Calcium/Calmodulin Kinase II Activity of Hippocampus in Kainate-Induced Epilepsy

This study investigated calcium/calmodulin kinase II (CaMKII) activity related to long-standing neuronal injury of the hippocampus in kainate (KA)-induced experimental temporal lobe epilepsy. Epileptic seizure was induced by injection of KA (1 μg/μL) dissolved in phosphate buffer (0.1 M, pH 7.4) into the left amygdala. Clinical seizures, histopathologic changes and CaMKII activity of the hippocampus were evaluated. Characteristic early limbic and late seizures were developed. Hippocampal CaMKII activity increased significantly 4 and 8 weeks after intra-amygdaloid injection of KA, when late seizures developed. The histopathologic changes of the hippocampus included swelling of neuronal cytoplasm with nuclear pyknosis and loss of neurons in CA3 during this period. The increased activity of CaMKII may correlate with appearance of distant damage in the hippocampus. The above results indicate that intra-amygdaloid injection of KA produces excitatory signals for ipsilateral CA3 neurons in the hippocampus and that subsequently increased levels of CaMKII in postsynaptic neurons induce neuronal injury via phosphorylation of N-methyl-D-aspartate type glutamate receptor.

Key Words: Ca(2+)-Calmodulin Dependent Protein Kinase; Epilepsy; Hippocampus; Kainic Acid; Neurons

**INTRODUCTION**

A wide range of pathologic insults to the brain induce depolarization of the neuronal cell membrane, and release neurotransmitters leading to the activation of aberrant cellular signaling pathways. Such insults include traumatic brain injury, ischemia, and seizures. Activation of voltage-sensitive ion channels and/or the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor increase cellular calcium (Ca²⁺), which plays a major role in the development of neuronal injury (1-3). Increased intracellular calcium activates many calcium-dependent enzymes, including protein kinase C (PKC), calmodulin kinase II (CaMKII), also often referred to as Ca²⁺/calmodulin kinase II or Ca²⁺/calmodulin-dependent protein kinase II), phospholipase A₂, nitric oxide synthase (NOS), and various proteases and endonucleases. These may have direct effects on structural proteins and may modify the function of enzymes, receptors or ion channels by altering phosphorylation. They may also produce toxic free radicals via various cascade mechanisms. Both PKC and CaMKII phosphorylate and activate the serum responsive factor (SRF) and Ca²⁺/cAMP response element binding protein (CREB), respectively (4). CREB can also be phosphorylated by cAMP-dependent kinase (protein kinase A, PKA). The increase in cellular levels of phosphorylated SRF and/or CREB results in the induction of immediate early genes (IEGs) (4). Neuronal exposure to excitotoxic levels of glutamate cause either acute toxicity (osmotic lysis) or delayed Ca²⁺-dependent neuronal death (5-7).

CaMKII is a major calcium messenger component that regulates many calcium-dependent processes in neurons. CaMKII phosphorylates and regulates receptor-gated ion channels (8, 9), neuroskeletal elements (10) and calcium-dependent ion channels (6). It is also involved in neurotransmission (11). CaMKII constitutes 1% of total forebrain protein and up to 2% of total hippocampal protein (12). In addition, CaMKII is predominantly expressed in neurons rather than glial cells (13). The ε-subunit is homologous to the major postsynaptic density (PSD) protein which constitutes up to 50% of the total PSD protein (14). The high synaptic expression of CaMKII suggests that this enzyme may be important for normal synaptic function. Since CaMKII is a neuronally enriched enzyme that regulates many important cellular functions, it is easily speculated that the inhibition of this enzyme have important effects on neuronal function.

Significant inhibition of CaMKII activity has been observed in many models of delayed neuronal cell death including animal models of ischemia (15) and glutamate excitotoxicity in neuronal cultures (16). Transient forebrain ischemia results...
in more than 50% inhibition of CaMKII activity in the hippocampus and cortex (17). The decrease in CaMKII activity observed after ischemia is an early (within 10 sec) and long-lasting phenomenon that precedes the development of delayed neuronal cell death (17). This inhibition of CaMKII activity has been implicated in the delayed neuronal cell death (15). Understanding the cellular mechanisms regulating the inhibition of CaMKII will provide an insight into the molecular mechanism of excitotoxicity-induced changes in neuronal transducing systems.

Kainate (KA) administration, an experimental model for human temporal lobe epilepsy (18), is known to engender abnormal excitation/inhibition in the limbic system (19), characteristic neuronal injury in the hippocampus, and spontaneous recurrent seizures (20). This study investigates CaMKII activity relative to long-standing neuronal injury in the hippocampus in KA-induced experimental temporal lobe epilepsy.

MATERIALS AND METHODS

Experimental model for temporal lobe epilepsy

Adult male Wistar rats, 250-300 g, were divided into two groups; KA injection for biochemical and histopathologic studies (75 rats), and sham operated control (6 rats). The experimental rats were anesthetized with pentobarbital sodium (Nembutal, Abbott, Osaka, Japan, 50 mg/kg i.p) fixed on a stereotactic frame (David-Kopf, USA), and stereotactic operations were performed. A stainless steel cannula with internal stylet for KA (Nacalai tesque, Kyoto, Japan) microinjection, 0.03 mm in internal diameter, was implanted in the left amygdala using sterile techniques. Coordinates for the implantation target were: AP +5 mm, ML +5 mm and DV +2 mm with respect to the interaural zero point (20). The cannula was fixed with dental cement. The experimental animals were left free for 7 days to recover from the operation.

KA was prepared immediately before each injection. KA crystals were dissolved in a 0.2 M phosphate-buffered solution (pH 7.4) at a concentration of 1 mg/mL and sterilized through a 0.45 μm microfilter. The injection was delivered following the injection of KA (1 μg/μL) into the left amygdala, successful administration was determined by the induction of clinical seizures. During the first hour after KA injection, animals exhibited “staring spells” followed by repetitive head nodding and “wet dog shakes”. During the next 2 hr, progressive motor seizures developed, including masticatory and facial movements, tremors of the forepaws, and rearing and loss of posture. Finally, animals suffered from limbic status epilepticus with continuous convulsions, lasting 1 to 2 days. The seizures disappeared spontaneously 3 days after KA injection, and motor seizures developed again at about 4 weeks after the injection. During the initial postictal period, animals demonstrated reduced motor activity, but were otherwise normal. Each of ten rats, which developed clinical seizures successfully, were sacrificed by decapitation at 1, 2, 4, 8, and 16 weeks after the injection. Five whole brains were taken immediately and fixed in 10% neutral buffered formalin for histopathologic study, and the other five brains and six brains of sham operated control rats were kept frozen in liquid nitrogen for CaMKII assay.

Histopathologic examination

Routine paraffin blocks were made from both hippocampi and observed histopathologic features in the H&E and cresyl violet stained slides. The right side of the brain was used as control.

Calcium/calmodulin kinase II (CaMKII) assay

CaMKII activity from the hippocampal tissue was measured using a modification of Soderling’s method (21). Frozen hippocampus, 100 mg, was washed and suspended with 1 mL ice-cold homogenization buffer comprised of 30 mM HEPES (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA), 27 TIU aprotinin, 0.1 mM [ethylene-bis(oxyethylenenitrilo)]tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM ATP. The suspension was transferred into a glass homogenizer (Kontes, Vineland, N J) and was disrupted (16). Homogenates were normalized for protein and were studied for endogenous protein phosphorylation.

Standard phosphorylation reaction solutions for kinase assay contained 3 μg protein, 30 mM HEPES (pH 7.4), 1 mM dithiothreitol, 10 mM magnesium acetate, 3.3 μM syn tide II and 1 mM [γ-32P]ATP. Final reaction volume was 100 μL. Standard reactions were performed in a shaking water bath at 30 °C. To measure calcium-dependent activity, reactions were initiated by the addition of 2 mM CaCl2 and 1.2 mM calmodulin; whereas, for calcium-independent activity, 2 mM EGTA was added instead of CaCl2 and calmodulin. The reactions continued for 1 min, and were terminated by the addition of 11 μL of stop solution containing 25 mM Tris, 10 mM Na2-pyrophosphate, and 10 mM β-glycerophosphate. Aliquots (80 μL) were spotted on phosphocellulose paper (Whatman p81). After washing the paper, the radioactivity of free, nonphosphorylated [γ-32P]ATP was quantified by liquid scintillation counter. CaMKII activity (cpm/μg protein/min, mean ± SD) was measured three times from each tissue sample and the average value was calculated. The data were analyzed by Student t-test.
RESULTS

Histopathologic features

The control hippocampus disclosed well defined, palisading neuronal arrangements, consisting of cornu ammonis 1 (CA1), CA2, CA3, CA4, and dentate gyrus. A few neurons were pyknotic, but no neuronal swelling or partial loss was noted. There were no identifiable histopathologic changes on the control side of the hippocampus until 16 weeks after the injection of KA (Fig. 1A and 2A).

The experimental hippocampus from the KA injection revealed no significant histopathologic change within the first week. Mild swelling of neuronal cytoplasm with a few pyknotic nuclei in CA3 was observed at 2 weeks after the injection. The changes progressed with time. A severe

Fig. 1. Coronal section of the brain examined by Nissl stain, 16 weeks after KA injection into the left amygdala, reveals relatively intact hippocampal neurons on the right side (A, ×25) and selective, significant loss of CA3 neurons in the left hippocampus (B, ×25).

Fig. 2. Coronal section of the brain, at 8 weeks after KA injection, examined by H&E stain revealed relatively intact CA2 and CA3 neurons of the right hippocampus (A, ×40), and moderate loss of CA3 neurons in the left hippocampus (B, ×80).
swelling of neuronal cytoplasm was observed at 4 weeks (Fig. 2B) and a decrease in the number of neurons in CA3 at 8 weeks (Fig. 2B) after the injection. The neuronal loss in CA3 persisted up to 16 weeks after KA injection into the amygdala (Fig. 1B).

Calcium/calmodulin kinase II (CaMKII) activity

The CaMKII values (cpm/µg protein/min) on the control side of the hippocampus ranged from 39,725 to 48,173 regardless of the experimental period. Mean values were 42,107 at 1 week; 44,934 at 2 weeks; 45,505 at 4 weeks; 43,432 at 8 weeks; and 41,363 at 16 weeks after KA injection. There were no statistically significant differences of CaMKII values in the hippocampus between the sham operated and the control side of the KA-injected rats. Mean CaMKII values on the experimental side of the hippocampus were 42,556 at 1 week; 46,037 at 2 weeks; 63,666 at 4 weeks; 65,448 at 8 weeks; and 50,050 at 16 weeks after KA injection. There were no statistically significant differences of CaMKII values in the hippocampus between the sham operated and the control side of the KA-injected rats.

Mean CaMKII values on the experimental side of the hippocampus were 42,556 at 1 week; 46,037 at 2 weeks; 63,666 at 4 weeks; 65,448 at 8 weeks; and 50,050 at 16 weeks after KA injection. There was no significant difference in CaMKII activity between the control and lesioned sides of the hippocampus at 1, 2 and 16 weeks after the KA injection. However, significantly increased CaMKII activity was noted at 4 and 8 weeks (p<0.001) on the left (KA-injected) side of the hippocampus. CaMKII activity is summarized in Fig. 3.

DISCUSSION

Intra-amygdaloid injection of KA produces initial limbic motor seizures lasting 2 or 3 days with staring, head nodding, wet-dog shakes, salivation, chewing, forepaw tremors, and rearing and loss of postural control (22). Continuous convulsions lasting over 30 sec are frequently seen. The seizures spontaneously disappear 3 days after KA injection. These are more complex seizures, involving initial lip and facial movements, barrel rotation, and circling movements indicating the secondary involvement of extra-amygdaloid structures. A significant loss of CA3 neurons in the ipsilateral hippocampus was noted after intra-amygdaloid injection of KA in this study, supporting results found in the literature (20, 23).

A simple hypothesis to explain the distant lesions would be that KA diffuses from the amygdala either directly or through the cerebrospinal fluid (CSF), and that sufficient concentrations of KA directly damage vulnerable neurons, such as the CA3 neurons of the hippocampus. However, sufficient doses of diazepam administration blocked distant damage in the CA3 even in the presence of direct damage to the amygdala by KA injection (24). Therefore, the results suggest that KA-induced hippocampal damage following intra-amygdaloid injection stems from two sources—local damage due to the direct toxic action of KA and distant injury mediated by the paroxysmal discharge accompanying convulsions. Other electrophysiologic, autoradiographic and histopathologic studies support the hypothesis that distant damage in the hippocampus is mediated by paroxysmal epileptiform discharge (25-27). Collectively, these findings strongly suggest that paroxysmal discharge after intra-amygdaloid injection of KA is generated in the entorhinal cortex, where the amygdala heavily projects (28). This discharge may deliver a powerful excitatory action on CA3 neurons primarily via the granule cells with their mossy fibers, and the perforating pathways from the entorhinal cortex to granule cells (18). The early and late expression of c-FOS, c-JUN and heat shock protein (HSP) 72 in the entorhinal cortex and hippocampus also indicate indirect damage of the hippocampus (29).

It is well known that glutamate synapses are subject to various forms of prolonged enhancement (30), including long-term potentiation (LTP). The activation of subtypes of glutamate receptors plays a major role in the induction of LTP, and such changes contribute to the induction of epileptic seizures. A number of postsynaptic kinases, PKC, CaMKII, tyrosine kinase (PKT) and PKA, are believed to be important for the induction of LTP (31, 32). Long-term alterations in these protein kinases have also been reported in kindling models for epilepsy (33). Among these kinases, PKA appears to contribute significantly to the induction of the most persistent and long-lasting components of LTP in the hippocampus (32).

In the present study, CaMKII activity in the hippocampus increased significantly 4 and 8 weeks after an intra-amygdaloid injection of KA, when late seizures developed dini-
全社会。在实验性颞叶癫痫中，CaMKII可能与神经元细胞损伤和核苷酸损伤有关。该损伤可能在该区域形成的16周后，特别是通过AMPA受体的信号，导致了CaMKII介导的钙离子内流的增加，可能对细胞产生损伤作用。因此，CaMKII在海马区的活动在急性神经营养素注射后增加。这种作用发生在海马区的ca电源上，从而引起钙离子内流增加，导致细胞损伤。

这些发现支持了部分其他研究的结果，即在CaMKII中诱导的磷酸化通过NMDA受体介导的钙通道在PSD通过兴奋性神经元在胎儿大鼠皮质培养中增加（34）。这可以导致增加钙离子内流，从而增加兴奋性神经元的释放。因此，CaMKII在海马区的活动在急性神经营养素注射后增加。



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