Neuropharmacology 55 (2008) 1383–1390

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

HSP70 expression protects against hippocampal neurodegeneration induced by endogenous glutamate *in vivo*

Gabriela X. Ayala, Ricardo Tapia*

Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, AP 70-253, 04510-México DF, Mexico

ARTICLE INFO

Article history:
Received 2 July 2008
Received in revised form 26 August 2008
Accepted 27 August 2008

Keywords:
Rat hippocampus
Heat shock protein 70
Glutamate release
4-Aminopyridine
Excitotoxicity

ABSTRACT

The K^+ channel blocker 4-aminopyridine (4-AP) stimulates the release of glutamate from nerve endings and induces seizures and neurodegeneration when perfused by microdialysis in rat hippocampus. In addition, there is a temporal correlation between the progress of neurodegeneration in the perfused hippocampus and the expression of the inducible cellular stress marker heat shock protein 70 (HSP70) in the non-damaged contralateral hippocampus. All these effects of 4-AP are prevented by the NMDA receptor antagonists 3-phosphonopropyl-piperazine-2-carboxilic acid (CPP) and (+)5-methyl-10,11-dyhydro-5*H*-dibenzo(a,d)cyclohepten-5,10-imine maleate (MK-801), indicating that they are due to NMDA receptor overactivation by excessive extracellular synaptic glutamate. We hypothesized that the induction of HSP70 in the non-damaged contralateral hippocampus should have a protective action against this excitotoxic effect. Here we demonstrate that 4-AP perfusion in one hippocampus prevented the neurotoxic effect of 4-AP when perfused by microdialysis in the contralateral hippocampus 24 h later. However, both the stimulation of glutamate release and the EEG epileptiform discharges, which occur immediately after 4-AP perfusion, were similar after the first and the second perfusions. When CPP was coperfused with 4-AP during the first microdialysis, HSP70 induction in the contralateral hippocampus was prevented and the protection against the second 4-AP perfusion was abolished in 50% of the rats. These results suggest that HSP70 induction is an important cellular mechanism to protect vulnerable neurons from excitotoxic overactivation of glutamate receptors by endogenous glutamate, and may be relevant to pathological conditions in which extracellular endogenous glutamate is augmented, such as ischemia.

1. Introduction

Excitotoxicity due to excessive glutamatergic neurotransmission is a well studied phenomenon that has been related to the mechanisms of neuronal death occurring in epilepsy and several neurodegenerative diseases. *In vivo*, systemic or intracerebral administration of glutamate receptor agonists is frequently used to induce neuronal damage in the central nervous system. In contrast to these approaches, we have studied a model of epilepsy and neurodegeneration induced by stimulation of glutamate release from nerve endings by the K^+ channel blocker 4-aminopyridine (4-AP). We have thus demonstrated that unilateral microdialysis perfusion of 4-AP in rat hippocampus induces an immediate transient increase of extracellular glutamate and intense epileptiform seizures, as well as delayed (about 3 h) neurodegeneration in CA1 and CA3 subfields, effects that were blocked by *N*-methyl-D-aspartate (NMDA) receptor antagonists and by tetrodotoxin (Peña and Tapia, 1999, 2000). Subsequently, we found that 4-AP intrahippocampal perfusion induces the expression of the inducible heat shock protein 70 (HSP70) in the ipsilateral cortex and in the contralateral undamaged hippocampus, reaching a peak of expression at 24 h, at the time when the neurodegeneration is maximal in the perfused hippocampus. Treatment with the NMDA receptor antagonists 3-phosphonopropyl-piperazine-2-carboxylic acid (CPP) or (+)5-methyl-10,11-dyhydro-5*H*-dibenzo(a,d)cyclohepten-5,10-imine maleate (MK-801) suppressed the expression of HSP70 and prevented the seizures and the neurodegeneration (Ayala and Tapia, 2003). All these effects seem to be mediated by Ca^{2+} entrance through the NMDA receptor channel, because the intracellular Ca^{2+} chelator 1,2-*bis*(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis (acetoxymethyl ester) blocked the expression of HSP70 in the contralateral hippocampus and the neurodegeneration of the perfused hippocampus (Ayala and Tapia, 2005). These results suggest that the glutamate-mediated excitotoxic effects of 4-AP are mediated by NMDA receptor overactivation and that the neuronal hyperexcitation propagates to the undamaged contralateral brain regions to induce neuronal stress that is reflected by the expression of HSP70.

* Corresponding author. Tel.: +52 55 56225642; fax: +52 55 56225607.
E-mail address: rtapia@ifc.unam.mx (R. Tapia).

0028-3908/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuropharm.2008.08.035

Upregulation of stress proteins, particularly HSP70, has been linked to protective mechanisms, because of its role in preventing protein denaturation and aberrant peptide aggregation during cellular injuries. HSP70 is expressed in the brain when subjected to several kinds of stressful conditions, such as hyperthermia, ischemia, trauma and excitotoxicity (Gonzalez et al., 1989; Marcuccilli and Miller, 1994; Planas et al., 1997; Sharp et al., 1999; Yenari et al., 1999; Ohtsuka and Suzuki, 2000; Rajdev et al., 2000; Brown, 2007; Obrenovitch, 2008).

The foregoing data prompted us to study whether the expression of HSP70 in the undamaged but stressed contralateral hippocampus, induced by microdialysis perfusion of 4-AP in the ipsilateral hippocampus, was capable of protecting the tissue from the excitotoxic effect of 4-AP. For this purpose, we perfused 4-AP in the contralateral hippocampus 24 h after the first intrahippocampal 4-AP perfusion, at a time of maximal HSP70 expression. The effect of this double microdialysis on the EEG and on the extracellular glutamate concentration was also evaluated. This approach allowed us to test whether HSP70 expression due to mild NMDA receptor overactivation by excess extracellular endogenous glutamate could protect against intense glutamate-mediated excitotoxicity.

2. Materials and methods

2.1. Microdialysis and EEG recording

Male Wistar rats weighing 200–230 g were used throughout and handled according to the Rules for Research in Health Matters (Mexico), with approval of the local Animal Care Committee, and all efforts were made to minimize animal suffering and reduce the number of animals used. For the microdialysis procedure, rats were anesthetized with halothane in oxygen, secured in a stereotaxic frame and maintained anesthetized on a thermal cushion. Previously water-flushed microdialysis cannulae (CMA/12, CMA Solna, Sweden) were positioned in the left dorsal hippocampus (AP −3.6, L 2.4, V −3.8; Paxinos and Watson, 1982). The probes were perfused with a Ringer–Krebs medium of the following composition (in mM): 118 NaCl, 1.2 KH_2PO_4, 4.7 KCl, 1.18 $MgSO_4$, 25 $NaHCO_3$, 10 glucose, 2.5 $CaCl_2$ (pH 7.4) at a rate of 2 μL/min, using a microinjection pump (CMA 100, Carnegie Medicin). After a 1 h equilibration period, 25 μL (12.5 min) consecutive fractions of perfusate were continuously collected. After the first three fractions (basal release of amino acids), 4-AP (Sigma, St. Louis, MO, USA) at a previously established concentration of 35 mM (Peña and Tapia, 2000; Ayala and Tapia, 2005) was perfused during 12.5 min (the NaCl concentration was proportionally reduced to maintain isoosmolarity), and three additional fractions with normal Krebs medium were collected. Animals were returned to their individual cages with food and water *ad libitum*. Twenty-four hours later the microdialysis procedure was repeated in the right (contralateral) hippocampus of each rat. In another group of animals, during the first microdialysis procedure 35 mM 4-AP was perfused together with the NMDA receptor antagonist CPP (Tocris, Ballwin, MO, USA), at the previously used 100 μM concentration (Ayala and Tapia, 2003), and 24 h later the second microdialysis perfusion with 4-AP alone was carried out as described above.

After each of the two microdialysis trials, the glutamate content of the 25-μL perfusate fractions was measured by HPLC as previously described (Massieu et al., 1995). The values reported were not corrected for the efficiency of the dialysis membrane, which was 7–11% for the amino acids (Massieu et al., 1995), and close to 11% for 4-AP (Morales-Villagrán and Tapia, 1996).

During both microdialysis procedures the hippocampal EEG was recorded using the microdialysis probes as electrodes (Peña and Tapia, 1999, 2000). A Grass polygraph, with low frequency filter as 3 Hz and high-frequency filter at 100 Hz, was used for the recording.

2.2. Histology and HSP70 immunocytochemistry

Twenty-four hours after the second microdialysis, rats were anesthetized with pentobarbital and transcardially perfused with 0.9% NaCl, followed by 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS) pH 7.4. Brains were removed, left in paraformaldehyde for 24 h and transferred successively to 10%, 20% and 30% sucrose at 4 °C for 24 h each one. Coronal sections (50 μm thick) were cut in a cryostat and alternate sections were used for cresyl violet staining and for HSP70 inmunocytochemistry to correlate the neurodegeneration and the HSP70 expression in the same rat. For quantitative analyses of the damage, in both perfused hippocampus the morphologically undamaged neurons (cells >15 μm diameter with clear cytoplasm) were counted in the cresyl violet-stained sections, with the help of an analyzer system (NIH Image 1.6). Three brain slices obtained from each of 8–10 rats for each experimental group were counted, covering the CA1 and CA3 areas; neuron counting in the CA1 region was made in three subzones, lateral, medial and internal, as indicated in Section 3.

For immunocytochemistry, free floating sections were immersed in PBS with 3% H_2O_2 and 0.25% Triton X-100 for 1 h. The slices were blocked with 5% albumin plus 0.1 M PBS for 2 h. Sections were incubated with monoclonal anti-HSP70 antibody (3 μg/ml, clone W27, Santa Cruz Biotechnology, CA, USA) for 72 h, and after incubation for 2 h with biotinylated anti-mouse were processed according to the ABC-biotin–avidin peroxidase procedure (Vector Laboratories, Burlingame CA), using diaminobenzidine tetrahydrochloride as substrate. Some 4-AP-treated sections were processed without primary antibody incubation, and no immunoreactivity was detected in these control sections.

Two-tailed *t*-test was used for statistical comparisons, as indicated in the figure legends.

3. Results

3.1. Extracellular glutamate and EEG

As shown in our previous studies (Peña and Tapia, 1999, 2000; Ayala and Tapia, 2005), perfusion of 35 mM 4-AP during 12.5 min induced at the next 12.5 min a peak transient increase of extracellular glutamate. During the first microdialysis this increase was 45.4% ± 12.4% and this value was not significantly different from that observed during the second microdialysis (60.8% ± 10.1%). This effect was not significantly modified by CPP when coperfused with 4-AP during the first microdialysis. In these experiments the peak increase during the first microdialysis was 65.6% ± 3.2% and 50.4% ± 13.1% during the second microdialysis, with 4-AP alone (Fig. 1).

As reported previously (Peña and Tapia, 1999, 2000), EEG epileptiform discharges produced by 4-AP appeared within the first 15 min after the beginning of 4-AP perfusion and were

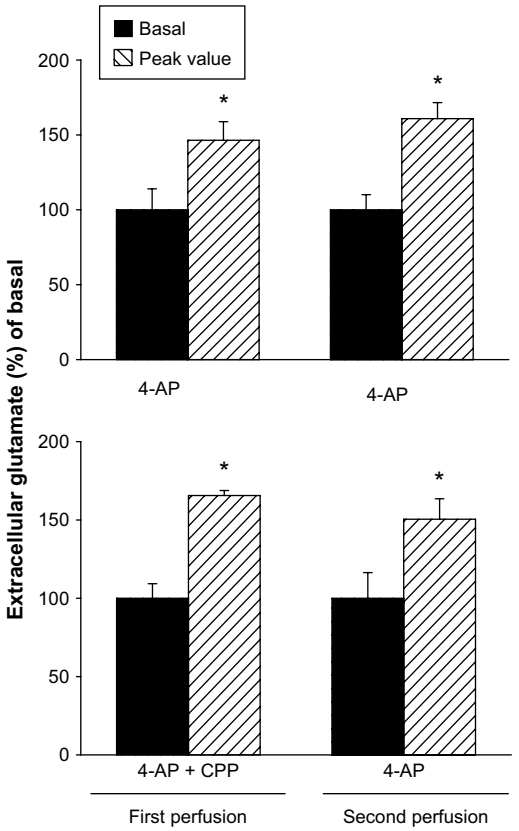

Fig. 1. Peak percent increase over the basal values in extracellular glutamate concentration after 4-AP intrahippocampal perfusions. (A) First and second perfusions with 4-AP. (B) First perfusion with 4-AP + CPP and second perfusion with 4-AP alone. Means ± SEM ($n = 10$ for 4-AP alone and $n = 8$ for 4-AP + CPP). *$p < 0.01$ vs the corresponding basal values (*t*-test).