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Mechanisms of seizure-induced 'transcriptional channelopathy' of hyperpolarization-activated cyclic nucleotide gated (HCN) channels

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Abstract

Epilepsy may result from abnormal function of ion channels, such as those caused by genetic mutations. Recently, pathological alterations of the expression or localization of *normal* channels have been implicated in epilepsy generation, and termed 'acquired channelopathies'. Altered expression levels of the HCN channels--that conduct the hyperpolarization-activated current, I_h--have been demonstrated in hippocampus of patients with severe temporal lobe epilepsy as well as in animal models of temporal lobe and absence epilepsies. Here we probe the mechanisms for the altered expression of HCN channels which is provoked by seizures. In organotypic hippocampal slice cultures, seizure-like events selectively reduced HCN type 1 channel expression and increased HCN2 mRNA levels, as occurs *in vivo*. The mechanisms for HCN1 reduction involved Ca^{2+}-permeable AMPA receptors-mediated Ca^{2+} influx, and subsequent activation of Ca^{2+}/calmodulin-dependent protein kinase II. In contrast, upregulation of HCN2 expression was independent of these processes. The data demonstrate an orchestrated program for seizure-evoked transcriptional channelopathy involving the HCN channels, that may contribute to certain epilepsies.

Keywords

Seizure; Epilepsy; I_h; Hyperpolarization; Ion channel; HCN channel; Animal model; Epileptogenesis; AMPA channels; Calcium; CaM Kinase II; Organotypic slice culture; Hippocampus

Introduction

Mutations in over 70 ion channel genes lead to human diseases including cardiac arrhythmia, ataxia, chronic pain, neuropathy and epilepsy (Noebels, 2003; Jentsch et al., 2004; Waxman et al., 2006). This fact has given rise to the concept of 'channelopathy' as a basis for neuronal dysfunction that culminates in disease (Waxman, 2001; Noebels, 2003). More recently, it has been increasingly recognized that abnormal expression levels of otherwise intact ion channels

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(Su et al., 2002; Ellerkmann et al., 2003; Peters et al., 2005), or channel expression in the 'wrong' location or cells, might also promote neurological disorders. These findings have been termed "transcriptional (or acquired) channelopathies" (Waxman, 2001). Focusing on Epilepsy, long-lasting changes in the expression levels of chloride channels ($GABA_A$ receptors; Brooks-Kayal et al., 1998), sodium channels (Ellerkmann et al., 2003), calcium channels (Su et al., 2002) and the hyperpolarization-activated cationic (HCN) channels (Chen et al., 2001; Brewster et al., 2002; Bender et al., 2003) have been found to promote pathological brain activity. Therefore, better understanding of the mechanisms that lead to abnormal expression patterns of these ion channels is important, because it should provide molecular targets for intervention in the pathological disease process.

The HCN channels conduct the hyperpolarization-activated cationic current, I_h, an important regulator of resting membrane potential of neurons (Maccaferri et al., 1996; Lupica et al., 2001) and their responses to network activity (Maccaferri et al., 1996; Magee, 1999; Poolos et al., 2002). The properties of I_h are governed, at least in part, by the subunit composition of HCN channels, that are encoded by a family of genes (Ludwig et al., 1998; Santoro et al., 2000; Santoro and Baram, 2003). In human and rodent hippocampus and cortex, two isoforms (HCN1 and 2) predominate (Poolos et al., 2002; Robinson et al., 2003; Brewster et al., 2007). HCN1 channels conduct a relatively fast-kinetics current with modest cAMP gating, consistent with currents recorded in hippocampal pyramidal cells and CA1 interneurons where HCN1 expression is high (Magee, 1999; Lupica et al., 2001; Poolos et al., 2002; Vasilyev and Barish, 2002; Surges et al., 2006; Brewster et al., 2002, 2007). In contrast, the HCN2 gene encodes a channel with slower kinetics and robust cAMP-evoked shifts in voltage dependence (Santoro et al., 2000). Changes in HCN channel expression have been found in surgical specimens from patients with temporal lobe epilepsy and severe hippocampal sclerosis (Bender et al., 2003). As mentioned above, in animal models, the epileptogenic process following long experimental febrile seizures appears to involve long-lasting downregulation of the HCN1 isoform (Brewster et al., 2002; Dubé et al., 2006). This is associated with altered properties of I_h, increasing the probability of rebound depolarization and repetitive neuronal firing in response to hyperpolarizing input at physiological frequencies (Chen et al., 2001). HCN1 expression is also abnormal in models of absence epilepsy, specifically in thalamus (Budde et al., 2005; Kuisle et al., 2006) and cortex (Di Pasquale et al., 1997; Strauss et al., 2004). Here we examine the molecular mechanisms that underlie these pathological expression patterns.

Materials and Methods

Animals

Experimental procedures were approved by the University of California Irvine Animal Care Committee, and were carried out in accordance with NIH guidelines. Sprague-Dawley derived rats were used and housed as described (Brewster et al., 2002, 2007).

Organotypic slice cultures and experimental agents

Hippocampal slice cultures were prepared and maintained using the interface technique as previously described (Chen et al., 2004; Bender et al., 2007). Briefly, 400 μm hippocampal slices from P8 rat pups were collected in cold preparation buffer, placed on moist membrane inserts in 6-well plates filled with 1 ml culture medium (50% Minimal Essential Medium, 25% Hank's balanced salt solution, 20% inactivated horse serum, 30 mM HEPES, 30 mM glucose, 3 mM glutamine, 0.5 mM ascorbic acid, 1 mg/ml insulin, 5 mM $NaHCO_3$, pH 7.3) and incubated in humidified, CO_2-enriched atmosphere at 36°C. Pairs of adjacent slices (sister cultures) were compared (used for control and experimental conditions). Seizure-like events were induced after 3 days *in vitro* by incubating cultures for 3 hours in medium containing kainic acid (KA 6 μM; Sigma, St. Louis, MO). Seizure-like events were terminated by returning

cultures to normal medium, resulting in cessation of epileptiform discharges (Routbort et al., 1999). Controls were treated identically, but media were devoid of convulsants. Calcium/calmodulin-dependent protein kinase II (CaM Kinase II) specific inhibitors included KN-93 (10 μM), KN-62 (10 μM) or autocamtide-2 related inhibitory peptide II (AIP-II, 10 μM; all Calbiochem, San Diego, CA). They were applied together with the excitant or immediately after the termination of the seizure-like events, and maintained for 24 and 21 hours, respectively. Inhibitors were added also to some control groups, to consider their potential effects on basal HCN channel expression. A protein kinase C (PKC) specific blocker, calphostin C (1 μM; Calbiochem) as well as a selective Ca^{2+}-permeable AMPA-receptor blocker, 1-naphthyl-acetyl spermine (NASPM, 100 μM; Sigma, St. Louis, MO) or a selective NMDA-receptor antagonist APV (40 μM, Tocris, Ellisville, MO), were similarly used. Cultures were harvested 48 hours or one week after KA exposure, fixed in 4% buffered para-formaldehyde (PFA), cryoprotected and frozen for *in situ* hybridization (ISH) and immunocytochemistry (ICC), or frozen on powdered dry ice for Western blot analyses.

Extracellular recordings

Seizure-like events were induced after 3 days *in vitro* by incubating cultures for 3 hours in medium containing 6 μM KA. In pilot studies, KA and low [Mg^{++}] both provoked seizure-like events within minutes of transfer of the cultures to the convulsant medium. Epileptiform discharges ceased within 2-3 minutes of removal of the cultures to media with normal [Mg^{++}] or lacking KA (Simeone T, unpublished, and Routbort et al., 1999, respectively). To examine these *in vitro* seizure-like events, selected slices were subjected to extracellular field recording. This was carried out on slices perfused with pre-warmed (35°C), oxygenated (95% O_2 / 5% CO_2) control medium, or with medium containing 6 μM KA. Field potentials were recorded in CA3 using conventional techniques as described previously (Dubé et al., 2000).

In situ hybridization (ISH)

Quantitative analyses of hippocampal HCN1&2 mRNA levels were accomplished as described previously (Brewster et al., 2002, 2007). Briefly, cultures were harvested 48 hours or one week after KA exposure, fixed in 4% buffered PFA, cryoprotected and frozen. Cultures were sectioned (20 μm) and slide mounted prior to the procedure. ISH was carried out as described elsewhere (Brewster et al., 2002, 2007, Bender et al., 2003), using antisense ^{35}S-cRNA probes, with the minor modification that specific activity of probes was 1.5- 4.2 × 10^9 cpm/μg.

Western blot procedures

Western blots for HCN channels and the GluR2 subunit of the AMPA type glutamate receptors were performed and analyzed as described (Brewster et al., 2005, 2007) pooling 2 slice cultures, from the 2 hippocampi of a single rat, for each sample. Briefly, samples were centrifuged at 1,000 × g, the supernatant was centrifuged at 16,000 × g and the membrane fraction pellet was resuspended in artificial cerebrospinal fluid. Protein concentration was determined (Bio-Rad, Hercules, CA) and equal amounts diluted in Laemmli buffer and separated on SDS-PAGE gels. Care was taken to include samples from all groups on a single gel. Immunoreactive bands were visualized using chemiluminescence. Values are expressed as optical density (OD) of the HCN-immunoreactive bands (× 100) divided by the optical density of the actin band, to account for potential differences in loading of the samples in the gel (n=4-5 cultures per group). All the values are expressed as means +/- SEM (Brewster et al., 2005, 2007). Antisera used for Western blots included rabbit anti-HCN1 or anti-HCN2 serum (1:500 each; Chemicon, Temecula, CA, and Alomone, Jerusalem, Israel, respectively), rabbit anti GluR2(3) (Cat. # 07-598; 1:2,000; Chemicon) and rabbit anti-actin (1:60,000; Sigma, St. Louis, MO; as a loading control). Antibody specificity was evaluated using mice lacking the appropriate HCN isoform.

Immunocytochemistry (ICC)

ICC was performed as described previously (Chen et al., 2004; Brewster et al., 2007). Briefly, free-floating brain or culture sections (40 μm) were collected in PBS + 0.3% Triton-X100 (PBS-TX), pre-incubated for 1 hour with 3% normal goat serum/PBS-TX, followed by incubation with polyclonal rabbit anti-HCN1 (1:2,500; Chemicon), rabbit anti-HCN2 (1:1,500; courtesy Dr. R. Shigemoto) or rabbit anti-c-fos (1:30,000; Calbiochem). After application of 2^{nd} antisera, binding was visualized using diamino-benzidine (DAB), using the avidin-biotin technology (Brewster et al., 2007). Specificity of the HCN channel antisera was evaluated by absence of signal in tissue derived from mice lacking the tested isoform (Brewster et al., 2007).

TUNEL-labeling

The procedure was generally carried out to the manufacturer's (Promega) instructions. Briefly, cultures were exposed 6 μM KA, (a dose used to elicit the seizure-like events), or 50 μM, a concentration known to provoke cell death (a “positive control”; Liu et al., 2001) for 3 hours, and fixed 24 or 48 hours later, sectioned (50 μm), mounted and dried. Sections were transferred to 0.1M Na-citrate (pH 6.0), treated with Proteinase K (20 μg/ml; 15 min), post-fixed and incubated with terminal transferase (TdT) and biotin-16-UTP (both from Promega, Madison, WI). TdT-labeled, apoptotic cells were visualized using the avidin-biotin procedure, with DAB as a reporter (see above). Apoptotic cells were counted without knowledge of treatment (“blindly”), and are presented as TUNEL-positive cells/mm^2 pyramidal cell layer.

Data acquisition and analyses

Data acquisition and analysis were performed without knowledge of treatment group. Quantification of *in situ* hybridization signals were carried out as described (Brewster et al., 2002, 2005, 2007) on sections run concurrently. Signal linearity was ascertained using ^{14}C standards. Western blot data acquisition and analysis were accomplished by measuring optical density of the HCN1, HCN2 or GluR2 immunoreactive bands (Brewster et al., 2005). Data were statistically analyzed with unpaired (*in vivo*) or paired (*in vitro*) t-tests, or single measure ANOVA, as required, using the GraphPad Prism software (San Diego, CA).

Results

HCN channel expression in organotypic hippocampal cultures recapitulates the in vivo pattern

Understanding the molecular events by which seizures coordinately reduce the expression of the HCN1 isoform and increase HCN2 channel expression requires a controllable *in vitro* model. The organotypic slice culture preserves hippocampal circuitry, permitting generation of seizure-like events *in vitro* (Gutierrez et al., 1999; Routbort et al., 1999). We first examined whether basal expression levels of the HCN1 and HCN2 channel isoforms in the cultures (explanted on P8 then kept *in vitro* for 3-4 days; DIV 11-12) approximated the *in vivo* pattern at an equivalent age (P10-12). HCN1 and HCN2 channels were expressed in the hippocampus *in vitro*, and the neuroanatomic distribution of their mRNAs resembled the *in vivo* patterns (Fig 1C vs 1A for HCN1 channels; Fig 1D vs 1B for the HCN2 isoform). Channel protein expression also mirrored the patterns found in intact hippocampus at an equivalent maturational state (Brewster et al., 2005, 2007): HCN1 localized preferentially to dendritic domains of CA1 pyramidal cells (arrows, Fig 1E), whereas at this age, HCN2 channels were still mainly confined to the principal cell layers (arrows, Fig 1F; see also Brewster et al., 2007).

Seizure-like events can be generated in the organotypic slice culture

Seizure-like events were generated using the glutamate receptor agonist KA (Routbort et al., 1999). Epileptiform discharges were examined using field recording, and neuronal activation was evaluated also by measuring the immediate early gene c-fos (Labiner et al., 1993). Using field recording, no epileptiform discharges were detected in normal medium, and were apparent upon application of 6 μM KA (Fig 1G). The epileptiform activity was characterized by an ictal-like burst (~30-60s) of population events that occurred at a ~30 Hz frequency within the burst (Fig 1G, bottom left trace). Near the end of the burst the events degenerated into low amplitude gamma oscillations (~30 Hz; Fig 1G, bottom right trace) and eventually ceased altogether. After the ictal-like burst, spontaneous interictal-like events or single population events occurred with a frequency of ~0.2 Hz. These seizure-like events cease within minutes when cultures are returned to normal medium (Routbort et al., 1999). Expression of the immediate early gene c-fos was also activated in principal cell layers of 7/7 cultures harvested immediately after a 3-hour exposure to KA. Fos expression did not occur in control cultures (0/5; Fig 1H). The relationship of fos expression to seizure-like events was supported by the induction of this gene also in cultures (5/5) exposed to low extracellular magnesium (not shown). In addition, the dose of KA employed here to generate seizure-like events did not provoke cell death, as measured at several time-points (Fig 1I), consistent with findings by others (Routbort et al., 1999).

Seizure-like events regulate HCN1 and HCN2 channel expression differentially

In vivo, 20-minute and 3-hour bouts of seizure activity down-regulate HCN1 mRNA and protein levels, and enhance HCN2 gene expression (Brewster et al., 2002, 2005), and these changes are detectable by 48 hours. The *in vitro* system recapitulated these effects: seizure-like events provoked by KA reduced HCN1 mRNA significantly in CA1 and CA3 (Fig 2A,B), accompanied by a substantial (47%) reduction of protein levels (KA 129.0 ± 43.8 OD/Actin vs. control 242.3 ± 55.0 OD/Actin; Fig 2C). HCN2 mRNA expression was upregulated (Fig 2D-E), but protein levels were not significantly changed (KA 113.9 ± 22.9 OD/Actin vs. control 98.9 ± 14.9 OD/Actin; Fig 2F), in line with previous finding *in vivo* (Brewster et al., 2005). The transcriptional regulation of HCN channel expression persisted *in vitro* for at least one week. Thus, HCN1 mRNA levels in area CA1 and area CA3 were 79 ± 2.9% and 82 ± 3.9% of control levels, respectively ($p < 0.01$; n=9 per group), in line with the enduring downregulation of HCN1 mRNA and protein expression found *in vivo* (Brewster et al., 2002, 2005). HCN2 mRNA levels were 122 ± 2.8% and 120 ± 1.9% of controls in CA1 and CA3 respectively ($p < 0.0001$; n=7 per group). Thus, seizure-like activity *in vitro* reproduced the *in vivo* effects of seizures provoked by KA or hyperthermia, rendering the *in vitro* system suitable for probing the underlying mechanisms.

Activation of CaM Kinase II is required for downregulation of HCN1 expression by seizure-like activity, but not for upregulation of HCN2 mRNA expression

Bursts of neuronal activity provoke many cellular changes, including a frequent alteration of intracellular Ca^{2+} (Hardingham and Bading, 1999). Ca^{2+} influx may involve several routes, and often leads to protein phosphorylation via the Ca^{2+}-activated CaM Kinase II, that makes up to 2% of all proteins in hippocampal neurons (Sola et al., 2001). Therefore, we studied the role of CaM Kinase II in the mechanisms by which seizure-like events regulated HCN channel expression.

Incubating the hippocampal cultures with a moderate dose of the selective CaM Kinase II blocker KN-93 (10 μM) abrogated the reduction of HCN1 mRNA levels provoked by seizure-like events (Fig 3A). The blocker alone did not influence HCN channel expression (Fig 3A), and did not lead to cell death (not shown). These data support the notion that seizure-like activity-dependent HCN1 downregulation is mediated by activation of CaM Kinase II. In

contrast to HCN1, the cascade of molecular events resulting in increased HCN2 channel expression did not require the actions of CaM Kinase II, because this increase still occurred in the presence of KN-93 (Fig 3B). The blocker itself did not significantly influence basal expression levels of HCN2 mRNA. Thus, the requirement for CaM Kinase II activation was specific to seizure-like activity-induced expression changes of the HCN1 isoform.

Persistent activation of CaM Kinase II following, rather than during, the seizure-like activity bursts is needed for regulation of HCN1 channel expression

Abrogation of seizure-induced downregulation of HCN1 mRNA expression by application of KN-93 together with KA might be interpreted as resulting from dampening of the seizure-like activity by the concurrent blocking of CaM Kinase II activation. To eliminate this possibility, we measured the effects of seizure-like events on HCN1 expression in hippocampi where KN-93 was applied after the seizure-like activity and maintained for 21 hours (schematic; Fig 4). This approach still abolished the downregulation of HCN1 expression (Fig 4A), whereas blocking CaM Kinase II activation for 4 hours only (3 hours concurrently with KA) did not interfere with their ability to reduce HCN1 mRNA levels (not shown). Similar results were obtained with two additional blockers, KN-62 (not shown), and the structurally distinct AIP-II (Fig 4B). In contrast, calphostin C, an inhibitor of PKC, did not prevent seizure-like activity-induced decrease of HCN1 expression (Fig 4C). These data suggest that enduring and selective activation of CaM Kinase II was involved in the mechanisms of this regulation of HCN1 gene expression.

Blocking Ca^{2+}-permeable AMPA channels abrogates the effects of in vitro seizures on HCN1 channel expression

The fact that CaM Kinase II blockade lasting for many hours was required to interfere with seizure-induced downregulation of HCN1 expression, suggested that the seizure-like activity led to protracted activation of this enzyme, starting a few hours after the activity. This raised the possibility that CaM Kinase II was activated through Ca^{2+}-entry via newly formed channels, and specifically through Ca^{2+}-permeable AMPA receptors. Ca^{2+}-permeable AMPA receptors are known to be formed newly after seizures, as shown in several models (e.g., Friedman et al., 1994; Sanchez et al., 2001). This occurs by seizure-induced reduction of GluR2 mRNA and protein levels, leading to formation of GluR2-lacking AMPA channels that are permeable to Ca^{2+}. Therefore, we queried whether seizure-like activity reduced GluR2 protein levels in the organotypic slice cultures by using Western blots (Fig 5). Consistent with mRNA data (Richichi et al., 2005), GluR2 protein levels were significantly reduced (30% and 47% respectively) in cultures exposed to KA and quantified 24 and 72 hours later (Fig 5A,B; $p < 0.05$).

If Ca^{2+} entry via Ca^{2+}-permeable AMPA receptors is a key event in the process by which seizures reduces expression of the HCN1 gene, then blocking these receptors should prevent this effect. Therefore, we investigated whether, in the current preparation, Ca^{2+} influx via Ca^{2+}-permeable AMPA receptors was required for down-regulation of HCN1 channel expression by seizure-like events. The selective Ca^{2+}-permeable AMPA-receptor blocker NASPM, (100 μM; schematic, Fig 6), abolished the seizure activity-induced downregulation of HCN1 mRNA expression. The time-course of NASPM actions, over many hours, was consistent with an effect on newly-generated Ca^{2+}-permeable AMPA-receptors. A similar treatment with the NMDA receptor blocker APV (40 μM) did not modify the downregulation of HCN1 by seizure-like events, suggesting that the latter receptors were not involved in this molecular pathway.

Discussion

The principal findings of these studies are: (1) As found *in vivo*, seizure-like activity *in vitro* regulates the expression of the HCN channels at the transcriptional level, differentially modulating the expression of the HCN1 and HCN2 isoforms. (2) Reduction of HCN1 channel expression involves activation of CaM Kinase II and requires Ca^{2+}-entry via newly formed Ca^{2+} permeable AMPA receptors. (3) Enduring CaM Kinase II activation during the hours following the seizure-like events is required for the regulation of HCN1 channels expression, consistent with activation of the enzyme by Ca^{2+} entry through newly formed Ca^{2+}-permeable AMPA channels. (4) The selectivity of the molecular pathway defined here is evident from the fact that it is not involved in seizure-like activity-evoked modulation of HCN2 channel expression. This further suggests that the "transcriptional channelopathy" of the different HCN channel isoforms involves multiple intra-cellular pathways.

Normal and pathological neuronal activity can be modulated at different time scales, and ion channels are major contributors to this modulation. The current conducted by the HCN channels, I_h, is rapidly and transiently modified by cAMP-mediated gating (Wang et al., 2002; Shin et al., 2004), altered channel phosphorylation (Zong et al., 2005; Poolos et al., 2006) and additional, undefined processes (Van Welie et al., 2004; Fan et al., 2005). Whereas these modulations are transient and reversible, transcriptional changes of HCN channel expression may last for months, alter neuronal firing patterns and contribute to a hyper-excitable hippocampal network (Chen et al., 2001; Brewster et al., 2002). Here we focused on the mechanisms by which these transcriptional changes in HCN channel expression occur.

Seizures orchestrate reciprocal changes in the expression of the HCN1 and HCN2 isoforms

Previous work found that individual hippocampal neurons express both HCN1 and 2 isoforms. Seizures induced coordinated reduction of HCN1 expression, and reciprocal enhancement of HCN2 mRNA expression (Brewster et al., 2002). This "molecular switch", accompanied by slowed kinetics and a shifted voltage-dependent activation curve of cellular I_h in the depolarizing direction, led to increased bursting behavior in response to hyperpolarizing input, and enhanced network excitability (Dubé et al., 2000; Chen et al., 2001; Brewster et al., 2002). The molecular and cellular basis for this differential regulation was difficult to study *in vivo*, because of variables (e.g., systemic hormones that influence channel expression), and difficulties in testing mechanistic hypotheses by blocking specific molecular pathways. The fact that these alterations were recreated by seizure-like events in slice cultures suggests that the mechanisms involved are independent of systemic factors and involve activity-dependent changes within hippocampal neurons.

The mechanisms for seizure-like activity-induced HCN1 regulation involve newly-formed functional Ca^{2+}-permeable AMPA channels

The current experiments demonstrate an interesting time-course of seizure-induced reduction of HCN1 expression. In essence, functional Ca^{2+}-permeable AMPA receptors and activation of CaM Kinase II were required for many hours following the seizures, and blocking them during the seizures did not interfere with the ability of seizures to regulate HCN1 expression. This sequence of events, coupled by the findings of the current work and evidence from other groups (Friedman et al., 1994; Sanchez et al., 2001), suggests the following scenario:

(a) First, seizure-like events lead to reduced expression of the GluR2 subunit of AMPA receptors (Friedman et al., 1994; Sanchez et al., 2001; Richichi et al., 2005). This reduction in mRNA and protein levels leads to preferential formation of GluR2-lacking, Ca^{2+}-permeable AMPA receptors, a process that takes several hours.

(b) Ca^{2+}-permeable AMPA-receptors constitute a small fraction of AMPA receptors, and their maximal opening does not depolarize the neuron (Yin et al., 2002). Ca^{2+} influx through this route may initiate specific intracellular cascades, including activation of CaM Kinase II (Hardingham and Bading, 1999) or, at a minimum, activation of the cellular pool of CaM Kinase II that is involved in seizure- induced regulation of HCN1 expression. In support of this step, reduction of HCN1 channel expression was selectively prevented by NASPM, a blocker of Ca^{2+}-permeable AMPA channels, and not by blocking Ca^{2+} entry via NMDA receptors.

(c) The hours-long process of formation of Ca^{2+}-permeable AMPA receptors led to protracted activation of cellular CaM Kinase II. The experiments reported here, using 3 distinct blockers of CaM Kinase II, demonstrated that reduction of HCN1 involved protracted Ca^{2+}-mediated activation of CaM Kinase II: blocking the enzyme only for an hour beyond the duration of KA application did not abolish the activity-evoked repression of HCN1 channel expression, but inhibiting the kinase for 21 hours, starting *after* the termination of the seizure-like events sufficed to block HCN1 downregulation. This suggests that ongoing activation of CaM Kinase II, via ongoing Ca^{2+} entry through Ca^{2+}-permeable AMPA receptors, is involved.

Finally, the current experiments indicate that the upregulation of HCN2 channel expression by seizure-like events was independent of CaM Kinase II. Seizures change expression of hundreds of genes (Becker et al., 2003) potentially through one or several key signal transduction pathways, such as those regulated by CaM Kinase II and the phosphatase calcineurin (Sola et al., 2001). Downstream from these enzymes, single transcription factors may coordinately modulate expression of receptor or channel families (Brooks-Kayal et al., 1998; Huang et al., 2002; Roberts et al., 2005). In the case of HCN1 and 2, the promoters of these 2 genes share few transcription factor sites (N. Patel, personal communication). This, together with the current experiments, suggests that the divergence in the regulation of HCN1 and 2 gene expression by seizure-like activity occurs “upstream” from specific transcription factors, i.e., at the level of Ca^{2+}-activated enzymes.

In summary, a ‘transcriptional channelopathy’ involving the HCN channels is emerging as an important factor in the pathogenesis of several epilepsies (Chen et al., 2001; Brewster et al., 2002; Strauss et al., 2004; Shah et al., 2004; Budde et al., 2005; Kuisle et al., 2006). The current study provides insight into the mechanisms of this transcriptional dysregulation, that may lead to therapeutic targets.

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References

Becker AJ, Chen J, Zien A, Sochivko D, Normann S, Schramm J, Elger CE, Wiestler OD, Blümcke I. Correlated stage- and subfield-associated hippocampal gene expression patterns in experimental and human temporal lobe epilepsy. Eur J Neurosci 2003;18:2792–2802. [PubMed: 14656328]

Bender RA, Soleymani SV, Brewster AL, Nguyen ST, Beck H, Mathern GW, Baram TZ. Enhanced expression of a specific hyperpolarization-activated cyclic nucleotide-gated cation channel (HCN) in surviving dentate gyrus granule cells of human and experimental epileptic hippocampus. J Neurosci 2003;23:6826–6836. [PubMed: 12890777]

Bender RA, Kirschstein T, Kretz O, Brewster AL, Richichi C, Ruschenschmidt C, Shigemoto R, Beck H, Frotscher M, Baram TZ. Localization of HCN1 channels to presynaptic compartments: novel plasticity that may contribute to hippocampal maturation. J Neurosci 2007;27:4697–4706. [PubMed: 17460082]

Brewster AL, Bender RA, Chen Y, Dubé C, Eghbal-Ahmadi M, Baram TZ. Developmental febrile seizures modulate hippocampal gene expression of hyperpolarization-activated channels in an isoform and cell-specific manner. J Neurosci 2002;22:4591–4599. [PubMed: 12040066]

Brewster AL, Bernard JA, Gall CM, Baram TZ. Formation of heteromeric hyperpolarization-activated cyclic nucleotide-gated (HCN) channels in the hippocampus is regulated by developmental seizures. Neurobiol Dis 2005;19:200–207. [PubMed: 15837575]

Brewster AL, Chen Y, Bender RA, Yeh A, Shigemoto R, Baram TZ. Quantitative analysis and subcellular distribution of mRNA and protein expression of the hyperpolarization-activated cyclic nucleotide-gated channels throughout development in rat hippocampus. Cereb Cortex 2007;17:702–712. [PubMed: 16648453]

Brooks-Kayal AR, Shumate MD, Jin H, Lin DD, Rikhter TY, Holloway KL, Coulter DA. Selective changes in single cell GABA(A) receptor subunit expression and function in temporal lobe epilepsy. Nat Med 1998;4:1166–1172. [PubMed: 9771750]

Budde T, Caputi L, Kanyshkova T, Staak R, Abrahamczik C, Munsch T, Pape HC. Impaired regulation of thalamic pacemaker channels through an imbalance of subunit expression in absence epilepsy. J Neurosci 2005;25:9871–9882. [PubMed: 16251434]

Chen K, Aradi I, Thon N, Eghbal-Ahmadi M, Baram TZ, Soltesz I. Persistently modified h-channels after complex febrile seizures convert the seizure-induced enhancement of inhibition to hyperexcitability. Nat Med 2001;7:331–337. [PubMed: 11231632]

Chen Y, Bender RA, Brunson KL, Pomper JK, Grigoriadis DE, Wurst W, Baram TZ. Modulation of dendritic differentiation by corticotropin-releasing factor in the developing hippocampus. Proc Natl Acad Sci USA 2004;101:15782–15787. [PubMed: 15496472]

Di Pasquale E, Keegan KD, Noebels JL. Increased excitability and inward rectification in layer V cortical pyramidal neurons in the epileptic mutant mouse Stargazer. J Neurophysiol 1997;77:621–631. [PubMed: 9065835]

Dubé C, Chen K, Eghbal-Ahmadi M, Brunson K, Soltesz I, Baram TZ. Prolonged febrile seizures in the immature rat model enhance hippocampal excitability long term. Ann Neurol 2000;47:336–344. [PubMed: 10716253]

Dubé C, Richichi C, Bender RA, Chung G, Litt B, Baram TZ. Temporal lobe epilepsy after experimental prolonged febrile seizures: prospective analysis. Brain 2006;129:911–922. [PubMed: 16446281]

Ellerkmann RK, Remy S, Chen J, Sochivko D, Elger CE, Urban BW, Becker A, Beck H. Molecular and functional changes in voltage-dependent Na($^{+}$) channels following pilocarpine-induced status epilepticus in rat dentate granule cells. Neuroscience 2003;119:323–333. [PubMed: 12770549]

Fan Y, Fricker D, Brager DH, Chen X, Lu HC, Chitwood RA, Johnston D. Activity-dependent decrease of excitability in rat hippocampal neurons through increases in I_h. Nat Neurosci 2005;8:1542–1551. [PubMed: 16234810]

Friedman LK, Pellegrini-Giampietro DE, Sperber EF, Bennett MV, Moshe SL, Sukin RS. Kainate-induced status epilepticus alters glutamate and $GABA_A$ receptor gene expression in adult rat hippocampus: an in situ hybridization study. J Neurosci 1994;14:2697–2707. [PubMed: 8182436]

Gutierrez R, Armand V, Schuchmann S, Heinemann U. Epileptiform activity induced by low Mg^{2+} in cultured rat hippocampal slices. Brain Res 1999;15:294–303. [PubMed: 9878794]

Hardingham GE, Bading H. Calcium as a versatile second messenger in the control of gene expression. Microsc Res Tech 1999;46:348–355. [PubMed: 10504212]

Huang Y, Doherty JJ, Dingledine R. Altered histone acetylation at glutamate receptor 2 and brain-derived neurotrophic factor genes is an early event triggered by status epilepticus. J Neurosci 2002;22:8422–8428. [PubMed: 12351716]

Jentsch TJ, Hubner CA, Fuhrmann JC. Ion channels: function unravelled by dysfunction. Nat Cell Biol 2004;6:1039–1047. [PubMed: 15516997]

Kuisle M, Wanaverbecq N, Brewster AL, Frere SG, Pinault D, Baram TZ, Luthi A. Functional stabilization of weakened thalamic pacemaker channel regulation in rat absence epilepsy. J Physiol 2006;575:83–100. [PubMed: 16728450]

Labiner DM, Butler LS, Cao Z, Hosford DA, Shin C, McNamara JO. Induction of c-fos mRNA by kindled seizures: complex relationship with neuronal burst firing. J Neurosci 1993;13:744–751. [PubMed: 8381172]

Liu W, Liu R, Chun JT, Bi R, Hoe W, Schreiber SS, Baudry M. Kainate excitotoxicity in organotypic hippocampal slice cultures: evidence for multiple apoptotic pathways. Brain Res 2001;916:239–248. [PubMed: 11597611]

Ludwig A, Zong X, Jeglitsch M, Hofmann F, Biel M. A family of hyperpolarization-activated mammalian cation channels. Nature 1998;393:587–591. [PubMed: 9634236]

Lupica CR, Bell JA, Hoffman AF, Watson PL. Contribution of the hyperpolarization-activated current (I_h) to membrane potential and GABA release in hippocampal interneurons. J Neurophysiol 2001;86:261–268. [PubMed: 11431507]

Maccaferri G, McBain CJ. The hyperpolarization-activated current (I_h) and its contribution to pacemaker activity in rat CA1 hippocampal stratum oriens-alveus interneurons. J Physiol 1996;497:119–130. [PubMed: 8951716]

Magee JC. Dendritic I_h normalizes temporal summation in hippocampal CA1 neuron. Nat Neurosci 1999;2:508–514. [PubMed: 10448214]

Noebels JL. The biology of epilepsy genes. Annu Rev Neurosci 2003;26:599–625. [PubMed: 14527270]

Peters HC, Hu H, Pongs O, Storm JF, Isbrandt D. Conditional transgenic suppression of M channels in mouse brain reveals functions in neuronal excitability, resonance and behavior. Nat Neurosci 2005;8:51–60. [PubMed: 15608631]

Poolos NP, Migliore M, Johnston D. Pharmacological upregulation of h-channels reduces the excitability of pyramidal neuron dendrites. Nat Neurosci 2002;5:767–774. [PubMed: 12118259]

Poolos NP, Bullis JB, Roth MK. Modulation of h-channels in hippocampal pyramidal neurons by p38 mitogen-activated protein kinase. J Neurosci 2006;26:7995–8003. [PubMed: 16870744]

Richichi C, Bender RA, Brewster AL, Simeone TA, Yin HZ, Weiss JH, Baram TZ. CaM Kinase II is required for seizure-evoked reduction of hyperpolarization-activated, cyclic nucleotide-gated (HCN) 1 channel expression. Soc Neurosci Abst 2005;377:16.

Roberts DS, Raol YH, Bandyopadhyay S, Lund IV, Budreck EC, Passini MA, Wolfe JH, Brooks-Kayal AR, Russek SJ. Egr3 stimulation of GABRA4 promoter activity as a mechanism for seizure-induced up-regulation of $GABA_A$ receptor α4 subunit expression. Proc Natl Acad Sci USA 2005;102:11894–11899. [PubMed: 16091474]

Robinson RB, Siegelbaum SA. Hyperpolarization-activated cation currents: from molecules to physiological function. Annu Rev Physiol 2003;65:453–480. [PubMed: 12471170]

Routbort MJ, Bausch SJ, McNamara JO. Seizures, cell death, and mossy fiber sprouting in kainic acid-treated organotypic hippocampal cultures. Neuroscience 1999;94:755–765. [PubMed: 10579566]

Sanchez RM, Koh S, Rio C, Wang C, Lamperti ED, Sharma D, Corfas G, Jensen FE. Decreased glutamate receptor 2 expression and enhanced epileptogenesis in immature rat hippocampus after perinatal hypoxia-induced seizures. J Neurosci 2001;21:8154–8163. [PubMed: 11588188]

Santoro B, Chen S, Luthi A, Pavlidis P, Shumyatsky GP, Tibbs GR, Siegelbaum SA. Molecular and functional heterogeneity of hyperpolarization-activated pacemaker channels in the mouse CNS. J Neurosci 2000;20:5264–5275. [PubMed: 10884310]

Santoro B, Baram TZ. The multiple personalities of h-channels. Trends Neurosci 2003;26:550–554. [PubMed: 14522148]

Shah MM, Anderson AE, Leung V, Lin X, Johnston D. Seizure-induced plasticity of h channels in entorhinal cortical layer III pyramidal neurons. Neuron 2004;44:495–508. [PubMed: 15504329]

Shin KS, Maertens C, Proenza C, Rothberg BS, Yellen G. Inactivation in HCN channels results from reclosure of the activation gate: desensitization to voltage. Neuron 2004;41:737–744. [PubMed: 15003173]

Sola C, Barron S, Tusell JM, Serratosa J. The Ca^{2+}/calmodulin system in neuronal hyperexcitability. Int J Biochem Cell Biol 2001;33:439–455. [PubMed: 11331200]

Strauss U, Kole MH, Bräuer A, Bahnke J, Bajorat R, Rolfs A, Nitsch R, Deisz RA. An impaired neocortical I_h is associated with enhanced excitability and absence epilepsy. Eur J Neurosci 2004;19:3048–3058. [PubMed: 15182313]

Su H, Sochivko D, Becker A, Chen J, Jiang Y, Yaari Y, Beck H. Upregulation of a T-type Ca^{2+} channel causes a long-lasting modification of neuronal firing mode after status epilepticus. J Neurosci 2002;22:3645–3655. [PubMed: 11978840]

Surges R, Brewster AL, Bender RA, Beck H, Feuerstein TJ, Baram TZ. Regulated expression of HCN channels and cAMP levels shape the properties of the h current in developing rat hippocampus. Eur J Neurosci 2006;24:94–104. [PubMed: 16882011]

van Welie I, van Hooft JA, Wadman WJ. Homeostatic scaling of neuronal excitability by synaptic modulation of somatic hyperpolarization-activated I_h channels. Proc Natl Acad Sci USA 2004;101:5123–5128. [PubMed: 15051886]

Vasilyev DV, Barish ME. Postnatal development of the hyperpolarization-activated excitatory current I_h in mouse hippocampal pyramidal neurons. J Neurosci 2002;22:8992–9004. [PubMed: 12388606]

Wang J, Chen S, Nolan MF, Siegelbaum SA. Activity-dependent regulation of HCN pacemaker channels by cyclic AMP: signaling through dynamic allosteric coupling. Neuron 2002;36:451–461. [PubMed: 12408847]

Waxman SG. Transcriptional channelopathies: an emerging class of disorders. Nat Rev Neurosci 2001;2:652–659. [PubMed: 11533733]

Waxman SG, Hains BC. Fire and phantoms after spinal cord injury: Na^+ channels and central pain. Trends Neurosci 2006;29:207–215. [PubMed: 16494954]

Yin HZ, Sensi SL, Ogoshi F, Weiss JH. Blockade of Ca^{2+}-permeable AMPA/kainate channels decreases oxygen glucose deprivation induced Zn^{2+} accumulation and neuronal loss in hippocampal pyramidal neurons. J Neurosci 2002;22:1273–1279. [PubMed: 11850455]

Zong X, Eckert C, Yuan H, Wahl-Schott C, Abicht H, Fang L, Li R, Mistrik P, Gerstner A, Much B, Baumann L, Michalakis S, Zeng R, Chen Z, Biel M. A novel mechanism of modulation of hyperpolarization-activated cyclic nucleotide-gated channels by Src kinase. J Biol Chem 2005;280:34224–34232. [PubMed: 16079136]

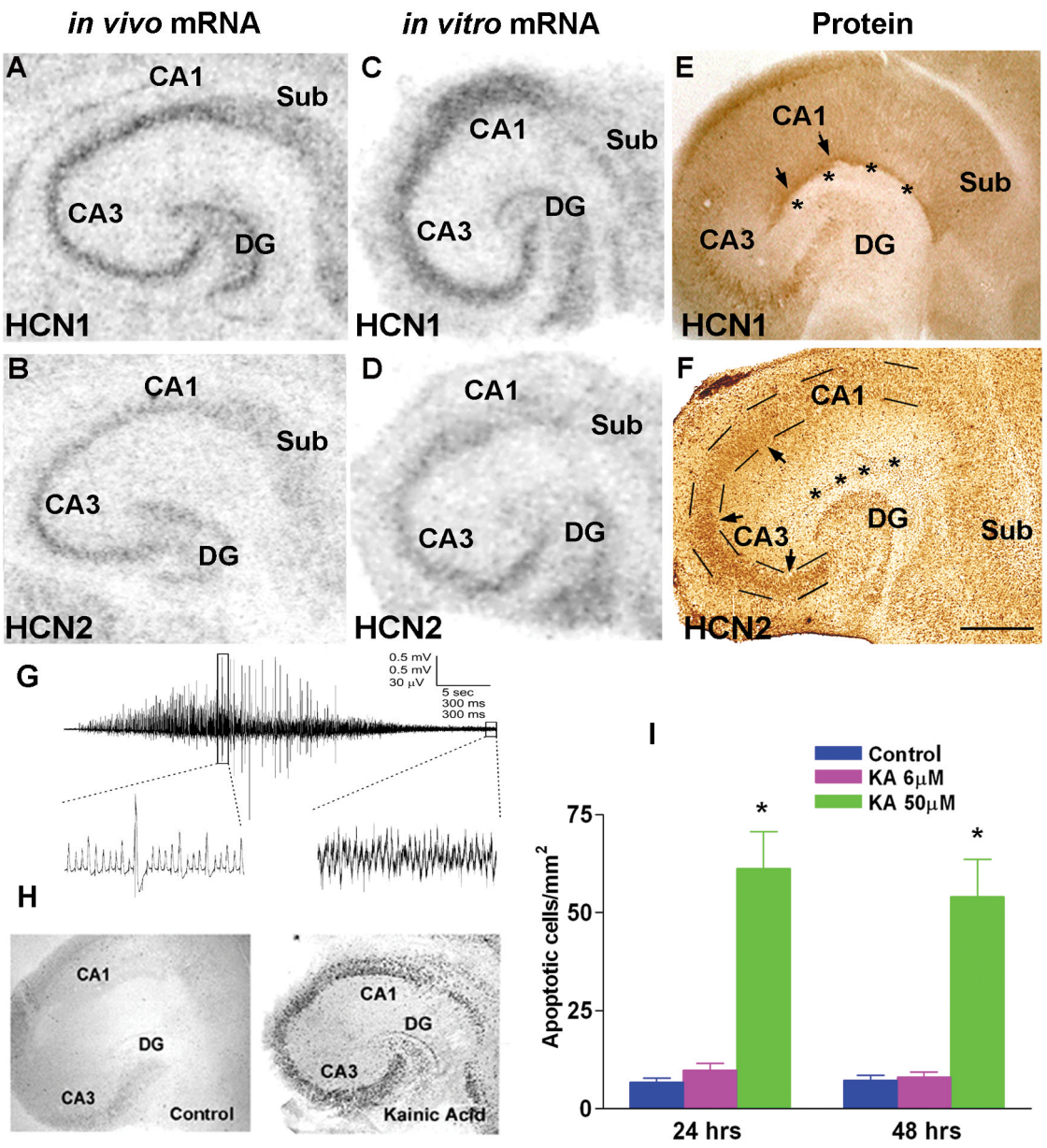

Fig. 1.

HCN channel expression and generation of seizure-like events in the hippocampal organotypic slice culture. (A-F) Expression of HCN1 and 2 *in vitro* recapitulated the *in vivo* patterns. (A,B) HCN1 and 2 mRNAs in horizontal hippocampal sections on P13, (C,D) in an age-equivalent slice culture (explanted on P8, 5 days *in vitro*), using *in situ* hybridization. Both isoforms (HCN1 > HCN2) were expressed in CA1 and CA3 pyramidal cell layers, subiculum (sub) and dentate gyrus (DG). (E) At the protein level, HCN1 channels were concentrated in dendritic fields of CA1 pyramidal cells (arrows). (F) HCN2 channels were mainly confined to the principal cell layers at this age (arrows); the pyramidal cell layer is delineated by broken lines, and asterisks demarcate the hippocampal fissure. (G, H) Seizure-like activity could be generated in the organotypic slice culture. (G) Example of a burst of epileptiform activity (seizure-like event) detected by extracellular recording in CA3 after application of 6 µM KA. The typical seizure-like burst consisted of population events (30-60s) that occurred at a ~30

Hz frequency within the burst (Fig 1G, bottom left trace). Near the end of the burst the events degenerated into low amplitude gamma oscillations (~30 Hz; Fig 1G, bottom right trace), then stopped. After the ictal-like burst, spontaneous interictal-like events or single population events occurred with a frequency of ~0.2 Hz. These seizure-like events cease within minutes when cultures are returned to normal medium. (H) Activation of hippocampal neurons by KA was evident via the expression of the immediate early gene c-fos. Fos-immunoreactive neurons were seen in CA1 and CA3 pyramidal neurons in 7/7 of cultures exposed to KA, and in none (0/5) of the control cultures. (I) Seizure-like events provoked by 6 μM KA did not induce neuronal death. Density of TUNEL-positive nuclei in pyramidal cell layer was not significantly different between KA-treated (purple bar) and control cultures (blue bar) at 24 hours (left series of bars) as well as at 48 hours (right series of bars). A known toxic dose (Liu et al., 2001) of KA, 50 μM, provoked neuronal death at both time-points (green bars) validating the TUNEL detection method (n=8-11 per group; * = significance). Scale bar: 300 μm (E,F).

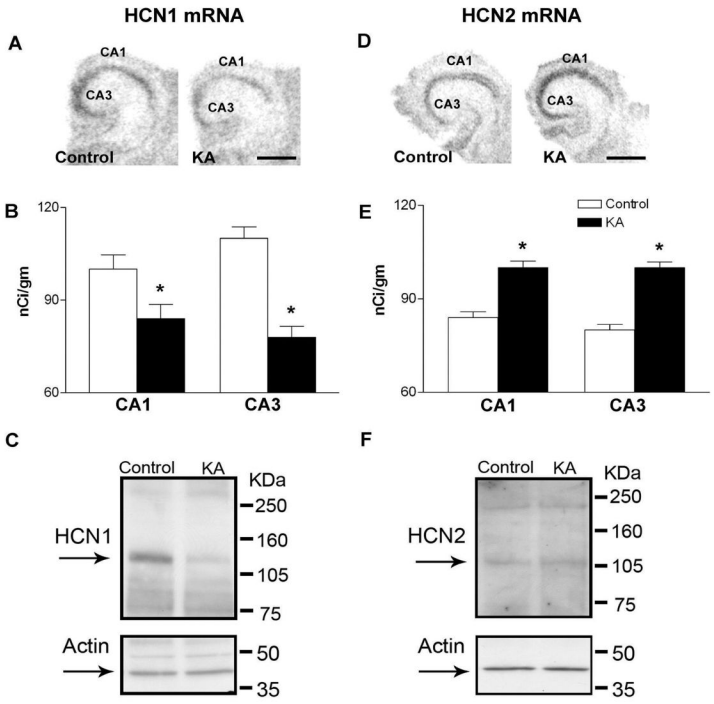

Fig. 2.

Seizure-like activity *in vitro* regulates HCN channel expression. (A-C) Representative autoradiographs (A) and quantitative analyses (B) showing HCN1 mRNA levels in hippocampal slice cultures 48 hours after a 3-hour exposure to 6 μM KA, compared to control sister cultures. A significant reduction in mRNA (measured in nCi/gm of hippocampal tissue) was apparent in both CA1 and CA3 pyramidal cell layers after the seizure-like activity (n=13 per group; * = significance). (C) Western blots showed that the mRNA changes in CA1 were reflected in reduced protein levels, i.e., reduced intensity of the HCN1-immunoreactive band in experimental cultures, compared to controls (KA: 129.0 ± 43.8 OD/Actin vs. control: 242.3 ± 55.0 OD/Actin, a 47% reduction). In contrast to HCN1 channels, HCN2 channels mRNA expression was increased by the seizure-like events (D, E). However, this was not accompanied by protein changes (F). (KA: 113.9 ± 22.9 OD/Actin vs. control: 98.9 ± 14.9 OD/Actin). Notes: Actin values are not comparable between HCN1 and HCN2; HCN2-immunoreactive band is of lower molecular weight compared to HCN1. Scale bar: 75 μm (A,D).

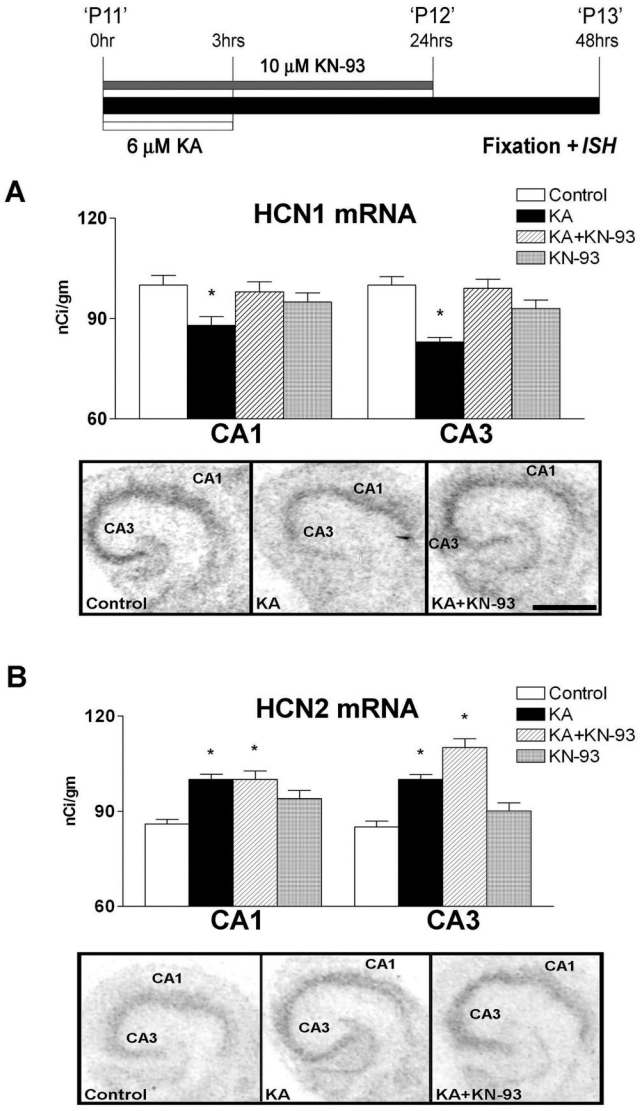

Fig. 3.

CaM Kinase II activation is required for seizure-like activity-induced reduction of HCN1, but not for upregulation of HCN2 mRNA expression. (A) Representative autoradiographs and quantitative analyses show abrogation of KA-induced reduction of HCN1 expression (measured in nCi/gm of hippocampal tissue) by co-exposure to the CaM Kinase II activation blocker KN-93 (10 μM; KA+KN-93, maintained for 24 hours; see schematic). Application of KN-93 alone had no effect. (B) In contrast, KA-induced increases of HCN2 mRNA levels still occurred when cultures were co-incubated with KN-93 (n=35-38 per group; * = significance vs controls). Scale bar: 75 μm.

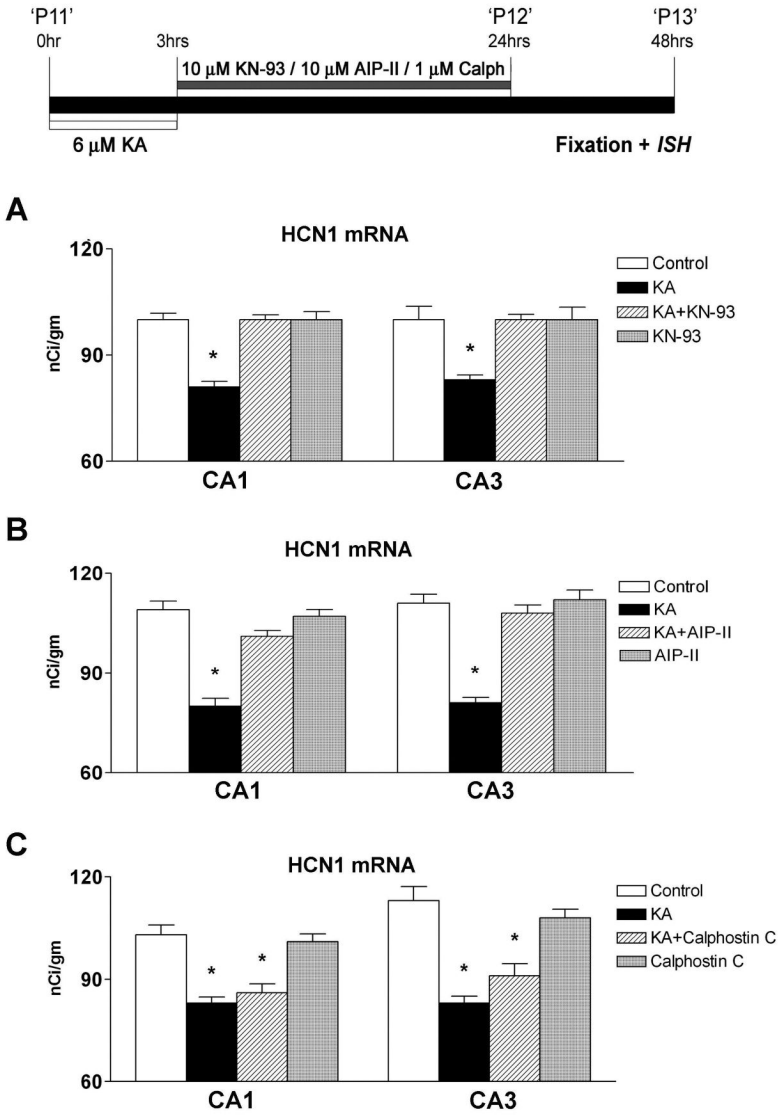

Fig. 4.

Sustained activation of CaM Kinase II is required for activity-induced downregulation of HCN1 channel expression. (A) The CaM Kinase II activation blocker, KN-93 (10 μM) or (B) the peptidic inhibitor of this enzyme, AIP-II (10 μM) were applied immediately after KA-induced seizure-like events, and maintained for 21 hours (schematic). This sufficed to abrogate the reduction of HCN1 expression (measured as nCi/gm), suggesting that delayed rather than acute CaM Kinase II activation were critical for this effect. When KN-93 was removed 1 hour after the end of KA-evoked activity, it failed to abolish activity-evoked reduction of HCN1 (not shown); n=25-33 per group; * = significance. (C) The protein kinase C blocker, calphostin C (Calph, 1 μM), failed to block HCN1 downregulation, excluding involvement of PKC in the mechanisms of this HCN1 regulation (n=10 per group; * = significance).

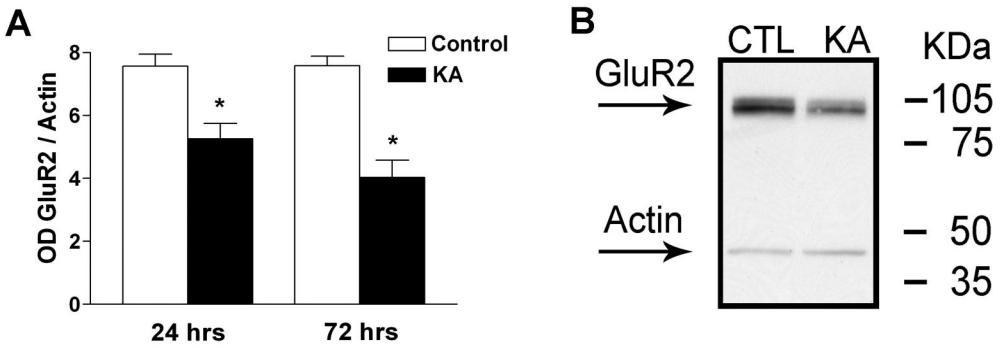

Fig. 5.

Seizure-like activity *in vitro* reduces GluR2 protein levels. (A) Quantitative Western blot analyses and (B) representative gel demonstrated a substantial reduction in the GluR2 protein levels in cultures exposed to 6 μM of kainic acid for 3 hours and analyzed 24 and 72 hours later, compared to controls. Optical density (OD) of GluR2-immunoreactive bands was normalized to that of actin. Reduction of GluR2 protein levels promotes formation of GluR2-lacking AMPA channels that are permeable to Ca^{2+} (n=3-6 per group; * = significance, ANOVA with Bonferroni's *post-hoc* tests).

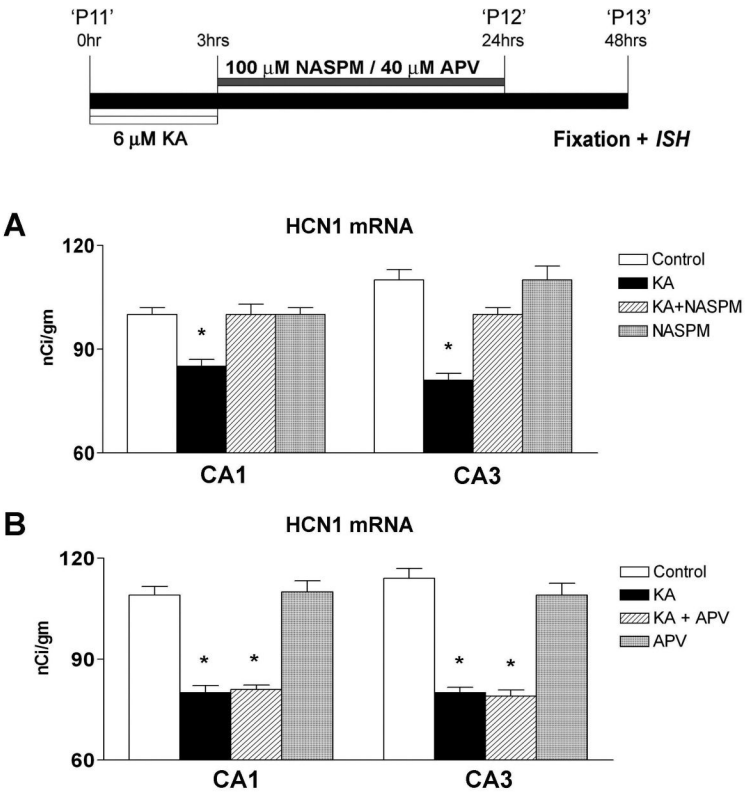

Fig. 6.

Ca^{2+}-permeable AMPA receptors but not NMDA receptors contribute to the seizure-like activity-induced regulation of HCN1 channel expression. (A) Application of the Ca^{2+}-permeable AMPA receptor blocker NASPM (100 μM) for 21 hours starting immediately after a 3-hour KA exposure (schematic) prevented the reduction of HCN1 mRNA levels in CA1 and CA3 pyramidal cell layers (n=10-14 per group). (B) In contrast, reduction of HCN1 expression (measured as nCi/gm) still occurred when Ca^{2+} entry through NMDA receptors was blocked using APV (40 μM) in the same manner (n=24 per group). These data suggest that Ca^{2+}-permeable AMPA receptors, but not NMDA receptors are essential for this activity-induced modulation of HCN1 expression (* = significance).