ELSEVIER

Epilepsy Research 58 (2004) 107–117

Epilepsy Research

www.elsevier.com/locate/epilepsyres

Prolonged changes in Ca^{2+}/calmodulin-dependent protein kinase II after a brief pentylenetetrazol seizure; potential role in kindling

Yu Dong, Howard C. Rosenberg*

Department of Pharmacology and Therapeutics, Medical College of Ohio, 3035 Arlington Avenue, Toledo, OH 43614-5804, USA

Received 1 October 2003; received in revised form 8 January 2004; accepted 14 January 2004

Abstract

This study evaluated the alteration of CaMKII autophosphorylation and distribution in rat brain following a single, brief pentylenetetrazol (PTZ) seizure and during PTZ kindling. Total CaMKII α subunit (α-CaMKII) and α-CaMKII phosphorylated at Thr^{286} were detected by immunoblot. A large decrease in CaMKII Thr^{286} phosphorylation, as well as CaMKII translocation from particulate to soluble fraction was observed in both cerebral cortex and hippocampus 0.5–4 h after the brief PTZ convulsion. These changes reverted to control values by 12 h. These long-lasting changes in CaMKII autophosphorylation and subcellular distribution after a brief seizure suggested that CaMKII could be involved in carrying forward the signal resulting from brief seizure activity, at least for a few hours, as would be required for kindling to occur. In PTZ kindled rats, convulsions produced changes in CaMKII Thr^{286} phosphorylation and distribution in the same direction and of similar magnitude as after the acute convulsion, but lasting for a much longer time. In fact, reduced Thr^{286} phosphorylation of α-CaMKII was observed up to 48 h, completely bridging the interval between PTZ injections. Similar, but intermediate changes were found in tissue from rats that were only partially kindled. These results implicate CaMKII as a molecular messenger in the acquisition of PTZ kindling.

Keywords: Pentylenetetrazol; Seizure; Kindling; CaMKII; Autophosphorylation; Subcellular translocation

1. Introduction

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional serine/threonine protein kinase. After binding to Ca^{2+}/calmodulin (Ca^{2+}/CaM) complex, CaMKII α and β subunits are autophosphorylated at Thr^{286} and Thr^{287}, respectively, resulting in autonomous activity, i.e. no longer requiring the continued presence of Ca^{2+}/CaM for activity. This property extends the duration of CaMKII activation long beyond the initial signal—the brief increase in intracellular Ca^{2+}. CaMKII is abundant in brain, accounting for as much as 1% of total protein (2% in hippocampus), and is widely distributed in cell bodies, dendrites, pre-synaptic membranes, and especially in postsynaptic density (PSD), where it comprises 20–40% of PSD protein (Hanson and Schulman, 1992). Its unique properties, abundance and location contribute to the role of CaMKII as a key transducer of extracellular signals,

Abbreviations: CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; α-CaMKII, CaMKII α subunit; Thr^{286}-pCaMKII, CaMKII phosphorylated on Thr^{286}; Ca^{2+}/CaM, Ca^{2+}/calmodulin complex; PTZ, pentylenetetrazol; PSD, postsynaptic density; LTP, long-term potentiation

*Corresponding author. Tel.: +1-419-383-4182; fax: +1-419-383-2871.

E-mail address: hrosenberg@mco.edu (H.C. Rosenberg).

0920-1211/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.eplepsyres.2004.01.003

which would be extensively activated during seizure activity.

It was reported that status epilepticus induced by bicuculline resulted in decreased CaMKII activity in crude synaptic membranes of cortex and hippocampus (Bronstein et al., 1988). Using the pilocarpine-induced seizure model, it was found that inhibition of CaMKII activity lasted for up to 5 days after the end of seizure activity, and for several weeks in rats that subsequently developed spontaneous recurrent seizures (Churn et al., 2000a; Kochan et al., 2000). In another study, an antisense oligonucleotide was used to inhibit α-CaMKII subunit expression in cultured hippocampal neurons, which resulted in epileptiform activity (Churn et al., 2000b). Transgenic mice carrying a null mutation (−/−) for the CaMKII α subunit exhibited profound hyperexcitability (Butler et al., 1995). From the above studies, it appears that CaMKII and seizure activities are highly related. Seizure activity caused depressed CaMKII activity, while a decrease in available CaMKII resulted in increased excitability.

The above evidence suggested a possible cause-and-effect relationship between seizures and CaMKII regulation. This relationship suggests that CaMKII might play a role in kindling, a phenomenon whereby repeated, initially subconvulsive stimulation (electrical or chemical) ultimately results in intense partial or generalized convulsive seizures (Goddard et al., 1969). Pentylenetetrazol (PTZ) is a prototype systemic convulsant used to study chemical kindling. Systemic administration of PTZ results in reliable induction of convulsive seizures in a dose-dependent pattern. A 45 mg/kg dose of PTZ in rats induces convulsions usually manifested by front limb clonus (Rosenberg et al., 1985). A single subconvulsive dose of PTZ, such as 30 mg/kg, usually is not sufficient to produce generalized convulsive activity (i.e. the observable motor component of the seizure). However, if given repeatedly, generalized clonic, or even tonic-clonic convulsions will eventually develop; i.e. PTZ kindling will occur (Pinel and Van Oot, 1975).

As little is known regarding what effect a brief, self-limited seizure might have on CaMKII, the effects of a single convulsive dose and a single subconvulsive dose of PTZ on CaMKII were investigated first in the present study. Then, the dynamic change of CaMKII during and after the PTZ kindling process was evaluated. By evaluating CaMKII regulation following a single seizure and following kindled seizures, a possible link between the single seizure episode and recurrent seizure activities could be assessed, which might shed light on the mechanism of epileptogenesis. Since PTZ mainly affects forebrain, and as the ratio of CaMKII α and β subunits in rat forebrain is 3–4:1 (Braun and Schulman, 1995), this study focused on changes in CaMKII α subunit.

2. Materials and methods

2.1. Materials

Male Sprague–Dawley rats (225–300 g for single PTZ injection, 150–175 g initial weight for PTZ kindling) were obtained from Harlan Laboratories (Indianapolis, IN, USA). All animal procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Medical College of Ohio's Institutional Animal Care and Use Committee. Reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies, MA1-048 for total CaMKII and MA1-047 for CaMKII phosphorylated on Thr^{286}, were obtained from Affinity BioReagents, Inc. (Golden, CO, USA). HRP-conjugated secondary antibody, anti-mouse IgG, was purchased from Chemicon International (Temecula, CA, USA). ECL Western blotting detection reagents were from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

2.2. Acute PTZ treatment

Male Sprague–Dawley rats (225–300 g) were given a single convulsive dose of PTZ, 45 mg/kg, i.p. in saline. Matched controls received saline. At this PTZ dose, convulsions typically begin in about 1 min, then progress to bilateral front limb clonus (usually not involving hind limbs). The convulsive episode usually lasts 30–40 s. Rats were decapitated at one of several times following PTZ: 5 min, 0.5, 1, 2, 4, 12, 24 or 48 h.

Since a 30 mg/kg PTZ dose was used for PTZ kindling, the effect of an acute 30 mg/kg dose on CaMKII was tested in a group of rats. A single dose of 30 mg/kg PTZ typically produced either no overt convulsive behavior, or twitches of face and ears. For ease of dis-

cussion, this is referred to as a "subconvulsive" dose. Rats were observed for 30–60 min after PTZ injection, then decapitated at the 1 h time point. Controls were handled the same way but received saline.

2.3. PTZ kindling procedure

Male Sprague–Dawley rats, initially 150–175 g, were housed under standard conditions, one per cage. These were assigned randomly to kindling or control (pair-handled, saline-injection) groups. Kindling was induced by repeated i.p. injection of a subconvulsive dose of PTZ (30 mg/kg) every 48 h between 8.00 and 10.00 a.m. (Atack et al., 2000; Corda et al., 1990, 1992; Suzuki et al., 2001). After each injection, the convulsive behavior was observed for 30 min and the kindled seizure stage assigned based on a scale slightly modified from that of Racine (1972): stage 0, no convulsive behavior; stage 1, ear/face twitches or prolonged generalized tremors; stage 2, myoclonic jerks or unilateral front leg clonus; stage 3, more than 20 severe myoclonic jerks of axial and limb muscles, or bilateral front leg clonus; stage 4, bilateral front leg clonus plus a fall (loss of postural control, falling); and stage 5, tonic-clonic convulsion, or clonus of all four legs and falling. A rat was considered to be fully kindled after having reached at least three consecutive convulsions of stage 4 or 5, which agrees with definitions in the various published systems. Controls were treated with the same number of saline injections. Fully kindled rats and their controls were decapitated 1, 24 or 48 h after the final PTZ or saline injection.

In another group of rats, PTZ treatment was arbitrarily stopped after 14 injections. These partially kindled rats were randomly assigned to be used either 1 or 24 h after the last PTZ injection. For the saline controls, animals were randomly assigned to two groups after 13 saline injections. After the usual 48 h interval, one group received saline as the final dose, while the others were given 30 mg/kg PTZ as the final dose. This latter group was observed for convulsive behaviors for 30 min then decapitated after 24 h.

2.4. Tissue preparation

Treated rats and pair-handled controls were decapitated at predetermined time points after PTZ injection, as described above. The brain was quickly isolated and immediately chilled in ice-cold saline for 1 min. Cerebral cortices and hippocampus were dissected within 1 min, rapidly frozen in liquid nitrogen, and stored at −76 °C until use. All further preparation was done at 4 °C.

Cerebral cortex or hippocampus was homogenized in 6 ml (3 ml for hippocampus) 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM EGTA, 1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml trypsin inhibitor, 2 mM benzamide–HCl, and 0.1 mg/ml bacitracin. For most experiments, homogenate was centrifuged at 14,000 × g for 30 min at 4 °C, and aliquots of supernatant and pellet as well as total homogenate were saved for Western blot (Moore et al., 1996). For a few experiments, more rigorous subcellular fractionation was performed. Cerebral cortex was homogenized in an isotonic sucrose buffer (the above Tris buffer plus 0.32 M sucrose) according to Fukuda et al. (1998). The total homogenate was centrifuged at 1000 × g for 15 min, which yielded supernatant (S1), and pellet (P1) that included nuclei and cell debris. The S1 fraction was further centrifuged at 14,000 × g for 30 min and separated into S2 and P2 fractions, the P2 containing mainly myelin, synaptosomes and mitochondia. The S2 fraction was then subjected to 100,000 × g centrifugation for 60 min to yield S3 and P3 fractions. S3 represents soluble cytosolic proteins, while P3 includes plasma membrane, rough/smooth endoplasmic reticulum, and Golgi bodies (Fukuda et al., 1998). All pellet fractions were resuspended in the above Tris–sucrose buffer. Aliquots of homogenate and all subcellular fractions were stored at −76 °C until use.

2.5. Immunoblot analysis

Protein samples from total homogenate and subcellular fractions were denatured, separated by 10% SDS–polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane. After blocking in 5% non-fat milk, membranes were either incubated with monoclonal antibody MA1-048 (1:100,000 dilution) to detect CaMKII α subunit total protein, or with monoclonal antibody MA1-047 (1:2000 dilution), which only recognizes α-CaMKII phosphorylated on Thr^{286}. Using HRP-conjugated secondary antibody, the protein bands were visualized by ECL chemiluminescence and exposed to light sensitive au-

toradiography film (Hyperfilm ECL, Amersham). Autoradiographs were analyzed with a Bio-Rad imaging densitometer (Model GS-670). For each set of data on an autoradiograph, the mean density of the control samples was set as 1.0, then the relative density of each band was taken as a fraction of this value.

2.6. Statistics

Most of the data were evaluated using one-way ANOVA. Significant results were further evaluated by Dunnett's test to determine which means differed significantly from the control. When appropriate, other data were evaluated by *t*-test. In all cases, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of a single convulsive dose of PTZ on CaMKII Thr^{286} phosphorylation and subcellular distribution

After an acute convulsive dose of PTZ (45 mg/kg), convulsions began in about 1 min and lasted for 30–40 s. Convulsions usually started with body jerks and progressed to bilateral front limb clonus; hind limbs were rarely involved. A post-ictal state was noted right after the convulsions and lasted no longer than 10 min. Only rats that developed the above typical clonic convulsions after 45 mg/kg of PTZ were used (about 90%). Rats not progressing to front limb clonus or those having more severe convulsive seizures were not used.

In cerebral cortex, there was a remarkable reduction in CaMKII Thr^{286} phosphorylation at 0.5 h (but not at 5 min), and this significant reduction remained up to 2 h. The maximum change, at 1 h, was an 80% loss of CaMKII Thr^{286} phosphorylation. An apparent partial reversal started 4 h after convulsion, with no residual effect or rebound at 12 h or thereafter (Fig. 1A). The total amount of CaMKII α subunit in cerebral cortical homogenate was not changed at any time point after the convulsion.

Total CaMKII α subunit in both supernatant and pellet (separated by 14,000 × *g*) was evaluated by MA1-048 antibody (Fig. 1B). There was a reduced amount of CaMKII in pellet from 0.5 to 2 h after convulsion, with partial resolution by 4 h, and no further effect or rebound at 12 h or beyond. Simultaneously, there was a corresponding time-dependent increase in the amount of CaMKII in supernatant. This pellet to supernatant translocation (Fig. 1B) had a time course that precisely matched that of the decreased CaMKII Thr^{286} phosphorylation (Fig. 1A).

Reduced CaMKII Thr^{286} phosphorylation in hippocampus (Fig. 2) was very similar to that found in cerebral cortex, except that the reduction was still significant in hippocampus 4 h after PTZ-induced convulsion. CaMKII translocation in hippocampus did not appear to be as pronounced as in cerebral cortex. In cerebral cortex, translocation of CaMKII from pellet to supernatant doubled the CaMKII signal intensity in the supernatant between 0.5 and 2 h. In contrast, though there was a trend for CaMKII to increase in hippocampal supernatant (0.5–4 h), none of these time points reached significance. On the other hand, significant decreases of CaMKII signal in the hippocampal pellet fraction were seen at 0.5 and 1 h.

To gain further insight into the translocation of CaMKII following the PTZ convulsion, a more rigorous subcellular fractionation was performed. Using tissue from rats 1 h after the PTZ convulsion, the time of peak change (Figs. 1 and 2), cerebral cortices were homogenized and subcellular fractions were obtained by a series of centrifugation steps using isotonic sucrose buffer (Fukuda et al., 1998). The results in Fig. 3 show the data from cortex of PTZ-treated rats as a percent of the values in the saline controls. Statistical analysis was done with the actual values from both treated and controls. CaMKII translocation from the P3 to the S3 fraction was obvious (Fig. 3). It was also clear that CaMKII was translocated from the P2 to the S2 fraction (Fig. 3). These results showed a clear translocation of CaMKII to the soluble compartment. Even though the buffer and fractionation method differed from that used in the experiments described above, the results indicated that the one-step 14,000 × *g* centrifugation was sufficient to show the direction and degree of CaMKII translocation from the particulate to the soluble fraction after PTZ convulsion. This was similar to the results reported by Kolb et al. (1995) comparing similar separation methodologies in a hippocampal ischemia model to study CaMKII translocation. Using anti-Thr^{286}-pCaMKII antibody, it was found that reduced CaMKII Thr^{286} phosphorylation

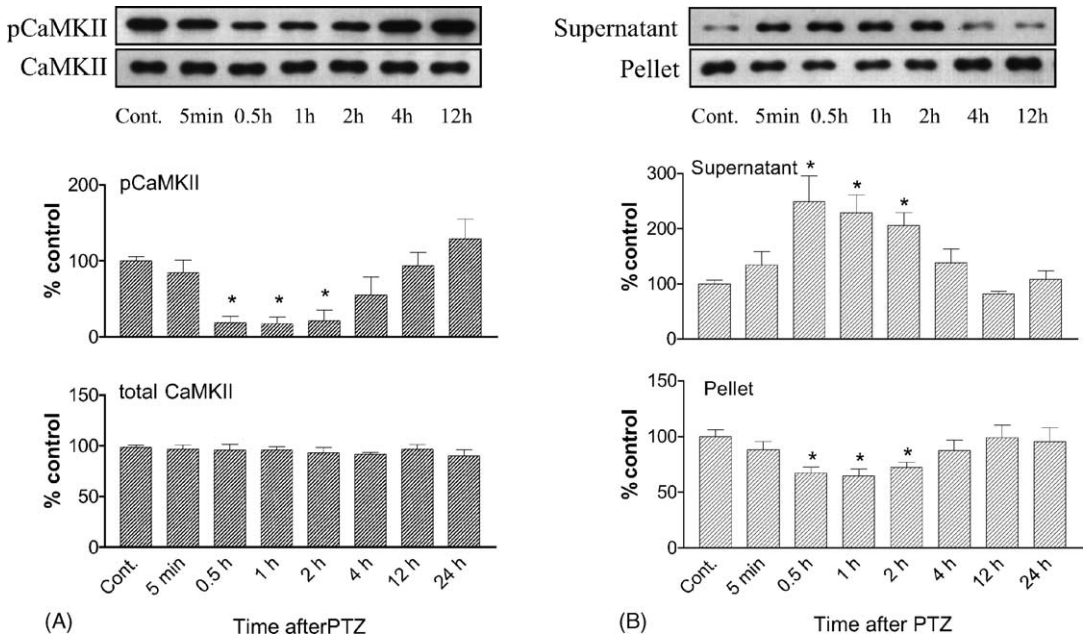

Fig. 1. Effect of an acute convulsive dose of PTZ (45 mg/kg) on CaMKII phosphorylation and translocation in cerebral cortex. Rats were given PTZ at time = 0, then decapitated at the times shown. Cerebral cortices were isolated and homogenized, and supernatant and pellet fractions were separated by 14,000 × *g* centrifugation. Samples were evaluated by Western blot. (A) The top of the figure shows sample results from cerebral cortical homogenates evaluated by specific monoclonal antibodies for total CaMKII α subunit, and for this same protein phosphorylated on Thr^{286} (pCaMKII). The bar graphs show the summary results at the various time points. Thr^{286}-pCaMKII was significantly decreased in homogenate of cerebral cortex at 0.5, 1 and 2 h; total CaMKII protein in homogenate was not changed. (B) The top part shows sample results of Western analysis for total CaMKII in pellet and supernatant fractions. There was a significant translocation of CaMKII from the pellet to the supernatant fraction at 0.5, 1 and 2 h after PTZ-induced convulsion ($n = 4$–5).

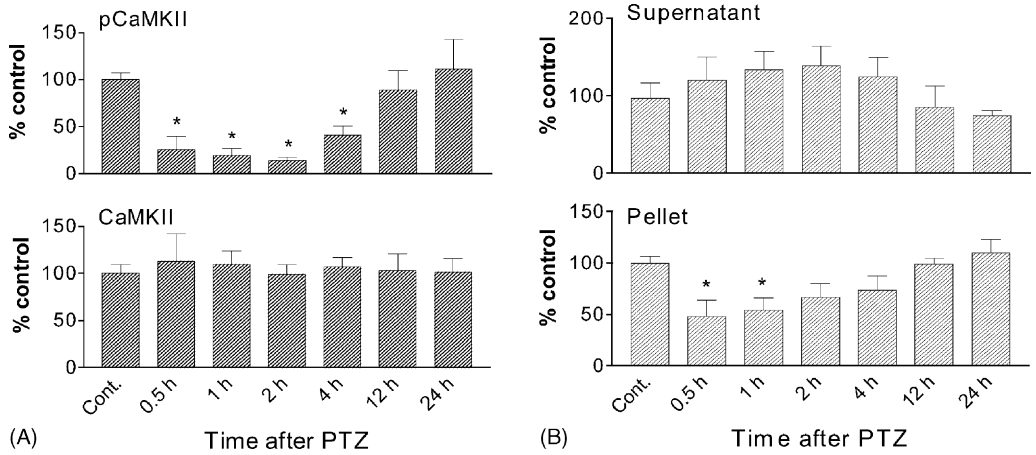

Fig. 2. Effect of an acute convulsive dose of PTZ (45 mg/kg) on CaMKII phosphorylation and translocation in hippocampus. Rats were given PTZ and tissues prepared as in Fig. 1. (A) CaMKII phosphorylation on Thr^{286} (pCaMKII) was significantly decreased in homogenate of hippocampus at 0.5, 1, 2 and 4 h after PTZ-induced convulsion, while total CaMKII in homogenate was not changed. (B) PTZ convulsion caused CaMKII translocation from 14,000 × *g* pellet to supernatant, with significant changes in pellet at 0.5 and 1 h. Changes in CaMKII phosphorylation and translocation had both recovered by 12 h and thereafter ($n = 4$–5).

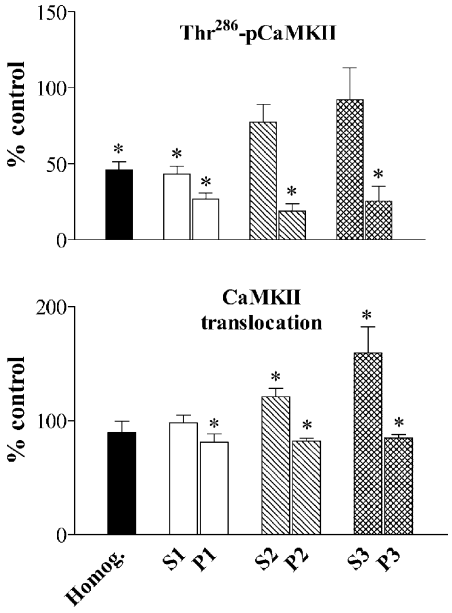

Fig. 3. CaMKII translocation and reduced phosphorylation in subcellular fractions of cerebral cortex 1 h after an acute convulsive dose of PTZ (45 mg/kg). In contrast to the experiments shown in other figures, cerebral cortices were isolated and subcellular fractions were obtained by a series of centrifugation steps using an isotonic sucrose buffer (see Section 2). Homogenate was separated into S1 and P1 ($1000 \times g$), S1 was separated into S2 and P2 ($14{,}000 \times g$), and S2 was separated into S3 and P3 ($100{,}000 \times g$). Results are shown as percent of the mean values for the corresponding saline treated controls, with actual data values from each group ($n = 4$) compared to the corresponding controls by t test. Top panel: Following an acute PTZ seizure, Thr^{286}-pCaMKII was significantly decreased in total homogenate, which was due to the concurrent Thr^{286}-pCaMKII reduction in both $1000 \times g$ supernatant and pellet (S1 and P1). CaMKII phosphorylation was also significantly reduced in the pellet fractions following the higher speed centrifugations (P2 and P3). Bottom panel: An hour after the PTZ seizure, there was a significant amount of CaMKII translocated from pellet to supernatant fraction which could be observed from both P2 to S2 ($14{,}000 \times g$) and P3 to S3 ($100{,}000 \times g$). (Homog., total homogenate; S, supernatant; P, pellet).

in both S1 and P1 fractions contributed substantially to the large decrease of CaMKII Thr^{286} phosphorylation in total homogenate (Fig. 3). However, after $14{,}000 \times g$ or $100{,}000 \times g$ centrifugation, significant loss of CaMKII Thr^{286} phosphorylation was only seen in pellet fractions (P2 and P3, Fig. 3). This was partially due to CaMKII translocation away from pellets, resulting in more CaMKII protein present in the supernatant in the tissue from PTZ treated rats.

Administering a benzodiazepine, flurazepam (20 mg/kg, i.p.), caused just threshold motor effects with reduced skeletal muscle tone and barely detectable ataxia. When given 20 min prior to 45 mg/kg PTZ, this pre-treatment not only prevented PTZ-induced convulsive activity, but also prevented CaMKII translocation and the loss of phosphorylation (data not shown), which suggested that the changes in CaMKII resulted from the occurrence of seizures.

3.2. Effect of an acute, subconvulsive PTZ dose on CaMKII Thr^{286} phosphorylation and subcellular distribution

Since a subconvulsive 30 mg/kg dose of PTZ was to be used for the PTZ kindling procedure, the effect of this dose on CaMKII was compared to that of the convulsive dose (45 mg/kg). Rats were studied 1 h after receiving 30 or 45 mg/kg PTZ, or saline, the time for peak changes noted above. In cerebral cortical homogenate, 30 mg/kg PTZ caused quite a large decrease (50%) in CaMKII Thr^{286} phosphorylation (Fig. 4A), though not as large as after 45 mg/kg PTZ (80%). Total CaMKII in homogenate was unchanged. In contrast to the translocation seen 1 h after the convulsion produced by 45 mg/kg PTZ, there was no significant translocation produced by the 30 mg/kg dose (Fig. 4B). It seemed that CaMKII translocation required a larger stimulation, i.e. more severe seizure activity. These results suggested that the loss of CaMKII Thr^{286} phosphorylation and translocation need not occur together, and so could have different bases.

3.3. CaMKII Thr^{286} phosphorylation and subcellular distribution in PTZ kindled brain

Since a single PTZ-induced convulsion, lasting only about 1 min, caused changes in CaMKII for hours (Figs. 1 and 2), it was thought possible that CaMKII might play a role in PTZ kindling. Rats were kindled with 30 mg/kg PTZ every 48 h until at least three consecutive stage four or five seizures were induced (Fig. 5A). In the first 2 weeks, stage 1 to 2 convulsions were frequently seen, which were comparable to those sometimes produced by an acute subconvulsive 30 mg/kg dose of PTZ. During weeks 3–4, stage 3 convulsions emerged, which mostly resembled the clonic convulsive effect of 45 mg/kg PTZ. After week 4, stage

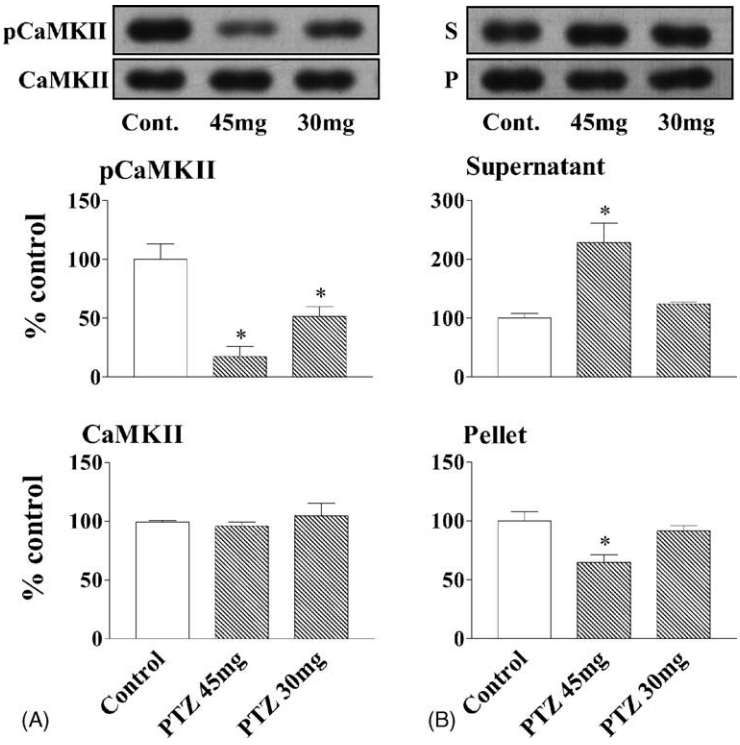

Fig. 4. Dose-related effects of PTZ on CaMKII phosphorylation and translocation. Rats given either 30 or 45 mg/kg PTZ or saline were used 1 h later. Cerebral cortices were isolated and homogenized, and homogenate, as well as 14,000 × *g* supernatant and pellet were subjected to SDS–PAGE. (A) The top panel shows examples of autoradiographs from Western blots using total homogenate. The lower part of the figure shows that 30 mg/kg PTZ caused a significant decrease in CaMKII phosphorylation, which was not as large as that following 45 mg/kg PTZ, while neither dose affected total CaMKII in homogenate. (B) The top panel shows sample results of total CaMKII in supernatant (S) and pellet (P) fractions. The lower portion shows that 30 mg/kg PTZ did not cause any significant CaMKII translocation from pellet to supernatant, in contrast to the effect of the 45 mg/kg dose ($n = 5$–6).

4 to 5 kindled convulsions became prominent. The seizures in kindling rats differed somewhat from those produced in naïve rats. These convulsions generally began later (typically 3–5 min after PTZ, but sometimes up to 30 min later), and often were marked by repeated episodes during the next 10–30 min. Rearing, usually with brief front limb clonus, was common during the kindling seizures, but almost never seen in the acute convulsions, and was typically repeated. When a tonic-clonic seizure occurred, it was briefer, and followed by more rapid recovery than those infrequently observed in naïve rats after the acute, higher dose.

CaMKII Thr^{286} phosphorylation and translocation were evaluated in cerebral cortex of PTZ kindled rats 1, 24 and 48 h after the last PTZ injection (Fig. 5B). Reduced CaMKII Thr^{286} phosphorylation was not only observed at 1 and 24 h, but was still present at 48 h after the convulsion, which completely bridged the 48 h interval used for kindling injections. No change in total CaMKII was detected. The translocation of CaMKII was also altered in PTZ kindled rat cerebral cortex. In contrast to the findings after an acute PTZ convulsion (Fig. 1), there was no apparent change in CaMKII subcellular distribution 1 h after the kindled seizure. However, there was a late-appearing CaMKII translocation noted at 24 h, when there was a significant increase in the supernatant (Fig. 5B).

The prolonged reduction (up to 48 h) in CaMKII Thr^{286} phosphorylation after the final PTZ kindled seizure was exactly reproduced in hippocampal homogenate. However, in hippocampus, there was no significant CaMKII translocation, but only a trend (data not shown). A similar difference between cortex and hippocampus was also noted after acute PTZ,

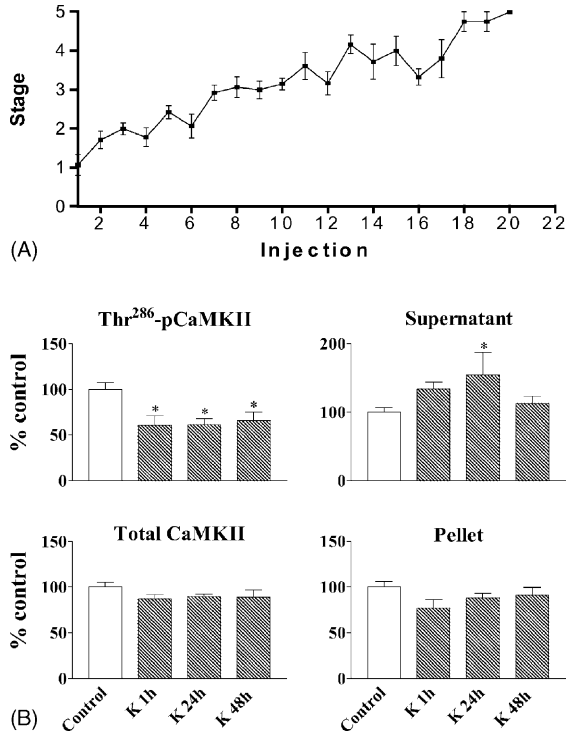

Fig. 5. CaMKII phosphorylation and translocation in cerebral cortex of PTZ kindled rats. Rats were kindled with 30 mg/kg PTZ every 48 h until reaching three consecutive stage 4 or 5 seizures. These fully kindled rats were randomly assigned to be decapitated at 1 h (K 1 h, $n = 4$), 24 h (K 24 h, $n = 4$), or 48 h (K 48 h, $n = 6$) after the last PTZ injection. Cerebral cortices were isolated and homogenized, and then separated into supernatant and pellet by 14,000 × g centrifugation. Total CaMKII and Thr^{286}-pCaMKII were detected and measured by Western blot. (A) All the rats reported here reached three consecutive stage 4 or 5 convulsions within 20 doses of PTZ. (B) CaMKII phosphorylation was decreased to about 60% of control after reaching the fully kindled state. This decrease was found at 1, 24 and 48 h after the last PTZ stimulation. Total CaMKII was not affected. Significant CaMKII translocation was not seen until 24 h after the final PTZ injection (significant change in supernatant).

when CaMKII Thr^{286} phosphorylation in hippocampus was decreased to a similar degree as in cerebral cortex, but CaMKII translocation was less (Figs. 1 and 2). This was also true in partially kindled hippocampus, described in the following section. It is not yet known if this is related to a difference in CaMKII expression, localization or function in hippocampus and cerebral cortex, or to differing involvement of these brain areas in the seizure discharge.

3.4. CaMKII Thr^{286} phosphorylation and subcellular distribution after partial PTZ kindling

Since kindling is a dynamic process, the prolonged changes in CaMKII seen in fully kindled brain might be the final result of a gradual change building during repeated PTZ stimulation. If so, changes in CaMKII Thr^{286} phosphorylation and translocation might be intermediate in the middle of the kindling process, and this was tested in partially kindled rats. Rats were given 30 mg/kg PTZ every 48 h for only 14 injections, resulting in average convulsions of stage 3 (Fig. 6A), before being decapitated at 1 and 24 h after the last PTZ injection. In cerebral cortex, CaMKII Thr^{286} phosphorylation was significantly decreased to about 70% of control level 24 h after 14 PTZ treatments, while there was no significant decrease at 1 h (Fig. 6B). Total CaMKII was not affected. Significant CaMKII translocation was also seen in the supernatant fraction, but only at 24 h. Changes in hippocampus were similar to those in cerebral cortex (data not shown).

Since changes in CaMKII were present 24 h after the last PTZ dose in partially kindled rats, a group of saline treated rats was given 30 mg/kg PTZ as the last dose to rule out the possibility of an unexpected effect of the last PTZ dose or of handling. After having received 13 saline injections, the 30 mg/kg dose of PTZ caused convulsive activity (ranked as stage 3) in only one of four rats, quite similar to the average effect of the initial dose of PTZ used for kindling (Figs. 5A and 6A). As shown in Fig. 6B (clear columns), this one dose of PTZ did not cause any change in either CaMKII Thr^{286} phosphorylation or translocation 24 h later, indicating that neither repeated handling and saline injection, nor the last dose of PTZ itself, could account for the prolonged changes in CaMKII.

4. Discussion

There have been a number of studies on CaMKII modulation by various types of experimental seizures, such as experimental status epilepticus or kindled seizures. Most have reported down-regulation of CaMKII enzymatic activity and expression following prolonged or recurrent seizures (Bronstein et al., 1988, 1990, 1992; Goldenring et al., 1986; Wu et al.,

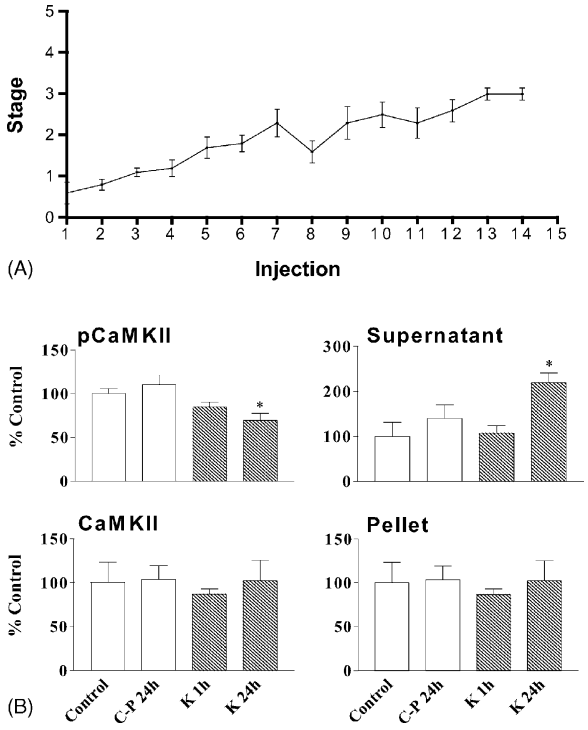

Fig. 6. CaMKII phosphorylation and translocation in cerebral cortex of partially kindled rats. Rats were given 30 mg/kg PTZ every 48 h for a total of 14 injections. These partially kindled rats were randomly assigned to be used at 1 h (K 1 h, $n = 5$) or 24 h (K 24 h, $n = 5$) after the last PTZ injection. Control rats were given 13 doses of saline and randomly assigned to two groups. One group (control) received saline as dose 14 before being used 1 h later. The other saline controls (C-P 24 h) received 30 mg/kg PTZ as the final injection, after which they were observed for convulsive behaviors, and then decapitated 24 h later (C-P 24 h). Cerebral cortices were isolated and homogenized, and then separated into supernatant and pellet by 14,000 × g centrifugation. Total CaMKII and Thr^{286}-pCaMKII were detected and measured by Western blot. (A) The partially kindled rats reached an average seizure stage of 3.00 ± 0.149 (mean ± S.E.M., $n = 10$) after 14 PTZ treatments. (B) Open columns represent mean + S.E.M. of saline controls. Groups designated as above. The single PTZ injection in saline controls did not cause any change in CaMKII phosphorylation or localization 24 h later. Hatched columns represent mean + S.E.M. of partially kindled rats, designated as above. CaMKII phosphorylation was significantly decreased to about 70% of control 24 h after 14 PTZ treatments, but there was no significant decrease at 1 h. Total CaMKII was not affected by any treatment. Significant CaMKII translocation was only seen in the supernatant fraction of cortex 24 h after the final PTZ injection in these partially kindled rats.

1990; Churn et al., 2000a; Kochan et al., 2000). However, very little is known about the effect of brief, self-limited seizure activity on CaMKII.

The present experiments, using a brief convulsive seizure induced by PTZ, found slowly appearing, but long-lasting changes in CaMKII autophosphorylation and subcellular distribution. As shown in Figs. 1 and 2, a brief PTZ seizure caused a decrease in CaMKII Thr^{286} phosphorylation for at least 4 h, as well as a similar temporal profile of CaMKII translocation from pellet to supernatant in both hippocampus and cerebral cortex. It is worth noting that the PTZ clonic convulsions typically lasted a minute or less, yet produced changes in CaMKII for several hours. Though electrographic seizure activity may have lasted a little longer than the behavioral convulsions, the results showed that the slowly developing change in CaMKII was sustained far beyond the initial brief seizure event. This suggested that CaMKII could be involved in carrying forward the signal resulting from a single seizure episode, at least for a few hours, as would be required for kindling to occur.

Before CaMKII was fully characterized, septal kindling had been shown to produce a decrease in endogenous Ca^{2+}/calmodulin-dependent phosphorylation of synaptic plasma membrane proteins measured 2 weeks after kindling. The major two proteins, approximately 50,000 and 60,000 Da, turned out to be Ca^{2+}/calmodulin-dependent protein kinase II subunits (Wasterlain and Farber, 1984; Goldenring et al., 1986). Further study showed decreased in vitro autophosphorylation of CaMKII in PSDs isolated from cerebral cortex of septal kindled rats (Wu et al., 1990). There was also a significant decrease in CaMKII α subunit immunoreactivity in hippocampus 2 weeks after septal kindling, and a significant decrease in CaMKII β subunit mRNA in both hippocampus and cerebral cortex (Bronstein et al., 1990, 1992). All of the above studies, from the same group of investigators, demonstrated that septal kindling produced a down-regulation of CaMKII autophosphorylation, activity as well as total protein. The fact that such changes in CaMKII were still present 8 weeks after the completion of septal kindling (Wasterlain and Farber, 1984) suggested a permanence required if CaMKII is involved in the kindled state. On the other hand, it was not known if CaMKII was down-regulated during the kindling process, which would

be important for formulating hypotheses concerning causality. To provide some information on this issue, the present study investigated the dynamic change of CaMKII during PTZ kindling (partial kindling) and right after establishing the PTZ kindled state (1 h to 2 days after kindling).

Once the rats were fully kindled, CaMKII Thr^{286} phosphorylation was significantly reduced in both hippocampus and cerebral cortex, and reached an apparent steady state, in which there was no difference in the reduction of CaMKII Thr^{286} phosphorylation at 1, 24 and 48 h after the final kindled seizure (Fig. 5). The reduction in CaMKII autophosphorylation observed for 2–4 h after a single seizure, appeared to have been prolonged during the kindling process. It was especially important to note the result at 48 h, which is the interval between PTZ injections during kindling. Since this change in CaMKII was maintained for the entire 48 h interval, it was possible for a seizure to reinforce the effects of the previous one, providing a continuous signal between PTZ doses. This could allow CaMKII to participate in the signaling mechanisms involved in kindling.

To investigate the dynamic change in CaMKII during PTZ kindling, rats were partially kindled (Fig. 6). After partial kindling, changes in CaMKII phosphorylation and translocation were sustained until 24 h after the last PTZ stimulation, which was much more prolonged than after a single PTZ treatment. However, in the partially kindled state, changes in CaMKII did not reach the time-independent, steady state as in fully kindled rats; i.e. significant changes were seen at 24 h but not at 1 h after the final PTZ dose. The results from partially kindled rats further supported the idea that CaMKII is regulated as a function of kindling, and could be involved in the step-by-step establishment of PTZ kindling.

Though the results of the present study suggest a role for CaMKII in PTZ kindling, they may also indicate an adaptive response to the repeated intense neural activity, which might tend to limit neural excitation upon subsequent stimulation. This could be involved in post-ictal depression or amnesia following a seizure. Such an interpretation would not preclude a signaling role for CaMKII in kindling. Other data also suggest an active role for CaMKII in kindling. One piece of evidence is that CaMKII appears to be a causal factor for seizure genesis in α-CaMKII (−/−) mutant mice, which exhibited profound excitability (Butler et al., 1995). On the other hand, these α-CaMKII (−/−) mutant mice could not be kindled (Butler et al., 1993), providing additional support for a relationship between CaMKII regulation and kindling. In addition, experiments in which CaMKII was reduced by the use of an antisense oligodeoxynucleotide (Churn et al., 2000b) also found that reduced CaMKII expression could lead to hyperexcitability. In the case of septal kindling, CaMKII in vitro autophosphorylation was decreased up to 8 weeks after completion of septal kindling (Wasterlain and Farber, 1984), suggesting that reduced CaMKII phosphorylation and expression might participate in maintaining the kindled state. These reports provide evidence that down-regulation of CaMKII activity and/or expression may play a role in increased neural activity, or increased seizure susceptibility. The present study further suggests that CaMKII subcellular re-distribution may also be involved in kindling. Thus, understanding the interaction between CaMKII translocation and phosphorylation may provide insight into mechanisms for prolonged CaMKII regulation following seizure activity.

An intrinsic property of CaMKII is maintenance of the autophosphorylated, autonomous active state for a period of minutes after an initial activation (Dupont and Goldbeter, 1998). However, it is not obvious what mechanism might maintain the long-lasting, dephosphorylated state of CaMKII for hours or days after seizure activity. One possibility could be through increased activity of one or more of the phosphatases for which CaMKII is a substrate. For example, in one study, CaMKII enzymatic activity was found to be increased 2 weeks after completion of septal kindling (Yamagata and Obata, 1996), in contrast to the decrease reported in other studies of septal kindling (Goldenring et al., 1986; Wu et al., 1990). One difference noted in these studies was that phosphatase inhibitors were included during tissue preparation in the more recent report that found increased CaMKII activity (Yamagata and Obata, 1996), suggesting that phosphatases might be involved in regulating CaMKII during kindling. In fact, in concurrent work on effects of an acute PTZ seizure (Dong and Rosenberg, 2004), we have noted that the reduced CaMKII Thr^{286} phosphorylation (but not the translocation) was apparently associated with enhanced phosphatase activity an hour after a brief PTZ seizure. Further studies will be re-

quired to determine the relationships among CaMKII activity, phosphatase activity, and cellular compartmentalization of these enzymes during kindling.

Acknowledgements

We are grateful to Eugene Orlowski for excellent technical assistance. This work was supported by research funds from the Medical College of Ohio and NIH grant RO1-DA02194.

References

Atack, J.R., Cook, S.M., Hutson, P.H., File, S.E., 2000. Kindling induced by pentylenetetrazole in rats is not directly associated with changes in the expression of NMDA or benzodiazepine receptors. Pharmacol. Biochem. Behav. 65, 743–750.

Braun, A.P., Schulman, H., 1995. The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. Annu. Rev. Physiol. 57, 417–445.

Bronstein, J., Farber, D., Wasterlain, C., 1988. Decreased calmodulin kinase activity after status epilepticus. Neurochem. Res. 13, 83–86.

Bronstein, J.M., Farber, D.B., Micevych, P.E., Lasher, R., Wasterlain, C.G., 1990. Kindling induced changes in calmodulin kinase II immunoreactivity. Brain Res. 524, 49–53.

Bronstein, J.M., Micevych, P., Popper, P., Huez, G., Farber, D.B., Wasterlain, C.G., 1992. Long-lasting decreases of type II calmodulin kinase expression in kindled rat brains. Brain Res. 584, 257–260.

Butler, L.S., Silva, A.J., Tonegawa, S., McNamara, J.O., 1993. Enhanced seizure susceptibility yet impairment of kindling development in α-calcium-calmodulin kinase II mutant mice. Soc. Neurosci. Abstr. 19, 1030.

Butler, L.S., Silva, A.J., Abeliovich, A., Watanabe, Y., Tonegawa, S., McNamara, J.O., 1995. Limbic epilepsy in transgenic mice carrying a Ca^{2+}/calmodulin-dependent kinase II alpha-subunit mutation. Proc. Natl. Acad. Sci. U.S.A. 92, 6852–6855.

Churn, S.B., Kochan, L.D., DeLorenzo, R.J., 2000a. Chronic inhibition of Ca^{2+}/calmodulin kinase II activity in the pilocarpine model of epilepsy. Brain Res. 875, 66–77.

Churn, S.B., Sombati, S., Jakoi, E.R., Sievert, L., DeLorenzo, R.J., 2000b. Inhibition of calcium/calmodulin kinase II alpha subunit expression results in epileptiform activity in cultured hippocampal neurons. Proc. Natl. Acad. Sci. U.S.A. 97, 5604–5609.

Corda, M.G., Giorgi, O., Longoni, B., Orlandi, M., Biggio, G., 1990. Decrease in the function of the γ-aminobutyric acid-coupled chloride channel produced by the repeated administration of pentylenetetrazol to rats. J. Neurochem. 55, 1216–1221.

Corda, M.G., Orlandi, M., Lecca, D., Giorgi, O., 1992. Decrease in GABAergic function induced by pentylenetetrazol kindling in rats: antagonism by MK-801. J. Pharmacol. Exp. Ther. 262, 792–800.

Dong, Y., Rosenberg, H.C., 2004. Brief seizure activity alters Ca^{2+}/calmodulin-dependent protein kinase II dephosphorylation and subcellular distribution in rat brain for several hours. Neurosci. Lett. 357, 95–98.

Dupont, G., Goldbeter, A., 1998. CaM kinase II as frequency decoder of Ca^{2+} oscillations. Bioessays 20, 607–610.

Fukuda, S., Harada, K., Kunimatsu, M., Sakabe, T., Yoshida, K., 1998. Postischemic reperfusion induces alpha-fodrin proteolysis by m-calpain in the synaptosome and nucleus in rat brain. J. Neurochem. 70, 2526–2532.

Goddard, G.V., McIntyre, D.C., Leech, C.K., 1969. A permanent change in brain function resulting from daily electrical stimulation. Exp. Neurol. 25, 295–330.

Goldenring, J.R., Wasterlain, C.G., Oestreicher, A.B., de Graan, P.N., Farber, D.B., Glaser, G., DeLorenzo, R.J., 1986. Kindling induces a long-lasting change in the activity of a hippocampal membrane calmodulin-dependent protein kinase system. Brain Res. 377, 47–53.

Hanson, P.I., Schulman, H., 1992. Neuronal Ca^{2+}/calmodulin-dependent protein kinases. Annu. Rev. Biochem. 61, 559–601.

Kochan, L.D., Churn, S.B., Omojokun, O., Rice, A., DeLorenzo, R.J., 2000. Status epilepticus results in an *N*-methyl-D-aspartate receptor-dependent inhibition of Ca^{2+}/calmodulin-dependent kinase II activity in the rat. Neuroscience 95, 735–743.

Kolb, S.J., Hudmon, A., Waxham, M.N., 1995. Ca^{2+}/calmodulin kinase II translocates in a hippocampal slice model of ischemia. J. Neurochem. 64, 2147–2156.

Moore, A.N., Waxham, M.N., Dash, P.K., 1996. Neuronal activity increases the phosphorylation of the transcription factor cAMP response element-binding protein (CREB) in rat hippocampus and cortex. J. Biol. Chem. 271, 14214–14220.

Pinel, J.P., Van Oot, P.H., 1975. Generality of the kindling phenomenon: some clinical implications. Can. J. Neurol. Sci. 2, 467–475.

Racine, R.J., 1972. Modification of seizure activity by electrical stimulation. I. After- discharge threshold. Electroencephalogr. Clin. Neurophysiol. 32, 269–279.

Rosenberg, H.C., Tietz, E.I., Chiu, T.H., 1985. Tolerance to the anticonvulsant action of benzodiazepines. Relationship to decreased receptor density. Neuropharmacology 24, 639–644.

Suzuki, K., Omura, S., Ohashi, Y., Kawai, M., Iwata, Y., Tani, K., Sekine, Y., Takei, N., Mori, N., 2001. FK506 facilitates chemical kindling induced by pentylenetetrazole in rats. Epilepsy Res. 46, 279–282.

Wasterlain, C.G., Farber, D.B., 1984. Kindling alters the calcium/calmodulin-dependent phosphorylation of synaptic plasma membrane proteins in rat hippocampus. Proc. Natl. Acad. Sci. U.S.A. 81, 1253–1257.

Wu, K., Wasterlain, C., Sachs, L., Siekevitz, P., 1990. Effect of septal kindling on glutamate binding and calcium/calmodulin-dependent phosphorylation in a postsynaptic density fraction isolated from rat cerebral cortex. Proc. Natl. Acad. Sci. U.S.A. 87, 5298–5302.

Yamagata, Y., Obata, K., 1996. Ca^{2+}/calmodulin-dependent protein kinase II in septally kindled rat brains: changes in protein level, activity and subcellular distribution in hippocampus and cerebral cortex. Neurosci. Lett. 211, 109–112.