Three-dimensional structure of the type 1 inositol 1,4,5-trisphosphate receptor at 24 Å resolution

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We report here the first three-dimensional structure of the type 1 inositol 1,4,5-trisphosphate receptor (IP$_3$R). From cryo-electron microscopic images of purified receptors embedded in vitreous ice, a three-dimensional structure was determined by use of standard single particle reconstruction techniques. The structure is strikingly different from that of the ryanodine receptor at similar resolution despite molecular similarities between these two calcium release channels. The 24 Å resolution structure of the IP$_3$R takes the shape of an uneven dumbbell, and is ~170 Å tall. Its larger end is bulky, with four arms protruding laterally by ~50 Å and, in comparison with the receptor topology, corresponds to the cytoplasmic domain of the receptor. The lateral dimension at the height of the protruding arms is ~155 Å. The smaller end, whose lateral dimension is ~100 Å, has structural features indicative of the membrane-spanning domain. A central opening in this domain, which is occluded on the cytoplasmic half, outlines a pathway for calcium flow in the open state of the channel.

Keywords: channel/cryo-electron microscopy/IP$_3$ receptor/single particle reconstruction

Introduction

Inositol 1,4,5-trisphosphate receptors (IP$_3$Rs) are ligand-gated calcium release channels for intracellular calcium stores in many eukaryotic cells (Berridge, 1993; Yoshida et al., 1997; Patel et al., 1999). They mediate a large area of calcium-regulated signal transduction events (Pozzan et al., 1994; Wilcox et al., 1998; Gailly and Colson-Van Shoor, 2001), from the secretion of granules in epithelial cells, to the control of gene expression, cell proliferation and cell death (Marks, 1997), to long-term depression in the nervous system (Inoue et al., 1998).

Three types of IP$_3$Rs have been cloned (Furuichi et al., 1989; Mignery et al., 1989; Südhof et al., 1991; Blondel et al., 1993; Maranto, 1994), and are expressed in varying abundance in different tissues (Newton et al., 1994), including smooth muscle, cardiac myocytes (Moschella and Marks, 1993; Perez et al., 1997; Lipp et al., 2000), kidney (Blondel et al., 1993), endothelial cells (Go et al., 1995) and neurons. IP$_3$Rs are tetramers of ~300 kDa subunits. Hydrophobicity analysis has predicted 6–8 hydrophobic regions in each subunit, and >85% of the mass of the receptor to be located on the cytoplasmic side. Biochemical and molecular biology studies (Patel et al., 1999) have located the IP$_3$-binding domain close to the N-terminus of the receptor subunit, and found that the long modulatory domain in the middle of the sequence contains the phosphorylation sites, splicing sites and the binding sites for various accessory proteins. Evidence has also accumulated to support the six transmembrane domain (TM) model for the receptor with a GXRXGGXGD motif in the proposed pore loop, which contains two short hydrophobic sequences (Ramos-Franco et al., 1999; Williams et al., 2001).

Earlier negative stain electron microscopy (EM) studies of detergent-solubilized receptors purified from smooth muscle (Chadwick et al., 1990) and cerebellum (Maeda et al., 1990) revealed significant variation of the protein particles in terms of size and shape. EM images of partially ordered IP$_3$Rs in the endoplasmic reticulum membranes of cerebellar Purkinje neurons (Katayama et al., 1996) provide an estimate of the lateral dimensions of the receptor on the cytoplasmic side of 12–16 nm. These studies failed to provide sufficient information to deduce the three-dimensional structure of the receptor.

A three-dimensional structure of the IP$_3$R can provide a foundation for assimilating various structure–function studies into a coherent molecular image. Due to the limited quantity of receptor protein in most tissues and the large size of single receptor molecules, growth of three-dimensional crystals for X-ray diffraction or NMR studies of solubilized receptors is problematic for the structure determination of IP$_3$R. Single particle reconstruction (SPR) from EM images of isolated receptors (Frank, 1996) can, however, be performed from picomole quantities of protein. To avoid the artifacts seen in negative stain images (Chadwick et al., 1990; Maeda et al., 1990; Frank, 1996), we chose to begin our SPR directly from cryo-EM images of the purified receptors. Thousands of images of detergent-solubilized receptors frozen in random orientations in vitreous ice were obtained by cryo-EM, and combined together by SPR to yield a 24 Å resolution structure. The structure is consistent with the predicted topology for the receptor, and predicts a possible pathway for calcium flow across the membrane.

Results

Purification and functional characterization of murine type 1 IP$_3$R

Cerebellum is the most abundant source for type 1 IP$_3$R, making this tissue the best source for the purification of the receptors (Ferris et al., 1989; Maeda et al., 1990; Hingorani and Agnew, 1992). We purified the receptors...
from frozen mouse cerebellum, following the process with a standard [3H]IP3 binding assay (Thrower et al., 2000). After heparin and concanavalin A (Con A) affinity chromatography, the purified receptors were run through a size exclusion FPLC column (Figure 1A). The receptor peak eluted immediately after the 2.0 MDa dextran peak, consistent with the purified receptors being tetramers.

To check whether it contained any type 2 or 3 IP3Rs or ryanodine receptors (RyRs), the receptor preparation was probed with antibodies specific for the various types of IP3Rs and an antibody against RyRs (both types 1 and 2). The positive control for the type 2 IP3R antibody was microsomes made from mouse cardiac muscle, and that for the type 3 IP3R antibody was made from renal cells (data not shown). The positive control (CTL) for the RyR antibody was the microsomes made from mouse skeletal muscle.

Functional assays showed that the purified receptors have properties characteristic of native IP3Rs. The IP3-binding affinity of the purified receptors was found to be ~40 nM (Figure 2A). The apparent specific activity was 100–150 pmol/mg, consistent with previous studies employing this assay (Chadwick et al., 1990; Thrower et al., 2000). Single channel recordings (Figure 2B) showed that once reconstituted into lipid bilayers, the purified receptors presented the expected IP3-gated...
channel activity with ~85 pS Ba2+ conductance, 4% maximum open probability and block by heparin. To verify their functional integrity further, the purified receptors were reconstituted into calcium-loaded lipid vesicles, and IP3-specific calcium efflux (Figure 2C) was measured by use of a Ca2+-sensitive dye. The results suggested that the majority of the receptors in vesicles could form functional calcium release channels. Moreover, as illustrated in the inset to Figure 2C, pharmacological identity of the receptors is satisfied by stereo-specific response to the D but not the L isoform of IP3.

**Cryo-EM images and three-dimensional reconstruction**

Isolated IP3R particles embedded in vitreous ice were imaged while being kept at liquid nitrogen temperature. Figure 3A shows some of the particle images. The receptor particles are regular in size (~150 Å), and isolated from each other. About 4500 particle images were selected interactively from digitized micrographs. The image processing was performed with the software package EMAN (Ludtke et al., 1999). A subset of 400 particle images was first used to generate a starting model. This was then refined against the remaining particle images by iterative cycles of reprojection from the model, multi-reference alignment, classification and three-dimensional reconstruction. After 10 iterations, a stably converged model was achieved. Reprojections from the converged model match very well with the class averages at the same orientations (Figure 3B). As a test for robustness of the three-dimensional reconstruction, four different starting models were used for the refinement; in each case, convergence to the same three-dimensional structure was achieved.

Correction for the contrast transfer function in the model was performed through Wiener filtering (Hawkes, 1980; Grigorieff, 1998). Sections across the resultant density map in planes normal to its 4-fold symmetry axis (C4 axis) are shown in Figure 4A. Based on the typical protein density (0.81 Da/Å3), a threshold density value was determined to account for the 1.2 MDa mass for each IP3R particle. The surface rendering of this model in two different orientations is shown in Figure 4B. The lateral dimensions of the structure vary along the C4 axis, one end smaller than the other, rendering the appearance of an uneven dumbbell. In Figure 4A, the first five slices from the bottom of the structure show an opening enclosed in the center, which corresponds to the deep indentation on the bottom of the small end as shown in Figure 4B (panel II).

**Discussion**

In this study, we present, for the first time, the three-dimensional structure of the type 1 IP3R. The structure was obtained from images of purified receptors, which were also shown to be functional by IP3 binding, single channel recording and IP3-specific Ca2+ efflux assays. The IP3R structure is significantly different from the 22 Å resolution structure of the RyR (Serysheva et al., 1995, 1999; Orlova et al., 1996), the other type of intracellular calcium release channel. At the current resolution (24 Å), the structure not only reveals the molecular morphology of the receptor, but also defines the spatial arrangement of its subdomains. Previously, only two-dimensional projection images of the receptor were available by negative stain and freeze etching EM (Chadwick et al., 1990; Maeda et al., 1990; Katayama, 1996), giving limited information about the receptor shape and size. By using cryo-EM, we have overcome the limitations of these former studies. The receptor images we obtained were regular in size and well preserved in shape, allowing a reliable three-dimensional structure to be generated from SPR.

The IP3R channel takes the shape of an uneven dumbbell with one end significantly larger than the other (Figure 4B, panels I and II). The purified receptor has a very low open probability, only 4% when maximally activated (Thrower et al., 2002). Considering the fact that the receptors were imaged in the absence of agonists, we assume that the structure reflects the IP3R in the closed state. Given the known topology of the receptor, the small end probably presents the TM (panels III and IV in
Figure 4B), and the large end the cytoplasmic domain (CD). The two domains join at a low density region (called the waist hereafter; Figure 4A). Such assignment gives a 14% density ratio of the TM to the whole structure; this is consistent with the −11% mass ratio of the TM to the whole receptor estimated from the primary sequence and the predicted topology. The TM has an opening to the bottom, i.e. the luminal side, which tapers to ~20 Å in diameter at its half height along its C4 axis, and is completely occluded at its cytoplasmic half. The occlusion of the opening in the TM represents the block of a putative central pathway for ion flux. Even though the gate and selectivity filter of the channel are not visible at the current resolution, consideration of the occlusion of the ion flux by the closed gate and the allowable size of the central opening in the selectivity filter suggests their locations in the cytoplasmic half of the TM. This contrasts with the proposed pore region architecture modeled after the KcsA potassium channel by Williams et al. (2001). Crossing the membrane from the luminal side, the TM density is tilted ~35–45° towards the C4 axis (Figure 4B, panel III), making the lateral dimension of the TM at the luminal surface almost 2-fold that on the cytoplasmic surface. The dimensions of the TM, both laterally and vertically, are similar to those of the membrane-integral portions of the voltage-gated sodium and potassium channels (Sato et al., 1998; Sokolova et al., 2001).

The density in the waist region (Figure 4A) is low except at four high density pillars. These pillars correspond to the major connections between the CD and the TM of the tetrameric receptor. The crevices between the pillars (the yellow arrow in Figure 4A pointing to one pillar) might allow ions to flow, reminiscent of the lateral flow pathways suggested by the four lateral ‘windows’ between the T1 and membrane-integral portion of the Shaker potassium channel (Kobertz et al., 2000; Sokolova et al., 2001). Furthermore, the channel gate rests between the waist and the central opening in the luminal half of the TM, an ideal position to control ion flow.

The CD resembles a bulb with four small arms protruding laterally by ~50 Å (Figure 4B, panels I and II). The four arms make the projection views of the receptor along the C4 axis similar to one of the views observed in an earlier negative stain EM study (Chadwick et al., 1990). The dimensions of this domain are consistent with estimates from a quick-freeze deep-etch EM study of bovine cerebellar IP$_3$Rs (Katayama et al., 1996). Currently, there is no function associated with the four protruding arms, though they could be hypothesized as regulatory domains or specific ligand-binding sites.

The various domains of the IP$_3$R structure fit well with the predicted topology of the IP$_3$R subunits (Figure 4B, panel IV; Michikawa et al., 1994; Ramos-Franco et al., 1999). The six TM model puts the N- and C-termini of the receptor on the cytoplasmic side, and a long loop (106 amino acid residues, including two short hydrophobic
sequences and two N-glycosylation sites) between the fifth and sixth transmembrane segments on the luminal side. A short sequence of this long loop was proposed to be the pore loop (Ramos-Franco et al., 1999; Williams, 2001). This arrangement of the total mass for each subunit of a receptor conforms well to the electron density distribution of the IP₃R structure.

The shape of the TM in the IP₃R structure leads us to believe that the structure for the channel portion of the IP₃R is distinct from that of the RyR (Serysheva et al., 1995, 1999; Orlova et al., 1996). Despite the conserved GXXRXGGGXGD motif, the sequence of the pore loop of the IP₃R is markedly different from that of the RyR. In three-dimensional reconstructions, the channel portion of the RyR in the closed state has no apparent opening on the luminal side, although its lateral dimension at the luminal side (~120 Å) is close to that of the IP₃R (Orlova et al., 1996; Samsö and Wagenknecht, 1998). The transmembrane density of the RyR structure tapers slightly towards the luminal side, while the opposite is true for that of the IP₃R. The different topologies proposed for the RyR (four TM2 to TM6) and the IP₃R (six TMs) may be the basis for the structural differences. Spherical reconstruction (Jiang et al., 2001) has the advantage of resolving the TMs of membrane proteins in a lipid environment, and may be applicable in the future for resolving the discrepancy between these two closely related calcium release channels.

The present depiction of the IP₃R structure will be valuable to the field of calcium signaling as a template for future structure–function studies of this important channel. It forms the basis for improving the structure to a better resolution, and sets the stage for investigating the structural alterations following the binding of specific ligands and various accessory proteins by tracing the resultant global conformational changes of the receptor complexes. For example, chromogranins bind to the luminal face of the receptor, dramatically increasing its open probability in the presence of IP₃ (Thrower et al., 2002), and may allow us to observe the receptor channel in its open state. The structure of the type 1 IP₃R probably represents a general model for all three types of IP₃Rs since they have 60–70% homology in amino acid sequence (Südhof et al., 1991), and share the same topology according to hydrophobicity analysis.

Materials and methods

Purification of type 1 IP₃Rs from mouse cerebellum

The preparation followed published procedures with many minor modifications (Ferris et al., 1989; Hingorani and Agnew, 1992; Thrower et al., 2000). Special care was taken to minimize the degradation of the receptors during the purification by making the procedure as short as possible and using protease inhibitors at every stage. Briefly, 30 frozen mice cerebellum (on average ~2.3 g; Pel Freez Biologicals, Rogers, AK) were used for each preparation. The cerebellum were put into 30 ml of cold buffer A (50 mM Tris–HCl pH 8.0, 1.0 mM EGTA and 1.0 mM β-mercaptoethanol supplemented with protease inhibitors). The tissue was homogenized manually by 15 strokes in a 40 ml Knotes glass homogenizer. The homogenate was then centrifuged at 100 000 g (SW28 rotor in a Beckman L8-70M ultracentrifuge) for 30 min. The pellet was homogenized again in buffer A, yielding the microsome preparation, and the final volume was adjusted to 70 ml with buffer A. The microsomes used for calcium flux experiments (Figure 2C) were prepared in EGTA-free buffer A plus 1.0 mM Ca²⁺.

For detergent extraction, CHAPS was added at 1.2% to the microsome preparation. The extract mixture was incubated for 25 min with intermittent inversions, and subsequently was centrifuged at 45 000 g (SS34 rotor in a Sorvall RC 5 plus centrifuge) for 10 min. The supernatant contained the solubilized receptors and was combined and dialyzed with 10 ml of heparin–agarose beads (Sigma) for 15 min with end-over-end rotation. Thereafter, the beads were collected, washed with 50 ml of buffer B (buffer A + 0.25 M NaCl + 1.0% CHAPS), and then eluted with 10 ml of buffer C (buffer A + 0.6 M NaCl + 0.5% CHAPS). The eluate was collected and incubated with 1.0 ml of Con A–Sepharose beads (Sigma) for 1.5 h. Finally, the beads were collected, washed with 10 ml of buffer D (buffer A + 0.5% CHAPS + 1.0 mM CaCl₂ + 1.0 mM MgCl₂) and eluted with 8 ml of buffer E (buffer A + 0.5% CHAPS + 1.0 M methyl-α-D-mannopyranoside + 4.0 mM EGTA). To remove small size impurities, the preparation was concentrated to 2.0 mg/ml in a Vivaspin G-100 concentrator (Vivascience,宾brook, Lincoln, UK), and then injected into a Superose 6 HR10/30 FPLC gel filtration column in an AKTA system (Amersham Pharmacia Biotech Inc., Piscataway, NJ), and eluted at a flow rate of 0.3 ml/min with buffer G (0.4% CHAPS, 5 mM Tris–HCl pH 8.0, 50 mM NaCl, 50 mM KCl, 1.0 mM EGTA and protease inhibitors). The IP₃R peak eluted as the first peak at 8.1 ml (Figure 1A).

Reconstitution of IP₃Rs into lipid vesicles

Small unilamellar vesicles (SUVs) of egg phosphatidylcholine (PC; Avanti Polar Lipids, Alabaster, AL) were prepared in dialysis buffer (0.1 mM Tris–HCl pH 8.0, 50 mM NaCl, 1.0 mM EGTA, 10 μM protease inhibitors). Purified receptors were concentrated to 0.4–0.5 mg/ml, and washed once with 2.0 ml of buffer G. The concentrated receptors were then mixed with a suspension of SUVs (1.0 mg/ml lipids) in equal volume. The solubilization of egg PC SUVs by CHAPS was characterized as described in Rigaud et al. (1995). The mixture was stirred for 30 min, and then loaded into a piece of pre-cleaned membrane tubing (10 mm wide, molecular weight cut 10 000–14 000, Spectrum Laboratories, Inc., Rancho Dominguez, CA), and dialyzed against 2000 vols of dialysis buffer for 24 h with two buffer changes in the middle. The vesicles were collected. Nycodenz (Sigma) was added thereafter to 15% in the vesicle suspension. The mixture was loaded into a centrifuge tube and covered with a small volume (~50 μl) of the dialysis buffer. Centrifugation at 200 000 g for 2 h (SW55S rotor in a Sorvall M150GX, Kendro Laboratory Products, Newtown, CT) concentrated the vesicles to the top buffer layer, leaving the non-incorporated IP₃Rs in the bottom. The vesicles were collected and used for bilayer recording. For calcium flux assay, the vesicles were prepared in the same way except that the dialysis buffer contained 1.0 mM Ca²⁺ and no EGTA.

Characterization of IP₃Rs

SDS–PAGE/immunoblotting. Gel analysis of the receptors was performed in a standard way (Bollag et al., 1996). For IP₃Rs and RyRs, a 7% resolution gel was used. For western blots, the protein was transferred from the gel to a sheet of Millipore Immobilon-P transfer membrane (Bedford, MA) in a mini-Trans-Blot cell (Bio-Rad, Hercules, CA). The membrane was then blocked overnight with 5% non-fat dry milk in buffer TBS-T (150 mM NaCl, 10 mM Tris–HCl pH 7.4 and 0.1% Tween-20). For type 1 IP₃R, the primary antibody was a laboratory-made rabbit anti-mouse monoclonal directed against the internal 20 amino acid residues of the receptor, and was incubated with the membrane for 1 h in TBS-T + 0.5% milk. The membrane was then washed and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Amersham Bio Sciences, Piscataway, NJ) in the same buffer. The final detection of HRP was performed with the Pierce ECL plus kit (Rockford, IL). Polyclonal rabbit anti-mouse antibody against type 2 IP₃R was purchased from Chemicon International (Temecula, CA). Type 3 IP₃Rs were probed with a mouse monoclonal antibody (Transduction Laboratories, Lexington, UK). The mouse monoclonal antibody for RyRs (types 1 and 2) was obtained from Affinity BioReagents (Golden, CO). An HRP-conjugated horse anti-mouse IgG (Amersham) was used for detection of the mouse antibodies. [³H]IP₃ binding assay. The [³H]IP₃ binding assay was performed according to the standard polyethylene glycol (PEG) precipitation procedure (Thrower et al., 2000). Briefly, the solubilized receptors or the reconstituted IP₃R vesicles were incubated with [³H]IP₃ for 15 min in the presence or absence of 1.0 μM IP₃. Then 0.5% γ-globulin (Sigma) and 30% PEG 8000 were added in sequence to precipitate the IP₃Rs with the bound IP₃. The mixture was centrifuged at 14 000 g for 5 min. The pellet
was rinsed once, resuspended and counted in a scintillation counter. For the competition test, different concentrations of cold IP$_3$ were used. Onto the perforated carbon film of these grids was laid a single particle reconstruction.

IP$_3$R vesicles. Ca$^{2+}$-loaded vesicles or microsomes were prepared as stated above. Membrane-impermeant Ca$^{2+}$-sensitive fluorescent dye, Indo-1 (Molecular probes, Eugene, OR), was prepared in calcium-free water, as were IP$_3$ isomers used in these experiments. Time-based fluorescence measurement was performed in a SPEX FluoroMax-3 spectrofluorometer (Jobin Yvon Inc., Edison, NJ) with a temperature control unit (Wavelength Electronics Inc., Bozeman, MT) to keep the cuvette at 10°C, which minimizes non-specific calcium leak. For each run, 40 μl of Ca$^{2+}$-loaded vesicles were quickly run through a 1.0 ml G-50 spin column equilibrated in the assay buffer (40 mM HEPES, 100 mM NaCl, 5 mM KCl, pH 7.6, 8 g/100 ml Chelex-100) to remove all Ca$^{2+}$ outside the vesicles. The vesicles were then added into the assay buffer containing 3.3 μM Indo-1 to start the experiment. At specific moments, agonists or antagonists were added into the cuvette, and time-based changes in the Indo-1 fluorescence intensity were recorded.

Cryo-EM imaging and three-dimensional reconstruction 

Cryo-electron microscopy. Freezing of the cryo-EM samples was performed as described by Jiang et al. (2001) except that the Quantifoil grids (Quantifoil Micro Tools GmbH, Jena, Germany) with 1.2–2.0 μm holes were used. Onto the perforated carbon film of these grids was laid a thin (~10 nm) carbon film. Solubilized, purified IP$_3$Rs were concentrated and loaded onto the carbon film of a TEApore chamber and mixed with stirring. Normally, within 5 min, insertion of the receptor into the bilayer would occur. The channel activity was recorded at 0 mV with 2 μM IP$_3$ added to the cis-side.

Calcium efflux from IP$_3$R vesicles. Ca$^{2+}$-loaded vesicles or microsomes were prepared as stated above. Membrane-impermeant Ca$^{2+}$-sensitive fluorescent dye, Indo-1 (Molecular probes, Eugene, OR), was prepared in calcium-free water, as were IP$_3$ isomers used in these experiments. Time-based fluorescence measurement was performed in a SPEX FluoroMax-3 spectrofluorometer (Jobin Yvon Inc., Edison, NJ) with a temperature control unit (Wavelength Electronics Inc., Bozeman, MT) to keep the cuvette at 10°C, which minimizes non-specific calcium leak. For each run, 40 μl of Ca$^{2+}$-loaded vesicles were quickly run through a 1.0 ml G-50 spin column equilibrated in the assay buffer (40 mM HEPES, 100 mM NaCl, 5 mM KCl, pH 7.6, 8 g/100 ml Chelex-100) to remove all Ca$^{2+}$ outside the vesicles. The vesicles were then added into the assay buffer containing 3.3 μM Indo-1 to start the experiment. At specific moments, agonists or antagonists were added into the cuvette, and time-based changes in the Indo-1 fluorescence intensity were recorded.

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