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Research Report

Prolonged seizure activity leads to increased Protein Kinase A activation in the rat pilocarpine model of status epilepticus

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ABSTRACT

Status epilepticus is a life-threatening form of seizure activity that represents a major medical emergency associated with significant morbidity and mortality. Protein Kinase A is an important regulator of synaptic strength that may play an important role in the development of status epilepticus-induced neuronal pathology. This study demonstrated an increase in PKA activity against exogenous and endogenous substrates during later stages of SE. As SE progressed, a significant increase in PKA-mediated phosphorylation of an exogenous peptide substrate was demonstrated in cortical structures. The increased activity was not due to altered expression of either regulatory or catalytic subunits of the enzyme. Through the use of phospho-specific antibodies, this study also investigated the effects of SE on the phosphorylation of the GluR1 subunit of the AMPA subtype of glutamate receptor. After the onset of continuous seizure activity, an increase in phosphorylation of the PKA site on the GluR1 subunit of the AMPA receptor was observed. These data suggest a potential mechanism by which SE may increase neuronal excitability in the cortex, potentially leading to maintenance of seizure activity or long-term neuronal pathology.

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1. Introduction

Seizure disorders are one of the most common neurological conditions worldwide. Among these patients are some that develop sustained seizure activity that does not self-terminate. This condition, known as status epilepticus (SE), is associated with significant morbidity and mortality, and requires immediate aggressive treatment (Chen and Wasterlain, 2006). As the duration of continuous seizure activity increases, the probability that the seizures will terminate without treatment decreases, as does the efficacy of many commonly used anti-epileptic therapies (DeLorenzo et al., 1999; Loscher, 2007). Additionally, increased duration of SE is associated with a higher risk of developing long-term neuronal pathology, including cognitive deficits and new-onset epilepsies (Holmes, 2002; Young, 2006). These observations suggest pathologic alterations in neuronal physiology that develop as SE progresses. A better understanding of these processes is essential, both for the development of more effective therapeutic options and for the insight it may provide into neuronal plasticity and epileptogenesis.

As SE progresses, pathologic activation of pre-existing cellular mechanisms that regulate synaptic strength, leading to an imbalance between excitatory and inhibitory neuronal

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inputs, may underlie the transition to self-sustaining seizure activity. One such mechanism involves the cyclic-AMP dependent Protein Kinase A (PKA). PKA is a ubiquitously expressed member of the serine–threonine kinase family and is known to modulate a multitude of cellular processes, many of which are involved in the regulation of neuronal excitability. PKA has been associated with mediation of short-term synaptic facilitation via reversible phosphorylation of ion channels such as its regulation of NMDA and AMPA receptors (Greengard et al., 1991). For example, PKA-mediated phosphorylation of AMPA-type glutamate receptors at serine-845 increases the open probability of the receptor channel and regulates receptor trafficking to the synapse (Carvalho et al., 2000).

Epileptogenesis and other long-term pathologic sequelae of SE suggest a persistent modulation of synaptic strength occurs in addition to the acute maintenance of seizure activity. Interestingly, PKA is also involved in such persistent synaptic modulation; for example, the coordinated activity of CaM kinase II and PKA is essential for the conversion of early to late phase long-term potentiation (LTP) (Blitzer et al., 1998; Huang and Kandel, 1994; Massicotte and Baudry, 2004; Smolen et al., 2006) and PKA also may play a role in LTP maintenance through modulation of gene expression and protein synthesis (Huang et al., 2000; Waltereit and Weller, 2003). The involvement of PKA in both acute pro-excitatory mechanisms and long-term modulation of synaptic strength make this system an intriguing target for study in the pathophysiology of SE.

The present study investigated the effects of SE on PKA activity in both whole cell homogenate and crude synaptoplasmic membrane fractions isolated from both hippocampus and cortex. Through the use of both a PKA-specific peptide substrate assay for PKA activity, and the use of phospho-specific antibodies directed against the PKA site of the GluR1 AMPA subunit, we demonstrated an SE-dependent increase in PKA activity against both exogenous and endogenous substrates. This increased kinase activity was not due to altered expression of either regulatory or catalytic subunits of the enzyme. These data suggest a mechanism by which SE may increase neuronal excitability in the cortex through increased receptor phosphorylation. The data present a cellular signaling system that may be involved in the maintenance of seizure activity and long-term changes in neuronal physiology due to SE.

2. Results

2.1. *SE results in increased homogenate PKA activity against exogenous substrate*

PKA activity was assessed in whole tissue homogenates using an exogenous substrate (Fig. 1). SE onset and duration were monitored electrographically as described previously (Singleton et al. 2005). At predetermined time-points the cortex and hippocampus were isolated, homogenized and assayed for PKA activity through a substrate phosphorylation procedure (see Experimental procedures).

The basal activity level of PKA in control cortical homogenates was 0.037 ± 0.004 pmol PO_4/μg protein (n = 6). Under maximally stimulated conditions, control cortical samples showed a roughly ten-fold increase in PKA activity, 0.300 ± 0.026 pmol PO_4/μg protein (n = 6). To confirm the specificity of the assay for measuring PKA activity we included KT-5720 (10 μM), a specific inhibitor of PKA, in parallel reaction vessels. Treatment of these samples produced significant decreases in both basal and maximally treated reactions (data not shown).

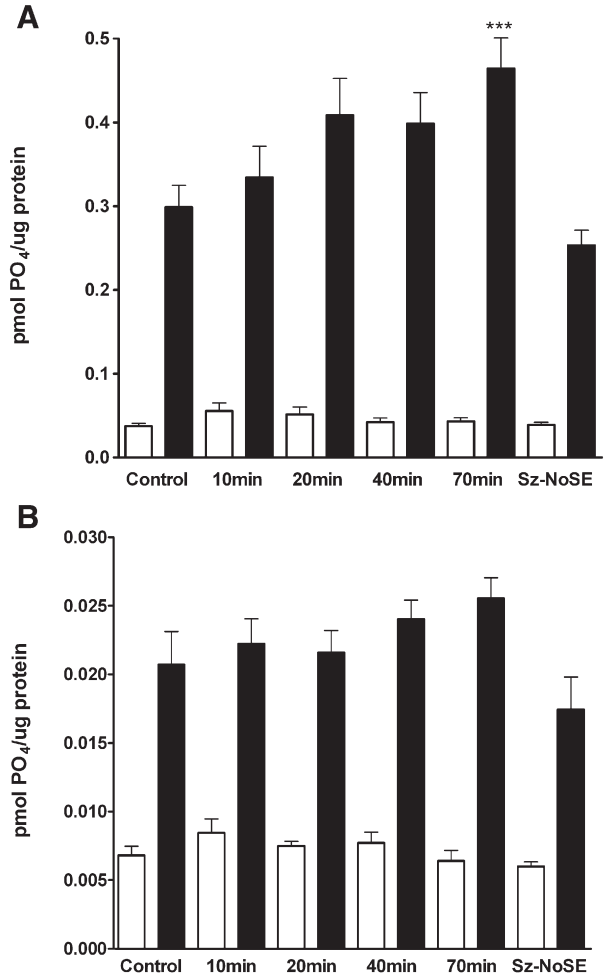

Fig. 1 – Prolonged SE activity results in increased PKA activity in cortical homogenates. Cortical (A) and hippocampal (B) homogenates were assayed for basal (white bars) and maximally stimulated kinase activity (black bars) in control, 10, 20, 40 and 70-minute animals post-first discrete seizure. In all cortical and hippocampal samples, a statistically significant cAMP-induced PKA activity was observed compared to basal phosphorylation levels (n = 6 for each group, p < 0.001, one-way ANOVA). (A) There was no SE-induced effect on basal cortical PKA activity at any time-point studied. Maximal kinase activity in cortical homogenates was not significantly different from control at the 10 and 20-minute post-first discrete seizure time-points; however, at 70 min a 53% increase in maximal activity was observed (*p < 0.001). (B) No significant difference from control in either basal or maximal PKA activity was observed in hippocampal homogenate at any time-point studied (p > 0.05).**

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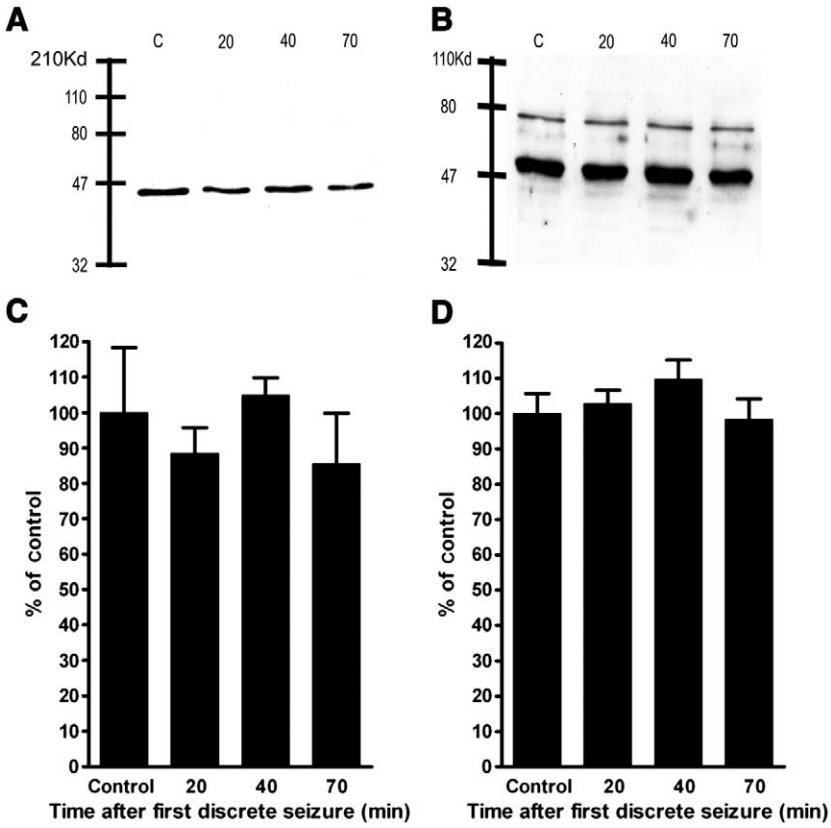

Fig. 2 – SE did not result in altered PKA catalytic or regulatory subunit expression in cortical homogenates. Western blot analysis was used to determine if the SE-induced increase in PKA activity observed in cortical homogenate was due to an alteration of PKA protein expression of either the catalytic subunit (42kD) or the regulatory subunit (52 KD). Representative blots are shown of PKA catalytic subunit (A) or regulatory subunit (B) expression in cortical homogenates from control animals and from fractions isolated 20, 40 and 70 min post-first discrete seizure. Immunoreactivity was quantified by computer-assisted densitometry for catalytic (C) and regulatory (D) subunits. No statistically significant differences were observed between control and 20, 40 and 70-minute time-points for either subunit, nor were significant differences observed when time-points were compared to one another ($n=4$ at each time-point).

SE did not alter the basal level of substrate phosphorylation at any time-point studied. However, prolonged seizure activity in SE did result in a duration-dependent increase in maximal (cAMP-dependent) substrate phosphorylation. While PKA activity trended higher than control values in animals experiencing as little as 20 min of continuous seizure activity, this difference did not become statistically significant until 70 min after the first discrete seizure. The level of activity seen in 70-minute samples was 0.464 ± 0.036 pmol PO_4/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 homogenate from animals that experienced discrete seizures, but not status epilepticus, isolated 70 min after the first discrete seizure. These animals had maximal PKA activity levels that were statistically indistinguishable from controls ($n=6$, $p>0.05$), indicating that the increased PKA activity was likely due to prolonged seizure activity and not a nonspecific effect of pilocarpine administration.

Hippocampal homogenates were examined for PKA activity as described above. Interestingly, and in contrast to the increase in kinase activity observed in the cortex, PKA activity was not significantly affected by seizure activity at any time-point studied. Additionally, both basal and cAMP-stimulated hippocampal PKA activities were significantly lower per mass of homogenized protein than the PKA activity in cortical homogenate. The basal PKA activity in control hippocampal homogenates was 0.007 ± 0.001 pmol PO_4/μg protein ($n=6$). Under maximal reaction conditions, control hippocampal samples showed an increase in PKA activity to 0.021 ± 0.002 pmol PO_4/μg protein ($n=6$). No significant difference from control ($p>0.05$) was observed in hippocampal homogenates at any duration of SE (Fig. 1B).

2.2. *SE does not acutely alter the expression of PKA subunits*

One possible mechanism that would account for the observed increase in PKA-dependent phosphorylation is a change in the relative expression of the regulatory subunit to the catalytic subunit. To determine whether the observed increase in PKA activity seen in whole cell homogenates was due to a relative decrease in regulatory subunit expression relative to that of the catalytic subunit, western analysis was performed on whole cell homogenates from the cortex and hippocampus

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using commercially available antibodies towards the catalytic and regulatory subunits of PKA (see Experimental procedures). Control, 20, 40 and 70-minute samples were examined to determine changes in protein levels during a time-course of SE. Expression of the catalytic subunit did not differ significantly from control at any of the time-points studied in cortical and hippocampal samples ($p>0.05$ for all points, $n=4$). Thus, the increased PKA activity observed as SE progressed could not be explained by a significant increase in catalytic subunit expression (Figs. 2A, C).

To determine if a selective loss of PKA regulatory subunit could explain the apparent increase in activity, immunoreactivity towards the regulatory subunit was assessed through western analysis. No cortical or hippocampal homogenates from any time-point displayed regulatory subunit immunoreactivity that was significantly different from controls or each other (Figs. 2B, D, $n=4$ at each time-point, $p>0.05$). The relative expression ratio of the catalytic and regulatory subunits of PKA in homogenates was not altered following various durations of SE and thus could not account for the increase in the observed PKA activity.

2.3. *SE causes an increase in PKA in cortical crude synaptosomal fractions*

In light of the SE-induced increase in PKA activity on the whole cell homogenate level, crude synaptic membrane fractions were examined to determine if there were any synaptically-selective changes in PKA activity. Subcellular fractions were isolated from animals at 10, 20, 40 and 70 min post-first discrete seizure (see Experimental procedures). Basal levels of substrate phosphorylation in the cortical crude synaptoplasmic membrane (SPM) samples were 0.005 pmol PO_4/μg protein (Fig. 3), which were considerably lower than the activity levels measured in cortical homogenates. Control maximal levels of PKA activity were 0.020±0.001 pmol PO_4/μg protein, a four-fold increase over the basal samples ($n=6$, $p<0.001$).

Similar to the effect observed in homogenates, cAMP-stimulated PKA activity increased over control values as SE progressed, becoming significantly higher than control at 40 min post-first discrete seizure. In 40-minute samples the maximal PKA activity was 0.034±0.004 pmol PO_4/μg protein, a 70% increase over the control value ($n=6$, $p<0.05$). 70-minute samples had an activity level of 0.032±0.005 pmol PO_4/μg protein ($n=6$, $p<0.05$) which was a 55% increase over control. As was noted in the cortical homogenates, basal levels of PKA activity in cortical SPM were not significantly different at any time-point when compared to control values ($n=6$, $p>0.05$).

Crude SPM fractions from the hippocampus did not show an increase in PKA activity at any time-point in the course of SE (Fig. 3B). Basal levels of PKA activity in control hippocampal crude SPM fractions were 0.004 pmol PO_4/μg protein while maximal activity levels were 0.013 pmol PO_4/μg protein. Basal substrate phosphorylation levels were not significantly different from the control value at any time-point. Similar to the results observed in the homogenate, maximal (cAMP-stimulated) PKA activity was not significantly different from control values in hippocampal crude SPM fractions ($n=6$, $p>0.05$).

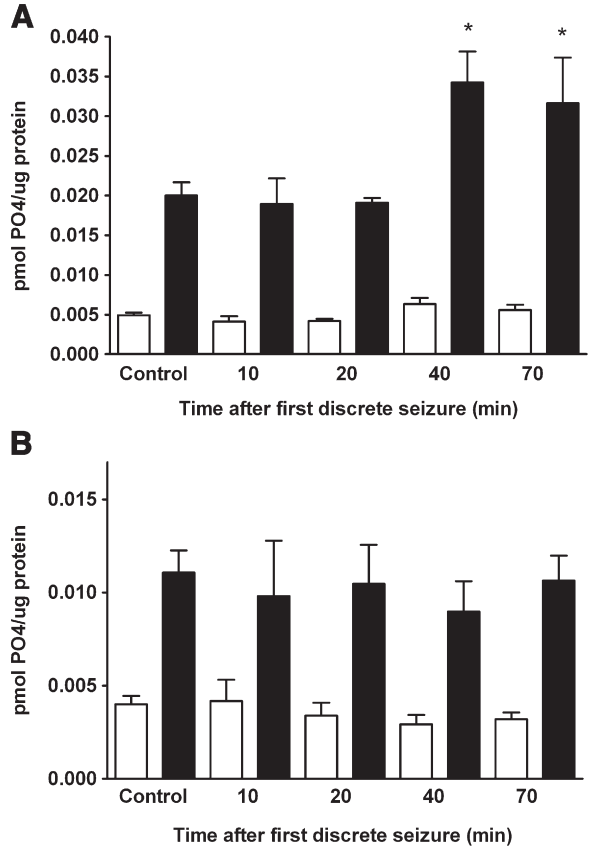

Fig. 3 – Prolonged SE activity results in increased PKA activity in cortical crude SPM. Crude SPM fractions were isolated from cortex and hippocampus and assayed for basal (white bars) and maximally stimulated (black bars) levels of PKA activity in control, 10, 20, 40 and 70-minute post-first discrete seizure animals. As observed for whole cell homogenate, there was no SE-induced effect on basal activity levels in any samples tested. A significant increase in PKA activity between basal and cAMP-stimulated samples was observed in all samples ($n=6$ for all groups, $p<0.001$, one-way ANOVA). (A) PKA activity was maintained over the early stages of SE in cortical crude SPM, but displayed a significant increase in activity over control at 40 and 70 min (*$p<0.05$). (B) No significant differences in PKA activity from control were observed at any time-point in hippocampal crude SPM.

2.4. *SE does not induce a change in the subcellular distribution of PKA subunits*

While expression of PKA was not affected by SE in whole cell homogenate, one potential explanation for the observed increase in PKA activity in crude SPM is translocation of the holoenzyme to the synapse or a paucity of regulatory subunits in synaptosomal fractions. This is of particular interest due to the well-documented regulatory role of PKA-mediated phosphorylation on several neurotransmitter receptors. Western analysis was performed to determine PKA protein levels in cortical crude SPM fractions at time-points both near the onset of SE and later in SE. Control, 20, and 70-minute post-first discrete seizure samples were probed for both catalytic and

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regulatory subunit expression (Fig. 4). When probing for catalytic subunits, immunoreactivity for 20- and 70-minute time-points was not significantly different from control. 20 min after the first discrete seizure, catalytic subunit immunoreactivity was 89.6±16.4% of control ($p>0.05$, $n=4$), while at 70 min after the first discrete seizure catalytic subunit immunoreactivity was 97.1±12.5% of control ($p>0.05$, $n=4$).

PKA regulatory subunit expression was examined in cortical SPM at the same time-points described above (Figs. 4 C, D). As with the catalytic subunit, SE did not induce any SPM-specific changes in regulatory subunit immunoreactivity. 20 min after the first discrete seizure, PKA regulatory subunit remained at 100.0±10.92% of control, while at 70 min immunoreactivity was 85.6±14.9% of control. Neither time-point was statistically different when compared to either control or each other ($n=4$, $p>0.05$).

Hippocampal crude SPM samples were also probed for PKA catalytic and regulatory subunit expression levels. When probing for catalytic subunits, hippocampal crude SPM fractions also did not demonstrate any SE-induced changes in immunoreactivity (data not shown). 20 and 70-minute samples had immunoreactivity of 101.8±3.6 and 94.3±4.8% of control respectively. Differences from control densities were not statistically significant ($p>0.05$, $n=4$). When analyzing regulatory subunit expression in the hippocampal fractions, 20 and 70-minute samples had immunoreactivities of 81.6±8.1 and 87.7±15.4% of control. No change in regulatory subunit expression at any time-point was statistically significant when compared to controls ($p>0.05$, $n=4$). Thus the increase in PKA activity was not due to a change in catalytic versus regulatory subunit expression.

2.5. *SE increases phosphorylation of the GluR1 subunit of the AMPA receptor*

To investigate the potential cellular relevance of the observed increase in PKA activity, we examined the effect of SE on an important PKA substrate, the GluR1 subunit of the AMPA receptor. The level of phospho-GluR1–Ser^{845} was determined by western analysis (see Experimental procedures). Cortical homogenates isolated from control animals were used as comparisons (Figs. 5A, B). As seizure activity progressed, there was a significant increase in GluR1–Ser^{845} phosphorylation when compared to control levels. In samples isolated from

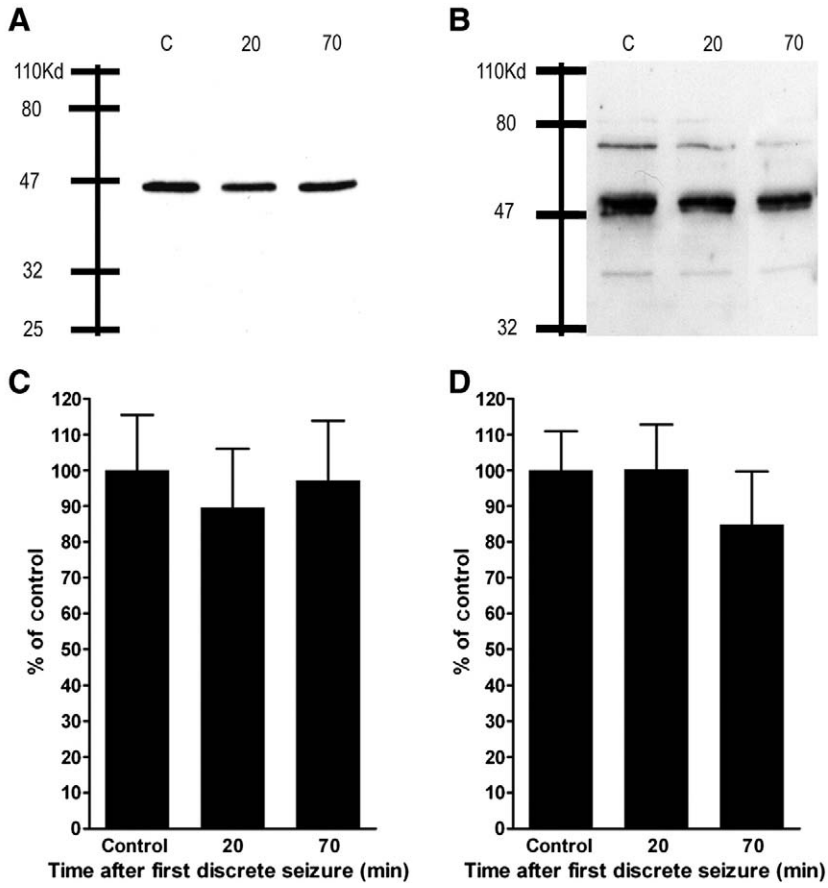

Fig. 4 – SE does not alter expression of PKA catalytic and regulatory subunit expression in cortical crude SPM fractions. Western blot analysis was used to determine if the SE-induced increase in PKA activity observed in cortical crude SPM was due to an alteration in synaptic expression or subcellular distribution of either the catalytic (42 kD) or the regulatory subunit (52 kD) of PKA. Cortical crude SPM fractions from control, 20 and 70-minute animals were assayed for PKA catalytic (A) and regulatory (B) subunit expression. Western blots revealed relatively stable expression of both subunits in the 20 and 70-minute samples. PKA catalytic (C) and regulatory (D) subunit expression in 20 and 70-minute samples were quantified by densitometry. No significant changes in the expression of either PKA subunit were noted when compared to control ($p>0.05$, $n=4$ at each time-point).

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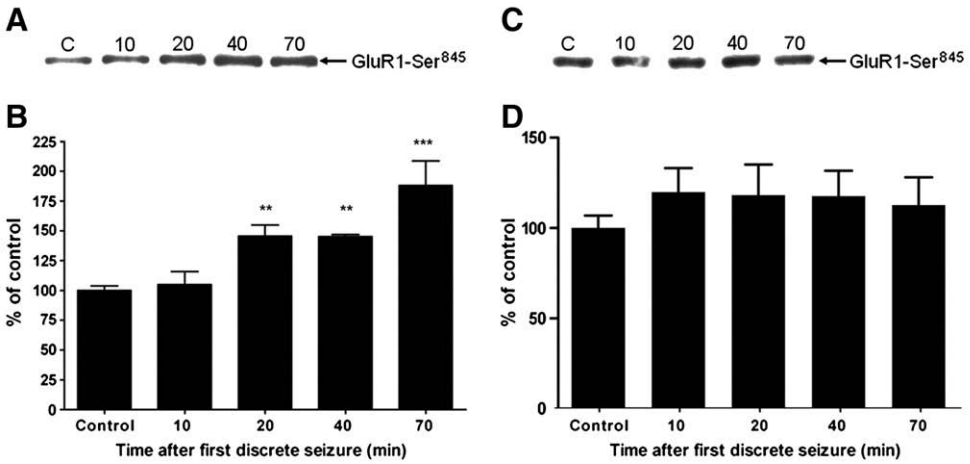

Fig. 5 – SE resulted in an increase in cortical GluR1–Ser^{841} phosphorylation that paralleled the SE-induced increase in PKA activity. A) Representative blot demonstrating cortical immunoreactivity of the anti-GluR1–Ser^{845} antibody in control animals, and animals that were 10, 20, 40 and 70 min post-first discrete seizure. B) Phospho-GluR1 was quantified by computer-assisted densitometry (see Experimental procedures). As seizure activity progressed, SE-induced a significant increase in phosphate incorporation into the GluR1 subunit. Significant increases were present at 20 min post-first seizure and at all subsequent time-points. Pretreatment with the NMDA-receptor antagonist, MK-801 did not block the SE-induced increase in GluR1 phosphorylation. ($^{**}p<0.01$, $^{***}p<0.001$ $n=4$ each time-point). C) Representative blot and D) computer-assisted densitometry demonstrating hippocampal immunoreactivity of the anti-GluR1–Ser^{845} antibody in control animals, and animals that were 10, 20, 40 and 70 min post-first discrete seizure. No significant difference from control was noted at any time-point studied.

animals 20 min post-first discrete seizure, GluR1 phosphorylation levels were 145.6+9.4% of control levels ($p<0.01$, $n=3$). The increased GluR1 phosphorylation was maintained at 40 min post-first discrete seizure (145.3±1.7% of control level, $p<0.01$, $n=3$) and at 70 min after the first discrete seizure (188.1±20.6 of control, $p<0.001$, $n=3$).

In contrast to the marked increase in GluR1 phosphorylation observed in cortical samples, phospho-GluR1–Ser^{845} immunoreactivity was statistically unchanged in hippocampal samples at all time-points (Figs. 5C, D, $p>0.05$, $n=3$).

Previous studies (Kochan et al., 2000; Kurz et al., 2001; Rice and DeLorenzo, 1998; Rice et al., 1998) have demonstrated that many pathologic behavioral and biochemical sequelae of SE are NMDA receptor-dependent. To determine if the observed increase in phosphorylation of the PKA site on the GluR1 subunit was NMDA-dependent (through stimulation of calcium-dependent adenylate cyclase, for example), a non-competitive NMDA-receptor antagonist, MK-801, was administered to animals prior to the induction of SE. Administration of MK-801 resulted in an increase in Ser^{845} phosphorylation to 280+12% of control at the 70-minute time-point. MK-801 did not affect Ser^{845} phosphorylation under sham conditions (95.3±13.4% of control, $n=3$).

3. Discussion

The present study demonstrated an SE-dependent increase in cortical PKA activity against both exogenous and endogenous substrates. Increased phosphorylation of a PKA-specific substrate was observed in both whole cell homogenate and synaptoplasmic membrane fractions. This increase in activity was not accompanied by any noticeable change in expression of the catalytic or regulatory subunits of the enzyme. Additionally, this study noted increased phosphorylation of an endogenous PKA substrate, the Ser^{845} site of the GluR1 AMPA receptor subunit. The timing of this increased GluR1 subunit phosphorylation coincided with the measured increase in PKA activity, providing further evidence for an SE-induced increase in the activity of the enzyme and suggesting a potential downstream pathological effect.

Evidence from several animal models suggests a possible role for PKA in the development and maintenance of seizure activity. Studies have shown that application of cAMP analogues increased neuronal excitability (Boulton et al., 1993), and demonstrated kindling by the repeated injection of cAMP into the rat amygdala (Yokoyama et al., 1989). Additionally, PKA inhibitors demonstrated anti-seizure effects in a model of picrotoxin-induced seizures (Vazquez-Lopez et al., 2005). In a model of auditory-evoked seizures in genetically epilepsy-prone rats, PKA activity was shown to be increased in both cortex and hippocampus (Yechikhov et al., 2001), and the magnitude of this increase was intensified by daily evoked seizure activity. Finally, after administration of cAMP into the inferior colliculus of genetically epilepsy-prone rats, previously discrete seizures culminated into SE (Ludvig and Moshe, 1989). These results suggest an acute pro-excitatory effect of PKA in several seizure models, however no study has yet elucidated the physiologic role of this enzyme in seizure activity.

One potential role for the increase in kinase activity observed in the present study involves a pathologic derangement of physiologic synaptic potentiation. Under normal physiologic conditions, potentiation of individual synapses occurs routinely throughout the brain. This long-term potentiation (LTP) of synaptic strength is an important part of many

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cellular models of learning and memory (Lynch, 2004). Neuronal kinases, such as PKA and CaMK II, are essential for physiologic LTP. For example, activation of synaptic CaMK II is one of the key events initiating early LTP; the kinase, activated by calcium, phosphorylates neurotransmitter receptors, both potentiating their function and inducing the recruitment of additional receptor subunits to synaptic sites. Transition of this early-stage synaptic potentiation to a longer lasting intermediate phase is thought to involve the activation of PKA. Synaptic PKA is capable of both phosphorylating additional sites on receptor subunits and prolonging the activation of synaptic CaMK II. This prolongation of CaMK II activity most likely occurs via inhibition of protein phosphatase 1-mediated dephosphorylation of autophosphorylated CaMK II (Michel et al., 2008; Smolen et al., 2006). This sequential activation of synaptic kinases, while essential for normal function at individual synapses or locally, would represent a significant pathology if it occurred globally. Existing studies suggest that this may be taking place in SE. For example, previous work by our laboratory suggests an increase in synaptic CaMK II activity coincident with the onset of SE (Singleton et al., 2005). The present study identifies an increase in synaptic PKA activity after 30 min of continuous seizure activity via exogenous enzyme assay, and after 20 min using endogenous substrate, which is presumably more sensitive to local changes in PKA activity. The later onset of this increase in PKA activity suggests that, as with physiologic LTP, it may be important for the maintenance of increased excitability. Acutely, this maintenance of excitability may help prevent self-termination of seizure activity. If the potentiation of excitability is persistent, increased PKA activity could play a role in epileptogenesis.

The cause of the SE-dependent increase in PKA activity does not appear to be due to alteration in the expression or subcellular distribution of the enzyme. The results of western analysis of SE and control homogenates indicate that overall expression of PKA subunits was unchanged throughout SE, suggesting that increased enzyme synthesis was not the cause of increased PKA activity. There was no change in the expression of PKA in crude SPM during SE, suggesting that bulk translocation of the enzyme to the synapse did not cause the increased receptor phosphorylation. Finally, while an increase in the ratio of catalytic to regulatory subunits of PKA can cause an increase in PKA-mediated phosphorylation, this ratio remained unchanged throughout SE in both homogenates and crude SPM.

While changes in PKA expression do not appear to explain the SE-induced increase in enzyme activity, several other potential mechanisms were considered. An increase in endogenous intracellular cAMP concentrations would not be expected to affect the substrate phosphorylation assay performed in this study, since dilution of the cellular extract in assay buffer would likely dilute out any local increases in cAMP. However, increased PKA activity was observed when the enzyme was stimulated by exogenously added cAMP, which is more suggestive of an increase in total enzyme concentration or efficacy. Activation of NMDA receptors – and the resulting influx of calcium – is essential for a number of pathological biochemical events in the pilocarpine model of SE (Kurz et al., 2001; Prasad et al., 2002; Rice and DeLorenzo, 1998). A plausible mechanism for NMDA-mediated calcium influx to activate PKA, via activation of calcium-dependent adenylate cyclase, can be proposed. However, administration of MK-801 did not block the increase in Ser^{845} phosphorylation; in fact the magnitude of the increase was larger in MK-801 treated animals than those treated with pilocarpine alone. This may be due to blockade of the NMDA-stimulated calcineurin activity that has been observed in SE (Kurz et al., 2001). Thus, the SE-mediated increases in PKA activity may be due to post-translational modification of the enzyme, or due to SE-induced changes in the interaction of PKA with regulatory and scaffolding proteins, such as members of the AKAP family (Dell'Acqua et al., 2006) or, in dopaminoceptive neurons, DARPP-32 (Svenningsson et al., 2004). The precise nature of the SE-induced alterations in cortical synaptic machinery leading to the observed increases in PKA-mediated phosphorylation represents an intriguing area for further study.

PKA activity towards an exogenous substrate was increased in the same brain regions, and with a similar time-course, as the increased GluR1 subunit phosphorylation. Therefore, an increase in PKA activity directed towards the GluR1 subunit was thought to be a likely mechanism for the observed increase in Ser^{845} phosphorylation. However, a number of other potential mechanisms were considered to account for the observed SE-dependent increase in phospho-Ser^{845} immunoreactivity. An increase in the total amount of GluR1 protein present was ruled out by western analysis, demonstrating that total subunit expression was unchanged by SE in cortical homogenates. A decrease in phosphatase expression or activity would also account for increased phosphorylation at this site. Dephosphorylation of this site has been shown to involve calcineurin, a calcium/calmodulin dependent enzyme highly enriched in neuronal tissue (Snyder et al., 2003). However, previous studies have demonstrated an SE-dependent increase, rather than a decrease, in synaptic calcineurin content and activity, making this mechanism unlikely (Kurz et al., 2003).

In contrast to the cortical data, no SE-induced alteration in PKA activity towards exogenous substrate or towards the GluR1–Ser^{845} phosphorylation site was observed in the hippocampus in this study. The absence of an effect in the hippocampus, a region generally thought to be particularly vulnerable to SE-induced neuronal damage (Turski et al., 1986), is intriguing. This effect may represent differential regulation of excitatory neurotransmitter systems between cortical and hippocampal neurons. Alternatively, increased GluR1 phosphorylation in the hippocampus may be masked by other pathological processes, such as alterations in receptor subunit composition. Regardless, the significant increase in cortical receptor subunit phosphorylation, and the increased neuronal excitability it implies, remains relevant to the pathology of SE, potentially allowing maintenance of generalized seizure activity or playing a role in decreased cognitive function after SE.

The data suggest that as SE progresses, an activation of PKA in cortical tissues occurs. One cellular effect of this activation of PKA is increased phosphorylation of the GluR1 subunit at Ser^{845}. This up-regulation of AMPA receptor phosphorylation may lead to increased neuronal excitability, potentially playing a role in maintenance of seizure activity or in SE-

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induced neuropathology. Further study of the mechanisms underlying this increase in kinase activity and AMPA subunit phosphorylation could improve our knowledge of the biochemical processes underlying SE, as well as provide further insight into neuronal plasticity.

4. Experimental procedures

4.1. Materials

All materials were reagent grade and purchased from Sigma Chemical Company (St. Louis, MO, USA) or 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-PKAβ catalytic subunit was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Purified goat polyclonal anti-PKA RII subunit was obtained from Upstate (Lake Placid, NY, USA). Rabbit anti phospho-GluR1–Ser^{845} and anti-GluR1 antibodies were obtained from Zymed (San Francisco, CA, USA).

4.2. Methods

4.2.1. 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. Adult male Wistar rats (90 days old) 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 (Singleton et al., 2005). 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.

4.2.2. Induction of status epilepticus

Twenty minutes prior to the injection of pilocarpine, methylscopolamine, a muscarinic antagonist, was administered by intra-peritoneal (i.p.) injection (1 mg/kg) to reduce adverse peripheral affects of the pilocarpine. Baseline EEG recordings were obtained for 10 min following scopolamine injection. SE was induced in experimental animals by i.p. injection of 375 mg/kg pilocarpine HCl. Behavioral and encephalographic activities were recorded throughout the procedure as described previously (Singleton et al., 2005). Once initial seizure activity was observed, the time was noted and rats were allowed to seize for predetermined durations (10, 20, 40 and 70 min post-first discrete seizure) before the animals were processed. Since we have previously characterized SE onset as being approximately 10 min after the first discrete seizure in this model (Singleton et al., 2005), these times approximate 0, 10, 30 and 60 min of SE, respectively. Analysis and review of EEG activity was performed using Insight II software (Persyst Corporation Prescott, AZ, USA).

4.2.3. Brain region isolation

Specific brain regions (cortex and hippocampus) were dissected away on ice and immediately homogenized into an iced-cold buffer containing 50 mM Tris–HCl (pH 7.4), 7 mM EGTA, 5 mM EDTA, 1 mM dithiothreitol (DTT), and 0.3 mM phenylmethylsulfonyl fluoride (PMSF). The osmolarity was adjusted to 325 mOsm using sucrose. Osmolarity was verified using a VAPRO 5520 (Westcor, Inc., Logan, UT, USA). For GluR1 phosphorylation analysis, 1 mM NaF was included to inhibit phosphatase activity. The brain regions were homogenized by 10 strokes (up and down) using a Teflon pestle at 12,000 rpm (Fordham Bethel, CT, USA). A portion of the sample was then aliquoted and frozen at −80 °C until processed for biochemical analysis.

4.2.4. Isolation of subcellular fractions

Subcellular fractions were isolated by a differential centrifugation procedure as described previously (Kurz et al., 2001). Brain region homogenates were centrifuged at 5000×*g* for 10 min (Beckman JA-17 rotor) to produce a crude nuclear pellet. The supernatant from this spin was then centrifuged for 30 min at 18,000×*g* to produce a crude synaptoplasmic membrane/mitochondrial pellet (crude SPM), which was re-suspended in homogenization buffer. All fractions were rapidly separated into aliquots and stored at −80 °C for later use.

4.2.5. Substrate phosphorylation

Brain region homogenates and crude SPM fractions were normalized for protein concentration. PKA-dependent phosphorylation reactions were carried out via the SignaTECT assay kit (Promega) according to the manufacturer's instructions. Briefly, all reactions contained the manufacturer's proprietary PKA Assay Buffer, [γ-^{32}P]ATP, and 100 mM PKA Biotinylated Peptide Substrate. ATP was prepared by adding 0.05 μL [γ-^{32}P]ATP (10 μCi/μL) to 0.5 mM 5 μL unlabeled ATP per reaction. cAMP was added to maximal reactions for a final concentration of 25 μM, an equivalent volume of dH2O was added to basal reactions. Final volume of all reaction tubes was 25 μL. The reaction tubes were incubated in a 30 °C water bath for 5 min, 150 μg/5 μL homogenate or crude SPM protein sample was added, and then tubes were allowed to incubate an additional 5 min at 30 °C. The reactions were stopped by the addition of 7.5 M guanidine hydrochloride (12.5 μL). All reactions were performed in triplicate. 10 μL aliquots were then taken from each tube and spotted on a biotin capture membrane. Membranes were then washed to remove unbound phosphates as described in the manufacturer's instructions. Following drying the biotin membrane sections were placed in scintillation vials, 2 mL of Cytoscint was added and the reactions were counted using a Beckman LS 6500 scintillation counter.

4.2.6. Immunodetection of PKA regulatory and catalytic subunits

Western analysis was performed essentially as described previously (Kurz et al., 2008). 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 (BioRad, Hercules, CA, USA). The nitrocellulose membranes were incubated with primary antibody in blocking solution at 4 °C overnight. Primary antibodies were diluted 1:1000. The nitrocellulose membranes were reacted with an appropriate horseradish-peroxidase-conjugated secondary antibody (1:5000) in blocking solution for 1 h. Blots were developed using a chemiluminescent substrate (Pierce, Rockford, IL, USA). 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). Finally, western blot membranes were probed with an anti-β-tubulin antibody (1:1000) to confirm that observed differences in immunoreactivity between experimental groups were not due to variability in protein loading. β-tubulin immunoreactivity for each experimental group did not vary more than ±10% from the control value (as assessed by densitometry) and this variability was not statistically significant in any group studied.

4.2.7. *Statistical analysis*

Data was analyzed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA). Student's *t* test was used for all single, parametric comparisons. For multiple comparisons, one-way ANOVA with a Tukey post hoc analysis with 95% confidence was used to reduce type-1 errors associated with multiple comparisons.

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