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Calcineurin-mediated $GABA_A$ receptor dephosphorylation in rats after kainic acid-induced status epilepticus

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ABSTRACT

Calcineurin (CaN) is a neuronally enriched, calcium-dependent phosphatase, which plays an important role in a number of neuronal processes including development of learning and memory, and modulation of receptor's function and neuronal excitability as well as induction of apoptosis. It has been established in kindling model that the status epilepticus (SE)-induced increase in CaN activity is involved in the development of seizures through down-regulation of γ-aminobutyric acid A receptor ($GABA_AR$) activation. However, the mechanism by which CaN mediates $GABA_A$ receptor dephosphorylation in SE is not fully understood. Here, using a model of kainic acid (KA)-induced SE and CaN inhibitor FK506, we observed the behaviors induced by KA and levels of CaN activity and CaN expression in hippocampus by immunobloting. The results showed that the SE-induced CaN activity was time-dependent, with a peak at 2 h and a return to basal level at 24 h, whereas a significant increase in CaN expression was seen at 24 h after SE. It is proposed that the rapid elevation in CaN activity after KA-induced SE is not likely due to an increase in CaN expression but rather an increase in CaN activation state or kinetics. In addition, we also demonstrated that pre-treatment with FK506 remarkably suppressed the SE-induced CaN activity and its expression, and reversed the SE-induced dephosphorylation of $GABA_AR$ 2/3 subunits. Taken together, our data suggest that down-regulation in inhibition of $GABA_AR$ 2/3 by CaN activity contributes to an elevation in neuronal excitability of hippocampus, which may be involved in development of chronic processes of seizures.

1. Introduction

Status epilepticus (SE) may cause long-term functional and structural consequences possibly resulting in brain dysfunctions such as chronic epilepsy.[1] There is physiological and neurochemical changes which take place during the seizure activity.[2] These cellular and molecular alterations may later manifest themselves as spontaneous recurrent seizure activity, through a process known as epileptogenesis.[2] Notably, calcium and calcium-regulated systems have been found to be closely associated with generation of seizures.[3] Of these, one calcium-regulated enzyme of particular interest is the calcium-stimulated phosphatase, calcineurin (CaN).

CaN is a member of the serine/threonine protein phosphatase family enriched in neural tissue.[4] It has been found that CaN-mediated dephosphorylation is an important process in the control of many cellular processes including development of learning and memory,[5] regulation of long-term potentiation,[6] modulation of neurotransmitter release,[7] as well as induction of apoptosis.[8] Additionally, CaN is reported to take its action in inhibiting the activity of the GABA receptor,[9] regulating gene transcription and affecting cytoskeletal architecture.[10,11] Recent studies have demonstrated that SE may lead to a rapid increase in CaN activity in hippocampus and cortex that may be necessary for the development of seizures in a kindling model.[12] However, the SE-induced increase in CaN activity is not consistent with that in CaN expression, suggesting that the delayed expression of CaN may contribute to a long-lasting neuronal excitability of the hippocampus.[12] A further study revealed that phosphorylation of $GABA_AR$ β2/3 subunits was down-regulated by hypoxia-induced seizures, whereas this effect was reversed by CaN inhibitor FK506. These findings suggest that CaN may mediate dephosphorylation of $GABA_AR$ β2/3 subunits, by which inhibition of $GABA_AR$ contributes to subsequent neuronal hyperexcitability after seizures.[13] Despite many cellular and molecular mechanisms linked to epileptogenesis have been intensively investi-

Abbreviations: SE, status epilepticus; KA, kainic acid; CaN, calcineurin; $GABA_AR$, γ-aminobutyric acid receptor; SD, standard deviation.

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gated, the generation and development of seizures is not fully elucidated.

In the present study, we characterize the alterations of CaN activity, protein expression and phosphorylation of $GABA_AR$ in hippocampus after KA-induced SE. Western blot analysis demonstrated that a time-dependent increase in CaN activity and protein expression, and a down-regulation of $GABA_AR$ phosphorylation induced by SE. These changes were shown to have different responses to FK506, with attenuation of CaN activity and its protein expression and inhibition of $GABA_AR$ dephosphorylation. This raises the possibilities that CaN plays an important role in epileptogenesis via dephosphorylation of $GABA_AR$, and that CaN inhibitor such as FK506 may have clinical potential in the treatment of refractory seizures.

2. Experimental procedures

2.1. Experimental animals

Adult male Wistar rats (Experimental Animal Center of Shandong University, China) weighing 180–270 g were used. Animals were maintained in a temperature-controlled room (26 °C) with a 12 h light/dark cycle and free access to food and water. The experimental procedures were approved by the Commission of Shandong University for the ethics of experiments on animals, in accordance with international standards. Seizures were scored using a modified Racine scale.[14]

2.2. Pre-treatment of animals

The animals were divided into five groups (25 rats in each group, $n = 125$): a control group (no pre-treatment, followed by saline, 0.9%); a KA group (no pre-treatment, followed by KA); a KA + saline group (saline pre-treatment, followed by KA after 0.5 h); an FK506 group (FK506 pre-treatment, followed by KA after 0.5 h); and a rapamycin group (rapamycin pre-treatment, followed by KA after 0.5 h). The CaN inhibitor, FK506 (Fujisawa, Japan) was administered as a single i.p. bolus at 2 mg/kg bw; and the immunosuppressant (but not CaN inhibitor) rapamycin (Sigma) was administered as a single i.p. bolus at 10 mg/kg bw.

2.3. Induction of status epilepticus

Seizures were induced by intracerebroventricular injection of KA (Sigma, USA). Briefly, rats were anaesthetized with intraperitoneal (i.p.) pentobarbital (50 mg/kg, Sigma) and were placed in a stereotaxic frame. Then 0.5 μg of KA dissolved in 1.0 μl of 0.9% NaCl was injected by microsyringe following the stereotaxic coordinates: 0.85 mm caudal, 1.5 mm lateral, and 4.3 mm deep, relative to the bregma. Only rats with sustained generalized motor seizures that scored 4–5 on the Racine scale were included in the experimental groups. Seizures were allowed to continue for 30 min and were terminated by an injection of diazepam (i.p., 10 mg/kg, Sigma). The proportions of animals having stage 4 and 5 seizures were 92%, 88%, 84%, and 88% in the KA, KA + saline, FK506, and rapamycin groups, respectively ($p > 0.05$).

2.4. Sample preparation

Four animals from each group were decapitated at 2, 6, 12, 24 and 72 h after SE induction to analyze for CaN activity, CaN protein expression and $GABA_AR$ dephosphorylation. Brains were dissected in Petri dishes on ice in order to preserve postmortem enzyme activity. Hippocampus was isolated and immediately homogenized in ice-cold homogenization buffer containing 5 mM HEPES (pH 7.0), 7 mM EGTA, 5 mM EDTA, 1 mM dithiothreitol (DTT), 0.3 mM phenylmethyl sulfonyl fluoride (PMSF), and 300 mM sucrose. The samples were then centrifuged at 12,000 rpm for 10 min at 4 °C, and stored at −80 °C.

2.5. CaN activity assay

CaN activity was assayed using the procedure detailed by Kurz et al.[12] All reaction tubes were prepared on ice and contained the following: 25 mM morpholinopropanesulfonic acid, pH 7.0, 1 mM DTT, 2 mM p-nitrophenol phosphate (pNPP), and 150 nM okadaic acid. Basal tubes also contained 2 mM EGTA and 2 mM EDTA, while the treated tubes contained 2 mM $MnCl_2$. Mn^{2+} activates CaN more strongly than Ca^{2+},[15] and Mn^{2+} was therefore used in the maximal reactions in order to maximize the cation-stimulated activity of the enzyme. Reactions were incubated at 37 °C for 30 min in a shaking water bath. Prior to use, protein concentration of all homogenates was determined using the method of Bradford.[16] Tubes were then removed from the water bath and placed on ice to stop the reaction. The absorbance of the reaction mixture was immediately measured at 405 nm in a plate reader, and absorbance units were converted to μg/ml of PNP (p-nitrophenol) produced by comparison with a pNP concentration standard absorption curve.

2.6. Immunoblotting

Forty micrograms of protein was separated by sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis and then transferred into nitrocellulose membranes. After blocking in 5% fat-free milk for 1 h, the membranes were incubated with primary antibodies to CaN (1:13,000, Sigma), and $GABA_AR$ 2/3 (1:500, Boehringer Mannheim, Germany) at 4 °C overnight. They were then incubated with horseradish peroxidase-conjugated second antibodies (1:3000, Santa Cruz Biotechnology, CA, USA) for 1 h. Immunoreactivity was enhanced using a chemiluminescence kit (Pierce, Rockford, IL, USA) and preparations were exposed to film. The bands were scanned and analyzed using an image analyzer (Alpha Innotech, San Leandro, CA, USA).

2.7. Data analysis

The data are shown as the means ± SEM. Statistical analysis was performed using one-way ANOVA followed by Newman–Keuls test with SPSS 12.0 (SPSS Inc., USA). The proportion of animals with stage 4 and 5 seizures was analyzed by χ^2 test. Statistical significance was accepted at the conventional $p < 0.05$ level.

3. Results

3.1. Behavioral episodes

Behavioral episodes induced by KA followed the typical course of seizures, with a gradual increase in intensity to SE. No differences in behavioral seizures were noted in these rats pretreated with or without FK506. Control animals did not exhibit any behavioral seizure activity.

3.2. Characteristics of CaN activity in rats with KA-induced SE

To determine if CaN levels were elevated after KA-induced SE, we measured CaN activity at 2, 6, 12, 24 and 72 h after SE. The results showed that a significant increase in both basal and maximal CaN activity was seen after SE, with a peak at 2 h ($p < 0.01$), and a return to basal levels after 24 h (Fig. 1A). We also observed that pre-treatment with FK506 remarkably decreased the SE-induced CaN activity at 2 h ($p < 0.01$) (Fig. 1A′). It is therefore