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Research report

Calcium/calmodulin-dependent protein kinase II activity and expression are altered in the hippocampus of Pb^{2+}-exposed rats

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Abstract

In the present study, we examined whether calcium/calmodulin-dependent protein kinase II (CaMKII) is affected by chronic developmental Pb^{2+} exposure. The effects of Pb^{2+} exposure on rat hippocampal CaMKII were assessed by measuring CaMKII activity, phosphorylation of CaMKII at threonine-286, and CaMKII α and β protein levels. In the hippocampus of Pb^{2+}-exposed 50-day-old rats known to exhibit deficits in hippocampal long-term potentiation (LTP) and spatial learning, there was a marked reduction (41%) in the apparent maximal velocity (Vmax) of CaMKII and a significant increase (22%) in apparent affinity of the enzyme. These Pb^{2+}-induced changes in CaMKII activity could not be explained by changes in enzyme phosphorylation at threonine-286 or sensitivity to calmodulin. In vitro incubation of hippocampal homogenates from control rats, but not from Pb^{2+}-exposed rats, with Pb^{2+} prior to assay recapitulated the increase in the affinity of the enzyme observed with in vivo exposure to Pb^{2+}. Western blots of cytosolic and membrane fractions from hippocampus showed a significant decrease in the levels of CaMKII-β but not α protein in the cytosolic fraction of Pb^{2+}-exposed rats. These findings indicate effects of developmental Pb^{2+} exposure on CaMKII, a component of calcium signaling associated with synaptic plasticity, learning, and memory.

Theme: Disorders of the nervous system
Topic: Neurotoxicity

Keywords: Calcium/calmodulin dependent protein kinase II (CaMKII); NMDA receptor (NMDAR); Lead (Pb^{2+}); Calcium signaling; Enzyme kinetics; Phosphorylation

1. Introduction

Deficits in cognitive function are the undisputed effects of childhood lead (Pb^{2+}) intoxication [5]. Although the exact molecular mechanisms of these Pb^{2+} effects are not fully understood, antagonism of the *N*-methyl-D-aspartate receptor (NMDAR), an ionotropic glutamate receptor, is emerging as a principle mechanism of Pb^{2+} neurotoxicity [33]. Pb^{2+} has been shown to be a potent and selective antagonist of the NMDAR both in vitro and in rats exposed to environmentally relevant levels of Pb^{2+} [2,20]. Additionally, exposure to Pb^{2+} during development alters the expression and proportion of NMDAR subtypes expressed in the hippocampus of rats exhibiting environmentally relevant blood Pb^{2+} levels [34,38,43]. Animals exposed to Pb^{2+} also express deficits in spatial learning and hippocampal long-term potentiation (LTP) [3,16,27,34]. The hippocampus is an area of the brain necessary for spatial learning and where molecular mechanisms of synaptic plasticity such as NMDAR-dependent LTP have been extensively studied [6,30,31].

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Based on these previous studies, we hypothesized that an alteration in calcium/calmodulin-dependent protein kinase II activity (CaMKII), a kinase activated in the postsynaptic density by calcium influx through the NMDAR [9], occurs in the hippocampus of Pb^{2+}-exposed animals. This hypothesis is supported by studies demonstrating a decrease in the phosphorylation of cyclic AMP response element binding protein (CREB), a nuclear transcription factor that is phosphorylated by several kinases activated by NMDAR-mediated calcium flux [29], in the hippocampus of Pb^{2+}-exposed rats [38,39].

CaMKII is a multimeric kinase composed of four distinct subtypes found in the adult brain [4,7]. Expression of these subunits is regulated developmentally and anatomically with CaMKII α and β expressed in neurons [4,7,40]. In the postsynaptic density, CaMKII is activated by a NMDAR-mediated rise in intracellular calcium which allows for the binding of CaMKII to a calcium/calmodulin complex [23]. The binding of this complex results in the autophosphorylation of threonine residue 286 and calcium-independent activity of CaMKII [23]. The expression of CaMKII and phosphorylation of the threonine-286 residue have been proven to be crucial for learning, memory, and synaptic plasticity [21]. For example, mice that possess a threonine to alanine mutation at residue 286 no longer possess the ability to phosphorylate the protein at this site [21]. These mice exhibit impairments in the induction of long-term potentiation and learning deficits in the Morris water maze and contextual fear conditioning, similar to the deficits observed in rats exposed to Pb^{2+} [21,27,34,36].

Previous studies have demonstrated that Pb^{2+} can bind and alter the activity of calmodulin, a cofactor necessary for the activation of many calcium-dependent enzymes such as CaMKII. Calmodulin-dependent calcium flux in in vitro systems has been shown to be affected by the presence of Pb^{2+} [13,25]. Further, calcineurin, a calmodulin -dependent phosphatase, demonstrates both a dose-dependent stimulation or inhibition when incubated with Pb^{2+} in vitro [24]. Rat brain calmodulin-dependent cAMP phosphodiesterase activity is also altered by Pb^{2+} exposure [17]. While the effect of Pb^{2+} on calmodulin-dependent enzymes and processes has been characterized by previous studies, it is unclear how in vivo Pb^{2+} exposure affects CaMKII activity. A recent study investigating the effects of in vitro Pb^{2+} exposure on catecholamine release from PC12 cells concluded that Pb^{2+}-evoked exocytosis is modulated by CaMKII [41]. While this in vitro study in PC12 cells is the first to demonstrate an effect of Pb^{2+} on CaMKII, no study has yet measured the activity of CaMKII in an animal model of in vivo Pb^{2+} exposure.

In the present study, we examined the effects of chronic Pb^{2+} exposure on CaMKII activity, phosphorylation, and expression in the rat hippocampus using our established in vivo model of Pb^{2+} neurotoxicity [27,34]. Our findings show that developmental Pb^{2+} exposure dramatically decreases the activity and alters the subcellular compartmentalization of CaMKII protein in the rat hippocampus.

2. Materials and methods

2.1. Animal protocol and Pb^{2+} exposure

Rats were exposed to Pb^{2+} during development as previously described [27,34]. Briefly, female Long–Evans rats were fed 0 or 1500 parts per million (ppm) Pb^{2+} acetate in the diet for 10 days before breeding to normal Long–Evans males. Litters were culled to 10 animals 1 day after birth and weaned at postnatal day (PN) 21. Male pups were sacrificed at 50 days of age by decapitation or focused microwave irradiation (to preserve phosphorylation state [35]; 10 kW Muromachi Microwave Fixation system, Stoelting Co., Wood Dale, IL) and the brain was dissected and stored at −80 °C until use. All pups remained on their respective dam's diet until time of sacrifice. Each litter of rats was considered one experimental unit for statistical purposes. All animal studies were reviewed and approved by the Johns Hopkins University Animal Care and Use Committee and have been carried out in accordance with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

2.2. Pb^{2+} analysis

Rat diet, blood, hippocampi, and Pb^{2+} solutions were analyzed for Pb^{2+} content by an independent laboratory (ESA Laboratories, Inc. Chelmsford, MA).

2.3. Hippocampal extract preparation for enzyme assays and Western blots

Frozen hippocampi from rats euthanized by decapitation or focused microwave irradiation were homogenized in 20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, Complete protease inhibitor tablets (Roche Biochemicals, Basel, Switzerland), and Phosphatase Inhibitor Cocktail I and II (Calbiochem, San Diego, CA) using a polytron. Homogenates were centrifuged for 5 min at 350 × *g* and the supernatant from decapitated rats was snap frozen for CaMK enzyme activity while the supernatant from rats euthanized by focused microwave irradiation was mixed 1:1 with 2× Laemmli buffer, boiled at 100 °C, and stored at −80 °C until use for phospho CaMKII expression.

Membrane and cytosolic fractions were prepared from fresh frozen tissue using a modified preparation described by Moyano et al. [32]. Briefly, frozen rat hippocampus was homogenized in 10 mM Tris–HCl (pH 7.4), 0.32 M Sucrose, 5 mM EDTA, 1 mM EGTA, and Complete protease inhibitor tablets. Homogenates were centrifuged

at 700 × *g* for 10 min at 4 °C. The supernatant was then centrifuged at 37,000 × *g* for 40 min. The supernatant was harvested and designated the cytosolic fraction. The pellet was resuspended in 0.1% Triton X-100, 5 mM Tris–HCl (pH 7.4), and gently rocked for 10 min at 4 °C after which the solution was centrifuged at 37,000 × *g* for 40 min. The supernatant was designated the membrane fraction. All samples were diluted 1:1 with 2× Laemmli buffer, boiled at 100 °C, and stored at −80 °C until use.

2.4. Immunoblotting and quantification

Samples were loaded in triplicate to a 10% SDS–PAGE gel. Each gel was loaded with a five-point protein curve that was used to determine the linearity of the enhanced chemiluminescence (ECL) intensity or fluorescence as a function of protein concentration. Proteins were electrophoretically transferred to a nitrocellulose membrane and then stained with a 1% (wt/vol) Ponceau S solution to determine equal loading of lanes. Membranes were blocked in PBS containing 3% (wt/vol) nonfat dry milk (PBS–MILK) for ECL or Li-Cor Blocking Buffer for fluorescence detection (Li-Cor Bioscience, Lincoln, NE) for 1 h at room temperature and then probed with anti-phosphoCaMKII (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-CaMKII α, or anti-CaMKII β (1:500, Zymed, San Francisco, CA) overnight at 4 °C in PBS–MILK (phospho CaMKII) or Li-Cor Blocking Buffer (CaMKII α and β). Membranes were then probed with an IgG-horseradish peroxidase-conjugated secondary antibody in PBS–MILK for ECL detection or an IgG-IRDye™ 800 conjugated secondary antibody (Rockland, Gilbertsville, PA) in Li-Cor Blocking Buffer for fluorescence detection. Immunoblots were visualized using ECL detection or the Li-Cor Odyssey Infrared Fluorescence Imaging system. ECL images were captured and analyzed for optical density by Inquiry (Loats Associates Inc, Westminster, MD). Fluorescent images were analyzed using the Li-Cor Odyssey Software. Optical density (OD) values were corrected for background, normalized to control, expressed as percentage of control and log transformed for statistical analysis.

2.5. CaMKII enzyme activity analysis

CaMKII enzyme activity analysis was performed using the SignaTECT Calcium/Calmodulin-Dependent Protein Kinase Assay System (Promega, Madison, WI). This kit uses a specific biotinylated substrate for CaMKII to assess CaMKII-dependent phosphorylation. Briefly, hippocampal homogenates were diluted to an approximate protein concentration of 2 μg/μl in 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM DTT, and 0.1 mg/ml BSA. Phosphorylation reactions were begun by the addition of the diluted hippocampal homogenate into a solution with a final concentration of 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM DTT, 100 μM ATP (0.5 μCi [γ-^{32}P-ATP]), and biotinylated substrate (concentration range 1 μM to 100 μM). Activation of the enzyme was achieved by running the reactions in the presence of 1 mM $CaCl_2$ and 1 μM calmodulin. The reaction was also run under basal conditions by the replacement of the activators with 1 mM EGTA (pH 7.2). Reactions were allowed to proceed at 30 °C for 2 min at which time they were terminated by the addition of guanidine hydrochloride to a final concentration of 2.5 M. Reactions were spotted to a streptavidin-impregnated membrane and washed 4 times with 2 M NaCl, 4 times with 2 M NaCl in 1% phosphoric acid, and 2 times in distilled water. Membranes were dried and then assessed for retained radioactivity by liquid scintillation counting. Radioactivity counts were converted to specific activity and analyzed using the Sigma Plot Enzyme Kinetics Module to determine apparent Km and Vmax values for each sample by nonlinear Michaelis–Menten or two-site isozyme iterative analysis. Specificity of the CaMKII assay was confirmed by performing the assay in the presence of KN-93, a specific inhibitor of CaMKII (data not shown).

The in vitro effect of Pb^{2+} on the activity of CaMKII was determined by performing the assay as described above with a 30-min preincubation of the hippocampal homogenates from control and Pb^{2+}-exposed rats with 0 or 10 μM Pb^{2+} acetate in 0.01% nitric acid. The sensitivity of CaMKII activity to calmodulin was determined by using the assay conditions described above at a saturating substrate concentration (100 μM substrate) in the presence of 10 nM $CaCl_2$, a calcium concentration empirically determined to maximize calmodulin-dependent CaMKII activity (data not shown), and calmodulin concentrations ranging from 3 nM to 54 μM.

2.6. Statistical analysis

All comparisons performed were analyzed for statistical significance by using Student's *t* test or paired *t* test (Intercooled Stata, Stata Corporation, College Station, TX). EC_{50} of CaMKII activation by calmodulin was calculated using a sigmoidal log EC_{50} model (BioDataFit 1.02 in BioToolKit 300, Chang Bioscience, Castro Valley, CA). All data are presented as mean ± standard error of the mean (SEM).

3. Results

3.1. Developmental Pb^{2+} exposure increases blood and hippocampal Pb^{2+} levels

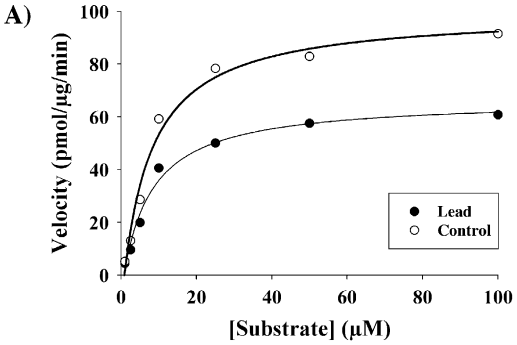
Developmental Pb^{2+} exposure increased blood and hippocampal Pb^{2+} levels in the absence of a change in body weight (Control = 267.1 ± 3.6 g vs. Pb^{2+} = 261.5 ± 2.8 g, $P > 0.05$, $n = 10$). At 50 days of age, control rats exhibited blood and hippocampus Pb^{2+} levels that were both below the limit of detection. However, blood Pb^{2+} concen-

trations of 36.1 ± 8.3 μg/dl and hippocampal Pb^{2+} concentrations of 478 ± 27 ng/g were measured in Pb^{2+}-exposed rats. These concentrations are similar to those observed in Pb^{2+}-exposed rats that exhibit cognitive and synaptic plasticity deficits [15,34].

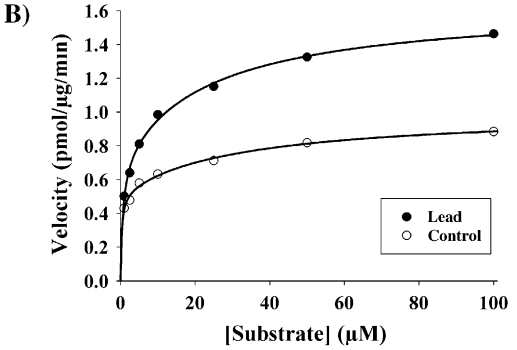
3.2. Developmental Pb^{2+} exposure alters CaMKII enzyme activity

Hippocampal CaMKII activity was examined by measuring the phosphorylation of a specific peptide substrate. The specificity of the assay for CaMKII activity was confirmed by preincubation of hippocampal extracts with KN-93, a specific inhibitor of CaMKII (data not shown). Fig. 1 shows representative Michaelis–Menten substrate vs. velocity curves and enzyme activity parameters for hippocampal CaMKII from control and Pb^{2+}-exposed rats. Hippocampal CaMKII activity from both control and Pb^{2+}-exposed rats reached maximal velocity within the concentration range of substrate peptide used in the assay (Fig. 1). However, apparent maximal velocity (Vmax) of hippocampal CaMKII in the presence of calcium and calmodulin was markedly reduced by 41% in Pb^{2+}-exposed rats relative to controls (Fig. 1A; Control = 93.5 ± 6.8 pmol/μg/min vs. Pb^{2+}-exposed = 55.2 ± 6.9 pmol/μg/min; $n = 7$; $P < 0.05$) as assessed by nonlinear Michaelis–Menten kinetics ($R^2 = 0.983$ for nonlinear Michaelis–Menten model). In addition, the apparent substrate concentration at half maximal velocity (Km) was decreased by 22% in Pb^{2+}-exposed hippocampus in the presence of activators (Fig. 1A; Control = 9.8 ± 0.7 μM vs. Pb^{2+}-exposed = 7.6 ± 0.6 μM; $n = 7$; $P < 0.05$).

On the other hand, when the same hippocampal CaMKII assay was performed in the absence of exogenously added calcium and calmodulin, a different effect of Pb^{2+} exposure on activity parameters was observed (Fig. 1B). Unlike the data generated under activating conditions, which was best modeled using a nonlinear Michaelis–Menten model, the activity data under non-activating conditions (i.e., no calcium or calmodulin added) were best fitted by a two-site isozyme model as determined by the Sigma Plot Enzyme Kinetics Module ($R^2 = 0.996$ for isozyme model vs. $R^2 = 0.794$ for nonlinear Michaelis–Menten model). Under these conditions, only the apparent Vmax, and not the apparent Km of the enzyme, was altered by developmental Pb^{2+} exposure (Fig. 1B). Under non-activating conditions, an increase in the apparent Vmax of both sites was demonstrated (Fig. 1B; Control Vmax1 = 0.52 ± 0.01 pmol/μg/min vs. Pb^{2+}-exposed Vmax1 = 0.72 ± 0.07 pmol/μg/min and Control Vmax2 = 0.72 ± 0.10 pmol/μg/min vs. Pb^{2+}-exposed Vmax2 = 1.31 ± 0.23 pmol/μg/min; $P < 0.05$ for both comparisons; $n = 5$), with no significant change in the apparent Km of either site (Fig. 1B; Control Km1 = 0.39 ± 0.11 μM vs. Pb^{2+}-exposed Km1 = 0.50 ± 0.16 μM and Control Km2 = 28.4 ± 7.8 μM vs. Pb^{2+}-exposed Km2 = 29.4 ± 2.3 μM).



Treatment	Km (μM)	Vmax (pmol/μg/min)
Control	9.8 ± 0.7	93.5 ± 6.8
Lead	7.6 ± 0.6 *	55.2 ± 6.9 *

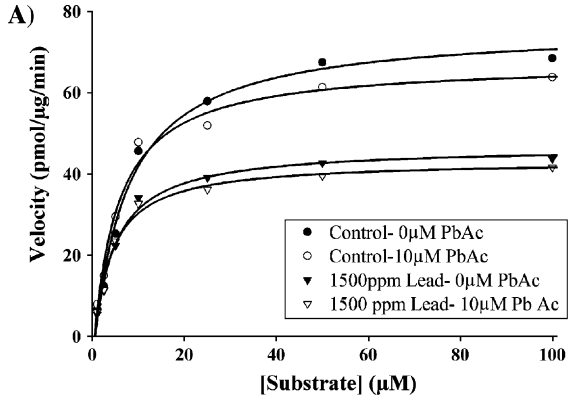


Treatment	Km 1 (μM)	Km 2 (μM)	Vmax 1 (pmol/μg/min)	Vmax 2 (pmol/μg/min)
Control	0.39 ± 0.11	28.4 ± 7.8	0.52 ± 0.01	0.72 ± 0.10
Lead	0.50 ± 0.16	29.4 ± 2.3	0.72 ± 0.07 *	1.31 ± 0.23*

Fig. 1. Developmental Pb^{2+} exposure alters the activity of hippocampal CaMKII. (A) Pb^{2+} exposure decreases CaMKII activity and increases affinity for substrate in the presence of calcium and calmodulin. Representative substrate vs. velocity curves demonstrating a 41% decrease in apparent Vmax and a 23% decrease in apparent Km values in Pb^{2+}-exposed rat hippocampi as measured by nonlinear Michaelis–Menten iteration using Sigma Plot Enzyme Kinetics Module ($R^2 = 0.983$). Apparent Km and Vmax values for hippocampal CaMKII are presented as mean ± SEM. *$P < 0.05$ as determined by t test; $n = 7$. (B) Developmental Pb^{2+} exposure increases apparent Vmax without altering apparent Km of hippocampal CaMKII in the absence of exogenously added calcium and calmodulin. Representative substrate vs. velocity curves of hippocampal CaMKII activity demonstrating an increase in apparent Vmax in Pb^{2+}-exposed rat hippocampi as measured by an iterative two-site isozyme analysis using Sigma Plot Enzyme Kinetics Module ($R^2 = 0.996$). Apparent Vmax1 and Km1 represent the kinetic values of a high affinity site. Apparent Vmax2 and Km2 represent the kinetic values of a low affinity site. The values presented are mean ± SEM. *$P < 0.05$ as determined by t test; $n = 5$.

3.3. Preincubation of hippocampal CaMKII with Pb^{2+}-acetate alters the apparent Km but not apparent Vmax under activating conditions

Preincubation of hippocampal extracts with 10 μM Pb^{2+}acetate resulted in a 20% decrease in the apparent Km of hippocampal CaMKII from control but not from Pb^{2+}-exposed rats (Fig. 2A; Control Rats—0 μM Pb^{2+}-



Diet	In Vitro Pb (µM)	Km (µM)	Vmax (pmol/µg/min)
Control	0	9.2 ± 0.5	77.8 ± 1.2
Control	10	7.4 ± 0.9*	75.1 ± 2.2
1500 ppm PbAc	0	6.3 ± 0.5	57.1 ± 4.6
1500 ppm PbAc	10	6.6 ± 0.7	56.7 ± 4.4

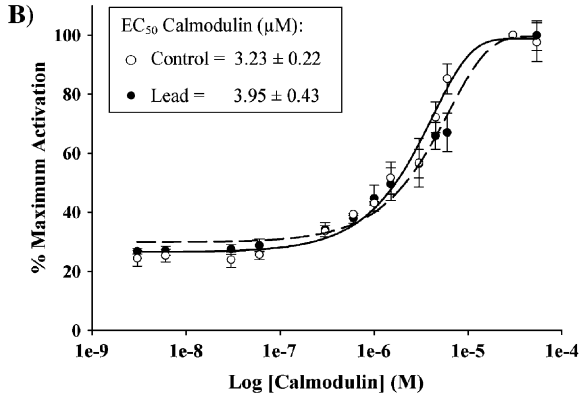


Fig. 2. Incubation of hippocampal extracts with Pb^{2+} acetate decreases CaMKII apparent Km without altering apparent Vmax. (A) Preincubation of hippocampal extracts with Pb^{2+} acetate increases CaMKII affinity for substrate in the hippocampus of control but not Pb^{2+}-exposed rats. Representative substrate vs. velocity curves for hippocampal CaMKII activity in the presence of 0 or 10 µM exogenously added Pb^{2+} acetate demonstrate a 20% decrease in apparent Km value for CaMKII from control rats (0 µM Pb^{2+} acetate = closed points; 10 µM Pb^{2+} acetate = open points). Apparent Vmax and Km values for hippocampal CaMKII from control or Pb^{2+}-exposed rats preincubated with 0 or 10 µM Pb^{2+} acetate are presented as mean ± SEM. *$P < 0.05$ as determined by paired t test; $n = 5$. (B) CaMKII sensitivity to calmodulin is not altered by developmental Pb^{2+} exposure. Hippocampal CaMKII activity from control and Pb^{2+}-exposed hippocampal homogenates as a function of calmodulin concentration (3 nM to 54 µM) is presented. All values are normalized to maximal activation and are presented as mean ± SEM. Control = open points; Pb^{2+} exposed rats = closed points. No significant difference is observed in the calmodulin sensitivity of hippocampal CaMKII from Pb^{2+}-exposed rats as measured by EC_{50}; $n = 4$.

acetate preincubation Km = 9.2 ± 0.5 µM vs. 10 µM Pb^{2+}-acetate preincubation Km = 7.4 ± 0.9 µM; $n = 5$, $P < 0.05$; Pb^{2+}-exposed rats—0 µM Pb^{2+} acetate preincubation Km = 6.3 ± 0.5 µM vs. 10 µM Pb^{2+}-acetate preincubation Km = 6.6 ± 0.7 µM, $n = 5$). The decrease of apparent Km after in vitro preincubation with Pb^{2+}-acetate is of the same magnitude (20%) when one compares the apparent Km of CaMKII from control and in vivo Pb^{2+}-exposed hippocampus under activating conditions (Fig. 1A). Preincubation of Pb^{2+}-acetate with hippocampal extracts does not affect the apparent Vmax of hippocampal CaMKII (Fig. 2A; Control rats—0 µM Pb^{2+}-acetate preincubation Vmax = 77.8 ± 1.2 pmol/µg/min vs. 10 µM Pb^{2+}-acetate preincubation Vmax = 75.1 ± 2.2 pmol/µg/min; Pb^{2+} exposed rats—0 µM Pb^{2+}-acetate preincubation Vmax = 57.1 ± 4.6 pmol/µg/min vs. 10 µM Pb^{2+}-acetate preincubation Vmax = 56.7 ± 4.4 µM).

3.4. Developmental Pb^{2+} exposure does not alter the sensitivity of CaMKII for calmodulin

We hypothesized that one possibility for the changes in CaMKII activity measured in hippocampal extracts from Pb^{2+}-exposed rats is an alteration in the sensitivity of the enzyme to calmodulin. It has been suggested that activation and subsequent autophosphorylation of CaMKII may alter the affinity of CaMKII for the calcium/calmodulin complex [11]. To test this hypothesis, we examined the activation of hippocampal CaMKII from control and Pb^{2+}-exposed rats as a function of calmodulin concentration. No significant shift in the sensitivity of hippocampal CaMKII from Pb^{2+}-exposed rats to calmodulin was observed since the activation curves with control tissue virtually overlap (Fig. 2B). Additionally, there was no significant difference in the EC_{50} of calmodulin for the activation of CaMKII between control and Pb^{2+}-exposed hippocampal homogenates (Fig. 2B—Control EC_{50} = 3.23 µM ± 0.22 vs. Pb^{2+}-exposed rat EC_{50} = 3.95 µM ± 0.43; $P > 0.05$, $n = 4$).

3.5. CaMKII phosphorylation and protein levels

An alternative explanation for the changes observed in CaMKII activity in hippocampal extracts from Pb^{2+}-exposed rats is a change in the phosphorylation state of the protein. Therefore, Western blotting for phospho-threonine-286 CaMKII was performed from hippocampal homogenates of rats sacrificed by focused microwave irradiation, a method of euthanasia shown to preserve in vivo protein phosphorylation [35]. No significant differences in the levels of phospho-threonine-286 CaMKII were measured in whole hippocampal homogenates from control or Pb^{2+}-exposed rats euthanized by focused microwave irradiation (Control = 100 ± 6.7% vs. Pb^{2+}-exposed = 90.1 ± 14.7%, $P > 0.05$, $n = 7$).

Expressions of the brain-specific CaMKII isoforms α and β were also measured in cytosolic and membrane fractions of rat hippocampi to determine if expression of these isoforms is altered by Pb^{2+} exposure. CaMKII α expression was not changed by Pb^{2+} exposure in cytosolic (Fig. 3; Control = 100 ± 10.3% vs. Pb^{2+}-exposed = 85.0 ± 17.6%, $P > 0.05$, $n = 7$) or membrane (Fig. 3; Control = 100 ± 9.9% vs. Pb^{2+}-exposed = 114.4 ± 17.9%, $P > 0.05$, $n = 8$) fractions of rat hippocampus. However, CaMKII β expression was decreased in cytosolic (Fig. 3; Control = 100 ±

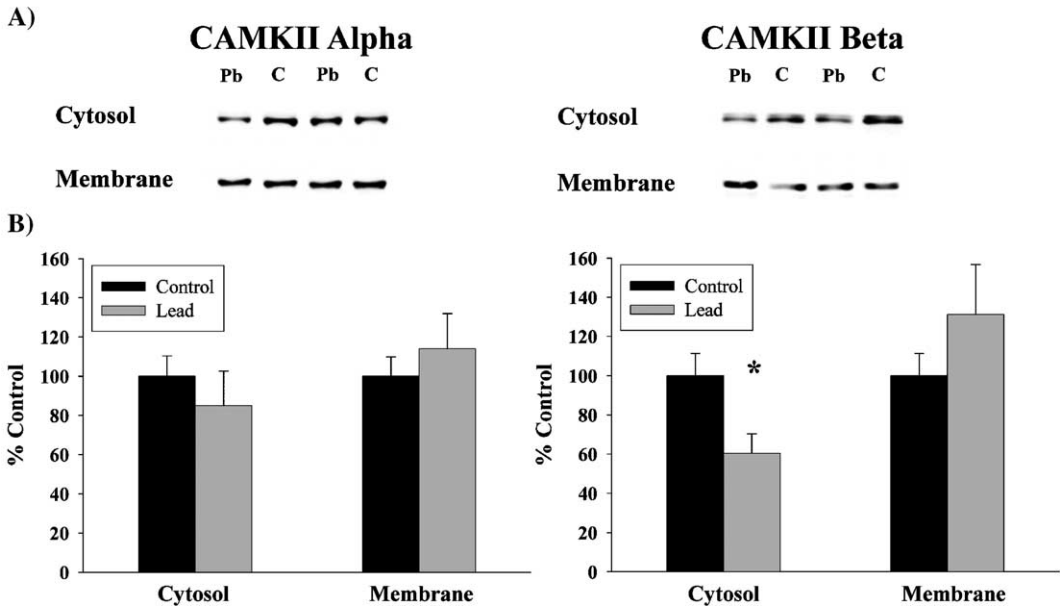


Fig. 3. CaMKII β expression is decreased in the cytosol of Pb^{2+}-exposed rat hippocampus. (A) Representative Western blot images of CaMKII α and β in cytosolic and membrane fractions of hippocampi from control and Pb^{2+}-exposed rats (Pb = lead; C = Control). (B) Developmental Pb^{2+} exposure alters the compartmentalization of CaMKII β, but not α, in the rat hippocampus. Hippocampal cytosolic and membrane extracts from control and Pb^{2+}-exposed rats were resolved by SDS–PAGE and probed for the expression of CaMKII α and β. Quantification of these immunoblots is provided as mean ± SEM of optical density normalized to percent control. No significant change is observed in the expression of CaMKII α in the hippocampus of Pb^{2+} exposed rats. $*P < 0.05$ as determined by t test; $n = 6–8$.

11.3% vs. Pb^{2+}-exposed = 60.4 ± 9.9%, $P < 0.05$, $n = 6$) but not changed in membrane (Fig. 3; Control = 100 ± 11.4% vs. Pb^{2+}-exposed = 131.1 ± 25.7%, $P > 0.05$, $n = 8$) fractions of rat hippocampus.

4. Discussion

The present study demonstrates that developmental Pb^{2+} exposure alters CaMKII activity, affinity for substrate, and compartmentalization of CaMKII in the hippocampus of young adult rats. Rats exposed to this chronic Pb^{2+} treatment have been shown previously to express deficits in spatial learning, hippocampal LTP, alterations in NMDAR subunit expression, and decreased CREB phosphorylation at the same age as used in the present study [34,38,39]. We now show that similarly treated animals exhibit a 41% decrease in CaMKII activity (Vmax) and a 22% increase in the apparent affinity of the enzyme for the substrate (Km) in the presence of calcium and calmodulin (Fig. 1A). An opposite effect on CaMKII apparent Vmax was measured if the same hippocampal homogenate was assayed in the absence of added activators (basal conditions; Fig. 1B). Therefore, under basal conditions, there is an apparent increase in CaMKII activity, but under activating conditions there is a decrease in enzyme activity in the hippocampus of Pb^{2+}-exposed rats.

The present work also shows that in vitro preincubation of Pb^{2+} with hippocampal homogenates from control, but not from Pb^{2+}-exposed rats, results in a 20% decrease in the apparent Km of CaMKII with no change in Vmax (Fig. 2). This finding demonstrates that Pb^{2+} can disrupt the normal functioning of CaMKII and may explain the increased affinity of the enzyme measured in the hippocampus of rats exposed to Pb^{2+} in vivo. Further, it is interesting that preincubation of hippocampal extracts from rats exposed to Pb^{2+} in vivo did not produce a change in enzyme affinity, suggesting that the site at which Pb^{2+} alters CaMKII function is already saturated with Pb^{2+} as a result of the in vivo exposure.

To further investigate potential mechanisms by which chronic Pb^{2+} exposure decreases CaMKII activity, we measured the phosphorylation of the enzyme at threonine 286, its sensitivity to calmodulin and protein levels of the α and β isoforms in hippocampal tissue. We found no significant effect of developmental Pb^{2+} exposure on threonine-286 phosphorylation or sensitivity to calmodulin (Fig. 2). However, CaMKII β protein expression was decreased by 40% in hippocampal cytosolic fractions of Pb^{2+}-exposed rats with no change in CaMKII α protein (Fig. 3). The lack of a change in CaMKII α protein is consistent with a previous finding that CaMKII α mRNA expression is not altered in the hippocampus of Pb^{2+}-exposed rats [19]. Together, these findings demonstrate that chronic developmental Pb^{2+} exposure alters the activity, affinity, and compartmentalization of an enzyme that is essential for synaptic plasticity, learning, and memory [8,9,21].

How does Pb^{2+} exposure alter the activity of hippocampal CaMKII in the absence of alterations in phosphorylation at threonine-286 and changes in calmodulin

sensitivity? One of the more potent Pb^{2+}–protein interactions that have been documented is between Pb^{2+} and zinc binding sites [18,22,42]. Studies have shown that CaMKII is able to bind zinc which alters its activity and phosphorylation [28]. Zinc can directly activate CaMKII when added at 400 μM in the absence of calcium and calmodulin [28], an observation similar to that observed in the current study where CaMKII from rats exposed to Pb^{2+} in vivo demonstrates an increase in activity in the absence of calcium and calmodulin (Fig. 1B). Further, zinc at higher concentrations (5 mM) has been demonstrated to inactivate the kinase activity of CaMKII [28]. Since Pb^{2+} is known to bind and replace zinc from zinc binding sites in several proteins and peptides [22,42], it is possible that Pb^{2+} may directly interact with CaMKII and alter its kinetic parameters. This direct alteration of CaMKII kinetics is supported by our observation that in vitro preincubation of hippocampal homogenates from control rats with Pb^{2+} alters the apparent Km of CaMKII in the presence of calcium and calmodulin (Fig. 1A).

Although preincubation of Pb^{2+} with hippocampal tissue from control rats is able to recapitulate the alteration in apparent Km, the decrease in CaMKII apparent Vmax in the presence of activators measured in animals exposed to Pb^{2+} in vivo cannot be explained by preincubation of Pb^{2+} with the enzyme. We believe that this effect of in vivo Pb^{2+} exposure on CaMKII activity could be due to post-translational modifications at sites other than threonine-286 or to the observed decrease in CaMKII β protein levels (Fig. 3). Phosphorylation at threonine 305 and 306 has been extensively characterized as inhibitory sites for CaMKII activity [11]. Similar to Pb^{2+}-exposed rats, mice expressing inhibited CaMKII mutants that resemble persistent phosphorylation at threonine 305 and 306 exhibit a decreased capacity for synaptic plasticity and learning [12,34]. Alternatively, the decrease in CaMKII β expression measured in the cytosolic fraction of hippocampal extracts from Pb^{2+}-exposed rats (Fig. 3) may account for the decrease in CaMKII activity in the presence of calcium and calmodulin.

The exact mechanism of the decrease in cytosolic CaMKII β protein expression in Pb^{2+}-exposed rats and its downstream effects is at present unclear. However, one possible effect may be related to a new described function of CaMKII β. CaMKII β is an F-actin binding protein that localizes CaMKII hetero-oligomers in dendritic spines [37] and its cytoskeletal localization can be regulated by glutamate stimulation of the NMDAR [37]. Recently, a study by Fink et al. [14] has defined a morphogenic effect of CaMKII that is specific for the β isoform. That is, increasing the expression of CaMKII β by a number of approaches enhances dendritic arborization by increasing the movement, branching, and extension of filopodia and fine dendrites in hippocampal neurons. Further, increasing CaMKII β levels also increase synapse formation [14]. On the other hand, decreased expression of CaMKII β protein resulted in an opposite effect. Therefore, increasing or decreasing the levels of CaMKII β, but not α, leads to a corresponding increase or decrease in dendritic arborization and synapse formation. These findings could explain the decreased levels of dendritic arborization and synapse formation that have been documented in the hippocampus of rats exposed to Pb^{2+} developmentally [1,10,26].

The effect of developmental Pb^{2+} exposure on CaMKII activity under basal and activating conditions may also have important implications for the ability of the enzyme to adequately respond to activity-dependent changes at the synapse. Based on the present findings, it is important for future studies to define CaMKII substrates whose level of phosphorylation may be affected by our present findings. We have previously shown that the level of phosphorylation of the transcription factor CREB is significantly decreased in cortical and hippocampal nuclear fractions from Pb^{2+}-exposed rats [38,39]. However, a direct correlation has yet to be demonstrated.

In summary, the current study provides valuable insights with respect to intracellular calcium signaling in Pb^{2+}-exposed rats and identifies alterations in the activity and expression of CaMKII, an enzyme known to be involved in synaptic plasticity and in regulating dendritic morphology [8,9,21]. Further, this is the first account of an alteration of CaMKII activity and expression observed in an in vivo model of developmental Pb^{2+} neurotoxicity.

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