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Ethanol disrupts NMDA receptor and astroglial EAAT2 modulation of Kv2.1 potassium channels in hippocampus

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Abstract

Delayed-rectifier Kv2.1 channels are the principal component of voltage-sensitive K^+ currents (I_K) in hippocampal neurons and are critical regulators of somatodendritic excitability. In a recent study, we demonstrated that surface trafficking and phosphorylation of Kv2.1 channels is modulated by NMDA-type glutamate receptors and that astroglial excitatory amino acid transporters 2 (EAAT2) regulate the coupling of NMDA receptors and Kv2.1 channels. Since ethanol is known to acutely inhibit NMDA receptors, we sought to determine if NMDA receptor and astroglial EAAT2 modulation of Kv2.1 channels is impaired by ethanol in rodent hippocampus. As expected, bath application of NMDA to hippocampal cultures reduced the size of Kv2.1 clusters and produced a hyperpolarizing shift in the voltage-dependent activation of I_K that was associated with dephosphorylated Kv2.1 channels. Ethanol, applied acutely, prevented the hyperpolarizing shift in activation of I_K induced by NMDA and restored Kv2.1 clustering and phosphorylation to near control levels. Ethanol also attenuated the dephosphorylation of Kv2.1 channels produced by the EAAT2 selective inhibitor dihydrokainic acid. These data demonstrate that acute ethanol disrupts changes in Kv2.1 channels that follow NMDA receptor activation and impairs astroglial regulation of the functional coupling between NMDA receptors and Kv2.1 channels.

Keywords

Kv2.1 channels; NMDA receptors; ethanol; astroglial EAAT2; phosphorylation; clustering

Introduction

Kv2.1 channels are the principal component of sustained outward K^+ currents (I_K) in hippocampus and cortex and are found in large, highly phosphorylated clusters on the soma and proximal dendrites of pyramidal neurons (Du et al., 2000; Lim et al., 2000; Murakoshi and Trimmer, 1999). It has been proposed that the cellular location of Kv2.1 channels allows for control of somatic sub-threshold excitatory responses, and ultimately, regulation of action potential initiation (Murakoshi and Trimmer, 1999). However, knock-down of Kv2.1 channels increases excitability of CA1 pyramidal neurons only during high-frequency, but not low-frequency stimulation (Du et al., 2000), suggesting that these channels function differently than typical delayed-rectifier channels (Surmeier and Foehring, 2004).

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Consistent with an emerging role for Kv2.1 channels in models of homeostatic plasticity, the surface trafficking, phosphorylation and functional properties of these channels are sensitive to changes in glutamatergic receptor activity (Misonou et al., 2006; Misonou et al., 2004). We have recently demonstrated that the astroglial excitatory amino acid transporter 2 (EAAT2) regulates the coupling between extrasynaptic NMDA receptors and Kv2.1 channels, suggesting a previously unknown role for glutamate transporters in mediating the ability of extrasynaptic NMDA receptors to induce changes in neuronal plasticity (Mulholland et al., 2008). Since the NMDA receptor is known to be a critical target of ethanol (Allgaier, 2002; Woodward, 2000), the present study investigated the actions of ethanol on NMDA receptor and EAAT2 regulation of Kv2.1 channel gating, surface trafficking and phosphorylation.

Materials and Methods

Materials

Sprague-Dawley[®] rat pups from a breeding colony established from breeders and dams originally supplied by Harlan (Indianapolis, IN) were used in these studies. Anti-Kv2.1 rat polyclonal and mouse monoclonal antibodies were generously provided by J.S. Trimmer (University of California, Davis) or were purchased from NeuroMab (UC-Davis and Antibodies, Inc, Davis, CA). All compounds and electrophysiology reagents were purchased from Sigma-Aldrich Co. except for tetrodotoxin (Calbiochem, San Diego, CA), dihydrokainic acid (DHK) (Tocris, Ellisville, MO), and absolute ethanol (AAPER Alcohol and Chemical Co., Shelbyville, KY).

Preparation and Treatment of Cultures

Hippocampal neuronal cultures and organotypic hippocampal slices were prepared according to previously reported methods (Carpenter-Hyland et al., 2004; Mulholland et al., 2008). All experiments were conducted with at least three replicates and data were collected from at least three different culture preparations. To examine changes in clustering, phosphorylation and functional properties of Kv2.1 channels, cultures were washed twice with 1 ml of 25 mM HEPES incubation buffer containing (in mM): NaCl (140), KCl (5.4), CaCl₂ (1.8), glycine (0.01), glucose (15), MgCl₂ (2), tetrodotoxin (0.002) (pH 7.4). After a brief acclimation period in HEPES buffer, cultures were then subjected to various treatments in HEPES buffer without Mg²⁺ as indicated in the results section. Extrasynaptic NMDA receptors were isolated using the MK-801 trapping technique to selectively inactivate synaptic NMDA receptors, as previously described (Mulholland et al., 2008)

Kv2.1 Clustering and Phosphorylation Analysis

Immunostaining of Kv2.1 clusters was performed using 14-17 day-old low-density hippocampal neurons following previously described methods (Mulholland et al., 2008). Briefly, Metamorph version 4.6 software (Molecular Devices, Downingtown, PA) was used to interactively define Kv2.1 clusters at twofold above cytoplasmic background for evaluation of cluster size. Data collected in Metamorph were exported to Prism (GraphPad Software, Inc.) for statistical analysis. Confocal images were prepared for display in Adobe Photoshop CS (Adobe Systems, San Jose, CA).

The shift in the electrophoretic mobility of Kv2.1 due to changes in the phosphorylation state of the channel was determined in organotypic hippocampal slices by standard immunoblotting procedures using mouse anti-Kv2.1 primary antibody, as previously described (Mulholland et al., 2008). Data are expressed as a ratio of the integrated density of phosphorylated Kv2.1 to total Kv2.1, and statistical analyses of integrated density values for Kv2.1 were performed using Prism.

Whole-Cell Patch-Clamp Electrophysiology

Measurement of voltage-dependent activation of neuronal I_K in hippocampal neurons was performed as previously described (Mulholland et al., 2008). Average peak outward K^+ currents were converted to conductance (G), and normalized G was plotted against each test potential and fit to a single Boltzman equation, as previously reported (Misonou et al., 2006; Misonou et al., 2004; Mulholland et al., 2008). For measurement of NMDA-induced currents before and after MK-801 trapping in cultured hippocampal neurons, a multibarreled Warner Perfusion Fast-Step system (~8 ms switching time; 2 psi; Warner Instruments) was used to apply agonist for 5 s by switching from the control barrel to the barrel containing NMDA/glycine and then back, and the last 0.5 s of steady-state amplitude was measured.

Kv2.1 Channel Expression and Electrophysiological Recordings

HEK 293 cells (American Type Culture Collection, Manassas, VA) were prepared according to previously described methods (Woodward, 2004). Briefly, cells were plated at a density of 5×10^4 cells in 35-mm dishes and were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS at 37°C in 5% CO₂/95% air. Twenty-four hours after plating, cells were transfected with cDNA coding for Kv2.1 (kindly provided by Dr. Dennis Wray, University of Leeds, Leeds, UK) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Cells were used for electrophysiological recordings 24 hr after transfection. Transfected cells were identified by eGFP fluorescence and voltage-clamped at -80 mV, and Kv2.1 currents were evoked every 30 s by application of a depolarizing voltage command to 0 mV lasting 200 ms. Currents were measured the last 50 ms of the voltage step, and the average steady-state current of 5 steps was determined before, during, and following washout of 100 mM ethanol exposure. Data were analyzed by repeated-measured one-way ANOVA using Prism.

Results

Previous studies have shown that activation of glutamate receptors alters the voltage-dependent gating, surface trafficking and phosphorylation of Kv2.1 channels (Misonou et al., 2006; Misonou et al., 2004; Mulholland et al., 2008). Since NMDA-type glutamate receptors are an important site of action for ethanol, we hypothesized that ethanol would block the effects of NMDA on Kv2.1 channels. As expected, local application of 10 μ M NMDA (10 min) produced a hyperpolarizing shift in the voltage-dependent activation curve for neuronal I_K , and this shift was attenuated by co-application of 100 mM ethanol (Fig. 1A). Bath application of 2.5 μ M NMDA also potently reduced cluster size of somatodendritic Kv2.1 channels (Fig. 1B,C). This effect was largely eliminated by co-exposure of neurons to NMDA and ethanol (50 – 100 mM) (Fig. 1B,C). Ethanol alone had no effect on Kv2.1 clustering (data not shown) or currents recorded in HEK 293 cells transfected with Kv2.1 channels (Mean current (nA): baseline = 0.896 ± 0.22 , ethanol = $0.845 \pm .20$, washout = 0.887 ± 0.23 ; $n = 3$; $p > .05$). We have recently shown that activation of extrasynaptic, but not synaptic NMDA receptors, affects Kv2.1 channels (Mulholland et al., 2008). Therefore, the next set of experiments examined the extent to which ethanol inhibits extrasynaptic NMDA receptor currents. As expected, whole-cell NMDA receptor currents were reduced by ~33% following selective blocking of synaptic NMDA receptors using the MK-801 trapping procedure. Ethanol (50 mM) produced an ~20% reduction in NMDA receptor currents even under conditions where extrasynaptic NMDA receptors were isolated using the MK-801 trapping procedure (Fig. 1D).

Recent evidence from our laboratory has demonstrated that selective inhibition of astroglial EAAT2 rapidly activates NMDA receptors leading to dephosphorylation of Kv2.1 channels (Mulholland et al., 2008). Thus, we next investigated whether ethanol prevented NMDA- and EAAT2-induced dephosphorylation of Kv2.1 in organotypic hippocampal slices that, unlike dispersed cell cultures, maintain a normal glial-neuronal component (Benediktsson et al.,

2005; Haber et al., 2006; Hailer et al., 1996). Treatment of organotypic hippocampal slices with ethanol (50 – 100 mM) alone did not affect Kv2.1 phosphorylation levels (Fig. 2A). As previously demonstrated, Kv2.1 channels were significantly dephosphorylated by bath application of 2.5 μ M NMDA (Fig. 2B) or 500 μ M DHK, an EAAT2 selective inhibitor (Fig. 2C). Kv2.1 dephosphorylation induced by either NMDA or DHK was significantly attenuated by ethanol (Fig. 2B,C).

Discussion

Results from our previous study showed that activation of extrasynaptic, but not synaptic NMDA receptors regulates surface trafficking and phosphorylation of Kv2.1 channels and gating of neuronal I_K (Mulholland et al., 2008). These studies also demonstrated that EAATs, by controlling levels of extracellular glutamate, tightly regulate the activity of extrasynaptic NMDA receptors and the subsequent modulation of Kv2.1 channels. In the present study, we show that in hippocampus, ethanol prevents the NMDA-induced hyperpolarizing shift in the voltage-dependent activation of neuronal I_K and reduces the unclustering and dephosphorylation of Kv2.1 channels. Perhaps most interesting, ethanol also blocked Kv2.1 dephosphorylation induced by the selective EAAT2 inhibitor DHK. The ability of ethanol to prevent NMDA receptor and EAAT2 regulation of Kv2.1 likely relates to the known inhibitory effects of ethanol on NMDA receptor activation (Allgaier, 2002; Woodward, 2000). Indeed, ethanol at concentrations of 50 – 100 mM produced ~ 50% reduction in amplitude of NMDA receptor currents in hippocampal neurons (Lovinger et al., 1989, 1990). Here, we demonstrated that ethanol inhibition of extrasynaptic NMDA receptors does not differ in sensitivity when compared with ethanol inhibition of whole-cell NMDA currents. The incomplete nature of ethanol's inhibition of NMDA receptor currents likely explains why ethanol produced only partial attenuation of DHK-induced Kv2.1 dephosphorylation. Acute ethanol is also known to inhibit glutamate uptake in cultured astrocytes and in acute nucleus accumbens slices prepared from adult rodents (Melendez et al., 2005; Othman et al., 2002). However, ethanol did not affect basal phosphorylation levels of Kv2.1 suggesting that the effect of ethanol on EAATs does not contribute to the partial attenuation of DHK dephosphorylation of Kv2.1. Nonetheless, these data demonstrate that ethanol significantly disrupts the actions of NMDA receptors on Kv2.1 channel function and impairs astroglial regulation of the functional coupling between NMDA receptors and Kv2.1 channels.

Somatodendritic clusters of Kv2.1 are found closely apposed to astrocytic processes (Du et al., 1998), and astrocytic EAAT2 is thought to control ~80% of glutamate uptake in hippocampus (Danbolt, 2001). Data from a variety of sources suggests that astrocytes contribute more to neuronal function than merely providing a supportive role. Indeed, spontaneous increases in intracellular Ca^{2+} is prevalent in astrocytes (Scemes and Giaume, 2006), and this rise can trigger the release of glutamate into the extracellular space (Montana et al., 2006). Recently, activation of extrasynaptic NMDA receptors has been linked with astrocytic glutamate transmission in hippocampus and nucleus accumbens (D'Ascenzo et al., 2007; Fellin et al., 2004; Le Meur et al., 2007). The extracellular glutamate arising from astrocytes contributes to extrasynaptic NMDA receptor-mediated tonic currents in CA1 neurons (Le Meur et al., 2007), that finely tunes synaptic integration and NMDA receptor responses (Lee et al., 2007; Sah et al., 1989) and contributes to synchronizing hippocampal neuronal activity (Angulo et al., 2004). Given that ethanol is capable of inhibiting NMDA receptor and EAAT2 dephosphorylation of Kv2.1, it is tempting to speculate that tonic NMDA currents that arise from astrocytic glutamate release are blocked during acute exposure to ethanol.

We have previously demonstrated that activation of extrasynaptic NMDA receptors and inhibition of EAAT2 alters Kv2.1 channels in hippocampus (Mulholland et al., 2008). In the present study, we have extended those findings and demonstrated that acute ethanol is capable

of disrupting NMDA receptor modulation of Kv2.1 channel gating, surface trafficking and phosphorylation in hippocampus. Ethanol also blocked DHK-induced dephosphorylation of Kv2.1 channels, suggesting that impairment of astroglial regulation of NMDA receptor coupling to Kv2.1 is an important action of ethanol in the brain.

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Fig. 1.

Acute ethanol prevents NMDA-induced shift in voltage-dependent activation of neuronal I_K and unclustering of Kv2.1 channels in hippocampal neurons. **(A)** Local application of NMDA produced a hyperpolarizing leftward shift in the voltage activation curve for I_K that was prevented by ethanol co-exposure. (half-maximal conductance of neuronal I_K : 3.31 ± 2.3 mV for control, -12.15 ± 1.9 mV for NMDA, and -0.46 ± 2.2 mV for NMDA+ethanol; ANOVA with Student Newman-Keuls (SNK), $p < .01$ for control vs. NMDA, $p < .05$ for NMDA vs. NMDA+ethanol, $n = 6-7$ /group). **(B)** Ethanol reversed NMDA-mediated somatodendritic unclustering of Kv2.1 channels. Scale bar = 5 μ m. **(C)** The reduction in Kv2.1 cluster size induced by bath application of 2.5 μ M NMDA was blocked by ethanol co-exposure ($*p < .001$

vs. CTRL, ** $p < .05$ vs. NMDA, *** $p < .001$ vs. NMDA; ANOVA with SNK; $n = 10$). **(D)** Ethanol (50 mM) produced a reduction in whole-cell and isolated extrasynaptic NMDA receptor currents induced by 5 s application of 50 μ M NMDA/10 μ M glycine in hippocampal neurons (scale bar: 2.5 s, 500 pA; % ethanol inhibition: 19.8 ± 1.6 for whole-cell current, 18.4 ± 1.5 for extrasynaptic currents; $p > .05$, t test; $n = 6$ /group). Ethanol was pre-applied to the neuron for 30 s prior to being co-administered with NMDA/glycine.

Fig. 2.

Ethanol blocks NMDA receptor- and EAAT2-induced dephosphorylation of Kv2.1 channels in organotypic hippocampal slices. **(A)** Acute exposure of ethanol (50 – 100 mM) to hippocampal slices did not affect basal phosphorylation levels of Kv2.1 channels. **(B)** Ethanol (75 – 100 mM) significantly attenuated NMDA (2.5 μ M) dephosphorylation of Kv2.1 channels (* p < .01 vs. CTRL, ** p < .05 vs. NMDA; ANOVA with SNK; n = 3). **(C)** Dephosphorylation of Kv2.1 by the EAAT2 inhibitor DHK (500 μ M) was significantly prevented by 100 mM ethanol co-exposure (* p < .01 vs. CTRL, ** p < .01 vs. DHK; ANOVA with SNK; n = 3).