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Skeletal muscle excitation–contraction coupling is independent of a conserved heptad repeat motif in the C-terminus of the DHPRβ_{1a} subunit

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ABSTRACT

In skeletal muscle excitation–contraction (EC) coupling the sarcolemmal L-type Ca^{2+} channel or 1,4-dihydropyridine receptor (DHPR) transduces the membrane depolarization signal to the sarcoplasmic Ca^{2+} release channel RyR1 via protein–protein interaction. While it is evident that the pore-forming and voltage-sensing DHPRα_{1S} subunit is essential for this process, the intracellular DHPRβ_{1a} subunit was also shown to be indispensable. We previously found that the β_{1a} subunit is essential to target the DHPR into groups of four (tetrads) opposite the RyR1 homotetramers, a prerequisite for skeletal muscle EC coupling. Earlier, a unique hydrophobic heptad repeat motif (L···V···V) in the C-terminus of β_{1a} was postulated by others to be essential for skeletal muscle EC coupling, as substitution of these residues with alanines resulted in 80% reduction of RyR1 Ca^{2+} release. Therefore, we wanted to address the question if the proposed β_{1a} heptad repeat motif could be an active element of the DHPR–RyR1 signal transduction mechanism or already contributes at the ultrastructural level i.e. DHPR tetrad arrangement. Surprisingly, our experiments revealed full tetrad formation and an almost complete restoration of EC coupling in β_1-null zebrafish *relaxed* larvae and isolated myotubes upon expression of a β_{1a}-specific heptad repeat mutant (LVV to AAA) and thus contradict the earlier results.

1. Introduction

Excitation–contraction (EC) coupling is understood as the signal transduction process linking membrane depolarization to the contraction of a muscle cell. In skeletal muscle this involves a direct crosstalk between two Ca^{2+} channels, the plasmalemmal high voltage-activated skeletal muscle Ca^{2+} channel or 1,4-dihydropyridine receptor (DHPR) and the intracellular Ca^{2+} release channel, the ryanodine receptor type-1 (RyR1) in the sarcoplasmic reticulum (SR) membrane. Membrane depolarization induces conformational changes in the voltage-sensing DHPRα_{1S} which are transduced to RyR1 via protein–protein interaction [1,2]. This leads to opening of RyR1 without the need of DHPR Ca^{2+} influx [3]. Both the channels are targeted into the muscle triad junctions where clusters of DHPR in the sarcolemma co-localize with clusters of RyR1 in closely juxtaposed SR membrane [4,5]. Ultrastructurally, DHPRs, visible as "particles" in freeze-fracture images, are arranged in groups of four (tetrads) to communicate with every other RyR1 homotetramer and thus are geometrically arranged in orthogonal arrays following the RyR1-arrays [6,7].

The skeletal muscle DHPR is a hetero-multimeric protein complex consisting of the central α_{1S} subunit and the auxiliary subunits β_{1a}, $\alpha_2\delta$-1, and γ_1 [8]. According to the current model, the pore-forming and voltage-sensing α_{1S} subunit transduces the opening signal to RyR1, essentially via the intracellular loop connecting homologous repeats II and III [9,10]. The intracellular β_{1a} subunit was shown to have multiple roles in targeting and modulating the central α_{1S} subunit [11,12]. The lack of β_{1a} is incompatible with skeletal muscle EC coupling, and leads to perinatal lethality in β_1-null mice due to respiratory paralysis [13] or to an immotile larval phenotype in the β_1-null zebrafish mutant *relaxed* [14,15]. As shown in zebrafish *relaxed* the absence of β_{1a} specifically leads to, (i) reduction of α_{1S} membrane targeting, (ii) severe reduction in α_{1S} charge movement, and (iii) complete absence of the ultrastructural arrangement of DHPRs into tetrads in orthogonal arrays, a prerequisite for skeletal muscle EC coupling [14].

Despite the inability to ventilate their gills, *relaxed* larvae are able to survive for few days due to oxygen and metabolite diffusion via the skin [16]. This in combination with the possibility

Abbreviations: EC, excitation–contraction; DHPR, 1,4-dihydropyridine receptor; RyR1, ryanodine receptor type-1; SR, sarcoplasmic reticulum; zf-, β_{1a}zebrafish β_{1a}; rb-, β_{1a}rabbit β_{1a}; GFP, green fluorescent protein; nt, nucleotide numbers; WT, wild-type; hpf, hours post-fertilization.

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of zygote injection into the externally developing embryos makes zebrafish mutant *relaxed* an excellent in vivo expression system. In a reconstitution study we showed that the expression of β_{1a} in the *relaxed* system restored the motile phenotype and that DHPR tetrad formation is an exclusive property of β_{1a} [17]. Thus, specific structural elements important for the formation of tetrads must exist in β_{1a}.

Earlier studies, with β_{1a}/β_{2a} chimeras and truncation mutants expressed in murine β_1-null myotubes, restricted the domain of β_{1a} that is essential for skeletal muscle EC coupling to its C-terminus [18–20]. More precisely, a β_{1a}-specific C-terminal hydrophobic heptad repeat motif (L478, V485, V492) was proposed to control the EC coupling activity [22]. A β_{1a} mutant with the heptad repeat LVV exchanged to alanines could only reconstitute 20% of intracellular Ca^{2+} transients and also induced a significant positive shift in their voltage-dependence. Leucine heptad repeat motifs are well known to mediate protein–protein interactions [21]. On this basis it was hypothesized that the heptad repeat of β_{1a} is involved in direct interaction with RyR1 [20,22] and thus plays an active role in the signal transduction from DHPR to RyR1 via β_{1a}.

Moreover, since we showed that β_{1a} is specifically required for tetrad formation [17] which is a prerequisite for EC coupling, it is also possible that already the arrangement of DHPRs into tetrads is dependent on this β_{1a}-specific heptad repeat motif. Therefore, we wanted to address this question by comparing the ultrastructural arrangement of DHPR with functional recordings of EC coupling. If, despite the lack of the LVV motif, tetrads but no intact EC coupling could be restored, the hypothesis of β_{1a} as a direct signal transducer – as an additional β_{1a} function – would be supported. On the other hand, if tetrads were not formed, the conclusion would be that the LVV motif is simply involved in the initial scaffolding process which ultrastructurally enables the interaction of DHPR and RyR1 by placing them in the appropriate relative configuration.

We took advantage of the β_{1a}-null zebrafish *relaxed* expression system that allows us to directly compare the effects observed in vitro to an intact in vivo muscle system. Mutant constructs from zebrafish β_{1a} (zf-β_{1a}AAA) and from a mammalian (rabbit) β_{1a} (rb-β_{1a}AAA), in which the conserved hydrophobic heptad repeat motif LVV was exchanged to alanines, were expressed in isolated *relaxed* myotubes and entire larvae. Our results with β_{1a}AAA show that knock out of the LVV motif did not interfere with correct targeting of DHPR into tetrads. Furthermore, and to our surprise, heptad repeat mutants were able to restore robust intracellular Ca^{2+} transients in *relaxed* myotubes and a fully motile phenotype in *relaxed* larvae, thus illustrating restoration of proper DHPR–RyR1 coupling. In contrast to the earlier proposals, our results indicate that the β_{1a}-specific C-terminal heptad repeat motif LVV is not a critical determinant of skeletal muscle EC coupling, because it is neither necessary for tetrad formation nor for DHPR–RyR1 signal transduction.

2. Materials and methods

Experimental procedures were essentially the same as described earlier in detail [17] and thus only a concise summary is given.

2.1. Zebrafish embryos

Rearing and breeding of zebrafish, heterozygous for the β_1-null mutation *relaxed* (*red*^{ts25}) was performed according to the established procedures [23,24]. Freshly spawned eggs were directly used for zygote RNA microinjection and/or raised at 28 °C to be used for experiments.

2.2. Expression plasmids

The cDNAs of the β subunits and mutants were N-terminally in-frame fused to GFP cDNA and cloned into expression vector pCI-neo (Promega). Nucleotide numbers (nt) are given in parenthesis. All sequences generated and modified by PCR were checked for integrity by sequence analysis (Eurofins MWG Operon, Martinsried, Germany).

2.2.1. zf-β_{1a}AAA

Fusion PCR was used to generate LVV/AAA substitutions (L478A, V485A, V492A) with zf-β_{1a} cDNA (GenBank AY952462) in pCI-neo as template. The sense primer was used for T to C transition which created triplet codons GCC (nt 1452–1454) and GCG (nt 1473–1475) both coding for alanine instead of valine. With the antisense primer, triplets CTG (nt 1431–1433) and GTC (nt 1452–1454) were mutated to GCG and GCC respectively, both coding for alanine instead of leucine and valine. To gain the final construct zf-β_{1a}AAA, the PCR-generated EarI–XbaI fusion fragment (nt 879–1803) was co-ligated with fragment EcoRV–EarI from zf-β_{1a} (nt −748 to 879) into the EcoRV/XbaI (nt −748 to1803) cleaved zf-β_{1a} clone.

2.2.2. rb-β_{1a}AAA

The LVV/AAA substitutions (L478A, V485A, V492A) were created by using the fusion PCR technique with rb-β_{1a} cDNA (GenBank NM_001082279) in pCI-neo as template. The sense primer substituted GTC (1452–1454) and GTG (1473–1475) to GCC and GCG respectively, both coding for alanine instead of valine. The antisense primer was used to replace CTG (1431–1433) and GTC (1452–1454) with GCG and GCC respectively, both coding for alanine instead of leucine and valine. The resulting BstXI–XbaI (nt 834–1801) fusion product was ligated together with fragment EcoRV–BstXI from rb-β_{1a} (nt −763 to 834) into the EcoRV/XbaI (nt −763 to 801) cut rb-β_{1a} to generate the final construct rb-β_{1a}AAA.

2.2.3. GFP

GFP alone was cloned into expression vector pCI-neo for standardizing experimental conditions [17].

2.3. Zygote injection of in vitro synthesized RNA

All β subunits and mutants were linearized with restriction enzyme XbaI, but GFP with NotI. Purified and linearized DNA templates were used for in vitro transcription followed by phenol/chloroform extraction and ethanol precipitation. RNA pellets were resuspended in RNAse-free water, fidelity checked on an agarose gel under denaturing conditions and the aliquots were stored at −80 °C until use. Eggs from heterozygous parental zebrafish in one-cell stage were injected within 20 min after spawning. Approximately 2.6 ng of RNA, containing 0.1% phenol red as an injection volume tracer [24] was injected per egg. The GFP fluorescence of 8-h-old healthy injected embryos was quantified using a photomultiplier system. Only proper developing injected embryos with a mean fluorescence signal exceeding 40% above uninjected control embryos were considered for further experiments.

2.4. Identification of rescued larvae

Differentiation of motility-restored homozygous *relaxed* larvae, used in motion analysis experiments, from the injected "normal" siblings (i.e. heterozygous and wild-type, WT) was done by keeping all injected larvae in isolation up to 5 days. During this period a gradual fallback to the paralyzed phenotype due to degradation of the injected β-RNAs and translated proteins was observed for restored *relaxed* but not normal larvae. Genotype confirmation of