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Brief seizure activity alters Ca^{2+}/calmodulin dependent protein kinase II dephosphorylation and subcellular distribution in rat brain for several hours

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Abstract

The effect of a brief pentylenetetrazol (PTZ) convulsive seizure on rat cerebral cortical Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) was investigated. By immunoblot, it was found that a single PTZ seizure, lasting less than a minute, caused translocation of CaMKII α-subunit (α-CaMKII) from the particulate to the soluble fraction for several hours, paralleled by a dramatic loss of α-CaMKII Thr^{286} phosphorylation. The reduced α-CaMKII Thr^{286} phosphorylation apparently resulted from enhanced phosphatase activity following PTZ seizure, especially in the particulate fraction. CaMKII translocation and phosphatase activation following a brief seizure episode can both contribute to long-lasting CaMKII regulation far outlasting the immediate effects of the seizure on neuronal function.

Keywords: Pentylenetetrazol seizure; Ca^{2+}/calmodulin dependent protein kinase II; Phosphatase; Autophosphorylation; Subcellular translocation

The present study examined the possibility that a brief seizure can alter brain Ca^{2+}/calmodulin dependent protein kinase II (CaMKII), which could affect CaMKII-mediated signaling. CaMKII is a multifunctional serine/threonine protein kinase. After binding Ca^{2+}/calmodulin (Ca^{2+}/CaM) complex, autophosphorylation on Thr^{286} of CaMKII α subunit (Thr^{287} for β subunit) results in Ca^{2+}/CaM independent activity, which is eventually terminated by Thr^{286} dephosphorylation [8]. Autophosphorylation also occurs on Thr^{305} and Thr^{306}, located within the CaM binding domain. Ca^{2+}-independent autophosphorylation on either Thr^{305} or Thr^{306} blocks CaM binding, preventing further Ca^{2+}/CaM dependent CaMKII activation. Protein phosphatase 1 and 2A (PP1 and PP2A) are the main phosphatases that act on these autophosphorylation sites [11].

There is evidence for CaMKII involvement in some types of experimental seizures. For example, 2 weeks after completing septal kindling, in vitro CaMKII autophosphorylation was decreased in hippocampus [7] and postsynaptic density (PSD) isolated from rat cortex [13]. It was also found that CaMKII α subunit immunoreactivity was significantly decreased in hippocampus, and CaMKII β subunit mRNA was decreased in both hippocampus and cerebral cortex [2,3]. Thus, it is not clear if reduced CaMKII activity was due to altered enzyme regulation or reduced CaMKII protein. A recent study of septal kindling found that protein level of CaMKII α (but not β) subunit was significantly decreased in hippocampus and parietal cortex [14]. However, unlike the earlier study, CaMKII enzymatic activity was increased in both brain regions 2 weeks after the animals were fully kindled [14]. One possible explanation for these opposite results might be the inclusion of phosphatase inhibitors in the latter [14], but not in the previous reports [2,3,7,13]. It is also worth noting that, in the recent study, CaMKII translocation from particulate to soluble fraction was suggested by greater recovery of CaMKII enzymatic activity in soluble fraction [14]. To date, CaMKII subcellular re-distribution following seizure activity has been little studied.

The present study used a brief pentylenetetrazol (PTZ)

Abbreviations: CaMKII, Ca^{2+}/calmodulin dependent protein kinase II; α-CaMKII, CaMKII α subunit; Thr^{286}-pCaMKII, CaMKII phosphorylated on Thr^{286}; CaM, calmodulin; CaMK phosphatase, Ca^{2+}/CaM-dependent protein kinase phosphatase; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PP2C, protein phosphatase 2C; PTZ, pentylenetetrazol; PSD, postsynaptic density.

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induced clonic seizure, which lasts only 30–40 s, and sought changes in CaMKII phosphorylation and subcellular re-distribution. A crucial consideration was if phosphatase activity might be involved in CaMKII regulation after a seizure. In spite of the important role of phosphatases in regulating CaMKII, this has received little attention to date. Since PTZ clonic seizures affect cerebral cortex [1], and α subunit is the main CaMKII subunit in cerebral cortex, this study focused on alterations of CaMKII α-subunit (α-CaMKII).

Male Sprague–Dawley rats (225–300 g) were given a single convulsive dose of PTZ, 45 mg/kg, i.p. in saline. At this PTZ dose, convulsion is manifest as bilateral front limb clonus (usually not involving hind limbs), and usually lasts 30–40 s. Only rats that developed the above typical clonic convulsions were used (about 90%). Those not progressing to front limb clonus or those having more severe convulsive seizures were discarded. Matched controls received saline. Rats were decapitated at one of several times following PTZ: 5 min, 0.5, 1, 2, 4, 12, 24 or 48 h. The brain was quickly isolated and immediately chilled in ice-cold saline for 1 min. Left and right cerebral cortices were quickly dissected within 1 min, rapidly frozen in liquid nitrogen, and stored at −76 °C.

Sample preparation was done at 4 °C following the procedures of Moore et al. [10]. Left hemicortices were homogenized in 6 ml 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. A portion of homogenate was saved, and the rest was centrifuged at 14,000 × *g* for 30 min at 4 °C. Aliquots were stored at −76 °C. The right hemicortex from each rat was prepared the same way, except that the buffer contained either a mixture of non-specific phosphatase inhibitor (25 mM Na_4PO_7, 50 mM NaF and 1 mM Na_3VO_4) or a specific PP1/PP2A inhibitor Microcystin-LR (1 μM). As PTZ was administrated systemically, no difference in the effect of PTZ between left and right cortices was anticipated, so that one hemicortex served as control for the other side to test the effects of post-mortem phosphatase activity on CaMKII phosphorylation and translocation.

Protein samples from total homogenate and subcellular fractions were denatured and separated by 10% SDS-PAGE. CaMKII α subunit total protein was detected by a monoclonal antibody MA1-048 (Affinity BioReagents, Inc; 1:100,000 dilution); α-CaMKII phosphorylated on Thr^{286} was detected by monoclonal antibody MA1-047 (Affinity BioReagents, Inc.; 1:2000 dilution).

As shown in Fig. 1, in the absence of phosphatase inhibitors, a prolonged time-dependent reduction in CaMKII Thr^{286} phosphorylation was found from 0.5 h to 4 h after a brief PTZ seizure. The maximum change was at 1 h, when CaMKII Thr^{286} phosphorylation was reduced to 17.4 ± 8.7% of control (mean ± SEM, $n = 5$). An apparent partial reversal started 4 h after convulsion (55.2% of control, $n = 4$), with no residual effect at 12 h or thereafter. Concurrently, there was an obvious CaMKII translocation from pellet to supernatant. CaMKII translocation was not affected by using phosphatase inhibitors; however, including either Microcystin-LR or non-specific phosphatase inhibitor mixture during tissue preparation totally abolished the change in Thr^{286} phosphorylation. Furthermore, the presence of phosphatase inhibitors provided an overall stronger phospho-CaMKII signal in both control and treated tissues (Fig. 1), suggesting constitutive phosphatase activity acting in vitro. However, such phosphatase activity alone could not explain the time-dependent loss and recovery of CaMKII Thr^{286} phosphorylation after a PTZ seizure (Fig. 1B). Rather, it was suspected that phosphatase activation was induced by PTZ seizure, gradually developing then diminishing over time.

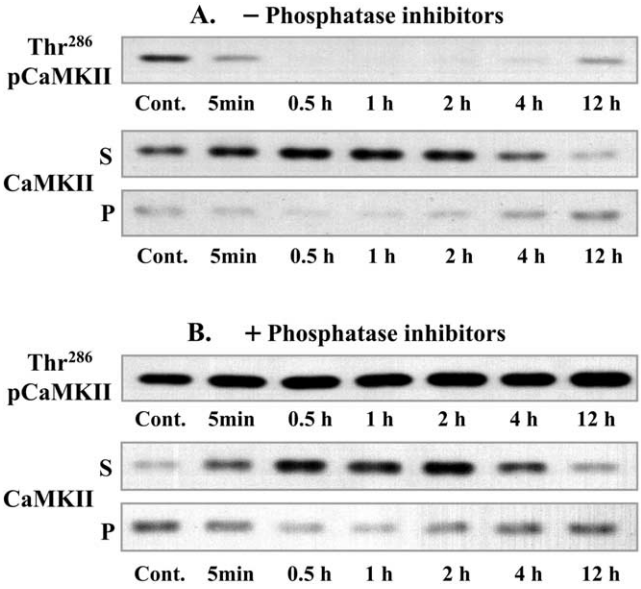

Fig. 1. CaMKII autophosphorylation and translocation in cerebral cortex after a brief PTZ seizure. (A) In the absence of phosphatase inhibitors, CaMKII phosphorylated on Thr^{286} (Thr^{286}-pCaMKII) was significantly decreased in homogenate of cerebral cortex at 0.5, 1 and 2 h (ANOVA; $P < 0.05$, $n = 4–5$); total CaMKII protein in homogenate was not changed (data not shown). Meanwhile, there was a translocation of CaMKII from the pellet to the supernatant fraction, which was significant at 0.5, 1 and 2 h after PTZ induced convulsion (ANOVA; $P < 0.05$, $n = 4–5$). (B) In the presence of phosphatase inhibitors, the reduction in CaMKII autophosphorylation on Thr^{286} disappeared; however, CaMKII translocation from pellet to supernatant was not affected.

To test the above hypothesis, endogenous phosphatase activity in cerebral cortex was evaluated by in vitro dephosphorylation. Rats were used 1 h after PTZ or saline, the time for peak decrease in CaMKII Thr^{286} phosphorylation. CaMKII in supernatant or pellet was pre-phosphorylated at 0 °C for 10 min with [γ-^{32}P] ATP [15]. Endogenous phosphatase activity was then evaluated in the absence of phosphatase inhibitor, or in the presence of PP1/PP2A inhibitor Microcystin-LR (1 μM) or okadaic acid (2.5 μM), or a non-specific phosphatase inhibitor mixture (25 mM Na_4PO_7, 50 mM NaF, and 1 mM Na_3VO_4). The reaction

buffer contained 10 mM piperazine-*N*,*N*′-(2-ethanesulfonic acid) (PIPES, pH 7.4), 10 mM $MgCl_2$, 5 mM $CaCl_2$, 20 μg/ml calmodulin, 7 μM [γ-^{32}P] ATP (10 Ci/mM), and 50 μg of protein in a volume of 100 μl. The reaction was allowed to proceed at 30 °C. Aliquots were withdrawn at 0, 5, 15, and 30 min and subjected to SDS-PAGE. ^{32}P-labeled α-CaMKII was detected by autoradiography. The time-dependent loss of ^{32}P-CaMKII indicated endogenous phosphatase activity.

Endogenous phosphatase activities were compared between supernatant and pellet, as well as between tissue from control and PTZ 1 h rats. PTZ treatment had no effect on endogenous phosphatase activity in supernatant; however, there was significant treatment effect in the pellet, i.e. greater loss of ^{32}P-labeled CaMKII in pellet from PTZ 1 h cortex (Fig. 2A). This indicated enhanced phosphatase activity 1 h after PTZ seizure, particularly in the pellet fraction. Strack et al. [12] have demonstrated that CaMKII is differentially regulated by protein phosphatases in distinct subcellular compartments. Soluble CaMKII is dephosphorylated predominantly by PP2A, whereas PSD-associated CaMKII is dephosphorylated predominantly by PP1 [12]. This suggested a role of compartmentalization in CaMKII regulation. Specifically, the enhanced phosphatase activity noted in the pellet fraction 1 h after PTZ seizure might have been due to increased PP1 activity.

PP1 and PP2A are the two major protein phosphatases that dephosphorylate CaMKII. Available phosphatase inhibitors, such as Microcystin-LR [10] and okadaic acid are non-selective between PP1 and PP2A, though okadaic acid at low concentration (nM range) is relatively selective for PP2A [4]. As shown in Fig. 2B, both 2.5 μM okadaic acid and 1 μM Microcystin-LR largely blocked CaMKII in vitro dephosphorylation. However, PP1/PP2A inhibitor alone could not completely block the loss of CaMKII phosphorylation, especially in the pellet fraction of cortex from PTZ-treated rats, suggesting involvement of other phosphatase(s) as well. Since a significantly greater CaMKII dephosphorylation was still present in pellets of PTZ 1 h cortex after adding PP1/PP2A inhibitor, the increased phosphatase activity in pellet fraction after PTZ seizure could not be fully explained by PP1/PP2A activation.

Okadaic acid-insensitive protein phosphatases were reported to be involved in CaMKII dephosphorylation [5]. Protein phosphatase 2C (PP2C), which is okadaic acid-insensitive, was shown to be relatively specific for dephosphorylation of CaMKII at Thr$^{286/287}$ and Thr$^{305/306}$ [6]. Another phosphatase specific for Ca^{2+}/calmodulin dependent kinases (I, II and IV) was discovered by Ishida et al. [9], and was named Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMK phosphatase). CaMK phosphatase was reported to be highly specific for autophosphorylated CaMKII, and could reverse its Ca^{2+}/calmodulin-independent activity [9]. CaMK phosphatase was not inhibited by 10 μM okadaic acid, but was sensitive to NaF [9]. Since both PP2C and CaMK phosphatase are okadaic acid-insensitive, it was suspected that these might have been responsible for the okadaic acid resistant phosphatase activity, and possibly contributed to the enhanced particulate phosphatase activity following PTZ seizure.

Thus, a mixture of phosphatase inhibitors was used in CaMKII in vitro dephosphorylation assay. This mixture contained pyrophosphate, a non-specific phosphatase inhibitor; NaF, an inhibitor for PP1, PP2A, protein phosphatase 2B and CaMK phosphatase; and orthovanadate

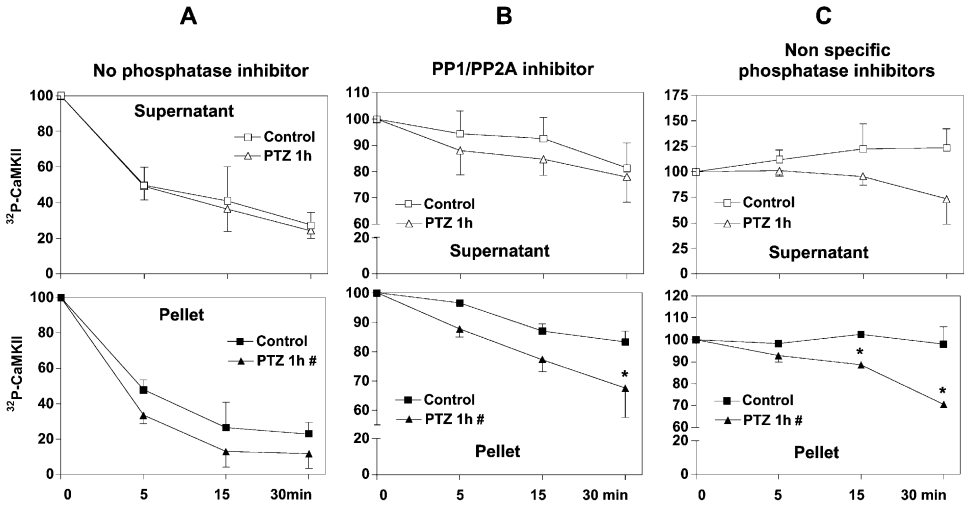

Fig. 2. In vitro CaMKII dephosphorylation. (A) Endogenous phosphatase activity in supernatant and pellet from cerebral cortex of control and PTZ treated rats (1 h). There was no difference between supernatant fractions, but a significantly greater loss of ^{32}P-CaMKII in pellet was seen after PTZ seizure (#, $P < 0.05$, 2-way ANOVA, $n = 4$). (B) Effect of PP1/PP2A inhibitor on dephosphorylation. Okadaic acid (2.5 μM) or Microcystin-LR (1 mM) was added after CaMKII prephosphorylation with [γ-^{32}P] ATP. Either inhibitor largely protected CaMKII from dephosphorylation in supernatants from both control and PTZ rats, but was less able to prevent dephosphorylation in pellets, and significantly less so after PTZ (#, $P < 0.05$, two-way ANOVA, $n = 4$; *, $P < 0.05$, post-hoc Tukey test). (C) Effect of non-specific phosphatase inhibitors. In supernatant, non-specific inhibitors totally blocked CaMKII dephosphorylation in both control and PTZ treated cortex. In pellet, CaMKII dephosphorylation was totally blocked in control rats, but there was still a significant loss in pellet from PTZ-treated rats (#, $P < 0.05$, two-way ANOVA, $n = 4$; *, $P < 0.05$, post-hoc Tukey test).

(1 mM), a potent tyrosine phosphatase inhibitor which also inhibits PP1, PP2A and PP2C at millimolar level [9]. As shown in Fig. 2C, this mixture of phosphatase inhibitors totally blocked CaMKII dephosphorylation in supernatant fractions from both control and PTZ-treated rat brain, as well as in pellet fraction from control rat brain. This indicated that PP2C or CaMK phosphatase might be responsible for the PP1/PP2A inhibitor-resistant phosphatase activity in these samples, though a contribution of other phosphatases that might have also been inhibited by this mixture could not be ruled out. Surprisingly, the broad phosphatase inhibitor mixture was still not able to completely inhibit CaMKII dephosphorylation in pellet fraction from PTZ seizure cortex. The effect of this mixture was not much different from the effect of PP1/PP2A inhibitor in the degree of inhibition, in that both blocked about 70% of CaMKII in vitro dephosphorylation when the reactions proceeded for 30 min. It seems that the increased phosphatase activity in the particulate fraction 1 h after a brief PTZ seizure could not be completely explained by any of the phosphatases inhibited by this mixture, suggesting that other, unknown phosphatase(s), not sensitive to the commonly used phosphatase inhibitors, might be involved. Other changes in kinetics of CaMKII enzymatic activity also could not be ruled out.

It should be noted that ^{32}P labeled CaMKII will reflect all autophosphorylation sites, including Thr^{286}, Thr^{305}, Thr^{306}, Ser^{314}. All these sites are sensitive to phosphatases, but have different roles. Dephosphorylation of Thr^{286} inactivates CaMKII. Dephosphorylation of Thr^{305} and Thr^{306} unlock the CaM-binding site so that CaM can bind, leading to CaMKII activation [11]. Therefore, dephosphorylation of ^{32}P-labeled CaMKII could not predict the overall effect on CaMKII activity. In a single preliminary trial, the same blots were used later to visualize CaMKII phosphorylated at Thr^{286} (with monoclonal antibody MA1-047). That limited data showed that loss of phosphate from Thr^{286} was generally more rapid than the overall loss of ^{32}P signal. Thus, it appears that the increased endogenous phosphatase activity shown in Fig. 2 is responsible for loss of the Thr^{286} phosphorylation.

In summary, we found enhanced phosphatase activity in particulate fraction and concurrent CaMKII translocation toward soluble fraction after a brief PTZ seizure. CaMKII translocation appeared independent of phosphatase activation, as shown in Fig. 1, in that the presence of phosphatase inhibitors had no effect on CaMKII translocation. Thus, it seems that CaMKII translocation and compartmentalized phosphatase activation after a brief PTZ seizure may synergistically contribute to prolonged CaMKII regulation that far outlasts the immediate effects of the brief seizure. In a concurrent study, similar, but more prolonged changes in CaMKII autophosphorylation and subcellular distribution were noted during and after PTZ kindling (unpublished observation). We now suggest that the compartmentalized phosphatase activation found in the present study might have relevance to prolonged CaMKII regulation accompanying kindling.

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