

Published in final edited form as:

Nature. ; 483(7387): 108–112. doi:10.1038/nature10751.

Structural and functional conservation of key domains in InsP₃ and ryanodine receptors

Min-Duk Seo^{1,*}, Saroj Velamakanni^{2,*}, Noboru Ishiyama¹, Peter B. Stathopoulos¹, Ana M. Rossi², Samir A. Khan², Philippa Dale², Congmin Li³, James B. Ames³, Mitsuhiro Ikura¹, and Colin W. Taylor²

¹Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, Ontario, M5G 1L7, Canada

²Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1PD, UK

³Department of Chemistry, University of California, Davis, California, 95616, USA

Abstract

Inositol 1,4,5-trisphosphate receptors (InsP₃R) and ryanodine receptors (RyR) are tetrameric intracellular Ca²⁺ channels¹. For each, the pore is formed by C-terminal transmembrane domains and regulated by signals detected by the large cytosolic structures. InsP₃R gating is initiated by InsP₃ binding to the InsP₃-binding core (IBC, residues 224–604 of InsP₃R1)² and it requires the suppressor domain (SD, residues 1–223)^{2–8}. We present structures of the N-terminal region (NT) of InsP₃R1 with (3.6 Å) and without (3.0 Å) InsP₃ bound. The arrangement of the three NT domains, the SD, IBC-β and IBC-α, identifies two discrete interfaces (α and β) between the IBC and SD. Similar interfaces occur between equivalent domains (A, B and C) in RyR⁹. The orientations of the three domains docked into a tetrameric structure of InsP₃R¹⁰ and of the ABC domains in RyR⁹ are remarkably similar. The importance of the α-interface for activation of InsP₃R and RyR is confirmed by mutagenesis and, for RyR, by disease-causing mutations^{9,11,12}. InsP₃ causes partial closure of the clam-like IBC, disrupting the β-interface and pulling the SD towards the IBC. This reorients an exposed SD loop (HS-loop) that is essential for InsP₃R activation⁷. The loop is conserved in RyR and includes mutations associated with malignant hyperthermia and central core disease^{9,11,12}. The HS-loop interacts with an adjacent NT, suggesting that activation re-arranges inter-subunit interactions. The A-domain of RyR functionally replaced the SD in a full-length InsP₃R, and an InsP₃R in which its C-terminal transmembrane region was replaced by that from RyR1 was gated by InsP₃ and blocked by ryanodine. Activation mechanisms are conserved between InsP₃R and RyR. Allosteric modulation of two similar domain interfaces within an N-terminal subunit re-orientates the first domain (SD or A-domain), allowing it, via interactions of the second domain of an adjacent subunit (IBC-β or B-domain), to gate the pore.

Correspondence and requests for materials should be addressed to C.W.T (cwt1000@cam.ac.uk) or M.I. (mikura@uhnres.utoronto.ca).

*These authors contributed equally.

Author Information The atomic coordinates of NT^{Cysless} of rat InsP₃R1 with (3UJ4) and without InsP₃ bound (3UJ0) have been deposited in the Protein Data Bank. The authors declare no competing financial interests.

Full Methods and associated references are available in the online version of the paper.

Supplementary Information is linked to the online version of the paper.

Author Contributions M.S., N.I., P.B.S., M.I. and C.L. determined and analysed the structure of NT. S.V. prepared and characterized the full-length InsP₃R and chimeras. A.M.R., S.A.K. and P.D. completed analyses of InsP₃ binding and related molecular biology. J.B.A., M.I. and C.W.T. supervised work in their respective laboratories, coordinated the project and, with input from other authors, wrote the paper.

The essential role of the SD in linking InsP₃ binding to InsP₃R gating highlights the need to define the structural consequences of InsP₃ binding to the NT (residues 1-604 of InsP₃R1) (Supplementary Fig. 1). Because our attempts to crystallize the NT yielded poorly diffracting crystals, we expressed a Cys-less form of the NT (NT^{Cysless}). Native and Cys-less forms of the NT and IBC behaved indistinguishably (Supplementary Fig. 2 and Supplementary Tables 1-2), but NT^{Cysless} provided crystals with much improved diffraction (Supplementary Table 3). We determined crystal structures of NT^{Cysless} with (3.6 Å) and without (3.0 Å) InsP₃ bound, showing three subdomains: the SD, IBC-β (residues 224-436) and IBC-α (residues 437-604) (Fig. 1a). The structures of these subdomains were nearly identical to those of isolated native SD and IBC^{2,3} (Supplementary Fig. 3).

The SD, IBC-β and IBC-α form a triangular structure, with the SD behind the InsP₃-binding site (Fig. 1a). The SD interacts via two interfaces with the IBC, one with IBC-β (β-interface) and another with IBC-α (α-interface). A 3¹⁰-like turn between the last strand of the SD and the first strand of IBC-β positions the IBC relative to the SD (Supplementary Fig. 4e). Within this connecting turn, a salt bridge (K225/D228) stabilizes the backbone conformation and so positions residues that form the β-interface. These interactions in the connecting turn and β-interface are augmented by a network of hydrophobic interactions within IBC-β (Fig. 1b). The α-interface forms a long ‘Velcro’-like structure that also involves a network of hydrophobic and electrostatic interactions (Fig. 1c). Intimate hydrophobic interactions between V33, and to a lesser extent L32, from the SD; and V452, F445, A449 and L476 from IBC-α are supported by bidentate salt bridges between R54/K127 in the SD and D444 in IBC-α (Fig. 1c). The V33K mutation at the α-interface almost abolished inhibition of InsP₃ binding by the SD^{3,4} and reduced channel open probability⁴, confirming its importance. Mutation of neighbouring residues that contribute less to the α-interface (L32K, D34K, R36E, K127E) had lesser effects on InsP₃ binding, while mutation of residues that do not contribute to the interface (D35K, K52E) had no effect (Supplementary Table 4)^{3,4}. Hydrophobic and electrostatic interaction networks at the α- and β-interfaces contribute to a buried surface between the SD and IBC (~2040 Å²) that forms a hub connecting InsP₃ binding to channel activation.

The structure of the NT is remarkably similar to that of the N-terminal of RyR1⁹. The three NT domains of InsP₃R1 (SD, IBC-β and IBC-α) can be individually superposed to corresponding domains of RyR1 (A, B and C) (Supplementary Fig. 3), and the relative orientation of the domains is nearly identical (Fig. 1d). Mutation of Y167A, located on an exposed loop of the SD opposite the IBC interfaces (‘HS-loop’¹¹, residues 165-180, boxed in Fig. 1d), attenuates InsP₃-evoked Ca²⁺ release⁸; and Ca²⁺, a co-regulator of InsP₃R¹⁴, causes the loop to become accessible.¹⁵ The disease-associated ‘hot spot loop’ of RyR1¹¹ sits at the same location within the ABC structure⁹ (Fig. 1d) and a mimetic peptide causes RyR2 to become leaky¹⁶. Furthermore, the backbone and side chain conformation of this loop region superposes well in the two receptors (Fig. 1e). The HS-loop provides a critical link between InsP₃ binding and gating.

The domain interfaces of InsP₃R1-NT and RyR1-ABC are also similar. The bidentate salt bridges between R54/K127 and D444 at the InsP₃R1 α-interface are preserved in RyR1 ABC, albeit in a reversed-charge manner between D40/D61 and R402 (Supplementary Fig. 4a). In RyR1, mutation of these residues (R402C, D61N) is associated with malignant hyperthermia and central core disease⁹, suggesting that disruption of the interaction perturbs RyR gating, as it does for InsP₃R. The structural similarities extend also to the β-interface of InsP₃R1 and corresponding A/B interface in RyR1 (Supplementary Fig. 4b-d).

Our structures of NT^{Cysless} with and without InsP₃ bound, together with that of the InsP₃-bound IBC² (Supplementary Fig. 5) reveal the structural changes evoked by InsP₃ (Fig. 2). Side chains of nine residues become organized around InsP₃ (Supplementary Fig. 5a), and the domain orientation angle between IBC-β and IBC-α is reduced (by ~8°) after InsP₃ binding (Fig. 2 and Supplementary Fig. 5a). This InsP₃-evoked ‘clam closure’, which is consistent with earlier predictions¹⁷ and small-angle X-ray scattering¹⁸, causes the distance across the entrance to the InsP₃-binding pocket to decrease (Supplementary Fig. 5b, c). A similar agonist-evoked domain closure occurs in some glutamate receptor channels¹⁹. The SD and IBC remain associated after closure of the IBC (Fig. 2). InsP₃ binding hardly changes the interactions across the extensive α-interface, but at the β-interface the SD residues move away from IBC-β (Supplementary Fig. 5d-f). With the SD glued to IBC-α by the α-interface, and the β-interface serving as a lubricant, InsP₃ binding causes the SD to twist (by ~9°) and move closer to the top of the IBC (Fig. 2). This causes an amplified translational movement of the conserved HS-loop in the SD (Supplementary Fig. 5g). While our work was under review, 3.8 Å structures of apo- and InsP₃-bound NT derived from a single crystal grown in excess InsP₃ were published, showing similar InsP₃-induced allostery in the interfaces between domains¹³. This confirms our observations, but our higher resolution structures reveal more detail of the α- and β-interfaces associated with this conformational change (Supplementary Discussion).

Docking the ABC structure into cryo-EM maps of RyR1 showed that the N-terminal domains form a central ring at the top of the mushroom-like RyR1⁹. Rigid-body docking of our apo-NT^{Cysless} structure into a cryo-EM 10 Å structure of a closed InsP₃R1¹⁰ reveals an arrangement remarkably similar to that of RyR1 with a high docking contrast (Fig. 3 and Supplementary Fig. 6). The three domains of the four NTs, which form the upper cytoplasmic surface of the mushroom-like InsP₃R, are arranged as four hillocks around a central bowl. This arrangement allows InsP₃ unrestricted access to the IBC from the side of the cap (Fig. 3), and it is consistent with accessibility studies and binding sites for regulatory proteins (Supplementary Fig. 6c and Supplementary Table 5). Within the tetrameric InsP₃R, the only contacts between NT subunits are via the critical HS-loop of the SD and a flexible loop (β20-β21, Supplementary Fig. 7) in IBC-β (Fig. 3c, d). The latter is longer in RyR and it lies ~10 Å further from the neighbouring hot spot loop⁹ (Fig. 3c, d). In InsP₃R, the arm domains (residues 67-109) of each SD are the only NT structures that extend beyond the cap towards the pore (Fig. 3a), but these domains are neither essential for InsP₃R activation⁸ nor conserved in RyR^{9,11}.

The structural similarities between the N-termini of RyR and InsP₃R prompted us to examine whether the domains are functionally interchangeable. In a chimeric N-terminal fragment comprising the A-domain of RyR2 and IBC from InsP₃R1 (RyR2A-IBC), the A-domain mimicked the SD by inhibiting InsP₃ binding (Fig. 4a, b). Mutations within the A-domain loop that forms the A-B interface in RyR⁹ or the equivalent InsP₃R loop in the SD attenuated this inhibition of binding (Fig. 4c, Supplementary Table 6 and Supplementary Fig. 8). InsP₃ stimulated Ca²⁺ release via InsP₃R1 or a chimeric InsP₃R1 in which the SD was replaced by the A-domain of RyR1 (RyR1A-InsP₃R1 (Fig. 4a, d and Supplementary Fig. 9). Both InsP₃Rs were similarly expressed and they released similar fractions of the Ca²⁺ stores and with similar sensitivity to InsP₃ (Supplementary Table 7). Opening of native InsP₃R or RyR is restrained by interactions between cytosolic domains^{20,21}. It is therefore significant that expression of InsP₃R1 or RyR1A-InsP₃R1 affected neither the Ca²⁺ content of the ER nor the Ca²⁺ leak from it (Supplementary Fig. 10), confirming that InsP₃R and RyR1A-InsP₃R1 have no detectable spontaneous activity. This demonstrates that the SD of InsP₃R can be functionally substituted by the A-domain of RyR.

An InsP₃R1 in which residues downstream of TMD1 were replaced by the equivalent region of RyR1 (InsP₃R1-RyR1) also responded to InsP₃ (Fig. 4a,e). Expression of InsP₃R1-RyR1 increased Ca²⁺ leak from the ER, and this was reversed by ryanodine, which blocks the RyR pore²². However, the increased leak was insufficient to affect the steady-state Ca²⁺ content (Supplementary Fig. 10), suggesting that InsP₃R1-RyR1 has minimal spontaneous activity. Expression of InsP₃R1-RyR1 matched that of other InsP₃Rs, but cells expressing InsP₃R1-RyR1 were ~20-fold less sensitive to InsP₃ (Supplementary Table 7). Because the TMDs minimally affect InsP₃ binding²³, this diminished response probably reflects a decrease in InsP₃ efficacy. The increased Ca²⁺ leak and reduced efficacy of InsP₃ suggest that within InsP₃R1-RyR, communication between the SD and channel are slightly less effective than in native InsP₃R. Nevertheless, it is remarkable that cytosolic domains of an InsP₃R should so effectively regulate the pore of a RyR when the two receptors share only modest sequence identity and differ in the number of residues separating the NT from TMDs (Fig. 4a), and in the lengths and sequences of their C-terminal tails and the loops linking TMDs (Supplementary Fig. 11).

Ryanodine (10 μM) had no effect on InsP₃R1 or RyR1A-InsP₃R, but it abolished InsP₃-evoked Ca²⁺ release via InsP₃R1-RyR1 (Fig. 4e, f). Because ryanodine binds selectively to active RyR²⁴, ³H-ryanodine binding is stimulated by agonists of RyR, like caffeine. Whereas caffeine had no effect on specific ³H-ryanodine binding to InsP₃R1-RyR1, InsP₃ stimulated it (Fig. 4g). InsP₃ therefore causes conformational changes to the channel of InsP₃R1-RyR1 that mimic those of native RyR in allowing binding of ³H-ryanodine.

Conservation of structure-function relationships between InsP₃R and RyR (Fig. 1-4) allows comparisons between them to suggest possible mechanisms of InsP₃R activation. For both receptors, gating requires that conformational changes in the large cytoplasmic structures pass to the TMDs^{10,22}, but the N-terminal domains of InsP₃R and RyR are at least 60 Å from these TMDs^{1,9,22} (Fig. 3a and Supplementary Fig. 6). Despite some evidence implicating direct interactions between the SD and TMD4-5 loop in gating InsP₃R (Supplementary Discussion), we suggest, and in keeping with results from RyR^{20,26,27}, that additional cytosolic domains couple the NT to opening of the pore. The exposed HS-loop in the SD (Fig. 1d and 3c,d) (hot spot-loop of RyR)^{9,11} is arranged similarly within the isolated N-terminal structures of InsP₃R and RyR (Fig. 1d) and it reorients after InsP₃ binding (Fig. 2). When the NT is docked into the InsP₃R structure¹⁰, the HS-loop forms (with an exposed loop of IBC-β) the only interface between adjacent NT, however, the equivalent loop is displaced in the RyR⁹ (Fig. 3c, d). InsP₃ binding closes the clam-like IBC, disrupting the β-interface and re-orienting the HS-loop (Fig. 2 and Supplementary Fig. 5). This, we suggest, disrupts interaction of the HS-loop with a neighbouring NT to cause a coordinated rearrangement of the apical InsP₃R structure (Fig. 3). The open state of RyR1 is associated with outward movement of protein density in regions that match the locations of docked ABC structures^{9,22} and with larger movements of peripheral ‘clamp domains’^{9,22} that are absent from InsP₃R¹⁰. Movement of these apical domains in RyR is accompanied by rearrangements within regions that taper towards the pore²² and which, in InsP₃R, include the most flexible parts of its structure¹⁰. We suggest that similar rearrangements of the apical surface of InsP₃R and RyR couple by shared mechanisms to additional cytosolic domains to gate the pore of each channel.

METHODS SUMMARY

The N-terminal (NT, residues 1-604) of rat InsP₃R1 in which all Cys were replaced by Ala (NT^{Cysless}) was expressed in *E. coli* and purified. Crystals of NT^{Cysless} were grown by the hanging-drop vapour diffusion method in 0.1 M Hepes pH 7.0, 0.8-1.0 M (NH₄)₂SO₄, and 3% (v/v) trimethylamine N-oxide for apo-state crystals, or 0.1 M Na citrate (pH 6.0), 8% (w/

v) PEG-6000, 70 mM Li₂SO₄, 3% dimethyl sulfoxide for InsP₃-bound crystals. Diffraction data were collected at 100 K on the 19-ID (apo-crystals) or 19-BM (InsP₃-bound crystals) beam lines at the Advanced Photon Source Synchrotron facility (Argonne, IL) and processed with HKL2000²⁸. Structures of apo-NT^{Cysless} at 3.0 Å resolution and InsP₃-bound NT^{Cysless} at 3.6 Å resolution were determined by molecular replacement using structures of the SD (PDB code: 1XZZ)³ and the IBC (1N4K)² as search models with the program Phaser²⁹. Iterative refinement and model building were performed with Refmac5 and Coot, respectively. Numbering of secondary structure motifs is in accord with Supplementary Figure 7. Binding of ³H-InsP₃ or ³H-ryanodine to full-length InsP₃R1, chimeras of InsP₃R1 and RyR, and to related N-terminal fragments was defined using equilibrium-competition binding assays⁴. Functional properties of InsP₃R1 and chimeras were characterized after stable expression in DT40 cells lacking endogenous InsP₃R⁴. A luminal Ca²⁺ indicator was used to record InsP₃-evoked Ca²⁺ release from the intracellular stores of permeabilized DT40 cells⁴.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Paul Allen (Harvard University) and David MacLennan (University of Toronto) for kind gifts of plasmids encoding RyR2 and RyR1, respectively. C.W.T. thanks Taufiq Rahman and Vera Konieczny (University of Cambridge) for discussions. M.I. acknowledges Katsuhiko Mikoshiba and Takayuki Michikawa (RIKEN) for long-standing support and discussions. This work was supported by grants from the Heart and Stroke Foundation of Ontario (T-7181) to M.I., and the Wellcome Trust (085295), Biotechnology and Biological Sciences Research Council (BB/H009736) and Medical Research Council (G0900049) to C.W.T. M.S. is supported by postdoctoral fellowships from the Canadian Institutes of Health Research and the National Research Foundation of Korea (2009-352-E00006). A.M.R. is a fellow of Queens' College, Cambridge. M.I. holds a Canadian Research Chair in Cancer Structural Biology.

Appendix

METHODS

Materials

InsP₃ was from Enzo Life Sciences (Exeter, UK). Adenophostin A was from A. G. Scientific (San Diego, CA, USA). Ryanodine was from Ascent Scientific (Bristol, UK). Cyclopiazonic acid was from Sigma (St. Louis, MO). Sources of other materials are specified in earlier publications^{2-4,18} or in the descriptions that follow.

Cloning, expression and purification of N-terminal fragments of InsP₃R1 and RyR2

The open reading frame (ORF) encoding the N-terminal fragment (NT, residues 1-604) of rat InsP₃R1 (GenBank: GQ233032.1) was amplified by PCR from the full-length clone lacking the S1 splice site using forward 5'-CGGGATCCATGTCTGACAAAATGTCTAGT-3' and reverse 5'-CGCGCTCGAGTCACTTTCGGTTGTTGTGGA-3' primers. The PCR product was ligated into a pGEX-6P-2 vector (GE Healthcare, Little Chalfont, Bucks, UK) as a BamHI/XhoI fragment to give pGEX(NT), which includes an N-terminal GST tag followed by a PreScission-cleavage site. To generate NT^{Cysless}, a QuikChange multisite directed mutagenesis kit (Agilent, Stockport, UK) was used to mutate all Cys residues to Ala using pGEX-6P-2-(NT) as the template and the primers listed in Supplementary Table 8. Residues are numbered by reference to rat InsP₃R1 containing the S1 splice site.

Plasmids encoding GST-tagged IBC (residues 224-604) and IBC^{Cysless} were generated using PCR to amplify the appropriate sequence from the ORF of full-length rat InsP₃R1 or NT^{Cysless} using the following primers: forward 5'-CGGGATCCATGAAATGGAGTAACAAAG-3' and reverse 5'-CGCGCTCGAGTCACTTTTCGGTTGTTGTGGA-3'. Each PCR product was ligated into a pGEX-6P-2 vector as a BamHI/Xho I fragment to produce pGEX-6P-2-(IBC) and pGEX-6P-2-(IBC^{Cysless}), respectively. For analysis of the effects of mutations within the SD on InsP₃ binding, the plasmids described previously were used to express His₆-tagged NT and IBC⁴ and the His₆-tag was cleaved prior to experiments⁴. Mutations were introduced using a QuikChange mutagenesis kit and the primers listed previously⁴ or in Supplementary Table 9.

The sequence encoding the A-domain of RyR2 was amplified by PCR from rabbit RyR2 (GenBank: GI164831)³⁰ in pcDNA3 using the following primers: forward 5'-ACTAGTCTCGAGGTGCTCTTCCAGGGCCCATGGCTGATGGGGCGAA-3' and reverse 5'-GATATCCTTCACTTCTGAGCTGATGGG-3'. The ORF for the IBC of InsP₃R1 was excised from pGEX-6P-2-(NT) as a BamHI/XhoI fragment and ligated into a pET41a vector to produce pET41a-(IBC). To generate a plasmid encoding a chimeric NT in which the A-domain of RyR2 (residues 1-210) was fused to the IBC of InsP₃R1 (residues 225-604) (RyR2A-IBC), the PCR product from above was ligated into pET41a-(IBC) as a SpeI/EcoRV fragment to produce pet41a-(RyR2A-IBC). Mutations within the ORF of the A-domain of RyR2A-IBC were generated by site-directed mutagenesis using the QuikChange Lightning mutagenesis kit (Stratagene) using the primers listed in Supplementary Table 9. The complete coding sequences of all constructs were confirmed by sequencing. The sequences of the proteins used are summarized in Supplementary Table 1 and Figure 4a.

For structural studies, NT^{Cysless} was expressed as a GST-fusion protein in BL21-CodonPlus(DE3) *E. coli* strain. Transformed cells were first grown at 37°C until the OD₆₀₀ reached ~1.0 and then induced with 0.5 mM IPTG at 15°C for ~18 h. Proteins were purified using glutathione sepharose 4B resin (GE Healthcare), and the GST tag was cleaved from the eluted proteins with PreScission protease (GE Healthcare) during overnight dialysis at 4°C in cutting buffer (20 mM Tris-HCl, pH 8.4, 300 mM NaCl, 5% glycerol, 2 mM DTT). The cleaved proteins were further purified with cation-exchange chromatography (Fractogel EMD SO3-resin, EM Industries Inc.) followed by size-exclusion chromatography (Superdex 200, GE Healthcare). Purified proteins were concentrated to 14 mg/ml in a buffer comprising 20 mM Tris-HCl, pH 8.4, 360 mM NaCl, 2.5% glycerol, 0.2 mM TCEP, 1 mM PMSF. Similar methods were used to express InsP₃R fragments for binding studies, but with the following modifications: bacteria were initially grown at 22°C, the GST-tag was cleaved by incubation of bacterial lysates immobilized on glutathione sepharose 4B resin with PreScission for 5 h in PreScission-cleavage buffer (GE Healthcare). The eluant was then used for ³H-InsP₃ binding analyses without further purification. Western blotting and silver-stained gels were used to verify expression and purification of NT fragments.

Western blotting

Western blotting of DT40 cells solubilized in TEM containing Triton-X100 (1% v/v) was performed as previously described³¹ using anti-peptide antisera corresponding to residues 240-253 within the IBC (AbNT, 1:1000) or 2733-2749 (AbCT, 1:500) of rat InsP₃R1. The secondary antibody was HRP-conjugated donkey anti-rabbit antibody (1: 5000, Santa Cruz Biotechnology).

³H-InsP₃ Binding

Equilibrium-competition binding assays were performed at 4°C in Tris-EDTA medium (TEM: 50 mM Tris, 1 mM EDTA, pH 8.3) containing ³H-InsP₃ (0.75 nM, Perkin-Elmer Life Sciences), purified protein (1–4 μg) and unlabelled InsP₃ in a final volume of 500 μl. After a 5-min incubation, during which equilibrium was attained, reactions were terminated by addition of 500 μl of TEM containing 30% poly(ethylene glycol) 8000 and γ-globulin (750 μg) followed by centrifugation (20,000 g, 5 min). For ³H-InsP₃ binding to IBC, the amount of ³H-InsP₃ was reduced to 0.25 nM, and incubation volumes were doubled. Pellets were solubilized in 200 μl of TEM containing 2% Triton-X 100 (v/v), mixed with EcoScintA scintillation liquid (National Diagnostics) and radioactivity was determined by liquid scintillation counting. Non-specific binding was determined in the presence of 10 μM InsP₃. Binding results were fitted to a Hill equation (GraphPad Prism, version 5) from which pIC₅₀ (-logIC₅₀, where IC₅₀ is the half-maximal inhibitory concentration) and thereby pK_D (-logK_D) values were calculated³².

³H-ryanodine binding

Microsomal membranes were prepared from DT40 cells by lysis with a glass homogenizer and sonication in cytosol-like medium (CLM) supplemented with protease inhibitors (Roche complete protease inhibitor cocktail), followed by centrifugation (50,000 g, 30 min). CLM had the following composition: 140 mM KCl, 20 mM NaCl, 1 mM EGTA, 20 mM Pipes, 2 mM MgCl₂, 375 μM CaCl₂ (free [Ca²⁺] ~ 220 nM), pH 7. Equilibrium-competition binding was performed with microsomal membranes (100 μg protein/ml) at 4°C in 200 μl of CLM supplemented with protease inhibitors and ³H-ryanodine (100 nM, Perkin-Elmer Life Sciences). Reactions were terminated after 90 min, and radioactivity was determined as described for ³H-InsP₃ binding. Non-specific binding was defined by addition of 10 μM unlabelled ryanodine.

³H-ryanodine binding to RyR typically requires many hours to reach equilibrium²⁴ because it binds only to the open state of the channel and spontaneous openings are rare. In our analyses of ³H-ryanodine binding to InsP₃R1-RyR1 (Fig. 4g) equilibrium was attained within 90 min, perhaps because the modestly increased spontaneous activity of the chimeric channel (Supplementary Fig. 10b) contributed to an increased rate of ³H-ryanodine binding to the open state. In parallel comparisons, specific binding of ³H-ryanodine to InsP₃R1-RyR1 expressed in DT40 cells and stimulated with InsP₃ (1 μM) was (d.p.m.; mean [range] for 2 independent experiments): 4241 [4073-4409] after 90 min, 4941 [4825-5058] after 3 h, and 4410 [4108-4712] after 14 h. It is, however, important to note that our conclusion that InsP₃ selectively stimulates ³H-ryanodine binding to InsP₃R1-RyR1 is not dependent on having measured binding under equilibrium conditions.

Crystallization and data collection

Crystals of apo-NT^{Cysless} were grown by the hanging-drop vapour diffusion method at 293 K by mixing 1 μl of protein with an equal volume of reservoir solution (0.1 M HEPES, pH 7.0, 0.8–1.0 M (NH₄)₂SO₄). Using an additives screen, 3% (v/v) trimethylamine N-oxide was identified as an important additive to obtain single rod-shaped crystals. After a series of microseeding trials, rod-shaped single crystals were obtained within 5 days. For crystallization of InsP₃-bound NT^{Cysless}, five molar excess of InsP₃ (~1 mM) was added before crystallization. Crystals of InsP₃-bound NT^{Cysless} were grown using the same method except for the reservoir solution containing 0.1 M Na citrate (pH 6.0), 8% (w/v) PEG-6000, 70 mM Li₂SO₄ and 3% dimethyl sulfoxide.

For data collection, crystals were equilibrated in 25% glycerol cryo-protective solutions containing reservoir buffer, and flash frozen in liquid nitrogen. Diffraction data were

collected at 100K on 19-ID beam line for apo-state crystals or 19-BM beam line for InsP₃-bound crystals at the Advanced Photon Source Synchrotron facility (Argonne, IL), and were processed with HKL2000. Crystals of apo-NT^{Cysless} belong to the space group P1 with cell dimension a=63.1 Å, b=77.2 Å, c=101.5 Å, α=105.4°, β=100.0°, γ=101.0°. Crystals of InsP₃-bound NT^{Cysless} belong to the space group C2 with cell dimension a=189.2 Å, b=78.7 Å, c=134.1 Å, α=90.0°, β=124.5°, γ=90.0°. Crystals of both apo- and InsP₃-bound NT^{Cysless} contained two molecules in the asymmetric unit (Supplementary Table 3).

Structure determination and refinement

Structures of apo-NT^{Cysless} at 3.0 Å resolution and of InsP₃-bound NT^{Cysless} at 3.6 Å resolution were determined by molecular replacement using structures of the SD (PDB code: 1XZZ)³ and the IBC (PDB code: 1N4K)² as search models with the program Phaser²⁹. Iterative refinement and model building were performed with Refmac5³³ and Coot³⁴, respectively (Supplementary Table 3). Structures of the two molecules in the asymmetric unit of apo-NT^{Cysless} are virtually identical (rmsd value = 0.543 Å) except for a minor variation in the loop between β20 and β21 which does not affect the interpretation of our results. The low rmsd between chain A and chain B is maintained through the regions of the molecule which make up interface-α and interface-β, thus increasing the validity of our description of the ‘open-clam’ structure. The two molecules in the asymmetric unit of InsP₃-bound NT^{Cysless} are more converged than those of the apo-structure (rmsd value = 0.134 Å), which also validates our description of the ‘closed-clam’ structure. The molecule of chain A for each state was used to generate figures, but the chain B molecule of apo-NT^{Cysless} was used for the side chain of D444 in Fig. 1c. All water molecules were modeled in Coot³⁴. Initially, water molecules were detected using the automatic “find waters” function in the program. A 2F_o-F_c map was used with a sigma cut-off value of 1.0, and minimum and maximum distances to protein atoms of 2.4 and 3.2 Å, respectively. We subsequently picked additional water molecules and deleted inappropriate water molecules by manually surveying the density in Coot. After refinement, all water molecules exhibiting negative electron density due to inconsistent modeling were deleted.

Circular dichroism (CD) analysis

CD spectra were collected on a Jasco J-720 spectrometer using a 1-mm path length cuvette at 20 °C. The NT and NT^{Cysless} (0.2 mg/ml) were prepared in a buffer (20 mM Tris-HCl, pH 8.4, 360 mM NaCl, 2.5% glycerol, 0.2 mM TCEP, 1 mM PMSF). CD spectra were obtained from 260 to 200 nm, with a 2-nm bandwidth, an 8-s response time, and scan speed of 50 nm/min. Data are averages of three consecutive scans.

Cloning and functional expression of chimeric InsP₃R

To generate the plasmid encoding a chimeric InsP₃R1 in which residues 2274-2748 of InsP₃R1 (all residues downstream of those immediately before TMD1) were replaced by the equivalent region from RyR1 (residues 4511-5037) (InsP₃R1-RyR1), the appropriate region of the ORF of rabbit RyR1 (GenBank: X15209)³⁵ was amplified by PCR from the expression vector pcDNA3.2 using the following primers: forward, 5'-CGCGGGTTCGAAGTCCCCGAGGCCACCAGAACCCCC-3', and reverse 5'-CGGGGCGTCTCGAGTCATTAGCTCAGCTGGTCCTCGTACTGCTTGCGGAAGC-3'. The PCR product was cloned in-frame as a BstBI/XhoI fragment into a pENTR1a vector containing nucleotides 1-6822 of rat InsP₃R1. This construct was transferred into the Gateway-compatible expression vector, pcDNA3.2, to generate pcDNA3.2-(InsP₃R1-RyR1). A plasmid encoding InsP₃R lacking the SD was generated from ORFs for the full-length InsP₃R1 lacking the S1 splice site (pENTR1A(InsP₃R1)) and the IBC (pENTR1A(IBC)). Both plasmids were digested with NheI and KpnI, and the fragment from pENTR1A(IBC) was cloned into pENTR1A(InsP₃R1). Site-directed mutagenesis was then

used to silence 3 internal BamHI sites within this construct without affecting the coding sequence to generate the plasmid pENTR1A(InsP₃R1^{ΔSD}). A plasmid encoding a chimera in which the SD of InsP₃R1 (residues 1-224) was replaced by the A-domain of RyR1 (residues 1-210) (RyR1A-InsP₃R) was prepared by isolating the coding sequence for the A-domain of RyR1 by PCR from the rabbit RyR1 ORF using the following primers: forward, 5'-GCTAGCATCATGGGTGACGGAGGA-3' and reverse 5'-GGATCCTTCACAGCAGGAGCAGATG-3'. The PCR product was cloned as a NheI/BamHI fragment into pENTR1A(InsP₃R1^{ΔSD}). The complete coding sequences of all plasmids were verified by sequencing. Domain boundaries of the chimeric proteins are summarized in Supplementary Table 1.

DT40-KO cells were transfected by electroporation with linearized plasmids (10 μg DNA/10⁶ cells) using the Neon (Invitrogen, Paisley, UK) or Amaxa (Lonza, Slough, UK) nucleofection systems. G418 (2 mg/ml) was used to select and amplify clones of G418-resistant cells. Stable cell lines were selected and InsP₃R expression was measured by Western blotting. DT40 cells were cultured in RPMI 1640 medium with L-glutamine (Invitrogen) supplemented with 10% foetal bovine serum, 1% heat-inactivated chicken serum (both from Sigma) and 10 μM 2-mercaptoethanol at 37°C in humidified air containing 5% CO₂. Cells (~2 × 10⁶ cells/ml) were passaged every 2-3 days. Similar methods were used for transient transfections with RyR1 and InsP₃R1^{CyslessNT}, but with 50 μg DNA/3 × 10⁶ cells.

Functional analyses of InsP₃R in DT40 cells

Uptake of Ca²⁺ into the intracellular stores of saponin-permeabilized DT40 cells and its release by InsP₃ were measured using a low-affinity Ca²⁺ indicator (Mag-fluo-4) trapped within the endoplasmic reticulum³⁶. All experiments were performed at 20°C in CLM supplemented with 1.5 mM MgATP to allow active Ca²⁺ uptake. After the intracellular stores had loaded to steady-state with Ca²⁺ (~150 s), InsP₃ was added with thapsigargin (1 μM) to prevent further Ca²⁺ uptake (Supplementary Fig. 9). The effects of InsP₃ were assessed after a further 10-40 s. InsP₃-evoked Ca²⁺ release is expressed as a fraction of the ATP-dependent Ca²⁺ uptake. Typical experiments are shown in Supplementary Figure 9.

Structural model of RyR2A-IBC

A structural homology model of RyR2A-IBC (Fig. 4c) was produced using UCSF Chimera³⁷ to first superpose the backbone structures of apo-NT^{Cysless} and the ABC of RyR1 (PDB, 2XOA), the only RyR subtype for which there is a complete N-terminal structure⁹. This A-domain structure of RyR1 was then used to allow superposition of the A-domain from RyR2 (PDB, 3IM5)¹², effectively achieving superposition of NT^{Cysless} onto a 'virtual' chimera of RyR2A with RyR1BC. The predicted structure of the RyR2A-IBC was then revealed by masking the SD of InsP₃R1 and the BC domains of RyR1 (Fig. 4c).

Computational docking

Rigid-body docking of the apo-NT^{Cysless} structure into a ~10 Å cryo-EM density map of InsP₃R1¹⁰ was implemented using the six-dimensional search procedure in the Situs Program package³⁸. The Laplacian filter was applied to the density maps to enhance the fitting contrast. Docking of the crystal structure of RyR1-ABC (PDB code: 2XOA) into a 9.6-Å cryo-EM density map (EMDB code: EMDB-1275)¹⁰, as previously described⁹, was repeated using the above procedure. The UCSF Chimera package³⁷ was used to visualize the docking results with the density maps (Fig. 3 and Supplementary Fig. 6).

References

1. Serysheva, I., editor. Structure and function of calcium release channels. Academic Press; San Diego:
2. Bosanac I, Alattia J-R, Mal TK, Chan J, Talarico S, Tong FK, Tong KI, Yoshikawa F, Furuichi T, Iwai M, Michikawa T, Mikoshiba K, Ikura M. Structure of the inositol 1,4,5-trisphosphate receptor binding core in complex with its ligand. *Nature*. 2002; 420:696–700. [PubMed: 12442173]
3. Bosanac I, Yamazaki H, Matsu-ura T, Michikawa M, Mikoshiba K, Ikura M. Crystal structure of the ligand binding suppressor domain of type 1 inositol 1,4,5-trisphosphate receptor. *Mol. Cell*. 2005; 17:193–203. [PubMed: 15664189]
4. Rossi AM, Riley AM, Tovey SC, Rahman T, Dellis O, Taylor EJA, Veresov VG, Potter BVL, Taylor CW. Synthetic partial agonists reveal key steps in IP₃ receptor activation. *Nat. Chem. Biol*. 2009; 5:631–639. [PubMed: 19668195]
5. Uchida K, Miyauchi H, Furuichi T, Michikawa T, Mikoshiba K. Critical regions for activation gating of the inositol 1,4,5-trisphosphate receptor. *J. Biol. Chem*. 2003; 278:16551–16560. [PubMed: 12621039]
6. Schug ZT, Joseph SK. The role of the S4-S5 linker and C-terminal tail in inositol 1,4,5-trisphosphate receptor function. *J. Biol. Chem*. 2006; 281:24431–24440. [PubMed: 16815846]
7. Chan J, Yamazaki H, Ishiyama N, Seo MD, Mal TK, Michikawa T, Mikoshiba K, Ikura M. Structural studies of inositol 1,4,5-trisphosphate receptor: coupling ligand binding to channel gating. *J. Biol. Chem*. 2010; 285:36092–36099. [PubMed: 20843799]
8. Yamazaki H, Chan J, Ikura M, Michikawa T, Mikoshiba K. Tyr-167/Trp-168 in type1/3 inositol 1,4,5-trisphosphate receptor mediates functional coupling between ligand binding and channel opening. *J. Biol. Chem*. 2010; 285:36081–36091. [PubMed: 20813840]
9. Tung CC, Lobo PA, Kimlicka L, Van Petegem F. The amino-terminal disease hotspot of ryanodine receptors forms a cytoplasmic vestibule. *Nature*. 2010; 585:585–588. [PubMed: 21048710]
10. Ludtke SJ, Tran TP, Ngo QT, Moiseenkova-Bell VY, Chiu W, Serysheva II. Flexible architecture of IP₃R1 by cryo-EM. *Structure*. 2011; 19:1192–1199. [PubMed: 21827954]
11. Amador FJ, Liu S, Ishiyama N, Plevin MJ, Wilson A, MacLennan DH, Ikura M. Crystal structure of type I ryanodine receptor amino-terminal β -trefoil domain reveals a disease-associated mutation “hot spot” loop. *Proc. Natl. Acad. Sci. USA*. 2009; 106:11040–11044. [PubMed: 19541610]
12. Lobo PA, Van Petegem F. Crystal structures of the N-terminal domains of cardiac and skeletal muscle ryanodine receptors: insights into disease mutations. *Structure*. 2009; 17:1505–1514. [PubMed: 19913485]
13. Lin CC, Baek K, Lu Z. Apo and InsP₃-bound crystal structures of the ligand-binding domain of an InsP₃ receptor. *Nat. Struct. Mol. Biol*. 2011; 18:1172–1174. [PubMed: 21892169]
14. Taylor CW, Tovey SC. IP₃ receptors: toward understanding their activation. *Cold Spring Harb. Perspect. Biol*. 2010; 2:a004010. [PubMed: 20980441]
15. Anyatonwu G, Joseph SK. Surface accessibility and conformational changes in the N-terminal domain of type I inositol trisphosphate receptors: studies using cysteine substitution mutagenesis. *J. Biol. Chem*. 2009; 284:8093–8102. [PubMed: 19141613]
16. Tateishi H, Yano M, Mochizuki M, Suetomi T, Ono M, Xu X, Uchinoumi H, Okuda S, Oda T, Kobayashi S, Yamamoto T, Ikeda Y, Ohkusa T, Ikemoto N, Matsuzaki M. Defective domain-domain interactions within the ryanodine receptor as a critical cause of diastolic Ca²⁺ leak in failing hearts. *Cardiovasc. Res*. 2009; 81:536–545. [PubMed: 18996969]
17. Sureshan KM, Riley AM, Rossi AM, Tovey SC, Dedos SG, Taylor CW, Potter BVL. Activation of IP₃ receptors by synthetic bisphosphate ligands. *Chem. Commun*. 2009; 14:1204–1206.
18. Chan J, Whitten AE, Jeffries CM, Bosanac I, Mal TK, Ito J, Porumb H, Michikawa T, Mikoshiba K, Trehwella J, Ikura M. Ligand-induced conformational changes via flexible linkers in the amino-terminal region of the inositol 1,4,5-trisphosphate receptor. *J. Mol. Biol*. 2007; 373:1269–1280. [PubMed: 17915250]
19. Mayer ML. Glutamate receptors at atomic resolution. *Nature*. 2006; 440:456–462. [PubMed: 16554805]

20. Hamada T, Bannister ML, Ikemoto N. Peptide probe study of the role of interaction between the cytoplasmic and transmembrane domains of the ryanodine receptor in the channel regulation mechanism. *Biochemistry*. 2007; 46:4272–4279. [PubMed: 17361990]
21. Ramos-Franco J, Galvan D, Mignery GA, Fill M. Location of the permeation pathway in the recombinant type-1 inositol 1,4,5-trisphosphate receptor. *J. Gen. Physiol.* 1999; 114:243–250. [PubMed: 10436000]
22. Samsó M, Feng W, Pessah IN, Allen PD. Coordinated movement of cytoplasmic and transmembrane domains of RyR1 upon gating. *PLoS Biol.* 2009; 7:e85. [PubMed: 19402748]
23. Iwai M, Michikawa T, Bosanac I, Ikura M, Mikoshiba K. Molecular basis of the isoform-specific ligand-binding affinity of inositol 1,4,5-trisphosphate receptors. *J. Biol. Chem.* 2007; 282:12755–12764. [PubMed: 17327232]
24. Chu A, Diaz-Munoz M, Hawkes MJ, Brush K, Hamilton SL. Ryanodine as a probe for the functional state of the skeletal muscle sarcoplasmic reticulum calcium release channel. *Mol. Pharmacol.* 1990; 37:735–741. [PubMed: 1692609]
25. Lai FA, Meissner G. The muscle ryanodine receptor and its intrinsic Ca^{2+} channel activity. *J. Bioenerg. Biomembr.* 1989; 21:227–246. [PubMed: 2546931]
26. Liu Z, Wang R, Tian X, Zhong X, Gangopadhyay J, Cole R, Ikemoto N, Chen SR, Wagenknecht T. Dynamic, inter-subunit interactions between the N-terminal and central mutation regions of cardiac ryanodine receptor. *J. Cell Sci.* 2010; 123:1775–1784. [PubMed: 20427316]
27. George CH, Jundi H, Thomas NL, Scoote M, Walters N, Williams AJ, Lai FA. Ryanodine receptor regulation by intramolecular interactions between cytoplasmic and transmembrane domains. *Mol. Biol. Cell.* 2004; 15:2627–2638. [PubMed: 15047862]
28. Otwinowski Z, Minor W. Processing X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* 1997; 276:307–326.
29. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser crystallographic software. *J. Appl. Crystallogr.* 2007; 40:658–674. [PubMed: 19461840]

References

30. Otsu K, Willard HF, Khanna VK, Zorzato F, Green NM, MacLennan DH. Molecular cloning of cDNA encoding the Ca^{2+} release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. *J. Biol. Chem.* 1990; 265:13472–13483. [PubMed: 2380170]
31. Tovey SC, Dedos SG, Rahman T, Taylor EJA, Pantazaka E, Taylor CW. Regulation of inositol 1,4,5-trisphosphate receptors by cAMP independent of cAMP-dependent protein kinase. *J. Biol. Chem.* 2010; 285:12979–12989. [PubMed: 20189985]
32. Kenakin, TP. Pharmacologic analysis of drug-receptor interactions. 3rd ed. Lippincott, Williams and Wilkins; New York: 1997.
33. Murshudov GN, Vagin AA, Dodson EJ. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr.* 1997; 53:240–255. [PubMed: 15299926]
34. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr.* 2004; 60:2126–2132. [PubMed: 15572765]
35. Zorzato F, Fujii J, Otsu K, Phillips M, Green NM, Lai FA, Meissner G, MacLennan DH. Molecular cloning of cDNA encoding human and rabbit forms of the Ca^{2+} release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 1990; 265:2244–2256. [PubMed: 2298749]
36. Tovey SC, Sun Y, Taylor CW. Rapid functional assays of intracellular Ca^{2+} channels. *Nat. Protocol.* 2006; 1:259–263.
37. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF Chimera - a visualization system for exploratory research and analysis. *J. Comput. Chem.* 2004; 25:1605–1612. [PubMed: 15264254]
38. Wriggers W, Milligan RA, McCammon JA. Situs: a package for docking crystal structures into low-resolution maps from electron microscopy. *J. Struct. Biol.* 1999; 125:185–195. [PubMed: 10222274]

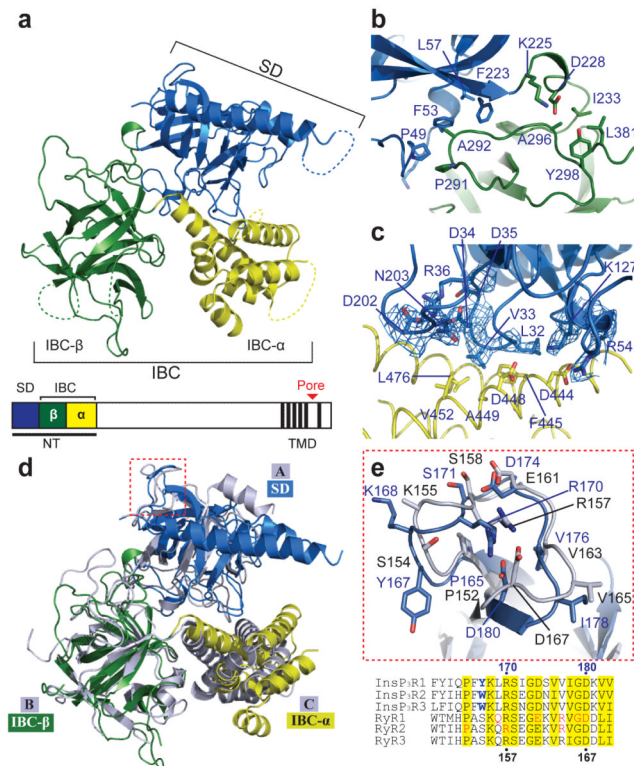


Figure 1. Structure of the N-terminal region of InsP₃R1 without InsP₃ bound

a, Structure of NT^{Cysless} at 3 Å resolution showing SD (blue), IBC- β (green) and IBC- α (yellow). Dashed lines show invisible regions in electron density. Positions of the three domains within a single InsP₃R subunit are shown. **b**, **c**, Interfaces between SD/IBC- β (β -interface) (**b**) and SD/IBC- α domains (α -interface, with the hydrophobic core boxed and the $2F_o - F_c$ electron density map of key residues (contoured at 1.0σ) shown as mesh) (**c**). **d**, Superposition of apo-NT^{Cysless} and RyR1-ABC (grey)⁹ structures by overlaying IBC- β and RyR1 B-domain. ‘Hot spot’ (HS) loop in RyR1 and corresponding region in InsP₃R1 are highlighted (red). **e**, Close-up views of HS regions of InsP₃R1 (blue) and RyR1 (grey, black lettering) with conserved residues depicted as sticks. Structure-based DALI Lite alignment of rat InsP₃R and rabbit RyR show conserved residues in yellow, RyR1 disease-associated mutations in red, and hydrophobic residues implicated in activation of InsP₃R in blue⁸.

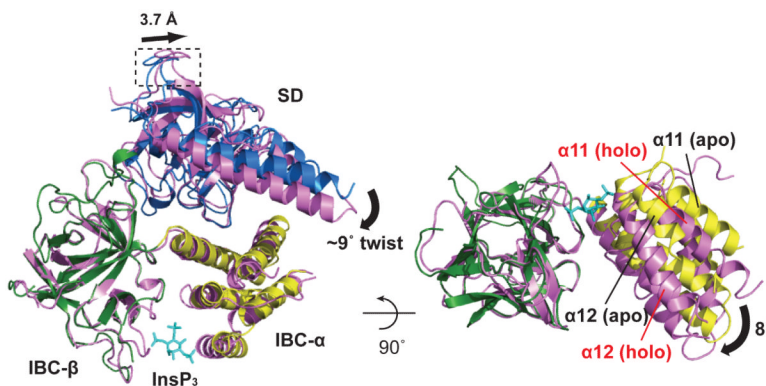


Figure 2. InsP₃-evoked conformational changes

Superposition of apo-NT^{Cysless} (SD, blue; IBC-α, yellow; IBC-β, green) and InsP₃-bound NT^{Cysless} (3.6 Å resolution, magenta) by overlaying IBC-β domain. InsP₃ binding causes the SD to rotate towards the IBC accompanied by a swing approximately perpendicular to the IBC 'clam closure'. This twist (curved arrow) is measured as the angular difference between the SD arm helices in the apo- and bound states (~9°). Movement of the HS-loop (boxed) shows the distance between α-carbons of Y167 (~3.7 Å). A view rotated 90° about the x-axis is shown at right with only IBCs represented. The interdomain (IBC-β and IBC-α) angular difference between the free and bound states is ~8° (black arrow). Further details of InsP₃ binding and its effects on the IBC and α- and β-interfaces in Supplementary Figure 5.

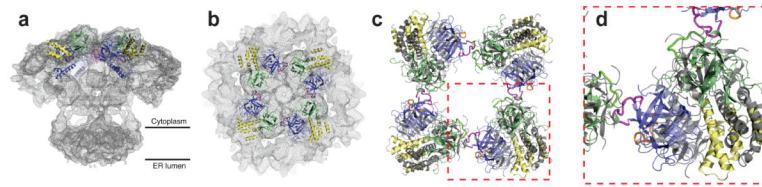


Figure 3. Docking of the apo-NT^{Cysless} structure into the cryo-EM map of InsP₃R1
a, b, Side (**a**) and top (**b**) views of apo-NT^{Cysless} structure docked into the cryo-EM map (grey mesh) of InsP₃R1 in a closed state¹⁰. Contour level corresponds to mass of InsP₃R1 tetramer of 1.3 MDa (protein density 0.8 Da/Å³). Four molecules of the NT (SD, blue; IBC-β, green; IBC-α, yellow) are located at top of the cytoplasmic portion of the InsP₃R1 tetramer. **c, d**, Dockings of the apo-NT^{Cysless} (coloured as in a) and ABC⁹ (grey) structures into cryo-EM structures of InsP₃R1¹⁰ and RyR⁹, respectively, are overlaid and presented to show only N-terminal structures. HS-loops of InsP₃R (magenta) and RyR (orange) are highlighted. Enlargement of boxed area (**d**). Locations of other binding sites within NT of InsP₃R1 are shown in Supplementary Figure 6.

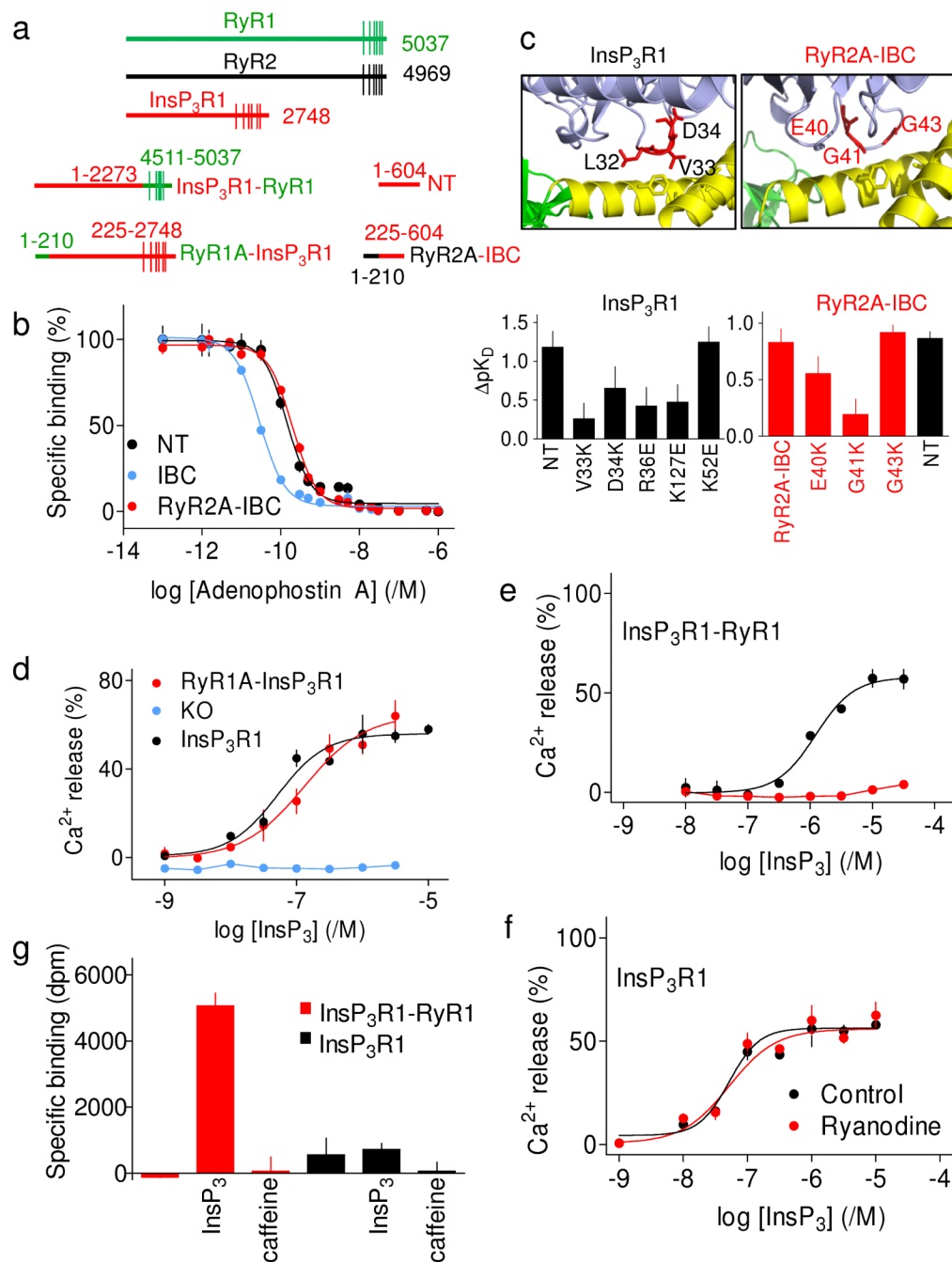


Figure 4. Functional chimeras of InsP₃R and RyR

a, Proteins used. **b**, Specific binding of ³H-InsP₃ in presence of adenophostin A. **c**, Inhibition of ³H-InsP₃ binding to IBC by SD or A-domain, and effects of mutations within equivalent loops. Affinities shown relative to IBC (ΔpK_D). Structures show key residues within SD or A-domain at α -interface. **d**, Ca²⁺ release from DT40 cells expressing InsP₃R1, RyR1A-InsP₃R1 or lacking InsP₃R (KO). **e**, **f**, Effect of ryanodine (10 μ M) on Ca²⁺ release from DT40 cells expressing InsP₃R1-RyR1 (**e**) or InsP₃R1 (**f**). Ryanodine (10 μ M) did not stimulate Ca²⁺ release via InsP₃R1-RyR1 suggesting that TMDs may not alone mediate stimulation of RyR²⁵. Results (**d-f**) are percentages of ATP-dependent Ca²⁺ uptake. **g**,

Specific ^3H -ryanodine binding (dpm, disintegrations/min) to membranes of DT40 cells expressing $\text{InsP}_3\text{R1}$ or $\text{InsP}_3\text{R1-RyR1}$ with caffeine (10 mM) or InsP_3 (1 μM). Non-specific binding was 2245 ± 211 dpm. Results (**b-g**) are means \pm s.e.m., n = 3.