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Structure of IP₃R Channel: High-resolution Insights from Cryo-EM

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Abstract

Inositol 1,4,5-trisphosphate receptors (IP₃Rs) are ubiquitously expressed intracellular Ca²⁺ channels and the major mediators of cellular Ca²⁺ signals generated by the release of Ca²⁺ ions from intracellular stores in response to a variety of extracellular stimuli. Despite established physiological significance and proven involvements of IP₃R channels in many human diseases, detailed structural basis for signal detection by these ion channels and their gating remain obscure. Recently, single particle electron cryomicroscopy (cryo-EM) has yielded a long-awaited near-atomic resolution structure of the entire full-length type 1 IP₃R. This structure provided exciting mechanistic insights into the molecular assembly of IP₃R, revealing the pronounced structural conservation of Ca²⁺ release channels and raising many fundamental and controversial questions on their activation and gating. Here we summarize the major technological advances that propelled our cryo-EM analysis of IP₃R to near-atomic resolution and discuss what the future holds for structural biology of Ca²⁺ release channels.

Introduction

The release of Ca²⁺ ions from intracellular stores, such as endoplasmic/sarcoplasmic (ER/SR) reticulum, is the most versatile cellular signaling mechanism that is responsible for numerous and markedly different cellular actions including gene transcription, secretion, muscle contraction, learning and memory, cell proliferation and cell death. Intracellular Ca²⁺ release is mediated by Ca²⁺ release channels, inositol 1,4, 5-trisphosphate receptors (IP₃Rs) and ryanodine receptors (RyRs), which are enormous tetrameric membrane protein assemblies. Dysfunction of these ion channels has been linked to a wide range of neurodegenerative and neuromuscular diseases characterized by abnormal intracellular Ca²⁺ homeostasis [1,2]. Defining the molecular mechanisms for Ca²⁺ signals generated upon activation of Ca²⁺ release channels is therefore a major goal in the quest for understanding health and pathology in virtually all living cells. Accomplishment of this goal is dependent

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upon our knowledge of the detailed protein structure, thus enabling mechanistic insights into channel function.

Understanding structural basis of ion transport through biological membranes is an important and challenging frontier in structural biology. Ion channels are integral membrane proteins that are embedded within the lipid bilayer membrane and responsible for molecular transport through the membranes. These proteins have been proven to be much more difficult targets for structural analysis than their soluble counterparts, mainly because of their instability outside biological membranes. To date, structural biology is taking advantage of recent technical advances in the cryo-EM field (reviewed in [3–5]), and single particle cryo-EM is increasingly becoming the most applied method for structure determination of dynamic multi-domain biological assemblies, which have not been amenable to the traditionally employed structural techniques of crystallography and NMR spectroscopy. In favorable cases, the achievable resolution for single particle cryo-EM is now comparable to that produced in atomic-resolution crystallographic studies, 2–3 Å [6–10]. Starting in 2013, single particle cryo-EM made remarkable strides in solving structures of a wide spectrum of integral membrane proteins at near-atomic (3–5 Å) resolutions [11–18] including the megadalton Ca^{2+} release channels, IP_3Rs and RyRs [19–25].

However, structural analysis of IP_3R poses unique challenges for single particle cryo-EM largely due to genuine heterogeneity of the channel complexes present in native tissues and their flexible multi-domain architecture. The IP_3R family is comprised of three homologous isoforms (types 1–3) encoded by separate genes [26,27]. Although all IP_3R isoforms exhibit strong sequence homology (~70% sequence identity), especially within the TM regions (~90% sequence identity), they harbor significant differences in the regulatory cytoplasmic domains. These differences likely underlie distinct isoform-specific channel properties in terms of channel modulation by endogenous and exogenous ligands and tissue distribution. IP_3Rs are expressed in virtually all mammalian tissues and their diversity is further expanded by the homo- and hetero-tetrameric assembly of the IP_3R protomers into functional channels, as well as by alternative gene splicing [26,27]. Moreover, the presence of glycosylation and phosphorylation sites at the cytosolic and luminal faces of the channel protein may also produce conformational variations *in vivo*. As such, preparations of completely structurally homogeneous IP_3R samples from native sources is not achievable given that most mammalian cell types express more than one IP_3R isoform, and expression of such large recombinant protein suitable for high-resolution structural analysis remains challenging. Advantageous to structural studies are cerebellar Purkinje cells that express predominantly the type 1 IP_3R isoform, which is the most functionally and structurally characterized member of the IP_3R family.

In this review, we summarize the technical and methodological advances that have enabled near-atomic resolution structure determination of $\text{IP}_3\text{R1}$ by single-particle cryo-EM [19]. We also discuss the latest structural insights into the IP_3R architecture, which uncover mechanistic details of Ca^{2+} release channels, and consider future prospects and challenges for structural studies of these channels.

Brief Retrospective on the IP₃R structural studies

During the past decade, structural information on the IP₃R protein has been obtained by three major structural techniques: X-ray crystallography [28–31], NMR spectroscopy [32,33] and single-particle cryo-EM [19,34–40]. However, given the exceptionally large size of the entire IP₃R channel (~1.2 MDa), only single particle cryo-EM was able to tackle the structure of the full-length tetrameric channel assembly. Over a decade ago, several low-resolution structures of the entire IP₃R1 were reported almost simultaneously [34–38] yet, none agreed, even with respect to the overall shape of the channel, leading to uncertainty over the true 3D structure of IP₃R1. Further cryo-EM studies allowed our group to unambiguously establish the 3D architecture of the entire IP₃R1 channel and clearly prove the veracity of the new structure to intermediate resolution (10–15 Å, depending on local flexibility in specific domains) by applying multiple validation methods [39,40], including tilt validation [41,42], class-average/map comparisons, independent refinements in multiple software packages and ‘gold standard’ resolution evaluation method [43]. Critical methodological improvements in protein purification and cryospecimen preparation protocols greatly aided the progress of structural studies of IP₃R1 and resulted in images with better contrast than those previously achieved. The root of discrepancies between the earlier conflicting EM studies of IP₃R1 [34–38] was confirmed to arise from insufficient contrast in the raw data underlying the 3D reconstructions [40,44]. These key studies [39,40] laid to rest a critical controversy on the IP₃R1 quaternary structure, however the lack of a high-resolution 3D structure of the entire IP₃R1 significantly limited our ability to understand the structure-function relationship underlying ligand-induced channel activation and to rationalize its interactions with multiple modulatory molecules in a cellular context. Most recently, significant technical advances in the cryo-EM field have enabled structure determination of IP₃R1 at near-atomic resolution allowing further critical insights into the gating machinery of this ion channel [19].

From nano-scale morphology to near-atomic resolution structure of IP₃R1: how did we get there?

A number of factors influenced the success of near-atomic structure determination of IP₃R1 by single particle cryo-EM. In general, single particle cryo-EM analysis requires that the protein of interest is prepared in the form of aqueous solution of monodisperse, identical particles. This requirement poses particular challenges in the purification and stabilization of integral membrane proteins given that their native environment is a lipid bilayer. Key to determining a high-resolution structure of IP₃R1 was an optimized purification procedure combined with biochemical and functional characterization of the purified channel protein. The cerebellum is the most abundant tissue for IP₃R1 protein and was used as the primary source for the channel purification [45,46]. Choice of the detergent used for the protein solubilization and protein-to-detergent ratio in the protein sample has been proven to be a critical factor in achieving optimal vitrification conditions including the protein stability, ice thickness, particle distribution on an EM grid, that together can affect the quality of cryo-EM data [44]. Another encountered problem for IP₃R1 is obtaining the protein concentrations sufficient for cryo-specimen preparations. Membrane proteins are particularly

prone to aggregating during purification and concentration of the detergent solubilized protein sample. In the case of IP₃R1 studies, a continuous layer of thin carbon support film was used to achieve adhesion and optimal distribution of channel particles on the EM grid. These careful considerations enabled the structure of IP₃R1 to be solved initially at nanometer [39] and later at near-atomic resolution. However, there are two major factors that allowed us to push the resolution limit in recent cryo-EM studies of IP₃R1. First, data quality was dramatically improved due to the use of the direct electron detector. This new camera produces substantially higher signal-to-noise ratio at both low and high resolution and allows correcting for beam-induced motion, thereby practically doubling detector resolution compared to previous CCD technology. Second, new powerful image processing algorithms and methods have been designed within the framework of single-particle reconstruction software allowed for sorting out sample heterogeneity due to genuine protein flexibility and conformational variability of IP₃R1 channel. These technical advances in the cryo-EM field have been extensively discussed in excellent comprehensive reviews [3–5,47].

All together these methodological and technical improvements yielded a 4.7 Å resolution 3D structure of IP₃R1 based on the gold standard FSC criterion (Fig. 1). The map veracity was further validated with a phase randomization test (Fig. 1d) [48] and by performing independent reconstructions using two different software packages, EMAN2.1 and Relion1.3 (Fig. 1 a–c). Notably, the local resolution varies from 3.6 Å to 6.5 Å through the entire 3D reconstruction but was of sufficient quality to trace the backbone topology for 2,327 of 2,750 amino acids and unambiguously identify ten domains that comprise the full-length IP₃R1 protein (Fig. 2). Importantly, observed structural features correlate with the resolution assessment excluding a possibility for noise over-fitting during iterative refinement and providing confidence to functional interpretations. TM domain densities were best resolved permitting to clearly define side-chain densities of bulky residues (*e.g.* F2575, F2586, I2590, D2591, R2597) in the Ca²⁺ permeation pathway. However, side-chain assignments in the cytoplasmic domains where the density is not sufficiently resolved for rotamer placement were excluded from the final model of IP₃R1 and not interpreted.

Key domains in IP₃R: structure-function conservation

Recent advent of near atomic resolution structures of IP₃R1 and RyR1 allows for many parallels to be drawn between the two channels serving to expand our understanding of molecular mechanisms for their activation and gating. IP₃R and RyR channels largely perform the same intracellular function of Ca²⁺ transport across the ER/SR membrane, however they respond to different cellular stimuli, and the intensity, shape and duration of Ca²⁺ signals generated by these channels are defined by their regulators. Each channel function - signal detection, ligand binding, gating - captures specific properties of the protein structure.

Topologically, Ca²⁺ release channels are members of the tetrameric cation channel superfamily, where each channel monomer contains six transmembrane helices (TM1–TM6), of which the last two helices (TM5 and TM6) from four subunits pack in a right-handed bundle forming a single ion-conducting pore in the center of the channel. Helices TM1–TM4 form a peripheral bundle connected to the pore bundle (TM5/TM6) via the

TM4-5 linker, which structure and position result in swapping of TM domains, *i.e.* TM1–TM4 bundle of one subunit interacts with the pore helices from the neighboring subunit. The swapped architecture of TM domains is conserved feature of the tetrameric cation channels. While the overall architecture of the TM domains in IP₃R1 is similar to that of RyR1 structure, closer inspection reveals important sequence variations (*e.g.* TM5-6 luminal and TM2-3 cytosolic loops), which may explain differences in the gating properties of IP₃R and RyR channels.

The basic structural framework of IP₃R1 is provided by a unique architecture of the C-terminal helical domains (CTDs, Fig. 2 and 3) that are packed together to form a left-handed bundle around the fourfold channel axis. This bundle is ~80 Å long and spans the entire cytoplasmic region in the tetrameric channel assembly. As a consequence of the structural organization of the CTDs, the N-terminal ligand-binding domains (LBDs: β-TF1, β-TF2 and ARM1) connect to the TM domains through a helical linker domain (LNK) lying at the cytosol-membrane interface. This is an unexpected and exciting aspect of IP₃R1 structure solved by cryo-EM, which was impossible to reconcile with the decade old consensus that signals evoked by IP₃ binding to the N-terminus of the channel protein are transmitted to the channel pore formed near the C-terminus via a direct link between the LBD and TM domains [49–51]. In contrast, our structural studies strongly argue against this model and support a mechanism whereby long-range allosteric interactions through the CTD and LNK domains provide the functional coupling between ligand binding and channel opening. On the basis of the IP₃R1 structure several additional inter- and intra-domain interfaces were identified that might be involved in ligand-induced signal transmission towards the pore. This provides an additional level of channel regulation where multiple regulatory signals merge to control channel gating. This observation is consistent with structural results for RyR [20–25]. However, our analysis reveals that the CTD of RyR1 is structurally homologous to the LNK domain of IP₃R1 (Fig. 3), suggesting differences in the coupling mechanism underlying allosteric modulation of these two receptors. Notably, the CTDs of tetrameric TRPA1 display the architecture similar to that of IP₃R1 (Fig. 3a) [17].

An intriguing feature shared by both families of Ca²⁺ release channels is that the cytoplasmic domains form multiple α-helical solenoid scaffolds (Fig. 3). Given that Ca²⁺ release channels assemble *in vivo* as multi-protein complexes with auxiliary regulatory proteins, it is clear that their functionality greatly benefits from the modular solenoid design providing the binding sites for channel binding partners. Sequence analysis indicates that the two channels substantially differ in the number of residues in the full-length protein (IP₃R1: 2750 amino acids and RyR1: 5037 amino acids) and share only modest sequence homology (~17%). However, the alignment of the two structures highlights significant similarity between the two receptors (Fig. 3).

While primary sequence alignments of IP₃R and RyR families have played a major role in the comparative analyses to tease out channel structure-function, the lack of high-resolution 3D protein structures substantially limited the ability to fully rationalize channel properties. 3D protein folds are generally more conserved than amino acid sequences. Consequently 3D structural comparisons can result in more accurate sequence alignments and thus can be more informative. In the case of IP₃R and RyR, sequence alignments between such large

proteins with low overall sequence homology and many stretches of non-homologous regions, are prone to many errors that are dependent on the users choice of algorithm and gap penalties. Recent structural studies of RyR1 clearly demonstrate this point [20,25].

The Ca^{2+} sensor domains in IP₃R1 and RyR1 were first described through efforts to associate conserved residues with their functional role. Highly conserved glutamate residues (E4032 in RyR1 and E2101 in IP₃R1) were previously identified to play a role in Ca^{2+} sensitivity for activation of IP₃R1 and RyR1 channels [52,53], and proposed to likely serve an equivalent structure-function role in both channel families. However, both Efremov *et al.* [20] and des Georges *et al.* [25] claim that these residues are not structurally equivalent based purely on sequence alignment. In contrast with this, our structural alignment of the ARM3 domain of IP₃R1 and C-solenoid domain of RyR1 has revealed a strongly conserved protein fold (C α -RMS deviation of 1.8 Å) with the conserved glutamate residues occupying the same spatial location within their respective domain (Fig. 4). The resulting sequence alignments based on the aligned structures showed additional conserved residues (Fig. 4c), including two residues recently proposed to coordinate Ca^{2+} for RyR1 (Fig. 4b) [25]. Whether these residues play a role in coordinating Ca^{2+} in the IP₃R1 channel is not yet known, and future high-resolution structures of the ligand-bound channel may answer this question.

Conclusions

Ca^{2+} release channels were among the first ion channels attempted by single particle cryo-EM due to their size and relative natural abundance. Remarkable advances in single particle cryo-EM have recently resulted in near-atomic resolution structures of both full-length IP₃R1 and RyR1 channels permitting the exploration of their unique gating characteristics [19–24]. A proposed mechanism of ligand-gating based on structural analysis of IP₃R suggests that structural rearrangements are rather necessary to account for the conductance properties of IP₃R1 in the open state, however it is not yet obvious how the TM and cytoplasmic domains structurally rearrange upon channel activation and gating. Thus, the current major goal is to solve high-resolution structures of IP₃R in different functionally relevant states, providing further insights into IP₃R channel molecular mechanisms.

Until now cryo-EM structural studies of IP₃R1 have been conducted on the tetrameric IP₃R1 purified from native source, yet the regions for which the IP₃R1 backbone structure has yet to be resolved overlap with the known locations for alternative splicing and regions of post-translational modifications. This level of heterogeneity has complicated structure-functional studies of IP₃R. Future structural studies will greatly benefit from the production of recombinant IP₃R proteins, which are expected to be more homogeneous and advantageous for achieving higher resolution structures by single-particle cryo-EM. Additionally, the nature of lipid and solubilizing detergent affects the stability of the protein and likelihood of high-resolution structure determination by cryo-EM [54,55]. It has been demonstrated that purified membrane protein nano-disc preparations are particularly stable and allow the protein to be maintained at high concentrations suitable for cryo-EM studies [8,12]. However, one must keep in mind that this approach is prone to a general limitation of single-particle cryo-EM when exploring structure-function of ion channels: both faces of the

channel assembly are exposed to the same environment, while *in vivo* ion channels function in the biological membrane and are exposed to an essentially asymmetric cellular milieu. From this perspective, structural studies of ion channels reconstituted into lipid vesicles will likely have a high impact on structural analysis as this approach reflects both the presence of lipids, which can effect activity, and also allows to explore the structural basis of unique gating characteristics in a native-like environment [56,57].

Furthermore, based on numerous functional studies multiple conformations with different lifetimes and stability likely underlie IP₃R's functional states. Approaches to reduce conformational flexibility of IP₃R include stabilization of the channel conformation by binding of specific ligands, which would promote formation of stable channel complexes suitable for high-resolution structure determination. Overall, the field is now taking advantage of the high-resolution and high-throughput single particle cryo-EM, which together with advanced biochemical and physiological studies, will lead to increased rate of structure-function discoveries in the near future.

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Highlights

- Full-length tetrameric structure of IP₃R1 has been solved at 4.7 Å resolution.
- The overall structure was validated by multiple methods.
- The central four-helix bundle provides a supporting scaffold for the cytosolic assembly.
- Direct structural coupling between the C-terminal and IP₃-binding domains is revealed.
- Versatility of Ca²⁺ signaling via IP₃R is founded in the architecture of cytosolic domains.

Figure 1. Resolution assessment and structure validation

Two 3D reconstructions of IP₃R1 were performed entirely independently with Relion1.3 (a) and EMAN 2.1 (b) software packages using the same cryo-EM data. (c) Shown are the gold-standard FSC curves [41,43] for the final 3D reconstructions generated with Relion1.3 (black line) and EMAN2.1 (green line). (d) The FSC profiles for the Relion1.3 reconstructions: the blue line shows the FSC curve computed for the data in which the phases were randomized from 6 Å to Nyquist; the gold standard FSC curve is shown with a black line and is nearly identical to the “true FSC” (red line) calculated as described in [48].

Figure 2. Structure of rat cerebellar IP₃R1 determined by single-particle cryo-EM

Orthogonal views for the IP₃R1 channel: **(a)** view along the membrane plane; the detergent-phospholipid belt is observed at low density threshold (semi-transparent gray mesh) and denotes the position of the channel complex in the membrane with the cytosolic domains facing up; **(b)** view down the four-fold axis from the cytosolic side. **(c)** Topological scheme of the ten domains identified in the individual IP₃R1 subunit.

Figure 3. Structural conservation of ion channels. (a)

Structures of IP₃R1 (3jav), RyR1 (5tb0) and TRPA1 (3j9p) viewed along the membrane plane. **(b)** Correspondence of domains between IP₃R1 and RyR1 based on their structural alignments. Reported are C α RMSDs for the aligned residue pairs. Structure comparison was performed on the entire domains with the following exceptions: ARM1 is aligned with NSol domains and partial JSol domain (residues 1657–1720), the remaining portion of the JSol domain (residues 1721–2144) is aligned with HD, and BSol residues 2145–2599 are aligned with residues 1119–1494 from ARM2. Structurally conserved domains are color-coded based on IP₃R1.

Figure 4. Structural conservation of putative Ca^{2+} sensor region. (a)

The armadillo repeat domain 3 (ARM3) of IP₃R1 (3jav) and the C-solenoid of RyR1 (5tb0) share structural and sequence conservation. IP₃R1 is colored chartreuse and RyR1 is colored tan. C α RMSD is 1.8 Å. Dashed line indicates Ca²⁺ binding pocket identified in RyR1 [25]. The conserved residues E2101 (IP₃R1) and E4032 (RyR1) that have been implicated in channel Ca²⁺ sensitivity are colored red (a–c). (b) Two zoomed-in views of the RyR1 Ca²⁺ binding pocket (left panels) and the structurally equivalent residues of IP₃R1 (middle panels) are shown. Right panels show the overlay of RyR1 and IP₃R1 putative Ca²⁺ binding pockets with all identical residues colored blue. (c) Sequence alignment based on the structural overlap for the ARM3 and C-solenoid domains is shown. Identical residues are colored blue and structurally equivalent residues in IP₃R1 based on the putatively identified RyR1 Ca²⁺ binding pocket are indicated with a box.