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Measuring Ca^{2+}-Dependent Modulation of Voltage-Gated Ca^{2+} (Ca_v) Channels in HEK-293T cells

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Abstract

Voltage-gated Ca^{2+} (Ca_v) channels regulate a variety of biological processes, such as muscle contraction, gene expression, and neurotransmitter release. Ca_v channels are subject to diverse forms of regulation, including those involving the Ca^{2+} ions that permeate the pore. High voltage-activated Ca_v channels undergo Ca^{2+}-dependent inactivation (CDI) and facilitation (CDF), which can regulate processes such as cardiac rhythm and synaptic plasticity. CDI and CDF slightly differ between Ca_v1 (L-type) and Ca_v2 (P/Q-, N-, and R-type) channels. Human embryonic kidney cells transformed with SV40 large T-antigen (HEK-293T) are advantageous for studying CDI and CDF of a particular type of Ca_v channel. HEK-293T cells do not express endogenous Ca_v channels, and Ca_v channels can be exogenously expressed at high levels in these cells through transient transfection. This protocol explains how to characterize and analyze Ca^{2+}-dependent modulation of recombinant Ca_v channels in HEK-293T cells.

Overview

Voltage-gated Ca_v channels mediate Ca^{2+} signals that regulate cellular excitability, muscle contraction, gene expression, and hormone/neurotransmitter release. Ca_v channels are multi-subunit complexes that consist of a pore-forming α_1 subunit and auxiliary subunits, $Ca_v\beta$ and $Ca_v\alpha_2\delta$ (Simms and Zamponi, 2014). Ten genes encode distinct α_1 subunits that display distinct pharmacological and biophysical properties ($Ca_v1.x$-$Ca_v3.x$). Mutations in these genes have been associated with disorders such as epilepsy, migraine, deafness, and congenital stationary night blindness (Pietrobon, 2010, Striessnig et al., 2010).

The α_1 subunit mediates Ca^{2+} entry, and the auxiliary subunits regulate trafficking and other properties of the channel. In addition, the Ca_v1 and Ca_v2 α_1 subunits are constitutively associated with calmodulin (CaM), which is essential for Ca^{2+}-dependent inactivation (CDI) and facilitation (CDF). The N- and C-terminal lobes of CaM each contain 2 EF-hand Ca^{2+} binding domains. Increases in global and local Ca^{2+} entry through Ca_v channels are sensed by the N- and C-terminal lobes of CaM, respectively, which play distinct roles in CDI of Ca_v1 and Ca_v2 channels. CDI of Ca_v1 channels is mediated by the C-lobe of CaM and is

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insensitive to strong intracellular Ca^{2+} buffering. In contrast, CDI of Ca_v2 channels is mediated by the N-lobe of CaM and is inhibited by strong intracellular Ca^{2+} buffering. Interestingly, CDF of $Ca_v2.1$ channels is mediated by the C-lobe of CaM, and is spared by high concentrations of intracellular EGTA. The complex regulation of Ca_v channels by CaM has been described in recent reviews (Christel and Lee, 2012, Ben-Johny and Yue, 2014).

It should be noted that factors other than Ca^{2+} can influence inactivation of Ca_v1 and Ca_v2 channels. For example, inactivation of Ca_v channels can be driven by a purely voltage-dependent mechanism (voltage-dependent inactivation, VDI), which is seen for I_{Ba}. "Fast" VDI occurs from the resting (closed) state (Patil et al., 1998), while "slow" VDI occurs not only from the fast-inactivated state but also the open state (Sokolov et al., 2000). Both forms of VDI are influenced by $Ca_v\beta$ subunits. Some $Ca_v\beta$ subunits, like $Ca_v\beta_{1b}$ promote VDI, while the membrane-associated $Ca_v\beta$, $Ca_v\beta_{2a}$, diminishes VDI (Buraei and Yang, 2010). Since CDI can be masked by strong VDI, the use of Ca_v channels containing $Ca_v\beta_{2a}$ is a common approach in studies of CDI (Lee et al., 2000, DeMaria et al., 2001).

The α_1 subunit of Ca_v channels is subject to alternative splicing, which increases the functional diversity of Ca_v channels (Lipscombe et al., 2013). Such splicing events can either heighten or dampen Ca^{2+}-dependent regulation of Ca_v channels. For example, inclusion of the alternatively spliced exon 42A of $Ca_v1.3$ channels results in enhanced CDI due to a truncation of a distal C-terminal autoregulatory domain (Singh et al., 2008). In addition, alternative splicing of exon 37 in $Ca_v2.1$ alters CDF (Soong et al., 2002, Chaudhuri et al., 2005). These examples illustrate the capability of splicing events to serve as molecular switches for CDI and CDF of Ca_v channels.

Other regulators of CDI and CDF of Ca_v channels include a family of Ca^{2+} binding proteins (CaBPs) that are related to CaM (Haeseleer et al., 2000). Unlike CaM, CaBPs contain at least one nonfunctional EF-hand, and are almost exclusively expressed in neurons. CaBPs may compete with and/or allosterically modulate CaM interactions with Ca_v1 channels, which inhibits CDI. For $Ca_v2.1$ channels, one CaBP family member, CaBP1 strongly inhibits CDI and VDI, and voltage-dependent activation. The regulation of Ca_v channels by CaBPs has been recently reviewed (Christel and Lee, 2012, Lee et al., 2014).

The methods described below present the basic framework for characterizing CDI and CDF of recombinant Ca_v channels in HEK-293T cells. These cells have proven ideal for studying the molecular determinants and biophysical mechanisms underlying CDI and CDF of Ca_v channels. Because of the presence of CDI/CDF regulatory factors such as CaBPs and alternative splicing in neurons, additional insights into the neurophysiological significance of CDI and CDF may be gained by studying these processes in various neuronal cell-types.

MATERIALS

Detailed recipes (<R>) are listed at the end of this protocol.

Reagents

Ca_v channel cDNAs (examples shown below)

$Ca_v2.1$ (GenBank : NM023035.1) or $Ca_v2.2$ (GenBank: AF055477)

$Ca_v\beta_{2a}$ (GenBank: NM053581)

$\alpha_2\delta_1$ (GenBank: NM21948.1)

pEGFP (Life Technologies)

Culture medium: DMEM (GIBCO, 11965) + 10% FBS (Atlanta Biologicals, S11150)

DMEM (GIBCO, 11965)

Extracellular recording solution <R>

FuGene 6 Transfection Reagent (Promega, E2691)

HEK-293T cells (ATCC, CRL-11268)

Internal recording solution<R>

Versene (GIBCO, 15010-066)

Equipment

Micropipettor (P1000)

Borosilicate glass capillaries (WPI, TW 150-4)

Cell Culture Dish (Corning, 35 mm × 10 mm)

Data acquisition and analysis software

Micropipette puller

Microforge

Patch-clamp electrophysiology setup including inverted fluorescence microscope, micromanipulators, amplifier, and perfusion setup

Tissue culture hood Tissue culture incubator (37°C and humidified atmosphere with 5% CO_2)

METHOD

Transient transfection

1 Plate HEK-293T cells in 35 mm culture dishes. Cells should be maintained in DMEM+ 10% FBS (2 mL) in a tissue culture incubator at 37°C with a humidified atmosphere and 5% CO_2.

2 When cells are ~ 70% confluent, begin transient transfection with the cDNAs corresponding to the Ca_v channel of interest.

3 About 30 minutes prior to transfection, aspirate culture medium and replace with fresh medium.

4 In a sterile microfuge tube (1.5 mL), combine FuGene 6 transfection reagent (6 μL) with DMEM medium (100 μL). Then gently mix.

Notes:

For every 3 μg of DNA, use 2 μL of FuGene.

Use DMEM without FBS for transfection mix.

5 To the same tube, add cDNAs corresponding to the α_1 subunit (1.5 μg), $Ca_v\beta$ and $\alpha_2\delta$ (0.5 μg each), and pEGFP (50 ng). Then gently mix.

Note: pEGFP is used to help identify transfected cells via GFP fluorescence.

6 Let mix sit at room temperature for 15 minutes.

7 Next, add transfection mix to the cells in a drop wise manner covering the entire dish.

8 Cells should be ready for recordings after incubation at 37°C for 24–72 hours. If waiting 48 hours, replace with fresh medium after 24 hours.

9 To isolate cells for electrophysiological recordings, aspirate culture medium from transfected cells and replace with Versene (1 mL) for 1 minute. After 1 minute, aspirate the Versene and gently triturate the cell by pipetting up and down 10 times in culture medium (1 mL) with a P1000 micropipettor. Finally, for low-density plating, add cell suspension (1 to 2 drops) to culture dishes containing culture medium (2 mL).

Notes:

Allow cells to rest for at least 2 hours prior to recording at 37°C.

Do not use trypsin-EDTA to isolate cells, as it may proteolyze channels

Whole-cell voltage-clamp recording

10 Prior to recording, use a micropipette puller to produce electrodes with a resistance of 4–6 Megaohms (MΩ) in the extracellular recording solution. Polish the tips with a microforge. Electrodes can be coated with Sylgard to reduce pipette capacitance, although this is generally unnecessary.

11 Remove culture medium and fill culture dish with extracellular recording solution (1 mL).

12 After identifying a GFP-positive cell that has a spherical/boxlike shape, fill electrode with internal recording solution and begin electrophysiological recordings.

Notes:

Avoid oval and triangular shaped cells, which may yield distorted currents due to an inability to properly clamp the membrane voltage.

The internal recording contains adenosine triphosphate (ATP) which may undergo auto-hydrolysis under some conditions. Therefore, while working

solutions may be maintained at room temperature, for prolonged experiments, maintain the internal recording solution on ice.

13 Obtain a gigaohm (GΩ) seal and change the holding voltage (V_h) to -80 mV.

14 Apply negative pressure to rupture the membrane to gain whole-cell access. Before running voltage protocols, reduce capacitance and series resistance electronically (60–70%).

Characterization of CDI

For Ca_v2 channels, the internal recording solution should contain a relatively low concentration of EGTA (0.5 mM), as higher concentrations inhibit CDI (Fig. 1A). By contrast, CDI of Ca_v1 channels can be recorded with internal recording solutions containing a higher concentration of EGTA (5 – 10 mM).

15 Ca^{2+} currents (I_{Ca}) can be evoked by a long (1 second) depolarizing test pulse from V_h (-80 mV) to voltages ranging from -20 mV to +20 mV (Fig. 1A). Inactivation can be measured as the residual current amplitude at the end of the pulse normalized to the peak current amplitude (I_{res}/I_{peak}). A U-shaped curve is observed when I_{res}/I_{peak} is plotted against test voltages (Fig. 1), with maximal inactivation at the test voltage evoking maximal inward I_{Ca}.

16 With Ba^{2+} as the permeant ion, Ca_v channels undergo inactivation that is voltage-rather than Ca^{2+} -dependent. To measure voltage-dependent inactivation (VDI), repeat step 16 with extracellular solution containing Ba^{2+} instead of Ca^{2+} (Fig. 1B). Ba^{2+}-containing solution can be exchanged for the Ca^{2+}-containing solution via a gravity-driven or pressurized perfusion system. Alternatively, population averages of cells recorded in Ca^{2+}- or Ba^{2+}-containing solution are often used, since the differences in CDI and VDI are robust.

Note: Unlike Ca^{2+}, Ba^{2+} binds poorly to calmodulin (CaM), an essential mediator of CDI.

17 A convenient metric for CDI is the difference between I_{res}/I_{peak} for I_{Ca} and I_{Ba} (Fig. 1B).

Characterization of CDF for $Ca_V2.1$ channels

18 To characterize CDF of $Ca_v2.1$ channels, the internal recording solution should contain a relatively high concentration of EGTA (10 mM), which will minimize CDI.

19 To measure CDF, a test pulse from V_h (-80 mV) to 0 mV (P1) is given 1 s before, and 5 ms after (P2), a 50-ms prepulse to various voltages (Fig. 2A).

20 Repeat step 21 with Ba^{2+} as the charge carrier (Fig. 2B).

Note: The test pulse for P1 and P2 should be set about -10 mV for I_{Ba} as compared to that used for I_{Ca}. Due to surface charge screening effects, Ba^{2+}

causes a negative shift in the voltage-dependence of activation, which should be compensated for in channel modulation protocols.

21 Facilitation (F) is measured as the ratio of P2 to P1 and plotted against prepulse voltage. If P2/P1 is greater than 1, then the current is facili0ated.

22 A convenient metric for CDF is the difference between facilitation of I_{Ca} and I_{Ba} at the prepulse voltage eliciting maximal facilitation of I_{Ca} (Fig. 2B).

RECIPES

Extracellular recording solution

Reagent	Final concentration (in mM)
$CaCl_2$ or $BaCl_2$	10
Tris	150
$MgCl_2$	1

pH to 7.3 with methanesulfonic acid. Osmolarity should be around 290–310. Filter and store at at 4 °C.

Intracellular recording solution

Reagent	Final concentration (in mM)
EGTA	0.5 or 10
N-methyl-D-glucamine (NMDG)	140
HEPES	10
$MgCl_2$	2
Mg-ATP	2

pH to 7.3 with methanesulfonic acid. Osmolarity should be around 290–310. Filter and store at Sterile filter and store in aliquots at -20°C.

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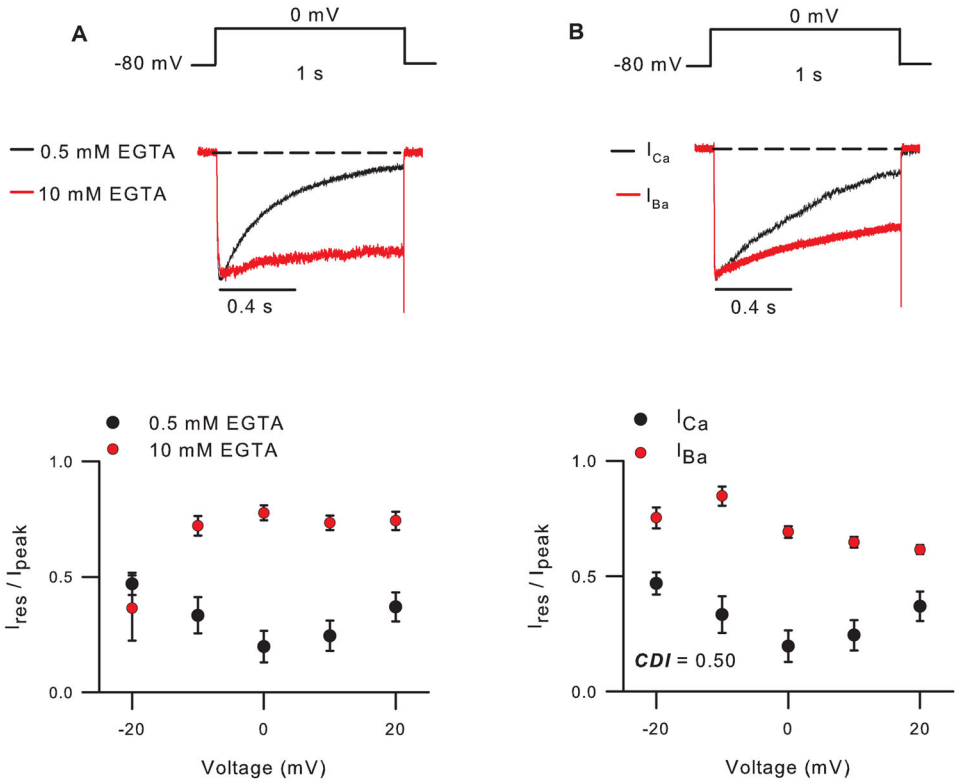

Figure 1. CDI of $Ca_V2.2$ channels

A,B) *Top*, Voltage protocols and representative current traces. Currents were leak-subtracted using the P/4 method. *Bottom,* I_{res}/I_{peak} represents residual current amplitude at the end of the pulse normalized to the peak current amplitude, and is plotted against test voltage. In **A,** internal recording solution contained 0.5 mM or 10 mM EGTA. In **B,** internal recording solution contained 0.5 mM and extracellular solution contained 10 mM Ca^{2+} (I_{Ca}) or Ba^{2+} (I_{Ba}). CDI = I_{res}/I_{peak} for I_{Ba} - I_{res}/I_{peak} for I_{Ca}, where I_{res}/I_{peak} for I_{Ba} = 0.69 ± 0.02 and I_{Ca} = 0.19 ± 0.07 for test pulse to 0 mV. Currents and averaged data for I_{Ca} (n=10) and I_{Ba} (n=8) are from different cells.

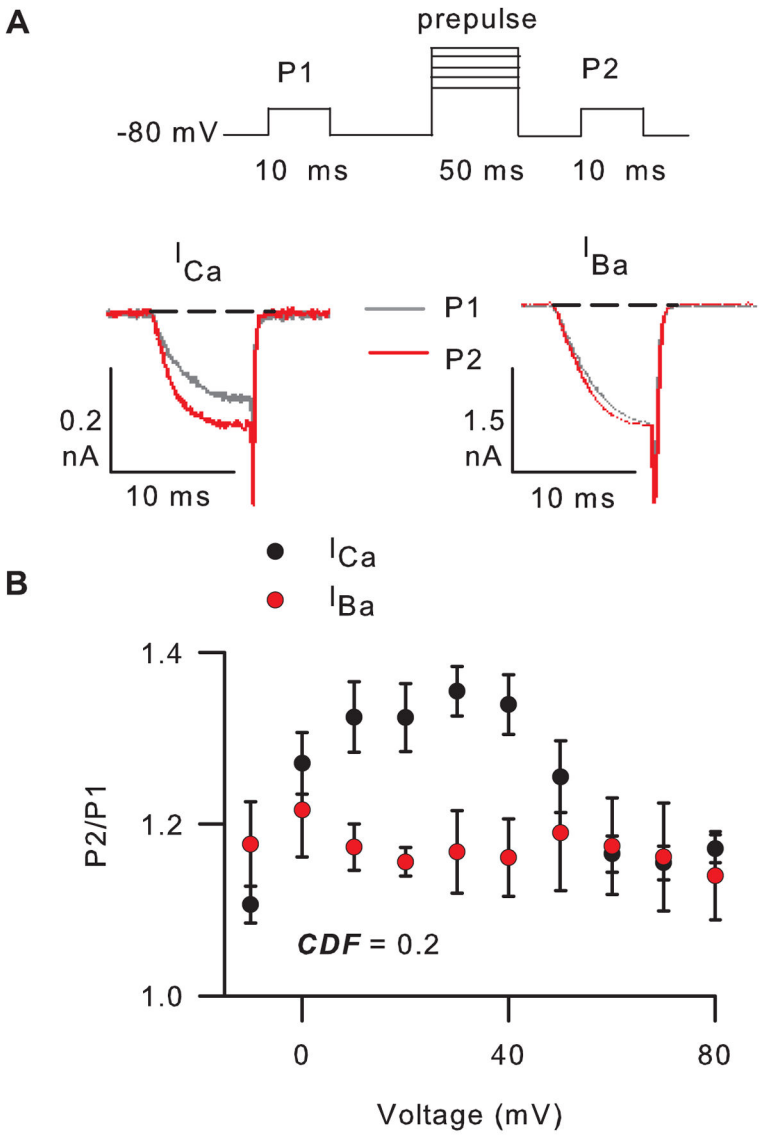

Figure 2. CDF of $Ca_v2.1$ channels

A) *Top,* voltage protocol and representative I_{Ca} and I_{Ba} evoked by 10-ms test pulses to 0 mV and -10 mV, respectively, before (grey) and after (red) a 50-ms prepulse to +30 mV. Currents were leak-subtracted using the P/4 method. **B)** The ratio of P2/P1 current amplitudes is plotted against prepulse voltage. CDF = the difference in P2/P1 for I_{Ca} and I_{Ba} using a +30-mV prepulse, where P2/P1 for I_{Ca} =1.4 ± 0.03 (n=4) and for I_{Ba} =1.2 ± 0.05 (n=4) for a +30-mV prepulse. Currents were recorded in 10 mM extracellular Ca^{2+} or Ba^{2+} and 10 mM intracellular EGTA. Currents and averaged data for I_{Ca} and I_{Ba} are from different cells.