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Research report

Effect of extracellular long-time microperfusion of high concentrations of glutamate and glycine on picrotoxin seizure thresholds in the hippocampus of freely moving rats

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Abstract

The effect of high concentrations of glutamate and glycine on picrotoxin seizure thresholds was investigated by perfusion through microdialysis probes in the hippocampus of freely moving rats. Microperfusion of glutamate at concentrations up to 1 mM, produced no changes in behavior or basal EEG recordings, but microperfusion of 200 μM glutamate was sufficient to lower the picrotoxin seizure threshold down to 50% in 60% of the animals studied and produced an increase of 180±23% in seizure duration. Microperfusion of 1 mM glutamate reduced seizure threshold in all animals, and markedly prolonged seizure duration (230±30%). Microperfusion of 200 μM or 1 mM glycine lowered picrotoxin seizure thresholds down to 50% in 70% of the animals and lengthened seizure duration up to 176±43%. Continuous microperfusion of the antagonist for the glycine binding site in NMDA receptors 5,7-dichlorokynurenic acid (100 μM) reversed the effect of both glutamate (1 mM) and glycine (1 mM) and suppressed seizures completely in 90% of the animals. These results indicate that although neurotoxicity is not achieved by perfusing glutamate and glycine at concentrations as high as 1 mM, neuronal excitability is modified by altering extracellular glutamate and glycine concentrations, and they suggest that glutamate-induced neuronal hyperexcitability is induced through mechanisms different from excitotoxicity. © 2001 Elsevier Science B.V. All rights reserved.

Theme: Disorders of the nervous system

Topic: Epilepsy: basic mechanisms

Keywords: Extracellular amino acid; Seizure; NMDA receptor; Hippocampus; Microdialysis; Extrasynaptic receptor

1. Introduction

The cellular mechanisms underlying the initiation and spread of epileptic seizures have been related to both increased glutamatergic excitation and decreased GABAergic inhibition [6]. Based on findings of several experimental models of epilepsy, increasing GABAergic transmission and decreasing glutamatergic transmission have been proposed as promising antiepileptic strategies with potential advantages compared to current therapies [22,24].

Glutamate excitotoxicity has been implicated in the pathophysiological process of epilepsy [31]. Extracellular concentrations of excitatory and inhibitory amino acids have been investigated during different types of epileptic seizures in humans and several animal models as indirect evidence of neurochemical modifications on the synaptic cleft. Human studies have shown an increase in several amino acids during epileptic seizures [8,15], but some animal experiments have provided conflicting results. Depending on the method used seizures may be correlated with an increase in the extracellular concentrations of excitatory amino acids [47], but other experiments show no modification [21,25] or a decrease [40,41] in glutamate and aspartate during epileptic seizures. These results are difficult to evaluate in terms of the excitatory effect on the postsynaptic receptors, because changes in glutamate concentration within the synaptic cleft are not reflected by modifications in the extracellular fluid [40].

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Direct application of excitatory neurotransmitters such as glutamate [28], or allosteric positive modulators such as glycine [29], failed to induce seizures or excitotoxicity in animals when directly infused into the brain. Glutamate excitotoxicity is more evident in stroke [9] and autoimmunity [45], but excitotoxicity seems to be a more complex phenomenon than a single increase in extracellular glutamate. Furthermore, most of the chronic models of epilepsy seem to need the conjunction of two or more factors. Electrically-induced kindling and kainate injections produce both a loss of inhibitory GABAergic neurons and excitatory synaptic changes in the rat hippocampus [5,44], and human studies indicate that both phenomena are also observed in the epileptic brain tissue [12]. In spite of this, glutamate and glycine perfused in extracellular fluid may not be enough to induce seizures, but could result in modified neuronal excitability.

Changes in the extracellular compartment do not seem to be involved directly in fast synaptic transmission, but are likely to have widespread effects mediated by extrasynaptic receptors. Amino acids released in the extracellular fluid do not easily reach synaptic NMDA and AMPA receptors [17,37], but prolonged high concentrations of glutamate and glycine may result in the activation of extrasynaptic NMDA receptor function [36,40], which may lead to the sustained hyperexcitability observed in epileptic tissue.

In this study we have investigated the effect of long-term microperfusion of glutamate and glycine in the hippocampus of freely moving rats, and how it affects picrotoxin seizure thresholds. We also studied the effect of the antagonist for the glycine binding site in NMDA receptors 5,7-dichlorokynurenic acid. For this purpose we have used a new experimental design on a new whole-animal model [43] 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 [43] for time periods as long as 6 months, thus providing a good model to study possible modifications in neuronal excitability. Picrotoxin, amino acids and the receptor antagonist were dialyzed through the probe to avoid possible dynamic effects imposed by the blood–brain barrier on amino acids and some systemic administrated drugs [1]. Our method is completely reversible, thus permitting the independent study in the same animal of each of the separate roles of excitatory and inhibitory neurotransmitters that may contribute to epileptic seizures.

A preliminary report of some of the findings has been published in abstract form [42]

2. Materials and methods

2.1. Drugs and reagents

Pentobarbital, picrotoxin and amino acids were purchased from Sigma (St Louis, MO, USA). 5,7-DKA was obtained from TOCRIS (Bristol, UK).

2.2. 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±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 our chronic animal protocols were designed to reduce the number of animals used [43]. 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 anesthetized 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 [32] 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 to 5 days.

2.3. Experimental procedure

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 to 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 [43]. Subthreshold picrotoxin doses (50% of threshold dose, which did not induce EEG or behavioral modifications) were used to investigate the effect of glycine and glutamate.

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 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 [43].

For the control experiments, Ringer solution was perfused at a constant flow rate of 2 ml/min during 2 h. Picrotoxin dissolved in Ringer solution was perfused at the same rate for 5 min. After picrotoxin administration, the perfusion of Ringer solution continued for one more hour.

In order to test the effect of glutamate and glycine, each was dissolved in Ringer solution at concentration 200 μM and 1 mM. Each solution was perfused continuously throughout the experiment in all the animals on different days, following the same protocol for Ringer solution and picrotoxin administration in the control experiments. In order to test the effect of 5,7-DKA, it was added to glutamate–Ringer solution or glycine–Ringer solution at a concentration 100 μM and also perfused continuously during the experiment in all the animals, administering picrotoxin as in the controls. Threshold control experiments were performed on all animals a week after glutamate or glycine administration to ensure that no permanent modification had been induced in the duration or number of seizures.

At the end of the experiments rats were anesthetized 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.

2.4. 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 glutamate, glycine and DKA 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.

3. Results

3.1. Seizure thresholds

Our results on individual seizure thresholds and seizure types were consistent with those described previously [43]. Seizure threshold among animals varied between 100 and 300 μM, although it remained unchanged in repeated between day experiments within individual rats.

3.2. Effect of glutamate microperfusion

Three hours microperfusion of glutamate at concentrations up to 1 mM did not produce any changes in behavior or basal EEG recordings. Microperfusion on 200 μM glutamate decreased picrotoxin seizure threshold down to 50% of the picrotoxin concentration needed to induce seizures in 60% of the animals studied (Fig. 1). The same concentration of glutamate produced an increase of 180±23% in seizure duration (Fig. 2B), and in the number of seizures induced after picrotoxin administration (Fig. 2A). In 40% of the animals seizure severity was also increased. Microperfusion of 1 mM lowered seizure threshold in all the animals (Fig. 1), and had a significant effect on seizure duration (230±30%) with several animals developing recurrent seizures.

3.3. Effect of glycine microperfusion

Microperfusion of 200 μM and 1 mM glycine did not modify behavior or basal EEG recordings. Picrotoxin seizures thresholds were reduced down to 50% in the 70%

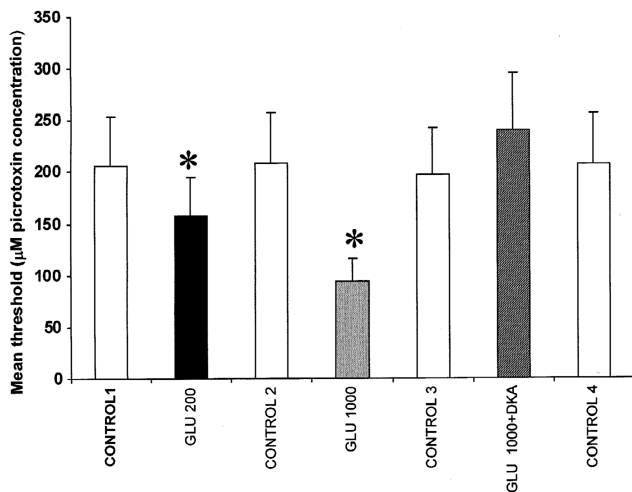

Fig. 1. Effect of glutamate continuous microperfusion (200 μM and 1 mM) in the hippocampus on picrotoxin thresholds. The significant decrease in seizure threshold (*$P<0.01$, by Student's paired t-test) induced by glutamate microperfusion was prevented by 5,7-DKA (100 μM). Data are mean±S.E.M. ($n=12$).

of the animals (Fig. 3) and seizure duration increased up to 176±43% after continuous perfusion of 200 μM glycine (Fig. 2B). The total number of seizures was also increased (Fig. 2A). Perfusion of 1 mM glycine had the same effect on seizure threshold, increasing seizure severity (40% of the rats developed recurrent seizures) and duration (an increase of 240±58%).

3.4. Effect of 5,7-DKA

Continuous microperfusion of the antagonist for the glycine binding site in NMDA receptors 5,7-DKA (100 μM) reversed the effect of both glutamate and glycine. 5,7-DKA suppressed seizures completely in 90% of the animals (Figs. 1 and 3).

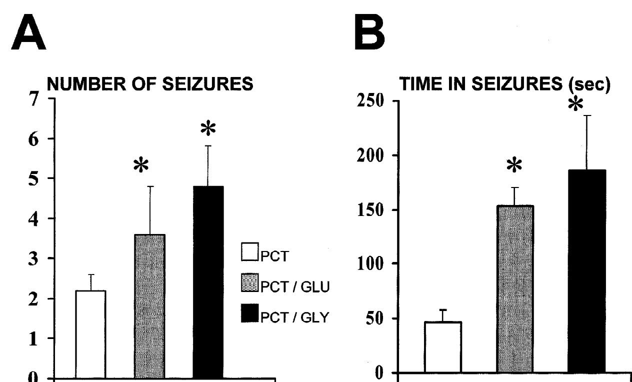

Fig. 2. Effect of increased glutamate and glycine extracellular concentrations on picrotoxin-induced seizures. Continuous microperfusion of glutamate (1 mM) or glycine (1 mM) increased significantly: (A) The number of seizures and (B) mean seizure duration (*$P<0.01$, by Student's paired t-test). Data are mean±S.E.M. ($n=12$).

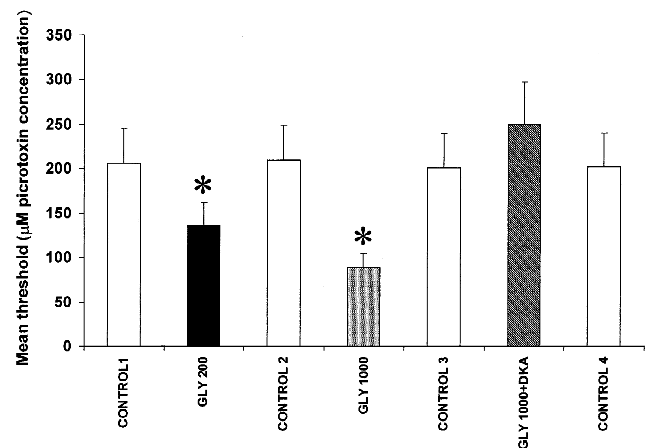

Fig. 3. Effect of glycine continuous microperfusion (200 μM and 1 mM) on picrotoxin thresholds. The significant decrease (*$P<0.01$, by Student's paired t-test) in seizure threshold induced by glycine microperfusion was also prevented by 5,7-DKA (100 μM) Data are mean±S.E.M. ($n=12$).

4. Discussion

In this present study we report the effect of high concentrations of glutamate and glycine on picrotoxin-induced seizure thresholds on freely moving rats. Our results show that high extracellular glutamate and glycine levels during prolonged microperfusion do not lead to excitatory neuronal damage or paroxysmal activity in the living rat brain. This is in agreement with previous reports where raising extracellular glutamate concentrations by microperfusion [28], or long-time pretreatment with glutamate transporter inhibitors [7,23,27,40], failed to produce seizures or neuronal damage. For glycine, Obrenovitch et al. [29] have also shown that no toxic effects are observed after perfusing concentrations up to 10 mM through microdialysis probes implanted in the striatum of halothane-anesthetized rats.

We have found, however, that elevating glutamate and glycine concentrations in the hippocampus significantly increases neuronal excitability. Glutamate and glycine acting alone are not enough to induce epileptiform seizures, but their continuous action may produce metabolic changes or receptor rearrangement which facilitate seizure onset when GABAergic inhibition is impaired. The fact that glutamate and glycine produce almost the same effect indicates that their action is likely to be mediated by NMDA receptors. This is also confirmed by the fact that 5,7-DKA, a competitive inhibitor of the glycine binding site in the NMDA receptor, completely prevents the effect of both glutamate and glycine.

The epileptiform synaptic potentials in the hippocampus are clearly dependent on the vesicular glutamate pool [33,48]. The effects observed could be explained by overactivation of synaptic NMDA receptors, however, it is difficult to understand the role of extracellular glutamate

and glycine concentrations based only on its synaptic effect.

Astroglial cells encapsulate synapses, and astroglial glutamate carriers have sufficient capacity to remove all glutamate released from the presynaptic site [19]. Glial cells respond to synaptically released glutamate by activation of electrogenic transporters, which generate a current that is directly proportional to the amount of glutamate released [14]. The high capacity and affinity for glutamate uptake by astrocytes in the synaptic region enables synaptic transmission for long periods of time with a high signal-to-noise ratio [34].

The need for astroglial cells working at the synaptic region as an effective system for clearance of the presynaptically released glutamate may be clearly understood by taking into account the role of the glutamate–glutamine cycle in both the presynaptic region and the glial cells [38]. Upon release into the synaptic cleft, the glutamate diffuses away, and the glial glutamate transporters internalize the extracellular glutamate. Inside the glial cells, glutamate is metabolized into glutamine, which is transported out of the glial cells and into the synaptic terminal for subsequent resynthesis of glutamate. Since glutamine does not act on the glutamate receptor, this release does not interfere with synaptic transmission [39]. Also, using knockout rats [35], it was shown that extracellular glutamate levels were unmodified in the animals which had lost neuronal glutamate transport, while those animals which had lost the glutamate transport in glia showed an increase in extracellular glutamate concentrations, suggesting a major role for glial glutamate transporters in the clearance of extracellular glutamate.

This special role of the astrocytes at the synaptic region may be explained by the astroglial heterogeneity [49]. We also need to take into account the morphological [13] and the pharmacological [11] evidence of synaptic barriers. Taken together, all these data suggest that the epileptiform synaptic potential is generated by the glutamate synaptic pool without the participation of the extracellular glutamate pool.

Recent research suggests that the brain extracellular environment appears to be an homeodynamic entity which maintains a steady intercellular milieu with regard to ions and small molecules, critical for neuronal function, under the control of neurons and glial cells [26]. Na^+-dependent glutamate transporters of the blood–brain barrier presumably assist in keeping the glutamate concentration low in the extracellular fluid of the brain [30]. Analysis of our results is simplified if we also take into account the concept of non-synaptic transmission [3,4,18,46] as a complementary mechanism to the glutamate action on synaptic transmission [40].

Fagni et al. [16] suggested that the L-glutamate added to the bath stimulated different glutamate receptors to those activated by the endogenous neurotransmitter, and postulated the existence of extrasynaptic receptors.

Evidence of extrasynaptic glutamate receptors has been reported [2,20], and a comparison of some properties of synaptic and non-synaptic NMDA receptors has been published [10]. Some data suggest that the extracellular glutamate and aspartate may act on neuronal NMDA non-synaptic receptors [17,36,37,40], as a possible mechanism of neuronal synchronization [40].

Although there is not direct evidence that extrasynaptic NMDA receptors participate in the development of seizures, the hypothesis described above seems the best suited to integrate our data with the majority of previously published experimental research.

The increase in extracellular glutamate and glycine does not produce neuronal damage or seizure activity. However, it may potentiate both when associated with other alterations, such as the loss of inhibitory neurons. In many brain injuries, both phenomena are likely to occur.

Furthermore, many drugs acting on NMDA receptors would bind easily to non-synaptic receptors, preventing the potentiation effect which may lead to epileptogenesis. NMDA extrasynaptic receptors thus remain a good target for new antiepileptic drugs.

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