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

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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 receptor-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 and Hains, 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 (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 and McBain, 1996; Lupica et al., 2001) and their responses to network activity (Maccaferri and McBain, 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 and Siegelbaum, 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

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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 h 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 the 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 h, 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 h or 1 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 h 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 min of removal of the cultures to media with normal [Mg^{++}] or lacking KA (T. Simeone 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 and 2 mRNA levels were accomplished as described previously (Brewster et al., 2002, 2007). Briefly, cultures were harvested 48 h or 1 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 $1.5–4.2 \times 10^9$ 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 1000×*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; 1:2000; 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 h with 3% normal goat serum/PBS-TX, followed by incubation with polyclonal rabbit anti-HCN1 (1:2500; Chemicon), rabbit anti-HCN2 (1:1500; courtesy Dr. R. Shigemoto) or rabbit anti-c-fos (1:30,000; Calbiochem). After application of 2nd 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 the absence of signal in tissue derived from mice lacking the tested isoform (Brewster et al., 2007).