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Altered phosphorylation and localization of the A-type channel, Kv4.2 in status epilepticus

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Abstract

Extracelluar signal-regulated kinase (ERK) pathway activation has been demonstrated following convulsant stimulation; however, little is known about the molecular targets of ERK in seizure models. Recently, it has been shown that ERK phosphorylates Kv4.2 channels leading to down-regulation of channel function, and substantially alters dendritic excitability. In the kainate model of status epilepticus (SE), we investigated whether ERK phosphorylates Kv4.2 and whether the changes in Kv4.2 were evident at a synaptosomal level during SE. Western blotting was performed on rat hippocampal whole cell, membrane, synaptosomal, and surface biotinylated extracts following systemic kainate using an antibody generated against the Kv4.2 ERK sites and for Kv4.2, ERK, and phospho-ERK. ERK activation was associated with an increase in Kv4.2 phosphorylation during behavioral SE. During SE, ERK activation and Kv4.2 phosphorylation were evident at the whole cell and synaptosomal levels. In addition, while whole-cell preparations revealed no alterations in total Kv4.2 levels, a decrease in synaptosomal and surface expression of Kv4.2 was evident after prolonged SE. These results demonstrate ERK pathway coupling to Kv4.2 phosphorylation. The finding of decreased Kv4.2 levels in hippocampal synaptosomes and surface membranes suggest additional mechanisms for decreasing the dendritic A-current, which could lead to altered intrinsic membrane excitability during SE.

Keywords

epilepsy; extracellular signal-regulated kinase; ion channels; mitogen-activated protein kinase; protein phosphorylation; seizures

Status epilepticus (SE) is a life-threatening condition defined as prolonged continuous seizures or intermittent seizures without recovery of consciousness. An episode of SE can be associated with the development of temporal lobe epilepsy (Leite *et al.* 2002). In several convulsant-induced models of limbic epilepsy (kainate and pilocarpine) in rodents, a prolonged episode of SE (90–120 min) is required for the development of epilepsy (Lemos and Cavalheiro

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1995; Loscher 2002). A number of molecular and biochemical alterations likely occur in the limbic structures during prolonged SE. Insights into these alterations may provide novel targets for therapeutic intervention in SE.

The MAPK cascade has been implicated in diverse cellular processes. The MAPK pathway consists of a superfamily of kinases, which includes the extracellular signal-regulated kinases 1 and 2 (ERK 1/2). The ERK pathway plays a critical role in hippocampal plasticity (Sweatt 2004; Thomas and Huganir 2004) and activation of the ERK pathway has been implicated in a number of pathological processes involving the CNS (Fukunaga and Miyamoto 1998; Costa *et al.* 2002). In particular, modulation of ERK signaling pathway activation has been demonstrated following seizures and SE in rodent models *in vivo* (Baraban *et al.* 1993; Gass *et al.* 1993; Kim *et al.* 1994; Garrido *et al.* 1998; Berkeley *et al.* 2002) and in hyperexcitability models *in vitro* (Fiore *et al.* 1993; Kurino *et al.* 1995; Murray *et al.* 1998; Merlo *et al.* 2004).

The ERK 1/2 pathway regulates a broad range of target molecules through proline-directed serine/threonine phosphorylation (Kennelly and Krebs 1991). The ERK pathway downstream effector molecules in physiologic and pathologic conditions in the nervous system are currently being investigated (Thomas and Huganir 2004). The voltage-dependent K^+ channel α-subunit, Kv4.2, is one of the substrates for ERK in hippocampus (Adams *et al.* 2000). Kv4.2 proteins localize to the somatodendritic regions of hippocampal neurons and contribute to the pore-forming regions of channels that express a transient, rapidly-activating K^+ current (A-current) (Baldwin *et al.* 1991; Sheng *et al.* 1992; Maletic-Savatic *et al.* 1995; Martina *et al.* 1998; Serodio and Rudy 1998). The A-current attenuates action potential initiation and back-propagating action potentials (B-APs) and reduces excitatory synaptic events in CA1 dendrites, thereby modulating neuronal excitability (Hoffman *et al.* 1997; Martina *et al.* 1998; Migliore *et al.* 1999; Johnston *et al.* 2000; Cai *et al.* 2004). Kv4.2 knockout mice have loss of the A-current and an increase in the B-APs in hippocampal CA1 pyramidal cell dendrites (Chen *et al.* 2006), which suggests that the Kv4.2 channel is the major contributor to the A-current in this region. It has previously been shown that inhibition of ERK activation causes a hyperpolarizing shift in the voltage dependence of activation of the A-current in CA1 dendrites (Watanabe *et al.* 2002). Activation of the upstream regulators of the ERK pathway, cAMP-dependent protein kinase (PKA) and protein kinase C leads to down-regulation of the A-current in CA1 dendrites (Hoffman and Johnston 1999). ERK pathway modulation of the A-current in hippocampal CA1 dendrites is thought to be due to direct phosphorylation of Kv4.2 channel subunits.

Relatively little is known about the targets of the ERK pathway during acute seizures and SE. In the studies presented here, we found an increase in ERK phosphorylation of Kv4.2 channels during SE. These changes were evident at a synaptosomal level. Furthermore, total levels of Kv4.2 proteins were decreased within the synaptosomal and surface membrane subcellular compartments, while Kv4.2 levels were unchanged at a whole-cell level. We predict that increased ERK phosphorylation of Kv4.2 and decreased levels of Kv4.2 channels in the post-synaptic membrane of hippocampal neurons would contribute to hyperexcitability during SE.

Materials and methods

Kainate-induced seizures: behavioral and video-EEG recording assessments

All experimentation involving animals was performed in accordance with the regulations of the Animal Welfare Policy. Male Sprague–Dawley rats (125–200 g) were injected with kainate (15 mg/kg) or vehicle intraperitoneally (i.p.). Convulsive seizure activity was assessed using behavioral observation and seizure scoring according to the Racine scale (Racine 1972). Scoring of behavioral seizures was performed independently by at least two investigators (AEA, LDJ, or LFB). Class 5 limbic motor seizures (rearing and falling) were considered

consistent with SE. Animals were killed at various time points after kainate injection for biochemistry studies.

In a subset of animals, cortical and hippocampal depth electrodes were implanted bilaterally using stereotaxic methods as described previously (Anderson *et al.* 1996). Briefly, animals were anesthetized using a ketamine/xylamine mixture (obtained from the Baylor College of Medicine animal facility) and positioned in a stereotaxic frame. Cortical surface and depth electrodes were made in the laboratory prior to implantation. Cortical surface electrodes were placed bilaterally over the somatosensory cortex and one additional surface electrode was placed over the frontal cortex (Anderson *et al.* 1996). Two additional electrodes were stereotaxically implanted into the hippocampus. An additional electrode used for ground was placed in the cervical paraspinous area. After a recovery period of 5–7 days, kainate or vehicle (sham control treatment) was administered as described above.

Baseline video-EEG recordings were taken for 30 min prior to kainate injection. We recorded three to four channels of EEG activity and video using an analog Nihon Kohden system (Tokyo, Japan) or a digital Stellate system. Behavioral seizures were scored according to the Racine Scale based on video assessments (post-acquisition review) and visual observation during the recording period. EEG traces were assessed for latency to onset of first seizure and SE following kainate by three trained investigators (LFB, RAH, and AEA). Repetitive spike and sharp wave activity occurring for at least 10 s in one or more channels was defined as an electrographic seizure. Onset of electrographic SE was defined as continuous seizure activity that occurred in all regions with no intervening episodes of seizure-free background activity more than 10 s. We administered pentobarbital (15 mg/kg subcutaneously) 3 h after kainate injection to stop SE, and we continued to monitor EEG activity for an additional 30 min or until no seizures were observed.

Tissue preparation and western blotting for ERK activation and ERK phosphorylation of Kv4.2

Immunoblotting was performed at various points in time after kainate administration. Animals that had EEG electrodes implanted were not used for the biochemistry studies. Animals were killed and the hippocampi rapidly dissected. CA1, CA3, and dentate regions were then microdissected in ice-cold cutting solution, frozen on dry ice, and stored at −80°C until tissue processing (Chen *et al.* 1992). Homogenates were prepared from the hippocampal subfields by brief sonication in ice-cold homogenization buffer (in mM: 50 Tris–HCl, pH 7.5, 50 NaCl, 10 EGTA, 5 EDTA, 4 p-nitrophenyl phosphate, 2 sodium pyrophosphate, 1 phenylmethylsulfonyl fluoride, 20 μg/mL leupeptin, and 4 μg/mL aprotinin). Subsequently, the samples were boiled in 4× sample buffer. The protein concentration of each sample was determined using a Bio-Rad Protein assay (Bio-Rad Laboratories Inc., Hercules, CA, USA). All samples were then normalized to the lowest sample concentration by adjusting the buffer solution amount to produce equal protein concentrations across all conditions. Normalized tissue homogenates were used for total and phospho-ERK 1/2 western blotting (Atkins *et al.* 1998). The following antibody dilutions were used: total ERK 1/2 1 : 10 000, phospho-ERK 1/2 1 : 5000 (Cell Signaling, Beverly, MA, USA) and anti-rabbit secondary 1 : 50 000 (Cell Signaling); PKA substrate antibody 1 : 1000 (Cell Signaling) and anti-rabbit secondary 1 : 20 000 (Chemicon, Temecula, CA, USA). Immunoreactive bands were visualized by enhanced chemiluminescence (Supersignal; Thermo Scientific, Rockford, IL, USA) and captured on autoradiography film (Phenix Research Products, Candler, NC, USA). The film was then developed and later densitized using a HP Scanjet 4470c (HP, Palo Alto, CA, USA).

Hippocampal membranes were prepared using the normalized CA1, CA3, and dentate gyrus homogenates. The hippocampal homogenates were centrifuged for 20 min at 4°C at 100 000 *g*. The pellet was resuspended and solubilized in 10% sodium dodecyl sulfate with 200 mM

1,4-dithio-DL-threitol, 10 mg/mL aprotinin and leupeptin, 10 mg/mL pepstatin, 80 μM phenylmethylsulfonyl fluoride, and 1 μM microcystin-LR. After the addition of 4× sample buffer the samples were sonicated as previously described (Adams *et al.* 2000; Anderson *et al.* 2000; Bernard *et al.* 2004). The triply phospho-Kv4.2 (Adams *et al.* 2000) and commercially available Kv4.2 (not phospho-selective) antibodies were used to probe membranes from the hippocampal subfields that recognizes the total pool of Kv4.2 channels. The following antibody dilutions were used: triply phospho-Kv4.2 1 : 300; total Kv4.2 1 : 200 (Chemicon); phospho-Kv4.2 – PKA CT 1 : 300 and NT 1 : 200 sites; and anti-rabbit secondary 1 : 20 000 (Cell Signaling).

For the crude synaptosomal preparations, we followed the methods previously described by Lu *et al.* (2003). Briefly, tissue (whole hippocampus) was homogenized in ice-cold homogenization buffer [0.32 M sucrose, 10 mM HEPES (pH 7.4)], with protease and phosphatase inhibitor cocktails (Sigma, St Louis, MO, USA). Some of the sample was saved as homogenate input, while the rest of the sample was used for synaptosome preparations. Synaptosomes were prepared by centrifugation at 800 *g* for 10 min at 4°C, and the supernatant was spun again at 7100 *g* for 15 min at 4°C. The pellet was resuspended in homogenization buffer and spun again at 7100 *g* for 15 min at 4°C again to yield the synaptosomal pellet, which was then resuspended in homogenization buffer. Sample buffer (4×) was then added to the homogenate and crude synaptosomal preparations. Enrichment of the synaptosomal compartment was confirmed by probing the different fractions with antibodies for synaptic marker proteins: PSD-95 (monoclonal; Affinity Bio-Reagents, Golden, CO, USA) and SV2 (monoclonal from the Developmental Studies Hybridoma Bank, Iowa City, IA, USA). We used the marker of neuronal nuclear proteins – neuronal nuclei (NeuN, monoclonal; Chemicon) to ensure that there was no contamination from this subcellular compartment in the synaptosomes. Homogenate samples and synaptosomes were probed with ERK, Kv4.2, phospho-ERK, and phospho-Kv4.2 antibodies using concentrations described in the previous sections. The blots were probed with an antibody against actin (polyclonal; Cell Signaling Technology) to ensure that equal amounts of protein were loaded in each lane.

Surface biotinylation assay

Surface expression of Kv4.2 in hippocampus was evaluated following SE. Rats received kainate to induce SE or vehicle (sham control group) as described above. Rats were decapitated 1 h after SE onset (roughly 3 h post-kainate injection) and sham controls were processed in parallel. The brain was quickly removed and placed in ice-cold cutting solution for 10 min containing: (in mM): 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 110 sucrose, and 5 D-glucose, 0.6 ascorbate. The ice-cold buffer was equilibrated with 95% O_2 and 5% CO_2. The hippocampi were then dissected and 400 μm sections were taken using a Vibratome 3000 (St Louis, MO, USA) in ice-cold dissection buffer. The slices were transferred into a reservoir chamber filled with the ice-cold cutting solution and the biotinylation assay was performed according to methods previously described (Goodkin *et al.* 2005; Nosyreva and Huber 2005). Specifically, the hippocampal slices were placed 3–4/well in 24 well plates containing 0.8 mL/well of sulfo-NHS-LC or sulfo-NHS-SS 1 mg/mL biotin (Pierce, Rockford, IL, USA) in phosphate-buffered saline (Invitrogen, Carlsbad, CA, USA) and incubated for 30 min at 4°C on a shaker. The slices were washed three times with cold phosphate-buffered saline then ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer was added to the tube containing (in mM): 50 Tris–HCl, pH 7.4, 0.25% Nadeoxycholate, 150 NaCl, 1 EDTA, along with the protease inhibitor cocktail (Sigma) and 1 Na_3VO_4. The slices were broken up by pipetting and the samples were sonicated and incubated on ice for 20 min to completely lyse the tissue, and then spun for 15 min at 14 000 *g*. Protein concentrations were measured with a RIPA Protein Assay (Pierce); 200–500 μg protein was added (at a concentration of 1 μm/μL) with 100 μL of UltraLink immobilized NeutrAvidin beads (Pierce) by rotating overnight at 4°

C. The beads were washed with RIPA lysis buffer and then eluted in nondenaturing sample buffer by heating samples at 100°C for 5 min. Both total and biotinylated proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to Immobolin-P membranes, and probed with anti-Kv4.2 antibody (1 : 500; Millipore, Billerica, MA, USA) or anti-actin antibody (1 : 1000; Sigma). Immunoreactive bands were visualized by enhanced chemiluminescence and captured on autoradiography film. Following densitometry, the surface/total protein ratio was calculated for each condition. When duplicate conditions were performed within one animal, the ratio values were averaged to obtain an animal average (n = 1). Therefore, the n values for the biotinylation experiments represent the number of rats used for each condition, as opposed to slices. Significant differences between surface/total ratios of treated slices and within-animal control slices were determined using an independent sample t-test (for ratio and p values).

Analysis

Each experimental point (n) for western blotting represents a single animal. Immunoreactivity was quantified by densitometry using Scion Image public domain software and analyzed using Graphpad PRISM® software (La Jolla, CA, USA). Samples from the control and experimental animals were run on the same gel for each set of experiments or time point. Following western blotting, the band from each protein of interest in the individual controls was initially normalized to the actin band for that given control (lane). These values were then averaged across the control bands and set to 100%. The experimental bands were first normalized to their actin level and then expressed as a percent of the average control level. This method permitted analysis across blots. The independent samples t-test was used to determine significance between experimental and control groups. Densitometry values were shown as mean ± SEM.

Results

Behavioral SE is associated with increased ERK activation in hippocampal areas CA1, CA3, and dentate gyrus

In previous studies, the time course of ERK activation and the brain regions demonstrating altered ERK signaling varied depending on the epilepsy model used and the timing of the investigations following convulsant stimulation. Furthermore, many of the previous studies evaluated ERK pathway activation semiquantitatively based on immunohistochemistry or in whole-cell hippocampal or hippocampal-cortical preparations (Baraban *et al.* 1993; Kim *et al.* 1994; Berkeley *et al.* 2002). We focused our initial studies on quantitative assessments of ERK pathway activation in microdissected hippocampal CA1 and CA3 subfields and in dentate gyrus following a 1-h episode of behavioral SE. For these studies, we prepared homogenates from the hippocampal regions and performed western blotting using antibodies against the dually phosphorylated, activated form of ERK 1/2 and total ERK 1/2. Densitometry of the changes in ERK2 (p42) demonstrated significantly increased ERK2 phosphorylation in hippocampal areas CA1 ($p < 0.01$, $n = 5$), CA3 ($p < 0.01$, $n = 5$), and dentate gyrus following SE ($p < 0.001$, $n = 5$) compared with controls ($n = 5$) (Fig. 1a and b) indicating increased ERK2 activation in these regions. No change in total ERK2 levels were identified for any of the hippocampal subfields: CA1 (108.0 ± 3.93% control), CA3 (96.71 ± 2.93% control), and dentate gyrus (98.23 ± 3.13% control) (Fig. 1a; lower panels for blots, densitometry not shown).

A subgroup of animals ($n = 4$) injected with kainate did not develop evidence of convulsive SE, although they did experience stage 1–3 seizures according to the Racine Scale. These latter events were transient and did not persist up to the 3 h time point. The hippocampi from these animals were subdissected and processed in parallel with the tissue from control and behavioral SE animals. There was a significant increase in percent change of phospho-regulated ERK2

(phospho-ERK2) in area CA1 (151.3 ± 15.92% control, $p < 0.01$) and area CA3 (136.3 ± 18.04% control, $p < 0.05$) but not in dentate gyrus (125.0 ± 14.71% control, not significant) compared with control animals. Previous work has suggested that in whole-cell hippocampal extracts, kainate-mediated increases in ERK activation are not evident at the 3-h time point unless there is associated behavioral SE (Kim *et al.* 1994). Our findings suggest that in areas CA1 and CA3, ERK2 activation occurs without the development of convulsive SE. The dentate gyrus has been described as having a 'gatekeeper' role in seizure generation [reviewed in (Coulter 2000)]. It is possible that an increase of ERK activation in the dentate gyrus may be necessary to generate SE. However, we cannot rule out the possibility that the animals were experiencing subclinical seizures.

ERK couples to Kv4.2 phosphorylation in hippocampus following behavioral SE

The role that the ERK signaling pathway plays in epilepsy is unclear. Based on previous studies defining the K^+ channel Kv4.2 as a downstream target of ERK in the hippocampus (Adams *et al.* 2000), we hypothesized that Kv4.2 channels may be one candidate target of the ERK pathway during SE. We have previously identified three ERK phosphorylation sites within the carboxy-terminal cytoplasmic domain of Kv4.2 (T602, T607, and S616) and developed a phospho-selective antibody that recognizes Kv4.2 when phosphorylated at all three sites (triply phospho-Kv4.2 antibody) (Adams *et al.* 2000). Using the triply phospho-Kv4.2 antibody in western blotting assays for the SE studies, we determined that there was a significant increase in ERK-phosphorylated Kv4.2 in hippocampal areas CA1, CA3, and dentate gyrus ($p < 0.001$, $n = 5$) following the development of behavioral SE (assayed in parallel with ERK assessments) compared with controls ($n = 5$) (Fig. 2a; upper panels and Fig. 2b). Parallel blots probed with the antibody to total Kv4.2 demonstrated no change in Kv4.2 protein levels in hippocampal whole-cell membranes following kainate (Fig. 2a; lower panels). These findings suggest that Kv4.2 is a candidate effector of ERK in kainate-induced SE and open up the possibility of ERK regulation of intrinsic membrane excitability in hippocampus during SE through modulation of K^+ channel activity.

In the previous section, we described significant increases in ERK2 activation at the 180 min time point following kainate in animals that had experienced transient stage 1–3 behavioral seizures but did not progress to stage 4 and 5 seizures (SE). In hippocampal membranes harvested from these animals we performed western blotting using the ERK triply phospho-Kv4.2 antibody. While there were significant increases in ERK2 activation in areas CA1 and CA3 in the animals that did not experience SE, there was no significant change in ERK phosphorylated Kv4.2 in any of the hippocampal subfields evaluated [area CA1 (72.84 ± 21.83% control, $n = 4$), area CA3 (121.2 ± 36.84% control, $n = 4$), or dentate gyrus (147.8 ± 41.38, $n = 4$)]. Together our findings suggest that coupling of ERK activation to Kv4.2 phosphorylation in hippocampus is a correlate of prolonged continuous seizures.

The triply phospho-Kv4.2 antibody recognizes composite phosphorylation of the ERK sites within the Kv4.2 carboxyl-terminal domain. Single-site antibodies also were generated against the three ERK sites within Kv4.2 (Adams *et al.* 2000). In order to determine whether there was selective phosphorylation at the Kv4.2 ERK sites, we screened CA1 membranes from control and SE animals with western blotting using the purified single-site phospho-Kv4.2 antibodies. There was a significant increase in immunoreactivity in SE compared with control animals at each of the mapped ERK phosphorylation sites within Kv4.2 [anti-serine 602 : 345.20 ± 32.63% control ($p < 0.01$), anti-threonine 607 : 169.90 ± 8.12% control ($p < 0.01$), anti-threonine 616 : 160.40 ± 17.64% control ($p < 0.05$); $n = 3$]. Thus, in area CA1 there was a greater increase in phosphorylation at the 602 site compared with the 607 and 616 sites during SE. These findings corroborate our findings with the triply phospho-Kv4.2 antibody and provide additional lines of evidence that Kv4.2 is a downstream target of the ERK pathway in SE.

Status epilepticus has been associated with modulation of multiple signal transduction pathways. We have previously described PKA phosphorylation sites at threonine 38 and serine 552 within the Kv4.2 animo- and carboxyl-terminal domains, respectively (Anderson *et al.* 2000). PKA activation in behavioral SE was evaluated by western blotting total hippocampal extracts with an antibody that recognizes PKA phosphorylation motifs. Kainate-induced behavioral SE was associated with modulation of the immunoreactivity of a number of bands using this antibody. Several of the bands demonstrated an increase in immunoreactivity supporting PKA activation in SE (Fig. 3a). We investigated whether there was altered Kv4.2 phosphorylation at the PKA sites. We used phospho-selective antibodies against the PKA sites: threonine 38 – α-phospho-Kv4.2 – PKA NT and serine 552 – α-phospho-Kv4.2 – PKA CT (Anderson *et al.* 2000) in western blotting of hippocampal membranes from control and SE animals (Fig. 3b). We found no significant change in Kv4.2 phosphorylation at these PKA sites in hippocampal areas CA1, CA3, and dentate gyrus from animals with behavioral SE compared with controls (densitometry not shown). These findings indicate that during SE there is evidence of PKA activation, but PKA does not couple to phosphorylation of Kv4.2. Thus, there does not appear to be convergence of the PKA and ERK pathways at the level of Kv4.2 during SE.

The time course of early and late electrographic and behavioral seizures following acute kainate administration

After we identified the robust increase in Kv4.2 phosphorylation by ERK during kainate-induced behavioral SE, we were interested in studying the time course of ERK activation and phosphorylation of Kv4.2 following kainate. Furthermore, we sought to determine whether there was a correlation of the biochemistry changes in ERK signaling with seizure activity. In a subset of animals with cortical and hippocampal depth electrodes implanted bilaterally, we used video-EEG recordings to measure latency of onset of electrographic versus convulsive seizures and SE following kainate administration (Fig. 4a). The latency to onset of the first electrographic seizure was significantly less than the first behavioral class 3 seizure ($p < 0.001$, $n = 8$; Fig. 4b). Similarly, the latency to onset of electrographic SE was significantly less than the first signs of class 4 and 5 behavioral SE ($p < 0.001$, $n = 9$; Fig. 4c). We also found that latency to onset of first electrographic seizure showed a strong correlation with latency to onset of SE ($r = 0.645$, $p < 0.05$, $n = 8$; Fig. 4d). A similar analysis with the onset to first behavioral seizure and behavioral SE showed little correlation (not shown). Sham-treated control animals ($n = 7$) were assessed in parallel using video-EEG; these animals demonstrated no electrographic or behavioral evidence of seizures. Following these studies, it became apparent that the 3-h time point following kainate administration that we had used for the assessment of ERK activation and phosphorylation of Kv4.2 (Figs 1 and 2, respectively) occurred after the animals had experienced, on average, over 2 h of electrographic SE. Thus, our biochemistry studies presented above represent a time point of prolonged, continuous seizure activity.

The time course of ERK activation and Kv4.2 phosphorylation following acute kainate administration

Kainate-induced ERK activation and phosphorylation of Kv4.2 was evaluated at 5, 15, 30, 60, and 180 min ($n = 4$ for each time point) following kainate injection and compared with vehicle-treated controls for each time point ($n = 4$ for each time point). The findings presented in the previous section were taken into consideration for the choice of time points. Because of the potential complication of implanted EEG electrodes on the biochemistry results, these animals were monitored for onset of behavioral seizures and did not have EEG recordings. The onset of early behavioral seizures (class 3) in this group of animals corresponded to the 60 min time point and by the 180 min time point all the animals had evidence of behavioral SE for at least 1 h.

The pattern of ERK2 activation following kainate was similar in all hippocampal areas studied. There was a significant increase in ERK2 activation in all hippocampal subfields 30 min following kainate injection ($p < 0.05$), which persisted throughout the remainder of the time course (Fig. 5). Levels of phospho-Kv4.2 (ERK sites) demonstrated a pattern of an early decrease in dentate gyrus and CA3 (5 and 15 min after kainate, respectively) and then a subsequent increase in all three hippocampal regions by the convulsive SE time point (180 min). The observation of ERK activation and no detectable levels of increased Kv4.2 phosphorylation suggest the possibility that ERK phosphorylation of Kv4.2 is slower than the kinetics of ERK activation itself following kainate administration. The significant decreases in ERK phosphorylation of Kv4.2 in dentate gyrus at the 5 min ($p < 0.01$) and in CA3 at the 15 min ($p < 0.05$) time points, may be due to phosphatase activation early after kainate administration, which possibly is the result of early seizure activity. No significant change in total ERK or Kv4.2 levels in total cellular membranes from experimental compared with control animals was observed (not shown).

Comparison of the biochemistry time course studies with the latency to onset of early and late kainate-induced electrographic and convulsive seizures suggests that the significant increase in ERK activation in hippocampus occurs after the animals have developed electrographic seizures but before the onset of SE. These changes persist with prolonged SE at which point the behavioral findings consistent with SE (Racine class 4 and 5 seizures) are evident. Significant increases in ERK phosphorylation of Kv4.2 appear to correlate with electrographic SE and onset of behavioral seizures. A statistical correlation analysis could not be performed as we did not use hippocampal tissue from the same animals used for video-EEG recordings.

Status epilepticus is associated with increased ERK phosphorylation of Kv4.2 and decreased total Kv4.2 levels in hippocampal synaptosomes

We evaluated the changes in ERK signaling and Kv4.2 phosphorylation in crude hippocampal synaptosomal preparations. Phospho-ERK2 was significantly increased in hippocampal synaptosomes at the 1 h (early SE) (330.2 ± 67.6%, $p < 0.05$, $n = 5$) and 3 h (late or prolonged SE) (174.2 ± 7.2%, $p < 0.05$, $n = 5$) time points following kainate compared with controls ($n = 5$). In line with these results, significant increases in ERK phosphorylated Kv4.2 were evident in hippocampal synaptosomes at the 1 h ($p < 0.01$, $n = 5$) and 3 h ($p < 0.05$, $n = 5$) time points following kainate compared with controls ($n = 5$) (Fig. 6a; lower blots and graph). As expected, ERK-phosphorylated Kv4.2 was significantly increased in whole-cell hippocampal homogenates at the 1 h (415.1 ± 64.7%, $p < 0.01$, $n = 5$) and 3 h (157.3 ± 8.5%, $p < 0.01$, $n = 5$) time points following kainate compared with controls (Fig. 6a; upper blots, densitometry not shown). Similarly, phospho-ERK2 was significantly increased in whole-cell homogenates at the 1 h (267.2 ± 32.9%, $p < 0.01$, $n = 5$) and 3 h (336.9 ± 121.0%, $p < 0.05$, $n = 5$) time points following kainate compared with controls ($n = 5$). There was no change in total ERK 1/2 seen in the 1 and 3 h time points after kainate using either synaptosomes or whole-cell homogenates (data not shown), which corroborated our earlier findings (Fig. 1).

Interestingly, we also found that total Kv4.2 levels were significantly decreased in hippocampal synaptosomes following prolonged SE (3 h after kainate) ($p < 0.05$, $n = 5$) (Fig. 6b; lower right panels and graph). The decrease only was evident after prolonged SE as total Kv4.2 levels were not significantly decreased in synaptosomes prepared from hippocampal tissue at the 1 h time point ($n = 5$) (Fig. 6b; lower left panels and graph). Similar to that seen in the CA1, CA3, and dentate membrane preparations (Fig. 2), no change in total Kv4.2 channels was evident at either the 1 or 3 h time point after kainate in whole-cell hippocampal homogenates (Fig. 6b; input, upper panels; densitometry not shown).

Behavioral SE is associated with a decrease in Kv4.2 localization at the surface membrane in hippocampus

We performed surface biotinylation studies in order to further evaluate localization changes in Kv4.2 proteins following SE. Using surface biotinylation of hippocampal slices prepared from animals with SE and controls we identified a significant decrease in the ratio of the surface to total Kv4.2 localization in slices from SE animals ($p < 0.05$, control: $n = 3$; SE: $n = 4$; Fig. 7). These findings suggest that there is a decrease in expression of Kv4.2 channel proteins at the cell surface membrane in hippocampus during prolonged SE.

Discussion

The findings presented here suggest that there are dynamic alterations in Kv4.2 channel post-translational modifications and post-synaptic localization during SE. Furthermore, we demonstrate that ERK activation during SE couples to phosphorylation of Kv4.2 in the hippocampus. The increases in ERK-phosphorylated Kv4.2 are reflected in the synaptosomal cellular compartment, which is expected to functionally down-regulate the A-current. An additional finding in our studies is that the pool of Kv4.2 channels is reduced in the synaptosomal and surface membrane subcellular compartments after prolonged SE. Given the critical role of Kv4.2 channels and its underlying A-current in hippocampal dendrites, we predict that the changes in Kv4.2 described here are likely to contribute to hyperexcitability and potentially recurrent seizures associated with SE.

The coupling of ERK activation to Kv4.2 phosphorylation during SE is predicted to have a significant impact on excitability in hippocampal neurons. ERK pathway activation leads to an increase in the amplitude of the B-APs in CA1 pyramidal cell dendrites, which is hypothesized to be via direct phosphorylation of Kv4.2 by ERK (Yuan *et al.* 2002). Indeed, studies *in vitro* using expression of phosphosite mutants at the mapped ERK sites within Kv4.2 compared with wildtype Kv4.2 channels in Xenopus oocytes resulted in functional alterations in Kv4.2 currents (Schrader *et al.* 2006). Phospho-mimetic mutation of the ERK sites resulted in an overall down-regulation of Kv4.2 currents, a depolarizing shift in channel activation, and slower recovery from inactivation compared with wildtype Kv4.2 currents. These findings were comparable with those described for the native A-current in hippocampal CA1 dendrites, where ERK pathway inhibition led to a hyperpolarizing shift in the activation curve for these currents (Watanabe *et al.* 2002). Work in the oocyte expression system also revealed differential functional effects of the three ERK sites within Kv4.2. Notably, phospho-mimetic mutation at the threonine 602 and 607 sites led to functional effects similar to that seen when all three sites were mutated to aspartate to mimic phosphorylation. Interestingly, phospho-mimetic mutation of the serine 616 site alone had the opposite effect. However, in the presence of phosphorylation at all three sites the effect of the threonine 602 and 607 sites seemed to override the functional effects of the serine 616 site and was similar to that identified when all three sites were mutated to aspartate (Schrader *et al.* 2006). Interestingly, our results using antibodies generated against the individual ERK phosphorylation sites within Kv4.2, revealed increases at all three ERK phosphorylation sites (greatest at threonine 602); thus, based on the results described above we predict that down-regulation of the A-current would be the predominant effect.

The mechanism underlying the decrease in Kv4.2 channels in the synaptosomal compartment in hippocampus following SE is currently undefined. Given that this change occurred relatively early following kainate-induced seizures and that Kv4.2 levels were unchanged in total cellular lysates and membranes, we hypothesize that there is altered trafficking or local translation of Kv4.2 channels during prolonged SE rather than a change in genomic transcriptional regulation of Kv4.2. Rapid alterations in $GABA_A$ receptor subunit trafficking have been reported in models of SE (Goodkin *et al.* 2005, 2007). $GABA_A$ receptors are regulated by PKA and

endocytosis is clathrin dependent (Kittler and Moss 2003; Terunuma *et al.* 2008). Recent work from Kim *et al.* (2007) described clathrin-dependent internalization of Kv4.2 channels in cultured hippocampal neurons during glycine-induced long-term potentiation. In this model, Kv4.2 internalization was associated with increased synaptic plasticity and dendritic excitability. It is possible that the alterations in Kv4.2 synaptosomal and surface expression during SE reflect alterations in clathrin-mediated endocytosis and subsequent targeting for degradation.

While A-current regulation is best characterized in hippocampal area CA1, Kv4.2 channel proteins are localized to dendritic fields within area CA3 and dentate gyrus (Maletic-Savatic *et al.* 1995; Rhodes *et al.* 2004), where they may contribute to the A-current and regulate dendritic excitability in these regions. Our findings from whole-cell membrane preparations obtained from subdissected hippocampus reveal that increased ERK activation and phosphorylation of Kv4.2 are evident in each of these regions. The changes in synaptosomal levels of ERK-phosphorylated Kv4.2 and total Kv4.2 channels were evident in preparations harvested from whole hippocampus. The evaluation of whether there are subfield-selective changes in the regulation of synaptosomal Kv4.2 is a potentially important area for future investigations.

Alterations in Kv4.2 regulation and dendritic excitability have been demonstrated in epileptic animals (Bernard *et al.* 2004). There was a decrease in Kv4.2 protein and mRNA expression in hippocampal area CA1 from epileptic animals (Bernard *et al.* 2004). This study also revealed an increase in ERK phosphorylation of Kv4.2 in area CA1 from epileptic animals. Parallel physiology studies in the epileptic animals revealed an increase in the amplitude of the B-AP in the CA1 dendrites compared with controls. The alterations in Kv4.2 in the epileptic animals were thought to underlie this finding. In both the pilocarpine and kainate chemoconvulsant models of epilepsy, a prolonged episode of SE is required for the development of long-term, spontaneously recurring seizures. Thus, alterations in Kv4.2 channel regulation and expression are evident at both early and late time points of epileptogenesis in chemoconvulsant models. However, during chronic epilepsy with decreased Kv4.2 mRNA levels in area CA1 of hippocampus it appears that there is a change in Kv4.2 transcriptional regulation or mRNA processing. During SE, Kv4.2 mRNA levels are not significantly altered (unpublished data). Thus, the mechanisms involved in Kv4.2 expression may be different at the early and late time points of epileptogenesis.

We were interested in determining whether Kv4.2 represents a molecular locus for the convergence of multiple signaling pathways involved in the regulation of hippocampal excitability. We have previously mapped phosphorylation sites within Kv4.2 for PKA (Anderson *et al.* 2000). Thus, we evaluated PKA activation and phosphorylation of Kv4.2 channels during SE. However, our results suggest that while the PKA pathway is activated during SE, it does not appear to couple to direct PKA phosphorylation of Kv4.2 channels. It is possible that PKA couples to upstream activators of ERK in SE through B-Raf activation (Sweatt 2001; Rueda *et al.* 2002) and thus indirectly regulates the phosphorylation of Kv4.2 through ERK. Like the ERK pathway, there are many other candidate effector molecules for PKA in SE.

In our studies correlating behavioral with electrographic seizure activity, we found a significant discrepancy between the early and late electrographic and behavioral seizure events. The onset of electrographic seizure activity and SE occurred significantly earlier than the onset of the behavioral seizures and SE parameters (Racine scale). These findings were taken into account when investigating the time course of ERK activation and Kv4.2 phosphorylation following kainate administration. The lack of correlation of the behavioral and electrographic seizures should be a consideration when evaluating SE-induced molecular and physiological events.

In the time course studies, onset of non-convulsive electrographic seizures and SE correlated with the time course of the biochemical changes. ERK activation was significantly increased in all areas of the hippocampus by 30 min after kainate administration, which was after the onset of electrographic seizures but before the development of behavioral seizures and SE. An appreciable increase in ERK triply-phosphorylated Kv4.2 was not evident until the onset of electrographic SE (45 min after kainate) in CA1 and dentate gyrus. However, in CA3 significant increases in phospho-Kv4.2 were not evident until the later behavioral SE time point (180 min). The molecular basis of the lack of temporal correlation of ERK activation and phosphorylation of Kv4.2 is not understood at this time. Future investigations will be directed at this question.

A number of previous studies have suggested that there are modifications in ERK pathway signaling early following convulsant stimulation (Berkeley *et al.* 2002; Otani *et al.* 2003). However, the downstream molecular targets of the ERK pathway in SE models are not well defined. While there are a number of potential targets for ERK in hippocampus, our studies provide evidence for Kv4.2 as an ERK pathway target in the hippocampus during kainate-induced SE. Through phosphorylation of Kv4.2, our findings reveal a direct mechanism whereby the ERK cascade could contribute to the hyperexcitability of hippocampal neurons during SE. Thus, therapeutic interventions targeting the mechanisms defined here may prove useful during acute seizures and SE.

Acknowledgements

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Abbreviations used

B-AP, back-propagating action potential; ERK 1/2, extracellular signal-regulated kinases; i.p., intraperitoneal; PKA, protein kinase; RIPA, radioimmunoprecipitation assay; SE, status epilepticus.

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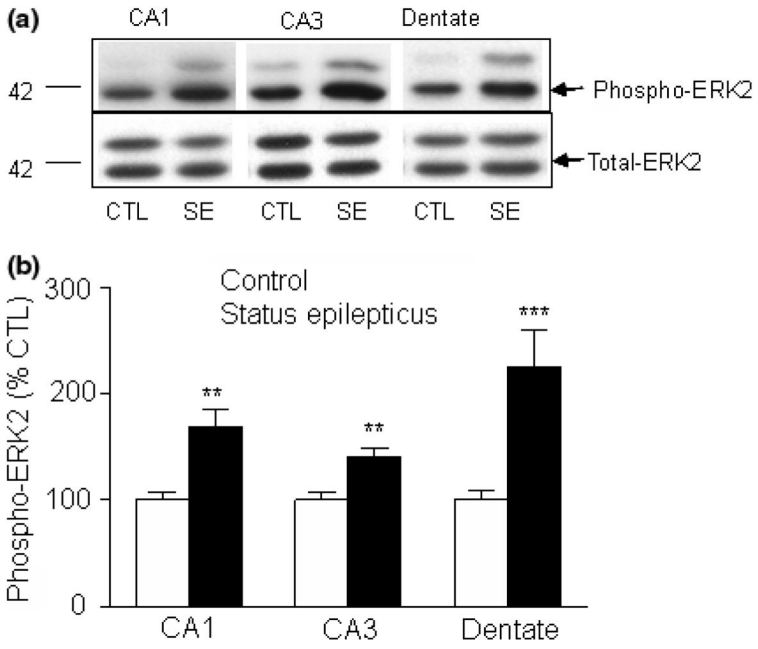

Fig. 1.

Status epilepticus (SE) is associated with extracellular signal-regulated kinase (ERK) activation in hippocampus. Following a 1-h episode of kainate-induced (15 mg/kg i.p.) behavioral SE, the hippocampal areas CA1, CA3, and dentate gyrus were subdissected and homogenates prepared for western blotting to evaluate total and activated ERK. (a) Representative blots with homogenates from control (CTL) and SE animals probed with antibodies against the activated, dually phosphorylated form of ERK 1/2 (the p44 and p42 isoforms, respectively) (top panel) and total ERK 1/2 (lower panel) are shown. (b) The levels of phosphorylated-ERK2 were densitized for areas CA1, CA3, and dentate gyrus from CTL and SE animals. There is a significant increase in activated (phosphorylated) ERK2 in CA1, CA3, and dentate gyrus during SE compared with CTL (**$p < 0.01$; ***$p < 0.001$, $n = 5$). Error bars represent the standard error of the mean.

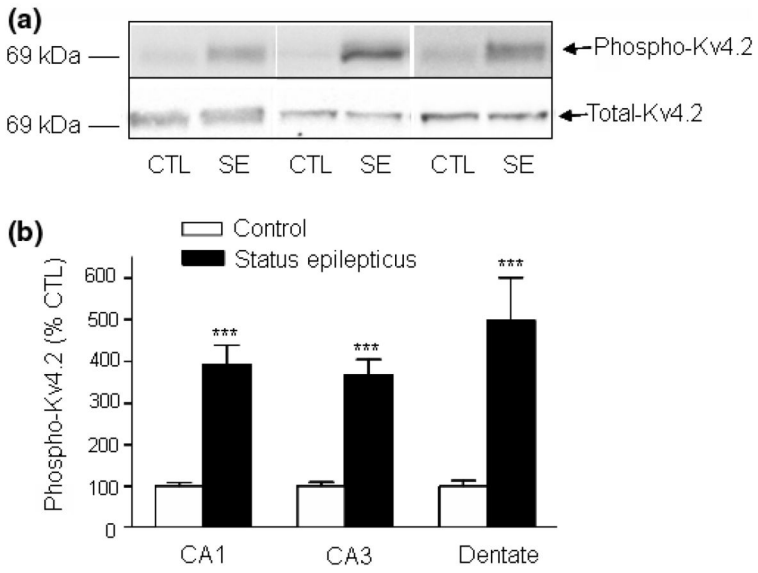

Fig. 2.

Status epilepticus (SE) is associated with an increase in extracellular signal-regulated kinase (ERK) phosphorylation of Kv4.2 in hippocampus. After 1-h of behavioral SE induced by kainate (15 mg/kg i.p.), hippocampi were subdissected and total cellular membranes prepared. Western blotting was performed with an antibody that recognizes Kv4.2 when triply phosphorylated at threonine 602 and 607 and serine 616 (ERK sites). (a) Representative blots are shown for CA1, CA3, and dentate gyrus that are probed with the triply phosphorylated Kv4.2 (upper panels) and the total Kv4.2 (lower panels) antibodies. (b) Densitometry for the blots probed with the triply phosphorylated Kv4.2 antibody is shown. There is a significant increase in ERK-phosphorylated Kv4.2 during SE in the hippocampal areas CA1, CA3, and dentate gyrus following SE compared with control (CTL; ***$p < 0.001$, $n = 5$). There were no significant changes in total Kv4.2 levels in membrane preparations during SE compared with controls (not shown).

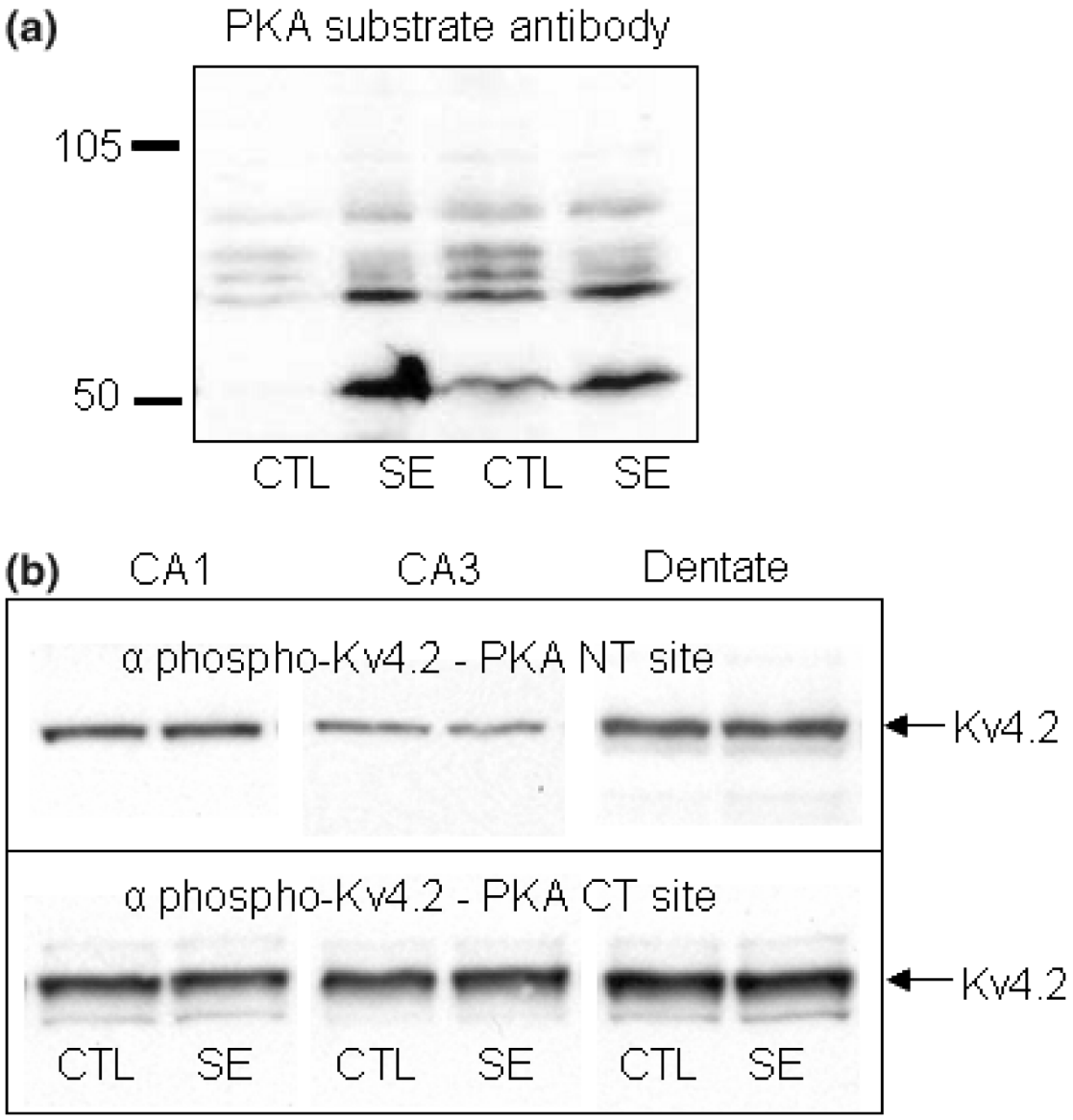

Fig. 3.

Protein kinase (PKA) activation in status epilepticus (SE) does not couple to increased phosphorylation at the PKA sites within Kv4.2. After 1-h of behavioral SE induced by kainate (15 mg/kg i.p.), the hippocampi were harvested and used to prepare total hippocampal or CA1, CA3, and dentate gyrus homogenates. The tissue was then prepared for western blotting of homogenate (a) and membrane preparations (b). (a) Representative blots probed with the PKA substrate antibody are shown for total hippocampal homogenates. Some bands in the hippocampal homogenates from the SE animals demonstrated an increase in immunoreactivity suggesting an increase in PKA activation in SE compared with controls (CTL). (b) Representative blots with CA1, CA3, and dentate gyrus membranes from CTL and SE animals probed with the phospho-selective antibodies against the PKA sites: threonine 38 – α-phospho-Kv4.2 – PKA NT (upper panel) and serine 552 – α-phospho-Kv4.2 – PKA CT (lower panel) are shown. PKA activation in SE did not couple to altered Kv4.2 phosphorylation at the PKA sites (densitometry not shown; not significant, $n = 4$).

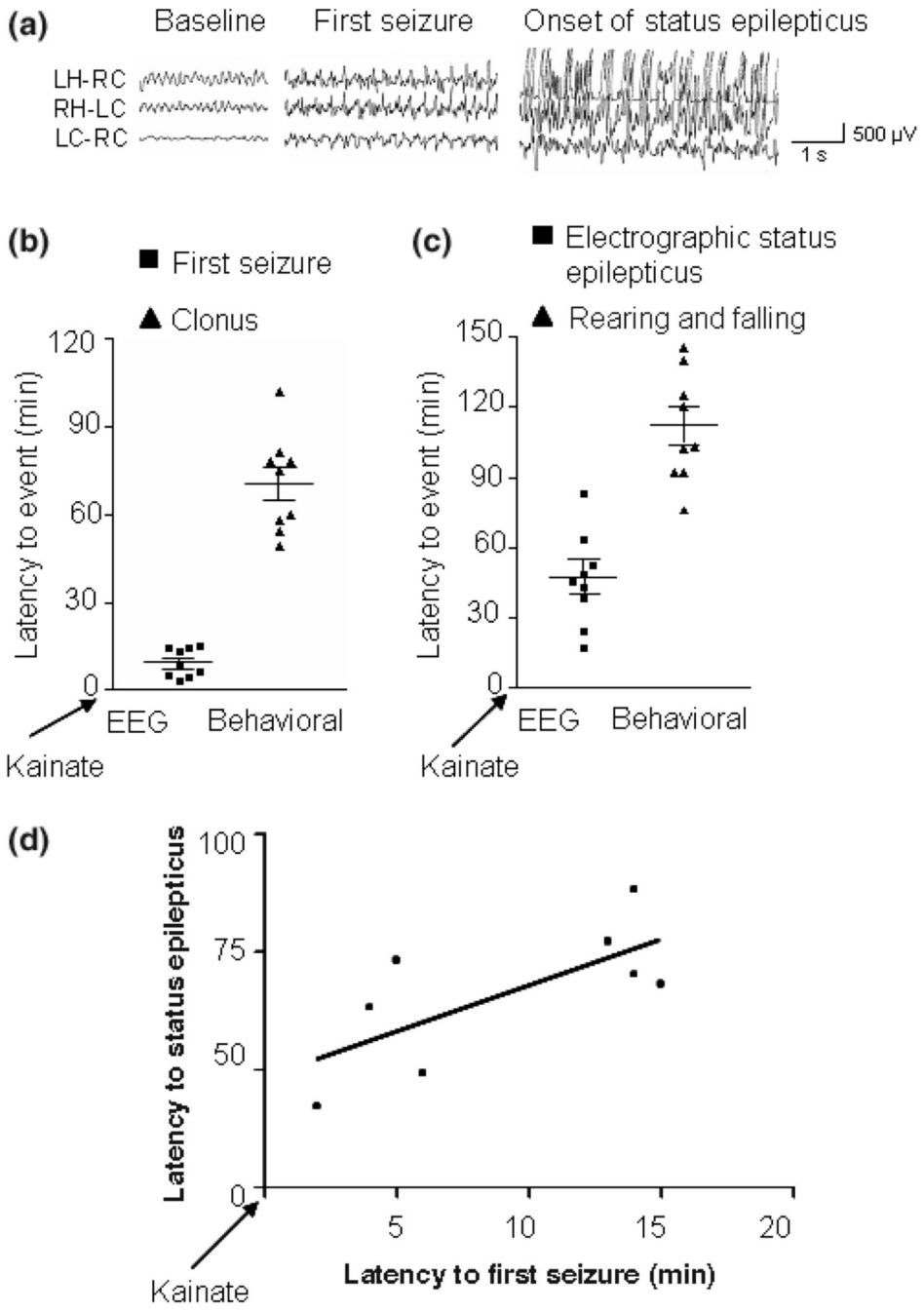

Fig. 4.

Video-EEG comparison of the latency to onset of electrographic versus behavioral seizures and status epilepticus (SE) following kainate administration. Kainate (15 mg/kg i.p.) was used to induce seizures and SE. A 30 min baseline video-EEG recording was performed prior to kainate or kainate vehicle (saline). The video-EEG recording was continued for 3–4 h following kainate administration. Video-EEG analysis to determine the onset of behavioral and electrographic seizures and SE was conducted independently by three investigators (RAH, AEA, and LFB). (a) Representative EEG traces are shown for baseline activity, the first electrographic seizure, and at the onset of electrographic SE. (b) Scatter plots demonstrate that the time to first electrographic seizure was significantly earlier than time to forelimb clonus.

The mean and standard error of the mean (SEM) are shown by the horizontal line and error bars, respectively ($p < 0.001$, $n = 8$). (c) The time to electrographic SE was significantly earlier than time to rearing and falling. The mean and SEM time are represented by the horizontal line and error bar, respectively ($p < 0.001$, $n = 9$). (d) Latency to first electrographic seizure was significantly positively correlated with latency to SE ($r = 0.645$, $p < 0.05$, $n = 8$). The line represents the line of best fit for the data points.

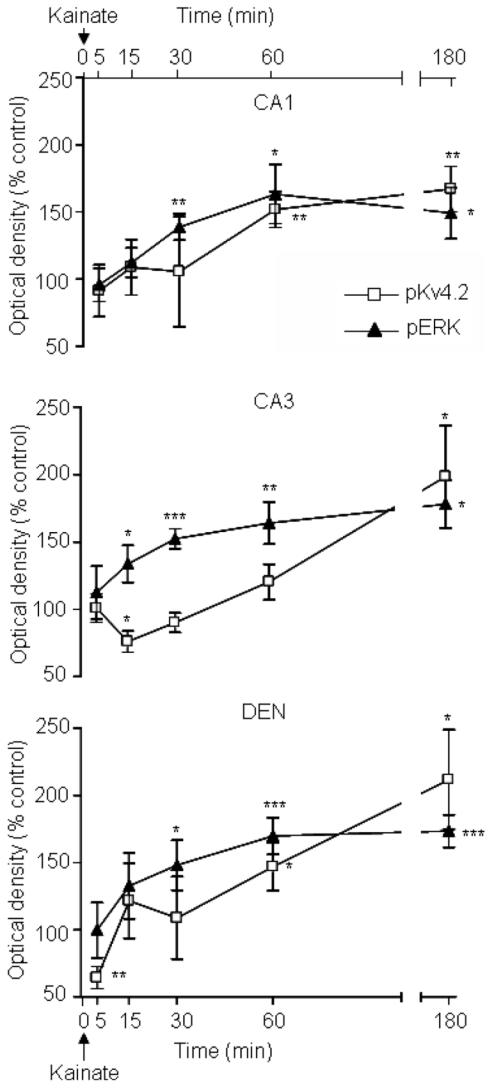

Fig. 5.

Time course of kainate-induced extracellular signal-regulated kinase (ERK) activation and phosphorylation of Kv4.2. The time course of kainate-induced ERK activation and phosphorylation of Kv4.2 was performed in areas CA1, CA3, and dentate subdissected from rats that were killed at five, 15, 30, 60, and 180 min following kainate (15 mg/kg i.p.) with a sample size of four per group per time point. Western blotting was performed using antibodies against activated, dually phosphorylated ERK1/2 and ERK-phosphorylated Kv4.2 (triply phospho-antibody). The time course for changes in activated, dually phosphorylated ERK and ERK phosphorylated-Kv4.2 (normalized to actin) are plotted for area CA1 (upper graphs), CA3 (middle graphs), and dentate gyrus (lower graphs). The data points represent the mean and the error bars represent the standard error of the mean (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, $n = 4$).

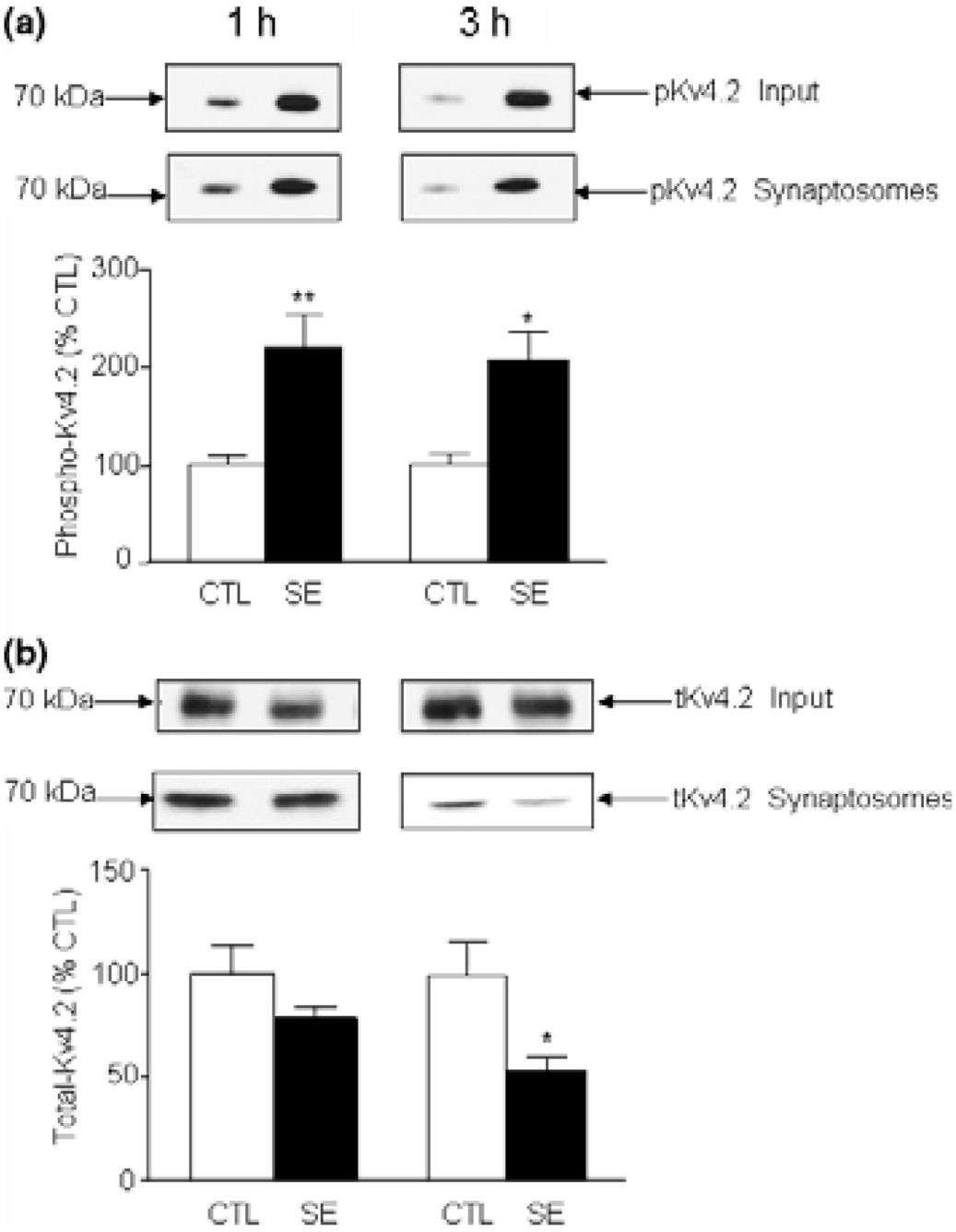

Fig. 6.

Status epilepticus (SE) is associated with increased extracellular signal-regulated kinase (ERK)-phosphorylated Kv4.2 and decreased total Kv4.2 channels in hippocampal synaptosomes. Animals were killed at 1 (early SE) and 3 h (prolonged SE) after administration of kainate (15 mg/kg i.p.). The hippocampi were used to prepare whole-cell homogenates (starting material for synaptosomes) and synaptosomes. Representative blots for both the input and synaptosomes were probed with the triply phospho-Kv4.2 (ERK sites) and total Kv4.2 antibodies (pKv4.2 and tKv4.2, respectively). The bar graphs below the blots summarize the 1 h (left) and 3 h (right) time points for the western blotting using pKv4.2 and tKv4.2 antibodies of synaptosomes from the SE and vehicle-treated control (CTL) animals processed in parallel.

(a) Representative blots are shown for triply phospho-Kv4.2 at the 1 h (left panels) and 3 h time points (right panels) for both input and synaptosomal preparations. Densitometry reveals that there is a significant increase in ERK-phosphorylated Kv4.2 1 h (early SE) (**$p < 0.01$, $n = 5$; left graph) and 3 h (prolonged SE) after kainate stimulation compared to controls (*$p < 0.05$, $n = 5$; right graph). (b) Representative blots probed with the total Kv4.2 antibody from input and synaptosomes prepared from tissue harvested at the 1 and 3 h time points following kainate are shown. Prolonged SE is associated with a reduction in total Kv4.2 levels in synaptosomes compared to controls (*$p < 0.05$, $n = 5$; right graph), while no change in total Kv4.2 levels is evident at the onset of SE (left graph). No change in total Kv4.2 was found using homogenate preparations at either time point (top panels). Error bars represent the standard error of the mean.

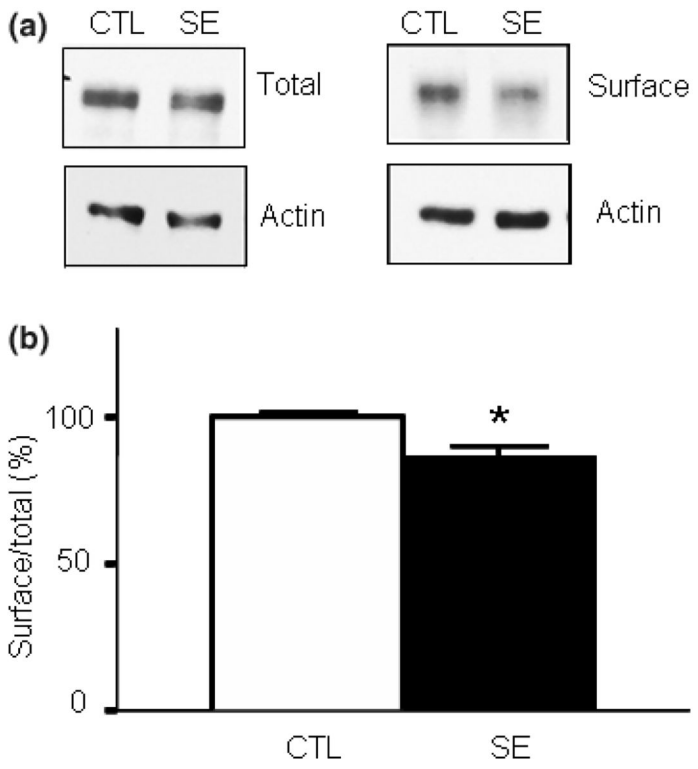

Fig. 7.

Status epilepticus (SE) is associated with a decrease in Kv4.2 localization at the surface membrane in hippocampus. Hippocampal slices were prepared from rats with kainate-induced SE and vehicle-treated controls (CTL). Surface biotinylation was performed. (a) Immunoblots probed with Kv4.2 followed by actin are shown for total compared with surface-biotinylated samples. (b) Summary data demonstrate a decrease in the ratio of surface to total Kv4.2 in SE compared with control (*$p < 0.05$; CTL: $n = 3$; SE: $n = 4$).