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STATUS EPILEPTICUS RESULTS IN A DURATION-DEPENDENT INCREASED
PROTEIN KINASE A ACTIVITY IN THE RAT PILOCARPINE MODEL

A thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University.

by

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Abstract

PROLONGED STATUS EPILEPTICUS RESULTS IN INCREASED PROTEIN KINASE A ACTIVITY IN THE RAT PILOCARPINE MODEL

By James McLeod Bracey, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University

Virginia Commonwealth University, 2005

Major Director: Dr. Severn B. Churn
Associate Professor Departments of Anatomy and Neurobiology,
Pharmacology and Toxicology, and Physiology
Director, Molecular Neuroscience Research Facility

This study was conducted to characterize cellular changes occurring during the progression of status epilepticus (SE) that could lead to the maintenance of increased membrane excitability. SE was induced by injection of pilocarpine after which rats were monitored both electrographically and behaviorally. After various lengths of time in SE, specific brain regions were isolated for biochemical study. SE resulted in an early

maintenance of PKA activity in both cortical homogenate and crude synaptoplasmic membrane (crude SPM) fractions. At subsequent stages of SE there was a significant increase in PKA activity in both homogenate and crude SPM fractions. Wester blot analysis showed that alteration of PKA protein expression was not responsible for the increase in PKA activity. These results show that SE has a significant duration-dependent effect on PKA activity. Combined with other cellular changes these findings, could represent a mechanism for the formation for potentiated seizure states like epilepsy.

INTRODUCTION

Epilepsy

Epilepsy is a common neurological disorder experienced by roughly 2.5 million people in the United States alone with greater than 150,000 people developing seizures and/or epilepsy each year. Overall, epilepsy occurs when there is an imbalance in signaling contribution between excitatory and inhibitory neurons. This imbalance leads to excessive excitatory signaling through an increase in glutamatergic (excitatory) responses, decreased γ -aminobutyric acid ([GABA]ergic) inhibitory responses, or possibly a combination of the two. Seizure activity is commonly attributed to a decrease in the inhibitory effects of GABA receptor mediated channels. Under normal physiologic conditions, GABA channels allow for the conductance of negatively charged chloride ions into the cell which counterbalance the effects of incoming positive ions associated with glutamatergic channels. This influx of chloride helps maintain the cell's normal electrical activity and membrane potential. When the GABA channel is somehow altered, the normally occurring inhibition it provides is lost resulting in an over excitation of the cell.

In epilepsy, most seizures are relatively short lived and end through a process of self termination. There are some pathologies that result in seizures that do not self-terminate but progress into a neurological insult known as status epilepticus (SE). It is possible that seizures that are capable of self-termination do so through the effects of

GABA-receptor mediated inhibition and that the inhibitory currents associated with these channels is the source of the seizure termination. Hosts of studies into antiepileptic drugs (AEDs) including, but not limited to, benzodiazepines, diazepam, lorazepam and barbiturates, have shown that these drugs target GABA_A receptors. These drugs work by enhancing the receptor-mediated inhibition through increased conductance through these channels. This suggests that these channels are intimately involved in the process of seizure termination.

Status Epilepticus

SE is generally defined as a seizure, either single or in an intermittent series, which lasts at least 30 minutes during which time the patient does not regain consciousness (Bassin et al. 2002). A more current definition states that SE is characterized by a continuous, generalized, convulsive seizure lasting greater than 5 minutes or two or more seizures during which the patient does not regain consciousness (Bassin et al. 2002; Lowenstein et al. 1999; Treiman et al. 1998). This shorter time window is supported by the fact that both clinically and experimentally, AEDs lose their efficacy over time as SE duration increases. Frontline AEDs must therefore be given as early as possible during the course of SE to ensure effectiveness.

SE is a highly common neurological emergency affecting greater than 100,000 individuals every year in the United States. SE is also a major medical emergency with a mortality rate of greater than 20% attributed primarily to anoxia, prolonged seizure and advanced age (DeLorenzo et al. 1996). It has also been shown that patients with epilepsy have a two to three fold greater chance of mortality when compared to the general population (Tomson 2000).

Prolonged and uninhibited excitation causes severe and irreversible damage in neuronal structures in a variety of pathologies such as cerebral ischemia, Alzheimer's disease, amyotrophic lateral sclerosis and seizure disorders (Anzai et al. 2003; Blennow et al. 1978; Butterfield and Boyd-Kimball 2005; Carriedo et al. 2000; Sankar et al. 1998), a process called excitotoxicity. These same types of effects are seen in SE as well with neuropathological changes induced by the seizures being confined to those locations experiencing excessive excitation. In addition, many proteins important for proper neuronal function have been shown to be affected by SE, including calcium/calmodulin dependent protein kinase II (CaMKII) (Churn et al. 2000) and protein phosphatase 2B or calcineurin (CaN) (Kurz et al. 2001).

Cyclic-AMP dependent Protein Kinase A

Another protein of interest is cyclic-AMP dependent protein kinase (PKA). PKA, a member of the serine/threonine kinase family, is ubiquitously expressed and mediates both intracellular signal transduction and intercellular signal transmission (Nguyen and Woo 2003). PKA is a heteromultimer composed of two regulatory subunits and two catalytic subunits. When cyclic-AMP (cAMP) binds to the regulatory subunits, the catalytic subunits are released activating the enzyme leading to the phosphorylation of endogenous substrates. This enzyme activation can then be maintained through several mechanisms such as the ubiquitination of the regulatory subunits or phosphorylation of the regulatory subunit by the catalytic subunit causing the two to have a decreased binding affinity. This decrease in subunit ratio or binding affinity results in sustained kinase activity.

Previous studies have shown that high frequency stimulation of synaptic pathways in the hippocampus can increase cAMP levels thus activating PKA (Chetkovich et al. 1991). In addition, adenylate cyclases have been shown to be activated by the D1 subtype of dopamine receptor resulting in increased cAMP formation (Barone et al. 1990). This increase in cAMP would also have the effect of stimulating PKA. Activation of PKA has been shown to be important for a variety of neuronal functions notably in the formation of late stages of long-term potentiation (LTP). Progression into late-LTP has the effect of changing synaptic systems from learning acquisition to memory consolidation. PKA facilitates this through increased ion channel function (Crump et al. 2001; Esteban et al. 2003; Meuth et al. 2002) and new protein synthesis, among others (Kang and Schuman 1996; Krug et al. 1984; Otani et al. 1989).

The Rat Pilocarpine Model of SE

A variety of models of SE are used, including anti-GABA drugs, glutamatergic drugs, cholinergic drugs and electrical stimulation (Churn et al. 2000). Damage from each of these models is similar and indicates that the pathological effects are not model specific, but due to seizure activity associated with SE. In the following study, the pilocarpine (pilo) model was used for SE induction in rats. Pilocarpine is a muscarinic agonist and mimics the action of acetylcholine. Prior to pilocarpine injection, methylscopolamine, a muscarinic antagonist, is injected to reduce the peripheral, adverse effects of pilocarpine. A short time following the injection of pilo, discrete seizure activity can usually be noticed. Typically within 8 to 12 minutes following the onset of discrete seizure activity, non-terminating seizure activity is noticed. This is defined as the onset of SE.

This research focuses on the effects of SE on PKA activity. This study was conducted to characterize the relationship between PKA activity and SE progression over time. SE was induced in adult rats and allowed to continue for predetermined times up to and including one hour of SE activity. Electroencephalographic (EEG) recordings were made throughout each experiment after which, animals were sacrificed and biochemical assays were performed. EEG was analyzed for total time in SE and time to the start of each SE phase. In addition to EEG recordings, biochemical assays were performed to determine if there is an SE duration dependent change in PKA activity. The data suggest that there is a delayed duration dependent increase in PKA activity seen in the cortex during SE. This is in concert with the decrease in CaMKII activity seen immediately upon the induction and the delayed increase in CaN activity seen during SE progression. The following chapter is a paper that has been submitted for publication in **Neurobiology of Disease**. The paper is formatted according to the guidelines set by the journal in which it will appear.

STATUS EPILEPTICUS RESULTS IN A DURATION-DEPENDENT INCREASE OF PROTEIN KINASE A ACTIVITY IN THE RAT PILOCARPINE MODEL

ABSTRACT

This study characterized the effects of status epilepticus (SE) on PKA activity in the rat pilocarpine model. SE was induced and seizure activity was monitored electrographically and cellular changes studied biochemically. PKA activity in whole tissue homogenates as well as crude synaptoplasmic membrane fractions was assessed through substrate phosphorylation reactions. No significant changes in PKA activity were observed during early stages of SE. However, as SE progressed, significant increases in both total and synaptic PKA activity were observed. In addition, there was no observed increase in PKA protein expression in either homogenate or synaptic samples. Because increased PKA and other protein kinase activities are associated with long-term potentiation (LTP), increased PKA activity during SE could result in maintained potentiation of large numbers of synapses on a whole-brain level. These data support the hypothesis that PKA plays a role in the progression of SE and possibly in the process of epileptogenesis

INTRODUCTION

Status Epilepticus (SE) is a life threatening emergency that affects approximately 100,000-150,000 people per year (Bassin et al. 2002; DeLorenzo et al. 1996; Fountain 2000; Goodkin et al. 2003) and has a significant mortality rate (Bassin et al. 2002; Tomson 2000; Waterhouse et al. 1998). SE is defined as either a continuous seizure lasting ≥ 30 minutes or continuous recurrent seizures without the recovery of consciousness (Fountain 2000). The definition has been modified in recent years to say that, in adults and children over 5 years of age, SE should be considered after ≥ 5 minutes of continuous seizure activity or two or more individual seizures between which there is an incomplete recovery to consciousness (Lowenstein and Alldredge 1998; Lowenstein et al. 1999). This revised definition takes into consideration the fact that most tonic clonic seizures self-terminate within a 5 minute window (Theodore et al. 1994). In addition, SE results in several secondary effects such as the loss of efficacy of seizure suppressing drugs, including benzodiazepines, with prolonged seizure episodes and the development of spontaneous recurrent seizures through a process known as epileptogenesis. This observation suggests that as seizure activity progresses, cellular changes occur that change neuronal physiology. Therefore, understanding the cellular changes that occur during SE may elucidate the processes that result in epileptogenesis.

Cyclic-AMP dependent Protein Kinase A (PKA) is a ubiquitously expressed member of the serine-threonine kinase family and is known to mediate intracellular signal transduction in a variety of animal systems. Notably, PKA has been associated with mediation of both short-term synaptic facilitation via reversible phosphorylation of ion channels as well as long-term facilitation through modulation of gene expression and

protein synthesis (Bailey and Kandel 1993; Hawkins et al. 1993; Kandel 2001). PKA has been shown to affect both excitatory and inhibitory neuronal signaling and exhibits complementary as well as antagonistic modulatory effects with respect to calcium/calmodulin dependent protein kinase II (CaMKII) and protein phosphatase 2B or calcineurin (CaN). For example, PKA has been shown to both enhance and inhibit GABA_A receptor currents (Kapur and Macdonald 1996; McDonald and Moss 1997) while the action of CaMKII increases agonist-evoked inhibitory currents (Churn and DeLorenzo 1998; Churn et al. 2002; Kapur and Macdonald 1996; Wang et al. 1995; Wei et al. 2004) as well as increases conduction through excitatory glutamate receptors (Fink and Meyer 2002; Poncer et al. 2002; Wang and Kelly 2001). In addition, PKA has been shown to have an inhibitory effect on protein phosphatase 1 (PP1) activation by CaN (Vinade and Dosemeci 2000). PKA has been extensively studied and has been shown to have altered expression or activity in a variety of pathologies. For instance, in cerebral ischemia models there is a differential activation of PKA and subsequent CREB phosphorylation in peri-ischemic and ischemic areas (Tanaka 2001). Disruption of hippocampal circuits, used as a model of neuronal reorganization, results in the up-regulation of several genes involved in signal transduction including PKA (Anguelova et al. 2000). In addition, PKA has been associated with the abnormal tau phosphorylation and neurofibrillary tangle formation seen in Alzheimer's disease (Bonkale et al. 1999). Despite these findings, little research has been done into PKA's role in status epilepticus.

This study utilized electrographic and behavioral monitoring in the Pilo-SE model and tracked changes in PKA activity during the progression of status epilepticus.

Following the development of SE, the data show that PKA activity is maintained while in

later stages of SE, there is a significant increase in PKA activity. The data suggest that the modulation of PKA activity is involved in cellular changes associated with prolonged seizure activity in SE.

MATERIALS AND METHODS

Materials

All materials were reagent grade and purchased from Sigma Chemical Company (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA) unless otherwise stated. [γ - ^{32}P]ATP was purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA, USA). Adult male Wistar rats were purchased from Harlan Laboratories (Indianapolis, IN, USA). SignaTECT PKA assay system components were purchased from Promega (Madison, WI, USA). KT-5720 was purchased CalBiochem (San Diego, CA, USA). Affinity purified rabbit polyclonal anti-PKA β catalytic subunit was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Purified goat polyclonal anti-PKA RII subunit and mouse monoclonal anti- β -tubulin (clone AA2) were obtained from Upstate (Lake Placid, NY, USA).

Methods

Pilocarpine Model of Status Epilepticus

All animal use procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. Wistar rats were handled following arrival from Harlan Laboratories, for acclimation prior to drug treatment. One week prior to the induction of SE, 4 surface electrodes were implanted into the skull of rats under ketamine anesthesia, as described previously (Churn 2005; Singleton et al. 2005b). Two frontal electrodes were implanted over frontal cortex [3.5mm ant. Bregma, \pm 2.5mm L/R (F1/F2)]. Two posterior electrodes were implanted over parietal cortex and hippocampus [2.0mm post. Bregma, \pm 2.5mm L/R

(P1/P2)]. A 5th electrode was fixed onto the surface of the skull as a ground. The electrodes were secured in place with dental acrylate and the animals were allowed at least 5 days to recover from surgery before experiments were performed.

Induction of Status Epilepticus

Control and experimental animals were hooked up to EEG machines (Viking IV, Nicolet, Madison, WI) and baseline EEG recordings were obtained for 10 minutes following scopolamine injection. 30 minutes prior to the injection of pilocarpine, methylscopolamine, a muscarinic antagonist, was administered i.p. (1 mg/kg) to reduce adverse peripheral affects of the pilocarpine. SE was induced in experimental animals by intraperitoneal injection of 375 mg/kg pilocarpine HCl (Pilo), a muscarinic agonist. Behavioral and encephalographic activity was recorded throughout the procedure using video and EEG recording. Once initial seizure activity was observed, the time was noted and rats were allowed to seize for specific amounts of time before the animals were processed. These time points consisted of 10, 20, 40 and 70 minutes post first discrete seizure. Since this lab has characterized SE onset as being approximately 10 minutes after the first discrete seizure (Singleton et al. 2005b), these times approximate 0, 10, 30 and 60 minutes of SE, respectively. Electrographic data was analyzed for SE susceptibility, number of discrete seizures, death rate, time to first seizure, time from first seizure to SE, average duration of SE for each animal, percent of time spent in each stage of SE for each animal, and once in SE, seizure severity. Spectral analysis of EEG activity was performed using Insight II software (Persyst Corporation Prescott, AZ). After induction of SE, seizure activity was characterized according to the system of Handforth and Treiman (Handforth and Treiman 1995a; Handforth and Treiman 1995b).

Seizure severity was determined based on the amount of time spent in each stage of SE. Behavioral seizures were assessed according to the scale of Racine (Racine 1972).

Brain Region Isolation

Brain region isolation was performed as previously described with slight modifications (Churn et al. 2000). Specific brain regions (cortex and hippocampus) were dissected away on ice and immediately homogenized into an iced-cold buffer containing 50mM Tris-HCl (pH 7.4), 7mM EGTA, 5mM EDTA, 320mM sucrose, 1mM dithiothreitol (DTT), and 0.3mM phenylmethylsulfonyl fluoride (PMSF). The brain regions were homogenized by 10 up and down strokes using a teflon pestle at 12,000 rpms (Fordham Bethel, CT). A portion of the sample was then aliquoted and frozen at -80°C until processed for biochemical analysis.

Isolation of Subcellular Fractions

Subcellular fractions were isolated by a differential centrifugation procedure as modified from Edelman *et al.* (Edelman et al. 1985). Brain region homogenates were centrifuged at 5,000 x g for 10 min (Beckman JA-17 rotor) to produce a crude nuclear pellet (P1). The supernatant from the spin (S1) was then centrifuged for 30 min at 18,000 x g to produce a crude synaptoplasmic membrane/mitochondrial pellet (crude SPM), which was resuspended in homogenization buffer. All fractions were rapidly separated into aliquots and stored at -80°C for later use.

Substrate Phosphorylation

Brain region homogenates and crude SPM fractions were normalized for protein concentration using the Bradford method with BSA as the standard (Bradford 1976). PKA dependent phosphorylation reactions contained sample (150 µg), PKA Assay Buffer

5X, [γ -³²P]ATP in 0.5mM ATP, PKA Biotinylated Peptide Substrate 100mM, \pm cAMP 0.025mM, \pm dH₂O. ATP mix was made by adding 0.05 μ L [γ -³²P]ATP ((3,000Ci/mmol) 10 μ Ci/ μ l) to 0.5mM cold ATP (5 μ l) per reaction. Final volume of all reaction tubes was 25 μ L. Following inclusion of PKA buffer, PKA substrate, dH₂O for basal reactions or cAMP for maximal reactions, [γ -³²P]ATP (5 μ L) was added and the reaction tubes allowed to incubate in a 30° C water bath for 5 minutes. After 5 minutes 5 μ L of protein sample was added to each tube. Tubes were then allowed to incubate in a 30°C water bath for an additional 5 minutes. The reactions were then stopped via the addition of 7.5M guanidine hydrochloride (12.5 μ L). Basal and maximally stimulated reactions were performed in triplicate. 10 μ L aliquots were then taken from each tube and spotted on a biotin capture membrane. Membranes were then washed to remove unbound phosphates. Washes were as follows: Four washes in 2M NaCl, 4 washes in 2M NaCl with 1% H₃PO₄ and 2 washes in deionized water. Following drying the biotin membrane sections were placed in scintillation vials, 2ml of cytosint was added and the reactions were counted using a Beckman LS 2800 scintillation counter.

Immunodetection of PKA Regulatory and Catalytic subunits

Western analysis was performed to quantify the protein levels of PKA regulatory and catalytic subunits essentially as described previously (Churn et al. 1995; Churn et al. 1992; Singleton et al. 2005b). Briefly, homogenate and crude synaptic cortical and hippocampal fractions were resolved on SDS-PAGE and transferred to a nitrocellulose membrane using the Trans-blot system (Bio-Rad, Hercules, CA, USA). The nitrocellulose membranes were then immersed for 1 h in blocking solution containing phosphate-buffered saline (PBS, pH 7.4), 0.05% (v/v) polyoxyethylene sorbitan

monolaurate (Tween-20), 50 g/L Bio-Rad blotting grade dry milk, and horse serum (Vector Laboratories Inc., diluted 50 mL/10 mL). The nitrocellulose membranes were then incubated with primary antibody in blocking solution at 4°C overnight. Primary antibodies were diluted 1:1000 in PBS with Tween-20 and 50g/L nonfat dry milk. Membranes were washed three times in a wash solution containing PBS and Tween-20. The nitrocellulose membranes were then reacted with an appropriate secondary antibody (1:5000) in blocking solution for 1 hour. Nitrocellulose was then washed three times in PBS for 10 min per wash. Blots were developed using ECL detection (Pierce, Rockford, IL). Chemiluminescence was detected by using Kodak autoradiographic film. Specific immunoreactive bands were quantified by computer-assisted densitometry using a BioRad GS-800 calibrated densitometer and the BioRad Quantity One software (Version 4.4.0) as described previously (Churn et al. 2000; Singleton et al. 2005b). As a protein loading control, western blot membranes were probed with an anti- β -tubulin antibody (1:1000).

Statistical Analysis

Data was analyzed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA USA). Student's t tests were used for all single, parametric comparisons. For multiple comparisons, one-way ANOVA with a Tukey post hoc analysis with 95% confidence was used.

RESULTS

Time-dependence of Pilo-induced SE

Monitoring of SE phases was performed through electrographic and video recordings (see Materials and Methods). Baseline electrographic recordings were obtained following injection of scopolamine with activity consisting of low amplitude, asynchronous spikes. Approximately 20 minutes after pilocarpine injection, electrographic activity slowed and amplitude increased finally culminating in overt ictal activity (Figure 1A). During seizure induction baseline data gave way to an increase in amplitude and decrease in frequency. Spectral analysis of ictal activity during the first discrete seizure indicated a near 10-fold increase in the delta component. This denoted the onset of the discrete seizure phase. A typical seizure progression was observed as reported previously including behavioral manifestations of decreased ambulation, uncontrolled salivation, tonic/clonic movements, rearing and falling and myoclonus (Singleton et al. 2005b). These characteristics represent behavioral scores of between 1 and 4 on the Racine scale (Racine 1972). Each discrete seizure was characterized by a gradual increase in electrographic activity which peaked for 10-40 seconds. This was followed by a sudden drop back to baseline levels (Singleton et al. 2005b). Typically, pilocarpine treated rats developed 2-3 discrete seizures lasting 20-50 seconds each. A small population of animals displayed discrete seizure activity but did not develop overt SE (Sz-NoSE). These animals were separated and used as a No-SE surgery control group as described before (Singleton et al. 2005a).

In animals that developed SE, EEG activity for each animal was analyzed for all SE seizure stages including Wax/Wane (W/W) (Figure 1B), Fast/Slow (F/S) (Figure 1C),

Early Continuous (EC) (Figure 1D) and Fast Spiking with Pauses (FS/P) (Figure 1E), as described (Singleton et al. 2005b). EEG from pilocarpine treated rats, experiencing 10 minutes to over one hour of time spent in SE, was reviewed and the times to the start of each seizure phase listed above were determined. Time to start of each seizure phase was calculated using the initiation of the first discrete seizure as time zero (Singleton et al. 2005b). Times for all rats were averaged together since the time each rat was sacrificed has no direct bearing on what seizure phase was entered. The average time for rats to enter W/W was 6 minutes 23 seconds (± 69 sec). The average time to the start of F/S was 10 minutes 57 seconds (± 79 sec). This average was slightly skewed due to the fact that one rat entered F/S prior to entering the W/W phase and did so only 41 seconds following the start of the first discrete seizure. Removing this data yielded a new average of 12 minutes 45 seconds to the onset of F/S.

Following the Fast/Slow period there was a significant decrease in the numbers of animals reaching later stages of SE. Animals that entered E/C did so in an average of 19 minutes 30 seconds (± 3 min. 5 sec. sec). Further, the animals that entered FS/P did so in an average of 28 minutes 34 seconds (± 4 min. 42sec.). These data show that, as SE seizure activity progresses, there is a positive correlation between length of time in SE and seizure phase attained.

SE-induced Activation of cAMP –dependent Protein Kinase

Maintenance of PKA Activity during SE in cortical and hippocampal homogenate

PKA activity during specific durations of status epilepticus was assessed in whole tissue homogenates using an exogenous substrate (Figure 2). Rats were allowed to seize for predetermined periods of time after which the cortex and hippocampus were isolated,

homogenized and used to test for PKA activity through a substrate phosphorylation procedure (see Materials and Methods).

The basal activity level of PKA in control cortical homogenates was approximately 0.034 pmol PO₄/μg protein (n=9) (Figure 2A). Under maximally stimulated conditions control cortical samples showed a roughly ten-fold increase in PKA activity, 0.30 pmol PO₄/μg protein (n=9) (Figure 2A). To confirm the specificity of the assay for measuring PKA activity we included KT-5720, a specific inhibitor of PKA, into parallel reaction vessels. Using control homogenate samples the inhibitor was added with its final concentration equaling 10 μM. Treatment of these samples produced significant decreases in both basal and maximally treated reactions (data not shown).

SE did not modulate basal PKA activity at any time point studied (Figure 2A). However, SE did result in altered maximal basal activity. Samples from animals experiencing 10 minutes of SE were observed to have roughly the same level of activity as is found in control samples (0.335 pmol PO₄/μg protein (p>0.05, One-way ANOVA)). In 20-minute animals, the maximal PKA activity was approximately 0.441 pmol PO₄/μg protein. While this is an increase of about 37%, it was not statistically significant when compared to control levels (n=8, p>0.05). Later in the progression of SE, maximal levels of PKA activity continue to remain higher than controls with 40-minute sample maximal PKA activity roughly 0.399 pmol PO₄/μg protein (33% increase) (n=6, p>0.05). The level of activity seen in 70-minute samples was observed to be approximately 0.46 pmol PO₄/μg protein, a 53% increase that was significantly different when compared to control samples (n=6, p<0.001). The data were also compared with data from animals that had discrete seizures but did not go into status epilepticus (Sz-NoSE). Sz-NoSE animals had

maximal PKA activity levels of roughly 0.26 pmol PO₄/μg protein, a 14% decrease but not significantly different from control animals (n=6, p>0.05). This is analogous to the previous findings that CaMKII activity does not change in animals displaying discrete seizures but not entering SE (Singleton et al. 2005a).

Hippocampal homogenates were examined for PKA activity as described above (Figure 2B). Basal PKA activity in the hippocampal homogenate samples was 0.006 pmol PO₄/μg protein (n=10). Maximally stimulated activity was approximately 4-fold higher than basal activity (0.021 pmol PO₄/μg protein) and was significantly different when compared to the basal reaction activity levels (n=10, p<0.001). Basal levels of PKA activity were not significantly different between control samples and any of the time points studied (n=39, p>0.05). As was observed in the cortical samples, maximal PKA activity was maintained over the early 10 and 20-minute time points (n=6, 0.022 pmol PO₄/μg protein and n=7, 0.022 pmol PO₄/μg protein, respectively, p>0.05). Samples from animals experiencing 40 and 70 minutes of SE showed slight increases in PKA activity when compared to control animals with 40-minute samples having maximal PKA activity levels around 0.024 pmol PO₄/μg protein and 70-minute samples PKA activity around 0.025 pmol PO₄/μg protein. Neither time point showed a significant change when compared to control samples (n=6 and n=6 respectively, p>0.05). Similar to the data observed for cortical homogenates, hippocampal samples from Sz-NoSE animals showed no significant change in maximal PKA activity when compared to control values (0.016 pmol PO₄/μg protein, n=4, p>0.05).

Immunodetection of PKA levels in cortex and hippocampus homogenate

A possible mechanism to modulate PKA activity can be accomplished through an increased enzyme expression or change in relative expression levels of regulatory subunit versus catalytic subunit. To determine whether the observed increase in PKA activity seen in whole cell homogenates was due to an increase in PKA protein levels or relative decrease in regulatory subunits versus catalytic subunits, western analysis was performed on whole cell homogenates from the cortex and hippocampus utilizing commercially available antibodies towards both the catalytic and regulatory subunits of PKA (see Materials and Methods). Control, 20, 40 and 70 minute samples were examined to determine any changes in protein levels during a time course of SE. When probing for PKA catalytic subunits in cortical homogenate (Figure 3 A, B), the relative density for control samples was 0.46. The 20-minute relative density was 0.36, a 12% change and the 40- and 70-minute relative densities were 0.43 and 0.35 respectively (4.8% and 15% changes $n=4$ each, $p>0.05$). In hippocampal samples (Figure 4 A, B), similar results were obtained. The relative density for control samples was 0.67 and all time points studied had optical densities of between 0.47 and 0.53. While the differences amounted to changes of 20-30% none were significant when compared to either control or each other ($n=4$ each, $p>0.05$). Thus, the net increase in PKA activity could not be explained by an increase in catalytic subunit expression.

To determine if a selective loss of PKA regulatory subunit could explain the apparent increase in activity, total subunit protein levels were assessed. In cortical homogenates (Figure 3 C, D), control samples had an average optical density of 1.54 while 20-minute samples had an optical density of 1.59, 40-minute samples 1.69 and 70-minute samples had an optical density of 1.50. No cortical homogenate samples

displayed a density that was significantly different when compared to either control or each other ($n=16$, $p>0.05$). Probing for PKA regulatory subunits in hippocampal homogenates also displayed results similar to those seen for the catalytic subunits (Figure 4 C, D). Control samples had an average optical density of 2.21 while 20, 40 and 70-minute samples had optical densities of 1.91, 1.85 and 1.70 respectively. These represent decreases of between 14% and 24% but were not statistically significant when compared to control levels or each other ($n=16$, $p>0.05$). Thus, altered catalytic subunit protein expression was not the primary mechanism to increase PKA activity.

Maintenance of PKA activity during SE in cortical and hippocampal brain subcellular fractions

Due to the early maintenance and subsequent increase of PKA activity on the whole cell homogenate level, we wanted to determine if there were any selective changes in PKA activity in the crude synaptic membrane fraction. Subcellular fractions were made from brain homogenates including those for 10, 20, 40 and 70 minute samples post first discrete seizure (See Materials and Methods). Basal levels of PKA activity in the cortical crude SPM samples was approximately $0.004 \text{ pmol PO}_4/\mu\text{g protein}$ (Figure 5A), a significantly lower level when compared to homogenate samples. Control maximal levels of PKA activity were $0.02 \text{ pmol PO}_4/\mu\text{g protein}$, a five-fold increase over the basal samples ($n=6$, $p<0.001$). Again, basal levels of PKA activity for all time points tested were not significantly different when compared to control values ($n=32$, $p>0.05$).

Similar to the observations made in the cortical homogenate, PKA activity levels were maintained during the initial phases of SE progression. Samples from 10-minute

animals had maximal PKA activity levels of 0.018 pmol PO₄/μg protein while 20-minute samples had activity levels of 0.019 pmol PO₄/μg protein. Neither of these were significant changes when compared to control values. As SE progressed into later stages, the crude SPM PKA activity level increased in a manner similar to the homogenate. In 40-minute samples the maximal PKA activity was 0.034 pmol PO₄/μg protein, a 70% increase over the control value (n=6, p<0.05). 70-minute samples had an activity level of 0.031 pmol PO₄/μg protein (n=6, p>0.05) which was a 55% increase over control.

Crude SPM fractions from the hippocampus displayed a similar maintenance of PKA activity throughout the time course of SE (Figure 5B). Basal levels of PKA activity in control hippocampal crude SPM fractions was 0.004 pmol PO₄/μg protein while maximal activity was 0.013 pmol PO₄/μg protein (n=8). Again both levels were significantly lower than those seen in homogenate samples. Basal levels for all experimental time points were not significantly different from the control value. Maximal PKA activity for all time points was not significantly different from the control maximal value. The 10-minute samples had an activity of 0.012 pmol PO₄/μg protein (n=5), 20-minute samples had activity of 0.011 pmol PO₄/μg protein (n=8), 40-minute samples had activity of 0.009 pmol PO₄/μg protein (n=4) and 70-minute samples had maximal PKA activity of 0.011 pmol PO₄/μg protein (n=10). Similar to all other fractions studied, hippocampal crude SPM samples showed a maintenance of PKA activity but without the increase in activity during later stages of SE progression.

Immunodetection of PKA in subcellular fractions

Since PKA affects many substrates found in the synapse and has been shown in this study to have an increased level of activity in subcellular fractions at later stages of

SE, it was important to determine if the increased activity was due to a translocation of kinase to the synaptic region. Western analysis was performed to determine PKA protein levels in the crude SPM fractions at specific time points post first discrete seizure. Control, 20, 40 and 70-minute samples were used to probe for both catalytic and regulatory subunit expression (Figures 6-7). When probing for catalytic subunits, optical density in cortical control crude SPM was 0.69 (Figures 6A, B). Densities for 20 and 70-minute time points were roughly the same as control with values of 0.61 and 0.67 respectively. These changes were only 3% and 12% respectively and were not statistically significant ($p > 0.05$). The 40-minute samples however, had an average optical density of 0.18. This was a decrease of about 75% ($p < 0.05$).

As was the case for homogenate samples, western blots were probed for PKA regulatory subunit expression over the time course of SE progression (figure 6 C, D). While optical density values were observed to be lower than those associated with the homogenate samples the results obtained were very similar to those for the PKA catalytic subunit expression in the same tissue. Control samples had an average optical density of 0.96 which was equaled by the 20-minute animals which also had an average optical density of 0.96. Similar to the PKA catalytic subunit, the optical density for 40-minute samples was observed to decrease by about 47% to 0.51. This was followed by a return near to control levels for the 70-minute samples with an average optical density of 0.82. While there is a large decrease at 40-minutes, none of the time points were statistically different when compared to either control or each other ($n=16$, $p > 0.05$).

Wanting to ensure that these decreases in 40-minute samples were not due to a protein loading error, blots were probed for levels of β -tubulin, a neuronal specific

marker. Average optical density for control samples was 1.23 while those for 20, 40 and 70-minute samples were 1.20, 1.11 and 1.13 respectively. None of these were statistically significant changes when compared to control samples. ($p > 0.05$, one-way ANOVA) This indicates that the loss of PKA regulatory and catalytic subunits seen after 40 minutes of SE represents some form of major cellular change in enzyme expression. Attempts were made to identify a subcellular pool with an increased level of PKA at 40-minutes but no significant changes could be seen. If PKA translocates to the cytosol it is possible that PKA native to the cytosol could mask any increase in PKA from the synapse.

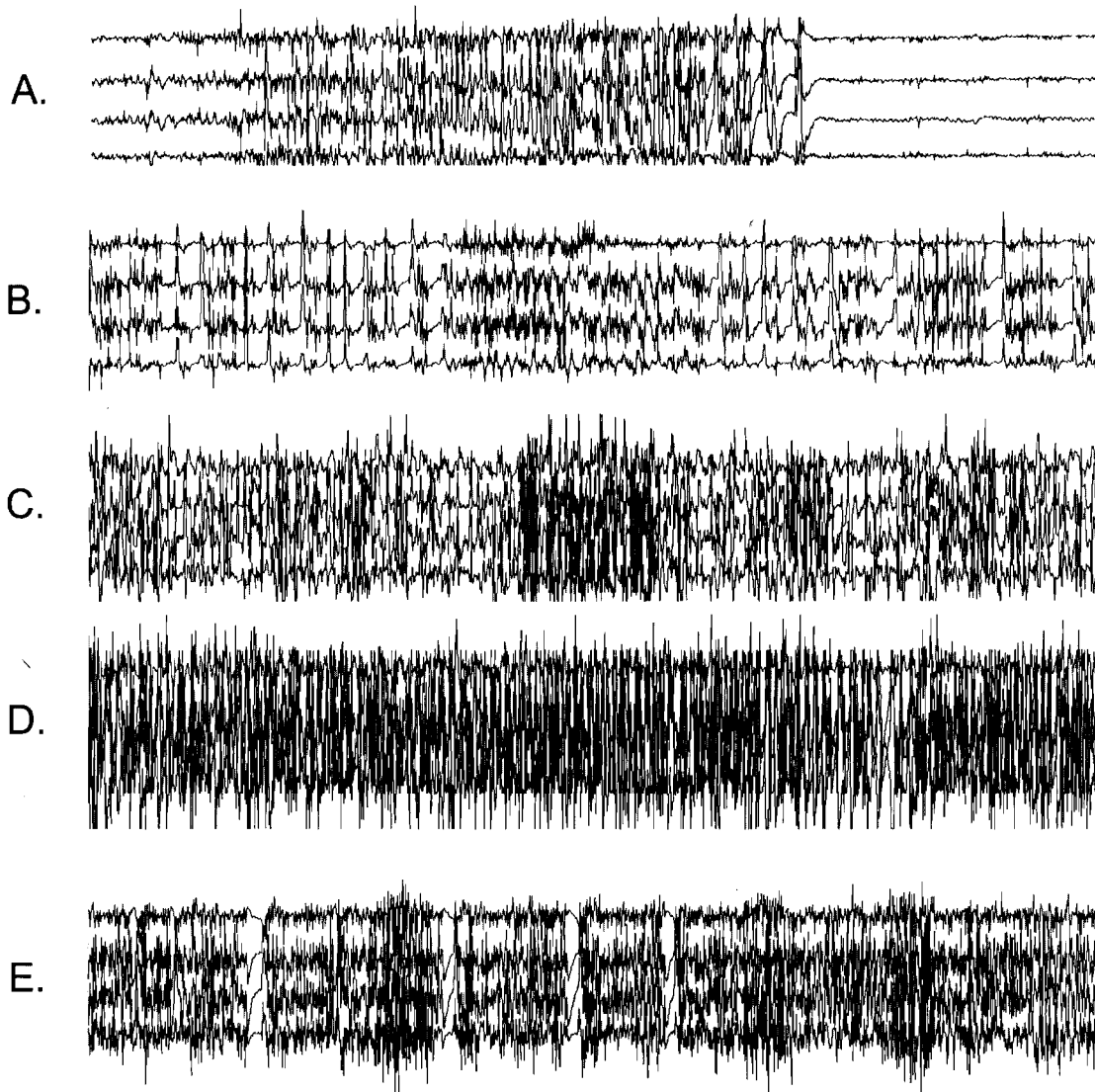
Despite having no significant changes in PKA activity, hippocampal crude SPM samples were probed for PKA catalytic and regulatory subunit expression levels. When probing for catalytic subunits, hippocampal crude SPM fractions displayed results similar to those seen in the homogenate samples (Figure 7 A, B). Average optical densities for control samples and experimental time points were around 1.0. Control samples had a density of 1.06 while 20, 40 and 70-minute samples had densities of 1.08, 0.94 and 1.00, respectively. These differences from control densities represented changes of between 1% and 12% only and were not statistically significant.

When analyzing regulatory subunit expression in the hippocampal fractions, a trend similar to that of the cortical samples was observed (Figure 7 C, D). Control densities were roughly equal to 0.98. As was noticed in the homogenate, a small decrease was observed at 20 minutes with a density of 0.79. A further decrease was seen at 40 minutes to a density of 0.64 while the 70-minute samples had a density of 0.85.

While these differences accounted for changes between 14% and 45%, none were statistically significant when compared to controls (n=16, p>0.05).

Figure 1. Seizure Progression in the Rat Pilo-SE model

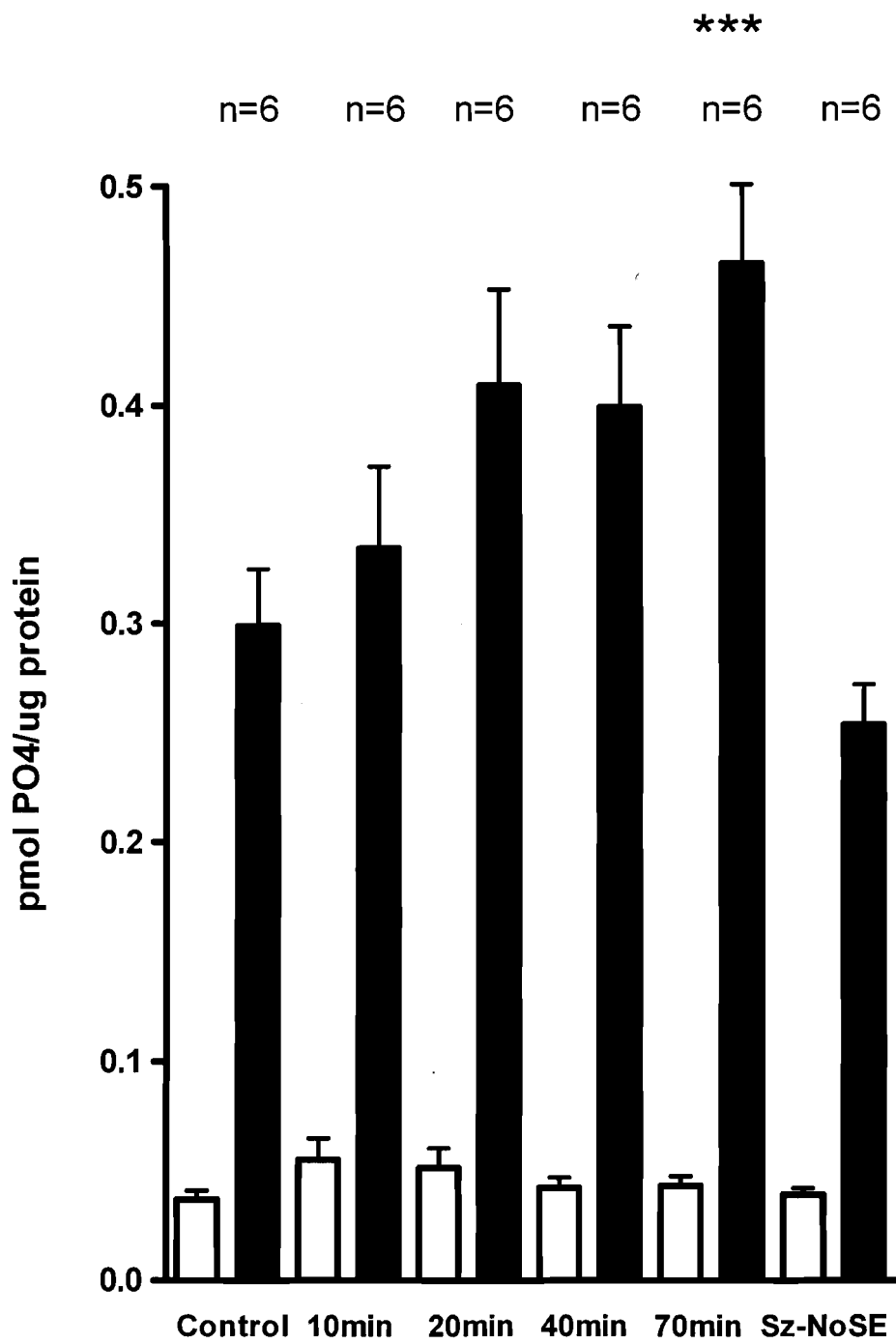
Typical electrographic progression during SE induction. A) Following pilocarpine injection, discrete seizure activity was observed consisting of small increases in amplitude over baseline progressing to higher amplitude, lower frequency activity. This was followed by an abrupt ending. B) Roughly 7 minutes after observation of the first discrete seizure, seizure activity progressed into a wax/wane spike frequency. This phase denoted the onset of SE. C) Following wax/wane rats typically entered a fast/slow phase within 13 minutes of the start of the first discrete seizure. This phase was characterized by high amplitude, high frequency spiking followed by low amplitude, low frequency spiking. D) The next phase, early continuous, typically commenced within 21 minutes and was characterized by continuous high frequency, high amplitude spiking. E) Fast spiking with pauses was the final phase of SE observed in this population of animals and commenced roughly 30 minutes post first discrete seizure.



Seizure progression in the Rat Pilo-SE model

Figure 2A. Prolonged SE activity results in increased PKA activity in cortical homogenates.

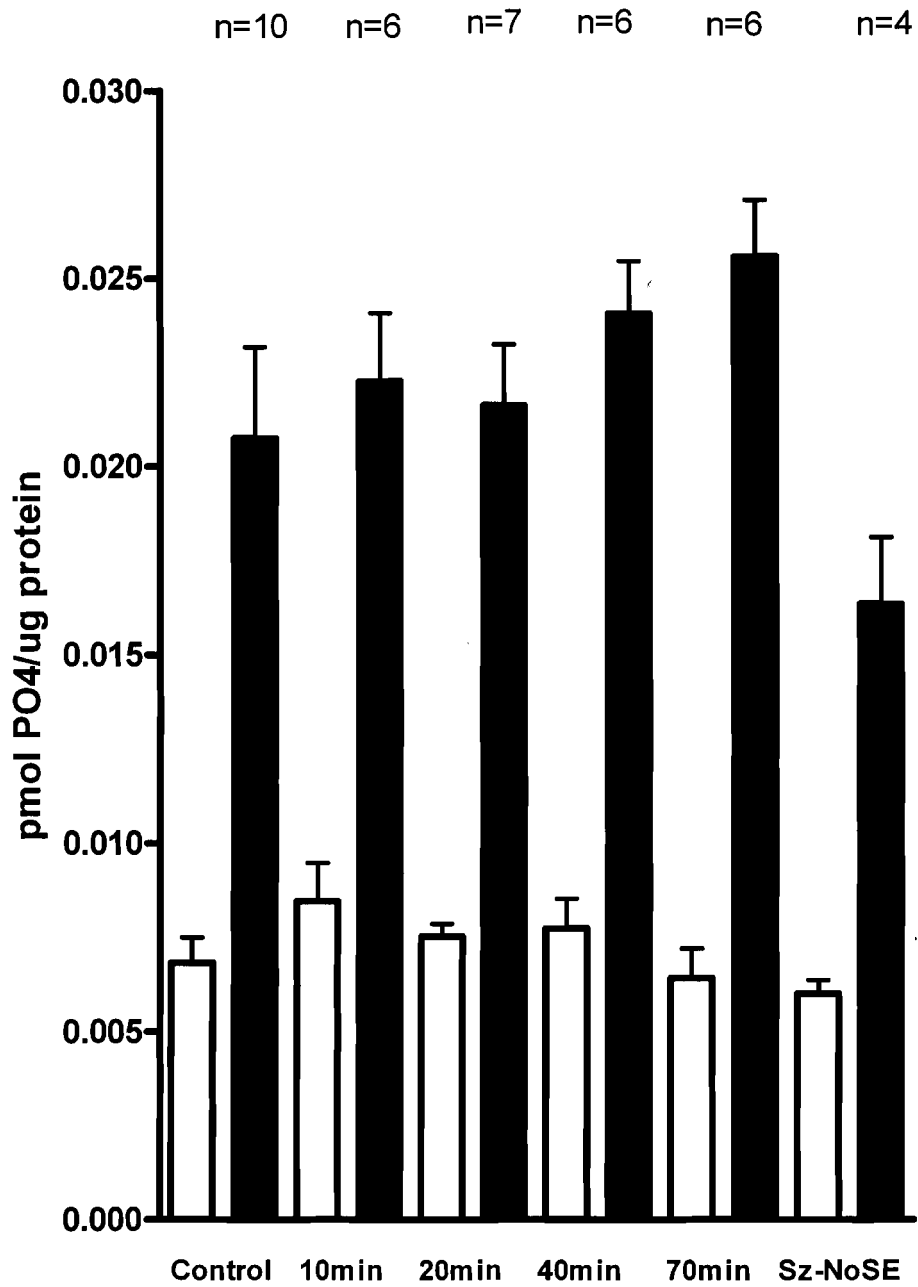
Cortical homogenates were studied for basal (white bars) and maximally stimulated kinase activity (black bars) in control, 10, 20, 40 and 70-minute animals post first discrete seizure. In all samples, a statistically significant cAMP-induced PKA activity was observed compared to basal phosphorylation levels ($p < 0.001$, one-way ANOVA). There was no SE-induced effect on basal PKA activity at any time point studied. Kinase activity in cortical homogenates was not significantly different from control during the early 10 and 20-minute time points of SE. An increasing trend was observed between 20, 40 and 70-minute animals with the 70-minute samples displaying a 53% increase in maximal activity. PKA activities of animals that displayed discrete seizures but did not progress into SE (Sz-NoSE) were also analyzed. (***) denotes $p < 0.001$ compared to control maximal values)



Prolonged SE results in increased PKA activity in cortical homogenates

Figure 2B. Prolonged SE results in maintenance of PKA activity in hippocampal homogenates.

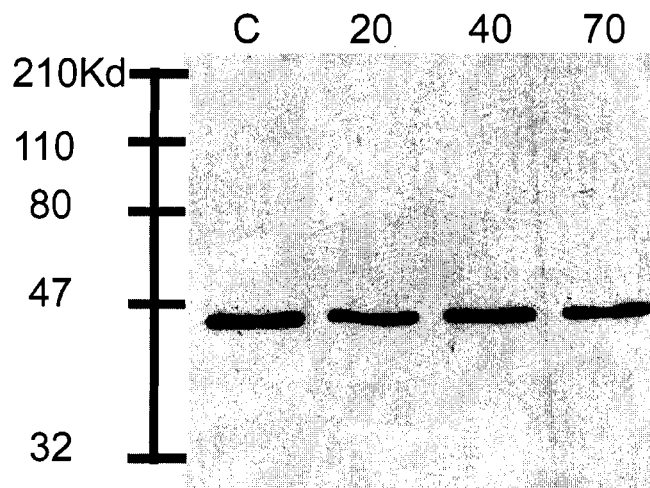
Hippocampal homogenates were studied for basal (white bars) and maximally stimulated kinase activity (black bars) in control, 10, 20, 40 and 70-minute animals post first discrete seizure. In all samples a statistically significant cAMP-induced PKA activity was observed compared to basal phosphorylation levels ($p < 0.001$, one-way ANOVA). There was no SE-induced effect on basal PKA activity at any time point studied. Hippocampal homogenates displayed the same relative trend in increasing PKA activity for maximally stimulated samples, however, none of these changes were significantly different from control activity. PKA activities for animals that displayed discrete seizures but did not progress into SE (Sz-NoSE) were also analyzed.



Prolonged SE results in maintenance of PKA activity in hippocampal homogenates

Figure 3A. SE did not result in an altered PKA catalytic subunit expression in cortical homogenates.

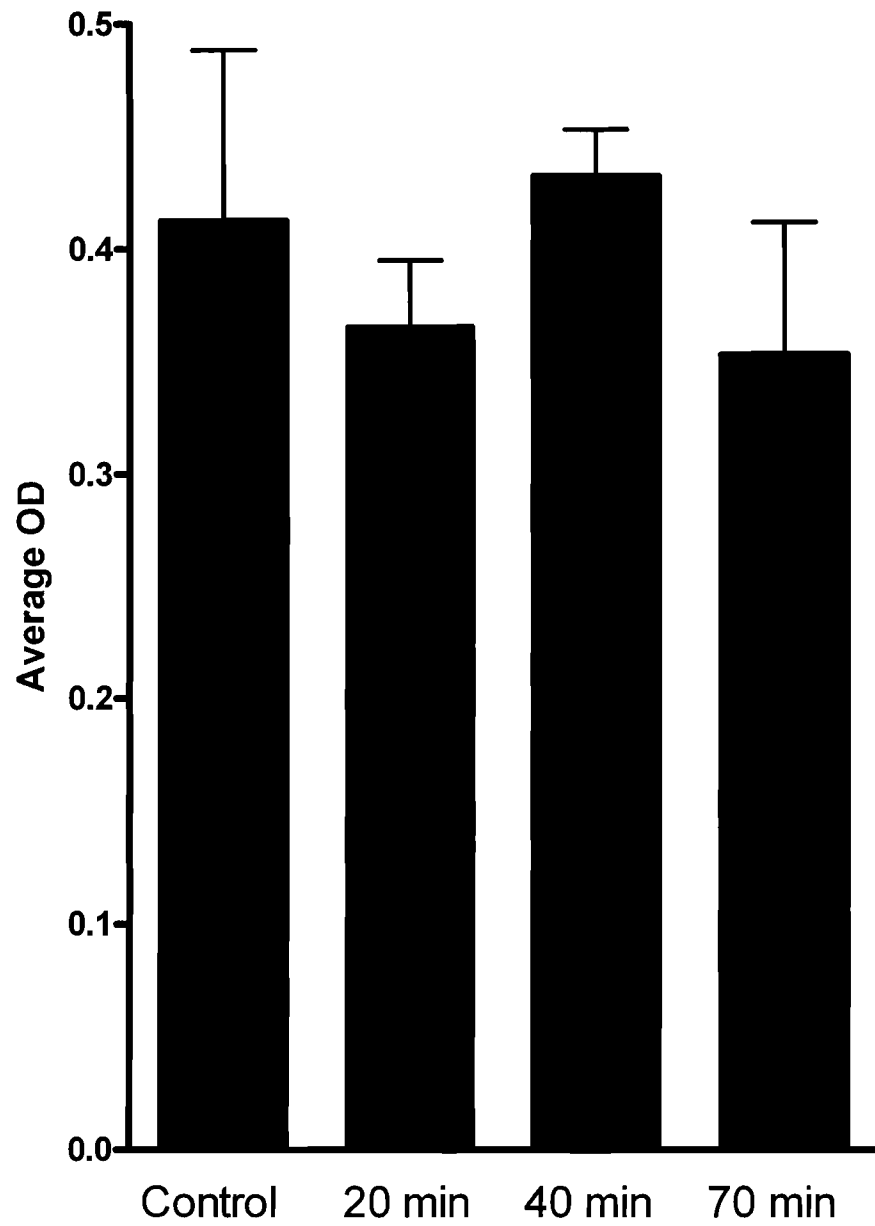
Western blot analysis was used to determine if the SE-induced increase in PKA activity was due to an increase in the catalytic subunit (42kD) expression. Figure 3A shows cortical homogenate samples for control, 20, 40 and 70-minute time points. As the blot indicates, there is no significant difference between control and seizure rats for any time point studied.



SE did not result in an altered PKA catalytic subunit expression in cortical homogenates

Figure 3B. SE did not result in significantly altered PKA catalytic subunit expression in cortical homogenates.

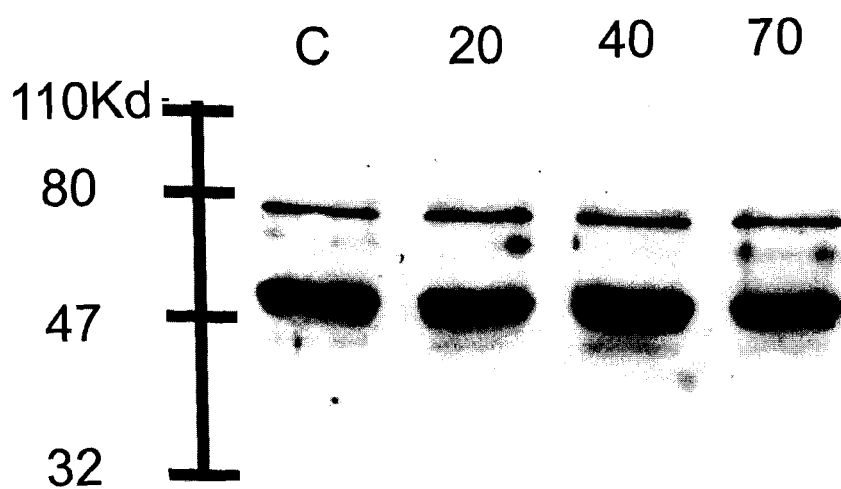
Slight variations between control and SE animals were observed for PKA catalytic subunit expression in cortical homogenates. However, none of these changes were statistically significant when compared to control, nor were they significant when compared to one another (n=4 for all samples). This demonstrates that the increase in PKA activity observed in cortical homogenate is not due to an increase in catalytic subunit expression.



SE did not result in significantly altered PKA catalytic subunit expression in cortical homogenates

Figure 3C. SE did not induce a decrease in PKA regulatory subunit expression in cortical homogenate.

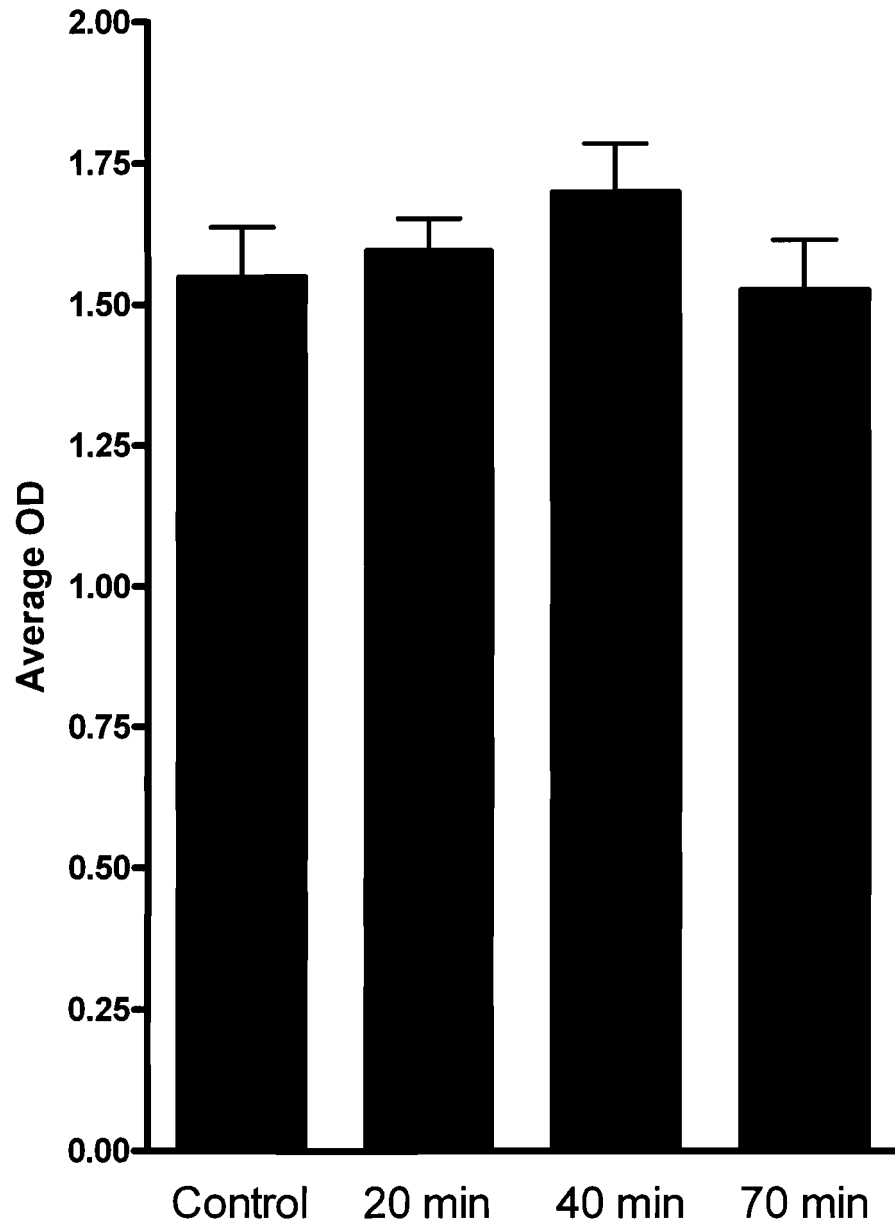
Western blot analysis was used to determine if the SE-induced increase in PKA activity was due to a relative decrease in PKA regulatory subunit (52kD) expression. Cortical homogenate samples for control, 20, 40 and 70-minute animals are shown here. The blot indicates that there is no significant difference in regulatory subunit expression between control and any time point studied.



SE did not induce a decrease in PKA regulatory subunit expression in cortical homogenates

Figure 3D. SE did not result in significantly altered PKA regulatory subunit expression in cortical homogenate.

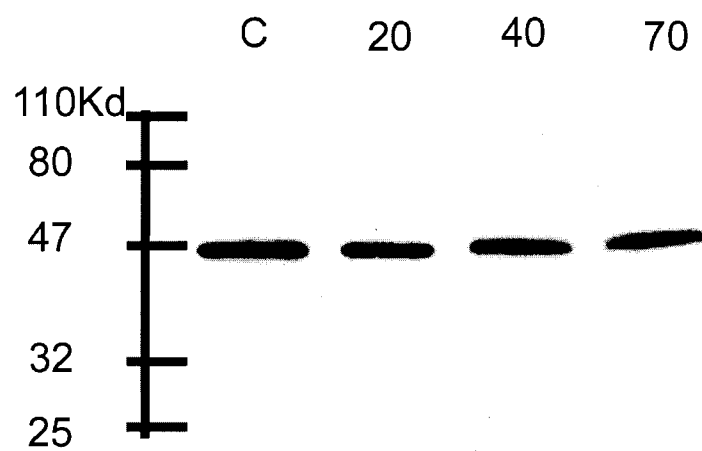
No statistically significant differences in PKA regulatory subunit expression were observed between control samples and any of the time points studied (n=4 for all samples). This indicates that a loss of regulatory subunit expression is not the cause of the increased PKA activity.



SE did not result in significantly altered PKA regulatory subunit expression in cortical homogenate

Figure 4A. SE did not result in altered PKA catalytic subunit expression in hippocampal homogenate.

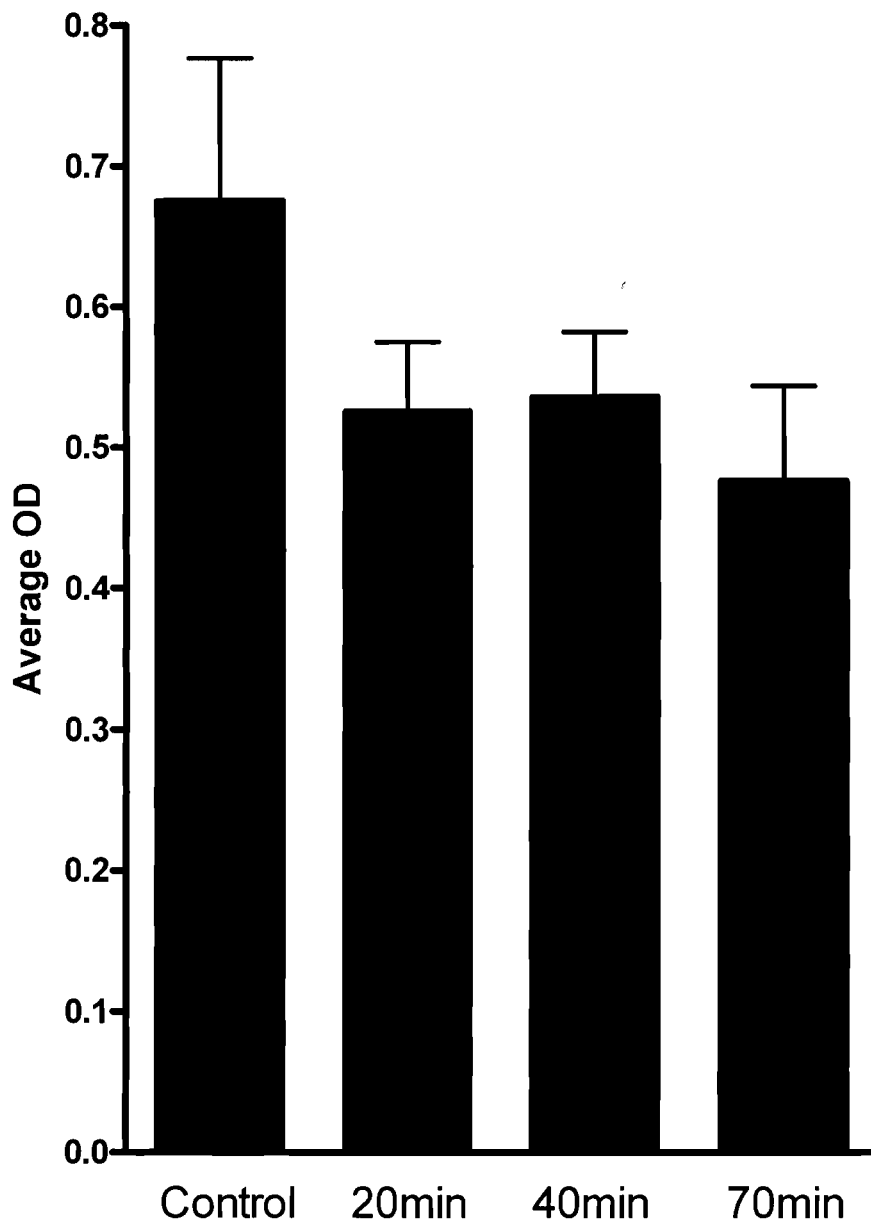
Western blot analysis was used to determine if SE-induced maintenance of PKA activity was due to an increase in PKA catalytic subunit expression. This figure shows hippocampal homogenate samples for control 20, 40 and 70-minutes time points. This blot indicates that there was no significant difference between control and seizure rats for any of the time points studied.



SE did not result in altered PKA catalytic subunit expression in hippocampal homogenate

Figure 4B. SE did not result in significantly altered PKA catalytic subunit expression in hippocampal homogenate.

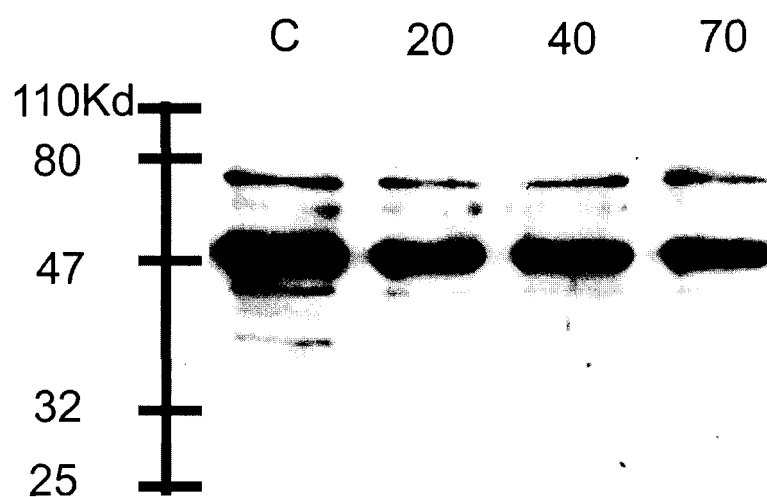
While there is an apparent trend towards decreasing levels of PKA catalytic subunit expression in hippocampal homogenates, there was no statistical significance between control and any time point studied. This shows that, while there was no increase in PKA activity in these samples, there was no significant change in the level of protein expression either. (n=4 for all samples)



SE did not result in significantly altered PKA catalytic subunit expression in hippocampal homogenate

Figure 4C. SE did not result in altered PKA regulatory subunit expression in hippocampal homogenate.

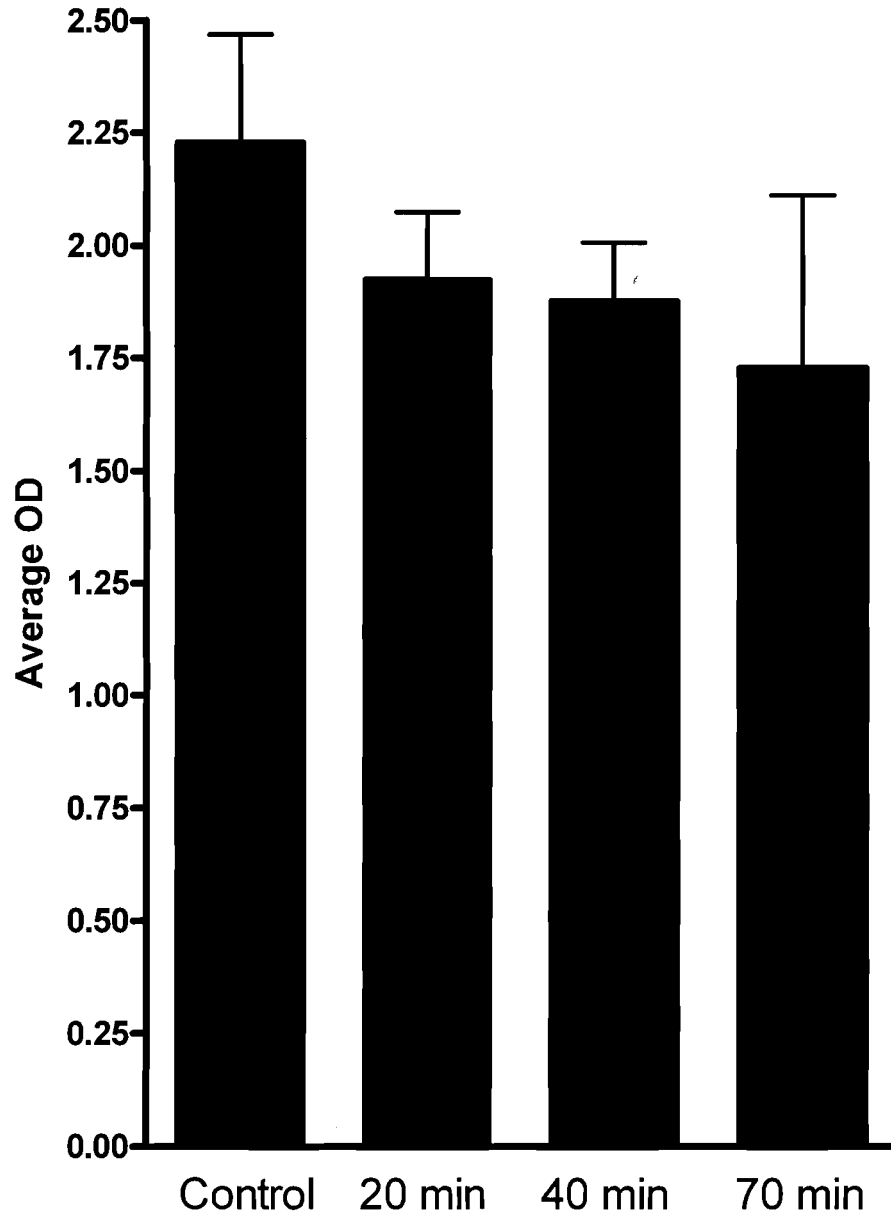
Western blot analysis was used to determine if the maintenance of PKA activity during SE seen in hippocampal homogenate was due to a decreased expression of PKA regulatory subunits. This blot shows hippocampal homogenate samples for control, 20, 40 and 70-minute animals. Probing for PKA regulatory subunits indicates that there is no significant difference between control samples and any time point studied.



SE did not result in significantly altered PKA regulatory subunit expression in hippocampal homogenate

Figure 4D. SE did not result in significantly altered PKA regulatory subunit expression in hippocampal homogenate.

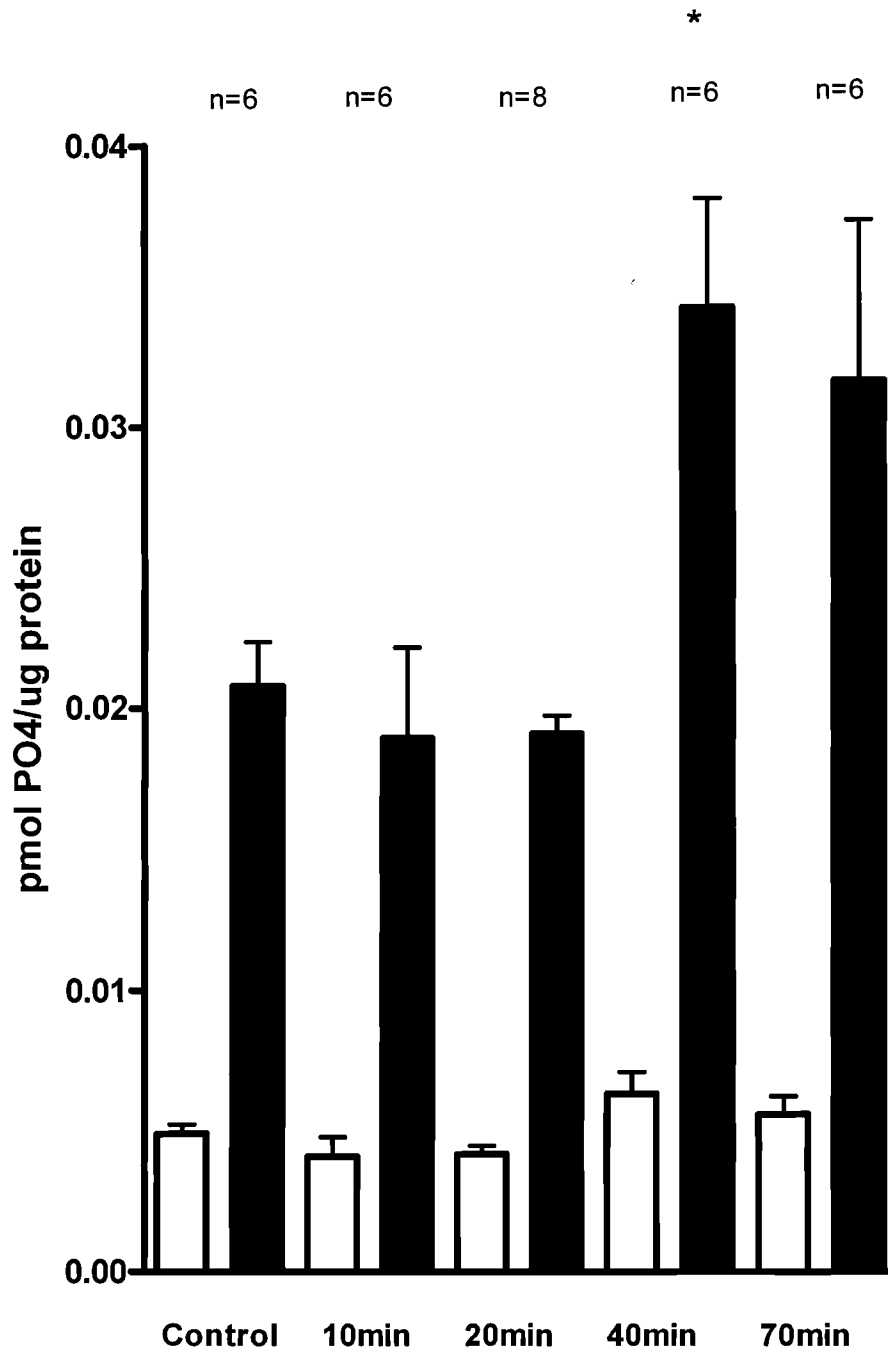
Presented here is a graphical representation of PKA regulatory subunit protein levels represented in the previous western blot. While a trend towards decreasing protein expression is noticed, none of the time points studied displayed a significant difference from control. (n=4 for all samples)



SE did not result in significantly altered PKA regulatory subunit expression in hippocampal homogenate

Figure 5A. Prolonged SE results in increased PKA activity in cortical crude SPM fractions.

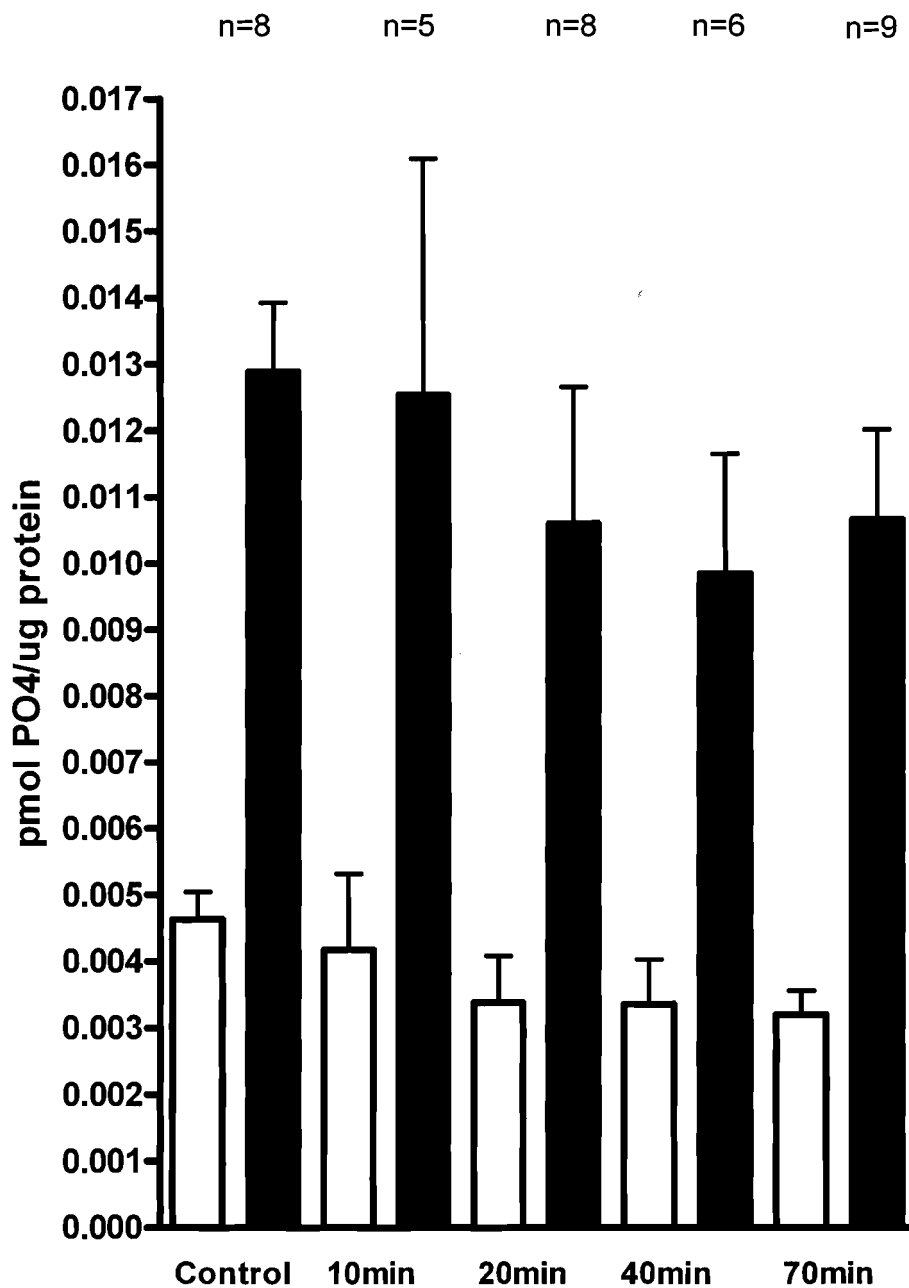
Cortical crude synaptic membrane (crude SPM) fractions were studied for basal levels (white bars) and maximally stimulated (black bars) levels of PKA activity in Control, 10, 20, 40 and 70-minute animals. There was no SE-induced effect on basal activity levels. All samples showed a significant increase in PKA activity between basal and cAMP stimulated samples ($p < 0.001$, one-way, ANOVA). PKA activity was maintained over the early stages of SE in the cortex but displayed a significant increase in activity at 40-minutes (* represents statistical significance of $p < 0.05$ when compared to control maximal values). An increase was also noted at 70-minutes but this was not significant.



Prolonged SE results in increased PKA activity in cortical crude SPM fractions

Figure 5B. SE results in maintenance of PKA activity in hippocampal crude SPM fractions.

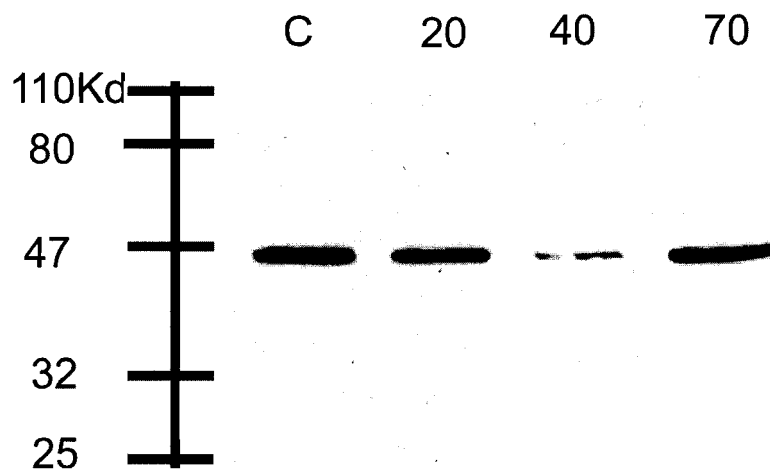
Hippocampal homogenates were studied for basal (white bars) and maximally stimulated kinase activity (black bars) in control, 10, 20, 40 and 70-minute animals post first discrete seizure. In all samples, a statistically significant cAMP-induced PKA activity was observed compared to basal phosphorylation levels ($p < 0.001$, one-way ANOVA). There was no SE-induced effect on basal PKA activity at any time point studied. Hippocampal homogenates displayed the same relative trend in increasing PKA activity for maximally stimulated samples; however, none of these changes were significantly different from control activity.



SE results in maintenance of PKA activity in hippocampal crude SPM fractions

Figure 6A. SE results in differential effects on PKA catalytic subunit expression in cortical crude SPM fractions.

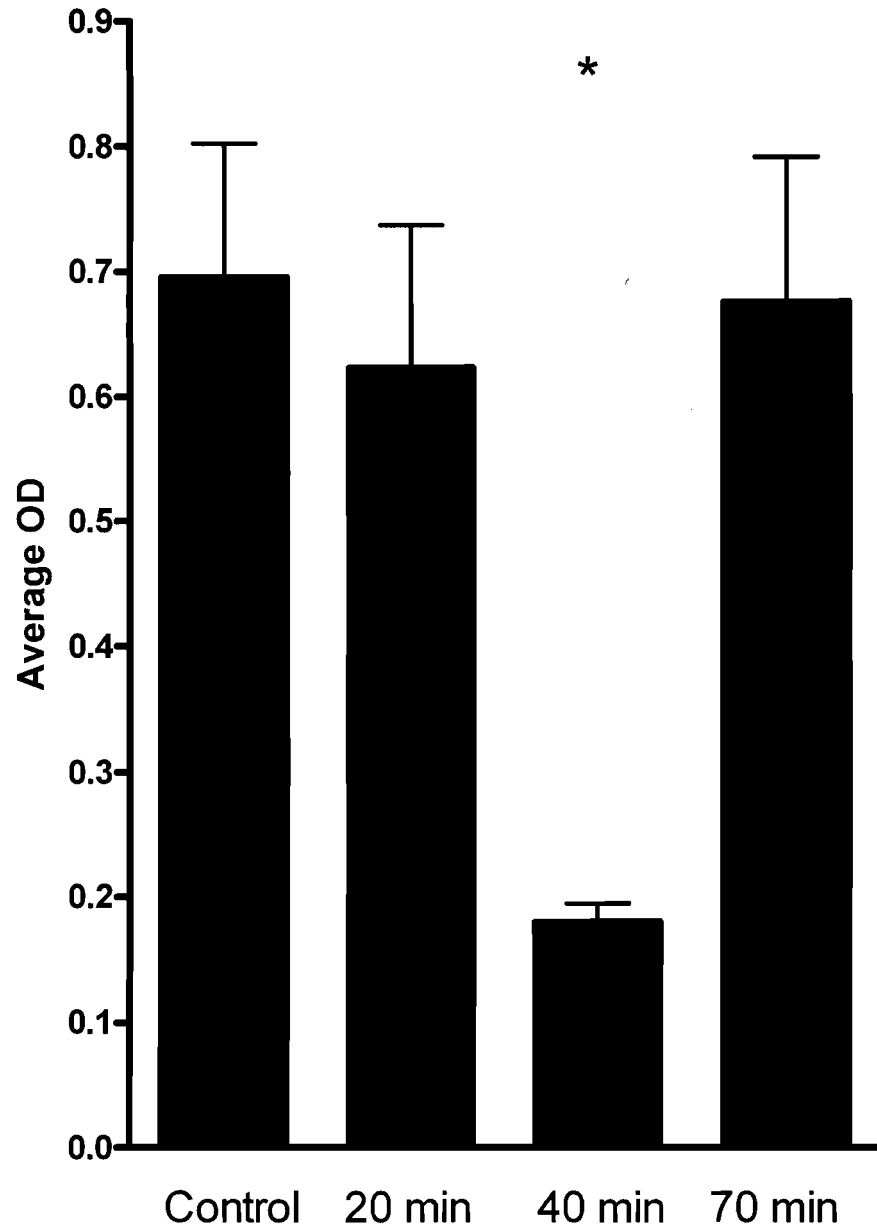
To determine if an increase in PKA catalytic subunit expression was responsible for the SE-induced increase in PKA activity in cortical crude SPM fractions, western blot analysis was performed. This blot shows cortical Crude SPM fractions for control, 20, 40 and 70-minute animals. PKA catalytic subunit expression remains relatively stable in the 20 and 70-minute samples. Interestingly, there is a 75% decrease in catalytic subunit expression at 40-minutes. This decrease in protein expression combined with the observed increase in kinase activity at the same time point indicates that PKA activity is significantly altered during the course of SE.



SE results in differential effects on PKA catalytic subunit expression in cortical crude SPM fractions

Figure 6B. Late stages of SE result in differential effects on PKA catalytic subunit expression in cortical crude SPM fractions.

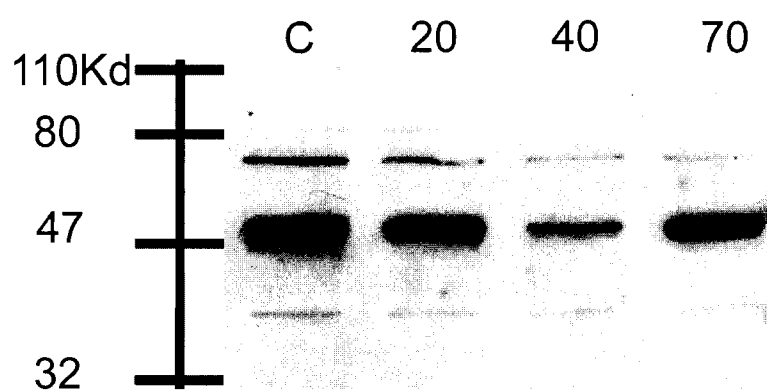
PKA catalytic subunit expression in 20 and 70-minute samples is shown to experience no significant change when compared to control. 40-minute samples, however, show a statistically significant 75% decrease in expression of catalytic subunits (All n=4 and * denotes $p < 0.05$). To ensure that this result was not due to protein loading error, blots were probed for β -tubulin. No differences were noted in β -tubulin expression for any time point studied. This confirms the decreased PKA concentration to be an accurate result. The return to near control values at 70-minutes indicates that there could be some form of PKA translocation away from the synapse at 40-minutes with a subsequent return of protein. Degradation and synthesis of new proteins should not be ruled out however.



Late stages of SE result in differential effects on PKA catalytic subunit expression in cortical crude SPM fractions

Figure 6C. SE results in differential effects of PKA regulatory subunit expression in cortical crude SPM fractions.

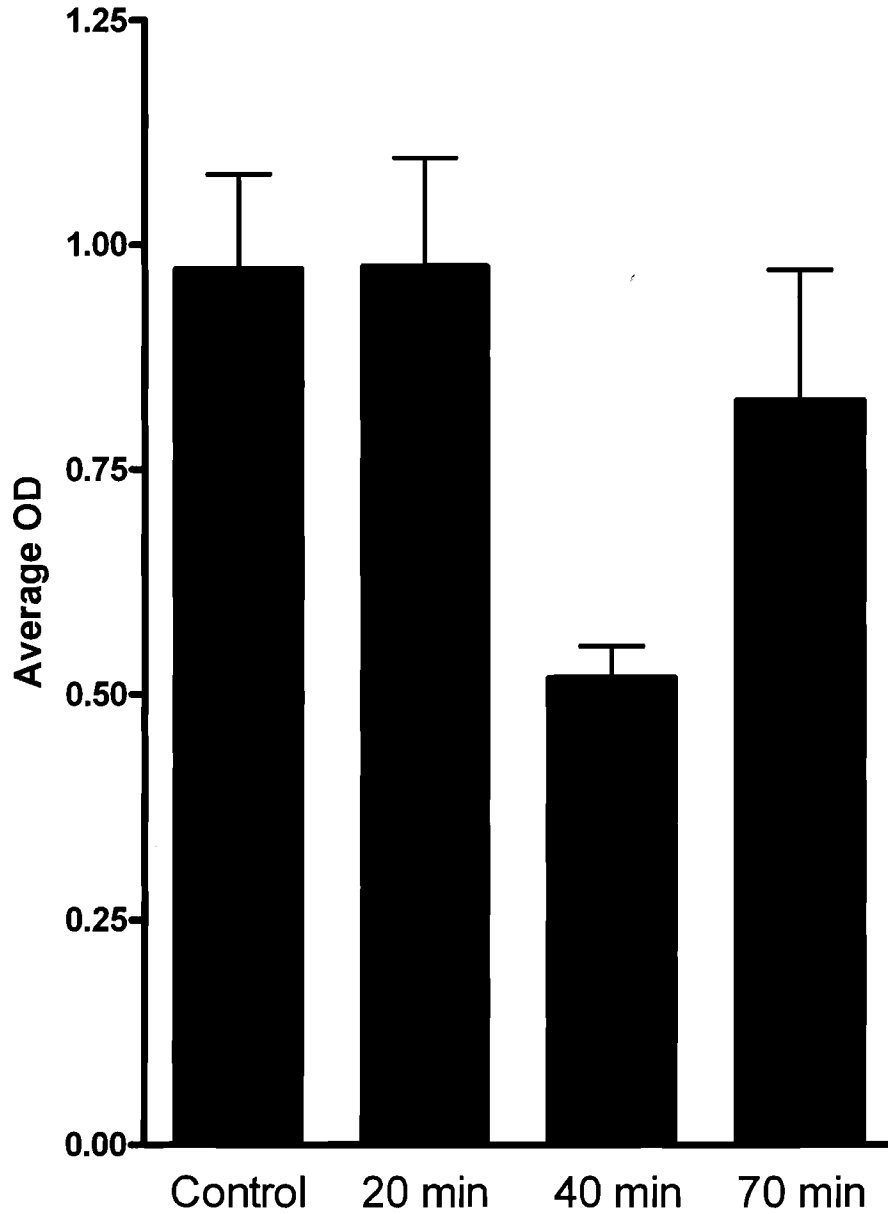
To determine if a decrease in PKA regulatory subunit expression was responsible for the SE-induced increase in PKA activity in cortical crude SPM fractions, western blot analysis was performed. This blot shows cortical Crude SPM fractions for control, 20, 40 and 70-minute animals. PKA catalytic subunit expression remains relatively stable in the 20 and 70-minute samples. Similar to the results seen for catalytic subunit expression, there is a 45% decrease in regulatory subunit expression at 40-minutes.



SE results in differential effects on PKA regulatory subunit expression in cortical crude SPM fractions

Figure 6D. Late stages of SE result in differential effects on PKA regulatory subunit expression in cortical crude SPM fractions.

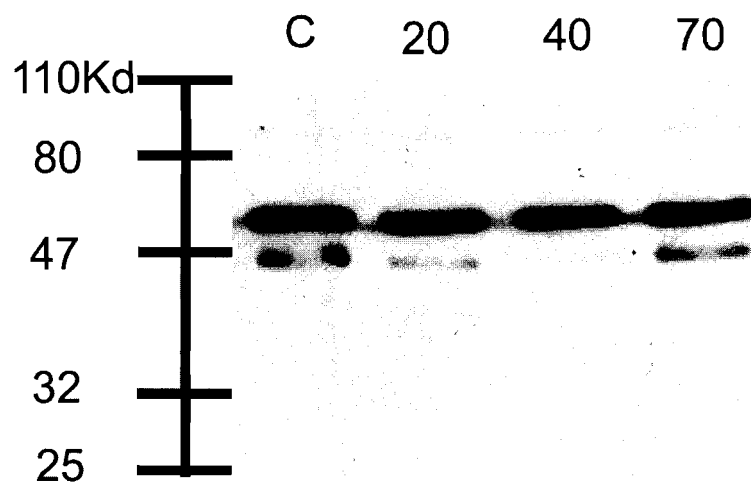
This figure shows that PKA regulatory subunit expression is maintained, compared to control, in 20 and 70-minute samples. Similar to the results seen for catalytic subunits, PKA regulatory subunit expression is observed to decrease at 40-minutes. This decrease (45%) is not significant when compared to control and is smaller than the decrease associated with catalytic subunits. Here too, the results suggest either a translocation of PKA away from the synapse with a subsequent return or the possibility of protein degradation and then synthesis.



Late stages of SE result in differential effects on PKA regulatory subunit expression in cortical crude SPM fractions

Figure 7A. Prolonged SE does not result in altered PKA catalytic subunit expression in hippocampal crude SPM fractions.

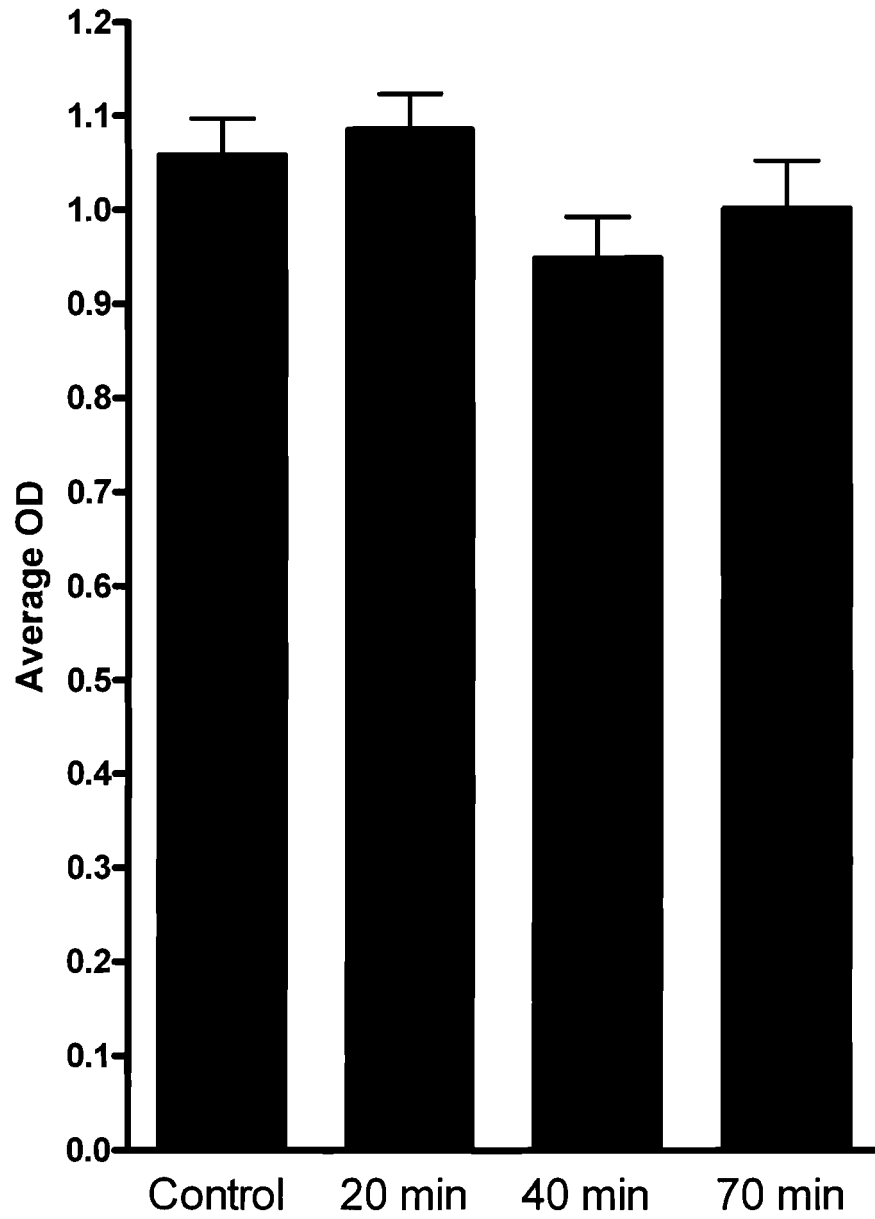
To determine if any alterations in PKA catalytic subunit expression were observed in hippocampal crude SPM fractions, western blot analysis was performed. Samples from control, 20, 40 and 70 minute samples showed no significant differences in levels of catalytic subunit expression when compared between one another.



Prolonged SE does not result in altered PKA catalytic subunit expression in hippocampal crude SPM fractions

Figure 7B. Prolonged SE does not result in significantly altered PKA catalytic subunit expression in hippocampal crude SPM fractions.

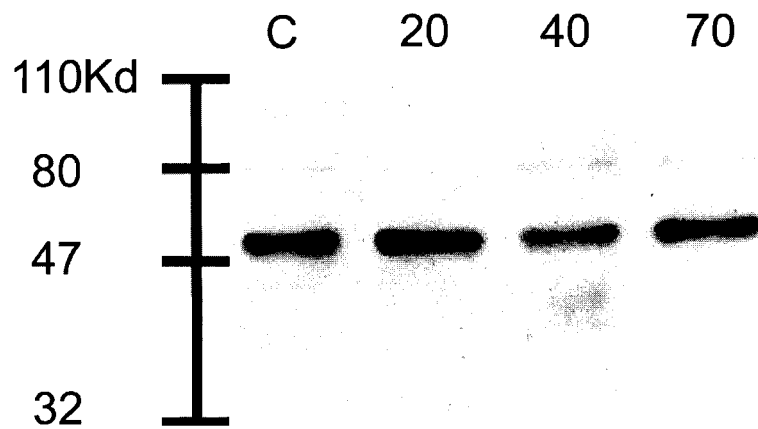
While there were some minor variations in PKA catalytic subunit expression in cortical crude SPM between control samples and each time point tested, none were significant. This data mirrors the findings that PKA activity is maintained but does not increase in these fractions.



Prolonged SE does not result in altered PKA catalytic subunit expression in hippocampal crude SPM fractions

Figure 7C. SE does not result in altered PKA regulatory subunit expression in hippocampal crude SPM fractions.

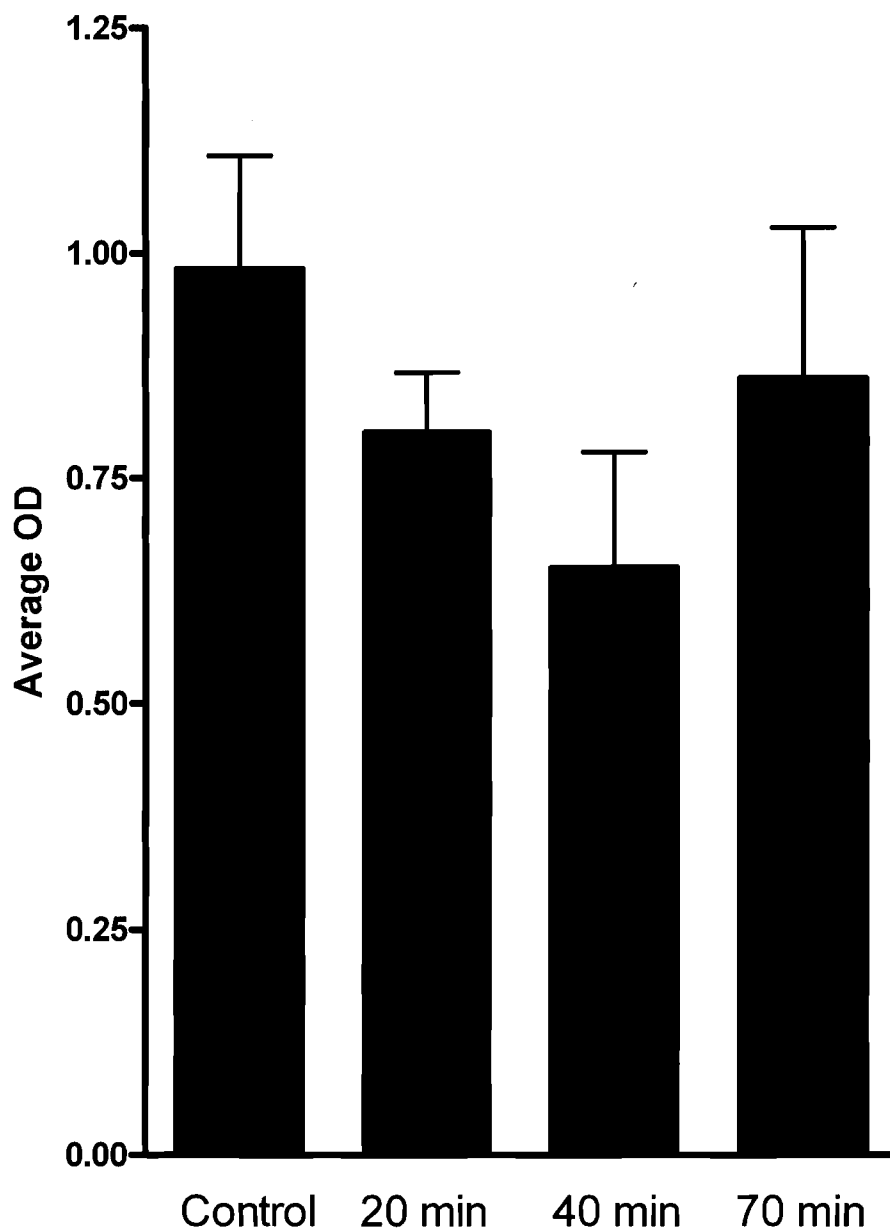
Western blot analysis was used to determine if any changes in expression of PKA regulatory subunits occurred in hippocampal crude SPM during SE. Comparing samples from control, 20, 40 and 70-minute animals indicated that there was no significant change in protein expression.



SE does not result in altered PKA regulatory subunit expression in hippocampal crude SPM fractions

Figure 7D. SE does not result in significantly altered PKA regulatory subunit expression in hippocampal crude SPM fractions.

There was some variation in expression of PKA regulatory subunits in hippocampal crude SPM fractions. However, no statistically significant differences were noted between control samples and samples from any time point studied. This data indicates that during the progression of SE, PKA expression is not significantly altered in the hippocampus.



SE does not result in significantly altered PKA regulatory subunit expression in hippocampal crude SPM fractions

DISCUSSION

Through electrographic mapping of seizure progression in the Pilo-SE model this study demonstrated an increase in PKA activity without an increase in total PKA protein. This suggests some form of post-translational modification occurring during the progression of SE which results in a dramatic increase in kinase activity. The results also showed a delayed increase in synaptic PKA activity over the progression of SE. This increase in activity was also not associated with an increase in protein concentration. Since prolonged seizure activity observed in SE has been shown to induce spontaneous recurrent seizures (SRS), the increase in PKA activity associated with late stages of SE could play a role in the development of these recurrent epileptic seizures. These observations suggest that these changes could lead to more permanent changes associated with seizure disorders.

One possible outcome from the cellular changes seen in SE is an imbalance in the regulation of membrane excitability. It has been shown previously that total CaMKII activity decreases significantly coincident with the onset of SE (Singleton et al. 2005a). In addition there is an early maintenance of PKA activity during SE with a subsequent increase in activity at later stages. This results in a change in regulation of a variety of cellular proteins. For example, CaMKII has been shown to positively regulate the function of GABA_A receptors causing increased inhibitory currents (Wang et al. 1995), and increased agonist (Churn and DeLorenzo 1998) and allosteric modulator binding (Churn et al. 2002). At the same time, PKA has been shown to inhibit GABA_A receptor channel function depending on the subunit composition (Kapur and Macdonald 1996). Together, these changes in CaMKII and PKA activities would result in a decreased

GABAergic inhibitory input thus creating an imbalance the regulation of membrane excitability. This data is mirrored by the findings that there is a complete loss of benzodiazepine efficacy during SE which act primarily on GABAergic receptors. Another outcome of the differential regulation of CaMKII and PKA is in the function of cyclic-AMP response element binding protein (CREB). Both PKA and CaMKII have been shown to phosphorylate CREB at Ser-133 (Shaywitz and Greenberg 1999). This activates the protein allowing for entry into the nucleus to induce gene transcription. At the same time CaMKII has been shown to phosphorylate an additional site on CREB, Ser-142, resulting in the inhibition of the enzyme (Dash et al. 1991; Sheng et al. 1991; Sun et al. 1994). Thus, CaMKII and PKA play antagonistic roles in the function of this protein and the subsequent synthesis of new proteins. The observed decrease in total CaMKII activity and increased PKA activity seen in SE could result in the net activation of CREB, thereby allowing for gene transcription and new protein synthesis. Support for this could lie in the apparent translocation of PKA away from the synapse seen after 40 minutes of SE. It is possible that PKA leaves the synapse and moves out into the cytoplasm where the majority of CREB is located. Further studies will be required to determine if there is an increased level of CREB phosphorylation and if a detectable increase in PKA protein is noticed in other subcellular fractions at this time.

An alternative hypothesis is that the cellular changes seen in the Pilo-SE model represent a pathological extension of long-term potentiation (LTP). Formation of LTP has been shown to be dependent on the actions of CaMKII and PKA. CaMKII activation has been shown to be required for the induction of early stages of LTP (Barria et al. 1997; Chen et al. 2001; Lisman et al. 2002; Soderling and Derkach 2000). This increase in

activity results in an increased phosphorylation levels of α -amino-3-hydroxy-5-methyl-4-isoxazoleproppionate (AMPA) receptors causing an increase in receptor function. This leads to altered membrane excitability and plasticity changes. Other factors are required to make the plasticity changes and alterations in membrane excitability more permanent. Progression into later stages of LTP has been shown to be dependent on an increase in PKA activity (Duffy and Nguyen 2003; Huang et al. 2000; Matsushita et al. 2001). This progression into late-LTP involves, among other events, an increase in neurotransmitter release (Kuromi and Kidokoro 2000; Thakur et al. 2004), increased ion channel insertion (Crump et al. 2001; Esteban et al. 2003), increased ion channel conductance (Meuth et al. 2002) and new protein synthesis (Kang and Schuman 1996; Krug et al. 1984; Otani et al. 1989). This shows that through multiple tetanic stimulations synapses can be selectively potentiated and progress from a state of learning to one of memory consolidation.

It is possible that the multiple discrete seizures and subsequent prolonged seizure activity in the Pilo-SE model represent a pathological extension of synaptic LTP. In this analogy, the expression of discrete seizure activity would act like whole-brain tetanic stimulation. This would result in increased CaMKII activity and induce prolonged seizure activity. Thus resulting in increased PKA activity leading to maintenance of a prolonged increase in membrane excitability. CaMKII activity in the synapse has been shown to increase coincident with the onset of SE (Singleton et al. 2005a). As seizure activity progresses, we have observed a significant increase in synaptic and total PKA activity. These discrete seizures could represent the tetanic stimuli that are required for the formation of LTP in synapses and single cells. However, instead of potentiating a

single cell or synapse, discrete seizures would potentiate all of the synapses of a given brain region.

If these changes result in the potentiation of thousands of synapses across whole brain regions, it could lead to the formation of a whole brain LTP-like event. This would result in the synchronization of the synapses and result in the formation of seizures. SE has been shown to be an ideal model for use in inducing epileptogenesis (Rice and DeLorenzo 1998; Turski 2000). Epileptogenesis is the process by which individuals develop spontaneous recurrent seizures (SRSs). Since SRSs have been shown to occur following SE and the data here show that changes associated with the formation of LTP also occur during SE, there is a possibility that SE results in the formation of a whole brain LTP-like event. The progression from early stages of SE to late stages with accompanying cellular changes could cement alterations in membrane excitability across the entire brain. This would result in an increased occurrence of spontaneous seizures referred to as epilepsy.

An additional point of interest is the relationship between the changes in PKA activity and the progression of the seizure profile seen in the Pilo-SE model. While PKA activity is maintained during the early phases of SE, PKA activity increases roughly coincident with the onset of the FS/P phase of SE. This indicates that a large-scale PKA activity change could either push the seizure progression into later stages or prevent SE activity from reverting back to earlier, less pathological phases. To further analyze this relationship, PKA activity should be studied in those animals that do not enter the later seizure profile stages of SE. It has been shown that young rats (postnatal day 30) are much less likely to enter late phases of SE primarily progressing only to the EC phase

(Holbert II et al. 2004). In addition, younger animals have also been shown to be less likely to develop spontaneous recurrent seizures following SE than older animals (Sankar et al. 2000). If no increase in PKA activity after 40 minutes of SE is noticed in these animals, it could indicate that the increase in PKA activity seen in adult animals may have a causative effect on the progression through late stages of SE. This would provide further evidence that SE treatments targeted towards PKA could have a positive impact on reducing seizure severity and possibly reduce the occurrence of spontaneous recurrent seizures.

The findings in this paper characterize both the EEG and phosphorylation studies of a time dependent SE-induced increase in PKA activity. During early periods of SE, when total CaMKII activity is observed to decrease, PKA activity is maintained. Later in the progression of SE, PKA activity is observed to increase significantly. This indicates that PKA could provide a neuronal marker for the development of later, more pathological stages of SE and possibly give an indication as to susceptibility of epileptogenesis. In addition to the increase in PKA activity seen in both the homogenate and crude SPM samples, there is no associated increase in PKA protein levels. This indicates that the increase in PKA activity is the result of some yet to be determined post-translational modification mechanism.

DISCUSSION

PKA changes associated with late stages of status epilepticus

This study was performed to examine the relationship between PKA activity and the development of status epilepticus (SE). Previously our lab has shown that coincident with the onset of SE, there is an immediate decrease in CaMKII activity on a whole cell level (Singleton et al. 2005a). Because it has been shown that CaMKII positively modulates the function of GABAergic receptor function (Churn and DeLorenzo 1998; Churn et al. 2002; Wang et al. 1995), a decrease in CaMKII activity may result in decreased inhibitory neuronal signaling. At the same time, it is known that PKA can also cause the inhibition of GABAergic receptors depending on subunit composition (Kapur and Macdonald 1996; Tehrani and Barnes 1995). Taken together, loss of CaMKII activity and either maintenance or an increase in PKA activity would result in the alteration of normal neuronal membrane excitability as is seen in seizure states. It was our hypothesis that during SE PKA activity is maintained to offset the decrease in total CaMKII activity thus resulting in altered regulation of membrane excitability. The results from this study indicate that not only is PKA activity maintained during the early stages of SE, there is an appreciable increase in PKA activity during later stages of SE progression. This interesting finding could be significant for a variety of reasons including a possible link between the developmental progression of SE and the induction of stages of long-term potentiation (LTP).

This study showed that in the rat pilocarpine model of SE, PKA activity is maintained during approximately the first 20-30 minutes of seizure activity. Following this time there is a significant increase in the level of PKA activity in cortical whole

tissue homogenates. Hippocampal samples showed a similar trend but did not reach significance in total PKA activity. Western blot analysis of cortex and hippocampal homogenates revealed no significant change in PKA subunit expression (either catalytic or regulatory) in any of the time points studied. This indicates that synthesis of new PKA protein is not responsible for the increase in kinase activity.

PKA activity assays in cortical crude synaptic fractions displayed results similar to those seen in the homogenate samples. Cortical crude SPM activity was observed to remain at control levels during the initial stages of SE. After 40-minutes of seizure activity, however, PKA activity is shown to increase significantly. Western analysis of crude synaptic fractions revealed slightly different results than those seen in the homogenate. PKA protein concentration for both catalytic and regulatory subunits remained relatively constant for early periods of SE. After 40 minutes of seizure activity, however, there is a significant decrease in protein concentration in the cortical synaptic fractions. This decrease appears transient due to the return of PKA protein levels to near control values after 70 minutes of seizure activity. PKA activity and protein concentration in hippocampal samples were maintained at near control levels for all time points studied. The data indicate that late stages of SE result in increased activation of PKA through some form of post-translational modification mechanism and also suggest that there is some level of PKA translocation away from the synapse. Combined with the fact that CaMKII activity decreases at the onset of SE, these data indicate that the cellular changes observed during SE could lead to improper regulation of membrane excitability.

LTP – SE relationship

Another hypothesis for the cellular changes associated with SE involves the formation of long-term potentiation. Extensive research has shown that CaMKII and PKA activation are required for the formation of different stages of LTP (Huang and Kandel 1994; Wikstrom et al. 2003; Yang et al. 2004). In early stages of SE, CaMKII is responsible for the positive modulation of AMPA receptor channels. Activated via Ca^{2+} influx through N-methyl-D-aspartate receptor channels (NMDA), CaMKII phosphorylates AMPA receptors resulting in increased channel function and the induction of LTP (Derkach et al. 1999; Soderling and Derkach 2000). Formation of later stages of LTP has been shown to be dependent primarily on new protein synthesis which is accomplished through a PKA dependent mechanism (Frey et al. 1988; Nguyen et al. 1994; Woo et al. 2002). PKA works to maintain early stages of LTP through reversible phosphorylation of membrane ion channels and triggers synthesis of new proteins through phosphorylation of transcription factors such as cyclic-AMP response element binding protein (CREB). This leads to the transcription of genes and the eventual translation of mRNA into proteins. This has been shown to be especially prevalent in dendritic spines during late-LTP, although the mechanism and identity of translated mRNAs is still largely unresolved (Frey et al. 1988; Nguyen et al. 1994; Tsokas et al. 2005; Woo and Nguyen 2003). The combination of the increased CaMKII and PKA activities results in the strengthening of synaptic signaling. This results in synapses converting from a state of learning to one of memory consolidation.

In light of the fact that both CaMKII and PKA show an increase in synaptic activity during the formation of LTP as well as in the Pilo-SE model, it makes one wonder if there is a relationship between the two and if SE could be a pathological

extension of LTP. High frequency stimulations have been shown to induce LTP through the activation of CaMKII in neurons (Fukunaga et al. 2002; Fukunaga et al. 1995; Havik et al. 2003). In an analogous manner, CaMKII activity is observed to increase in synaptic fractions coincident with the onset of SE. SE is typically preceded by a series of 2-3 discrete seizures. These seizures are brief, 10-50 seconds, and contain high amplitude spiking (Singleton et al. 2005b). When repeated, these discrete seizures could be acting to stimulate CaMKII activation on a whole-brain level in the same manner as high frequency stimulation of synapses in LTP paradigms. Interestingly animals that experience discrete seizures, but fail to enter into SE do not display a change in either CaMKII or PKA activity. This could suggest that either a certain severity of discrete seizure is required for the increase in CaMKII activity or that CaMKII activity increases as a result of the transformation from discrete seizures to the continuous seizure activity of SE. In addition this study shows that PKA activity increases after prolonged seizure activity and after the increase in CaMKII activity subsides. This increase in PKA activity could lead to the synthesis of new proteins required for the induction of later stages and maintenance of LTP. SE has been shown to be an accurate model to induce epileptogenesis (Rice and DeLorenzo 1998; Turski 2000). Due to this, the occurrence of discrete seizures followed by an increase in CaMKII and PKA activities could result in the transformation of SE into a potentiated seizure condition, epilepsy, in an LTP-like manner.

Possible cellular changes associated with SE

During SE there are many changes occurring within neurons producing a myriad of cellular alterations. Included in these are changes in the activity levels of several

protein kinases and phosphatases, notably, CaMKII, PKA and calcineurin (CaN). Coincident with the onset of SE, total CaMKII activity is observed to decrease while synaptic activity increases before decreasing over time (Singleton et al. 2005a). This can result in a variety of changes in the cell. CaMKII has been shown to phosphorylate AMPA receptors at the Ser-831 site. This causes a positive regulation of ion influx through these channels resulting in increased inward Na^+ and Ca^{2+} currents. Activation of CaMKII has also recently been shown to transiently phosphorylate the translation factor cytoplasmic polyadenylation element binding protein (CPEB) (Atkins et al. 2005). This results in new protein synthesis including new dendritic spine formation. If CaMKII activation is not sustained however, this activation of CPEB is lost after a short time (Atkins et al. 2005). In addition, CaMKII has been shown to phosphorylate CREB (primarily associated with PKA) at two sites and is traditionally thought to inhibit CREB function (Dash et al. 1991; Sheng et al. 1991; Sun et al. 1994). Loss of CaMKII activity would disinhibit CREB thus allowing it to take over for the now inactivated CPEB. Since loss of CaMKII is seen during the progression of SE, membrane excitability and new protein synthesis must be regulated in other ways. An increase in PKA activity seen at later stages of SE is a plausible option.

PKA has been shown to cause an increase in protein synthesis and increased membrane excitability in a variety of ways. First, PKA's main path to protein synthesis is through the phosphorylation of CREB. This allows CREB to enter the nucleus, bind CRE sites, and initiate gene transcription. PKA can also affect the CaMKII activation of CPEB, by maintaining its level of phosphorylation. Normally CPEB is dephosphorylated by protein phosphatase 1 (PP1). PKA acts to inhibit PP1 through the activation of

dopamine and cAMP-regulated phosphoprotein -32 (DARPP-32) and inhibitor 1. Activation of this family of proteins causes the inhibition of PP1 resulting in a maintenance of phosphorylation of CPEB. This mechanism is also responsible for maintaining the activation of AMPA receptors regulated through phosphorylation by CaMKII (Vinade and Dosemeci, 2000). These two events result in an increase in ion conductance as well as new protein synthesis.

In addition to the SE-induced changes in CaMKII and PKA activity, there are significant changes observed in protein phosphatase 2B or calcineurin (CaN). CaN has been associated with the modulation of function of both GABA and NMDA receptors (Huang and Dillon 1998; Krupp et al. 2002; Lu et al. 2000; Umemiya et al. 2001) and is also involved in regulating the release of neurotransmitters (Cordeiro et al. 2000), gene transcription (Clipstone and Crabtree 1992; Jain et al. 1993) and cytoskeletal architecture (Drewes et al. 1993; Goto et al. 1985; Mandelkow et al. 1995). Roughly coincident with the increase in PKA activity, CaN activity is observed to increase in both basal and maximal reactions (Kurz et al. 2001). This increase has several effects within the cell. First, CaN causes the activation of cofilin which leads to actin depolymerization in dendritic spines (Wang et al. 2005). This is often seen in neuronal degeneration associated with traumas such as seizures. In addition, CaN acts to reduce AMPA receptor ion conductance through the disinhibition of PP1. This is in direct contrast to the action of PKA on PP1. Since seizures are observed to continue after this 30-40 minute time point, PKA activity could overpower that of CaN and cause the continued excitation of glutamatergic receptor function. CaN also plays a role in the regulation of new protein synthesis. CaN is able to inactivate CREB through the disinhibition of PP1,

however, this only occurs following weakly stimulated synapses and cells (Hagiwara et al. 1992; King et al. 1984). It has also been reported that inhibition of CaN results in the enhancement of learning and memory and LTP (Malleret et al. 2001). This indicates that the increase in CaN activity seen during SE progression could be an attempt to reduce the long term effects of the seizures which are possibly caused through a PKA dependent mechanism.

Regulation of PKA activity

Regulation of PKA activity through NMDA receptor mediated Ca^{2+} entry is complex. Ca^{2+} can cause the activation of adenylate cyclases resulting in the formation of cAMP which leads to the activation of PKA. At the same time, this influx of Ca^{2+} can lead to the activation of calmodulin dependent phosphodiesterases (PDEs) which leads to a decrease in cAMP levels. This phenomenon is counterbalanced through the activation of CaMKII which inhibits the PDEs (Hashimoto et al. 1989). Since CaMKII activity is observed to decrease early in SE progression it is unlikely that this mechanism is what maintains PKA activity during the later stages of SE. Further reports suggest that PKA can be autoregulated. Activation of PKA can lead to phosphorylation of the regulatory subunits by the catalytic subunits thereby reducing their binding affinity (First et al. 1988). Attempts were made during this study to look at the levels of PKA regulatory subunit phosphorylation, however, no reliable results were ever obtained. Additionally, it has been shown that there is selective degradation of PKA regulatory subunits through ubiquitination (Chain et al. 1999; Chain et al. 1995; Hegde et al. 1993; Hegde et al. 1997). This results in the reduction of the regulatory to catalytic subunit ratio which would lead to prolonged activation. This is plausible considering the decrease in PKA

regulatory subunit expression seen in the crude SPM after 40-minutes of SE, but does not explain the return to control levels at 70-minutes. Further studies are required to determine if the PKA protein is translocating away from the synapse then back between 40 and 70-minutes or, alternatively, if the protein is being degraded by some mechanism and then resynthesized.

The cellular changes listed here are but a few of the many effects that these kinases and phosphatases have on the cell at any given time. Further research is required to fully elucidate the relationship between all of these enzymes and the impact that the progression of SE has on each.

Future Areas of Research

To date there has been little research that focuses on the role PKA plays in the progression of SE and its affect on the formation of spontaneous recurrent seizures. This study focuses on a temporal relationship between SE progression and PKA activity in adult animals. The data suggest that the significant increase in PKA activity could result in further progression through SE and that PKA may play a role in the formation of more permanent seizure states. In light of this, there are several additional areas of interest for future study.

The PKA activity assays used in this study employed an exogenous substrate. To characterize more thoroughly the effects of the increased PKA activity, a variety of endogenous substrates could be observed for changes in phosphorylation level during the progression of SE. For example, new protein synthesis has been shown to be required for the induction and maintenance of late-LTP (Huang et al. 2000). This is a PKA dependent process acting through cyclic-AMP response element binding protein (CREB). An

increase in the level of phospho-CREB at late stages of SE would provide further evidence that the increase in PKA activity is playing a role in the long-term maintenance of a pathological extension of LTP. Phospho-CREB specific antibodies are commercially available to facilitate this study. At the same time, there are a variety of ion channels that are directly or indirectly regulated by PKA. AMPA channels are known to be phosphorylated by CaMKII at Ser-831. This level of phosphorylation is regulated by the action of protein phosphatase 1 (PP1) which is activated through a disinhibition by calcinuerin. PKA regulates this process by phosphorylating Inhibitor 1/DARPP-32 family members which inhibits the activity of PP1 (Vinade and Dosemeci 2000). SE could produce an increased phospho-inhibitor 1 or phospho-DARPP-32 level which would lead to maintained ion currents through AMPA channels and this sustained excitability and altered membrane potential. This mechanism would also cause the maintenance of CPEB activation, a CaMKII dependent transcription factor, which is inactivated via dephosphorylation by PP1.

Additionally, there are amino acid residues on both AMPA and NMDA channels reported to be specific for interaction with PKA. On AMPA channels, Ser-845 is a PKA specific site. Phosphorylation at this site causes, by some accounts, increased receptor insertion and increased anchoring of the receptor to the postsynaptic density (Vinade and Dosemeci 2000). It would be of interest to determine if, during the course of SE, there is an increased phosphorylation level of AMPA receptors at the PKA specific site. This would give a further causative mechanism for the increased and continued membrane excitability associated with prolonged seizures. Furthermore, PKA has been shown to increase insertion and clustering of NMDA channels (Crump et al. 2001; Tingley et al.

1997). Similar to AMPA channels, analysis of phosphorylation levels of NMDA channels at PKA specific sites during SE could shed further light on the mechanism behind the imbalanced regulation of neuronal membrane excitability. Again phospho-AMPA and phospho-NMDA specific antibodies are available for this purpose.

Conclusions

This study shows that prolonged episodes of status epilepticus result in an increase in protein kinase A activity without the synthesis of significant amounts of new PKA protein. This indicates that during SE some form of post-translational modification mechanism is occurring that results in the increased activity. This increase in PKA activity is subsequent to a decrease in total CaMKII activity and initial increase in synaptic CaMKII activity. Decreased total CaMKII activity combined with increased PKA activity can lead to an imbalance in membrane excitability regulation as is seen in seizure states. An early increase in synaptic CaMKII activity combined with a subsequent PKA activity increase in the Pilo-SE model indicates that progression through SE could be acting as a pathological extension of LTP. These results support the hypothesis that PKA is involved in the progression of SE and may play a role in the process of epileptogenesis.

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VITA

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