Direct interaction and functional coupling between voltage-gated CaV1.3 Ca\textsuperscript{2+} channel and GABA\textsubscript{B} receptor subunit 2

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Abstract

Although Ca\textsubscript{V}1.2 and Ca\textsubscript{V}1.3 are two subtypes of L-type Ca\textsuperscript{2+} channels expressed in the CNS, functions of Ca\textsubscript{V}1.3 have not been well elucidated compared to Ca\textsubscript{V}1.2. Here, we found that Ca\textsubscript{V}1.3-NT associates with GABA\textsubscript{B}R2-CT using yeast two-hybrid, GST pull-down and co-immunoprecipitation assays. We also demonstrated co-localization of Ca\textsubscript{V}1.3 and GABA\textsubscript{B}R2 in HEK293 cells and cultured hippocampal neurons. Whole-cell patch-clamp and Ca\textsuperscript{2+}-imaging experiments revealed that activation of GABA\textsubscript{B}R increases Ca\textsubscript{V}1.3 currents and intracellular Ca\textsuperscript{2+} via Ca\textsubscript{V}1.3, but not Ca\textsubscript{V}1.2. These results show a physical and functional interaction between Ca\textsubscript{V}1.3 and GABA\textsubscript{B}R, suggesting the potential pivotal roles of Ca\textsubscript{V}1.3 in the CNS.

1. Introduction

Voltage-gated Ca\textsuperscript{2+} channels (VGCCs) control numerous cellular responses such as muscle contraction, neurotransmission and gene expression. VGCCs can be distinguished by their electrophysiological properties: high-voltage-activated L-, N-, P/Q-, and R-type Ca\textsuperscript{2+} channels and low-voltage-activated T-type Ca\textsuperscript{2+} channels [1]. Among them, L-type Ca\textsuperscript{2+} channels, also known as Ca\textsubscript{V}1 family, comprise Ca\textsubscript{V}1.1, Ca\textsubscript{V}1.2, Ca\textsubscript{V}1.3 and Ca\textsubscript{V}1.4. In particular, Ca\textsubscript{V}1.2 and Ca\textsubscript{V}1.3 are widely expressed in neurons, where the both are found in cell bodies and proximal dendrites [2,3]. However, their biophysical and pharmacological properties are different [4].

Ca\textsubscript{V}1.3 is activated at more hyperpolarized membrane potentials and less sensitive to dihydropyridines than Ca\textsubscript{V}1.2. While Ca\textsubscript{V}1.2 has been extensively studied [5–8], the neuronal functions of Ca\textsubscript{V}1.3 are poorly understood.

To investigate the functions of Ca\textsubscript{V}1.3 in the central nervous system (CNS), we employed a yeast two-hybrid screening in the rat brain cDNA library with the N-terminus of rat Ca\textsubscript{V}1.3 \textalpha\textsubscript{1} subunit (Ca\textsubscript{V}1.3-NT) as bait, and found the C-terminus of \textgamma\textsubscript{a} aminobutyric acid (GABA) type B receptor subunit 2 (GABA\textsubscript{B}R2) as a binding partner. As a member of G\textsubscript{a} family, it has been well known that activation of GABA\textsubscript{B}R causes inhibition of N- and P/Q-type Ca\textsuperscript{2+} channels and subsequent suppression of neurotransmitter release [9]. Besides these prominent inhibitory effects, activation of GABA\textsubscript{B}R has also been shown to augment L-type Ca\textsuperscript{2+} channels in various neurons [10,11].

In the present study, we demonstrated a physical interaction between Ca\textsubscript{V}1.3 \textalpha\textsubscript{1} subunit and GABA\textsubscript{B}R2 subunit using various methods and further showed that activation of GABA\textsubscript{B}R induced...
the increase in L-type Ca\(^{2+}\) channel currents via a direct interaction with Ca\(v\).1.3 using whole-cell patch-clamp and intracellular Ca\(^{2+}\) imaging techniques.

2. Materials and methods

2.1. Yeast two-hybrid assay

To construct bait, a cDNA fragment encoding Ca\(v\).1.3-NT (amino acids 1–151) was subcloned into the NdeI/SalI site of the GAL4 DNA-binding domain vector, pGBK7. Bait plasmid was then transformed into the yeast strain AH109. The rat brain cDNA library in the GAL4 activation domain vector, pACT2 (prey) was transformed into the yeast strain Y187. The yeast two-hybrid assay was performed as described previously [12].

2.2. GST pull-down assay

GST pull-down assays were performed with purified GST and GST-Ca\(v\).1.3-NT proteins using the ProFound Pull-Down GST Protein–Protein Interaction Kit (Pierce, Rockford, IL). The immobilized GST or GST-Ca\(v\).1.3-NT proteins were incubated with cell lysates prepared from HA-GABABR2-expressed HEK293 cells or rat brain. Glutathione-bound proteins were eluted by boiling at 95 °C in SDS-sample buffer for 10 min, and detected by immunoblotting with anti-GST (1:5000; Novagen, Madison, WI), anti-HA (1:1000; Cell Signaling, Beverly, MA) and anti-GABABR2 (1:1000; Chemicon, Temecula, CA) antibodies.

2.3. Co-immunoprecipitation assay

Co-immunoprecipitation assays were performed using the ProFound Mammalian Co-Immunoprecipitation Kit (Pierce). Briefly, lysates from adult rat hippocampi were precleared, and immunoprecipitated with 10 μg of a specific antibody and 50 μl of AminoLink™ Plus Coupling Gel at 4 °C for 6 h or overnight, and washed five times. Immune complexes were eluted using IgG Elution Buffer (Pierce) or by boiling at 95 °C in SDS-sample buffer for 10 min, and detected by immunoblotting with anti-Ca\(v\).1.3 (1:200) or anti-GABABR2 (1:1000) antibodies.

2.4. Immunocytochemistry

For immunocytochemistry, cells were fixed in 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. After blocked with 3% bovine serum albumin, cells were incubated with rabbit anti-Ca\(v\).1.3 and guinea pig anti-GABABR2 antibodies, and then with FITC- and rhodamine-conjugated secondary antibodies, respectively. Cells were then mounted on a glass slide, and viewed on a confocal FV 1000 laser scanning microscope (Olympus, Tokyo, Japan).

2.5. Electrophysiological recording

Ca\(v\).1.3 currents were measured using Ba\(^{2+}\) as a charge carrier with standard whole-cell patch-clamp techniques. Borosilicate glass electrodes were filled with the internal solution containing (in mM): 135 CsCl, 10 EGTA, 4 MgCl\(_2\), 10 HEPES and 4 Mg-ATP (pH 7.4). The external solution contained (in mM): 135 CholineCl, 5 BaCl\(_2\), 1 MgCl\(_2\), 10 glucose and 10 HEPES (pH 7.4). Cells were held at a holding potential of ~80 mV, and currents were elicited by a depolarizing test step to ~10 mV for 200 ms. All the data were obtained and analyzed using an EPC-10 amplifier and Pulse/Pulsefit software program (HEKA, Germany).

2.6. Hippocampal neuron cultures, transfection and intracellular Ca\(^{2+}\) imaging

Hippocampal neuron cultures, transfection with small interfering RNA (siRNA), and fura-2-based intracellular Ca\(^{2+}\) imaging were carried out using the protocols from Kim et al. [12]. Hippocampal cultures were isolated from 16 to 18-day-old fetal Sprague–Dawley rats, and hippocampal neurons were cultured and transfected with siRNA after 5 days in vitro. Each siRNA at 5 pmol/μl concentration was transfected into the primary cultured rat hippocampal neurons using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

3. Results

3.1. Identification of GABABR2 as a Ca\(v\).1.3 binding protein using the yeast two-hybrid system

To elucidate the function of Ca\(v\).1.3 L-type Ca\(^{2+}\) channels and its cellular mechanism in the CNS, we performed a yeast two-hybrid screening using Ca\(v\).1.3-NT. Through the screening of the rat brain cDNA library, we found that GABABR2 interacts with Ca\(v\).1.3-NT. To confirm this specific interaction, we carried out a yeast two-hybrid assay using Ca\(v\).1.3-NT cDNA (bait, amino acids 1–151) transformed into the AH109 yeast strain and the C-terminus of GABABR2 (GABABR2-CT) cDNA (prey, amino acids 741–941) transformed into the Y187 yeast strain. When AH109 and Y187 yeast strains were mated, a blue color was detected by the β-galactosidase colony-lift filter assay as shown in Fig. 1A. This result suggests that the observed interaction between Ca\(v\).1.3-NT and GABABR2-CT is positive.

3.2. In vitro and in vivo interaction between Ca\(v\).1.3 and GABABR2

Next, we attempted to determine whether the association of Ca\(v\).1.3 and GABABR2 occurs in native cells. As shown in Fig. 1B, GST-Ca\(v\).1.3-NT interacts with HA-tagged GABABR2 (HA-GABABR2) in HEK293 cells while GST, a negative control, alone was shown not to interact with HA-GABABR2. Subsequently, we confirmed the association of Ca\(v\).1.3 and GABABR2 in adult rat brain lysates. GST-Ca\(v\).1.3-NT interacts with endogenous GABABR2 in rat brain lysates (Fig. 1C). Although these results suggest an interaction between Ca\(v\).1.3-NT and GABABR2, it is necessary to demonstrate the association between endogenous Ca\(v\).1.3 and GABABR2 in native tissues. We therefore examined further to see whether Ca\(v\).1.3 binds to GABABR2 in adult rat hippocampus using an in vivo co-immunoprecipitation assay. Cell lysates prepared from adult rat hippocampi were immunoprecipitated with anti-Ca\(v\).1.3 antibodies, and subsequently immunoblotted with anti-GABABR2 antibodies. In Fig. 1D, GABABR selectively binds to endogenous Ca\(v\).1.3 in rat hippocampus whereas no signal was detected in an IgG control. When co-immunoprecipitation was performed in reverse with anti-GABABR2 antibodies followed by immunoblotting with anti-Ca\(v\).1.3, the result was the same (Fig. 1E). These results strongly suggest that a specific interaction between Ca\(v\).1.3 and GABABR2 occurs in mammalian cells as well as rat brain, especially in the hippocampus.

3.3. Cellular localization and functional interaction of Ca\(v\).1.3 with GABABR2 in HEK293 cells

For a functional interaction between the two proteins, Ca\(v\).1.3 and GABABR2, they must be positioned in close proximity. We therefore tested whether the both proteins are localized in the same subcellular compartment in heterologously expressed HEK293...
cells. HEK293 cells transfected with CaV1.3 along with GABABR1a and GABABR2 were immunostained with anti-CaV1.3 and then with FITC-conjugated secondary antibodies for CaV1.3, and immunostained with anti-GABABR2 and then with rhodamine-conjugated secondary antibodies for GABABR1a. Immunofluorescence analysis revealed that CaV1.3 was predominantly localized on plasma membrane, and co-localized with GABABR as shown in the merged image of Fig. 2A. Based on the evidence for a physical interaction between CaV1.3 and GABABR1a and GABABR2, we examined whether the activation of GABABR functionally modulates CaV1.3 activity. HEK293 cells transfected with CaV1.3 and GABABR2, we examined whether the activation of GABABR functionally modulates CaV1.3 activity. HEK293 cells transfected with CaV1.3 a1 and 3 subunits and GABABR1a and GABABR2 subunits were used for whole-cell patch-clamp recordings. The transfection efficiency was indirectly confirmed using co-transfected GFP. Ba2+ currents via CaV1.3 were increased when a selective GABABR agonist, baclofen, was applied. In ~70% of GFP-positive and current-recordable cells, we observed baclofen-mediated increase in Ba2+ currents. In Fig. 2B, a representative trace of the CaV1.3 recordings shows a rapid increase in Ba2+ currents upon the application of 100 μM baclofen. Baclofen significantly increased peak currents of CaV1.3 (156.9 ± 9.4% of control, n = 4, Fig. 2C), but did not cause any shift in a current–voltage curve in Fig. 2D (n = 3). These data provide the first experimental evidence showing that activation of GABABR increases CaV1.3 L-type Ca2+ currents in HEK293 cells.

3.4. Cellular localization and functional interaction of CaV1.3 with GABABR2 in cultured hippocampal neurons

We next examined whether co-localization and functional interaction of CaV1.3 with GABABR2 also occur in neurons. CaV1.3 and GABABR2 were immunostained in cultured rat hippocampal neurons using the same antibodies as used in HEK293 cells. As shown in Fig. 3A, CaV1.3 was localized on plasma membrane of the cell body as well as dendrites, and co-localized with GABABR2. The double-labeling experiments suggested co-localization of CaV1.3 and GABABR2 in hippocampal neurons. To study further the functional interaction, we tested the effect of baclofen on high-K-induced intracellular Ca2+ increase using fura-2-based
intracellular Ca\textsuperscript{2+} imaging techniques. Hippocampal neurons were depolarized by a brief application (10 s) of 60 mM KCl, and N- and P/Q-type Ca\textsuperscript{2+} currents were blocked by co-application of N-type Ca\textsuperscript{2+} channel blocker, \(\alpha\)-conotoxin MVIIA (1 \mu M), and P/Q-type Ca\textsuperscript{2+} channel blocker, \(\alpha\)-agatoxin IVA (100 nM). Under these conditions, 100 \mu M baclofen augmented high-K\textsuperscript{+}-induced intracellular Ca\textsuperscript{2+} increase by 32.4 ± 7.5% (\(n=5\), Fig. 3B).

Because there are two types of L-type Ca\textsuperscript{2+} channels in neurons, we further attempted to identify a specific role of CaV1.3 using a siRNA-mediated silencing method in cultured hippocampal neurons. Selective knockdown of CaV1.2 or CaV1.3 with siRNA was proven in our previous study [12]. Briefly, CaV1.2- and CaV1.3-siRNA decreased CaV1.2 and CaV1.3 mRNA, respectively without affecting the gene expression of the other channel. Using the CaV1.2 or CaV1.3 knockdown cells, the effect of baclofen on the high-K\textsuperscript{+}-induced intracellular Ca\textsuperscript{2+} increase was studied. In Fig. 3C, baclofen had no effect in CaV1.3 knockdown cells (\(n=6\), Fig. 3B).

On the other hand, augmentation in the high-K\textsuperscript{+}-induced intracellular Ca\textsuperscript{2+} increase was still observed upon baclofen application in CaV1.2 knockdown cells (32.3 ± 12.2%, \(n=4\), Fig. 3D). Fig. 3E illustrated the pooled data from CaV1.2- or CaV1.3-siRNA-treated cells as well as scrambled siRNA-treated cells as a control. These results clearly support a specific role of CaV1.3 in GABAB\textsubscript{R}-mediated facilitation of L-type Ca\textsuperscript{2+} currents both in HEK293 cells and in hippocampal neurons.

### 4. Discussion

In the present study, we first identified a direct interaction between CaV1.3 and GABAB\textsubscript{R}, and further examined functional modulation of CaV1.3 activity by GABAB\textsubscript{R} in a heterologously expressed system as well as in cultured hippocampal neurons. Here, we report the three principal findings on a physical and functional interaction between CaV1.3 and GABAB\textsubscript{R}: first, the present study is an original report that demonstrates the formation of the CaV1.3/GABAB\textsubscript{R} protein complex. We initially employed a yeast two-hybrid screening in the rat brain cDNA library with CaV1.3-NT cDNA, and found GABAB\textsubscript{R2}-CT as a binding partner. This specific interaction was further confirmed using a yeast two-hybrid assay, a GST pull-down assay, co-immunoprecipitation, and cellular localization in HEK293 cells, and also in rat hippocampal neurons. Secondly, the present work is the first report showing the facilitative effect of baclofen on CaV1.3 currents through GABAB\textsubscript{R}. It has been known that the activation of GABAB\textsubscript{R} augments L-type Ca\textsuperscript{2+} channel currents in various neurons [10,11]. However, this phenomenon is hard to be observed in the CNS because inhibition of N- and P/Q-type Ca\textsuperscript{2+} channels by GABAB\textsubscript{R} is dominant [9]. Based on the evidences for a physical association and cellular localization of CaV1.3 with GABAB\textsubscript{R2}, we further examined a functional coupling between the two proteins, and found that the activation of GABAB\textsubscript{R} increases CaV1.3 currents without modulating either channel kinetics or voltage dependency. Further experiments using the CaV1.2 expressing cell line would be interesting to carry out to see whether GABAB\textsubscript{R} has the effects on CaV1.2. The third and the last piece of novel discoveries in the present work is functional modulation of CaV1.3 activity by GABAB\textsubscript{R} in cultured rat hippocampal neurons. Although CaV1.3 is activated at more hyperpolarized potentials and less sensitive to dihydropyridines than CaV1.2 [4], these differences are not big enough to distinguish a specific role of each channel, especially in hippocampal neurons where the both CaV1.2 and CaV1.3 are expressed. In the present study, we observed baclo-
fen-induced increase in intracellular Ca\(^{2+}\) only in Ca\(_{\text{V}1.2}\) knock-down cells, but not in Ca\(_{\text{V}1.3}\) knockdown cells in the presence of N- and P/Q-type Ca\(^{2+}\) channel blockers, which suggests that increase in L-type Ca\(^{2+}\) channel currents by baclofen is dependent on Ca\(_{\text{V}1.3}\), but not on Ca\(_{\text{V}1.2}\). Under our experimental conditions, however, R-type Ca\(^{2+}\) currents were unblocked, which may explain the smaller facilitative effect of baclofen in hippocampal neurons than that in HEK293 cells. To confirm the role of GABA\(_{\text{B}}\)R on Ca\(_{\text{V}1.3}\) activation, whole-cell patch-clamp recordings are being undertaken in the presence of blocker cocktails that inhibit all non-L-type Ca\(^{2+}\) channels including R-type Ca\(^{2+}\) channels.

In the CNS, neuronal L-type Ca\(^{2+}\) channels are particularly important due to their roles in translating synaptic activity into gene expression and neuronal function [5–8]. Recent studies have suggested the roles of Ca\(_{\text{V}1.2}\) in synaptic plasticity and modulation of gene expression-related factors, and have also proposed the detailed mechanism of Ca\(_{\text{V}1.2}\)-mediated gene expression [7,8]. On the contrary, the roles of neuronal Ca\(_{\text{V}1.3}\) are only beginning to be investigated. Our results therefore would provide an evidence for potential roles of Ca\(_{\text{V}1.3}\) in neuronal activities and synaptic plasticity via a novel linkage with GABA\(_{\text{B}}\)R in the hippocampus.

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References


