Conformational motions and ligand-binding underlying gating and regulation in IP$_3$R channel

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Inositol-1,4,5-trisphosphate receptors (IP$_3$Rs) are ubiquitous Ca$^{2+}$ channels predominantly localized to the endoplasmic reticulum (ER) membranes. IP$_3$Rs play fundamental roles in the physiology of a diverse array of organisms as they mediate the release of Ca$^{2+}$ from ER Ca$^{2+}$ stores in response to diverse physiological stimuli. Decades of studies have revealed the central role that IP$_3$Rs have in generating complex intracellular Ca$^{2+}$ signals, which are responsible for many complex physiological processes ranging from gene transcription and secretion to the more enigmatic brain activities such as learning and memory. Dysfunction of IP$_3$Rs leads to aberrant Ca$^{2+}$ signaling that is associated with a multitude of human diseases such as Alzheimer’s disease, hereditary ataxias, cardiac hypertrophy, heart failure, Parkinson’s and Huntington’s diseases, atherosclerosis, hypertension and some migraines$^{1-3}$.

In mammals, there are three subtypes of IP$_3$R defined by characteristic features encoded by separate genes, and among them the type 1 IP$_3$R (IP$_3$R1) is predominantly expressed in the central nervous system, especially in Purkinje cells of the cerebellum. All IP$_3$R channels are tetrameric assemblies of monomers of either identical or different subtypes, resulting in channel complexes with a wide range of functional properties. Each IP$_3$R subunit comprises the pore-forming transmembrane domains (TMDs) and cytoplasmic (CY) domains containing binding sites for ligands and multiple modulators of the channel. These nonselective Ca$^{2+}$ channels are tightly linked to essential phospholipase C-signaling pathways that mediate generation of IP$_3$ and control a wide range of Ca$^{2+}$-dependent cellular processes in a highly coordinated manner. Complex cross talk occurring between these pathways and IP$_3$R channels leads to precise regulation of intracellular Ca$^{2+}$ levels. However, an in-depth understanding of the
IP₃Rs work as signaling hubs through which diverse cellular inputs like IP₃, Ca²⁺, ATP, thiol modifications, phosphorylation and interacting proteins are processed and then integrated to result in cytosolic Ca²⁺ signals with precise temporal and spatial characteristics. IP₃ and Ca²⁺ are the primary agonists of IP₃R channels and both are required for channel opening. Moreover, to fulfill their many physiological roles in vivo, IP₃Rs associate with an array of regulatory molecules ranging from ions and small chemical compounds to proteins. Unique interactions with these intracellular messengers contribute to the specificity, duration and shape of Ca²⁺ signals generated by IP₃Rs and the channel’s capacity to integrate signals from different pathways. A single IP₃ binding site exists at the N-terminus on each IP₃R subunit and functional studies suggest that binding of IP₃ to each monomer is required for channel opening. Important, IP₃ has little effect in the absence of its co-agonist Ca²⁺. Multiple Ca²⁺ binding sites have also been predicted. IP₃R channel opening is modulated with a biphasic dependence on cytosolic Ca²⁺ concentrations, suggesting that the channel has distinct types of Ca²⁺ binding sites for activation and inhibition. Electrophysiological studies showed that the IP₃-evoked activity of IP₃R is enhanced by modest increases in cytosolic Ca²⁺ concentration in the 10–100 μM range (<1 μM), while higher cytosolic Ca²⁺ concentrations are inhibitory to IP₃ responses [6–9]. ATP is known to enhance the IP₃-induced Ca²⁺ release, by specific binding to IP₃R [10–11]. Despite more than three decades since the discovery of IP₃Rs, the detailed molecular and structural mechanisms underlying the complex interplay between ligands, allosteric modulators and channel gating remains unresolved. With the resolution revolution in single particle cryo-electron microscopy (cryo-EM), there have been remarkable advances in structural characterization of IP₃Rs [12–13]. These studies reported that the IP₃R structure undergoes conformational changes upon ligand binding, suggesting structural flexibility that allows the channel to switch from a closed state, capable of interacting with its ligands such as IP₃ and Ca²⁺, to an open state, capable of transferring Ca²⁺ ions across the ER membrane. However, all of these 3D cryo-EM structures represent defined static conformations of the channel, and the mechanistic insights are derived based on interpolations between discrete structures, each of them likely a mixture of states from a dynamic conformational ensemble. Therefore, mechanistically it remains poorly understood how ligands engage the ligand-binding domains and how multiple signals are coordinated and processed within the IP₃R channel. This implies the necessity to consider the dynamic conformational landscape of the channel protein in determining the molecular mechanisms underlying its function.

In this work, we determined the structures of the IP₃R channel trapped in physiologically relevant states using key ligands that target channel gating. Here, we report cryo-EM structures of IP₃R1 bound to IP₃, ATP, Ca²⁺ and IP₃R1 bound to Ca²⁺ alone at overall resolutions of 3.50 Å and 3.26 Å, respectively. In addition, we used a deep neural network approach and 3D variability analysis to extract functionally relevant conformational motions of the IP₃R domains directly from 2D cryo-EM images. The ratcheting mechanism for IP₃ binding was built via deep-learning approach and corroborated by experimental single-particle cryo-EM analysis. This combined approach allowed for a mechanistic understanding of how conformational motions of the ARM2 domain are coupled with structural changes in the IP₃-binding pocket in the context of the tetrameric IP₃R channel. From these studies, we correlate protein structural changes that connect the ligand-binding regulatory sites to the channel gate via the IP₃R conformational landscape. Our structural findings and hypothesis were validated through mutagenesis and electrophysiology (see also [14]). Our study provides a structural framework for understanding the allosteric mechanisms underlying ligand-mediated IP₃R activation and regulation.

**Results**

**Inherently dynamic architecture of IP₃R1**

To elucidate allosteric coupling mechanisms underlying ligand-binding and gating kinetics of the IP₃R channel, we isolated native, Ca²⁺ permeable IP₃R channels and used cryo-EM single particle analysis to solve the channel structures in distinct ligand-bound conformations. As the membrane environment is relevant for the functionality of channels, we studied IP₃R1 reconstituted into lipid nanodiscs [15]. IP₃ and Ca²⁺ are imperative ligands to trigger the channel opening and ATP increases the open probability of IP₃R1 by synergizing with the activating effect of these two primarily ligands [16–17]. Purified IP₃R1 binds IP₃ in a stoichiometric manner (one IP₃ per IP₃R1 monomer) with an affinity of ~2–100 nM while functional IP₃ affinities vary from the nanomolar to low micromolar range with mean values for maximum effect around 10 μM, dependent on the technique used [18–20]. Ca²⁺ regulates IP₃R1 gating with positive and negative feedback acting in a bell-shaped manner with maximum channel open probability at low μM free Ca²⁺, while high μM Ca²⁺ inhibits the channel [21,22].

Hence, the cryo-EM 3D reconstructions of the nanodisc-reconstituted IP₃R1 channels were determined in the presence of saturating IP₃ concentration (10 μM), ~2 μM Ca²⁺ and 1 mM ATP (IP₃/ Ca²⁺/ ATP-bound IP₃R1: CIA-IP₃R1), and using 20 μM Ca²⁺, as the sole intracellular cation (IP₃R1 bound IP₃R1: Ca²⁺R1, estimated overall resolutions 3.50 and 3.26 Å, respectively (Table 1; Supplementary Figs. 1-4). The final reconstructions are in excellent agreement with the previously solved cryo-EM structures of IP₃R1 [15], and of sufficient quality to allow constructing the atomic structural models for almost the entire protein enabling reliable assignment of most of the side chains (Supplementary Figs. 1–4). Cross-validation between the atomic models and the final density maps suggested that the average resolution of structures was around 3.50 Å and 3.80 Å for Ca²⁺R1 and CIA-IP₃R1, respectively (Supplementary Figs. 2, 4).

The IP₃R1 structure has a large solenoid CY assembly built around the central helical bundle made of the C-terminal domains from four IP₃R1 subunits (Supplementary Fig. 6a, b). The solenoid scaffold includes domains responsible for binding of ligands and regulatory proteins and is connected via an allosteric nexus at the cytosolic-membrane interface to the TM channel assembly. Six TM helices from each subunit form the central ion-conduction pore and are well resolved in both the Ca²⁺- and CIA-IP₃R1 structures owing to the stabilizing effect of lipid nanodisc (Supplementary Fig. 7a, b).

Local resolution analysis showed that the central TM helices and ligand-binding domains (LBDs) had the highest resolution at 2.4–3 Å, while the cytoplasmic armadillo domain 2 (ARM2; L1103-G1567) had the lowest resolution, suggesting that this domain possesses structural flexibility (Supplementary Figs. 2c, 4c). Of note, this domain was similarly poorly resolved in all previous cryo-EM studies of IP₃Rs [23–24]. To improve map quality and local resolution in the ARM2 domain, we performed iterative focused refinements using a mask encompassing the ARM2 domain (Supplementary Figs. 1–4). The resulting cryo-EM maps are substantially improved, enabling atomic modeling of the ARM2 domain (Supplementary Figs. 1–4). One conformation, hereafter called ‘extended’, was observed in the apo- (ligand free) and Ca-IP₃R1 structures. By contrast, in the CIA-IP₃R1 structure, the ARM2 domain is moved ~30 Å towards the helical armadillo domain 1 (ARM1) from the same subunit (Fig. 1e) and exhibits a conformation distinct from that observed in the Ca-IP₃R1 or apo-IP₃R1, which we have termed ‘retracted’.

To gain insights into the conformational landscape of IP₃R1 underlying its discrete ligand-bound states, we made use of a deep-learning based gaussian mixture model (GMM) [25]. When taken together, the set of raw 2D particles, each in a known orientation, does not just define a single structure, but a complete set of related structures embodying the motion and composition of the macromolecule in
Table 1 | Cryo-EM data collection and refinement statistics

|                      | Ca-IP3R1 (EMD-27982) (8EAQ) | CIA-IP3R1 (EMD-27983) (8EAN) |
|----------------------|-----------------------------|-----------------------------|
| **Data collection and processing** |                             |                             |
| Magnification        | 130k                        | 130k                        |
| Voltage (kV)         | 300                         | 300                         |
| Exposure time (s)    | 7                           | 7                           |
| Electron exposure (e⁻/Å²) | 49                        | 49                           |
| Dose fractionation   | 35                          | 35                          |
| Defocus range (µm)   | -0.8 to -2.5                | -0.8 to -2.5                |
| Pixel size (Å)       | 1.07                        | 1.07                        |
| Symmetry imposed     | C4                          | C4                          |
| Initial particle images (no.) | 1,955,320                 | 1,452,797                   |
| Final particle images (no.) | 346,731                    | 133,740                     |
| Map resolution (Å)   | 3.26                        | 3.50                        |
| FSC threshold        | 0.143                       | 0.143                       |
| Map sharpening B factor (Å) | 2.4-6                    | 2.4-6                       |
| **Reefinement**      |                             |                             |
| Initial model used (PDB code) | 7LHE                      | 7LHE                        |
| Model resolution (Å) FSC = 0.5 | 3.5                      | 3.8                         |
| FSC threshold        | 0.5                         | 0.5                         |
| Map sharpening B factor (Å) | -129                     | -157                        |
| Model composition    |                             |                             |
| Non-hydrogen atoms   | 77960                       | 78516                       |
| Protein residues     | 9464                        | 9552                        |
| Ligands              |                             |                             |
| IP₃                  | -                           | 4                           |
| ATP                  | -                           | 4                           |
| Ca²⁺                 | 20                          | 16                          |
| Zn²⁺                 | 4                           | 4                           |
| lipids               | 28                          | 28                          |
| B factors (Å)        |                             |                             |
| Protein              | 104.42                      | 155.44                      |
| Ligand               | 72.32                       | 123.40                      |
| R.m.s. deviations    |                             |                             |
| Bond lengths (Å)     | 0.003                       | 0.003                       |
| Bond angles (°)      | 0.627                       | 0.602                       |
| Validation           |                             |                             |
| MolProbity score     | 1.79                        | 1.84                        |
| Clashscore           | 9.44                        | 10.74                       |
| Ramachandran plot    |                             |                             |
| Favored (%)          | 95.86                       | 95.78                       |
| Allowed (%)          | 3.97                        | 4.05                        |
| Outliers (%)         | 0.17                        | 0.17                        |

solution. Using a set of Gaussian spheres in space as a proxy, deep-learning makes it possible to relate these 2D particles most similar to each of these 3D states. We can then reconstruct 3D maps using particle subsets, representing actual points along motion pathways, directly from the particle data without any structural interpolations. That is, each structure along a motion pathway was reconstructed purely from experimental data and the computational model is used only to select which data to include.

As expected, the IP₃R₁ protein exhibits substantial dynamic motions involving both the cytoplasmic and pore domains (Supplementary Movie 1). In particular, our analysis captured that the ARM2 domain undergoes concerted motions in both ligand-bound states of IP₃-R₁ characterized in the current study, as well as in apo-IP₃-R₁ structure from our previous study. It is notable that ARM2 exhibits similar motion trajectories in all three states, but with increased motion amplitude in the presence of IP₃ (Supplementary Movie 2). This observation suggests that the reversible IP₃-binding process in the IP₃-R tetrameric channel is coupled to conformational motions of the ARM2 domain in some way. Furthermore, the helical domain (HD; E693-L1102) exhibits pronounced apical motions, and the linker domain (LNK; T2610-A2680) and intervening lateral domain (ILD; T2193-W2276) that form a nexus at the cytosolic-membrane interface, dilate. Noteworthy, these conformational changes are propagated to the HD-ARM3 interaction interface also undergoing structural rearrangements. In the TMD region the global movements are quite notable exhibiting a rotation of the TM assembly relative to the cytosolic LNK/ILD nexus (Supplementary Movie 3). Based on these results, we envision that the ARM2 domain undergoes structural transitions within the dynamic conformational ensemble and this domain flexibility affects the IP₃-binding pocket properties (discussed below). Overall, the observed conformational motions are in good agreement with our earlier proposed model for long-range allosteric conformational coupling between ligand-binding and activation of the channel gate.

Conformational dynamics of ARM2 govern IP₃ binding

The IP₃-binding pocket is formed at a cleft between the β-trefoil domain, BT2 (W226-V435) and the ARM1 domain (Fig. 2). The preceding BT1 domain (M1-K225) works as an IP₃ binding suppressor. These three domains (also known as ligand-binding domains, LBDs) form a triangular architecture at the apex of the β cylinder. In the CIA-IP₃R₁ structure, we observe a strong density bridging the BT2 and ARM1 domains at the site where IP₃ is expected to bind (Fig. 2). This density accommodates the IP₃ molecule well. The P1 and P5 phosphate groups of IP₃ are predominantly coordinated by residues from the ARM1 domain and the P4 phosphate group interacts with the BT2 residues. The CIA structure reveals that P4 forms hydrogen bonds with T267, R269, S274. P5 is accommodated within the binding pocket by residues: K508, R511, Y567, K569. In contrast, P1 points outside the IP₃-binding cleft and interacts with only R504 and R568, which is consistent with earlier studies demonstrating that this group is significant for IP₃ binding. The three hydroxyl groups, O2, O3 and O6 can form hydrogen bonds with the ARM1 residues, but have a secondary role in binding of IP₃. The overall geometry and composition of the IP₃-binding pocket are consistent with observations in crystallographic studies of expressed LBDs and cryo-EM studies of IP₃-R₅s (Supplementary Fig. 8) and have been validated by extensive site-directed mutagenesis.

Superimposition of a single subunit from the apo-, Ca²⁺- and CIA-bound structures shows that IP₃-binding causes rigid-body movements of all three LBDs resulting in a 4 Å closure of the cleft between the ARM1 and BT2 domains. Notably, there are small intra-domain rearrangements in both βT2 domains upon IP₃ and/or Ca²⁺ binding (1.2 Å Ca RMSD) (Fig. 2b; Supplementary Fig. 6). In contrast, the α-helices in the ARM1 domain undergo substantial shifts in the CIA structure with the entire domain moving towards the IP₃-binding pocket (Fig. 2b, c). In particular, the first α-helix in ARM1 (P437-A457) shifts 12° from its position in the Ca-IP₃R₁ structure, and its C-terminal segment (G458-K462) becomes unraveled. Additionally, the two helices in the ARM1 domain (h3: R504-E512; h7: R568-K576), which residues directly contribute to the coordination of IP₃, move -20° when engaged with IP₃.

Inspection of the interfaces between the LBDs and the ARM2 domain shows that ARM2 interacts with BT1 and ARM1 domains from the neighboring subunit in Ca-IP₃-R₁, CIA-IP₃-R₁ and apo-IP₃-R₁ structures (Fig. 1, Supplementary Fig. 6). Specifically, in both
apo- and Ca-IP3R1 structures the βTF1-ARM2 interface is stabilized by interactions between A75-D85 and R137-A146 from βTF1 with N1336-S1337 and T1301-H1302 from ARM2 (Fig. 1c, Supplementary Fig. 6d). Additionally, an interface between the ARM1 and ARM2 domains is formed at M535-A547 and G463-R471 and S1399-R1408, N1346-Q1352 and N1336-E1339, respectively. The ARM1 residues R545, M535, R471 are in position to form hydrogen bond with residues N1346, R1348, E1339 from the ARM2 domain. We found that these specific inter-subunit interfaces are perturbed in the CIA-IP3R1 structure, resulting in the formation of a smaller interaction area at a new interface located at residues V1391-S1399 and D1433-M1438 of ARM2 and K136-N145 of βTF1 (Fig. 1d).

ARM2 inter-subunit interfaces are indicated by boxes and enlarged in the lower panels. Zoomed in boxed areas depict the amino acids present at the molecular interfaces. The red asterisk marks the same helix in each structure. e HD and ARM2 domains from an aligned subunit from Ca-IP3R1 (tan) and CIA-IP3R1 (colored by domain) are superimposed. Arrows indicate 32 Å rotation of ARM2 and a 10 Å translation of HD.
The importance of coupling between binding of IP3 and Ca2+ in the regulation of IP3R gating activity is evident in multiple prior studies. However, a mechanistic description of IP3-Ca2+ interplay in modulating IP3R1 activity is still missing. In this study, to understand the structural basis for the modulation of the channel gating with IP3, we determined IP3R1 structures under activating conditions in the presence of 2 μM Ca2+ (CIA-IP3R1) and in the presence of 20 μM Ca2+ that produces channel inhibition (Ca-IP3R1). A comparison of the cryo-EM density maps of Ca-IP3R1 and apo-IP3R1 revealed strong non-protein densities in the putative Ca2+-binding sites predicted in earlier mutagenesis and functional studies. The ascribed Ca2+ densities were validated as described in Methods, establishing the locations of five occupied Ca2+ binding sites in each IP3R1 subunit (Fig. 3). The side chain densities are well resolved in the detected Ca2+ binding sites permitting assignment of coordinating residues for the bound Ca2+ ions.

Two Ca2+ binding sites are identified within the ligand-binding domains across the inter- and intra-subunit interfaces between βTF2 and βTF1 domains. The first site, Ca1(III), is located at an interface between the βTF2 and βTF1 domains from two neighboring subunits. The Ca2+ ion in this site is predominately coordinated by carboxyl groups from D426 and D180 residues in βTF2 and βTF1 domains, respectively. The second site, Ca-IIIS, is formed across the interface between the βTF2 and βTF1 in the same subunit. This intra-subunit site is composed of two carboxylate oxygen atoms from E283 and backbone carbonyl oxygen atoms from residues K51, K52, F53 and R54 of βTF1 domain (Fig. 3). The locations of these two Ca2+ bound ions match well to two predicted Ca2+ binding sites in the crystal structure of LBDs.

Another strong non-protein density is observed in the putative Ca2+ sensor region in ARM3 domain (R1582-H2192), which we assigned to a bound Ca2+ ion, and this Ca2+-binding site is referred to here as Ca-IIIb (Fig. 3). This Ca2+ ion is stabilized by side chain oxygen atoms from residues E1978 and E2042, Q2045, N1981, and N1971, main chain oxygen from T2654 and a secondary coordination shell may be contributed by H1980 and R1982. Noteworthy, none of the cytosolic Ca2+ binding sites described here is similar to that of helix-turn-helix (EF-hand motif) Ca2+-binding proteins. Moreover, alignment of 3D structures of the Ca-IIIb site in IP3R1, IP3R3 and RyR channel confirms a structural conservation of this Ca2+ binding site across both families of Ca2+ release channels as demonstrated in our previous studies. In our companion study, mutations E1978 or E2042 in the Ca-IIIb site markedly affected single channel activity such that altering the negative charge on either aspartate residue shifted the [Ca2+] dependence for activation to the left, consistent with an important role of this site in Ca2+ activation of IP3R1 activity.
appears to exert an effect via allosteric interactions with surrounding residues.

By contrast, in the CIA structure, only two cytosolic Ca\(^{2+}\) binding sites, Ca-II\(_{LBD}\) and Ca-II\(_{LS}\), were occupied with Ca\(^{2+}\) ions. The structures of the two cytosolic sites are nearly identical to those identified in Ca-IP\(_{R1}\) except for subtle rearrangements in the loop regions (Supplementary Figs. 6, 9). Given that Ca\(^{2+}\)-occupied Ca-II\(_{LBD}\) and Ca-II\(_{LS}\) sites are detected at both activating (2 μM) and inhibitory (20 μM) Ca\(^{2+}\) concentrations, it would thus appear that Ca\(^{2+}\) should bind with higher affinity to these two Ca\(^{2+}\)-binding sites compared to the Ca-I\(_{LBD}\) site.
which is occupied only at an inhibitory Ca\textsuperscript{2+} concentration. We found that in the CIA-IP\textsubscript{3}R1 structure, the D426 side chain moved away from the Ca-ILBD binding pocket and the positively charged R170 shifted toward the location of bound Ca\textsuperscript{2+}. This raises the possibility that the conformational differences in Ca-ILBD site between Ca-IP\textsubscript{3}R1 and CIA-IP\textsubscript{3}R1 structures might reflect changes in Ca\textsuperscript{2+}-binding affinities. Consistent with our data, mutagenesis of D426 in the Ca-ILBD site abolished Ca\textsuperscript{2+} binding\textsuperscript{42}. Hence, one could speculate that the Ca-ILBD site is a possible candidate for an inhibitory Ca\textsuperscript{2+}-binding site, and two other cytosolic Ca\textsuperscript{2+}-binding sites (Ca-IILBD and Ca-IIIS) are activating. As it might have been anticipated, binding of IP\textsubscript{3} "tunes" Ca\textsuperscript{2+}-inhibition of the IP\textsubscript{3}R channel\textsuperscript{36,37}.

In both the Ca-IP\textsubscript{3}R1 and CIA-IP\textsubscript{3}R1 structures, two clear densities, which were interpreted as the bound Ca\textsuperscript{2+} ion, are found in the luminal vestibule leading to the selectivity filter (SF) and the central cavity above the SF. We assigned these densities to bound Ca\textsuperscript{2+} ions (see Methods). One of these Ca\textsuperscript{2+}-binding sites, named Ca-IVL, is shaped by the side chains of residues D2551, G2550, R2544, H2541, S2545 contributed across the interface between two neighboring subunits (Fig. 3). The second Ca\textsuperscript{2+}-binding site (Ca-VL) is located in the central cavity, and Ca\textsuperscript{2+} ion bound at this site is ~2.9 Å away from the amide group in the side chain of N2583 suggesting that the bound Ca\textsuperscript{2+} ion is stabilized in a fully hydrated state. Both identified luminal sites, Ca-IVL and Ca-VL, hint at a high affinity binding site as they are occupied at

**Fig. 5 | Ion conduction pathway in IP\textsubscript{3}R1 upon ligand binding.** a Solvent-accessible pathways in CIA-IP\textsubscript{3}R1, Ca-IP\textsubscript{3}R1 and apo-IP\textsubscript{3}R1. The left panel plots the pore dimensions along the ion conduction pathway and the right panels reveal the solvent accessible volume. b The ion conduction pathways in CIA-IP\textsubscript{3}R1 (blue-green), Ca-IP\textsubscript{3}R1 (pink), apo-IP\textsubscript{3}R1 (PDB ID: 7LHE, tan) are aligned and overlaid. The backbone structures for two opposing subunits with several solvent lining side chains are shown and labeled. Red arrow indicates the change in side chain position of F2586 observed in CIA-IP\textsubscript{3}R1. Upper right panels show zoomed in views of the area indicated by the box in the left panel. Distances across the ion conduction pathway at I2590 and F2586 are measured from side-chain atoms from two opposite subunits. c Cryo-EM densities overlaid with atomic models for the TM6 gate residue F2586. Two rotamers of F2586 (orange and blue-green) are shown for CIA-IP\textsubscript{3}R1. Densities in Ca-IP\textsubscript{3}R1 and apo-IP\textsubscript{3}R1 permit only one rotamer fit for F2586. d Luminal view of the selectivity filter (2544-RSGGGVGD-2551) for CIA-IP\textsubscript{3}R1, Ca-IP\textsubscript{3}R1, and apo-IP\textsubscript{3}R1 at the region indicated by black arrows in `b' and colored respectively. Distance across the SF is measured between the G2546 Ca atoms from two opposite subunits.
relatively low Ca\(^{2+}\) concentration. Moreover, the N2583 residue in rodent IP3R1 corresponds to N2543 in humans, and it has been found that the N2543I mutation is closely associated with Gillespie syndrome\(^{43}\).

**Structural dynamics of the ATP-binding pocket**

ATP is known to be a crucial extracellular signaling molecule that increases the open probability of the IP3-R channels\(^{31,36,44,45}\). Previous sequence analysis identified three glycine-rich regions (GXGXXG) in IP3R, reminiscent of the Walker-A motif found in many ATP-binding proteins\(^{46,47}\). These motifs are involved in the Walker-A motif and were presumed to account for the ATP-sensitivity of IP3-Rs based on functional studies\(^{10,11}\). Although binding of ATP to these motifs has been shown with expressed polypeptides, site-directed mutagenesis and single channel measurements revealed that the Walker-A motifs are not required for ATP modulation of IP3R1 or IP3R3. At present, there is a substantial lack of structural knowledge about how ATP regulates the channel gating.

In this quest, using single-particle cryo-EM, we characterize the IP3R1 channel bound to ATP in the presence of its primary activating ligands (CIA-IP3R1). The CIA-IP3R1 structure reveals a previously unseen ATP-binding site located at the interface between the ILD and LNK domains, where clear density is seen (Fig. 4). The ATP molecule fits well into the observed elongated density. Consistent with this assignment, both the apoprotein and Ca\(^{2+}\)-bound structures lack this density. The resolution in the ATP-binding site was sufficient to unambiguously identify the pose of the ATP molecule as well as coordinating residues in the binding pocket. This pose allows for the formation of hydrogen bonds between the negatively-charged ATP triphosphate tail and the central hydrophilic cavity of the pore is narrowest in Ca2+-bound IP3R1, where clear density is seen (Fig. 4). The ATP molecule in the binding pocket was imaged with a resolution of 2.5 Å and widened to ~9.4 Å and 10.7 Å in apo- and CIA-bound structures, respectively (Fig. 5). Immediately above the SF there is a water filled cavity, where hydrated Ca\(^{2+}\) ions can reside.

Both the Ca2+ and CIA-structures exhibit two physical sites of conformation in the ion channel pore at the conserved F2586 and I2590 residues of the pore-lining TM6 helix, consistent with previous studies\(^{16,17,20}\). The F2586 and I2590 side chains form two hydrophobic seals in the middle of the TM6 helix and near the cytosolic side of the pore, respectively (Fig. 5). Superimposition of IP3R1 structures in apo- versus Ca2+-bound and CIA-bound states shows that IP3R binding is associated with coordinated structural changes within the ion conduction pathway. In the Ca2+-IP3R1 structure, the cross-sectional area at the pore constrictions has the diameter of 6 Å and 5 Å, respectively, as estimated by the distance between side chains from two opposite subunits at F2586 and I2590. These pore dimensions are similar to that in the nanodisc reconstituted apo-IP3R1 structure, in which the pore is closed, representing non-conducting channel state\(^{16}\). In contrast, in the CIA-IP3R1, the diameter of the ion pathway at F2586 constriction is ~11 Å (Fig. 5). This pore expansion is achieved by 50° rotation of the F2586 side chain away from the four-fold axis and down toward the ER lumen. This side chain rearrangement makes the constricted pore permeable to hydrated Ca\(^{2+}\) ions, with a diameter of 8–10 Å\(^{44,45}\). However, the pore diameter at the position of I2590 remains ~5 Å as seen in the Ca2+-IP3R1 and apo-IP3R1 structures (Fig. 5a, b). The ion pathway is too narrow at this location for passage of hydrated Ca2+. Consistent with this observation, the pores in structures of several K+ channels and the human CLC-I chloride channel are also not wider than a hydrated ion under activating conditions\(^{50,53}\). Thus, it is conceivable that a slightly narrow tunnel lined with dynamic side chains apparently provide a good pass for hydrated ions.

Furthermore, the pore-lining TM6 helix in the CIA- and Ca2+-bound structures of IP3R1 adopts the α-helix conformation at residues M2576-V2581 residue and confers flexibility to the ion conduction helix bundle (Supplementary Fig. 7c). By contrast, the apo-structure of ND-reconstituted IP3R1 exhibits straight TM6 helices at this region\(^{16,20}\). On the basis of these observations, we propose that gating in IP3R involves an α-to-π secondary structure transition.

In both the Ca2+-IP3R1 and CIA-IP3R1 structures, the SF and a central cavity are continuous with the luminal solvent, and hydrated Ca\(^{2+}\) ions can diffuse into and out of the water filled luminal vestibule of the ion conduction pathway (Fig. 5b, d). Thus, the Ca-VI site is easily accessible for binding of Ca\(^{2+}\). However, it seems that the occupancy of this Ca\(^{2+}\) binding site is dependent on conformation of the TM6 helix. The TM6 rearrangement upon the channel activation is accompanied by movement of the F2586 side chain in the hydrophobic constriction and shift of the N2583 side chain, which coordinates Ca\(^{2+}\) ion in the Ca-VI site (Fig. 3; Supplementary Fig. 9). This can potentially weaken Ca\(^{2+}\)-coordinating interactions or even force loss of Ca\(^{2+}\) into the water filled central cavity from where it can travel down the electrochemical gradient in vivo.

Overall, the conformation of the ion conduction pore in the CIA-IP3R1 structure is compatible with that in the IP3R1 channel characterized in the presence of adenophostin A and Ca\(^{2+}\)\(^{16,17}\). We note, however, an additional strong density at the location of F2586 in the pore-lining TM6 helix, where the F2586 side chain can be alternatively docked in the CIA-IP3R1 structure. This density points to the central 4-fold axis of the pore and is consistent with the conformation of the F2586 side chain observed in the apo- or Ca2+-IP3R1 structures representing the IP3R channel in closed (non-conducting) state (Fig. 5b, c). In addition, the structural rearrangements of the SF observed in CIA-IP3R1 structure stand in contrast to what is seen in the IP3R1 visualized in the presence of activating concentrations of adenophostin A and Ca\(^{2+}\) (AdA-IP3R1), which SF is narrower (~8.5 Å) than that in apo-IP3R1. These structural differences might reflect the separate contexts in which these structures were determined, CIA-bound in a nanodisc and the other in the detergent. Noteworthy, the narrow diameter of the SF in apo-IP3R1 likely does not reflect any physiologically relevant conformation as the luminal vestibule of the channel pore is normally

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exposed to the ER lumen containing calcium in the low millimolar range. While the Ca\(^{2+}\) binding to the luminal sites may have a regulatory function, it remains to be established whether Ca\(^{2+}\) store depletion could lead to dissociation of Ca\(^{2+}\) from the luminal vestibule.

To further investigate functionality of the amino acids at the constriction site, mutants of residues F2586 (F2586K) and I2590 (I2590N and I2550T) were generated in human IP3R1 (hR1). The F2586K mutation replaces a large, hydrophobic Phe with a positively charged Lys, while the I2590N and I2550T mutations are associated with human disease. The mutations were stably overexpressed in cells engineered via CRISPR/Cas9 to lack all three endogenous IP3Rs (HEK-3KO) and endogenous hIP3R1 (Endo. hIP3R1) were generated by CRISPR/Cas9 technology, the former is null for all IP3R subtypes while the latter expresses only IP3R1. Mutations and exogenously expressed IP3R1 (Exo. hR1) were stably expressed in HEK-3KO cells. All stable clonal cell lines result in expression levels above that of Endo. hIP3R1, as quantified in b. Data are presented as mean values \(\pm\) SEM \(\times 3\) independent experiments. HEK cell lines are color coded: blue—HEK-3KO, purple—endo hIP3R1, green—exo hIP3R1, pink—hIP3R1 F2546K #25, magenta—hIP3R1 F2546K #47, orange—hIP3R1 I2550T #116, red—hIP3R1 I2550N #61.5, brown—hIP3R1 F2550N #616. 

**Fig. 6** | Functional validation of gating residues in ion conduction pathway. 
a) Immunoblots of lysates prepared from the indicated HEK cell lines. HEK-3KO and endogenous hIP3R1 (Endo. hIP3R1) were generated by CRISPR/Cas9 technology, the former is null for all IP3R subtypes while the latter expresses only IP3R1. Mutations and exogenously expressed IP3R1 (Exo. hR1) were stably expressed in HEK-3KO cells. All stable clonal cell lines result in expression levels above that of Endo. hIP3R1, as quantified in b. Data are presented as mean values \(\pm\) SEM \(\times 4\) independent experiments. HEK cell lines are color coded: blue—HEK-3KO, purple—endo hIP3R1, green—exo hIP3R1, pink—hIP3R1 F2546K #25, magenta—hIP3R1 F2546K #47, orange—hIP3R1 I2550T #116, red—hIP3R1 I2550N #61.5, brown—hIP3R1 F2550N #616. b) Representative single cell Ca\(^{2+}\) traces in fura-2 loaded HEK cell lines stimulated with increasing concentrations of the muscarinic agonist carbachol (CCh). d) Basal fluorescence, prior to stimulation in HEK cell lines. e) Pooled data depicting the response of cell lines to CCh stimulation. Data in d–e are presented as mean values \(\pm\) SEM \(\times 60\) cells over three independent experiments. *significantly different from Endo. hIP3R1. #significantly different from Exo. hIP3R1. For 3\(\mu\)M CCh: ***p < 0.0001; **p = 0.0007; *p = 0.0395; for 30\(\mu\)M CCh and 100\(\mu\)M CCh: **p = 0.0001 One-way ANOVA with Tukey’s post-hoc test. Source data are provided as Source Data file.
charged K creates electrostatically unfavorable environment for the ARM2 switches between activating ligands propagating from the LBDs forming the apical portion of the channel opposing subunits colored by domains. Domain motions are indicated with arrows. Structures are viewed along the central 4-fold axis from the cytosol with one subunit outlined in black.

**Discussion**

The high-resolution structures of Ca\(^{2+}\) and Ca\(^{2+}\)/IP\(_3\)/ATP-bound IP\(_R\) presented in this study highlight the correlation between the protein dynamics and its propensity for accepting multiple cellular signals that regulate the channel activities, such as ligand-binding, gating and regulation. Considering the remote distances between the cytoplasmic ligand-binding sites and ion conduction pathway in the channel, the intrinsically flexible 3D architecture of the IP\(_R\) provides the basic premise behind the allosteric regulation of the channel activity that involves the transfer of ligand-evoked signals from the distinct ligand-binding domains to the ion conducting channel gate (Fig. 7). This process is mediated through molecular interactions at inter- and intra-domain interfaces embedded in the conformational landscape of the protein. While some structural features of the ligand-bound IP\(_R\) that we highlighted here confirm our previous observations\(^{60,61}\), the present study reveals mechanisms for the conformational transitions underlying IP\(_R\) activity.

Using deep-learning based modeling\(^{22}\) and 3D variability analysis\(^{29}\), we observe that the ARM2 domain exhibits the unique motions within the dynamic conformational ensemble, resulting in two predominant conformations seen in CIA- and Ca\(^{2+}\)-bound states. In the IP\(_3\)-binding process in the context of the tetrameric channel assembly, intrinsic flexibility of the ARM2 domain is highly important as its dynamic behavior perhaps allows to release structural constraints imposed at interaction interfaces between LBD and ARM2 domains that facilitates the conformational selection from a pre-existing ensemble of IP\(_3\)-binding pocket conformations and promotes specific binding of the IP\(_3\) molecule. Based on our results, the dynamic interactions between the ARM2 and LBDs can be envisaged as a reversible ratchet mechanism with the gear (ARM2) held in place by its contacts with βTF1 and ARM1 domains from the neighboring subunit (Fig. 7a). This dynamic re-arrangement allows direct (clockwise) as well as reverse (counterclockwise) movements of the ARM2 between ‘extended’ and ‘retracted’ positions with the latter favorable for specific binding of IP\(_3\). Altogether, these results allow us to delineate distinct steps in IP\(_3\)-binding conformational cycling, including interfacial rearrangements. It appears that IP\(_3\) may work by tuning the Ca\(^{2+}\) sensitivity of IP\(_R\)R1 by decreasing the Ca\(^{2+}\) affinity of the Ca\(^{2+}\)-ILBD site and stimulating Ca\(^{2+}\) binding to the Ca\(^{2+}\)-ILBD site. The conformational changes observed in the IP\(_3\)-binding domains unequivocally propagate through other cytoplasmic domains to the TMDs that form the channel pore (Fig. 7).

It is important to note that our high-resolution CIA-IP\(_R\)R1 structure demonstrates that under saturating activating conditions used in this study, the gating state of the channel pore likely relies on conformational dynamics of the F2586 side chains that can potentially alter the relative stability of conformational states of the pore. Hence, instead of directly going from closed to open conformation, the pore-forming elements can adopt a mixture of conformations resulting in different levels of IP\(_R\) channel activity observed under constant experimental conditions in electrophysiological studies\(^{59,60}\). This phenomenon known as modal gating has been observed in many other ion channels including RyR Ca\(^{2+}\) release channel\(^{62-64}\).

The observed degree of pore opening at I2590 in the CIA-IP\(_R\)R1 structure is not as wide as a hydrated Ca ion, however it might provide a good passage for Ca\(^{2+}\) permeation using a mechanism relying on dynamic side chains shaping the ion conduction pathway. Note-worthy, the structures of many other ion channels studied under activating conditions exhibit the pore opening to less degree than a hydrated ion\(^{59,63}\). The presence of the π-gating hinge in IP\(_R\) channel is consistent with structures of IP\(_R\)R3 channel, solved by cryo-EM\(^{65-67}\), suggesting that...
gating in both channel types is prone to α-to-τ transitions. However, the detergent-solubilized IP3R3 channel exhibits the τ-helix in TM6 in the non-conducting (closed) state, which transitions to an α-helix in the presence of IP3 and Ca2+. Strikingly, unlike IP3R3, the ligand-bound structures of IP3R1 reconstituted into lipid nanodisc solved in this study, appear to favor the τ-helix upon the channel activation. Together, these data suggest that the lipid environment plays an essential role in formation and maintenance of τ-helix structure.

Furthermore, the results of the present study are supportive of the importance of side chain dynamics in ATP binding by IP3R1 protein. Studies suggest that the three IP3R isoforms exhibit different sensitivity to ATP with IP3R1 being enhanced by micromolar ATP and IP3R3 being augmented by millimolar ATP, whereas IP3R2 is more sensitive to ATP but only at sub-micromolar IP332,33,44. Our analysis revealed a side chain conformational preference for W2639 that contributes to ATP binding. This observation supports the hypothesis that ATP-binding specificity of IP3R3s might be mediated by the recognition of specific rotameric states of key residues in the ATP-binding pocket. Additionally, the presence of conserved lysine residues responsible for the coordination of ATP phosphates in IP3R1 likely contribute to isoform specific ATP binding affinity. Decoding the structural basis of exquisite isoform specificity with respect to a modulatory role of ATP in the IP3R family will certainly be critical in future studies.

Overall, our analysis exposes a structural mechanism for the susceptibility of IP3R1 to binding of IP3, based on the conformational selection of the ligand-binding pocket, which may adapt different conformations in its unbound state for which IP3 binds selectively to the cytoplasmic domains towards the pore-forming TMDs (Fig. 7 and Supplementary Movies 1–3). The present results highlight a key role of specific interactions in the side chain network surrounding the ion conduction path in regulating gating behavior of IP3R channels. Notably, the structural rearrangements in the cytoplasmic domains are substantial relative to the transmembrane domain suggesting that the structural flexibility allows the protein domains to adapt to their individual molecular binding partners facilitating the binding process. Our study provides a firm structural framework for interpreting large amounts of functional and biochemical data accumulated during more than three decades of research targeting IP3-mediated Ca2+ release. However, further systematic work combining structural analysis with site-directed mutagenesis and electrophysiological characterization is still needed to test connections between functional paths and protein motions underlying ligand-mediated allosteric information transfer within the channel.

Methods

**IP3R purification and reconstitution into nanodiscs**

Purification of neuronal IP3R1 reconstituted was performed as previously described20. Briefly, rat cerebellar membranes were solubilized in 2 mM Lauryl Maltose Neopentyl Glycerol (LMNG, Anatrace) and 0.1% (w/v) 1-α-phosphatidylcholine (PC, Sigma) for 2 h at 4 °C. Non-solubilized material were cleared by ultracentrifugation (100,000 X g) and the supernatant was applied to an immunoaf

**CryoEM sample preparation and data acquisition**

The nanodisc reconstituted purified IP3R1 (2 mg/ml) was vitrified after 30 min in incubation with 20 μM free Ca2+ to obtain the structure of IP3R1 bound with Ca2+ (Ca-IP3R1) or with 2 μM free Ca2+, 10 μM IP3 and 1 mM ATP to obtain Ca/IP3/ATP-bound IP3R1 (CA-IP3R1). Vitrification of the IP3R1 samples was performed using a Vitrobot Mark IV (ThermoFisher Scientific, Inc.) as described earlier20. Blotting was done with filter paper Whatman 542 containing 8–14 μg Ca/g paper. The final free Ca2+ concentrations were determined using MaxChelator (http://maxchelator.stanford.edu/oprog.htm). CryoEM data acquisition was performed with a Titan Krios microscope (ThermoFisher Scientific, Inc.) operated at 300 keV, equipped with a BioQuantum energy filter (Gatan, Inc.) with zero-loss energy selection slit set to 20 eV. All data sets were acquired using the EPU software (ThermoFisher Scientific, Inc.) at a nominal TEM magnification of 130,000X and recorded on a K2 Summit direct electron detector (Gatan, Inc.) operated in super-resolution counting mode with a calibrated physical pixel size of 1.07 Å. The data acquisition parameters (total movie stacks, dose rate, total dose, defocus range, etc.) are summarized in Table 1 and Supplementary Figs. 1, 3, 5.

**Image processing**

Movie stacks were motion corrected with dose weighting and binned 2 × 2 by Fourier cropping resulting in a pixel size of 1.07 Å using MotionCor2.68. The motion-corrected micrographs were evaluated by ‘e2evalimage.py’ in EMAN269 and were used for all the following image processing. Contrast transfer function (CTF) determination was performed using Gctf70. Particles were picked using the NeuralNet autopicking procedure implemented in EMAN2 and the .box sets were acquired using the EPU software (ThermoFisher Scientific, Inc.) as described earlier20. Blotting was done with filter paper Whatman 542 containing 8–14 μg Ca/g paper. The final free Ca2+ concentrations were determined using MaxChelator (http://maxchelator.stanford.edu/oprog.htm), CryoEM data acquisition was performed with a Titan Krios microscope (ThermoFisher Scientific, Inc.) at a nominal TEM magnification of 130,000X and recorded on a K2 Summit direct electron detector (Gatan, Inc.) operated in super-resolution counting mode with a calibrated physical pixel size of 1.07 Å. The data acquisition parameters (total movie stacks, dose rate, total dose, defocus range, etc.) are summarized in Table 1 and Supplementary Figs. 1, 3, 5.

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**Gaussian Mixture Model (GMM) based heterogeneity analysis in EMAN2**

Overall analysis was performed as described in22, with adaptations to combine the three particle populations and C4 pseudosymmetry. Particles of IP3R1 under three different conditions (apo, CIA and Ca) were combined and a single averaged structure with C4 symmetry was reconstructed from the particles, which was used as the neutral state model for ARM2 domain, and the particles from the best featured classes were subjected to local refinement (Supplementary Figs. 1–5). The expanded consensus particles were imported to EMAN222,69 and cryoSPARC23,24 to analyze the conformational heterogeneity of the particles. Flow charts for data processing steps are presented in Supplementary Figs. 1, 3, 5.
ARM2 domain could only translate/rotate together as a rigid body, and only a single subunit of IP3R1 was allowed to deviate from the neutral state position. After training the network model, the first eigen-vector on the conformational space exhibited a clear extended-retracted motion of the ARM2 domain. The three distinct particle subsets all exhibited motion on the same pathway in the ARM2 domain, but to differing degrees, with the CIA data set exhibiting more motion than the other two states.

3D variability analysis in cryoSPARC
Symmetry expanded consensus particles were analyzed using the 3D Variability Analysis (3DVA) module. For each cryo-EM data set, five modes of variability were calculated. Ten maps for each mode were generated using a 4 A low-pass filter. In each state, the ARM2 domain displayed the largest motions of similar trajectories, regardless of symmetric and symmetry-breaking variability. To visualize the conformational variability across the channel protein assembly, the maps from the modes representing C4 motion (mode 0 of CIA-IP3RI, mode 4 of Ca-IP3RI, and mode 1 of apo-IP3RI) were examined in UCSF Chimera as volume series and recorded as movies (Supplementary Movies 1–3).

Model building
We utilized a consistent approach for protein model building in both CIA-IP3RI and Ca-IP3RI cryo-EM density maps. Our previously published cryo-EM structure of apo-IP3RI in nanodisc (PDB ID: 7LHE) was used for rigid body fitting in UCSF Chimera followed by an initial round of flexible fitting of the IP3R1 subunit in COOT. Density modification “phenix.resolve_cryo_em” was performed on the half-maps resulting in improved resolvability in the density maps allowing for further model optimization. AlphaFold was used to generate optimized models for each of the ten domains within an IP3RI subunit using the initial IP3RI model for each domain as a template. The results were then concatenated and tetramerized in Chimera, refined against the density map with “phenix.real_space_refine” with default options and manual optimization in Coot to maximize fit to density, minimize Ramachandran angle outliers and eliminate steric clashes. Ligands, including lipids, calcium, IP3, and ATP, were identified using “pw_ligands.py”, a stand-alone ligand identification tool based on Pathwalking. Briefly, “pw_ligands.py” identifies all non-protein density, analyzes the geometry of the un-modeled density and masks out ligands and water/ion density from the cryoEM map. At the reported resolutions, identification of non-ion/non-water ligands is robust, though it is difficult to distinguish waters from ions based purely on density profile. All prospective water/ion densities were examined; only clearly distinguishable densities with obvious coordinating chemistry were considered as ions. The ligands were fit to the masked densities and refined along with the protein model using “phenix.real_space_refine”. Model validation was carried out using EMRinger and MolProbity in PHENIX. Maps are described in the paper were identified with PDBBePisa, ChimeraX, LigPlot, and HOLEx. Figures and movie were prepared using UCSF Chimera; ChimeraX and VMD 1.9.4.

Generation of IP3R mutants
A two-step QuikChange mutagenesis protocol was used to introduce amino acid substitutions into cDNA encoding the human IP3R1 (hIP3R1; NM_001099952 in pDNA3.1 (Obtained from Annetta Wronska at Columbia University)). Mutagenesis and all DNA modifications were carried out using ffla Ultra II Hotstart 2X Master Mix (Agilent). Mutagenesis primers for hIP3RI F2586K forward (TTCCTGCTACTAGTGGTAAACCAGAAATGTGATTTGCTCAA), hIP3RI I2590T forward (TTTGGGTTACCTGAGTTAAGGGGTTATCATTGAGACT), and hIP3RI I2590T reverse (CTGGTTCCTACCTGACTGAGAAATGTGATTTGCTCAA) were synthesized by Integrated DNA Technologies (IDT). In addition to encoding the specified mutations, primers also silently introduced a restriction site for verification purposes. The coding regions for all constructs were confirmed by sequencing.

Cell culture and generation of HEK cell lines stably expressing IP3R monomers
HEK-293s were originally obtained from ATCC (CRL-3022). HEK-3KO cells, HEK293 cells engineered through CRISPR/Cas9 for the deletion of the three endogenous IP3R isoforms and HEK293 cells modified by CRISPR/Cas9 to only express endogenous hIP3RI (Endo. hR1) were grown at 37°C with 5% CO2 in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco/Life Technologies). Transfection of HEK 3KO cells to exogenously express desired hIP3RI WT or mutant constructs stably was performed as previously described. In brief, five million cells were pelleted, washed once with PBS, and resuspended in either Nucleofector Solution T (Lonza Laboratories) or a homemade transfection reagent (362.88 mM ATP-disodium salt, 590.26 mM MgCl2, 6.1479 mM KH2PO4, 23.81 mM NaHCO3, and 3.7 mM glucose at pH 7.4). 4–6 µg of DNA was mixed with the resuspended cells and electroporated using the Amann cell nucleofector (Lonza Laboratories) program Q-001. Cells were allowed to recover for 48 h before passage into new 10 cm plates containing DMEM media supplemented with 2 mg/ml Geneticin sulfate (G418; VWR). Following 7 days of selection, cell colonies were either picked and transferred to new 24-well plates or diluted into 96 well-plates, both of which contained DMEM media supplemented with 2 mg/ml G418. Diluted wells in the 96-well plate were screened after 7 days for the presence of a single colony of cells growing in each well. Those wells that exhibited multiple colonies growing were excluded from further consideration. 10–14 days after transfection, wells—both 96 and 24—that exhibited growth were expanded and those expressing the desired constructs were confirmed by western blotting.

Cell Lysis and SDS-PAGE analyses
HEK-3KO cells and HEK-3KO cells stably expressing IP3R constructs were harvested by centrifugation (200 x g for 5 min), washed once with PBS, and solubilized in membrane-bound extraction lysis buffer containing 10 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, 1 mM Na2, 20 mM Na3PO4, 2 mM Na2HPO4, 1% Triton X-100 (v/v), 0.5% sodium deoxycholate (w/v), and 10% glycerol supplemented with a cocktail of protease inhibitors (Roche, USA). Lysates were incubated for a minimum 30 min on ice and cleared by centrifugation (16,000 x g for 10 min) at 4°C. Protein concentrations in cleared lysates were determined using D2 protein assay kit (Bio-Rad) and 4x SDS gel loading buffer was subsequently added to 5 µg of lysate. Proteins were resolved using 8% SDS-PAGE and subsequently transferred to a nitrocellulose membrane (Pall Corporation). Membranes were probed with a rabbit polyclonal antibody against the C-terminal 19aa of IP3R1 (custom generated by Antibody Research Corporation) at a 1:1000 dilution, GAPDH (#AM4300, Invitrogen) at a 1:75,000 dilution, and the appropriate Dylight™ 800CW secondary antibodies at a 1:10,000 dilution (SA53571 and SA53521; Invitrogen). Membranes were imaged with an Odyssey infrared imaging system and quantified using Image Studio Lite (LI-COR Biosciences).

Single Cells Ca2+ imaging: measurement of cytosolic Ca2+ in intact cells
Single cell Ca2+ imaging was performed in intact cells as described previously. Glass coverslips were plated with HEK-3KO, Endo. hR1,
Exo. hRI, or HEK-3KO cells stably expressing IP3R mutant constructs at least 18 h prior to imaging experiments. Subsequently, the glass coverslips were mounted onto a Warner chamber and the cells were loaded with 2 μM Fura-2/AM (Molecular Probes) in Ca2+ Imaging Buffer (Ca2+ IB; 10 mM HEPES, 1.26 mM Ca2+, 137 mM NaCl, 4.7 mM KCl, 5.5 mM glucose, 1 mM NaHPO4, 0.56 mM MgCl2 at pH 7.4) at room temperature for 25 min. Following loading, cells were then perfused with Ca2+ IB which provided a basal 340/380 ratio of the [Ca2+] and stimulated with 3 μM, 30 μM, and 100 μM carbacol (CCh) to obtain measurements of agonist-induced Ca2+ release into the cytoplasm.

Ca2+ imaging was performed using an inverted epifluorescence Nikon microscope with a 40x oil immersion objective. Cells were alternately excited at 340 and 380 nm, and emission was monitored at 505 nm. Images were captured every second with an exposure of 15 ms and 4 × 4 binning using a digital camera driven by TILL Photonics software. Image acquisition was performed using TILLvision software and data was exported to Microsoft Excel where the means were calculated. Statistical analysis of at least n = 3 experiments for each cell line was performed in GraphPad Prism (one-way ANOVA with Tukey’s test).

**Reporting summary**
Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

**Data availability**
Data supporting the findings of this manuscript are available from the corresponding author upon reasonable request. The cryo-EM density maps have been deposited in the Electron Microscopy Data Bank (EMDB) under accession codes EMD-27983 (CIA-IP3R1), EMD-27982 (Ca-IP3R1). The coordinates have been in the RCSB Protein Data Bank (PDB) under accession codes EAR (CIA-IP3R1), EAQ (Ca-IP3R1). Data from our previously resolved cryo-EM map of apo-IP3R1 (EMD-23337 and coordinates 7LHE) were used in this study. Source data underlying Fig. 6 is available as a Source Data file. Source data are provided with this paper.

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Author contributions
I.I.S. and D.I.Y. conceived the project; G.F., M.R.B., A.B.S. prepared samples of IP3R1; G.F. collected cryo-EM data and determined structures; G.F., M.R.B. and M.L.B. built and refined the atomic models; G.F., M.R.B., M.L.B. and I.I.S. analyzed the structures; S.J.L., M.C. and G.F performed variability analysis; L.E.T and V.A made mutations in IP3R and performed functional analyses of IP3R activity; I.I.S. wrote the paper with the help from M.R.B. and G.F., and all other co-authors.

Competing interests
The authors declare no competing interests.

Additional information

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