Proper Restoration of Excitation-Contraction Coupling in the Dihydropyridine Receptor \( \beta_{1a} \)-null Zebrafish Relaxed Is an Exclusive Function of the \( \beta_{1a} \) Subunit*

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Johann Schredelseker,1,2 Anamika Dayal,3 Thorsten Schwerte, Clara Franzini-Armstrong, and Manfred Grabner

From the 1Department of Medical Genetics, Clinical and Molecular Pharmacology, Division of Biochemical Pharmacology, Innsbruck Medical University, A-6020 Innsbruck, Austria, the 2Institute of Zoology, University of Innsbruck, Innsbruck, A-6020 Austria, and the 3Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

The paralyzed zebrafish strain relaxed carries a null mutation for the skeletal muscle dihydropyridine receptor (DHPR) \( \beta_{1a} \) subunit. Lack of \( \beta_{1a} \) results in (i) reduced membrane expression of the pore forming DHPR \( \alpha_{1S} \) subunit, (ii) elimination of \( \alpha_{1S} \) charge movement, and (iii) impediment of arrangement of the DHPRs in groups of four (tetrads) opposing the ryanodine receptor (RyR1), a structural prerequisite for skeletal muscle-type excitation-contraction (EC) coupling. In this study we used relaxed larvae and isolated myotubes as expression systems to discriminate specific functions of \( \beta_{1a} \) from rather general functions of \( \beta \) isoforms. Zebrafish and mammalian \( \beta_{1a} \) subunits quantitatively restored \( \alpha_{1S} \) triad targeting and charge movement as well as intracellular \( Ca^{2+} \) release, allowed arrangement of DHPRs in tetrads, and most strikingly recovered a fully motile phenotype in relaxed larvae. Interestingly, the cardiac/neuronal \( \beta_{2a} \) as the phylogenetically closest, and the ancestral housefly \( \beta_{M} \) as the most distant isoform to \( \beta_{1a} \) also completely recovered \( \alpha_{1S} \) triad expression and charge movement. However, both revealed drastically impaired intracellular \( Ca^{2+} \) transients and very limited tetrad formation compared with \( \beta_{1a} \). Consequently, larval motility was either only partially restored (\( \beta_{2a} \)-injected larvae) or not restored at all (\( \beta_{M} \)). Thus, our results indicate that triad expression and facilitation of 1,4-dihydropyridine receptor (DHPR) charge movement are common features of all tested \( \beta \) subunits, whereas the efficient arrangement of DHPRs in tetrads and thus intact DHPR-RyR1 coupling is only promoted by the \( \beta_{1a} \) isoform. Consequently, we postulate a model that presents \( \beta_{1a} \) as an allosteric modifier of \( \alpha_{1S} \) conformation enabling skeletal muscle-type EC coupling.

Excitation-contraction (EC) coupling in skeletal muscle is critically dependent on the close interaction of two distinct \( Ca^{2+} \) channels. Membrane depolarizations of the myotube are sensed by the voltage-dependent 1,4-dihydropyridine receptor (DHPR) in the sarcolemma, leading to a rearrangement of charged amino acids (charge movement) in the transmembrane segments S4 of the pore-forming DHPR \( \alpha_{1S} \) subunit (1, 2). This conformational change induces via protein–protein interaction (3, 4) the opening of the sarcoplasmic type-1 ryanodine receptor (RyR1) without need of \( Ca^{2+} \) influx through the DHPR (5). The release of \( Ca^{2+} \) from the sarcoplasmic reticulum via RyR1 consequently induces muscle contraction. The protein–protein interaction mechanism between DHPR and RyR1 requires correct ultrastructural targeting of both channels. In \( Ca^{2+} \) release units (triods and peripheral couplings) of the skeletal muscle, groups of four DHPRs (tetrads) are coupled to every other RyR1 and hence are geometrically arranged following the RyR-specific orthogonal arrays (6).

The skeletal muscle DHPR is a heteromultimeric protein complex, composed of the voltage-sensing and pore-forming \( \alpha_{1S} \) subunit and auxiliary subunits \( \beta_{1a}, \alpha_{\delta}, \), and \( \gamma_{1} \). While gene knock-out of the DHPR \( \gamma_{1} \) subunit (8, 9) and small interfering RNA knockdown of the DHPR \( \alpha_{\delta} \) subunit (10–12) have indicated that neither subunit is essential for coupling of the DHPR with RyR1, the lack of the \( \alpha_{1S} \) or of the intracellular \( \beta_{1a} \) subunit is incompatible with EC coupling and accordingly null model mice die perinatally due to asphyxia (13, 14). \( \beta \) subunits of voltage-gated \( Ca^{2+} \) channels were repeatedly shown to be responsible for the facilitation of \( \alpha_{1} \) membrane insertion and to be potent regulators of \( \alpha_{1} \) current kinetics and voltage dependence (15, 16). Whether the loss of EC coupling in \( \beta_{1} \) null mice was caused by decreased DHPR membrane expression or by the lack of a putative specific contribution of the \( \beta \) subunit to the skeletal muscle EC coupling apparatus (17, 18) was not clearly resolved. Recently, other \( \beta \) functions were identified in skeletal muscle using the \( \beta_{1} \)-null mutant zebrafish relaxed (19, 20). Like the \( \beta_{1} \)-knock-out mouse (14) zebrafish relaxed is characterized by complete paralysis of skeletal muscle (21, 22). While \( \beta_{1} \)-knock-out mouse pups die immediately after birth.

* The abbreviations used are: EC, excitation-contraction; DHPR, 1,4-dihydropyridine receptor; RyR1, ryanodine receptor type-1; hpf, hours post fertilization; GFP, green fluorescent protein; nt, nucleotide(s); RE, restriction enzyme; WT, wild type; rb-\( \beta_{1a} \), rabbit \( \beta_{1a} \); zf-\( \beta_{1a} \), zebrafish \( \beta_{1a} \); MOPS, 4-morpholinepropanesulfonic acid.
due to respiratory paralysis (14), larvae of *relaxed* are able to survive for several days because of oxygen and metabolite diffusion via the skin (23). Using highly differentiated myotubes that are easy to isolate from these larvae, the lack of EC coupling could be described by quantitative immunocytochemistry as a moderate ~50% reduction of $\alpha_{1S}$ membrane expression although $\alpha_{1S}$ charge movement was nearly absent, and, most strikingly, as the complete lack of the arrangement of DHPRs in tetrads (19). Thus, in skeletal muscle the $\beta$ subunit enables EC coupling by (i) enhancing $\alpha_{1S}$ membrane targeting, (ii) facilitating $\alpha_{1S}$ charge movement, and (iii) enabling the ultrastructural arrangement of DHPRs in tetrads.

The question arises, which of these functions are specific for the skeletal muscle $\beta_{1a}$ and which ones are rather general properties of Ca$^{2+}$ channel $\beta$ subunits. Previous reconstitution studies made in the $\beta_{1s}$-null mouse system (24, 25) using different $\beta$ subunit constructs (26) did not allow differentiation between $\beta$-induced enhancement of non-functional $\alpha_{1s}$ membrane expression and the facilitation of $\alpha_{1s}$ charge movement, due to the lack of information on $\alpha_{1s}$ triad expression levels. Furthermore, the $\beta$-induced arrangement of DHPRs in tetrads was not detected as no ultrastructural information was obtained.

In the present study, we established zebrafish mutant *relaxed* as an expression system to test different $\beta$ subunits for their ability to restore skeletal muscle EC coupling. Using isolated myotubes for *in vitro* experiments (19, 27) and complete larvae for *in vivo* expression studies (28–31) and freeze-fracture electron microscopy, a clear differentiation between the major functional roles of $\beta$ subunits was feasible in the zebrafish system. The cloned zebrafish $\beta_{1a}$ and a mammalian (rabbit) $\beta_{1a}$ were shown to completely restore all parameters of EC coupling when expressed in *relaxed* myotubes and larvae. However, the phylogenetically closest $\beta$ subunit to $\beta_{1a}$, the cardiac/neuronal isoform $\beta_{2a}$ from rat, as well as the ancestral $\beta_{1s}$ isoform from the housefly (*Musca domestica*), could recover functional $\alpha_{1S}$ membrane insertion, but led to very restricted tetrad formation when compared with $\beta_{1a}$, and thus to impaired DHPR-RyR1 coupling. This impairment caused drastic changes in skeletal muscle function.

The present study shows that the enhancement of functional $\alpha_{1S}$ membrane expression is a common function of all the tested $\beta$ subunits, from $\beta_{1a}$ to even the most distant $\beta_{2a}$, whereas the effective formation of tetrads and thus proper skeletal muscle EC coupling is an exclusive function of the skeletal muscle $\beta_{1a}$ subunit. In context with previous studies, our results suggest a model according to which $\beta_{1a}$ acts as an allosteric modifier of $\alpha_{1S}$ conformation. Only in the presence of $\beta_{1a}$, the $\alpha_{1S}$ subunit is properly folded to allow RyR1 anchoring and thus skeletal muscle-type EC coupling.

**EXPERIMENTAL PROCEDURES**

Zebrafish Embryos—Adult zebrafish, heterozygous for the $\beta_{1s}$-null *redt* (25) (relaxed) mutation were maintained and bred under standard aquarium conditions (28, 29). Freshly spawned eggs were directly used for zygote RNA microinjection (see below) and/or raised until 25–32 h post-fertilization (hpf) at 28 °C to be used for experiments.

Expression Plasmids—All $\beta$ subunit cDNAs were N-terminally fused in-frame to GFP cDNA and cloned into expression vector pCI-neo (Promega) that allows both, *in vitro* RNA synthesis for zygote injection as well as transient expression in cultured *relaxed* myotubes. Constructs were designed as follows, with nucleotide numbers (nt) given in parentheses and asterisks indicating restriction enzyme (RE) sites introduced by the PCR technique using proofreading *Pfu* Turbo DNA polymerase (Stratagene). The integrity of cDNA sequences generated by PCR was confirmed by sequence analysis (Eurofins MWG Operon, Martinsried, Germany).

*zf-\beta_{1a}*—Total RNA from adult wild type (WT) zebrafish muscle was isolated using the RNeasy Mini kit (Qiagen) and reverse transcribed using the Ready-To-Go T-primed first-strand kit (Amersham Biosciences). From the first-strand cDNA, the zf-\beta_{1a} open reading frame (GenBank™ AW592462) was PCR-generated in three fragments: HindIII*-Xhol (nt $-5$–502), Xhol-HindIII (nt 502–1352), and HindIII-BamHI* (nt 1352–1577). A subclone was created by co-fragments Xhol-HindIII (nt 502–1352) and HindIII-BamHI* (nt 1352–1577) into the Xhol/BamHI polylinker RE sites of pBlueScript SK*+ (pBS) (Stratagene). For N-terminal GFP tagging, fragment HindIII*-Xhol (nt $-5$–502) was in-frame ligated together with the excised fragment Xhol-BamHI* (nt 502–1577) into the HindIII/BamHI polylinker RE sites of the proprietary expression plasmid pGFP (32). From this subclone GFP-zf-\beta_{1a} cDNA was excised with PstI-Xhol (nt $-734$–502) and Xhol-BamHI* (nt 502–1577) and ligated into the PstI/BamHI cut pBS. For the final construct zf-\beta_{1a} the Sall-BamHI* (nt $-771$–1577) insert was co-ligated with the 226-bp poly(A) tail excised with BamHI-NotI from the proprietary transcription plasmid pNKS2 (a gift of O. Pongs) into the Xhol/NotI cut polylinker of pCI-neo.

*rb-\beta_{1a}*-The open reading frame of rabbit $\beta_{1a}$ cDNA (GenBank NM_01082279) was isolated from plasmid pDNA3 (33) as the HindIII-BstXI fragment (nt $-20$–834) and as the BstXI-BamHI fragment (nt 834–1575) PCR fragment, reintroducing its original stop codon at nt 1572. For N-terminal GFP tagging, both fragments were co-fragmented into the HindIII/BamHI polylinker RE sites of pGFP (37). From this subclone GFP-rb-\beta_{1a} cDNA was excised with PstI-Kpnl (nt $-734$ to $-10$) and Kpnl-BamHI* (nt $-10$–1577) and ligated into the PstI/BamHI cut pBS. To gain the final construct for rb-\beta_{1a}, the Sall-BamHI* (nt $-771$–1575) insert was co-ligated with the BamHI-NotI-excised poly(A) tail (see above) into the Xhol/NotI cut polylinker of pCI-neo.

*rb-\beta_{2s}*-The open reading frame of rat $\beta_{2s}$ cDNA (GenBank M80545) was isolated from plasmid p91023(B) (34) as the HindIII-Xhol fragment (nt $-11$–1064) and the Xhol-BamHI* (nt 1064–1816) PCR fragment. For GFP tagging, fragments were co-ligated into the HindIII/BamHI polylinker RE sites of pGFP. From this subclone GFP-rb-\beta_{2s} cDNA was excised with PstI-Kpnl (nt $-740$–1816) and ligated into the PstI/BamHI opened pBS. As a final step, the Sall-BamHI* (nt $-777$–1816) insert was co-ligated with the BamHI-NotI cut poly(A) tail into the Xhol/NotI opened polylinker of pCI-neo.

$\beta_{M}$—Musca $\beta$ ($\beta_{M}$) cDNA (GenBank X78561) was isolated from plasmid $\beta_{M}$-pNKS2 (35) as HindIII-DraIII (nt 8–2369) and DraIII-Xbal (nt 2369–3506) fragments and was co-ligated.
in-frame with GFP cDNA into the HindIII/XbaI polylinker of pGFP\(^{37}\). GFP-\(\beta_{1a}\) cDNA was isolated as PstI-DraIII (nt \(-721–2369\)) and DraIII-XbaI (nt 2369–3506) fragments and ligated into the PstI/XbaI opened pBS. To generate the final \(\beta_{1a}\) construct, fragments Sall-DraIII (nt \(-758–2369\)) and DraIII-XbaI (nt 2369–3506) were ligated into the XhoI/XbaI cut polylinker of pCI-neo.

**GFP**—For standardizing experimental conditions, GFP alone was cloned into expression vector pCI-neo in the following way: the GFP cDNA was excised EcoRI-HindIII (nt \(-24–716\)) from subclone GFP-zf-\(\beta_{1a}\) in PBS (see above; GFP-open reading frame numbering) and was co-ligated with the HindIII-NotI cut poly(A) tail into the EcoRI/NotI-cleaved polylinker of pCI-neo.

**Primary Culture of Zebrafish Myotubes**—For the isolation of myoblasts, 25–28 hpf chorionated embryos derived from heterozygous relaxed parental fish were surface-sterilized using 0.5% sodium hypochlorite for 2 min and then enzymatically dechorionated using 2 mg/ml Pronase (Protease, Type XIV, Sigma) (28) for 20 min at 28 °C and collected in 0.5% sodium hypochlorite for 2 min and then enzymatically digested for 1 h in 200 units/ml collagenase type I in Hanks’ buffered salt saline (Sigma). Homozygous relaxed larvae were identified by their inability to move despite tactile stimulation. Motile “normal” siblings (i.e. heterozygous and WT) were used for control experiments. 100–150 larvae were anesthetized with 0.02% tricaine (MS-222; Sigma), decapitated, and the tails digested for 1 hi in 200 units/ml collagenase type 1 in Hanks’ buffered salt saline (Sigma) at 28 °C in a thermomixer with continuous trituration. Collagenase digestion was stopped by adding 7 ml of zebrafish culture medium containing 60% L-15 medium (Sigma) with 3% fetal calf serum, 3% horse serum (both Invitrogen), and 4 mM L-glutamine (Sigma). After centrifugation for 5 min at 200 \(\times\) g cells were resuspended and transfected with 2 \(\mu\)g of expression plasmid cDNA using the AMAXA\(^{TM}\) rat neonatal cardiomyocyte nucleofector kit (AMAXA Biosystems, Köln, Germany) according to the manufacturer’s manual. Myocytes were resuspended in 200 \(\mu\)l of zebrafish medium supplemented with 4 units/ml penicillin/streptomycin (Invitrogen) (“full zebrafish medium”) and plated on carbon, gelatin, and collagen-coated glass coverslips (for immunocytochemical experiments) or as droplets in the center of collagen-coated plastic dishes (for electrophysiological experiments). After 20 min, 1.5 ml of full zebrafish medium was added and cells were cultured at 28 °C for 4 to 6 days.

**Immunocytochemistry**—Myotubes cultured on glass coverslips were washed in phosphate-buffered saline supplemented with 100 \(\mu\)M N-benzyl-p-toluene sulfonamide. Cells were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 20 min, permeabilized, and blocked by incubating with 5% normal goat serum in phosphate-buffered saline supplemented with 0.2% bovine serum albumin and 0.2% Triton X-100 (PBT) for 30 min, followed by incubation with primary antibodies in PBT overnight at 4 °C. Primary antibodies used were monoclonal antibody 1A against \(\alpha_{1S}\) (Affinity Bioreagents) at 1:2,000 (36, 37) and rb-anti-GFP (Invitrogen) at 1:5,000 dilutions. After several washes with PBT, secondary antibodies, goat anti-mouse Alexa Fluor 594, and goat anti-rabbit Alexa Fluor 488 (Invitrogen) at a concentration of 1:4,000 in PBT were applied for 1 h at room temperature. Specimens were mounted in 90% glycerol, 0.1 M Tris with 5 mg/ml p-phenylenediamine to retard photobleaching (38). Images were taken with a cooled CCD camera (Diagnostic Instruments) mounted on a Zeiss Axiopt microscope equipped with a \(\times63\), 1.4 NA objective lens, using MetaVue image-processing software (Universal Imaging, West Chester, PA). For quantification of \(\alpha_{1S}\) triad expression, images were acquired with identical exposure times, followed by background subtraction and shading correction. Transfected cells were identified by positive anti-GFP staining.

Quantification of \(\alpha_{1S}\) triad expression was determined by measuring the average fluorescence intensity of Alexa Fluor 595 along a line across a row of \(\alpha_{1S}\) clusters (triadic junctions; see Fig. 1A) in 5 measurements on each myotube, which were obtained from at least 2 different cultures. Myotubes that barely expressed GFP-\(\beta\) (and as a consequence also \(\alpha_{1S}\)) and were only visible because of signal amplification by anti-GFP/Alexa Fluor 488 staining were excluded from the \(\alpha_{1S}\) quantitation to allow a quantitative link to our patch-clamp data. To this aim we determined the percentage of expressing myotubes, either identified by direct GFP fluorescence (patch-clamp approach) or by GFP-antibody enhancement (immunocytochemical approach) from the total number of myotubes. Calculations were done from 2 different preparations for both approaches. Fractions of expressing cells were 7 ± 1%, \(n = 460\); and 21 ± 6%, \(n = 325\), for the patch-clamp and immunocytochemical approach, respectively. Thus, to enable a link between both approaches, only the values of the highest \(2/3\) of expressing myotubes were considered for \(\alpha_{1S}\) fluorescence quantification.

**Whole-cell Patch Clamp Analysis**—Immobilization-resistant intramembrane charge movement, as a measure of functional \(\alpha_{1S}\) expression (39), as well as intracellular Ca\(^{2+}\) transients were recorded from myotubes cultured for 4–6 days after transfection. GFP fluorescing myotubes were patch clamp analyzed on an Olympus IX70 inverted fluorescence microscope equipped with Hoffmann modulation contrast. Patch pipettes were pulled from borosilicate glass (Harvard Instruments), fire-polished (Microforge MF-830, Narishige), and had resistances of 3.5–5 MΩ after back-filling with pipette solution containing 100 mM Cs-aspartate, 10 mM HEPES, 0.5 mM CsEGTA, 3 mM MgATP, and 0.2 mM Fluo-4 (pH 7.4 with CsOH). The bath solution consisted of 10 mM Ca(OH)\(_2\), 100 mM l-aspartate, and 10 mM HEPES (pH 7.4 with tetraethylammonium hydroxide). Contractions of myotubes were blocked by adding 100 \(\mu\)M of the myosin-II blocker N-benzyl-p-toluene sulfonamide (Sigma) to the bath solution (40). Recordings were performed with an Axopatch 200B amplifier controlled by pClamp software (version 7.0; Axon Instruments Inc., Foster City, CA) and leak currents were subtracted by a P/4 prepulse protocol. To inactivate endogenous T-type currents all test pulses were preceded by a 1-s prepulse to \(-30\) mV (39). Recordings were low-pass Bessel-filtered at 1 kHz and sampled at 5 kHz. DHPR charge movement was measured in 20-ms depolarization test pulses starting from a test potential of +70 mV down to \(-60\) mV in 10-mV increments. Total charge movement was calculated by integrating the ON-component of gating currents. 0.2 mM Fluo-4 was added to the patch pipette solution to measure intracellular Ca\(^{2+}\) release. Fluo-4 fluorescence was recorded using a P1814 photomultiplier system (PTI, S. Brunswick, NJ). Average fluorescence intensity (\(F\)) of a rectangular region on the patched
myotube was recorded in 200-ms depolarizing test pulses from +80 to −50 mV in 10-mV increments with a holding potential of −80 mV. The average fluorescence was normalized to the resting fluorescence and expressed as ΔF/ΔF_{0}. The voltage dependence of charge movement (Q) and maximum intracellular Ca^{2+} release for each test potential were fitted according to the following Boltzmann distribution,

$$A = A_{\text{max}}/[1 + \exp \left(-\frac{(V - V_{1/2})}{k}\right)]$$

(Eq. 1)

where A is Q or ΔF/ΔF_{0}, V_{1/2} is the potential at which A = A_{max}/2, and k is a slope factor. Data were analyzed using ClampFit 9.0 and 10.0 (Axon Instruments) and SigmaPlot 9.0 and 10.0 (SPSS Science, Chicago, IL) software.

**Zygote Injection of In Vitro Synthesized RNA**—For in vitro RNA synthesis 50 μg of all β subunit cDNAs and GFP cDNA were linearized with restriction enzymes XbaI and NotI, respectively, purified with phenol/chloroform and precipitated with 3 M NH_{4}Ac in 70% EtOH and the pellet redissolved in RNase-free water. Linearized DNA templates were fidelity checked on a 7% formaldehyde-agarose gel in MOPS running buffer. RNA fidelity and concentration were checked on a 7% formate/urea gel in MOPS running buffer.

For RNA injection, eggs from heterozygous parental zebrafish in the one-cell stage were collected immediately after spawning and positioned in a 0.9-mm groove of an agarose tray to be microinjected within 20 min. Injection needles were pulled from heat-sterilized borosilicate glass capillaries (Harvard Instruments) and front-filled with RNA solution (0.2 μg/μl), containing 0.1% phenol red as an injection volume tracer (29). Injection volume of RNA solution was −1/5 of total zygote volume (calculated 13 nl) and was injected using a motorized micromanipulator DC3001 and the pneumatic PicoPump PV830 (both WPI, Germany). Eight hours after injection, GFP fluorescence of healthy embryos was quantified using a PTI 814 photomultiplier system. Only proper developing injected embryos with a mean fluorescence signal exceeding 40% above uninjectected control embryos were considered for freeze-fracture electron microscopy or digital motion analysis.

**Identification of Rescued Relaxed Larvae**—Discrimination of the 25% of motility restored homozygous relaxed larvae used in motion analysis experiments from the injected normal siblings was done by keeping all injected larvae separated and thus identifiable for up to 5 days and by observing a gradual fallback to the paralyzed phenotype due to degradation of the injected β-RNAs and translated proteins. Only in the case where larval tail muscle tissue was used for freeze-fracture electron microscopy, motility restored relaxed larvae had to be identified by a restriction fragment length polymorphism test on the larval heads. For this, larvae were anesthetized, decapitated, and the tails fixed as identifiable specimen as described below. Genomic DNA was extracted by incubating the larval heads in DNA extraction buffer containing: 10 mM Tris (pH 8.2), 10 mM EDTA, 200 mM NaCl, 0.5% SDS, and 200 μg/ml protease K for 1 h at 55 °C with intermittent vortexing, followed by ethanol precipitation. After washing in 70% ethanol, the pellet was redissolved in water (29) and the DNA was used as PCR template to amplify a 459-bp fragment containing the relaxed mutation. The relaxed genotype was identified by restriction enzyme digest of the PCR product with BsrI. The PCR product was cleaved into 279- and 180-bp fragments only in the presence of WT alleles.

**Freeze-fracture Electron Microscopy**—Immediately after decapitating, tails of the injected motile larvae at 27–30 hpf were fixed with 6% glutaraldehyde in 0.1 M cacodylate buffer at neutral pH (both Sigma) and incubated for 30 min at room temperature. Tails were mechanically skinned in 3% glutaraldehyde in 0.1 M cacodylate buffer and stored at 4 °C until processing for freeze-fracture. Tails from motility restored relaxed larvae were cryoprotected in 30% glycerol, mounted between two copper holders covered with a thin layer of 20% polyvinyl alcohol in 30% glycerol, and frozen in liquid nitrogen-cooled propane. Finally, tails were freeze fractured by separating the two holders under vacuum, shadowed with platinum at 45 °C, and replicated with carbon in a freeze-fracture unit (model BFA 400; Balzers SpA). Replicas were analyzed in an electron microscope (model 410; Philips) and searched for myotomes that contained myotubes at the developmental stage when peripheral couplings between the sarcoplasmic reticulum and the surface membrane were present. At the age used, these comprise a group of 3–4 myotomes in the middle of the tail. Sites of peripheral couplings were identified by the clusters of unique large particles representing the position of DHPRs.

**Digital Motion Analysis**—Motion analysis was performed on custom made computer programs using different image and data analysis platforms: Optimas 6.5, Image Pro 6, and LabView 8.5. For quantification of larval motility, larvae were decolorized (see above) and transferred to 24-well plates to keep individuals identifiable for several days. 2-Min video sequences with 25 frames/s were acquired with a Sony CCD AVC-D7CE b/w camera and stored as multisaped TIFF stacks. The TIFF stacks were converted into stacks of differential images by subtracting subsequent images (41). To eliminate pixel noise, all differential images were 3 × 3 median filtered. The mean luminance of every image reflecting larval movement was quantified by automated counting of total dynamic pixels per image and plotting against time. On this processed signal, peak detection was performed. Peak detection is based on an algorithm that fits a quadratic polynomial to sequential groups of data points. The number of data points used in the fit was specified by the width of typical peaks found in the acquired signals. For each peak, the quadratic fit was tested against the threshold level that in turn was determined for each individual larva separately and depended on small differences in illumination and larval orientation. Peak amplitudes below the threshold level (e.g. small peaks induced by passive movements of the larva) were ignored. Peaks were detected only after the procession of approximately...
DHPR $\beta_{1a}$ and EC Coupling

width/2 data points beyond the location of the peak (42). The cumulative dynamic pixels per peak were calculated and the mean value for all larval movements of each experimental group was determined.

Statistics—Statistical significance from experimental approaches was determined by unpaired Student’s t test and data are reported as mean ± S.E., unless noted otherwise.

RESULTS

A prerequisite to be able to use muscle cells and the entire larvae of the $\beta_1$-null zebrafish mutant *relaxed* as expression systems is the complete phenotype rescue with the homologous zebrafish $\beta_{1a}$. Therefore, we cloned $\beta_{1a}$ cDNA from WT zebrafish skeletal muscle (19) and expressed it in *relaxed* myotubes and larvae. To test if the zebrafish expression system permits species-independent conclusions we also used a mammalian (rabbit) skeletal muscle $\beta_{1a}$ subunit (43). These two $\beta_{1a}$ subunits, which share 76% all-over amino acid identity, were tested against two non-skeletal muscle isofoms to dissect specific functions of $\beta_{1a}$ from general $\beta$ functions. For this aim, we tested the cardiac/neuronal $\beta_{3a}$ subunit, as the phylogenetically closest isofom to $\beta_{1a}$ and a $\beta$ subunit that is phylogenetically basal to all four mammalian $\beta$ subunit isofoms (35), namely the neuronal $\beta_m$ from the housefly (*M. domestica*) (77 and 60% amino acid identity of the core region (35) to rabbit $\beta_{1a}$, respectively).

**Triad Targeting of $\alpha_{1S}$: A Common Feature of $\beta$ Subunits—** Expression and targeting of the heterologously expressed $\beta$ subunits and their influence on the targeting of the endogenous $\alpha_{1S}$ subunit was investigated with immunocytochemistry on transfected, primary cultures of *relaxed* myotubes. Normal and *relaxed* myotubes transfected with pure GFP showed a diffuse pattern upon anti-GFP staining and a punctuate pattern of foci aligned along transverse stripes with the sarcomeric spacing upon anti-$\alpha_{1S}$ staining (Fig. 1A, 1st and 2nd rows). As previously shown (19) the foci correspond to the location of triads. By quantifying $\alpha_{1S}$ immunofluorescence we could determine $\alpha_{1S}$ membrane expression in *relaxed* as 44 ± 2% (n = 90) compared with GFP mock-transfected normal myotubes (100 ± 1%, n = 359, p < 0.001) (Fig. 1B), and thus confirm previous results (19). *Relaxed* myotubes transfected with either GFP-tagged $zf-\beta_{1a}$ or $rb-\beta_{1a}$ showed co-clustering of $\beta_{1a}$, detected by anti-GFP antibody, with the endogenous $\alpha_{1S}$ (Fig. 1A, 3rd and 4th rows) in a pattern indistinguishable from normal myotubes (not shown, but see Ref. 19). Quantification of $\alpha_{1S}$ immunofluorescence revealed a complete rescue of $\alpha_{1S}$ triad expression comparable or slightly above normal myotubes or of *relaxed* myotubes transfected with $zf-\beta_{1a}$ (98 ± 3%; n = 17; p > 0.05) or $rb-\beta_{1a}$ (112 ± 2%; n = 47; p < 0.01) (Fig. 1B). To test whether this facilitation of $\alpha_{1S}$ triad targeting, as a muscle-specific targeting feature, is an exclusive function of the skeletal muscle $\beta_{1a}$, we also tested the cardiac/neuronal $\beta_{2a}$ and $\beta_{3a}$ subunits. Interestingly, both heterologous $\beta$ subunits correctly colocalized with $\alpha_{1S}$ in triadic clusters (Fig. 1A, 5th and 6th rows). Importantly, $\alpha_{1S}$ triad targeting was restored by both, $\beta_{2a}$ and $\beta_{3a}$ to levels (88 ± 11%, n = 16 and 97 ± 5%, n = 17, respectively; p > 0.05) comparable with normal myotubes (Fig. 1B).

**Facilitation of $\alpha_{1S}$ Charge Movement: A Common Feature of $\beta$ Subunits—** *Relaxed* myotubes showed almost complete lack of immobilization-resistant intramembrane ($\alpha_{1S}$) charge movement, despite the fact that $\alpha_{1S}$ immunofluorescence was still ~44% of normal myotubes (19) (Figs. 1B and 2). This diver-
intramembrane charge movements (Fig. 2A) with $Q_{\text{max}}$ values of $11.13 \pm 1.72 \text{ nC/\mu F}$, $n = 12$, for zf-$\beta_{1a}$ and $11.07 \pm 0.77 \text{ nC/\mu F}$, $n = 22$, for rb-$\beta_{1a}$ (Fig. 2B). Interestingly, $\beta_{2a}$ and the phylogenetically even more distant $\beta_{4a}$ were also able to recover $\alpha_{1S}$ intramembrane charge movements to a level comparable with that of the homologic $\beta_{1a}$ subunits ($9.94 \pm 2.06, n = 26$, for $\beta_{2a}$ and $11.09 \pm 1.04, n = 9$ for $\beta_{4a}$) (Fig. 2C). $Q_{\text{max}}$ values recorded from all expression experiments were somewhat higher than those recorded from normal myotubes mock-transfected with GFP (7.76 $\pm 0.48 \text{ nC/\mu F}$, $n = 31$), pointing to a moderate $\beta$ overexpression.

The voltage dependence of charge movement was similar to that of normal myotubes ($p > 0.05$) for all constructs (half-maximal activation in mV: normal + GFP, $-4.99 \pm 0.94$; zf-$\beta_{1a}$, $-3.97 \pm 2.19$; rb-$\beta_{1a}$, $-4.49 \pm 0.85$; $\beta_{2a}$, $-5.16 \pm 1.04$; $\beta_{4a}$, $-3.47 \pm 2.75$).

**Skeletal-type EC Coupling: A Specific Feature of $\beta_{1a}$**—After trial targeting and functional expression of $\alpha_{1S}$ were shown to be non-exclusive features of $\beta_{1a}$, we raised the question if this nonspecificity also holds true for the restoration of proper DHPR-RyR1 coupling. Unlike skeletal muscle of other vertebrates, zebrafish skeletal muscle shows no DHPR inward Ca$^{2+}$ current (19) and thus a possible contamination of the measurements of intracellular RyR1 Ca$^{2+}$ release by influx of extracellular Ca$^{2+}$ and thus a cardiac-type EC coupling component (44–46) is not present. Both skeletal muscle isoforms, zf-$\beta_{1a}$ and rb-$\beta_{1a}$, were able to restore intracellular Ca$^{2+}$ transients (Fig. 3A) with similar voltage dependence and with maximum $\Delta F/F_0$ values of $1.91 \pm 0.39$ for zf-$\beta_{1a}$ ($n = 9$) and $2.07 \pm 0.17$ for rb-$\beta_{1a}$ ($n = 23$), indistinguishable ($p > 0.05$) from normal transients with a maximal $\Delta F/F_0$ value of $1.87 \pm 0.2$ ($n = 30$) (Fig. 3B). However, in relaxed myotubes transfected with the non-skeletal muscle isoform $\beta_{2a}$, intracellular Ca$^{2+}$ transients were dramatically different in kinetic and voltage dependence (Fig. 3, A and C). First, contrary to normal or $\beta_{1a}$ expressing myotubes, $\beta_{2a}$ expressing myotubes were unable to maintain stable Ca$^{2+}$ release over the entire pulse duration of 200 ms (Fig. 3A). Following an initial upstroke of intracellular Ca$^{2+}$, the transient rapidly decayed. Second, the voltage dependence of transients was shifted toward more positive potentials with a half-maximal activation at $22.02 \pm 3.59$ mV compared with $-0.66 \pm 2.52$ mV in normal myotubes ($p < 0.001$). Third, the maximum intracellular Ca$^{2+}$ release had a $\Delta F/F_0$ value of $1.49 \pm 0.17$ ($n = 23$; $p = 0.18$) that is $80 \pm 9\%$ of normal myotubes (Fig. 3C). In the case of the ancestral $\beta_{M}$, the aberrant shape of Ca$^{2+}$ transients and the voltage shift (half-maximal activation: $31.41 \pm 2.52$ mV) were even more pronounced. Maximum $\Delta F/F_0$ reached only $0.93 \pm 0.16$ ($n = 6$; $p = 0.05$) that is $50 \pm 9\%$ of normal myotubes. These substantial differences in voltage-dependent intracellular Ca$^{2+}$ release point to a less efficient

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J. Schredelseker, M. Shrivastav, A. Dayal, and M. Grabner, manuscript in preparation.
DHPR β₁a and EC Coupling

FIGURE 4. Impaired tetrad formation with non-skeletal muscle β isoforms in relaxed myotubes. Freeze-fracture electron microscopy on tail muscle tissue from 30–32 hpf relaxed larvae, zygote-injected with in vitro synthesized RNA coding for different β isoforms, revealed assembly of DHPRs in triadic clusters, indicated by yellow ellipses. In control experiments on normal larval (upper row, left) DHPR particles were predominately found in tetrad-like groups of 3 or 4 (indicated by red dots), indistinguishable (p > 0.05) from relaxed larvae injected with zf-β₁a (upper row, right). No particles could be found between the tetrads. In contrast, arrangement of DHPRs in β₂a- (center row) or β₆ (bottom row) injected larvae was less organized. Arrangement of DHPR particles in tetrads was lacking in many of the DHPR clusters (β₁a and β₆, left images) or was very limited (right images).

DHPR-RyR1 coupling upon expression of the non-skeletal muscle β subunits compared with the β₁a subunits.

DHPR Tetrat Formation: A Specific Feature of β₁a—To assess whether the impaired functional DHPR-RyR1 coupling in relaxed larvae expressing non-skeletal β subunits could be explained by failure to restore the appropriate spatial DHPR-RyR1 association we performed freeze-fracture electron microscopy of tail myotomes from injected larvae. This guarantees that our studied β subunits are expressed in every muscle cell. In normal larvae and zf-β₁a-injected larvae the DHPRs were normally arranged into groups of four (tetrads, marked by a central red dot in Fig. 4, upper row) and the centers of tetrads in turn were disposed in an orthogonal array related to the array of underlying RyRs. Note that tetrads may be incomplete, i.e. they may lack one or more DHPR particles, but the majority of DHPRs were located in the appropriate position relative to the predicted centers of tetrad within an array. The percentages of total particles in a cluster that constitute complete or almost complete tetrads (3 or 4 clearly visible particles) are essentially the same (p > 0.05) in normal (68 ± 19%, mean ± S.D.; n, number of clusters = 38) and zf-β₁a-injected relaxed larvae (67 ± 26%, n = 17). The position of 88 ± 11 and 82 ± 12% of the particles pertained to orthogonal arrays related to those of RyRs. In contrast, in myotubes from β₂a- and β₆-injected relaxed larvae DHPRs were arranged in clusters (38, 47, 48), but a small and very variable portion of the particles formed tetrads (β₂a, 24 ± 23% n = 34; β₆, 28 ± 30% n = 21; p < 0.001 compared with normal) (red dots, Fig. 4, center and bottom rows). Although none of the particles in the β₂a-expressing tails showed any indication of organization into arrays, a small and variable portion of the β₆ particles (48 ± 35%) seemed to form limited arrays.

Full Restoration of Larval Motility: A Specific Feature of β₁a—Expression of the heterologous β subunits, β₂a and β₆, restored aberrant intracellular Ca²⁺ release with a pronounced right shift of its voltage dependence, and almost completely failed to rescue DHPR tetrad formation (Figs. 3 and 4). The next question addressed was, what are the effects, if any, of these in vitro observed changes on skeletal muscle function in an intact in vivo muscle expression system? Thus, we analyzed the motility of β subunit-injected relaxed larvae at 30–32 hpf. These larvae spontaneously and repeatedly twitch their tails by bending them in a tight arch, holding the bend position for a very brief period of time and then relaxing to the straight position. The movements involve the simultaneous activity of the myotomes on one side of the tail. For analysis of larval movements 2-min videos of single larvae were recorded and converted into sequences of differential images (Fig. 5A, see “Experimental Procedures”). The total number of dynamic pixels per frame, i.e. the pixels that showed a displacement relative to the previous image in the sequence, was plotted against time (Fig. 5, B and C). Single larval twitches display double-peaks representing larval muscle contraction and relaxation (Fig. 5B). The mean value of cumulative dynamic pixels per movement for each experimental group was calculated and standardized to that of normal larvae (100 ± 3%, n = 160). The movement extent of relaxed larvae injected with zf-β₁a or rb-β₁a was 94 ± 4 (n = 103) and 91 ± 3% (n = 37), and was indistinguishable (p > 0.05) from that of normal larvae (Fig. 5D). Relaxed larvae, injected with the cardiac β₂a subunit displayed movements with a comparable profile to that of normal or β₁a-injected relaxed larvae (data not shown), but movement extent was significantly reduced to 26 ± 2% (n = 65, p < 0.001) compared with normal larvae (Fig. 5, C and D). An even more severe failure to restore larval motility was found with the most heterologous β₆. This β subunit was completely unable to recover a motile phenotype in relaxed larvae (Fig. 5D).

DISCUSSION

In the present study we established the β₁-null mutant zebrafish relaxed as an expression system to investigate the specific role of the DHPR β₁a subunit for skeletal muscle-type EC coupling. We could show that both, the homologous zf-β₁a as well as the mammalian rb-β₁a were equally able to completely restore all parameters of skeletal muscle-type EC coupling in vitro and in vivo approaches, thus demonstrating a species inde-
pendence of the relaxed expression system. Exogenously expressed β1α subunits led to triad expression of the DHPR α1S subunit qualitatively and quantitatively indistinguishable from normal myotubes. Immobilization-resistant intramembrane charge movement of the α1S, as the first step in the EC coupling signaling pathway, was as well properly restored as it was for the downstream intracellular sarcoplasmic reticulum Ca2+ release. Thus, together with the correct targeting of the DHPRs into tetrads opposite to the RyR1, the structural and functional prerequisites were fulfilled to allow the complete transient restoration of motility in β1α-RNA-injected relaxed zebrafish larvae.

Expression of the phylogenetically nearest isoform to β1α, the cardiac/neuronal β2a subunit or the ancestral β subunit, βM, in relaxed myotubes and larvae, was likewise able to completely restore functional α1S membrane insertion and charge movement. However, myotubes expressing β2a and βM in contrast to β1α, revealed drastic impairments in intracellular Ca2+ release. Only a minor fraction of DHPRs were grouped into tetrads that are essential for direct EC coupling. Therefore, the very weak motility of β2a-expressing relaxed larvae, and the complete absence of motility in βM-expressing relaxed larvae did not come unexpectedly. Thus, the newly established zebrafish relaxed expression system allowed us to clearly differentiate between functions of β subunits that seem common to all of them (α1S triad targeting, charge movement restoration) and functions that are essentially β1α-specific, like supporting proper intracellular Ca2+ release and effective tetrad targeting.

Previous results from β1knock-out mice demonstrated a complete lack of intracellular Ca2+ release, strongly reduced DHPR currents, charge movements, and isradipine membrane binding (17) but the exact reason for the loss of EC coupling capability remained enigmatic. The β1-null zebrafish mutant relaxed system (19, 20) allows a higher differentiated view on isolated functions of β1α, as an elaborated set of appropriate methodological approaches is practicable with this model system. In contrast to mouse myotubes, zebrafish myotubes show a higher degree of differentiation in culture and thus allow quantification of α1S membrane expression in the absence of β1α or upon expression of different β subunits. Because quantification of the α1S protein expression in the mouse myotube-typical peripheral peripheral couplings was never performed, a clear differentiation between non-functional α1S expression and functional α1S expression in the membrane (charge movement) was not feasible. Thus, the β1α-induced facilitation of α1S charge movement was not detected in the mouse system and consequently experimental attempts on the ultrastructural level were not pushed forward (14, 17, 18). Due to the lack of these essential informations, the data of a large series of β-expression experiments (24, 25, 49–52) were in general interpreted in a way that domains of the DHPR β1α subunit, similar to elements present in the α1S subunit, might be directly involved in activation of RyR1 channels (26).

The β1α Subunit as a Signal Transducer in EC Coupling?—However, previous observations in studies with chimeric α1 subunits (53) do not support a model with β1α as a signal-transducing DHPR element, e.g. the α1S II-III loop (44, 54–58). Substitution of the α1S II-III loop by the heterologous II-III loop of a housefly (M. domestica) α1 subunit completely erased EC coupling in heterologous expression experiments in dysgenic (α1S-null) mouse myotubes (57, 58). Surprisingly, this II-III loop chimera (SkLM) was perfectly targeted into tetrads opposite the RyR1 (53) and fully restored charge movement (57). Thus, except the deletion of the II-III loop RyR1-interaction domain (critical domain) (57), chimera SkLM fulfilled all basic requirements for proper skeletal muscle-type EC coupling. To our judgment, if the β1α subunit has any intrinsic signal transducing function in EC coupling, this should have been revealed in the above experiments in which all other factors were optimal.

The β1α Subunit as a Scaffold for DHPR α1S Tetrad Targeting?—Now the question arises, if the β1α subunit could act as a scaffold to anchor the DHPR α1 subunits into tetrads opposite the RyR1? Again, earlier chimeric studies on α1 subunits (53) rather disagree with such a hypothesis. In experiments, where the cardiac α1c subunit was expressed in dysgenic mouse myotubes, the endogenous β1α subunit interacted with α1c and supported functional membrane expression (59), but it was not capable to promote DHPR tetrad formation (53). Thus, it seems rather obvious that in combination with the β1α subunit intracellular components of α1S are also essential for proper tetrad formation (53).
The $\beta_{1a}$ Subunit as an Additional RyR1 Binding Protein or as an Allosteric Modifier of $\alpha_{1S}$ Conformation?—To sum up previous and recent results, two alternative models for the role of the $\beta_{1a}$ subunit for tetrad formation and subsequent EC coupling are possible. (i) The $\beta_{1a}$ subunit might be understood as a RyR1-anchoring/binding protein acting in addition to the anchoring functions of the II-III loop of other $\alpha_{1S}$ regions, like e.g. the III-IV loop (60) or the C terminus (61). Consequently, the $\alpha_{1S}$ subunit alone, despite its active binding domains, cannot bind sufficiently firm to the RyR1. It needs additional $\beta_{1a}$ binding site(s) to complete RyR1 binding of the $\alpha_{1S}$-$\beta_{1a}$ couple and thus allow tetrad formation. This model would be in accordance with a previous study (62) that described a $\beta_{1a}$-binding domain on RyR1. Because in dysgenic myotubes no $\beta_{1a}$/RyR1 co-localization was observed (63) we have to assume also that $\beta_{1a}$ alone would not be able to target to RyR1. (ii) An attractive alternative model that would more completely subsume our recent data is that the $\alpha_{1S}$ subunit lacking $\beta_{1a}$ as a partner protein is in a state of massive conformational distortion. In this state $\alpha_{1S}$ is unable to bind to RyR1 with its anchoring sites sterically hindered to appropriately interact with RyR1 (Fig. 6A). With this model also the lack of charge movement and thus of any EC coupling signal generation can be well explained by a possible misfolding of the hydrophobic core region of the voltage sensor (symbolized by the tilted cylinders in Fig. 6A). The $\alpha_{1S}$-$\beta_{1a}$ interaction would lead to a conformational correction of $\alpha_{1S}$ subunit protein folding, which can now perform charge movement and also sterically orientate and therefore activate its binding domains to enable tetrad formation by accurate RyR1 anchoring. Resetting both functions would now allow proper skeletal muscle-type EC coupling (Fig. 6B). Hence, in this model $\beta_{1a}$ would function not primarily as an additional binding entity (though this has not to be excluded) but as an allosteric modifier to restore functional $\alpha_{1S}$ conformation.

Non-skeletal muscle $\beta$ isoforms in this model are able to endorse only a partial conformational restoration (Fig. 6C). These isoforms would fully reinstate the voltage-sensing hydrophobic $\alpha_{1S}$ core region because charge movement is completely restored (Fig. 2C), but the intracellular anchoring domains seem to undergo only a very limited conformational correction. This would lead to an only partial (fuzzy) targeting of the $\alpha_{1S}$-$\beta$ pair to the RyR1 without allowing accurate tetrad formation and thus only weak EC coupling interaction is possible (Figs. 3C, 4, 5D, and 6C).

For future studies it will be of high interest which molecular regions of $\beta_{1a}$ do promote this specific influence on the $\alpha_{1S}$ conformation and thus are responsible for proper skeletal muscle-type EC coupling? Previous loss-of-function expression studies in the $\beta_{1}$-null mouse system revealed no effect on intracellular Ca$^{2+}$ transients, upon deletion of the $\beta_{1a}$ hook region, but a drastic reduction when either the C or N terminus of $\beta_{1a}$ were deleted (49). However, follow up truncation studies were done solely on the C terminus and the gradual loss of EC coupling was interpreted that the C terminus of $\beta_{1a}$ is the critical determinant of skeletal muscle-type EC coupling (52). However, loss-of-function studies always raise concerns that the observed effects are solely due to a more general loss of function due to induced global protein misfolding. The ancestral $\beta_{M}$ subunit is able to perform all basic $\beta$-functions like triad targeting and charge movement restoration but not the specific $\beta_{1a}$ functions. Because $\beta_{M}$ lacks major parts of the variable C- and N-terminal, and hook regions, it will serve as a valuable tool for gain-of-function approaches using the elaborate set of methods practicable with the newly established zebrafish relaxed system. Thus the next aim will be to identify in detail the crucial regions of the $\beta_{1a}$ subunit responsible for correct $\alpha_{1S}$ subunit protein folding as it is required for proper skeletal muscle EC coupling.

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