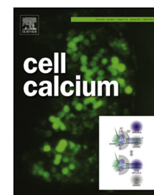
Cell Calcium 61 (2017) 22–31

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca



The mammalian skeletal muscle DHPR has larger Ca^{2+} conductance and is phylogenetically ancient to the early ray-finned fish sterlet (*Acipenser ruthenus*)

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ARTICLE INFO

Article history:
Received 15 September 2016
Received in revised form 21 October 2016
Accepted 21 October 2016
Available online 23 October 2016

Keywords:
L-Type calcium channel
Skeletal muscle
Sterlet DHPR
Excitation-contraction coupling
Calcium influx evolution

ABSTRACT

The L-type Ca^{2+} channel or dihydropyridine receptor (DHPR) in vertebrate skeletal muscle is responsible for sensing sarcolemmal depolarizations and transducing this signal to the sarcoplasmic Ca^{2+} release channel RyR1 via conformational coupling to initiate muscle contraction. During this excitation-contraction (EC) coupling process there is a slow Ca^{2+} current through the mammalian DHPR which is fully missing in euteleost fishes. In contrast to ancestral evolutionary stages where skeletal muscle EC coupling is still depended on Ca^{2+}-induced Ca^{2+}-release (CICR), it is possible that the DHPR Ca^{2+} conductivity during mammalian (conformational) EC coupling was retained as an evolutionary remnant (vestigiality). Here, we wanted to test the hypothesis that due to the lack of evolutionary pressure in post-CICR species skeletal muscle DHPR Ca^{2+} conductivity gradually reduced as evolution progressed. Interestingly, we identified that the DHPR of the early ray-finned fish sterlet (*Acipenser ruthenus*) is phylogenetically positioned above the mammalian rabbit DHPR which retained robust Ca^{2+} conductivity, but below the euteleost zebrafish DHPR which completely lost Ca^{2+} conductivity. Remarkably, our results revealed that sterlet DHPR still retained the Ca^{2+} conductivity but currents are significantly reduced compared to rabbit. This decrease is due to lower DHPR membrane expression similar to zebrafish, as well as due to reduced channel open probability (P_o). In both these fish species the lower DHPR expression density is partially compensated by higher efficacy of DHPR-RyR1 coupling. The complete loss of P_o in zebrafish and other euteleost species was presumably based on the teleost specific 3rd round of genome duplication (Ts3R). Ts3R headed into the appearance of two skeletal muscle DHPR isoforms which finally, together with the radiation of the euteleost clade, fully lost the P_o.

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1. Introduction

Excitation-contraction (EC) coupling links the electrical excitation of the sarcolemma to the contractile activation of the myofilaments. In vertebrate skeletal muscle, this mechanism depends on the close interplay of the sarcolemmal voltage-gated L-type Ca^{2+} channel or dihydropyridine receptor (DHPR) and the SR Ca^{2+} release channel or ryanodine receptor type-1 (RyR1). Depolarization of the sarcolemma generates conformational changes in the voltage-sensing DHPRα_{1S} subunit [1] which via inter-channel protein – protein interaction opens the RyR1 to release Ca^{2+} from SR stores, required for muscle contraction [2,3]. Simultaneous to this conformational DHPR-RyR1 interaction, there is a small and slowly activating inward Ca^{2+} current through the DHPR, found from lower vertebrates like sharks to higher vertebrates like mammals. The physiological significance of this L-type Ca^{2+} current which is not immediately required for EC coupling [4], is still a matter of uncertainty even after more than 40 years of continuous research [5–7]. In contrast to the skeletal DHPR, activation of the cardiac DHPR isoform leads to a large and rapidly activating inward Ca^{2+} current. Subsequently, this DHPR Ca^{2+} influx triggers opening of the Ca^{2+}-sensitive cardiac ryanodine receptor type-2 (RyR2) to release Ca^{2+} ions from the intracellular SR Ca^{2+} stores. This Ca^{2+}-induced Ca^{2+}

Abbreviations: EC, excitation-contraction; DHPR, dihydropyridine receptor; RyR1, ryanodine receptor type-1; SR, sarcoplasmic reticulum; CICR, Ca^{2+}-induced Ca^{2+} release; GLT, immortalized DHPRα_{1S}-null murine myotubes; P_o, open probability; Ts3R, teleost-specific 3rd round of genome duplication; st-α_{1S}, sterlet DHPR α_{1S} subunit; rb-α_{1S}, rabbit DHPR α_{1S} subunit; zf-α_{1S}, zebrafish DHPR α_{1S} subunit; GFP, green fluorescent protein; nt, nucleotide numbers.

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http://dx.doi.org/10.1016/j.ceca.2016.10.002
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release (CICR) mechanism consequently leads to cardiac muscle contraction [8–10].

Very interesting from the phylogenetic view is that the EC coupling mechanism in skeletal muscle fibers of invertebrates and also of early chordates like tunicates or cephalochordates (amphioxus) resembles the cardiac-type EC coupling of vertebrates, since the activation fully depends on external Ca^{2+} influx through the DHPR [11–18]. In skeletal muscles of hagfish and lamprey which are higher up on the phylogenetic tree, conformational (vertebrate skeletal-muscle-type) DHPR-RyR1 coupling is already established [13,19] but maximum force generation still depends on external Ca^{2+} influx [20]. On the evolutionary trajectory to the vertebrates EC coupling became fully Ca^{2+}-influx independent (see Fig. 6 in Ref. [18]). Despite loss of this direct role of Ca^{2+} influx in skeletal muscle EC coupling, robust DHPR currents were recorded from shark muscles [21], which belong to the ancient class “cartilaginous fish” (Chondrichthyes) (Fig. 1). Ca^{2+} influx through the skeletal muscle DHPR continued to exist at least until the phylogenetic branch of the “four limbed vertebrates” (Tetrapods) which is the most successful and species-rich clade within the ancient group of Sarcopterygii (Fig. 1). Finally, evolution led to a complete abolition of DHPR Ca^{2+} influx in the class of “ray-finned fishes” (Actinopterygii), more precisely in their highest developed infraclass of “modern bony fishes” (Euteleostei) containing the model organism zebrafish (*Danio rerio*). Hence, euteleost fish DHPRs fully lost their conductance and solely work as voltage sensors to detect membrane depolarization and as transmitters of this signal to the RyR1 [18]. Therefore, it is very likely that evolutionary replacement of the CICR mechanism in skeletal muscles of early chordates by conformational EC coupling in vertebrates eliminated the evolutionary pressure on the DHPR to conduct Ca^{2+}. Thus, it is feasible that the remaining DHPR Ca^{2+} influx in tetrapods is just an evolutionary remnant (vestigiality) of the ancient CICR stage.

One possibility to reach the state of DHPR non-conductivity in euteleost fishes could be a gradual diminution in conductance during the course of vertebrate phylogeny, due to random mutations in channel regions previously essential for Ca^{2+} conductance. Alternatively, a sharp transition from fully conducting skeletal muscle DHPRs all the way throughout vertebrate phylogeny until a stage of sudden non-conductivity only on the trajectory to the euteleost branch is likewise possible. However, such an abrupt switch would rather suggest novel physiological requirements for DHPR Ca^{2+}-non-conductivity in euteleost fishes, for so far unknown reasons.

To shed light on the mechanism how the complete loss of DHPR Ca^{2+} conductivity in skeletal muscle of euteleost fishes arose – by “fading out” or “switching off” – we investigated DHPR current properties in a selected species, which is evolutionary quite away from the Ca^{2+}-conducting tetrapods but somewhere on the trajectory towards the non-Ca^{2+}-conducting euteleost fishes (Fig. 1). Therefore, we cloned and sequenced the channel-pore-forming DHPRα_{1S} subunit of sterlet (*Acipenser ruthenus*) which is one of the 27 species from the family of sturgeons, close to the basis of Actinopterygii (Fig. 1). Sturgeons, together with the other basal groups, namely bichirs, gars, and bowfins consist of less than 50 extant species and are often considered as “living fossils” since their phylogenetic development has been very slow and their morphology and physiology has remained unchanged since earliest times [22,23]. However, it was unknown if DHPR Ca^{2+} conductivity already disappeared in those early ray-finned fishes.

In the present study, we focused on the biophysical characterization of the skeletal muscle DHPR Ca^{2+} conductivity and EC coupling parameters of sterlet in comparison to the mammalian rabbit (*Oryctolagus cuniculus*) and the euteleost zebrafish (*Danio rerio*). Sterlet DHPRα_{1S} was cloned and expressed in the DHPRα_{1S}-null murine muscle cell line GLT [24]. Interestingly, sterlet DHPRα_{1S} was found to conduct Ca^{2+} currents but at lower expression density and open probability (P_o) compared to the mammalian control DHPR. However, reduced DHPR expression density combined with enhanced DHPR-RyR1 coupling efficacy in sterlet was identical to zebrafish. Overall, our results suggest that DHPR Ca^{2+} conductivity “fades out” on the phylogenetic track from the mammals to euteleost fishes. But the final abrupt “turn off” in euteleost fish was presumably based on an additional extraordinary evolutionary event – the 3rd round of teleost specific genome duplication (Ts3R) at the basis of the teleost branch (Fig. 1). Ts3R led to the emergence of two skeletal muscle DHPRα_{1S} isoforms in teleosts which finally turned non-Ca^{2+} conducting in euteleost fish.

2. Materials and methods

2.1. Molecular cloning of the sterlet DHPRα_{1S} subunit

Sterlet DHPRα_{1S} subunit (st-α_{1S}) cDNA was created as follows, with nucleotide numbers (nt) given in parentheses and asterisks indicating restriction enzyme (RE) sites introduced by PCR using proofreading Phusion DNA polymerase (Finnzymes reagents). Total RNA from adult sterlet skeletal muscle was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed using the Ready-To-Go T-Primed First-Strand Kit (Amersham Pharmacia Biotech). Three cDNA fragments covering the entire open reading frame (ORF) of st-α_{1S} were PCR generated from first-strand cDNA with primers designed according to the zebrafish DHPRα_{1S}-a sequence (GenBank accession no. NM 001146150): SalI*-KpnI (nt −11 to 1119), KpnI-EcoRI (nt 1119–3004), EcoRI-XbaI* (nt 3004–5643). Two subclones were created by co-ligating fragments SalI*-KpnI (nt −11 to 1119) plus KpnI-EcoRI (nt 1119–3004), as well as ligating fragment EcoRI-XbaI* (nt 3004–5643) into the corresponding polylinker RE sites of pBluescript SK+ (pBS) (Stratagene). For the final construct and N-terminal GFP tagging, excised fragments SalI*-EcoRI (nt −11 to 3004) and EcoRI-XbaI* (nt 3004–5643) were in-frame co-ligated into the SalI/XbaI polylinker RE sites of $pGFP^{37}$ [25]. For an exemplar scheme of the GFP-α_{1S} construct and its expression observed as GFP fluorescence in α_{1S}-null myotubes, see Figs. 1 and 2 of Ref. [25]. After confirming the integrity of the cDNA by sequence analysis (Eurofins MWG Operon, Martinsried, Germany), sterlet DHPRα_{1S} subunit cDNA was deposited in GenBank database (GenBank accession no. HQ636615).

2.2. Expression plasmids

Identical to st-α_{1S}, rabbit and zebrafish DHPRα_{1S} subunits (rb-α_{1S}, zf-α_{1S}-a, and zf-α_{1S}-b, respectively) were N-terminally GFP-tagged by cloning the respective cDNAs into expression plasmid $pGFP^{37}$ as described previously [18,25].

2.3. Zebrafish

Wild-type (wt) zebrafish were bred and maintained under standard laboratory conditions according to the established protocols [31], approved by the Tierethik-Beirat of the Medical University Innsbruck and the Bundesministerium für Wissenschaft und Forschung.

2.4. Culture and transfection of GLT and primary zebrafish myotubes

Murine DHPRα_{1S}-null (GLT) myotubes were cultured on 35-mm culture dishes at 37 °C and 10% CO_2 as described previously [24]. Approximately 4 days after plating, at the onset of myoblast fusion, myotubes were transfected with 1 μg of N-terminally GFP-tagged DHPRα_{1S} cDNA constructs using FuGENE®HD transfection

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