Characterization of auto-regulation of the human cardiac $\alpha_1$ subunit of the L-type calcium channel: Importance of the C-terminus

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Abstract

The carboxyl terminal of the L-type calcium channel $\alpha_{1C}$ subunit comprises approximately one third of the primary structure of the $\alpha_1$ subunit (> 700 amino acids residues). This region is sensitive to limited posttranslational processing. In heart and brain the $\alpha_{1C}$ subunits are found to be truncated but the C-terminal domain remains functionally present. Based on our previous data we hypothesized that the distal C-terminus (approximately residues 1650–1950) harbors an important, predominantly inhibitory domain. We generated C-terminal-truncated $\alpha_{1C}$ mutants, and after expressing them in combination with a $\beta_3$ subunit in HEK-293 cells, electrophysiological experiments were carried out. In order to dissect the important inhibitory part of the C-terminus, trypsin was dialyzed into the cells. The data provide evidence that there are multiple residues within the inhibitory domain that are crucial to the inhibitory process as well as to the enhancement of expressed current by intracellular application of proteases. In addition, the expression of the chimeric mutant $\alpha_{1C}^{\Delta 1673}$ demonstrated that the C-terminal is specific for the heart channel. (Mol Cell Biochem 250: 81–89, 2003)

Key words: calcium channel, carboxyl-tail, electrophysiology, mutagenesis, trypsin

Abbreviations: L-VDCC – L-type voltage dependent Ca$^{2+}$ channels; $I_{Ca}$ – calcium current; hHT-$\alpha_1$ – human heart calcium channel $\alpha_1$-subunit; HEK-293 cells – human embryonic kidney cells; $I_{Ba}$ – barium currents; WT – wild-type

Introduction

Molecular cloning studies have revealed that the Ca$_{1.2}$ channels consisting of $\alpha_1$, $\beta_1$, $\beta_2$ or $\beta_3$ and $\alpha_2\delta$ subunits are responsible for L-type Ca$^{2+}$ currents in cardiac myocytes [1–4]. Ca$^{2+}$ entry through L-type voltage dependent Ca$^{2+}$ channels (L-VDCC) of cardiac and smooth muscle is critically important for contraction. The $\alpha_1$-subunit contains approximately 2,100 amino acids harboring the pore, the gating mechanism(s) and the binding sites for the Ca$^{2+}$ channel modulatory drugs; the accessory subunits appear to have a modulatory role and are probably involved in trafficking processes. The carboxyl-tail is the largest of the cytoplasmic domains of the Ca$^{2+}$ channel $\alpha_1$-subunit, containing more than 700 amino acid residues.

It seems reasonable to conclude that this region plays a role in regulating the activity of the Ca$^{2+}$ channel itself and therefore the amount of trigger Ca$^{2+}$ entering the cell. Recent data, in fact, suggest that the carboxyl tail is involved in subcellular localization via interactions with other cellular proteins [5] and regulation of the channels by protein phosphorylation [6, 7]. Based on previous observations that have been made in several laboratories, deletion of 46–70% of the C-
terminus of the α subunit results in a facilitation of the coupling between voltage gating and channel opening [8–10]. This implies that the carboxyl terminus ‘folds over’ to inhibit the activity of structural elements involved in channel activation. Therefore, it is possible that removal of this inhibitory modulator domain regulates channel activity in a positive manner. Gao et al. [7, 10] have discussed a mechanism by which the C-terminal α fragments of 30–50 kDa may be able to associate with and regulate the conductance of L-VDCC containing the C-terminal truncated α subunits. The amplitude and inactivation characteristics of calcium current are influenced by the conformation changes of the Ca channel protein. Protease perfusion has proven to be a useful methodology, though controversial, for studying Ca channel functional domains. Armstrong et al. [11] first used this approach and reported that intracellular pronase removes voltage-dependent inactivation of the Na current of the squid giant axon. Others have successfully utilized pronase and trypsin for the same purpose in a variety of cells. Hescheler and Trautwein [12] showed in single guinea pig ventricular cells that intracellular application of trypsin dramatically increased the amplitude of I Na. Inactivation kinetics appeared to be largely dependent on the charge carrier and the source of the channels. You et al. [13] presented data indicating the presence of two distinct inactivation regions that perform different functions. The so-called ‘slow ball’ contains allosterically coupled Ca binding and phosphorylation sites for modulation while the ‘fast ball’ in this model is only sensitive to [Ca2+]. Both ‘balls’ are susceptible to proteolytic digestion. The ‘slow-ball-long chain’ inactivation particle could be part of the C-terminal end of the Ca2+ channel. It is conceivable that the proteolytic action of trypsin removes much more of the obstacle that normally limits current flow. Wei et al. [8] investigated the characteristics of five deletion mutants of the carboxyl tail of cardiac α subunits after expression in Xenopus oocytes. They reported a 3–6 fold increase in peak Ba2+ current density. However, some of the first 193 amino acids following IVS6 seem to be crucial for the functional expression of the Ca2+ channel. In a paper from our laboratory, Klockner et al. [9] reported that a carboxyl-terminal deletion mutant (deletion of amino acids > 1673), in contrast to the wild-type form of the cardiac α subunit, no longer responded to trypsin application with an increase in peak Ba2+-currents, however, a 3-fold higher current density as well as faster inactivation were observed. The aim of our present study was to obtain specific information that bears on the functional importance of the intracellular carboxyl-terminus of the cardiac muscle α subunits in regulating the activity of the Ca2+ channel. Our hypothesis is that the proximal C-terminal (residues between aa1650–1950) harbors an important, predominantly inhibitory domain. Based on our previous observation [9], we constructed a series of C-terminal deletion mutants of the human heart calcium channel α1 subunit (hHT-α1) and expressed them in human embryonic kidney cells (HEK-293 cells). To refine the boundaries of the C-terminal inhibitory region, we used a different approach from others [10, 14, 15]. We applied trypsin intracellularly, which preferentially targets the 78 amino acids containing arginine and lysine in the C-terminus. Our data provide evidence that the C-terminal fragment is able to associate with the C-terminal cleaved hHT-α1-subunit expressed in HEK-293 cells.

**Materials and methods**

Construction of mutants and expression of channel subunits and C-terminal deletion mutants of the hHT-α1 subunit in mammalian cells

hHT-Δ1709

A cassette harboring the altered region of the hHT-α1 clone [3] was constructed using a PCR mutagenesis procedure. Preparative amplifications utilized the following primers for 20 cycles, the forward primer: 5′-GGGCCAGCTAGAGTGCAGC-3′ and the reverse primer: 5′-CTCTAGACTGAGCATGGCCTGGCTTC-3′. The restriction sites Sse8387I and XbaI are underlined, respectively. The amber stop codon introduced is shown in boldface type. The amplification product was isolated from a 2% sieving agarose gel, subcloned into pBluescript KS (+) via blunt end ligation, and was sequenced. The verified cassette was then used to replace the Sse8387I (6143)-XbaI (polylinker) fragment of hHT-α1.

hHT-Δ1784

A cassette harboring the altered region of the hHT-α1 clone was constructed using the PCR mutagenesis procedure. Preparative amplifications utilized the following primers for 20 cycles, the forward primer: 5′-GGCAAAGGGCCAGCCGCAGGC-3′ and the reverse primer: 5′-GCGGGCCCATGGAGAGCTGAGCTTCAGGCC-3′. The restriction site EagI is underlined. The amber stop codon introduced is shown in boldface type. The amplification product was isolated from a 2% sieving agarose gel, subcloned into pBluescript KS (+) via blunt end ligation, and was sequenced. The verified cassette was then digested with EagI, and this product was introduced into the EagI (6464) site of hHT-α1.

hHTα1-Δ1935

A cassette harboring the altered region of the hHT-α1 clone was constructed using the PCR mutagenesis procedure. Preparative amplifications utilized the following primers for 20 cycles, the forward primer: 5′-GGCAAGGGCCAGCAGG-3′ and the reverse primer: 5′-CTCTAGACTATAGTGAGCC-AGAGCCGGAG-3′. The restriction site EagI is underlined.