The β4 subunit of the voltage-gated calcium channel (Cacnb4) regulates the rate of cell proliferation in Chinese Hamster Ovary cells

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ABSTRACT

The β subunits of Voltage-Gated Calcium Channel (VGCC) are cytosolic proteins that interact with the VGCC pore-forming subunit and participate in the trafficking of the channel to the cell membrane and in ion influx regulation. β subunits also exert functions independently of their binding to VGCC by translocation to the cell nucleus including the control of gene expression. Mutations of the neuronal *Cacnb4* (β4) subunit are linked to human neuropsychiatric disorders including epilepsy and intellectual disabilities. It is believed that the pathogenic phenotype induced by these mutations is associated with channel-independent functions of the β4 subunit. In this report, we investigated the role of β4 subunit in cell proliferation and cell cycle progression and examined whether these functions could be altered by a pathogenic mutation. To this end, stably transfected Chinese Hamster Ovary (CHO-K1) cells expressing either rat full-length β4 or the rat C-terminally truncated epileptic mutant variant β$_{1-481}$ were generated. The subcellular localization of both proteins differed significantly. Full-length β4 localizes almost exclusively in the cell nucleus and concentrates into the nucleolar compartment, while the C-terminal-truncated β$_{1-481}$ subunit was less concentrated within the nucleus and absent from the nucleoli. Cell proliferation was found to be reduced by the expression of β4, while it was unaffected by the epileptic mutant. Also, full-length β4 interfered with cell cycle progression by presumably preventing cells from entering the S-phase *via* a mechanism that partially involves endogenous B56δ, a regulatory subunit of the phosphatase 2A (PP2A) that binds β4 but not β$_{1-481}$. Analysis of β4 subcellular distribution during the cell cycle revealed that the protein is highly expressed in the nucleus at the G1/S transition phase and that it is translocated out of the nucleus during chromatin condensation and cell division. These results suggest that nuclear accumulation of β4 at the G1/S transition phase affects the progression into S-phase resulting in a decrease in the rate of cell proliferation. Regulation of the cell cycle exit is a critical step in determining the number of neuronal precursors and neuronal differentiation suggesting that mutations of the β4 subunit could affect neural development and formation of the mature central nervous system.

1. Introduction

Variation in intracellular Ca^{2+} concentration is a widespread second signaling system regulating a diversity of key physiological functions. In response to action potentials, Ca^{2+} influx through VGCC leads to different organ-specific cellular events including muscle contraction, neurotransmitter release, cell division, gene transcription and more generalized biochemical processes (Flavell and Greenberg, 2008; Deisseroth et al., 2003; Greer and Greenberg, 2008). VGCC are found at the plasma membrane of most excitable cells such as muscle cells and neurons, and in non-excitable cells including glial cells and lymphocytes. Therefore, they represent the key signal transducers of membrane depolarization into Ca^{2+} influx (Catterall, 2011).

Since the first description of VGCC in skeletal muscle cells, a

number of studies have characterized their structure, function and regulation. VGCC are multimeric protein complexes composed of the main pore-forming α_1 subunit associated with the auxiliary subunits β, $\alpha_2\delta$ and γ. Among the auxiliary subunits, β subunits stand out as supporting an important set of regulatory functions. Indeed, they regulate the trafficking and the expression levels of the channel at the plasma membrane (Bichet et al., 2000) and the gating properties and kinetics of the main α_1 subunit (De Waard and Campbell, 1995), which in turn modulates many physiological processes including gene expression (for review see (Barbado et al., 2009)), excitation/secretion and excitation/contraction coupling (Catterall, 1999; Rousset et al., 2005). The VGCC-β subunits comprise a group of proteins characterized by similar protein structure but different tissue distribution. The β_4 subunit is primarily expressed in the brain and is highly expressed in Purkinje and granule cells of the cerebellum. The expression levels of β_4 are developmentally regulated and increase with age in rat brain (Castellano et al., 1993; Ludwig et al., 1997; Powers et al., 1992). In addition to its role in the regulation of calcium currents, the importance of β_4 in neural function is underscored by the fact that mutations of this subunit are associated with a variety of human pathologies including juvenile myoclonic epilepsy, which occurs as a result of a C-terminal 38 amino-acid deletion of β_4 (β_{1-481}) (Escayg et al., 2000).

Until recently, it was believed that β subunits were only able to carry out functions associated with their interaction with the calcium channel. However, there is now evidence that auxiliary β subunits may be directly involved in other cellular events that take place far away from the plasma membrane (Rima et al., 2016). Indeed, the short β_{4c} splice variant interacts with the nuclear protein heterochromatin protein 1 gamma (HP1γ) and controls its repressive effect on gene transcription (Hibino et al., 2003; Xu et al., 2011). In addition, the full-length β_3 subunit interacts and regulates the activity of an alternative spliced isoform of the transcription factor Pax6 (Zhang et al., 2010). Finally, Tadmouri et al. demonstrated that by interacting with the regulatory subunit of phosphatase 2A (PP2A) B56δ, β_4 translocates into the nucleus and organizes a protein complex that participates in transcriptional regulation (Tadmouri et al., 2012). This protein complex comprises a transcription factor, the thyroid receptor α, which provides gene-specificity for the epigenetic regulation (Tadmouri et al., 2012). The common point among these studies is that calcium channel-independent functions require the translocation of β subunits from the cytoplasm to the nucleus in order to partake in gene regulation. Even though control of gene expression seems to be a common feature to all β subunits, exceptions have been recently elucidated. More precisely, it was demonstrated that nuclear translocation of β_4 is splice variant-dependent; therefore, the ability of β subunits to interact with the epigenetic machinery depends on their nuclear-targeting capability (Etemad et al., 2014). A previous analysis of the role of β_4 in the regulation of gene expression in HEK293 cells and in mice brain highlighted significant changes in the expression level of a set of genes involved in cell proliferation (Ronjat et al., 2013). While β_{1-481} nuclear targeting properties are impaired, it remains able to modulate a set of genes but that were notably different from the genes regulated by the full-length β_4. Based on these observations, we investigated the possible role of the β_4 subunit in cell proliferation by stably expressing β_4 C-terminally fused to eGFP (β_4-eGFP) and eGFP in CHO-K1 cells and studying the impact of the protein on the cell cycle. In addition, we investigated the effect on cell proliferation of the epileptic mutant β_{1-481}-eGFP, which is defective in B56δ/PP2A interaction and regulation of gene expression.

2. Material and methods

2.1. CHO cell culture

Chinese Hamster Ovary K1 (CHO-K1) cells were obtained from ATCC (ATCC CCL-61) and handled following ATCC recommendations.

CHO-K1 cells were cultured in complete cell culture media containing Dulbecco's modified Eagle medium/Nutrient Mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (10 μg/mL) and maintained at 37°C in a humidified environment with 5% CO_2.

2.2. Plasmids and transfection

Expression vectors and cDNA constructs coding for eGFP, rat β_4-eGFP, rat β_4-myc, rat β_{1-481}-eGFP, mouse B56δ-myc and mouse B56δ-shRNA were previously described (Tadmouri et al., 2012). Briefly, parental CHO-K1 cells were seeded onto 100 mm Petri dishes and transfected with the desired plasmid construct at 50% confluence using a previously described calcium-phosphate protocol (Melchers, 2001). The plasmids used were: 1) the pEGFP-N1 vector (Clontech), 2) a pEGFP-N1 vector in which the full-length rat β_4 cDNA (CACNB4, accession number L02315) was cloned in frame upstream of eGFP to generate β_4 fused to the N-terminus of eGFP (β_4-eGFP), and 3) the rat β_{4b}R482X/pEGFP-N1 vector coding for β_4 epileptic mutant (β_{1-481}-eGFP) also C-terminally fused to eGFP.

2.3. Generation of stably transfected CHO-K1 cell lines

Parental CHO-K1 cells were transfected with the vectors coding for eGFP, β_4-eGFP or the β_{1-481}-eGFP mutant. Forty-eight hours after transfection, the cells were detached, sorted by eGFP fluorescence with a BD Fluorescence Activated Cell Sorter (FACS) ARIA and collected in single tubes. CHO-K1 cells expressing eGFP, β_4-eGFP, or β_{1-481}-eGFP were grown in tissue culture plates in complete culture medium supplemented with geneticin (Gibco) (1 mg/mL). The cells were seeded twice a week at low density for approximately 4 weeks and then dispatched in 96 well-plates (96WP) at a calculated density of 1 cell/well. After 7 days in culture, wells containing a single cell colony were examined with a fluorescence microscope and eGFP-positive cells were replated in 6-well plates (6WP). Cells derived from single colonies were then analyzed by flow cytometry and cultures containing above 95% eGFP-positive cells were expanded, aliquoted and frozen in complete cell culture medium containing 10% DMSO, and stored in liquid nitrogen. Thereafter, cells were grown without geneticin and regularly examined by flow cytometry to determine whether the cells kept expressing eGFP, β_4-eGFP or β_{1-481}-eGFP. Best expressing clonal cell lines were used for eGFP and β_{1-481}-eGFP, while three clonal cell lines were used for β_4-eGFP for proliferation data and as single one for all other experiments.

2.4. Ratiometric cell proliferation test

Parental CHO cells were mixed with those stably expressing eGFP or β_4-eGFP at a ratio of 1:4. From each cell suspension, four others were prepared by serial dilution (1:10, 1:100, 1:1 000, 1:10 000) and cultured in 6-well plates at 37°C, 5% CO_2. Aliquots of the cell suspensions were fixed with 4% paraformaldehyde (PFA) for 20 min on ice, washed with phosphate-buffered saline (PBS) and centrifuged at 110 × g for 3 min. The pellet was then resuspended in PBS and stored at 4°C in the dark for further analysis. When the cells reached 90% confluence, they were harvested, fixed, washed, resuspended in PBS, and stored at 4°C in the dark. Cell suspensions were analyzed by flow cytometry with a BD Accuri C6 system with a 488 nm excitation laser. Relative fluorescence values were determined by analyzing 100,000 events for each sample.

2.5. Cell counting

5×10^5 cells were seeded into 100 mm Petri dishes with complete medium. Twenty-four and 48 h after plating, cells were washed, harvested, resuspended in PBS, and counted using a Malassez cell counting chamber. Alternatively, the BD Accuri C6 system was also used with