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RESEARCH ARTICLE

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Kindling phenomena induced by the repeated short-term high potassium stimuli in the ventral hippocampus of rats: on-line monitoring of extracellular glutamate overflow

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Abstract We observed in this study that transient periodic stimuli in response to high potassium (40 mM, 5 min at 40-min intervals, 13–15 stimuli) perfusion in the ventral hippocampus of rats led to the appearance of a kindling-like phenomenon. In this kindling-like phenomenon, we confirmed the augmentation of glutamate release and the prolongation of spike discharge. Changes in the extracellular glutamate levels before and after the stimuli were monitored by the application of in vivo microdialysis combined with on-line enzyme fluorometric detection of glutamate. This kindling-like phenomenon was not observed when microdialysis was carried out using a Ca^{++}-free medium. The augmentation of glutamate release and the prolongation of spike discharge with epileptic convulsions are completely Ca^{++} dependent. These data show that repeated short-term increases in extracellular glutamate levels results in the enhancement of excitatory neuronal systems, causing an excessive propagation of seizure activity and culminating in secondary generalized seizures.

Key words Glutamate · On-line detection · Hippocampus · Potassium · Kindling

Introduction

Kindling is a widely accepted model of secondary generalized seizure in which repeated subconvulsive electrical stimulation results in progressively more intense seizure (Goddard et al. 1969; Maru and Goddard 1987). Repetitive microinjection of glutamate into the amygdala causes seizure propagation similar to that of the kindling model (Croucher and Bradford 1989; Mori and Wada 1987), suggesting that the enhancement of excitatory neurotransmitter levels, especially those of glutamate in the limbic system, plays a critical role in seizure propagation (Maru and Goddard 1987; Minamoto et al. 1992; Ueda and Tsuru 1994, 1995; Zhang et al. 1991). However, exogenous glutamate administration into the limbic system is not physiological, and it is important to investigate the relationship between endogenous glutamate release and convulsions to identify the basic mechanism responsible for the epileptogenesis in the kindling model. In addition, the main disadvantage of electrical kindling is the use of high-frequency electrical stimulation to evoke the status epilepticus. Such stimulation is too artificial to discuss the natural development of the epileptogenesis. Increases in extracellular potassium cation levels ($[K^+]_o$) occur during the intense synaptic activity occurring under some pathological conditions (Fritz and Gardner-Medwin 1976; Perez-Pinzon et al. 1995; Somjen and Giacchino 1985). Therefore, stimulating a specific area by increasing $[K^+]_o$ is thought to be more natural than high-frequency electrical stimulation. For these reasons, Semyanov et al. (Semyanov and Godukhin 1997; Semyanov et al. 1997) have produced a kindling-like state in rat hippocampal CA1 slices by repeated short-term $[K^+]_o$ increases in vitro. They were, however, unable to observe the development of epileptic convulsions because their studies were performed in vitro.

It is easy to repeatedly increase $[K^+]_o$ at a specific area of the brain for short periods to depolarize the area of dialysis if utilizing a liquid switch to perfuse high K^+ medium in an in vivo microdialysis system. In this study, we were interested in exploring the following under in vivo conditions: (1) the link between stimulation of the ventral hippocampus for short periods by repeated high K^+ perfusion and the inducement of a kindling-like phenomenon, (2) time-dependent changes in the extracellular glutamate concentration that follow repeated high K^+ perfusion, (3) changes in the behavioral and electroen-

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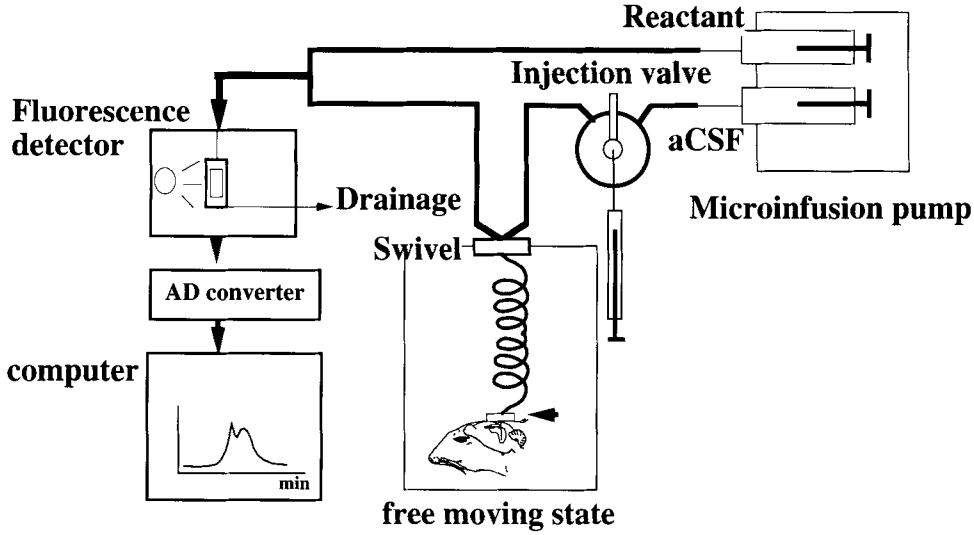

Fig. 1 Schema of on-line enzyme fluorometric detection of glutamate. Modifications made in the present study were as follows: the speed of the reactant was set at 6 µl/min, the volume of the mixing tube 48 µl, the incubation time 6 min, and the injection valve for microinjection of high K^+ aCSF was equipped as shown. *aCSF* Artificial cerebrospinal fluid

cephalographic (EEG) findings during stimuli, and (4) the role of calcium (Ca^{++}) influx in the development of kindling-like phenomenon induced by high K^+ perfusion. We examined the questionable points of (1)–(4) at the ventral hippocampus of rats, which is thought to be the crucial region for the development of kindling (Jarvie et al. 1990; Minamoto et al. 1992).

Materials and methods

Twelve male Wistar rats weighing 380–420 g at the time of surgery were anesthetized with pentobarbital sodium (37.5 mg/kg, i.p.). In this experiment, stereotaxic coordinates were determined by the rat brain atlas of Paxinos and Watson (1986). The incisor bar was set 3.3 mm below the intraaural line. Each rat was also stereotaxically implanted with a 22-gauge guide cannula for an in vivo microdialysis probe. The stereotaxic coordinates were 5.6 mm posterior, 5.0 mm right to the bregma, and 3.0 mm below the surface of the skull. Both the electrode plug for EEG recording of the dialysated portion and the 22-gauge guide cannula along with dummy cannula were firmly anchored to the skull with miniature screws and dental cement. Twelve rats were divided into groups A (n=6) and B (n=6).

Seven days after implantation and in the conscious state, the dummy cannula was replaced with a dialysis cannula consisting of a 24-gauge introducer needle (the tip was covered with a 5.0-mm length of permeable hollow fiber, 11 µm thick, 0.2 mm outside diameter, molecular weight cut-off 7000–8000; Cuprophan, Nikkiso, Japan). The microdialysis procedure was basically the same as that described by Nakahara et al. (1989). The dialysis cannula was connected to a microinfusion pump (EP-60; Eicom, Kyoto, Japan) and continuously perfused with artificial cerebrospinal fluid (aCSF) at 2.0 µl/min. The aCSF compositions used for continuous perfusion in group A were normal for aCSF as follows: 119.8 mM NaCl, 2.57 mM KCl, 1.36 mM $CaCl_2$, 0.47 mM $MgCl_2$, 27.2 mM $NaHCO_3$, 0.67 mM NaH_2PO_4, 1.27 mM Na_2HPO_4, 0.5 mM Na_2SO_4; pH 7.4. Those used in group B were free Ca^{++}, high Mg^{++} aCSF as follows: 82 mM NaCl, 2.57 mM KCl, 20 mM $MgCl_2$, 27.2 mM $NaHCO_2$, 0.67 mM NaH_2PO_4, 1.27 mM Na_2HPO_4, 0.5 mM Na_2SO_4, 2 mM EGTA.2Na; pH 7.4. In vivo microdialysis was carried out in a conscious and free-moving state. On-line enzyme fluorometric detection of glutamate was performed using essentially the same method as that described elsewhere (Matsuda et al. 1998). The modified portions of the system used in this study were as follows: the speed of the reactant was set at 6 µl/min, the volume of the mixing tube 48 µl, the incubation time 6 min, and the injection valve for microinjection of high K^+ aCSF was equipped as shown in Fig. 1. Changes in the hippocampal extracellular glutamate concentrations were monitored by detection of the fluorometric intensities of β-nicotinamide adenine dinucleotide (NADH) resulting from the reaction of glutamate and NAD^+ catalyzed by glutamate dehydrogenase (GLU-DH; Graham and Aprison 1966). The emerging dialysate was mixed with the reactant containing GLU-DH. Reactant solution consisted of 5.4 U/ml GLU-DH (Boehinger Mannheim, Mannheim, Germany), 0.34 mM NAD^+ (Sigma, St. Louis, MO., USA), 1.5 mM adenosine diphosphate, and 0.29% v/v hydrazine hydrate (Sigma) in 0.1 M TRIS-HCl buffer (pH 8.5). NADH was detected using a 12-µl flow cell in a fluorescence detector (Type RF-10AXL; Shimadzu, Kyoto, Japan) with 340–450 nm excitation-emission wavelengths.

Calibration was carried out by dipping the probe into 50 µM glutamate dissolved in normal aCSF for 10 min prior to every in vivo experiment. The fluorescence intensity of NADH was converted into glutamate concentrations based on calibration using the dipping test into 50 µM glutamate.

After a 3-h stabilization period following the probe insert, depolarization was induced at the right ventral hippocampus by the injection of high K^+ aCSF (10 µl) through an injection valve at 40-min intervals, and we then observed the time-dependent changes in glutamate levels, the EEG findings, and behavior changes. Transient periodic stimuli were repeated at least 15 times. The seizure stage was determined by Racine's scale (Racine 1972). The composition of high K^+ aCSF used in group A was Ca^{++}-containing high K^+ aCSF as follows: 86 mM NaCl, 40 mM KCl, 1.36 mM $CaCl_2$, 0.47 mM $MgCl_2$, 27.2 mM $NaHCO_3$, 0.67 mM NaH_2PO_4, 1.27 mM Na_2HPO_4, 0.5 mM Na_2SO_4; pH 7.4. That in group B was free Ca^{++}, high Mg^{++}, high K^+ aCSF as follows: 42 mM NaCl, 40 mM KCl, 20 mM $MgCl_2$, 27.2 mM $NaHCO_3$, 0.67 mM NaH_2PO_4, 1.27 mM Na_2HPO_4, 0.5 mM Na_2SO_4, 2 mM EGTA.2Na; pH 7.4.

Statistical analysis for the seizure stage between groups A and B was carried out using 2-way (Group×Stimuli) ANOVA with repeated measures followed by Tukey's test for multiple comparisons. All animal experiments were reviewed and approved by the Animal Welfare Committee of Miyazaki Medical College (1998–051–2).

Results

Transient periodic depolarization induced by high K^+ aCSF perfusion in the ventral hippocampus led to augmentation of the increasing ratios of glutamate levels

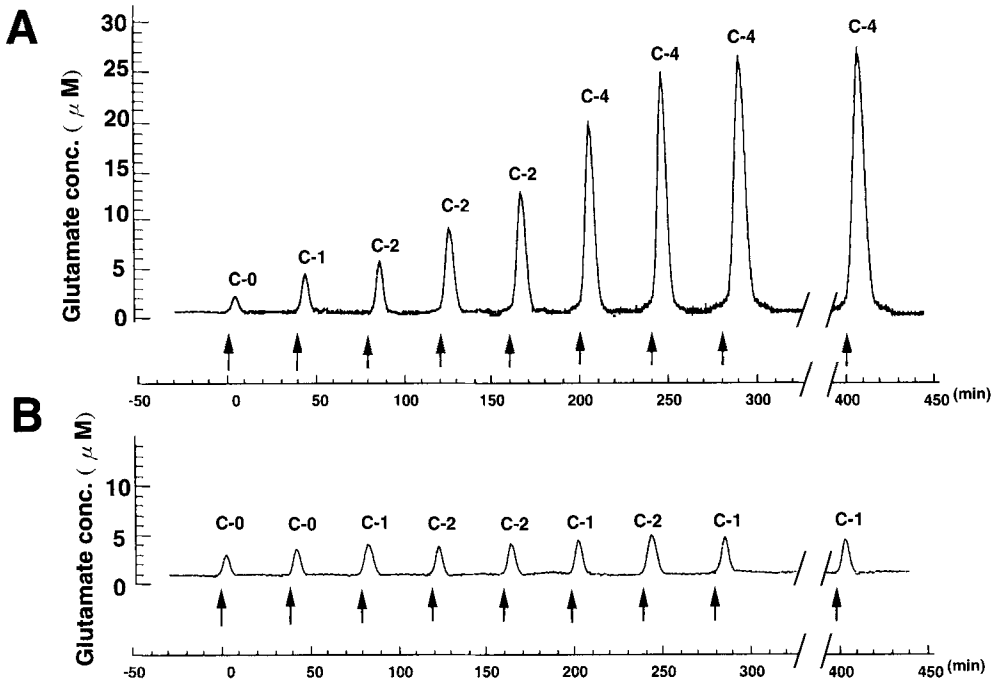

Fig. 2A,B The release of glutamate in group A (**A**) was augmented by the repetitive short-term depolarization by high K^+ perfusion as shown in Fig. 1. *Arrows* indicate the point when high K^+ aCSF was injected. C-0, -1, -2, and -4 in this figure represent the seizure stage according to Racine's scale. In contrast to group A, the augmentation of glutamate release following stimuli was not observed in group B (**B**), and seizure stage in group B did not progress

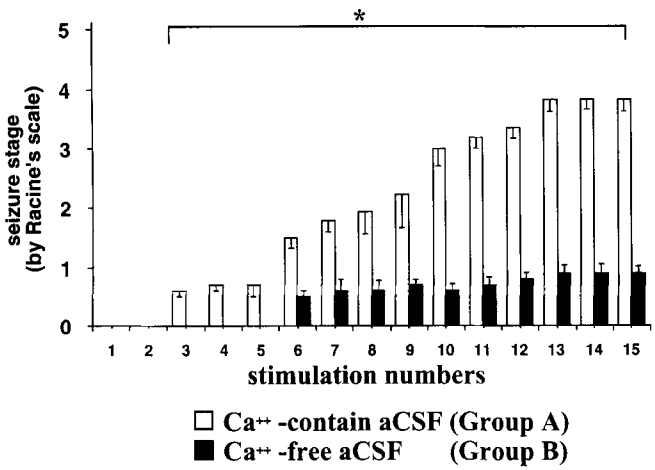

Fig. 3 The relationship between the number of high K^+ stimulations and the acquired seizure stage is presented (mean ± SEM). Rats in group B, who were perfused continuously with free Ca^{++}, high Mg^{++} aCSF, and depolarized with free Ca^{++}, high Mg^{++}, high K^+ aCSF, did not exhibit an augmentation of glutamate overflow or any kind of generalized convulsions such as those of C-3, -4, and -5. *Asterisk* $P<0.01$ [compared to group B at each stimulation; F values for group effect: $F1,87=217.891$, $P=0.0001$, statistical analysis was carried out by two-way (Group×Stimuli) ANOVA followed by Tukey's for multiple comparison]

(Fig. 2), more severe grade of seizure stages (Fig. 3), and the prolongation of spike discharge (Fig. 4) in all rats of group A. With regard to the basal glutamate levels in group A, we could not find any significant elevation of basal extracellular glutamate levels (Fig. 2). Although the rats were non-convulsive at the first stimulation, the epileptic convulsions following the stimuli gradually grew more severe (Fig. 3). Stage 4 or 5 was observed at about the13th–15th high K^+ stimulus in group A. The relationship between the number of high K^+ stimuli and the acquired seizure stage is presented in Fig. 3 (mean ± SEM). The seizure stage for each stimulus was statistically higher in group A than in group B, which was the group perfused continuously with free Ca^{++}, high Mg^{++} aCSF, and depolarized with free Ca^{++}, high Mg^{++}, high K^+ aCSF. An augmentation of glutamate overflow and convulsions was not observed in any of the group B rats. Regarding the EEG, the prolongation of spike discharges from the perfusion area in the hippocampus of group A rats was observed as shown in Fig. 4, but not in group B rats.

Discussion

In this study, through the on-line detection of glutamate with simultaneous monitoring of changes in behaviors and EEG findings, we observed that a kindling-like phenomenon can be initiated by stimulation of the ventral hippocampus by repeated short-term high K^+ perfusion.

Several investigators have already reported that the repetitive microinjection of exogenous glutamate into the limbic system causes seizure propagation similar to that of the kindling model (Croucher and Bradford 1989; Mori and Wada 1987); it is therefore reasonable to assume that a rise in extracellular glutamate levels in the limbic system plays an important role in seizure phenomena. Experimental study of patients with temporal lobe epilepsy and the amygdaloid kindling model suggest that the transient rise in extracellular glutamate at the time of an epileptic event in the hippocampus would precipitate seizures and that the concentrations reached may cause cell death (During and Spencer 1993; Ueda and Tsuru 1994, 1995). Although a result similar to that

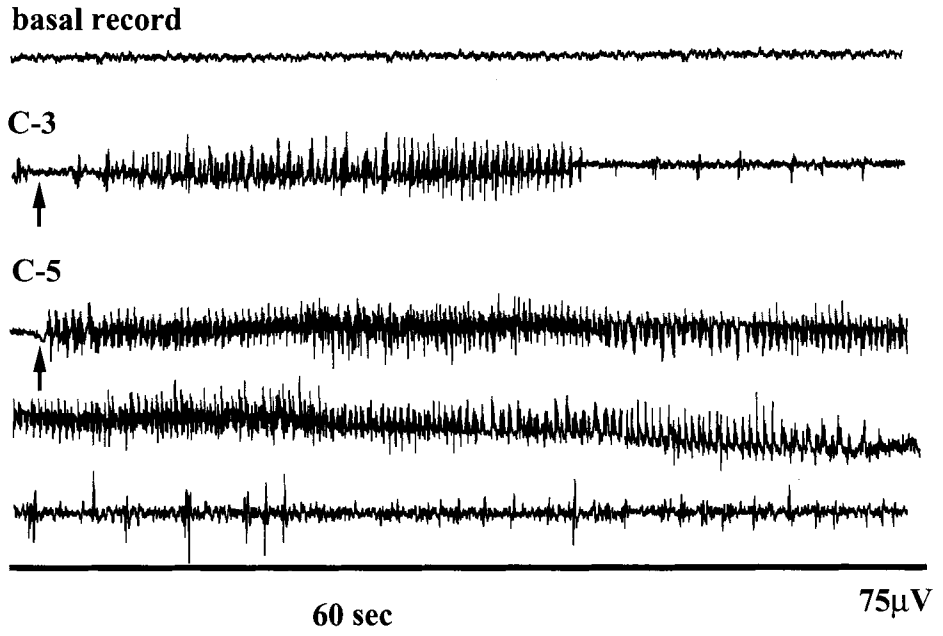

Fig. 4 Changes in electroencephalography (EEG) findings before and after high K^+ perfusion for 5 min at the respective seizure stages in group A. EEG at the *top* is basal EEG recording before the first stimulation, the *middle* is that when rats showed a C-3 seizure, and the *bottom* is that when rats showed a C-5 seizure. The *arrow* indicates the point when 10 μl high K^+ aCSF was injected from the injection valve

described above was obtained in this study, the characteristic feature of the increases in glutamate level in this study was an augmentation of the increasing ratios of extracellular glutamate levels just after the stimuli, together with seizure propagation and a prolongation of the spike discharge from the hippocampus.

In contrast to the rat perfused with Ca^{++}-containing aCSF (group A), the rat dialysated with free Ca^{++}, high Mg^{++} aCSF (group B) did not show any generalized convulsions. These results suggest that an augmentation of seizure-like kindling, a transient periodic increase in extracellular glutamate levels, and the prolongation of spike discharge are all phenomena dependent on Ca^{++} influx. There are two supposed pathways for the propagation of Ca^{++} signals to the cell nucleus that are evoked by the activation of *N*-methyl-D-aspartate (NMDA) receptors and L-type Ca^{++} channels (Bading et al. 1993; Ghosh and Greenberg 1995). Although the detailed mechanism for the Ca^{++} influx associated with this kindling-like phenomenon was not evaluated in this study, it has already been confirmed in an in vitro study that the selective blocker of L-type Ca^{++} channels, nimodipine, abolishes the development of the kindling-like phenomenon (Semyanov and Godukhin 1997). It is possible that the development of the in vivo high K^+-evoked kindling phenomenon shown in this study is also regulated by Ca^{++} influx through an L-type Ca^{++} channel. However, the Ca^{++} influx activated by NMDA receptors should also not be disregarded. According to a report by Morimoto et al. (1991), MK-801 (a non-competitive antagonist of NMDA receptor) strongly retards the amygdaloid electrical kindling, resulting in the early stages of kindled seizures and the growth of after discharges recorded from the amygdala being significantly suppressed. After the establishment of kindling, however, MK-801 only reduces the previously amygdaloid kindled seizure stage without shortening the after discharge duration. Hence, the Ca^{++} influx activated by NMDA receptors would be primary related to the early course of the development of the high K^+-evoked kindling-like phenomenon.

Jarvie et al. (1990) have investigated the Ca^{++}-dependent release of glutamate in the entorhinal kindling model. In this model, the statistically higher extracellular glutamate levels were maintained for longer periods in regions of the hippocampus than in other regions, suggesting that a permanent alteration in the excitatory presynaptic process had evolved, and that this alteration was located primarily in the ventral hippocampus. Their studies indicate that the ventral hippocampus in the area we stimulated in this study is one of the crucial areas for the development of kindling in rats. As shown in the glutamate release of rats in group B, the abolished increases in glutamate levels resulted in a failure of the kindling-like phenomena of the seizure stage to develop. This result indicates that this high K^+-evoked kindling-like phenomenon correlates with increases in the glutamatergic synaptic efficiency in the ventral hippocampus.

With regard to the basal glutamate levels, although other investigators have reported chronic increases in the basal glutamate levels in the interictal state following progressive epileptogenesis (Lothman et al. 1987; Minamoto et al. 1992; Zhang et al. 1991), we could not find any significant elevation of basal extracellular glutamate levels (Fig. 2). We have described that the basal levels of hippocampal extracellular glutamate either are not elevated or are decreased compared with those before the first electrical stimulus in the amygdaloid kindling model (Ueda and Tsuru 1994, 1995). These findings essentially follow the pattern in this study. Considering these results together with our previous results from the electrical amygdaloid kindling study and the results of During and Spencer (1993), it can be hypothesized that the evoked transient and periodic glutamate

increases in the limbic system (viz. hippocampus or amygdaloid body) play a more important role than the chronic elevation of extracellular glutamate concentrations.

Hence, the basic mechanism for this kindling-like phenomenon induced by transient periodic depolarization is deeply related to the electrical amygdaloid kindling phenomenon. The development of a kindling-like phenomenon as shown in this study, that is the process by which an epileptic circuit is acquired, is thought to require long-lasting forms of synaptic plasticity; to be more precise, a progressive increase in synaptic transmission efficacy (Goddard et al. 1969; Herron et al. 1986; Jarvie et al.1990; Maru and Goddard 1987). In this experiment, we further confirmed the role of glutamate in the hippocampus; that the transient periodic depolarization in the hippocampus appears to be accompanied by a progressive, transient, stimulus-induced enhancement of extracellular glutamate levels.

Consequently, we believe that these findings illustrate an important neurophysiological alteration in the presynaptic response that is closely related to neuronal plasticity and epileptic activity. It also appears that the enhanced glutamate release after the stimulus would provide a repetitive activation of NMDA receptors (Herron et al. 1986; Jarvie et al. 1990) and contribute to the development of the kindling-like phenomenon. As shown in the present study, following hippocampal depolarization, released glutamate leads to the excessive propagation of seizure activity through the hippocampus to other brain structures and causes a secondary generalized seizure similar to electrical kindling.

Additional study such as co-perfusion of antagonists of the Ca^{++} channel and/or NMDA receptors is necessary. However, this model, referred to as "K^+-kindling", is easily established and is useful in clarifying the basic mechanisms of secondary generalized epilepsy and in analyzing the potential of newly developed anti-epileptic drugs.

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