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Formation of heteromeric hyperpolarization-activated cyclic nucleotide-gated (HCN) channels in the hippocampus is regulated by developmental seizures

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Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels mediate hyperpolarization-activated currents (I_h). In hippocampus, these currents contribute greatly to intrinsic cellular properties and synchronized neuronal activity. The kinetic and gating properties of HCN-mediated currents are largely determined by the type of subunits–for example, HCN1 and HCN2–that assemble to form homomeric channels. Recently, functional *hetero*meric HCN channels have been described in vitro, further enlarging the potential I_h repertoire of individual neurons. Because these heteromeric HCN channels may promote hippocampal hyperexcitability and the development of epilepsy, understanding the mechanisms governing their formation is of major clinical relevance. Here, we find that developmental seizures promote co-assembly of hippocampal HCN1/HCN2 heteromeric channels, in a duration-dependent manner. Long-lasting heteromerization was found selectively after seizures that provoked persistent hippocampal hyperexcitability. The mechanism for this enhanced heteromerization may involve increased relative abundance of HCN2-type subunits relative to the HCN1 isoform at both mRNA and protein levels. These data suggest that heteromeric HCN channels may provide molecular targets for intervention in the epileptogenic process.

Keywords: Ion channel; Epilepsy; HCN; Hyperpolarization; I_h; Febrile seizures; Animal model; Epileptogenesis; Heteromeric; Co-immunoprecipitation; Rat; Kainic acid

Introduction

HCN channels mediate hyperpolarization-activated (I_h) currents (DiFrancesco, 1993; Pape, 1996). In hippocampus, these currents contribute to maintenance of resting membrane potential (Lupica et al., 2001), shape rhythmic and synchronized neuronal activity (Maccaferri and McBain, 1996; Magee, 1999) and regulate the temporal summation of dendritic depolarization (Magee, 1998; Poolos et al., 2002). Short-term modulation of HCN channel function is mediated by cAMP, influencing channel kinetics and voltage dependent activation curves (DiFrancesco, 1993; Waigner et al., 2001). Recently, long-term modulation of the properties of I_h currents has been suggested to result from regulated changes in channel subunit expression (Bräuer et al., 2001; Brewster et al., 2002; Santoro and Baram, 2003).

Of the four characterized genes encoding HCN channels, two (HCN1 and 2) are substantially expressed in rodent hippocampus (Bender et al., 2001; Moosmang et al., 1999; Santoro et al., 2000) and both isoforms can reside in a single neuron (Brewster et al., 2002; Franz et al., 2000). HCN4 is expressed almost exclusively in subcortical regions, whereas levels of HCN3 expression within neurons are generally low (Bender et al., 2001; Moosmang et al., 1999; Santoro et al., 2000). As described for other members of the voltage-gated cation channel family, four HCN subunits assemble to form a channel (for recent reviews, see Robinson and Siegelbaum, 2003; Santoro and Baram, 2003). Homomeric HCN1 channels conduct fast-kinetic currents with modest cAMP gating, consistent with currents recorded in hippocampal pyramidal cells and CA1 interneurons where HCN1 expression is high (Bender et al., 2001; Brewster et al., 2002; Santoro et al., 2000). By contrast, homomeric HCN2 channels conduct I_h currents with slower kinetics and robust cAMP-evoked shifts in voltage dependence (Ludwig et al., 1998; Robinson and Siegelbaum, 2003). More recently, the formation of heteromeric channels in vitro (Chen et al., 2001b; Er et al., 2003; Much et al., 2003; Proenza et al., 2002; Ulens and Tytgat, 2001; Xue et al., 2002) as well as in vivo (Er et

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al., 2003; Much et al., 2003) has been described. However, the mechanisms promoting heteromerization have remained unclear.

Because the isoform composition of both homomeric and heteromeric HCN channels determines their physiological characteristics (Robinson and Siegelbaum, 2003; Santoro and Baram, 2003), the relative abundance of the HCN isoforms, as well as the degree to which heteromerization occurs, will contribute to the properties of the I_h of an individual neuron (Franz et al., 2000; Santoro et al., 2000; Vasilyev and Barish, 2002), and thus to its intrinsic firing patterns and network responses. Differential regulation of HCN1 and HCN2 mRNA expression has been found in pathological states (Bender et al., 2003; Bräuer et al., 2001; Brewster et al., 2002), including experimental febrile and kainate-induced seizures, and has been associated with altered properties of the I_h (Chen et al., 2001a). Interestingly, the properties of I_h after experimental febrile seizures resembled neither those of heterologously expressed homomeric HCN1 or HCN2 channels, nor their arithmetic intermediates (Chen et al., 2001a,b; Ulens and Tytgat, 2001). Therefore, we considered the possibility that these currents may be a result of heteromeric HCN1/HCN2 channels, and tested the hypothesis that pathological neuronal activity such as developmental seizures promotes formation of heteromeric HCN1/HCN2 channels, at least in part by altering the relative abundance of cellular HCN1 and HCN2 subunits at the mRNA and protein level. This constitutes a novel mechanism for activity-induced plasticity of the ion channel complement of a neuron. In addition, it highlights an as yet undescribed molecular foundation for the evolution of neurological disease, such as seizure-evoked epilepsy.

Materials and methods

Seizure induction

Experimental procedures were approved by the UCI Animal Care Committee in accordance to National Institutes of Health (NIH) guidelines. Immature (postnatal day 10) Sprague–Dawley rats were subjected to experimental prolonged febrile seizures as previously described (Brewster et al., 2002; Dubé et al., 2000; Toth et al., 1998). Briefly, hyperthermia was induced using a stream of warm air directed ~30 cm above the animals. Core temperatures of pups were measured prior to hyperthermia induction (33.9 ± 0.3°C), at 2-min intervals, and at the onset of hyperthermia-provoked seizures (40.8 ± 0.9°C). Hyperthermia was maintained for 30 min resulting in seizure duration of approximately 24 min. Following hyperthermia, animals were moved to a cool surface for 10–15 min, then returned to their home cages. Hyperthermic controls were generated by subjecting littermates to the same hyperthermia procedure, but blocking the resulting seizures using diazepam (10 mg/kg). Longer and more intense seizures were evoked in rats of the same age by intraperitoneal injection of the glutamate receptor agonist kainic acid (KA, 1.2 mg/kg) as described previously (Brewster et al., 2002). Experimental groups (Feb, $n = 37$; KA, $n = 15$; diazepam + Feb, $n = 4$; diazepam alone, $n = 4$) were compared to littermate controls ($n = 40$).

Tissue harvesting

For immunoprecipitation and Western blot analyses (1, 2, 4 and 8 weeks after seizures) rats were rapidly decapitated, the hippocampus and thalamus were quickly dissected and homogenized in a glass/Teflon homogenizer in ice cold 0.32 M Sucrose, 0.1 M Tris–HCl (pH 7.4) containing Protease Inhibitor Cocktail (PIC Complete™; Roche). Following homogenization, samples were centrifuged at 1000 × *g* for 10 min at 4°C. The supernatant was centrifuged at 16,000 × *g* for 20 min at 4°C and the pellet was resuspended in radioimmunoprecipitation (RIPA) buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 1% Triton X-100, 1 mM EDTA, 150 mM NaCl, 1× PIC) for immunoprecipitation procedures or in artificial cerebrospinal fluid (aCSF; 124 mM NaCl, 3 mM KCl, 1.25 mM KH_2PO_4, 2.5 mM $MgSO_4$, 3.4 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose) for direct Western blot analyses. Protein concentration was determined using Bio-Rad Protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). For in situ hybridization procedures, rats were quickly decapitated and brains dissected and placed on powdered dry ice as described (Brewster et al., 2002; Eghbal-Ahmadi et al., 1999).

Immunoprecipitation

An equal amount of protein for each hippocampal and thalamic sample (1 mg/ml), diluted in 750 μl of RIPA buffer, was incubated with rabbit anti-HCN1 or anti-HCN2 antisera (AB5884 HCN1, AB5378 HCN2; 5 μl of 15 μg/μl, Chemicon, Temecula, CA) on a rotator overnight at 4°C, following previously described procedures (Kramar et al., 2002). After incubation with primary antibodies, samples were incubated with 80 μl of Protein A Agarose (Upstate Biotech, Lake Placid, NY) on a rotator for 2 h at 4°C and collected in spin-filter columns (CytoSignal Research Products, Irvine, CA). Samples were then washed twice with RIPA+ 1× PIC, followed by three washes in phosphate buffered saline (PBS)+ 1× PIC. Following the washes, samples were eluted from the columns with 50 μl of 2× Laemmli buffer and processed for Western blot analyses (Kramar et al., 2002).

Western blotting

Protein samples were denatured at 100°C for 5 min in Laemmli buffer and separated on a 4–12% gradient SDS-PAGE gel. After SDS-PAGE, the proteins were transferred to Hybond-P Polyvinyl Difluoride (PVDF) membranes (Amersham Pharmacia Biotech) at 100 V for 1 h at 4°C. Membranes were probed with rabbit anti-HCN1 or anti-HCN2 serum (1:5000 and 1:3000, respectively) in PBS with 5% non-fat milk overnight at 4°C followed by washes in PBS-1% Tween (PBS-T) (3 × 5 min). Membranes were then incubated with anti-rabbit immunoglobulin-horseradish peroxidase (1:10,000 Amersham Pharmacia Biotech) in PBS for 1 h at room temperature, followed by incubation with the enhanced chemiluminescence ECL-Plus kit (Amersham Pharmacia Biotech) for 5 min. Immunoreactive bands were visualized by apposing membranes to Hyperfilm™ ECL (Amersham Pharmacia Biotech). To control for equal sample loading, membranes were probed with an antibody to beta actin.

In situ hybridization

Semi-quantitative analysis of HCN1 and HCN2 mRNA levels was accomplished using in situ hybridization (ISH) of ^{35}S-cRNA probes and slide-mounted frozen sections (20 μm) as previously described (Brewster et al., 2002; Eghbal-Ahmadi et al., 1999), with the most stringent wash at 0.03 × SSC, at 62°C for 60 min.