Published in final edited form as:

Neuroscience. 2010 January 13; 165(1): 130–137. doi:10.1016/j.neuroscience.2009.10.024.

Inhibition of glutamate transporters couples to Kv4.2 dephosphorylation through activation of extrasynaptic NMDA receptors

Patrick J. Mulholland* and L. Judson Chandler

Department of Neurosciences, 67 President Street, Medical University of South Carolina, Charleston, SC 29425, USA

Abstract

Activation of glutamate receptors is known to modulate K⁺ channel surface trafficking, phosphorylation, and function, and increasing evidence has implicated K⁺ channels in plastic changes in glutamatergic synapses. Kv4.2 channels control the amplitude of back-propagating action potentials and shape postsynaptic responses in hippocampus, and synaptic glutamate receptor activation leads to increased phosphorylation of Kv4.2 channels that is associated with enhanced synaptic plasticity. Thus, we investigated the possibility that activation of extrasynaptic NMDA-type glutamate receptors couples to Kv4.2 channel dephosphorylation. In hippocampal neurons, we found that selective activation of extrasynaptic NMDA receptors dephosphorylates Kv4.2 channels, and driving synaptic activity increases phosphorylation of Kv4.2. We also observed that Ca²⁺ entry through NMDA receptors is necessary for dephosphorylation of Kv4.2 channels. Consistent with a synaptic and extrasynaptic localization at hippocampal synapses, a fraction of Kv4.2 channel clusters was found to localize outside of pre- and postsynaptic markers. Excitatory amino acid transporters (EAATs) regulate ambient extracellular glutamate levels that activate extrasynaptic NMDA receptors, and inhibition of glutamate uptake by blocking EAATs with the non-selective transporter inhibitor TBOA or the EAAT1/3 selective inhibitor SOS dephosphorylates Kv4.2 channels. These findings in conjunction with previous reports support the interesting possibility that synaptic and extrasynaptic NMDA receptors bi-directionally regulate phosphorylation levels of Kv4.2 channels in hippocampus. Moreover, we observed that EAAT activity controls extrasynaptic NMDA receptor modulation of Kv4.2 channel dephosphorylation.

Keywords

Hippocampus; A-type K⁺ channels; Excitatory amino acid transporters

Introduction

Kv4.2 channels underlie the transient A-type K⁺ current (I_A) in hippocampal dendrites where they control the amplitude of back-propagating action potentials and shape postsynaptic responses (Hoffman et al., 1997, Cai et al., 2004, Kim et al., 2005). Recent studies have shown

*Corresponding author. Tel: +1-843-792-5159; fax: +1-843-792-7353. mulholl@musc.edu.

Section Editor: Dr. L. Kaczmarek

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that Kv4.2 channels in hippocampus undergo activity-dependent internalization in response to activation of synaptic glutamate receptors resulting in altered I_A and enhanced magnitude of long-term potentiation (LTP) (Kim et al., 2007, Hammond et al., 2008, Jung et al., 2008, Lei et al., 2008). This is consistent for a role of Kv4.2 channels in controlling postsynaptic responses that contribute to synaptic plasticity (Kim and Hoffman, 2008).

Activation of NMDA-type glutamate receptors regulates synaptic plasticity and the phosphorylation and surface trafficking of some voltage-dependent K^+ channels (Misonou et al., 2004, Mulholland et al., 2008a, Park et al., 2008). Indeed, status epilepticus increases extracellular signal-regulated kinase (ERK) phosphorylation of Kv4.2 channels, and this post-translational modification of Kv4.2 channels was associated with a reduction in surface expression of Kv4.2 (Lugo et al., 2008). Here, we examined the effects of synaptic or extrasynaptic NMDA receptor activation on Kv4.2 channel phosphorylation in hippocampus. We observed that driving synaptic activity increases phosphorylation of Kv4.2 channels, and that inhibition of glutamate transporters or activation of extrasynaptic NMDA receptors couples to dephosphorylation of Kv4.2 channels.

Experimental Procedures

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. All compounds were purchased from Sigma-Aldrich Co. except for tetrodotoxin (TTX) (Calbiochem, San Diego, CA) and *DL-threo*- β -benzyloxyaspartic acid (TBOA) (Tocris Bioscience, Ellisville, MO). The triply phospho-Kv4.2 antibody at ERK sites was generously provided by A.E. Anderson (Baylor College of Medicine, Houston, TX). Care of all animals was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996) in an effort to minimize the number of animals used and their suffering. All experiments were conducted in accordance with the Medical University of South Carolina Institutional Animal Care and Use Committee guidelines.

Preparation and Treatment of Cultures

Organotypic hippocampal slices cultures were prepared according to previously described methods with minor modifications (Stoppini et al., 1991, Mulholland et al., 2008a). Five- to six-day-old Sprague-Dawley rat pups were rapidly decapitated and whole brains were aseptically removed and placed in chilled (4°C) dissection medium, consisting of Basal Medium Eagle (with Earle's salts) plus 25 mM HEPES, 2 mM GlutaMAX, and 100 μ g/ml streptomycin (pH 7.2). Bilateral hippocampi were removed and placed in chilled culture medium comprised of dissection medium plus 36 mM glucose, 25% (v/v) Earle's balanced salt solution, and 25% platelet-poor horse serum (PPHS). Each hippocampus was coronally sectioned at 400 μ m using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK), yielding approximately 13 slices (26 per animal), and placed into fresh chilled culture medium. Four slices were transferred onto Millicell-CM 0.4- μ m biopore membrane inserts and placed in 35-mm six-well culture plates in pre-incubated culture medium. Cultures were placed in a 37°C incubator with an atmosphere of 7.5% CO_2 /92.5% air for 10 days ensuring complete development of intrinsic network pathways prior to the start of the experiments. Media was changed every 3-4 days. After 3 days in culture, the media was changed to one containing reduced amounts of PPHS (5%).

Hippocampal neuronal cultures were prepared according to previously reported methods (Carpenter-Hyland et al., 2004). Briefly, newborn rat pups (< 24 hr old) were cold anaesthetized

and brains were removed and placed into isotonic saline (pH 7.4) containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. Pooled hippocampi were digested in 2 ml of 0.25% trypsin (w/v) in isotonic saline, treated with 20 µg/ml DNase I and dissociated by trituration through a fire-polished Pasteur pipette. The volume of the dissociated cell suspension was increased to 50 ml with Dulbecco's Modified Eagle's Medium (DMEM) containing PPHS and centrifuged for 10 min at $600 \times g$. The resulting pellet was resuspended in DMEM/PPHS and plated at a density of either 135,000 (low-density) or 750,000 cells (high-density) in poly-L-lysine-precoated 35 mm culture dishes. Three hours after plating, the DMEM/PPHS media was replaced with Neurobasal media that contained B-27 supplements, penicillin G/streptomycin and 0.5 mM GlutaMax. The Neurobasal media was astroglia conditioned (without B27) by incubation for 48 hr in astroglia-confluent flask (24 ml/155 mm flask) prior to the addition of hippocampal cultures. Three days after plating, 5 µM β -cytosine arabinoside (AraC) was added to each dish. Cultures were maintained in an incubator at 37°C in 7.5% CO₂/92.5% air for 14-17 days *in vitro* with a ¼ media change performed every 7 days.

All experiments were conducted with at least three replicates and data were collected from at least three different culture preparations. To examine alterations in Kv4.2 channel dephosphorylation, 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. Additional slices were co-exposed to 10 µM NMDA and a cocktail of phosphatase inhibitors (1 mM sodium fluoride, 1 mM sodium orthovanadate, 5 µM FK520).

In experiments examining the selective activation of extrasynaptic NMDA receptors in high-density primary neurons, synaptic NMDA receptors were first blocked in Mg²⁺-free buffer with 10 µM MK-801 in the presence of 2 µM TTX (Lu et al., 2001, Tovar and Westbrook, 2002). After 10 min, the MK-801-containing buffer was replaced with MK-801- and Mg²⁺-free buffer, and the unblocked extrasynaptic receptors were activated by bath application of 10 µM NMDA. In addition to a reduction in whole-cell NMDA currents after synaptic trapping, we have previously demonstrated in our cultures that the NMDA component of spontaneous excitatory postsynaptic currents (sEPSC) was markedly reduced and remained absent for more than 30 min following MK-801 washout (Carpenter-Hyland et al., 2004, Mulholland et al., 2008b, Mulholland et al., 2009).

Kv4.2 Phosphorylation and Clustering Analysis

Immediately following treatment, hippocampal slices or high-density neurons were washed once with ice-cold HEPES treatment buffer containing 2 mM Mg²⁺ and were then scraped into 100 µl of ice-cold homogenization buffer (50 mM Tris-HCl, 50 mM NaCl, 10 mM EGTA, 5 mM EDTA; 2 mM sodium pyrophosphate, 1 mM activated sodium orthovanadate, 0.2 mM AEBSF, 1 µg/ml aprotinin, 1 mM benzamide, 10 µg/ml leupeptin, 10 µg/ml pepstatin, pH 7.5). Isolated slices from two inserts or cells from three dishes were combined, probe sonicated for ~ 5 sec, and centrifuged at $23,100 \times g$ for 30 min at 4°C. The resulting supernatant was removed and the pellet was resuspended in 2% sodium dodecyl sulfate (SDS) and probe sonicated for ~ 5 sec. An aliquot was taken for determination of protein concentration by the bicinchoninic acid assay (Pierce Biotechnology, Inc., Rockford, IL). The remaining pellet was stored at -80°C until determination of Kv4.2 phosphorylation.

An aliquot of each sample was diluted with 1× and 6× sample buffer yielding final concentrations of 50 mM Tris-HCl, 4% glycerol (w/v), 2% SDS, 600 mM dithiothreitol and 0.02% bromophenol blue, pH 6.7. Samples were boiled for 5 min, and 15 µg of sample was separated on either 7.5% SDS- or lauryl sulfate-polyacrylamide gels using the buffer system of Laemmli and transferred to Millipore Immobilon-P PVDF membranes (Bedford, MA). After

transfer, blots were washed with phosphate-buffered saline containing 0.1% Tween 20 (PBST) and then blocked with PBST containing 5% nonfat dried milk (NFD) for 1 h at room temperature with agitation. After blocking, the membranes were then incubated overnight at 4°C with mouse anti-Kv4.2 (NeuroMab, Davis, CA) or triply phospho-Kv4.2 (Adams et al., 2000) diluted 1:1000 in PBST containing 0.5% NFD. The membranes were then washed in PBST prior to 1 h incubation at room temperature with horseradish peroxidase conjugated goat anti-mouse or anti-rabbit secondary antibody diluted 1:1000 in PBST containing 0.5% NFD. Membranes received a final wash in PBST and the antigen-antibody complex was detected by enhanced chemiluminescence.

The electrophoretic shift in the relative molecular mass (M_r ; referred to as dephosphorylation) of Kv4.2 channels due to a change in the phosphorylation state was determined in organotypic hippocampal slices by standard immunoblotting procedures. Lane plot profile analyses demonstrates that AP or NMDA treatment did not shift the peak intensity values compared with untreated slices (see Fig. 1A). Thus, data are expressed as a ratio of the area under the curve (AUC) scores created from lane plot profiles for phosphorylated (AUC score above the peak) and dephosphorylated (AUC score below the peak) Kv4.2, and statistical analyses of the AUC ratio was performed using Prism version 4.03 (GraphPad Software, Inc.). Lane plot profiles were created using Image J 1.38x software (National Institutes of Health, USA). For comparison of total Kv4.2 expression, optical density values were generated and analyzed using Image J and Prism software, respectively.

Because of the dense neuropil of organotypic slices, immunostaining of Kv4.2 clusters was performed using low-density hippocampal neurons following previously described methods (Carpenter-Hyland et al., 2004). Briefly, neurons were rinsed twice in ice-cold phosphate-buffered saline (PBS) (in mM: NaCl, 136; KCl, 2.6; KH_2HPO_4 , 1.8; Na_2HPO_4 , 10; pH 7.4) and fixed for 15 min in 4% paraformaldehyde/4% sucrose. Neurons were then permeabilized for 5 min in PBS with 0.2% Triton X-100. Following incubation in 5% normal donkey serum (NDS) for 1 hr, neurons were incubated overnight with primary antibodies at 4°C. After overnight incubation using CoverWell incubation chambers (Invitrogen Corporation, Carlsbad, CA), the chambers were removed and the dishes were incubated with Alexa 488 and 594 secondary antibodies at room temperature for 1 hr. Dishes were then mounted with 1,4-daizabicyclo(2,2,2)octane-containing mounting media and stored at 4°C until imaging. Neurons were colabeled with Kv4.2 (1:100) and the pre-synaptic marker synapsin (1:500; Sigma-Aldrich, Co.) or the postsynaptic density scaffolding protein PSD-95 (1:100; Invitrogen Corp.). Imaging of neurons was performed with a Leica SP5 confocal scope using a 63x NA 1.2 water-immersion objective and a 0.1 μm z-stack distance. Images were analyzed and prepared for display using ImarisXT 6.2.1 3D image analysis software, 64-bit version (Bitplane AG, Zurich, Switzerland). Prior to analysis, point-spread functions in the images were reduced using the adaptive blind 3D deconvolution method (AutoQuant X, version 2.1.3; Media Cybernetics, Inc., Bethesda, MD). Kv4.2 and synapsin clusters were then identified using Imaris software, and the number of Kv4.2 clusters that were closely opposed (<0.5 μm) to synapsin clusters was calculated.

Pentylenetetrazole-induced Seizures

Adult male C3H/He mice (80–100 d of age; 25–28 g) obtained from Charles River Laboratories were used to examine the effect of seizure activity on Kv4.2 phosphorylation. Animals were housed three to four per cage in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility under a 12 h light/dark cycle (lights on at 6:00 A.M.) with *ad libitum* access to food and water. Mice were injected (intraperitoneally) with 35 mg/kg pentylenetetrazole (PTZ) or vehicle (saline) in a dose volume of 10 mg/ml, and the number of myoclonic jerks was recorded for 20 min after injection. The mice were rapidly decapitated,

the brain was removed, and a membrane fraction from bilateral excised hippocampi were prepared for analysis of Kv4.2 phosphorylation following procedures described for neurons and slices.

Assessment of NMDA Excitotoxicity

Excitotoxicity (propidium iodide staining of neurons and glia with compromised membranes) in the CA1 region was detected using propidium iodide (PI) staining. PI staining significantly correlates with other reliable measures of cell death (Zimmer et al., 2000), and likely reflects staining of necrotic or end-stage apoptotic cells (Wolbers et al., 2004). Fluorescence of PI was visualized with a Leica SP5 confocal scope using a 2.5 $\times$ objective and a 2 $\times$ zoom setting. Intensity of PI fluorescence, in arbitrary optical units, was determined in the CA1 region using Image J 1.42q software (National Institutes of Health, Bethesda, MD). Data were analyzed using one-way analysis of variance to compare PI fluorescence across groups.

Results

While a number of studies have demonstrated internalization of Kv4.2 channels following activation of glutamate receptors, closer examination of the immunoblots (e.g., Lei et al., 2008 – Figs. 2 and 3) suggests that there may be an electrophoretic shift in the M_r of Kv4.2 channels. A shift in the M_r of Kv4.2 is likely due to dephosphorylation of the channel through a Ca^{2+} -dependent signaling event following glutamate receptor activation, similar to what has been described for Kv2.1 channels (Misonou et al., 2004, Mulholland et al., 2008a). To test this hypothesis, we first treated homogenate prepared from organotypic hippocampal slices in the presence or absence of alkaline phosphatase (AP; 100 U/ml) for 1 hr at 37°C and examined the electrophoretic shift in the M_r of Kv4.2 channels by gel electrophoresis. Consistent with a previous report (Misonou et al., 2004), AP treatment of the homogenate dephosphorylates the major band of Kv4.2 channels (Fig. 1A). Comparison of lane plot profiles demonstrates that AP treatment did not shift the peak intensity value; instead, a second band appears at a lower molecular weight (Fig. 1A). Analysis of the ratio of AUC scores above and below the peak intensity value of the lane plot profiles reveals that AP treatment significantly dephosphorylates Kv4.2 channels (Fig. 1B).

We next bath applied NMDA (5 – 25 μM) to organotypic hippocampal slices for 20 min. Acute NMDA exposure significantly dephosphorylates Kv4.2 channels (Fig. 2A), and this effect was prevented by co-exposure of slices to 10 μM NMDA and 10 μM MK-801, an NMDA receptor channel blocker, or a cocktail of phosphatase inhibitors (Fig. 2B). We also observed that 10 μM NMDA treatment significantly reduces total expression of Kv4.2 in the crude membrane fraction (Fig. 2C). Although it is clear that NMDA treatment produces an electrophoretic shift in the M_r of Kv4.2 channels, a distinct dephosphorylated Kv4.2 band is not apparent under resolving conditions using SDS gels. We then examined NMDA-induced dephosphorylation of Kv4.2 using lauryl sulfate gels that are better suited for resolving phosphorylated and dephosphorylated proteins (Shibata et al., 2003), and under these resolving conditions, NMDA treatment produces a distinct dephosphorylated Kv4.2 band of lower molecular weight and significantly reduces phosphorylated levels of Kv4.2 channels (Fig. 2D).

To determine if Ca^{2+} entry through NMDA receptors is necessary for dephosphorylation of Kv4.2 channels, we first treated slices with the Ca^{2+} ionophore ionomycin (5 μM) and TTX for 20 min. As expected, ionomycin treatment of slices markedly dephosphorylates Kv4.2 channels (Fig. 3A). In addition, removal of extracellular Ca^{2+} from the treatment buffer prevents NMDA dephosphorylation of Kv4.2 channels (Fig. 3B). To exclude the possible involvement of AMPA-type glutamate receptors, we exposed slices to NMDA in the presence of TTX and the AMPA receptor antagonist NBQX (50 μM) for 20 min, and observed that NMDA significantly dephosphorylates Kv4.2 channels in the presence of NBQX (Fig. 3B).

The dephosphorylation of Kv4.2 induced by NMDA treatment returned to basal values following a 4 h washout period (data not shown), suggesting that this effect is not due to excitotoxic insult. To directly test if this NMDA treatment caused toxicity in our model, we measured propidium iodide uptake in the CA1 region following either 20 min or 24 h 10 μ M NMDA treatment. Twenty-four h, but not 20 min NMDA treatment significantly increases propidium iodide uptake in the CA1 region (Fig. 3C).

NMDA receptors exist in synaptic and extrasynaptic domains (Newpher and Ehlers, 2008), and activation of synaptic NMDA receptors appears to increase phosphorylation of Kv4.2 channels (Kim et al., 2007, Hammond et al., 2008, Lugo et al., 2008). This raises the interesting possibility that activation of extrasynaptic NMDA receptors couples to dephosphorylation of Kv4.2 channels. We first tested whether synaptic activity increases phosphorylation of Kv4.2 channels using a phospho-specific antibody at three ERK sites (Adams et al., 2000). As expected, driving synaptic NMDA receptor activity by inhibiting GABAergic tone using the GABA receptor antagonist bicuculline (50 μ M) increases phospho-Kv4.2 levels (Fig. 4A) but did not dephosphorylate Kv4.2 channels (Fig. 4B). We also observed an increase in phospho-Kv4.2 levels 20 min following injection of the pro-convulsant PTZ (35 mg/kg) (Fig 4C). These mice treated with PTZ averaged 15 ± 5.1 myoclonic jerks during 20 min of observation. In contrast, selectively activating extrasynaptic NMDA receptors using the MK-801 technique in primary hippocampal neurons produces a significant dephosphorylation of Kv4.2 (Fig. 5A) and a reduction in phospho-Kv4.2 levels (Fig. 5B). Consistent with evidence suggesting that Kv4.2 channels localize to synaptic and extrasynaptic sites (Jinno et al., 2005, Burkhalter et al., 2006), we observed that a portion of Kv4.2 clusters does not colocalized with clusters for synapsin or PSD-95 (Fig. 5C). Indeed, $37.7 \pm 2.1\%$ of Kv4.2 clusters were not closely opposed to synapsin clusters. To further address the role of extrasynaptic NMDA receptors in modulating Kv4.2 channel dephosphorylation, we inhibited glutamate uptake by blocking excitatory amino acid transporters (EAATs) using the non-selective inhibitor TBOA (200 μ M) or the EAAT1/3 inhibitor *L*-serine *O*-sulfate (SOS; 1 mM). Exposure of slices to either TBOA (Fig. 5D) or SOS (Fig. 5E) significantly dephosphorylates Kv4.2 channels, even in the presence of 50 μ M NBQX (data not shown).

Discussion

The findings presented here suggest that activation of extrasynaptic, but not synaptic NMDA receptors produces an electrophoretic shift in the M_r of Kv4.2 channels. Both AP and ionomycin treatment produces a shift in the M_r of the channel, suggesting that the effect of extrasynaptic NMDA receptors on Kv4.2 channels likely reflects channel dephosphorylation through a Ca^{2+} -dependent mechanism. Indeed, removal of Ca^{2+} from the treatment buffer prevented NMDA and TBOA dephosphorylation of Kv4.2 channels, and the inclusion of the AMPA receptor antagonist NBQX did not block NMDA- or TBOA-induced Kv4.2 dephosphorylation. Consistent with previous work demonstrating that Kv4.2 channels are expressed at extrasynaptic domains of excitatory synapses (Shibata et al., 2003, Jinno et al., 2005, Burkhalter et al., 2006), we found that a fraction of Kv4.2 channel clustering was not associated with the glutamatergic postsynaptic density scaffolding protein PSD-95 or the presynaptic marker synapsin. We also demonstrated that blocking EAAT activity with the non-selective transporter inhibitor TBOA or the EAAT1/3 inhibitor SOS markedly dephosphorylated Kv4.2 channels. There is substantial evidence for a direct link between inhibition of EAATs and activation of extrasynaptic NMDA receptors (Fellin et al., 2004, Cattani et al., 2007, Le Meur et al., 2007), and it appears that uptake of glutamate by EAATs regulates the coupling of extrasynaptic NMDA receptors and Kv4.2 channels.

Interestingly, previous work suggest that Kv4.2 channel function and surface expression are reduced following AMPA receptors stimulation via Ca^{2+} -influx through synaptic NMDA

receptors and PKA phosphorylation of the channel (Kim et al., 2007, Hammond et al., 2008). Moreover, kainate-induced status epilepticus was associated with an increase in ERK phosphorylation and reduction in surface expression of Kv4.2 channels in CA1 (Lugo et al., 2008), and theta burst stimulation of CA1 pyramidal neurons induced long-lasting LTP and increased ERK phosphorylation of Kv4.2 that leads to a reduction in channel conductance, an increase in neuronal depolarization and a facilitation of complex spike bursting (Watanabe et al., 2002, Yuan et al., 2002, Morozov et al., 2003). We observed that driving synaptic activity in culture by inhibiting GABAergic tone using bicuculline or mild seizure activity induced by PTZ treatment of adult mice increases Kv4.2 phosphorylation at ERK and possibly other phosphorylation sites. Together these studies demonstrate that elevations in synaptic NMDA receptor activation lead to an increase in phosphorylation of Kv4.2. Moreover, selective activation of extrasynaptic NMDA receptors decreases phosphorylation of Kv4.2 at ERK sites. These data are not surprising given evidence that extrasynaptic NMDA receptors couple to a dominant ERK shut-off pathway in hippocampus and cortex (Ivanov et al., 2006, Mulholland et al., 2008b), and further suggest that basal activity under normal conditions maintains partial ERK phosphorylation of Kv4.2. These data together with previous reports suggest that synaptic and extrasynaptic NMDA receptor activity bi-directionally regulates the phosphorylation status of Kv4.2 channels in hippocampus. While increased phosphorylation of Kv4.2 by activation of synaptic NMDARs is associated with enhanced excitability and plasticity, it would be interesting to determine how extrasynaptic NMDA receptor-mediated dephosphorylation of Kv4.2 affects synaptic plasticity of glutamatergic synapses.

NMDA receptors play a critical role in experience-dependent synaptic plasticity, but they also contribute to neuronal Ca^{2+} overload during pathological conditions, such as stroke and neurodegenerative diseases (Rothman and Olney, 1995, Waxman and Lynch, 2005). A key determinant of whether activation of NMDA receptors leads to synaptic plasticity or cell death is their subcellular localization. NMDA receptors are present at excitatory synapses and at extrasynaptic domains (Newpher and Ehlers, 2008), and studies have demonstrated that selective activation of extrasynaptic NMDA receptors couples to a pro-apoptotic pathway and contributes to loss of mitochondrial membrane potential and to excitotoxicity (Hardingham et al., 2002, Leveille et al., 2008). In our study, we demonstrated that 20 min exposure of slices to 10 μM NMDA did not produce significant CA1 neurotoxicity, but was associated with dephosphorylation of Kv4.2 channels. If phosphorylation of Kv4.2 channels leads to an increase in excitability and prevalence of synaptic plasticity, it is tempting to speculate that dephosphorylation of Kv4.2 channels increases surface expression as an early mechanisms for reducing excitability and potentially preventing the subsequent excitotoxicity associated with activation of extrasynaptic NMDA receptors. However, it was previously reported that bath application of glutamate and NMDA reduced total expression of Kv4.2 channels (Lei et al., 2008). Consistent with this finding, we also demonstrated that NMDA exposure reduces expression of Kv4.2 channels in a total membrane fraction. This would suggest that Kv4.2 channels are internalized and rapidly degraded following activation of extrasynaptic NMDA receptors. Alternatively, it is possible that the NMDA-induced electrophoretic shift in the M_r of Kv4.2 channels is affecting the ability to accurately measure total protein levels.

In summary, we observed that activation of extrasynaptic NMDA receptors dephosphorylates Kv4.2 channels and reduces phosphorylation levels of Kv4.2 at ERK sites. Ca^{2+} entry through extrasynaptic NMDA receptors even in the presence of AMPA receptor blockers is necessary for dephosphorylation of Kv4.2 channels. Our data suggest that dephosphorylation of Kv4.2 channels by extrasynaptic NMDA receptors is regulated by EAATs. Consistent with previously published studies, elevating synaptic activity is associated with an increase in phosphorylation of Kv4.2 at ERK sites. Together, these data suggest that regulation of Kv4.2 phosphorylation is dependent upon the level of synaptic and extrasynaptic NMDA receptor activity.

Acknowledgments

This work was supported by NIAAA grants AA017922 (P.J.M.) and AA010983 (L.J.C.). We thank Nick Luong, Kaylin Currie and Melissa Wiel for their technical assistance in performing some of these experiments.

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Abbreviations

I_A	A-type K ⁺ currents
AP	Alkaline phosphatase
DMEM	Dulbecco's Modified Eagle's Medium
EAATs	Excitatory amino acid transporters
ERK	extracellular signal-regulated kinase
LTP	long-term potentiation
PI	Phosphatase inhibitors
PPHS	platelet-poor horse serum
PI	Propidium Iodide
M_r	Relative molecular mass
NBQX	2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline -7-sulfonamide
SOS	L-serine O-sulfate
SNK	Student Newman-Keuls
TBOA	DL-threo- β -benzyloxyaspartic acid
TTX	Tetrodotoxin

Figure 1.

Alkaline phosphatase (AP) dephosphorylates Kv4.2 channels in organotypic hippocampal slices. **(A)** AP (100 U/ml) treatment of homogenate prepared from hippocampal slices induces an electrophoretic shift in the M_r of Kv4.2 channels. Lane profile plots created using Image J software demonstrate that AP treatment did not shift the peak intensity value (arrow), but produces the appearance of a second band of lower molecular weight (arrow head). **(B)** AP treatment significantly dephosphorylates Kv4.2 channels ($*p < .01$ vs. CTRL; Student's t-test; $n = 3$).

Figure 2.

Activation of NMDA receptors leads to dephosphorylation of Kv4.2 channels in organotypic hippocampal slices. **(A)** Bath application of NMDA (0 – 25 μM) in the presence of 2 μM TTX dephosphorylates Kv4.2 channels, and 10 μM MK-801 co-exposure with 10 μM NMDA completely prevents this effect (* $p < .01$ vs. CTRL; ANOVA with Student Newman-Keuls (SNK); $n = 4-5$). **(B)** Co-exposure of slices with 10 μM NMDA and a cocktail of phosphatase inhibitors (PI) prevents NMDA-induced dephosphorylation of Kv4.2 channels (* $p < .01$ vs. CTRL; ANOVA with SNK; $n = 3$). **(C)** NMDA treatment reduces total expression of Kv4.2 channels in a crude membrane fraction (* $p < .05$ vs. CTRL; Student's t -test; $n = 4$). **(D)** A dephosphorylated band of lower molecular weight induced by NMDA treatment can be

resolved using lauryl sulfate gels. Under these resolving conditions, NMDA treatment significantly reduces levels of phosphorylated Kv4.2 band ($*p < .01$ vs. CTRL; Student's t -test; $n = 4$).

Figure 3.

Ca²⁺ entry is necessary for NMDA dephosphorylation of Kv4.2 channels. (A) Bath application of slices with the Ca²⁺ ionophore ionomycin significantly dephosphorylates Kv4.2 channels (**p* < .01 vs. CTRL; Student's *t*-test; *n* = 3). (B) Omission of extracellular Ca²⁺ from the treatment buffer prevents NMDA-induced dephosphorylation of Kv4.2. However, NMDA significantly dephosphorylate Kv4.2 in the presence of the AMPA receptor antagonist NBQX (50 μM) (**p* < .05 vs. CTRL; ANOVA with SNK; *n* = 3). (C) Twenty-four h, but not acute (20 min) exposure of slices to 10 μM NMDA produces significant PI uptake in the CA1 region (**p* < .001 vs. CTRL & 20 min NMDA; ANOVA with SNK; *n* = 4-8).

Figure 4.

Synaptic activity increases phosphorylation of Kv4.2 at ERK sites. Driving synaptic activity by inhibition of GABAergic tone using 50 μ M bicuculline (**A**) increases phospho-Kv4.2 levels ($*p < .05$ vs. CTRL; Student's *t*-test; $n = 4$) and (**B**) does not dephosphorylate Kv4.2 channels ($p > .05$; Student's *t*-test; $n = 3$). (**C**) Treatment of adult mice with 35 mg/kg PTZ markedly increases phosphoKv4.2 levels in hippocampus ($*p < .01$ vs. saline; Student's *t*-test; $n = 4$).

Figure 5.

Glutamate transporters regulate extrasynaptic NMDA receptor modulation of Kv4.2 channel dephosphorylation. (A) After MK-801 trapping of synaptic NMDA receptors, bath application of 10 μ M NMDA (20 min) resulting in selective activation of extrasynaptic NMDA receptors significantly dephosphorylates Kv4.2 channels ($*p < .01$ vs. CTRL; ANOVA with SNK; $n = 4$). (B) Activation of extrasynaptic NMDA receptors reduces phosphorylation of Kv4.2 channels ($*p < .05$ vs. CTRL; Student's *t*-test; $n = 4$). (C) As expected, a portion of Kv4.2 clusters does not co-localize with the presynaptic marker synapsin ($n = 4$, $100 \times 100 \times 3.5 \mu$ m sections totaling ~4500 Kv4.2 clusters) or PSD-95. Arrows indicate clusters of Kv4.2 channels

that do not colocalize with synapsin or PSD-95. **(D,E)** Bath application of the non-selective glutamate transporter inhibitor TBOA (200 μ M) or the EAAT1/3 inhibitor SOS (1 mM) in the presence of TTX dephosphorylates Kv4.2 channels (* $p < .01$ vs. CTRL; ANOVA with SNK for TBOA experiment; Student's t -test for SOS experiment; $n = 3-4$).