The molecular architecture of dihydropyridine receptor/L-type Ca\(^{2+}\) channel complex

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Dihydropyridine receptor (DHPR), an L-type Ca\(^{2+}\) channel complex, plays an essential role in muscle contraction, secretion, integration of synaptic input in neurons and synaptic transmission. The molecular architecture of DHPR complex remains elusive. Here we present a 15-Å resolution cryo-electron microscopy structure of the skeletal DHPR/L-type Ca\(^{2+}\) channel complex. The DHPR has an asymmetrical main body joined by a hook-like extension. The main body is composed of a “trapezoid” and a “tetrahedroid”. Homologous crystal structure docking and site-specific antibody labelling revealed that the \(a1\) and \(a2\) subunits are located in the “trapezoid” and the \(\beta\) subunit is located in the “tetrahedroid”. This structure revealed the molecular architecture of a eukaryotic Ca\(^{2+}\) channel complex. Furthermore, this structure provides structural insights into the key elements of DHPR involved in physical coupling with the RyR/Ca\(^{2+}\) release channel and shed light onto the mechanism of excitation-contraction coupling.
Results and Discussion

Improvement of DHPR sample for cryo-EM. Due to the relatively smaller size (450 kDa) and lack of any asymmetry, it would be difficult to obtain high-resolution structure by cryo-EM and single particle analysis if the sample is inhomogeneous. One of the possible reasons that previous structural studies did not obtain a high-resolution structure of DHPR is due to sample heterogeneity. To overcome this difficulty, we first improved DHPR purification procedure (see Methods) and purified DHPR using this new method. The protein purified by our new procedure consists of 5 bands with molecular weights 176-, 147-, 56-, 34- and 24-kDa, respectively, as identified by SDS-PAGE in reducing conditions, which correspond to the α1, α2, β, γ and δ subunits, respectively (Fig. 1a, left panel). In non-reducing conditions, however, the α2 and δ bands vanished; instead, a band slightly above α1 with an apparent molecular weight ~180-kDa appeared which corresponds to the α2/δ complex connected by a disulphide bond. The identities of subunits were confirmed by Western blotting analysis (Fig. 1a, right panel). The chemical purity of the protein sample can be confirmed by the cleanness of the SDS-PAGE gel— apart from the five bands belonging to the DHPR complex, there are no other bands apparently visible. Western blot identified the band below α2 being part of α1 (Fig. 1a, right panel, lane 1), presumably resulted from endogenous protease cleavage. Native PAGE gel of the purified protein sample showed only a single band with an apparent molecular weight ~450-kDa (Fig. 1a, left panel, lane 4), indicating the structural integrity of DHPR complex— all the subunits are within a multiple-subunit complex, including the peptide below α2. The chemical purity of the protein sample is further confirmed by this native PAGE gel— apart from the 450-kDa complex band, there are no other bands apparently visible. Negative-staining electron microscopy revealed that the purified DHPR sample contains uniform particles, indicating the DHPR particles are physically homogeneous (Fig. 1b). The chemically pure and physically homogeneous DHPR sample paved the way for high-resolution structural analysis by cryo-EM and single particle analysis.

The ion-conduction channel. Several groups have performed structural studies of DHPR14–18, and their results have varied; the proposed membrane topology of DHPR is controversial, and the ion-conduction channel in these studies is not resolved due to the low resolution achieved (>20 Å). Using our new purification procedure, we obtained chemically pure and physically homogeneous DHPR sample (Fig. 1). Cryo-EM images of DHPR particles were collected (Fig. 1c) and a total of 25,300 individual DHPR particle images were analysed using single-particle image processing software EMAN1.8 (Ref. 15), enabling us to break the 20-Å resolution barrier and obtain a structure at 15-Å according to the gold-standard criterion (Supplementary Fig. 1). The final three-dimensional (3D)-EM map is shown in Figure 2a. The map has an asymmetrical main body with dimensions of 90Å × 88Å × 125Å and a hook-like extension. The main body is composed of a “trapezoid” and a “tetrahedroid”, with the “tetrahedroid” attached to the bottom of the “trapezoid”; at the tip region of the “tetrahedroid” there are two “legs”. The map is asymmetric. In one particular side view, it looks like a “diamond” (Fig. 2a, left panel); when viewed from the top and the bottom, it has a ~88 × 88Å rectangular-shaped main body with a hook-like extension attached to one of the corners of the rectangle. In the centre of the rectangle, the top and bottom have a depression with a diameter of ~10 Å and ~14 Å (at the threshold of 1.8, indicated by red arrows) respectively (Fig. 2a, middle and right panels).

The presence of depressions on both sides of the rectangular-shaped main body of DHPR hints that they could be the openings of the ion-conduction channel. To see if this is the case, we analysed the 3D-EM map by sectioning the map along the axis connecting the centres of the top and bottom depressions. We first cut the map in vertical direction (Figure 2b, upper panel). As can be seen from the cut-through view, a channel with characteristics of a Ca2+/Na+ channel appears14: it has a funnel-shaped opening at the top, then narrows down to a neck with the smallest diameter (~6 Å) of the channel, presumably corresponding to the ion selectivity filter; after that, it runs into a vestibule (central cavity), gradually narrowing down to a narrow opening (~10 Å), presumably corresponding to the gate of the channel. To see these characteristics in detail, we then sectioned the map in horizontal direction using a 2 pixel step (Figure 2b, lower panel). The data show that a central hole runs through all the slices of the “trapezoid” part in the main body, forming a channel. The channel diameter gradually increases from its narrowest point, ~6 Å, in the 5th–6th slices, which presumably corresponds to the selectivity filter, to its widest point in the 12th–13th slice, and then it narrows back down to ~10 Å in the 18th slice, which presumably corresponds to the gate. After that, it runs out of the map. The length of this channel, from the putative selectivity filter to the gate, is ~3.7 nm, long enough to extend across a lipid bilayer. Also, the channel is surrounded by densities displaying pseudo-4-fold symmetry, which is consistent with the model that Ca2+ channels are composed of 4 homologous domains surrounding a central channel2. We therefore conclude that this channel we
observed corresponds to the ion-conduction channel of the DHPR/L-type Ca$^{2+}$ channel complex.

The membrane topology. To confirm that the channel we revealed is indeed the actual ion-conduction channel of DHPR, we then determined the membrane topology of the DHPR complex by subunit-specific antibody labelling followed by key subunit localization using cryo-EM and single particle analysis. Previous studies have tried to determine the membrane topology of DHPR and the locations of subunits by antibody and/or YFP-fusion protein or ConA labelling. In these studies, negative staining electron microscopy instead of cryo-EM, and in most cases, only projection images were used, this could potentially produce artefacts and lead to fault interpretation. To unambiguously determine the locations of subunits and membrane topology, we used cryo-EM and 3D reconstruction. Two monoclonal antibodies were chosen for our purpose—the first, against the $\alpha$2 subunit (anti-$\alpha$2) and the second, against the $\beta$ subunit (anti-$\beta$) of DHPR. We incubated antibodies with DHPR in a molar ratio of 10:1 to ensure that all DHPRs would form DHPR-antibody complexes and then collected cryo-EM images. These images were analysed using EMAN1.8, and the final 3D-EM maps are presented in Figure 3a. As shown in this figure, the antibody against the $\alpha$2 subunit is located on top of the "trapezoid" in the main body, while the antibody against the $\beta$ subunit is located at the tip region of the "tetrahedroid" in the main body. Previous studies have established that the $\alpha$2 subunit of DHPR is located on the extracellular side, and the $\beta$ subunit of DHPR is located on the cytoplasmic side. This enabled us to determine the membrane topology of the DHPR complex as follows: the top of the "trapezoid" in the main body faces the extracellular side, and the tip region of the "tetrahedroid" in the main body faces the cytoplasmic side. This membrane topology is consistent with the direction and orientation of the ion-conduction channel we showed and demonstrates that this is indeed the actual ion-conduction channel of the DHPR/L-type Ca$^{2+}$ channel.

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channel of DHPR enabled us to dock homologous crystal structures of recently solved constructed bacterial Ca\(^{2+}\) channel, Ca\(_{Ab}\) (a homologue of the \(\alpha_1\) subunit of DHPR), and the \(\beta\) subunit of the cardiac DHPR \(\beta_{2a}\) (a homologue of skeletal \(\beta\) subunit) complex with an AID segment of \(\alpha_1\) (Ref. 6) into our 3D cryo-EM map (Fig. 3b). We first docked the crystal structure of \(\beta_{2a}\)-AID (PDB-ID 1T0J) into the DHPR cryo-EM map. The crystal structure of the \(\beta_{2a}\) subunit has a tetrahedroid shape, which fits well into the “tetrahedroid” region of the DHPR cryo-EM map (Fig. 3b, middle panel). We then, using the AID as a guide, docked the crystal structure of Ca\(_{Ab}\) (PDB-ID 4MS2) (Ref. 1) into the DHPR cryo-EM map, making use of the fact that the AID segment is an extension of the 6th transmembrane helix in repeat I of the DHPR \(\alpha_1\) subunit. As can be seen from Figure 3b, the crystal structures of both the constructed bacterial Ca\(^{2+}\) channel and the cardiac DHPR \(\beta\) subunit fit the cryo-EM map quite well. From this docking, we identified the putative positions of the ion selectivity filter, located at its narrowest point (~6 Å), and the gate of the Ca\(^{2+}\) channel, located at the end of the ion-conduction channel. The positions of both the ion selectivity filter and the gate are consistent with the sectioning analysis (see Fig. 2b). Based on the positions of these two crystal structures and subunit-specific antibody labelling, we assigned the locations of the DHPR subunits \(\alpha_1\), \(\alpha_2\), \(\beta\) and \(\delta\) are indicated.

Figure 3 | Membrane topology determination, crystal structural docking and subunit assignments of DHPR complex. (a) Membrane topology. The 3D EM maps of DHPR are coloured in cyan, the anti-\(\alpha_2\) and anti-\(\beta\) antibodies are coloured in magenta and orange, respectively. The position of putative lipid bilayer is marked according to Figure 2b. As the \(\alpha_2\) subunit is located on the extracellular side and the \(\beta\) subunit is located on the cytoplasmic side\(^{12,14}\), the membrane topology of DHPR can be determined from the subunit-specific antibody labelling as shown here, which is consistent with the direction and orientation of ion-conduction channel shown in Figure 2b. (b) Crystal structure docking and subunit assignments. Crystal structure of Ca\(_{Ab}\) (PDB-ID 4MS2), a constructed bacterial Ca\(^{2+}\) channel and a homologue of the \(\alpha_1\) subunit of DHPR, and the cardiac DHPR \(\beta_{2a}\) (a homologue of skeletal \(\beta\) subunit) complex with AID of the \(\alpha_1\) subunit (PDB ID:1T0J) were fit into the pseudo-4-fold “trapezoid” and the “tetrahedroid” regions of the DHPR EM-map, respectively. Coloured ribbons represent the atomic models of the I–IV subunits of Ca\(_{Ab}\) (red, yellow, cyan and blue) and the DHPR \(\beta\) subunit (green). Left: top view, viewed from the extracellular side, down the putative ion-conduction channel. Middle: side view (“diamond”), viewed parallel to the membrane plane, obtained by a rotation of 90° upwards of the top view. Right: bottom view, viewed from the cytoplasmic side, obtained by a down-wards rotation of 180° of the top view. The putative locations of subunits \(\alpha_1\), \(\alpha_2\), \(\beta\) and \(\delta\) are indicated.
partner in the same corner region of the square-shaped RyR cytoplasmic assembly, thereby transmitting the signal from DHPR to RyR and vice versa during an E–C coupling process (Fig. 4).

Methods

DHPR purification. T-tubule membranes were prepared from rabbit skeletal muscle and homogeneous DHPR/L-type Ca\(^{2+}\) channels were purified by two-step chromatography—WGA affinity and DEAE-Sepharose ion exchange as described with modifications. Briefly, T-tubule membranes (3 mg/ml) were solubilized with 1% digitonin in 200 mM NaCl, 10 mM Hepes, pH 7.4, for 45 min, the detergent to protein ratio was 5:1 (w/t/w). The insoluble material was removed by centrifugation for 30 min at 120,000 × g. The solubilized membranes were loaded onto a 5-mL WGA fast-flow column specifically designed and custom-made by BIA Separations (Villach, Austria) and equilibrated with 0.1% digitonin, 200 mM NaCl, 20 mM Hepes, pH 7.4. The detergent concentration in all subsequent buffers was 0.1% digitonin. The unbound proteins were removed by washing with buffer I (0.1% digitonin, 50 mM NaCl, 20 mM Hepes, pH 7.4) for 10 column volumes. The DHPR/L-type Ca\(^{2+}\) channels were then eluted with 3 volumes of buffer I with additional 200 mM N-acetylglucosamine (GlcNAc). The GlcNAc-eluted solution was loaded onto a DEAE-Sepharose column equilibrated with buffer I, and the DHPR/L-type Ca\(^{2+}\) channels were eluted with a step gradient of NaCl from 100 mM to 300 mM in buffer I. The fractions were analysed by SDS-PAGE and the fractions containing purified proteins were concentrated and mixed with glycerol (final concentration ~25%), quickly frozen in liquid nitrogen and stored at −80 °C. All solutions contain protease inhibitor cocktail (Sigma-Aldrich, P8340) and all operations were performed at 4 °C.

SDS-PAGE and Western blotting analysis. Purified protein sample was analysed by 4 ~ 20% linear gradient polyacrylamide gel at reducing and non-reducing (without 2-mercaptoethanol) conditions. Native gel was run in 3 ~ 15% linear gradient polyacrylamide without SDS and 2-mercaptoethanol. Gels were silver stained with 0.1% AgNO\(_3\).

The proteins separated by 4 ~ 20% SDS-PAGE at reducing and non-reducing conditions were transferred to 0.45-μm polyvinylidene fluoride membranes (Millipore, Billerica, MA) at 400 mA for 2 ~ 4 h at 4 °C. Western blotting analysis was performed with monoclonal antibodies anti-α1 (ab2862, Abcam, Cambridge), anti-β2 (ab2864, Abcam, Cambridge) and anti-β3 (VD21/B12, DHSB, Iowa City, Iowa).

Cryo-specimen preparation and cryo-EM data collection. The purified DHPRs were diluted in buffer II (200 mM NaCl, 0.05% digitonin, 20 mM Hepes, pH 7.4) to an optimal concentration of ~0.5 mg/mL. DHPR-antibody complexes were prepared by incubating anti-α2 and anti-β antibodies with DHPR at a molar ratio 10:1 at 4 °C for at least 1 hour. A 2-μl aliquot of the above-prepared samples was applied on a glow-discharged continuous carbon film coated on a 200-mesh R1.2/1.3 Quantifoil grid (Quantifoil Micro Tools GmbH, Jena, Germany). The grid was blotted for 1 ~ 3 seconds and rapidly frozen in liquid ethane using an FEI Vitrobot Mark IV plunge (FEI Company, Hillsboro, Oregon) at 4 °C and 100% humidity, and stored in liquid nitrogen prior to data collection. The samples were imaged in an FEI Tecnai F30 field emission gun electron cryo-microscope operated at 300 kV, and image frames were recorded on a Gatan 4k × 4k CCD (Gatan Inc. Pleasanton, CA) at a nominal magnification of 78,000, equivalent to a pixel size of 1.43 Å after calibration. The particle images for the 15-Å resolution map of DHPR were collected from over 600 CCD image frames, and the number of cryo-EM frames recorded for DHPR/anti-α2 and DHPR/anti-β complexes were 95 and 191, respectively, with a defocus range of 2.5 ~ 4 μm. Whole dataset contained 25,300, 18,940 and 23,590 particle images for DHPR, DHPR/anti-α2 and DHPR/anti-β complexes, respectively. Overall ~70% of particle images were used for final map generation.

Cryo-EM data processing. All the particle images were selected manually using the EMAN2 program zebower.py. Reference-free 2D averages showed that the DHPRs are conformationally uniform. The contrast transfer function (CTF) parameters of these particle images were manually fitted using the EMAN program cftf. All particles were phase-flipped by cftf, and then an initial model was generated from 2D class averages using EMAN program startAvy. This model was further refined against the corresponding particle images using the standard EMAN iterative reconstruction algorithm without imposing any symmetry operations.

Gold-standard co-test of resolution assessment. The recently developed gold-standard Fourier shell correlation criterion was used to assess the resolution of the final EM map\(^30\). The DHPR data were randomly divided into two independent data sets. Initial models were generated by random phasing at 40 Å independently. The two maps produced were aligned by program FOLDERHUNTER before averaging up as a final map. The resolution of 15-Å for the final map was estimated by the 0.143 criterion of FSC curve between map-1 and map-2 without any mask (Supplementary Fig. 1).

Cryo-EM map post-processing and visualization. “Optimally filtered” map was generated as follows: first, the map was rescaled by a one-dimensional structure factor computed from raw particle images; second, a 15-Å Gauss low-pass filter was applied to the rescaled map. Fimal map visualization and rigid-body docking of crystal structures of Ca\(_{2+}\) (PDB-ID: 1MS2) and Ca\(_{2+}\)–β (PDB-ID: 1T0F) was performed in Chimera\(^1\). Comparison between different EM maps was carried out at the same display threshold after map normalization and alignment.

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**Author contributions**

C.C.Y. designed research; R.S.W. modified experimental procedures; H.L.H. and R.S.W. prepared sample; H.L.H. and G.Z.F. collected data; Q.L.W. and K.M.Z. did biochemical analysis; Z.W. and C.C.Y. analysed data; C.C.Y., Z.W. and H.L.H. wrote the manuscript with inputs from other authors.

**Additional information**

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