and killed by decapitation immediately after the onset of seizures (latency 17.3 ± 6.8 min). Brains were immediately removed and the hippocampus was microdissected and frozen to -30°C . PKA assay was performed according to the instructions of the ProFluor™ PKA assay kit. Pooled hippocampal areas (300 μ g) were homogenized in ice-cold buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM EGTA, 1 mM sodium orthovanadate, 1 mM PMSF, 20 μ g/ml leupeptin, and 4 μ g/ml aprotinin) and centrifuged at 40,000 rpm for 1 h to remove particular matter. Supernatants were added to the reaction buffer from the kit (containing a bisamide rhodamine 110 peptide substrate), 25 μ l of 10 μ M ATP solution, and incubated for 30 min. at 25°C . Following the kinase reaction, a termination buffer contains a protease which simultaneously stops the kinase reaction and removes amino acids specifically from the non-phosphorylated substrate, resulting in the production of highly fluorescent rhodamine 110. The phosphorylated substrate, however, is resistant to digestion by the protease reagent and remains non-fluorescent. Thus, the fluorescence intensity measured in the assay is inversely correlated with kinase activity. In order to dif-

ferentiate PKA activity from other protein kinases, controls without added ATP were performed. Negative controls were also used.

Fluorescence was measured with a LS50B Luminiscence Spectrometer (Perkin Elmer, Wellesley, MA, USA) in dark 96-well plates, at excitation wavelength 485 nm and emission wavelength 530 nm.

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, ascromycin and H-9 dihydrochloride administration. The 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. For enzyme activity measures, data were analyzed using one-way ANOVA, and $P < 0.05$ was considered significant.

RESULTS

Our results on individual seizure thresholds and seizure types were consistent with those described previously (13). Seizure threshold among animals varied between 100 and 300 μ M, 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 automatism and clonus of the contralateral forepaw; (iii) Rearing with alimentary automatism, 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 ranged from 14 to 36 min after the end of picrotoxin perfusion. However, latency showed no significant variation in repeated experiments with individual rats.

No significant differences were found in mean PKA activity during threshold picrotoxin seizures as compared to control rats ($94.9 \pm 26.8\%$ of controls) (Fig. 1). PKA activity measures show a remarkable variability between individual control rats (data not shown), but no significant differences were found in PKA activity according to seizure type or severity. However, continuous perfusion of H-9 dihydrochloride (100 μ M) protected completely against picrotoxin seizures in 50% of the animals (Fig. 2) and significantly reduced the mean number of seizures (from 2.38 ± 0.52 to 1.6 ± 0.3 , $P < 0.01$, Fig. 3) and the mean seizure duration (from 43.8 ± 14.2 to 21.2 ± 6.9 , $P < 0.01$, Fig. 4).

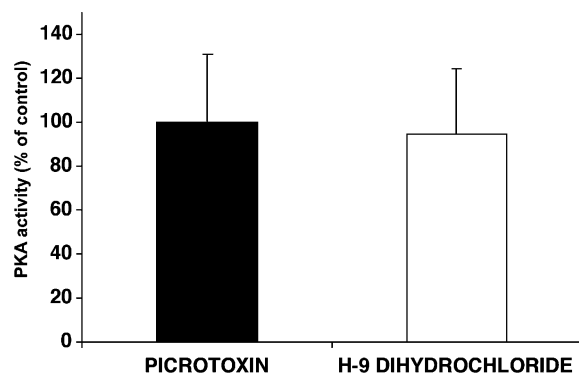


Fig. 1. Activity of cAMP-dependent protein kinase in control rats and during picrotoxin (4 mg/kg)-induced seizures. Data are presented as a percentage of control and represent mean \pm SEM of eight animals for each group. No significant differences were found in PKA activity during seizures ($P > 0.05$ by one-way ANOVA).

DISCUSSION

This study reports on the previously uninvestigated *in vivo* effect of PKA on picrotoxin-induced seizures. We have found that H-9 dihydrochloride, a PKA inhibitor, shows an antiepileptic effect against picrotoxin seizures when perfused into the rat hippocampus at a 100 μ M concentration, supporting the

idea that PKA phosphorylation may be involved in acute seizure activity.

PKA modulates receptor trafficking and gating (18) underlying synaptic plasticity, participates in several mechanisms involved in neurotransmitter release through its action on synaptic vesicle associated proteins (28–30) and regulates both glutamate (17,18) and GABA (22–24) receptor function.

An abnormal increase of protein kinase A activity in tottering mouse brain contributes to an impairment of GABA_A receptor function, and it was suggested that the resulting loss of inhibition could play a role in the induction of those seizures which characterize the mutant phenotype (15). Yechikhov et al. (16) compared the functioning of the cAMP-dependent system of protein phosphorylation in homogenates of neocortex and hippocampus in genetically prone to audiogenic seizures (GPAS) rats, GPAS rats exposed to daily repeated audiogenic seizures and non-epileptic Wistar rats. They found significant differences in phosphorylation of 270, 58, 54 and 42 kDa proteins in neocortex and hippocampus of GPAS rats in comparison with Wistar ones, and daily repeated seizures induced modifications in the phosphorylation of these proteins in the hippocampus.

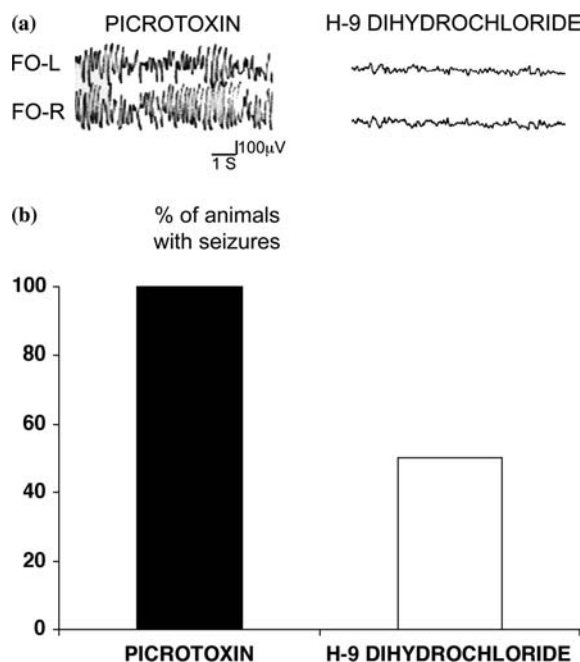


Fig. 2. (a) EEG recordings from a rat after intrahippocampal microperfusion of picrotoxin (seizure induced with a threshold dose), and the protective effect of H-9 dihydrochloride (100 μ M) on different days using the same picrotoxin dose. (b) Percentage of the animals showing at least one seizure after intrahippocampal microperfusion of picrotoxin alone ($n = 8$), and picrotoxin + H-9 dihydrochloride ($n = 8$).

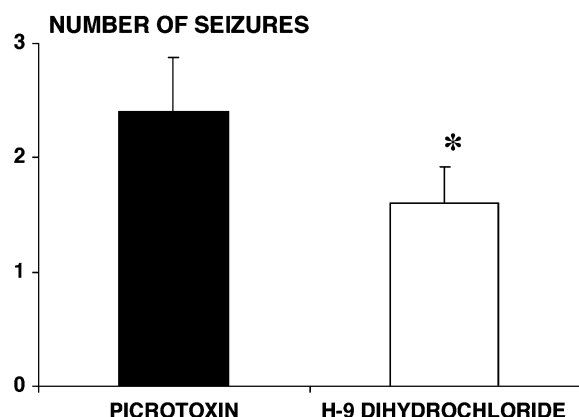


Fig. 3. H-9 dihydrochloride (100 μ M) significantly reduces the number of seizures (mean \pm S.E.M. from eight rats, $*P < 0.01$ by Student's paired *t*-test) when perfused in the hippocampus of freely moving rats.

These previous instances of PKA involvement in epileptic seizures came from genetic models of epilepsy in rodents. Using our model of acute picrotoxin-induced seizures, we found no significant modifications in hippocampal PKA activity during picrotoxin-induced seizures. The different results found in our animals compared with previous data are probably due to the different seizure mechanisms in the genetic and picrotoxin models of epilepsy.

It was pointed out that a positive finding with a kinase does not necessarily prove the involvement of the given kinase in the regulation of neuronal excitability (21), but the effect of the *in vivo* perfusion of H-9 dihydrochloride indicates that PKA may somehow regulate GABA_A-dependent neuronal excitability in the rat hippocampus. A probable

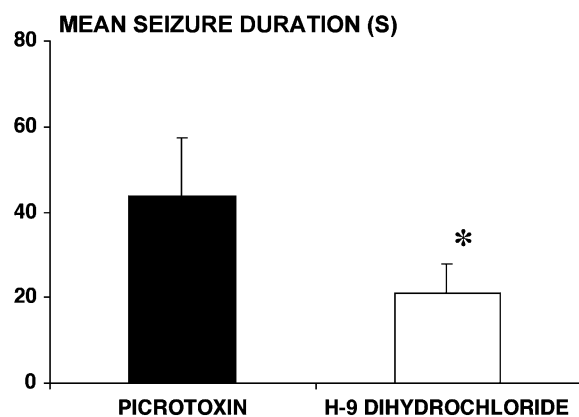


Fig. 4. H-9 dihydrochloride (100 μ M) significantly decreases the mean seizure duration in picrotoxin-treated rats (data are mean \pm S.E.M. from eight rats, $*P < 0.01$ by Student's paired *t*-test), when perfused in the hippocampus of freely moving rats.

explanation of our findings might come to light from the data on GABA_A receptors modulation by PKA. Poisbeau et al. (21) have shown that the function of GABA_A receptors at CA1 pyramidal cell inhibitory synapses is downregulated by PKA activation. They conclude that in these neurons a continuous phosphorylated site may keep GABA receptors consistently below their full inhibitory potential. Thus, continuous microperfusion of PKA inhibitors may increase basal GABA_A receptor activation, reducing the convulsant effect of picrotoxin. This would be consistent with a reduction in PKA activity during seizures, however, this reduction might be too small to be detected by our methods. Also, fast seizure arrest may be induced by other mechanisms, different from GABA receptor phosphorylation. Furthermore, PKA activity might be differently regulated in different neuronal types (20,21) during epileptic seizures, resulting in no significant differences in the mean total activity of PKA when measured in hippocampal homogenates. A more specific method will be needed in order to study a possible differential, region-specific activity of PKA in the hippocampus.

The action of H-9 dihydrochloride on other protein kinases cannot be ruled out. H-9 dihydrochloride, while inhibiting PKA with a K_i of 1.9 μ M, is also an inhibitor of PKC and PKG (K_i of 18 and 0.87 μ M, respectively). In addition, at the concentrations used, it may also inhibit calmodulin kinase II and casein kinases I and II.

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

Further research will be required to determine the relationships among protein kinases and amino acid neurotransmitter receptors during epileptic seizures.

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