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Cell Calcium 39 (2006) 227–236

cell calcium

www.elsevier.com/locate/ceca

Non-sense mutations in the dihydropyridine receptor β1 gene, *CACNB1*, paralyze zebrafish *relaxed* mutants

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Received 15 September 2005; received in revised form 21 October 2005; accepted 26 October 2005
Available online 20 December 2005

Abstract

Contractions by skeletal muscle require proper excitation–contraction (EC) coupling, whereby depolarization of the muscle membrane leads to an increase in cytosolic Ca^{2+} and contraction. Changes in membrane voltage are detected by dihydropyridine receptors (DHPR) that directly interact with and activate ryanodine receptors to release Ca^{2+} from the sarcoplasmic reticulum into the cytosol. A genetic screen for motility mutations isolated a new allele of the immotile zebrafish mutant *relaxed*. Muscles in *relaxed* embryos do not contract in response to potassium chloride (KCl) thus appear unresponsive to membrane depolarization, but do contract when stimulated by caffeine, an agonist of ryanodine receptors. This suggests that *relaxed* mutant muscles are defective in EC coupling. Indeed, immunohistochemical analysis demonstrated that mutants lack DHPRs in skeletal muscles. The mutant phenotype results from non-sense mutations in the zebrafish *CACNB1* gene that encodes the DHPR β1 subunit. The zebrafish *CACNB1* gene is expressed in skeletal muscles, PNS and CNS. Electrophysiological recordings showed no obvious abnormalities in the motor output of *relaxed* mutants, presumably due to redundancy provided by other β subunits. The structural and functional homology of *CACNB1* in zebrafish and mammals, suggests that zebrafish can be useful for studying EC coupling and potential neuronal function of *CACNB1*.

Keywords: Zebrafish; Muscle; Dihydropyridine receptor; EC coupling; Voltage-dependent calcium channel

1. Introduction

A critical component of the generation of motor behaviors is the precise control of muscle contraction. L-type voltage dependent calcium channels, also known as dihydropyridine receptors (DHPR), play an essential role in skeletal muscle contraction [1,2]. Muscles are depolarized upon binding of acetylcholine from motor nerve terminals by the acetylcholine receptors at the neuromuscular junction. The depolarization induces a conformational change in the voltage sensing regions of DHPRs located at the triads of t-tubules. The change in DHPRs induces ryanodine receptors (RYR) in the sarcoplamic reticulum (SR) membranes that directly interact with DHPRs to open and release Ca^{2+} ions from the SR into the muscle cytosol. The elevated cytoplasmic Ca^{2+} in turn activates contractile proteins to give muscle contraction. This mechanism by which depolarization leads to contraction is termed excitation–contraction (EC) coupling.

In skeletal muscles, the DHPR consists of five subunits: the pore-forming α1S subunit, the cytoplasmic β1a subunit, and the integral membrane α2, δ and γ1 subunits. The α1S subunit resembles the α subunit of the voltage-gated sodium channels with four repeated domains each having six transmembrane segments. The focus of this paper, the β1a subunit (CAB1a), is encoded by a muscle specific variant of *CACNB1* gene and regulates both membrane targeting of the α1S subunit and channel kinetics [3]. The α2 and the δ subunits are encoded by a single gene and are post-translationally processed into two proteins, which can modulate the kinetics of the

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doi:10.1016/j.ceca.2005.10.015

channel [4]. The γ1 subunit is not required for EC coupling [5].

Zebrafish is a useful vertebrate model system for the study of motor behaviors due to its stereotyped behaviors, amenability to electrophysiological analysis and the ease of performing genetic manipulations [6,7]. We carried out an ENU-induced mutagenesis screen for zebrafish motility mutants. In this report we describe a recessive mutation (*mi90*) that lacks motility and is a new allele of the previously identified *relaxed* mutant [6]. The immotile phenotype of *relaxed* mutants is likely due to a loss of function of the *CACNB1* gene resulting from non-sense mutations in the gene.

2. Materials and methods

2.1. Animals

Zebrafish (*Danio rerio*) were bred and maintained in a breeding facility according to established procedures that meet the guidelines set forth by the University of Michigan Animal Care and Use protocols. *mi90* was isolated in our behavioral screen of mutant fish that were generated according to published mutagenesis procedures [8]. *relaxed* (*red*^{ts25}) was generated in the Tübingen mutant screen [6].

2.2. Video recording of zebrafish behavior

Zebrafish were placed in a Petri dish (60 mm) with 10 ml of fresh fish water and tactile stimulation of the tail was delivered with a fine tungsten wire (125 μm) or with forceps. Behaviors were recorded by video microscopy using a CCD camera attached to a dissection microscope. Images were captured with a Scion LG-3 video card on a Macintosh G4 computer. The images and videos were viewed with the Scion Image software and processed with NIH Image (NIH).

2.3. Potassium chloride and caffeine induced muscle contractions

The response of embryos to KCl and caffeine were recorded with video microscopy. Embryos (48 hours post-fertilization (hpf)) were anesthetized in 0.02% tricaine in Evan's solution (134 mM NaCl, 2.9 mM KCl, 2.1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES at 290 mOsm and pH 7.8; [9]), and pinned on their sides to a Sylgard dish with two tungsten wires through the notochord at somite 6–7 and somite 15–16. The skin on one side of the trunk between two pins was removed with a pair of forceps to expose approximately 10 somites. The tungsten wires were then removed and the fish re-pinned dorsal side up on to the dish by placing two pins through the head. The tricaine solution was washed off and replaced with Evan's solution containing 6 μM D-tubocurarine to partially curarize embryos. An isotonic KCl solution (150 mM) or an Evan's solution containing 15 μM of caffeine was applied to the exposed side of trunk by ejection from a micropipette attached to a Picospritzer. Video images were captured before, during and 30 s after the application.

2.4. Genetic and physical mapping

A *mi90* female carrier (Michigan local strain) was crossed with a wild-type WIK male (Zebrafish Resource Center, Eugene, OR) to generate a mapping family. One female and one male carrier were identified from this family by crossing to known carrier fish and the progeny from the cross between these two fish were collected and subjected to bulk segregant analysis [10] using 20 mutants and 20 wild type sibling embryos described in the Zon lab protocol (http://zfrhmaps.tch.harvard.edu). Candidate genes were physically mapped to the LN54 radiation hybrid panel by PCR [11].

2.5. Cloning of CACNB1 cDNA and mutant rescue

Total RNA was isolated from 24 to 30 hpf embryos by using Tri-reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed using oligo dT primers and Superscript II reverse transcriptase (Invitrogen) following the manufacturer's protocol (Superscript II manual, version 11-11-203). A cDNA clone (Genbank Acc CK018153) was identified for the zebrafish *CACNB1* gene by performing a Blast search of the Genbank database with the mouse CAB1a protein sequence (Genbank Acc NM_031173). The entire coding sequence of the gene was cloned by RT-PCR in both wild-type and mutant fish by using primers specific for the cDNA sequence. The PCR products were gel-purified, cloned into the T-easy vector (Promega, Madison, WI, USA) and sequenced at the University of Michigan Sequencing Core. At least four independent clones were sequenced for each product to avoid sequencing errors.

To clone the z-b1c splice variant, a genomic contig (Genbank Acc CR388097) was identified by a Blast search of the Ensemble Zebrafish Genomic Sequence database (http://www.ensembl.org/Danio_rerio/) with the human CAB1c protein sequence. The following primers were designed based upon the genomic sequence for zebrafish *CACNB1*: forward primer: 5′-CTCGAGCTCAA-ATGTCCAGGACGCCTTCCA CCTC-3′; reverse primer: 5′-TGGGTTAGTGTATCGTCCTCAAAA-3′. The cDNA sequence for z-b1c was cloned by RT-PCR with these two primers. The exon–intron splicing sites were determined by comparing cDNA sequences and the genomic sequences obtained from the Ensemble database. Sequence alignment and phylogenetic analysis was done with the Lasergene software (DNAStar, Madison, WI, USA).

For mutant rescue experiments capped RNA for *CACNB1* was synthesized with the mMessage mMachine Sp6 Kit (Ambion, Austin, TX, USA) using the cDNA clone as template. The capped RNA was diluted to 100 ng/μl in nuclease-