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How to

An expert protocol for immunofluorescent detection of calcium channels in tsA-201 cells

Peter Koch *, Stefan Herzig, Jan Matthes

Department of Pharmacology, University Clinic Cologne, Gleuelerstrasse 24, D-50931 Cologne, Germany

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ABSTRACT

Introduction: Pore-forming subunits of voltage gated calcium channels (VGCC) are large membrane proteins (260 kDa) containing 24 transmembrane domains. Despite transfection with viral promoter driven vectors, biochemical analysis of VGCC is often hampered by rather low expression levels in heterologous systems rendering VGCC challenging targets. Especially in immunofluorescent detection, calcium channels are demanding proteins.
Methods: We provide an expert step-by-step protocol with adapted conditions for handling procedures (tsA-201 cell culture, transient transfection, incubation time and temperature at 28 °C or 37 °C and immunostaining) to address the L-type calcium-channel pore $Ca_v1.2$ in an immunofluorescent approach.
Results: We performed immunocytochemical analysis of $Ca_v1.2$ expression at single-cell level in combination with detection of different markers for cellular organelles. We show confluency levels and shapes of tsA-201 cells at different time points during an experiment. Our experiments reveal sufficient levels of $Ca_v1.2$ protein and a correct $Ca_v1.2$ expression pattern in polygonal shaped cells already 12 h after transfection.
Discussion: A sequence of elaborated protocol modifications allows subcellular localization analysis of $Ca_v1.2$ in an immunocytochemical approach. We provide a protocol that may be used to achieve insights into physiological and pathophysiological processes involving voltage gated calcium channels. Our protocol may be used for expression analysis of other challenging proteins and efficient overexpression may be exploited in related biochemical techniques requiring immunolabels.

1. Introduction

Voltage gated calcium channels are expressed in the cardiovascular system, skeletal muscles, endocrine cells and the nervous system and are involved in a variety of different crucial physiological processes (Catterall, 2011). Since the 1970s researchers have examined calcium channel kinetics in electrophysiological approaches in various biological systems, e.g. hek-293 cells, tsA-201 cells, hippocampal neurons and *Xenopus laevis* oocytes. The Patch clamp technique is a highly sensitive method allowing analysis of calcium-channel function, down to even a single molecule. However, for biochemical analysis rather large amounts of protein are required and in general overexpression of membrane proteins has been reported to be quite demanding (for review see Wagner, Bader, Drew, & de Gier, 2006). For obtaining efficient membrane protein (over-) expression, the expression system, method of transfection, incubation time and conditions must be chosen carefully in a protein specific manner, which is a labor- and time intensive approach (Bernaudat et al., 2011). Calcium-channel expression is especially challenging as coexpression of the pore protein with auxiliary β and α2δ subunits is necessary to increase calcium channel cell membrane expression and to provide a more physiological context (for review see Dolphin, 2003). Furthermore, calcium-channel expression is affected by proteasomal degradation of proteins being not processed to the cell membrane (Altier et al., 2010). Poor expression in heterologous expression systems thus is one major reason hampering our understanding of subcellular processes involving calcium channels.

Immunocytochemistry is a method to demonstrate the (co-)localization of proteins of interest. In classic protein purification approaches, poor protein expression is simply overcome by increasing the amount of biological material and extending the incubation period. However, immunofluorescent labeling of calcium channels is particularly challenging. In immunocytochemistry cellular confluency must be avoided to allow single cell analysis while cells require a long incubation period to accumulate $Ca_v1.2$ protein levels sufficient for immunodetection. $Ca_v1.2$ immunocytochemical experiments thus require a careful orchestration of conflicting parameters like the amount of deployed biological material, transfection method, incubation time and temperature. We harmonized parameters by specifically adjusting conditions for tsA-201 cell culture, transient transfection, and incubation as well as immunostaining. Here, we provide a protocol particularly suitable for immunofluorescent detection of calcium channels. Our protocol is a powerful tool to analyze calcium channel protein expression,

* Corresponding author.
E-mail addresses: peter.koch@uk-koeln.de (P. Koch), stefan.herzig@uni-koeln.de (S. Herzig), jan.matthes@uk-koeln.de (J. Matthes).

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subcellular localization or coexpression. This protocol may be utilized in other approaches requiring immunolabels like protein quantification or live cell imaging of fusion proteins. Furthermore, our protocol may be adopted in assays approaching the overexpression of other challenging proteins.

We established an elaborated sequential arrangement of protocol modifications taken from several previous studies yet performed in different methodological contexts:

In brief, we seed small tsA-201 cell volumes on glass coverslips (Senatore, Boone, & Spafford, 2011) to obtain low confluency levels prior to transfection (Beedle et al., 2004) by the calcium phosphate method (Graham & van der Eb, 1973). Using small cell volumes initially abolishes the necessity for splitting cells post transfection, which in return avoids stress-induced loss of cells while supporting cell attachment, polygonal shape and protein synthesis. Finally, for immunocytochemistry we extended the antibody incubation time to most efficiently immunolabel $Ca_v1.2$. Our experiments confirm that incubation of transfected cells at non-physiological 28 °C (Altier et al., 2010; Beedle et al., 2004; Lin et al., 2015; Senatore et al., 2011) for 48 h results in $Ca_v1.2$ protein levels that can be detected by immunocytochemistry (Altier et al., 2010). However, our immunofluorescent experiments also demonstrate, that $Ca_v1.2$ can be detected already after incubating cells for 12 h at 37 °C. Thus we also provide a quicker and less complex protocol allowing calcium channel expression analysis at physiological conditions.

2. Methods

2.1. Pre-transfection cell culture

Four squared coverslips (Menzel-Gläser 15 × 15 mm) were briefly heated in the blue flame of a Bunsen burner and then briefly rinsed in ethanol. The coverslips were washed twice in sterile water. After removing the water 200 μl poly-D-lysine solution 0.1% (w/v) (Sigma) were added covering each coverslip. Subsequently the coverslips were incubated at 37 °C for 8 h. After removing the poly-D-lysine solution the coverslips were washed twice with sterile water.

tsA-201 cells were cultured at 37 °C and 5% CO_2 in a humidified environment to 85% confluency (Fig. 1A) in a 60 mm diameter cell culture dish in 4 ml DMEM GlutaMAX medium (Gibco) with 10% FCS (Biochrom) and penicillin-streptomycin (Sigma). Medium was aspirated and cells were washed briefly two times in prewarmed PBS (Gibco). PBS was aspirated and 900 μl sterile filtered PBS (EDTA 0.02% w/v) was added. The dish was tilted several times for equal distribution of the PBS/EDTA buffer. Cells were incubated for 2 min at room temperature. 3.1 ml supplemented DMEM medium was added and tsA-201 cells were resuspended very carefully. We applied as little mechanical stress as necessary when singularizing cells.

Four poly-D-lysine coated glass coverslips were placed in a fresh 60 mm diameter culture dish and 3.8 ml of supplemented DMEM medium prewarmed to 37 °C was added. We ensured that the coverslips did not float also during all following steps. 200 μl of resuspended tsA-201 cells were added.

The cells were evenly distributed in the dish. Cells preferred the coverslip surface for attachment. Cells were grown for one day and transfected. Transfection can be performed when the tsA-201 cells display polygonal morphology (Fig. 1C).

2.2. Transient transfection

The calcium phosphate transfection method was first described by Graham and van der Eb (1973).

We added 1.33 μg $Ca_v1.2$ plasmid, 0.66 μg β2b plasmid, 0.99 μg α2δ1 plasmid and 0.5 μg Rab5-GFP plasmid to 380 μl chilled HBS buffer (21 mM HEPES, 137 mM NaCl, 49 mM KCl, 5.5 mM Dextrose and 0.75 mM Na2HPO4) and vortex briefly. Add 20 μl chilled $CaCl_2$ (2.5 M) drop wise to the HBS buffer containing the plasmids. To obtain fine CaPO4/DNA particles, we quickly vortexed the transfection mix after adding each droplet. The transfection mix was incubated for 30 min at room temperature. The transfection mix was added to the cell medium

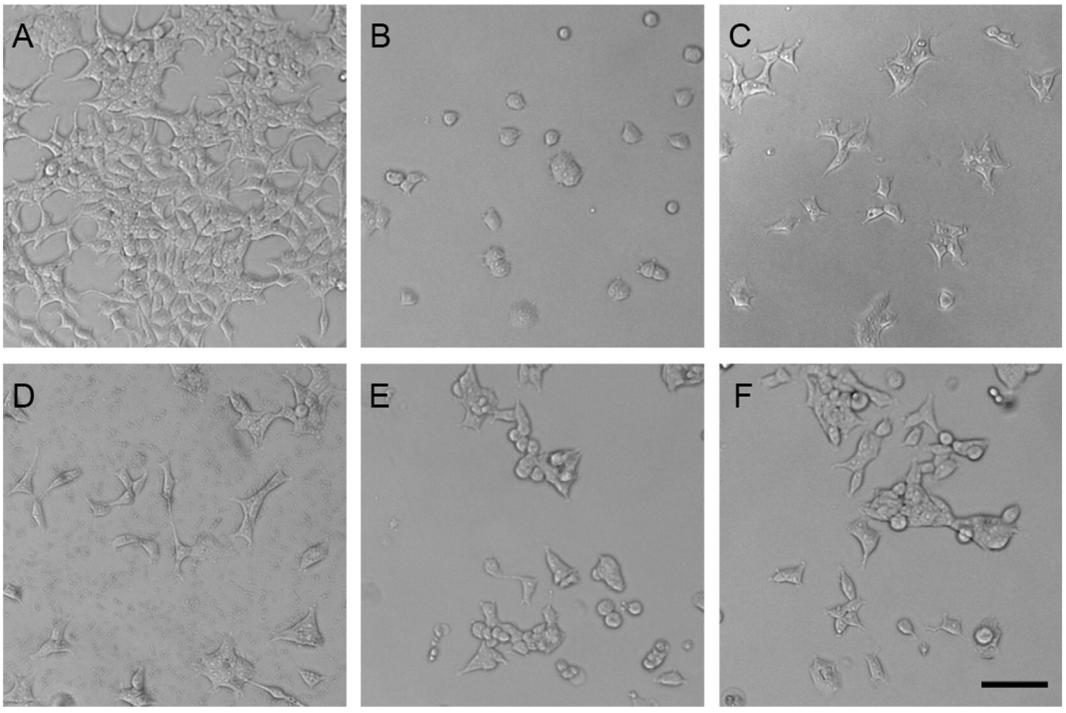

Fig. 1. tsA-201 cell morphology, confluency levels and stress recovery at selected time points. A. Cells at 85% confluency immediately before splitting. B. Singularized, reattached cells 3 h after splitting. C. Cells before transfection at 5% confluency with readopted polygonal morphology. D. Cells after 7 h incubation with calcium phosphate/DNA precipitate. E. Stressed cells after 48 h incubation at 28 °C. F. Cells after 7 h of recovery at 37 °C following 48 h incubation at 28 °C. Pictures were taken using a Leica DM IL LED microscope and a Leica DFC400 camera. Magnification 10×; Scale bar 50 μm.