Neuroscience Letters 513 (2012) 89–94

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

$GABA_B$ receptor-mediated $ERK_{1/2}$ phosphorylation via a direct interaction with $Ca_V1.3$ channels

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ARTICLE INFO

Article history:
Received 4 January 2012
Accepted 6 February 2012

Keywords:
L-type Ca^{2+} channels
Baclofen
Intracellular Ca^{2+}
FDSS6000

ABSTRACT

Neuronal L-type Ca^{2+} channels play pivotal roles in regulating gene expression, cell survival, and synaptic plasticity. The $Ca_V1.2$ and $Ca_V1.3$ channels are 2 main subtypes of neuronal L-type Ca^{2+} channels. However, the specific roles of $Ca_V1.2$ and $Ca_V1.3$ in L-type Ca^{2+} channel-mediated neuronal responses and their cellular mechanisms are poorly elucidated. On the basis of our previous study demonstrating a physical interaction between the $Ca_V1.3$ channel and $GABA_B$ receptor ($GABA_BR$), we further examined the involvement of $Ca_V1.2$ and $Ca_V1.3$ in the $GABA_BR$-mediated activation of $ERK_{1/2}$, a kinase involved in both CREB activation and synaptic plasticity. After confirming the involvement of L-type Ca^{2+} channels in baclofen-induced $ERK_{1/2}$ phosphorylation, we examined a specific role of $Ca_V1.2$ and $Ca_V1.3$ channels in the baclofen effect. Using siRNA-mediated silencing of $Ca_V1.2$ or $Ca_V1.3$ messenger, we determined the relevance of each channel subtype to baclofen-induced $ERK_{1/2}$ phosphorylation in a mouse hippocampal cell line (HT-22) and primary cultured rat neurons. In the detailed characterization of each subtype using HEK293 cells transfected with $Ca_V1.2$ or $Ca_V1.3$, we found that $GABA_BR$ can increase $ERK_{1/2}$ phosphorylation and $Ca_V1.3$ channel activity through direct interaction with $Ca_V1.3$ channels. These results suggest a functional interaction between $Ca_V1.3$ and $GABA_BR$ and important implications of $Ca_V1.3/GABA_BR$ clusters for translating synaptic activity into gene expression alterations.

1. Introduction

Neuronal Ca^{2+} channels have multiple functions in the central nervous system (CNS) such as neurotransmitter release, neuron excitability, gene expression, and neuronal plasticity. Among several types of neuronal Ca^{2+} channels, neuronal L-type Ca^{2+} channels are particularly important for their roles in translating synaptic activity into gene expression and neuronal function [4,14]. L-type Ca^{2+} channels, also known as the Ca_V1 family, comprise $Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$, and $Ca_V1.4$. In particular, $Ca_V1.2$ and $Ca_V1.3$ are the 2 main types of L-type Ca^{2+} channels in the CNS [10,18]. However, the specific roles of $Ca_V1.2$ and $Ca_V1.3$ in L-type Ca^{2+} channel-mediated neuronal responses and their cellular mechanisms are poorly elucidated.

The metabotropic γ-aminobutyric acid (GABA) type B receptor ($GABA_BR$) is one of the main neurotransmitter receptors to regulate neuronal network activity and synaptic functions [8,20]. Heterodimeric $GABA_BRs$ are formed by the $GABA_{B1}$ receptor and $GABA_{B2}$ receptor subunits. While the $GABA_{B1}$ receptor subunit contains the agonist binding domain, the $GABA_{B2}$ receptor subunit is responsible for G-protein activation [2,7]. As a member of the $G_{i/o}$ subtype G-protein-coupled receptor family, it is well known that activation of $GABA_BR$ causes inhibition of N- ($Ca_V2.2$) and P/Q-type ($Ca_V2.1$) Ca^{2+} channels and subsequent suppression of neurotransmitter release at presynaptic loci [16]. Besides these prominent inhibitory effects, activation of $GABA_BRs$ has also been shown to increase L-type Ca^{2+} channels in various neurons [5,17]. However, much less is known about the signal transduction pathway involved in the enhancement of L-type Ca^{2+} channels by $GABA_BRs$ in mammalian neurons. Compared to the presynaptic inhibition of $GABA_BRs$ through N- and P/Q-type Ca^{2+} channels, L-type Ca^{2+} channels are located on the soma and dendrites of neurons, suggesting that the augmentation of L-type Ca^{2+} channels by $GABA_BRs$ is mediated through a different mechanism.

Interestingly, it is reported that $GABA_BR$ induces the phosphorylation of extracellular signal-regulated protein kinases 1/2 ($ERK_{1/2}$) in the hippocampus and cerebellum [19,21], but the molecular mechanism underlying this event has not yet been clearly characterized. In our previous study, we demonstrated a physical interaction between the $Ca_V1.3$ α_1 subunit and the $GABA_{B2}$ receptor using yeast two-hybrid, GST pull-down, co-immunoprecipitation, and immunocytochemistry assays [15]. On the basis of this

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doi:10.1016/j.neulet.2012.02.014

previous study, we examined the involvement of $Ca_V1.2$ and $Ca_V1.3$ channels in $GABA_BR$-mediated $ERK_{1/2}$ phosphorylation and further examined the functional modulation between $Ca_V1.3$ and $GABA_BR$ on $ERK_{1/2}$ phosphorylation by using a mouse hippocampal cell line (HT-22), HEK293 cells, and cultured rat neurons.

2. Materials and methods

2.1. Materials

Baclofen, CGP54626, and nifedipine were purchased from Tocris Bioscience (Ellisville, MO). Anti-$ERK_{1/2}$ and anti-phospho-$ERK_{1/2}$ antibodies were purchased from Cell Signaling Technology (Beverly, MA). All secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

2.2. Cell culture and transfection

The immortalized mouse hippocampal–neuronal precursor cells, HT-22, and HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Primary cortical or hippocampal neurons were prepared from 18-day-old fetal Sprague-Dawley rats. The dissected cortex or hippocampi were incubated with 0.25% trypsin in Hanks' balanced salt solution (HBSS) for 15 min at 37 °C. After 3 washes with HBSS, neurons were then mechanically dissociated with fire-polished Pasteur pipettes by trituration and plated on poly-L-lysine coated plates. The neurons were seeded into 6-well plates at a density of 1×10^6 cells/well in Neurobasal/B27 medium (Invitrogen) containing 0.5 mM L-glutamine, 25 μM 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin. Experiments were carried out on neurons after incubating for 7–14 days in vitro (DIV). For transient transfection of either cell lines or neurons, cells were plated at a density of 1×10^6 per well on 6-well plates and transfected with $Ca_V1.2$-siRNA, $Ca_V1.3$-siRNA, $Ca_V1.2$, or $Ca_V1.3$ using Lipofectamine 2000 (Invitrogen, CA). $Ca_V1.2$- and $Ca_V1.3$-siRNA sequences were reported in our previous studies [12,15].

2.3. Phospho-$ERK_{1/2}$ assays

HT-22 or HEK293 cells were kept in serum-free DMEM for 3 h, the cells were treated with baclofen in the absence or presence of the indicated drugs and gently lysed in the lysis buffer containing 1 mM DTT, 5 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, and protease inhibitor cocktail. Equal amounts of lysates were prepared and immunoblotted with anti-$ERK_{1/2}$ (1:2000) and anti-p-$ERK_{1/2}$ (1:2000) antibodies. For western blot analysis, the proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) after 10% SDS-PAGE, and the membrane was blocked with 1× Tris-buffered saline (TBS) containing 0.1% Tween 20, and 5% BSA or 5% skim milk for 1 h at room temperature. After blocking, the membranes were incubated overnight at 4 °C with the respective primary antibodies. The membranes were washed 3 times, and incubated with diluted horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000, Jackson ImmunoReserch, West Grove, PA) for 1 h at room temperature. After 3 washes, the membranes were visualized using the enhanced chemiluminescence (ECL) kit (Millipore, Bedford, MA).

2.4. Measuring intracellular Ca^{2+} using an FDSS6000 system

For measuring Ca^{2+} channel-mediated intracellular Ca^{2+} changes in a 96-well format, we used an FDSS6000 96-well fluorescence plate reader. The cells were loaded with 5 μM fluo-4-AM and 0.001% Pluronic F-127 and incubated for 60 min at 37 °C in a HEPES-buffered solution (115 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 20 mM HEPES, and 13.8 mM glucose, pH 7.4). Cells were washed 3 times with a HEPES buffer and maintained with a volume of 80 μl per well. During the whole procedure, cells were washed using a BIO-TEK 96-well washer (BIO-TEK instruments, Winooski, VT). After incubation for at least 10 min, the cells were assayed using an FDSS6000 system (Hamamatsu Photonics, Japan). All data were collected and analyzed using an FDSS6000 system and the related software (Hamamatsu Photonics). On the basis of our previous studies [11,13], $Ca_V1.3$ channel activity was measured using high concentration of KCl (70 mM) in HEK293 cells transiently transfected with $Ca_V1.3$, β_3, $GABA_{B1}$, $GABA_{B2}$, and Kir2.1. Saline with 70 mM KCl was made by replacing an equivalent amount of NaCl.

2.5. Statistical analysis

The intensity of the bands was measured using the AlphaEase program (Version 5.1, Alpha Innotech, San Leandro, CA) and analyzed using the GraphPad Prism Version 4 program (GraphPad Software Inc., San Diego, CA). All numeric values are represented as the mean ± S.E. The statistical significance of the data was determined using ANOVA followed by Dunnett's test or Student's unpaired t test. Significance was set at $p < 0.05$, and the values of p for significant differences are indicated in the text and figure legends.

3. Results and discussion

3.1. Involvement of L-type Ca^{2+} channels in baclofen-mediated $ERK_{1/2}$ phosphorylation

We first examined whether activation of $GABA_BR$ induces phosphorylation of $ERK_{1/2}$ in the mouse hippocampal HT-22 cell line. As shown in Fig. 1A, a selective $GABA_BR$ agonist, baclofen, produced a rapid and transient increase in $ERK_{1/2}$ phosphorylation in HT-22 cells. The maximum effect was observed after 1 min of treatment with 100 μM baclofen, and phosphorylation returned to the basal level after 15 min of treatment. To examine whether this effect occurs via the specific activation of $GABA_BR$, we evaluated baclofen-mediated $ERK_{1/2}$ phosphorylation in the presence of a selective $GABA_BR$ antagonist, CGP54626, at 10 μM. Pretreatment with CGP54626 for 15 min significantly blocked baclofen-mediated $ERK_{1/2}$ phosphorylation (Fig. 1B). It has been shown that $GABA_BR$ activated $ERK_{1/2}$ in cultured cerebellar granule neurons [19], but the time of on-set of baclofen-mediated $ERK_{1/2}$ phosphorylation differed markedly from our results. They observed maximum effects when GABA or baclofen was pretreated for 10–20 min, while there was no significant change after 1 min of pretreatment, at which we observed the maximum effect in the present study. This suggests that a faster signal transduction pathway is involved in HT-22 cells. We next examined the involvement of L-type Ca^{2+} channels in baclofen-mediated $ERK_{1/2}$ phosphorylation, because activation of $GABA_BR$ is also known to modulate L-type Ca^{2+} channels in various neuronal types [5,17]. Cells were pretreated with a selective Ca^{2+} channel blocker, nifedipine (10 μM), for 15 min, and then stimulated with baclofen (100 μM) for 1 min. We found that nifedipine significantly blocked baclofen-induced $ERK_{1/2}$ phosphorylation without altering ERK expression levels (Fig. 1B), suggesting that L-type Ca^{2+} channels contribute to baclofen-induced $ERK_{1/2}$ phosphorylation in HT-22 cells.