Background: The 3D molecular structure of the L-type channel is poorly understood.

Results: The 3D locations of the alpha and beta subunits, and the II-III loop, are identified.

Conclusion: Key membrane targeting and signal transduction elements are placed in the context of the channel and cell membrane.

Significance: This work provides novel insight in the L-type channel structure-function relationships.

SUMMARY

The L-type Ca\textsuperscript{2+} channel (dihydropyridine receptor, DHPR) in skeletal muscle acts as the voltage sensor for excitation-contraction coupling. To better resolve the spatial organization of the DHPR subunits (\(\alpha_{1s}\), or Ca\textsubscript{v}1.1, \(\alpha_\beta\), \(\beta_1\alpha\), \(\delta_1\), and \(\gamma\)), we created transgenic mice expressing a recombinant \(\beta_1\alpha\) subunit with YFP and a biotin acceptor domain attached to its N- and C- termini, respectively. DHPR complexes were purified from skeletal muscle, negatively stained, imaged by electron microscopy, and subjected to single-particle image analysis. The resulting 19.1 Å resolution 3D reconstruction shows a main body of 17x11x8 nm with five corners along its perimeter. Two protrusions emerge from either face of the main body: the larger one attributed to the \(\alpha_2\delta_1\) subunit that forms a flexible hook-shaped feature, and a smaller protrusion on the opposite side that corresponds to the II-III loop of Ca\textsubscript{v}1.1 as revealed by antibody labeling. Novel features discernible in the electron density accommodate the atomic coordinates of a voltage gated sodium channel and of the \(\beta\) subunit in a single docking possibility that defines the \(\alpha_1\beta\) interaction. The \(\beta\) subunit appears more closely associated to the membrane than expected which may better account for both its role in localizing the \(\alpha_{1s}\) subunit to the membrane and its suggested role in excitation-contraction coupling.

Voltage-gated Ca\textsuperscript{2+} channels are key transducers in excitation-contraction (EC) coupling, secretion, regulation of gene expression, integration of synaptic input, and synaptic transmission (1). The skeletal muscle L-type Ca\textsuperscript{2+} channel (dihydropyridine receptor, DHPR) is a heteropentameric membrane protein complex formed by a Ca\textsuperscript{2+} channel pore forming subunit.
(CaV1.1) and four auxiliary subunits (α2-δ,β1a,γ). The DHPR senses depolarization in the sarcolemma and induces RyR1 to open and release Ca\(^{2+}\) stored in the sarcoplasmic reticulum (SR) into the cytoplasm for muscle contraction (2,3). Because in skeletal muscle the activation of RyR1 by the L-type Ca\(^{2+}\) channel is independent of extracellular Ca\(^{2+}\) entry, it is widely held that signaling between these two channels involves “conformational coupling” (reviewed in (4)). This conformational excitation-contraction (EC) coupling is mediated by a highly organized macromolecular complex whereby the large cytoplasmic domain of RyR1 acts as a hub for the assembly of up to four DHPR units (5).

DHPR’s α\(_{1S}\) subunit, or CaV1.1, is the largest subunit (176 kDa) mainly composed of a transmembrane domain that constitutes both the Ca\(^{2+}\) channel pore and the voltage sensor. It belongs to the voltage-gated ion channel superfamily, consisting of four repeats with six transmembrane helices each (S1-S6) (6,7), with each repeat connected to the next by a cytoplasmic loop. The I-II loop contains a highly conserved alpha interaction domain (AID domain) that has been proposed as the binding site of the β subunit (8). The largest cytosolic loop (the II-III loop, with 138 residues) has a major role in EC coupling (9-12). In solution the II-III loop displays an intrinsically disordered structure (13-15). The III-IV loop has also been suggested to interact with RyR1 (16,17) although without a direct role in EC coupling (18). The α2-δ subunit is the disulfide linked, cleavage product of a single gene (19): α2 (147 kDa), extracellular and highly glycosylated, and δ (24 kDa), which anchors α2 to the membrane through its single transmembrane domain. The β\(_{1a}\) subunit (56 kDa) is a soluble cytoplasmic protein unless bound to the AID of the CaV1.1 subunit. In addition to facilitating the trafficking of CaV1.1 to the plasma membrane, β\(_{1a}\) plays a key role in skeletal EC coupling (9,20-23). The structure of its core region has been solved to atomic resolution for several isoforms (24-26). The γ subunit (34 kDa) has four transmembrane domains with cytosolic C- and N-termini, and interacts with CaV1.1 (27) acting as a Ca\(^{2+}\) channel antagonist (28).

The structure of the DHPR complex has been mainly studied by transmission electron microscopy (TEM) and single particle analysis. While two initial 3D reconstructions differed in shape (29,30) a consensus structure consisting of a globular structure with an appendage is clearly emerging for L- and T- type channels (31,32), with the α2 subunit assigned to the large appendage (31,33) and the β subunit assigned to a region within the globular structure (31). Further discernment of subunits has been difficult due to inherent flexibility of the macromolecule, potential loss of subunits during the purification procedure, relatively small size for TEM, and presence of detergent surrounding the membrane-embedded region. In addition, isolation of a sufficient amount of material for single-particle image processing of this channel complex has been challenging.

Here we devised a comprehensive approach to simplify the purification of intact macromolecular complex by creating a transgenic mouse that was used to express a functional, recombinant DHPR complex with YFP and biotin bound to its β\(_{1a}\) subunit (34). Using negative stain, TEM and single particle analysis methodology we obtained a 19.1 Å 3D reconstruction of the recombinant DHPR complex. The 3D structure consists of a flat irregular main body with five pronounced corners, a flexible extension emerging from one of the faces, and a smaller protuberance extending from the opposite face, and its high definition enabled simultaneous docking of the atomic coordinates of a voltage-gated cation channel, a β subunit, and YFP. Decoration of the DHPR with an anti-II-III loop antibody allowed the mapping of this functional domain within the 3D structure. Our results significantly refine the structure of the DHPR complex and demonstrate the advantage of the use of animal transgenesis for the production and isolation of native heteromeric transmembrane protein complexes for structural analysis.

**EXPERIMENTAL PROCEDURES**

**Construction of the Recombinant cDNA** – The YFP-β\(_{1a}\)-BAD cDNA construct was made as previously reported (34). Targeted expression to skeletal muscle was achieved by cloning YFP-β\(_{1a}\)-BAD cDNA under control of the human skeletal actin (HSA) promoter (35). For this a 2882 bp fragment from pHSAvpA vector (gift from Dr. J. Chamberlain) containing 2409 bp of the HSA promoter, 293-bp of the 5’ end of HSA intron 1.
and 473bp of the splice acceptor sequence from SV40 (VP1 intron) was cloned upstream of the YFP-\(\beta_{1a}\)-BAD coding sequence.

**Transgenic Animals** – The complete 5648-bp YFP-\(\beta_{1a}\)-BAD expression construct, including a skeletal actin promoter and a SV40 polyA region, was excised and microinjected in fertilized mouse eggs (FVB/N) by the MGH transgenic facility, Charlestown, MA with approval of the Harvard University IACUC. Genotyping of transgenic mice was performed by PCR analysis of genomic tail DNA using the forward primer 5’-GAAGGGTTGCAAATATCATTTGTCGGG-3’, corresponding to bp 2624-2648 of the HSA promoter sequence and the reverse primer 5’-GTCGTGCTGCTTCATGTGGTCGGGG-3’, corresponding to bp 224-249 of the YFP cDNA sequence. Transgenic mice expressing HSA-YFP-\(\beta_{1a}\)-BAD were then crossed with heterozygous \(\beta_{1}\)-null mice (F1) and subsequently backcrossed (F2) to select for homozygous \(\beta_{1}\)-null animals expressing only the transgenic YFP-\(\beta_{1a}\)-BAD subunit.

**Gold-streptavidin Labeling and Confocal Microscopy** – FDB muscle fibers were isolated and bathed in an “internal solution” of (in mM): 140 Cs-Aspartate, 10 Cs2EGTA, 5 MgCl2, and 10 HEPES, pH 7.3 with CsOH. The cells were permeabilized by exposure for 30 s to saponin (Sigma; 12 mg/ml in internal solution). Following wash in internal solution, the cells were incubated in Alexa594-nanogold streptavidin (Invitrogen, 1:500 in internal solution) for 30 min, washed for 30 min with internal solution, and then viewed with a Zeiss LSM 510 Confocal microscope. YFP was visualized with 488 nm excitation and 505–530 nm band-pass emission; Alexa594 was visualized with 543 nm excitation and 560 nm long-pass emission. Images were acquired as the average of 4-8 line scans per pixel and digitized at 8 bits.

**DHPR Purification** – DHPR was purified from the skeletal muscle of mice expressing DHPR containing \(\beta_{1a}\) subunit with YFP and a BAD domain attached to its N- and C- termini respectively. Purification was performed under the presence of protease inhibitors at 4°C. Skeletal muscle was excised from the animal, homogenized, and whole membrane vesicles were isolated by differential centrifugation. The vesicle fraction (16-75 mg total protein) was solubilized with 1% (w/v) digitonin (Sigma) in equilibration buffer (50 mM Tris-HCl pH 7.4, 0.5 M NaCl, 1 mM iodoacetamide) and a protein/detergent (w/w) ratio of 5-7 for 40 min followed by centrifugation at 100,000 x g for 70 minutes (31,36,37). Glycosylated proteins, including the \(\alpha_{2}\)-\(\delta\)-1-containing DHPR complex, were then enriched using a WGA chromatography column. For this the supernatant was loaded to a wheat-germ agglutinin Sepharose affinity column (Sigma) (37), and the bound proteins were eluted with 0.5 M N-acetyl-glucosamine. Peak fractions were pooled and loaded onto a HiTrap streptavidin column (GE). Binding occurred by the affinity between the streptavidin resin and the metabolically biotinylated BAD domain of the \(\beta_{1a}\) subunit (34). Bound proteins were eluted with 5 mM D-desthiobiotin and peak fractions were concentrated ~10-fold with a centrifugal filter (MWCO 100 kDa).

**Antibody Labeling of the DHPR II-III Loop** – Western blots were performed with monoclonal antibodies against CaV1.1 (mA3-920 Thermo) (38), \(\alpha_{2}\) (abcam), \(\beta\) (Developmental Studies Hybridoma Bank, University of Iowa), and GFP (Clonetech). The secondary antibody (goat anti-mouse antibody, Pierce) was detected via chemiluminescence. For TEM, purified DHPR was incubated with a 10-fold molecular excess of mA3-920 antibody against the II-III loop of CaV1.1 for 12 hours at 4°C.

**Electron Microscopy** – Samples were imaged by negative stain TEM. The sample (5 µL) was applied to the carbon-coated side of a glow-discharged grid and let to adsorb for 70 seconds, the grid was washed twice with Milli Q water and then negatively stained with 0.75% uranyl formate as previously described (39). Images were collected on a FEI Tecnai T12 TEM operated at 120kV and a magnification of 67,000× at 0°, 45°, or 60° tilt angles under low-dose conditions (< 20 e-/Å²). Images were recorded on image plates. The antibody-labeled DHPR specimen was imaged on a Phillips CM10 TEM at a magnification 39,000× under low-dose conditions and images were recorded on a 1k x 1 k Gatan CCD camera.

**Image Processing** – Particles were selected from the micrographs using the software Boxer (40). A total of 11,929 particles were collected for
the DHPR dataset and a total of 625 particles for the antibody-labeled DHPR. 2D and 3D image processing was performed using the SPIDER software suite (41) for the unlabeled and antibody labeled datasets. Images were binned down to a final pixel size 4.48 Å and particles were windowed with final window sizes of 130² pixels for the DHPR complex and 125² pixels for the DHPR-antibody dataset. The windowed particles underwent alignment, classification and multi-reference alignment to produce class averages (42). 3D reconstruction of the DHPR complex was produced by projection matching using a 3D reference reconstruction (31) filtered to 40 Å resolution, followed by 3D refinement. Particles with low cross correlation were discarded, with 2,693 particles in the final 3D reconstruction. Resolution was calculated according to the Fourier shell correlation curve (FSC) between two half datasets, providing a value of 19.1 Å at the 0.5 cutoff criterion. The 3D reconstruction was filtered to 19.1 Å using a Gaussian low-pass filter. Image rendering was performed with Chimera (43).

RESULTS

Expression of a Recombinant β₁a Subunit in a β₁a null Mouse – We constructed a transgenic mouse expressing a recombinant β₁a subunit under control of the HSA promoter for specific expression in skeletal muscle (44). The recombinant β₁a subunit was engineered as a fusion protein (YFP-β-BAD) with YFP fused to the N-terminus and a biotin acceptor domain (BAD) fused to the C-terminus. Transgenic mice overexpressing the recombinant β₁a subunit were crossed with heterozygous β₁a null mice and bred to homozygosity for both the transgene and the β₁a null allele. Whereas homozygous β₁a null mice have a birth-lethal phenotype (22), the mice homozygous both for the β₁a null allele and for the YFP-β-BAD transgene were viable and appeared healthy, indicating that DHPR complexes containing the recombinant β₁a subunit were functional for EC coupling.

Figure 1 illustrates the subcellular distribution of the YFP-β-BAD transgene in a flexor digitorum brevis (FDB) fiber that had been permeabilized with saponin, and then exposed to 1 nm Fluoro-nanogold streptavidin. The yellow fluorescence arising from the YFP tag was arrayed in double rows having a center-to-center spacing of ~2 μm, consistent with a T-tubular localization. Furthermore, the red fluorescence arising from the bound Fluoro-gold streptavidin co-localized with the yellow fluorescence, indicating both that there is effective biotinylation of the BAD attached to the β₁a C-terminus and that its disposition in triad junctions makes it accessible to a moderate sized (1 nm) probe, consistent with previous work on myotubes (34,45,46). This previous work also showed that EC coupling was not impaired when streptavidin bound to YFP-β-BAD in myotubes (45).

Protein Purification – As expected for a muscle specific promoter, the YFP-β-BAD construct was expressed primarily in skeletal muscle, although a modest expression was also observed in liver and heart (Fig. 2A). The DHPR was purified from skeletal muscle as indicated in the Experimental Procedures section. Silver staining of SDS gels indicated the purity of the preparation and the presence of the larger subunits identified on the basis of their mass (Fig. 2B); the identity of the α₁, α₂, β₁a subunits and of YFP was further confirmed by Western Blot analysis (Fig. 2C). Note that the MW of β₁a subunit was higher than that of wt β₁a (96 kDa instead of 56 kDa) due to the presence of the YPF and BAD moieties. Altogether these results indicate that our methodology provides highly pure DHPR complexes from a relatively small amount of starting material (between 10 and 20 grams).

3D Reconstruction of the DHPR – TEM images of negatively stained DHPRs show individual particles of homogeneous size with a main body and a bulky appendage attached to it (Fig. 3A-B). In some views a smaller protuberance is visible on the opposite side. A total of 11,929 individual DHPRs was analyzed using single-particle image processing (47). Two-dimensional alignment and classification (42) produced stable 2D class averages with defined features and orientational information (Fig. 3C). At least seven of our twelve 2D averages (1, 5, 7, 9-12) strongly resemble most of the DHPR 2D averages published previously (31) and averages 8 and 14, formed by a thick line and a small appendage, strongly resemble one of three 2D averages published by another group (Fig. 4b of (33)). For
3D reconstruction we used a reference-based approach. Given that all the 2D averages presented in Wolf et al. (2003) (31) were represented in our dataset, we performed a reference alignment using their 23 Å resolution 3D reconstruction filtered to 40 Å resolution as a starting reference. This produced a first 3D reconstruction with novel prominent features such as pronounced corners (two of them square corners), a flatter shape of the main globular domain, and an irregular pentagon shape of 17x11x8 nm. Further refinement of the 3D reconstruction yielded a resolution of 19.1 Å according to the FSC criterion with a cutoff of 0.5 (Fig. 3D), indicating a net increase in detail from 40 Å and that our dataset had genuine signal that was absent in the reference. The angular distribution plot (Fig. 3E) indicates a full coverage of the angular range and consequently, the absence of the missing cone artifact (48).

The refined 3D reconstruction reveals that the main body is formed by two distinct regions, one forming a square and one forming a triangle, separated by a mass deficiency between them (Fig. 4A). The long appendage attaches to one edge of the square portion (Fig. 4A). The surface contour level was adjusted to the mid-point between the background density level and the highest density level at which the volume presents no discontinuities. Under these conditions the surface encloses a mass that is 23% larger than the expected molecular weight (49), nevertheless it is important to point out that in addition to the protein moiety there is also detergent present. While change of contour level between these limits had little effect on the appearance of the main body, the hook-shaped feature changed in shape, which is an indicator of flexibility. At the contour level used the hook shaped structure forms an L shape with a thickness between 2 and 4 nm that extends 7 nm from the main body.

Antibody Labeling of the II-III Loop – An antibody specific for the II-III loop (38) recognizes the CaV1.1 subunit of the DHPR complex by Western blot (Fig. 2C). Negative staining TEM of the DHPR complex previously incubated with the anti II-III loop antibody shows DHPR particles with antibody bound (Fig. 5A). The raw images of the DHPR were matched to the more similar 2D projection of the DHPR 3D reconstruction, which yielded the orientation of the DHPR with respect to its support and thus the localization of the epitope in the 3D model of the DHPR (Fig. 5B-C). All individual determinations converge on the small protrusion of the main body, indicating that this protrusion corresponds to the II-III loop.

3D Location and Docking of the YFP Atomic Structure – By employing affinity chromatography using the biotinylated BAD tag (see Experimental Procedures), our purification protocol ensured that all purified DHPR complexes used for analysis contained the recombinant YFP-β1a-BAD subunit. The presence of this YFP can account for an extra mass that was present in the 3D reconstruction of the recombinant DHPR but not in the 3D reconstruction of wt DHPR (31) (Fig. 4C). This protrusion situated along the main globular part of the complex presents a size and shape compatible with a YFP molecule (a beta barrel 45 Å long and 25 Å in diameter), and enabled a straightforward docking of the YFP atomic structure (Fig. 6). YFP can be seen as a peripheral addition to the DHPR macromolecule with a thickness compatible with the diameter of YFP. Therefore, at the current resolution it appears that the YFP tag is accommodated along the β1a subunit without noticeable changes in its native orientation. Such a location is probably the most benign in the context of the interaction between DHPR and RyR1 in the triad junction, and is consistent with the functional viability of the YFP recombinant β1a subunit.

Docking of the Atomic Structure of a Voltage Gated Ion Channel – Consistent with the considerable conservation of membrane topology among voltage-gated channels, with four subunits or repeats, each with six transmembrane domains (7), the main body of our 3D reconstruction can be closely fitted with existing atomic structures of voltage-gated cation channels such as the full-length potassium channel Kv1.2 (50) or the bacterial Na+ channel, NaVAb (51), by placing the channel with the selectivity filter towards the extracellular side and by aligning two of the corners of the channel to two square corners of the DHPR 3D structure. Because the fitting of these two atomic structures is equivalent at our resolution, we only present the fitting with the Na+ channel (Fig. 6).

3D Location of the β Subunit – CaV β subunits have hyper-variable N- and C-termini, which flank a core comprised of SH3, HOOK and GK domains
DISCUSSION

Use of a Transgenic Animal to Obtain Recombinant Membrane Proteins for Structural Determination – The DHPR presents a formidable challenge for its structural determination. On one hand, being a membrane protein it requires the use of detergent and a more complex purification process than soluble proteins. In addition it has a hetero-oligomeric composition, and the presence of detergent throughout the purification could promote subunit dissociation. The use of animal transgenesis as a method to produce recombinant proteins made it possible to introduce several features that facilitated the protein purification process and increased the quantity, integrity, and preservation of this hetero-oligomeric membrane protein complex. First, the introduction of metabolic biotinylation as a purification tag (56) enabled a straightforward 1-day purification from a relatively small quantity of starting material and the use of two consecutive affinity chromatographies ensured the presence of two key subunits in the complex. The shorter and faster purification protocol very likely promoted a better preservation of the macromolecule. Second, it was possible to insert a decoration tag (YFP) in one subunit for its recognition in the 3D reconstruction. Third, the use of an animal organ (skeletal muscle) where all of the subunits are normally expressed made it possible to obtain sufficient material for TEM. This rapid method of purification contributed to a 3D reconstruction of the DHPR that has more definition, enabling the docking of relevant atomic structures into the main body of the structure.

Novel Features in the 3D Reconstruction of the skeletal muscle L-type Channel Complex – The general outline of DHPR complex with a main body and a large appendage revealed by our studies is compatible with some earlier DHPR 3D reconstructions (31-33) but not with others (29,30). Below we summarize and interpret the new features revealed in our 3D reconstruction for each subunit.

Cav1.1 Subunit – Our studies localize Cav1.1 to the 11x11x8 nm square prism in the main body, with the voltage sensing modules (S1-S4) aligned with two outer corners of our 3D reconstruction, and Cav1.1’s II-III loop to the protuberance on the opposite side of the appendage. Because the II-III loop is downstream of S6 and thus adjacent to the...
intracellular helical bundle formed by the four S6 helices, our antibody labeling provides an additional unambiguous assignment of the orientation of the 3D reconstruction in the cell membrane, with the selectivity filter oriented towards the extracellular space as defined by the irregular appendage (Fig. 6), and the S6 helical bundle towards the intracellular space. The four CaV1.1 repeats are positioned clockwise as seen from the extracellular side (57). Finally, the assignment of the II-III loop to the cytosolic protuberance is consistent with the demonstrably important role of the II-III loop in EC coupling (11,16,58-64), and would allow the loop to extend towards the RyR1 in triad junctions of skeletal muscle.

**α2-δ1 Subunit** – Previous studies indicate that α2, on the extracellular side of the channel, corresponds to the hook-shaped structure (31). Our localization of the intracellular II-III loop on the opposite face of the main body further substantiates this assignment. We find that changes in the threshold for surface representation affect the shape of α2 more dramatically than that of the rest of the 3D structure, suggesting not only that α2 is a flexible domain, but also that its size is larger than what is represented by our 3D reconstruction. Indeed, while α2 represents 30% of DHPR’s mass, the hook-shaped structure represents only 13% of the mass of the DHPR 3D reconstruction. The δ1 subunit, disulfide-bonded to α2 (19), must cross the membrane near the point of attachment of the hook-shaped structure.

**β1α Subunit** – The β1α subunit has a known role in trafficking the α1β subunit to the membrane, is intracellular, and is important in EC coupling (9,20). The core domain (8) has been crystallized for several beta subunit isoforms resulting in very similar atomic maps, which strongly suggests that this domain has a conserved and rigid structure. The core domain has also been co-crystallized bound to AID, its alpha helical target in Cav1.1, 22 residues after the inner helix (S6) of repeat I (8,25). Since these intervening residues have a predicted alpha helix structure, the prevalent model has a physical separation between Cav1.1 and β1α (~3nm) situating the β subunit below Cav1.1 (24,25). However our 3D reconstruction is incompatible with this model. First, our insertion of YFP, which along with GFP constitutes a commonly used method for 3D sequence localization (65,66), localizes the β subunit beside Ca1.1. Furthermore, the core of the β subunit fits very well when it is docked adjacent to Ca1.1 (Fig. 6), which is compatible with an earlier TEM antibody labeling study with intact DHPR (31) (Fig. 4B-C) and with a DHPR α1-β subcomplex (33). The adjacent positioning keeps the β subunit close to the cytoplasmic domain of RyR1, consistent with its direct role in skeletal muscle EC coupling (9,20-23). However in this adjacent position the AID is situated 5 nm away from the center of the pore of Cav1.1, which is difficult to conceive if the intervening peptide between S6 and the AID forms an alpha helix. This position is possible if the peptide is flexible at least in some section, or if in vivo, the interaction with the closely adjoining RyR1 could reorient Cav1.1’s cytoplasmic loops. It is also possible that with the membrane context being lost after its solubilization with detergent, the β1α subunit could relocate slightly to a position that was unavailable in vivo although the clarity of the β subunit boundaries, characteristic of a non mobile region, argue against this point. In this localization the β subunit appears partially embedded in the membrane, a localization that was also implicit in a previous electron microscopy study (31), which is something unexpected given the cytosolic location suggested for β1α (21,24,67-69). Given the recognizable Cav1.1 subunit, α2 subunit, and II-III loop in our 3D reconstruction, we have rotated the main body of the structure by 10° with respect to that previous 3D reconstruction, which we believe provides a more reasonable orientation and results in a more cytosolic β subunit (see Figs. 4 and 6). While this represents a new interesting possibility, further testing with the full DHPR in the intact membrane is necessary to ascertain the exact location of the beta subunit in vivo.

**γ Subunit** – The γ subunit has four transmembrane domains with intracellular C- and N-termini, and interacts both with CaV1.1 (27) and the β subunit (70). After accommodating the atomic structure of a voltage-gated cation channel, the YFP and the core of the β subunit, there remains extra volume in the interphase of the Cav1.1/β subunits that could accommodate the γ subunit (asterisk in Fig. 6 top right panel).
In conclusion, to better ascertain the molecular organization of DHPR we have expressed a DHPR with a recombinant $\beta_{1a}$ subunit using a transgenic animal both for an improved purification strategy as well as to provide a 3D decoration tag. Using TEM and 3D image reconstruction we have obtained a 19.1 Å resolution 3D map of the full DHPR complex, which represents the highest resolution obtained for this complex to date. This reconstruction shows that the six transmembrane voltage-gated ion channel outline is recognizable in the full DHPR complex and antibody labeling shows that the protuberance on the cytosolic side of Ca$\text{v}$1.1 corresponds to the II-III loop. The atomic coordinates of the $\beta$ subunit core structure can be docked in a location that indicates a closer association to the membrane than previously reported, thus defining a new relative positioning of the Ca$\text{v}$1.1-$\beta$ subunits.

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3D organization of the dihydropyridine receptor


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FOOTNOTES

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The abbreviations used are: AID, alpha interaction domain of the beta subunit of the L-type channel; BAD, biotin acceptor domain; cryoEM, cryo electron microscopy; DHPR, dihydropyridine receptor; EC coupling, excitation-contraction coupling; FDB, flexor digitorum brevis; GK, guanylate kinase; HSA, human skeletal actin promoter; TEM, transmission electron microscopy.

FIGURE LEGENDS

FIGURE 1. Confocal microscopy of skeletal muscle tissue expressing a transgenic DHPR β1a subunit, YFP-β-BAD. A. YFP fluorescence displaying a T-tubular localization. B. Fluoro-nanogold streptavidin fluorescence of the same region. C. The overlay of the YFP and fluoro-nanogold fluorescence images indicates full co-localization of the two fluorophores and the binding of streptavidin demonstrates that the BAD protein is biotinylated. Scale bar, 2 µm.

FIGURE 2. Biochemical characterization of the purified DHPR complex. A. Western blot using anti-GFP antibody showing the expression of YFP-β1a-BAD in different tissues. B. SDS-PAGE silver stained gel (10% separating gel under reducing conditions) confirms the presence of the high molecular weight components of the purified DHPR complex: α1 (176 kDa), α2 (147 kDa), and YFP-β1a-BAD (96 kDa) subunits. C. Western blots of the skeletal muscle vesicle fraction incubated with antibodies against α1s-II-III loop, α2, β, and YFP. Both the anti-β and anti-GFP antibodies label the same band corresponding to the YFP-β1a-BAD construct, with a MW of 96 kDa.

FIGURE 3. TEM and image processing of the purified DHPR complexes. A. Raw electron micrograph displaying a homogeneous distribution of L-type channels; the more representative images are highlighted with circles. B. Windowed DHPR particles showing a main body with an appendage. C. Gallery of the 2D averages of DHPR after several rounds of classification and multi-reference alignment. The main body (m), hook- or leg- shaped feature (l), and the small protuberance (p) are indicated in averages 1 and 8. Scale bars for A-C, 50 nm. D. Fourier shell correlation curve indicating a resolution of 19.10 Å according to the 0.5 cutoff criterion. E. Plot of the angular distribution of the particles used in the final 3D reconstruction, showing a uniform distribution of Euler angular orientations of the DHPR. The size of the small circles represents the number of particles in that orientation and the center of the circular plot corresponds to a theta angle of 0°.

FIGURE 4. New 3D reconstruction of the DHPR complex and comparison with a previous 3D reconstruction and β1a subunit localization. A. 3D reconstruction of the recombinant DHPR in different orientations. B. 3D reconstruction of DHPR with the region labeled by antibodies against the β1a subunit.
colored in magenta from (31). B. Superimposition of both 3D reconstructions, (A) in semitransparent orange and (B) in blue mesh. An extra mass of the recombinant (YFP-β1α-BAD)DHPR, presumably the β1α-adjoining YFP, emerges from the location labeled by anti-β1α antibodies in the previous study. The blue mesh DHPR reconstruction has been rotated 10° with respect to the plasma membrane to match the new DHPR reconstruction in the new proposed orientation. Left panels show a side view (perpendicular to the plane of the membrane). Scale bar, 10 nm.

**FIGURE 5. Electron microscopy of the purified DHPR complex bound to anti II-III loop antibody.** A. Raw images of L-type channel labeled with anti-α1s-II-III loop antibody. B. Schematic representation of the antibody (blue) labeling the DHPR complex (white) in the orientation found in row A. C. 3D reconstruction of the DHPR in the corresponding orientation. The blue sphere represents the location found for the antibody. Scale bar, 10 nm.

**FIGURE 6. Interpretation of the 3D reconstruction of DHPR.** The dashed black line indicates the approximate boundary of the plasma membrane. The region corresponding to the II-III loop is indicated in blue shade. The atomic structures of a Na+ channel (dark green), the β subunit (SH3 in light blue and GK in purple) bound to the AID (light green), and YFP (yellow) are docked into the DHPR structure. The black dots are two features of the density map that match with the atomic structure of the β subunit and the asterisk shows an empty density that could allocate the N-, C-terminal and HOOK regions of the β subunit and the γ subunit. YFP has been omitted in the bottom panels for clarity. Scale bar, 10 nm.
Figure 1
Figure 2

A

Muscle Muscle Heart Diaphragm Brain Liver

Wt YFP-β-BAD transgenic

B

250- 150- 100- 50-

α₁S α₂ YFP-βRAD

C

α₁S α₂ β YFP

250- 150- 100- 75-
Figure 3

A

B

C

D

E

3D organization of the dihydropyridine receptor

Figure 3
Figure 4
Figure 5
Figure 6
3D Localization of the Alpha and Beta Subunits and of the II-III loop in the Skeletal Muscle L-type Ca2+ Channel
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