# Structure and function of inositol 1,4,5-trisphosphate receptors

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**The $InsP_3R$ is an intracellular calcium release channel that forms an important component of the $InsP_3$ signalling pathway and is ubiquitously expressed in multicellular organisms. Molecular studies have identified three separate genes in mammals that code for the three isoforms of $InsP_3Rs$. These three isoforms are shown to have different ligand affinities and modulatory properties. These differences are presently thought to generate the heterogeneity required in calcium dynamics to regulate processes as diverse as secretion, synaptic plasticity, immune responses in T cells and fertilization events in sea urchin eggs. The understanding of these processes mandates an understanding of the single channel properties of the three isoforms as well as splice variants that are expressed in a tissue-specific manner. Recent single channel studies aimed at understanding the functional properties of the $InsP_3Rs$ using point mutants in the channel have uncovered important residues that affect the calcium dependence and ion selectivity of the channel. Genetic studies using knockouts of the mammalian $InsP_3Rs$ appear inadequate to relate the channel function to the physiological behaviour at an animal level most likely due to compensation by other isoforms of the channel. In this scenario, study of $InsP_3R$ in *Drosophila melanogaster*, that encodes a single gene for $InsP_3R$ should prove useful in correlating single channel properties of the channel with phenotypes of the whole organism.**

CALCIUM ($Ca^{2+}$) is an intracellular messenger that governs almost everything that we do, starting from how we see things around us, how our hearts beat, how our brains acquire and store information to how life is propagated during fertilization and ultimately to how our cells die. To perform all of these functions, $Ca^{2+}$ signals are precisely co-ordinated at the level of both a single cell and as groups of cells. Specialized channels that allow release of calcium from intracellular stores and entry of extracellular calcium are present in cells. In most organisms the endoplasmic reticulum forms a major storehouse for intracellular $Ca^{2+}$ while several classes of $Ca^{2+}$ channels on the plasma membrane allow the entry of extracellular $Ca^{2+}$ upon activation[1].

Various mechanisms of intracellular and intercellular communication have been uncovered. Many signalling molecules secreted by one cell, which go and bind to and bring about downstream events in another cell, have been identified. The general theme involves binding of a ligand secreted by one cell to its cognate receptor on another cell. The binding of the ligand to the receptor triggers activation of intracellular molecules that bring about cellular events like secretion, neuronal action potentials, muscle contractions and gene transcription. One of the common mechanisms involves binding of ligands like hormones, growth factors or neurotransmitters to membrane receptors. These can be broadly classified as either seven transmembrane domain receptors which couple to heterotrimeric G proteins or single pass transmembrane receptors, which result in modulation of target molecules by tyrosine phosphorylation. In the case of the inositol 1,4,5-trisphosphate ($InsP_3$) signalling pathway, both these signalling mechanisms activate isoforms of phospholipase C. It cleaves membrane-bound phosphatidyl inositol 4,5 bis-phosphate to release $InsP_3$ and diacyl glycerol (DAG). These effector molecules can signal independently and activate different downstream pathways (Figure 1). DAG remains membrane anchored and activates protein kinase C to bring about an array of downstream events[2]. $InsP_3$ diffuses into the cytosol and binds the inositol 1,4,5-trisphosphate receptor ($InsP_3R$) which is located on the membranes of intracellular organelles, among which the most well studied is the endoplasmic reticulum membrane.

The $InsP_3R$ is a member of an increasing group of intracellular ion channels, which include the ryanodine receptor[3] and nicotinic acid adenine dinucleotide phosphate receptor (NAADPR[4]). Upon ligand-binding at the N-terminus, the channel domain present at the C-terminus opens to release calcium ($Ca^{2+}$) from intracellular stores. $Ca^{2+}$ on entering the cytosol can bind various $Ca^{2+}$ binding proteins that could function as downstream effectors or as buffers that help in restoring free $Ca^{2+}$ levels in the cytosol to the basal level of ~100 nM. The dynamics of intracellular $Ca^{2+}$ release can also bring about effects like $Ca^{2+}$ spikes and oscillations. These have been well studied in pancreatic acinar cells where they lead to fluid and pancreatic enzyme secretion[5]. Changes in levels of intracellular $Ca^{2+}$ can also allow entry of extracellular $Ca^{2+}$ through activation of plasma membrane store-operated channels[6], mediate neurotrophin secretion[7], long-term depression (LTD) in Purkinje cells[8] and gene transcription in T-cells[9,10].

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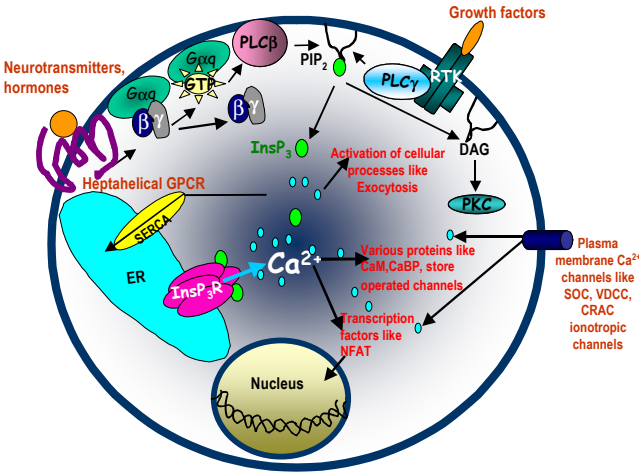


**Figure 1.** Overview of the signalling pathways that generate $InsP_3$ as the second messenger. The pathways are activated by different agonists in different cell types (refer to text for details) and has been summarized and represented in one cell for ease of presentation. Abbreviations: RTK, receptor tyrosine kinases; PLC, phospholipase C; PIP2, phosphatidyl inositol 4,5-bisphosphate; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, inositol 1,4,5-trisphosphate receptor; SERCA, sarcoendoplasmic reticulum ATPase; GPCR, G-protein coupled receptor; DAG, diacyl glycerol; SOC, store-operated channels; VDCC, voltage dependent cation channels; CRAC, calcium release activated channels; PKC, protein kinase C.

This review aims to provide an overview of the current knowledge of $InsP_3R$ channel function. First, there is a brief history of $InsP_3R$ protein purification and gene identification followed by a recent understanding of how modulation of channel function can affect differential cellular phenotypes. Recent data, on the crystal structure of the N-terminal region of the protein and electron micrograph data on the structure of type I $InsP_3R$, which sheds light on how structure affects function of the $InsP_3R$, will be discussed. We then describe the phenotypes exhibited by known mutants of the $InsP_3R$ in genetic model systems, including *Drosophila melanogaster*. Finally, we discuss how a study of single channel properties of $InsP_3R$ mutant alleles would help in understanding their phenotypes in cells and whole organisms.

## $InsP_3R$ genes in mammals and their domain organization

$InsP_3$ was identified as a second messenger, traversing from the cell surface to the intracellular mileu in insect salivary glands[11]. This increase in $InsP_3$ was sufficiently rapid to suggest that it may bring about the subsequently observed phenomenon of intracellular $Ca^{2+}$ release[11]. The first experimental evidence of $InsP_3$-mediated $Ca^{2+}$ release from the intracellular stores was shown in pancreatic acinar cells and the intracellular store was identified as a non-mitochondrial store[12]. The $InsP_3R$ was first identified as a protein binding to $^{32}$P-$InsP_3$ in permeabilized guinea pig hepatocytes and rabbit neutrophils[13]. Adenine nucleotides were found to enhance $InsP_3$-mediated intracellular $Ca^{2+}$ release[14]. $^3$H and $^{32}$P-labelled $InsP_3$-binding studies along with biochemical experiments of $InsP_3$ mediated $Ca^{2+}$ release from microsomal vesicles derived from the cerebellum, identified the cerebellum as the tissue where $InsP_3$ binding protein was expressed at levels 100–300 fold higher than peripheral tissues[15]. The protein was first purified from the cerebellum and its molecular weight was found to be ~260 kDa[16]. The gene coding for mammalian type I $InsP_3R$ was first cloned from a rat brain cDNA library[17]. Expression of the purified $InsP_3R$ and gel filtration studies identified the functional protein as a tetramer[17]. The first insight into the domain organization of the $InsP_3R$ came by expressing the $InsP_3R$ gene in COS cells with partial deletions that gave truncated proteins leading to the identification of the ligand-binding and tetramerization domains[18]. Subsequently, two independent genes coding for two other types of $InsP_3R$ (type 2 and type 3) were cloned from rat[19,20], mouse[21] and humans[22,23].

Further studies using monoclonal antibodies, single point mutants or expression of truncated proteins showed that the $InsP_3R$ has an N-terminal ligand-binding domain comprising of first ~650 amino acid residues, a C-terminal channel domain spanning the last ~600 amino acids and central modulatory domain that includes the middle ~1500 amino acids[24–27], Figure 2). All the three types of $InsP_3Rs$ share sequence identity in the range of 60–70% and a common domain organization.

Further investigations led to isolation of splice variants of the $InsP_3Rs$. The type I $InsP_3R$ in rat is known to have three splice sites named, SI, SII and SIII. SI splicing leads to the addition of 15 amino acids within the core $InsP_3$-binding domain at position 318 in the rat type I $InsP_3R$[28]. Splicing at the SII site results in addition of 40 amino acids to the protein within the modulatory domain at amino acid position[28] 1692. The SII splice site is made up of three separate sites[29] (A, 24 residues, B, 1 residue; and C, 15 residues). SIII splicing adds 9 amino acids in the region between the SI and SII sites at amino acid position[30] 910, Figure 2).

## Cellular and sub-cellular localization of $InsP_3R$ isoforms

The $InsP_3R$ isoforms have different cellular and subcellular distributions[31]. $InsP_3R$ levels in a cell vary widely between different tissues and cell lines. For example, cerebellar Purkinje cells express almost exclusively type I $InsP_3Rs$, whereas the type II and III isoforms predominate in hepatocytes and pancreatic cells respectively[32–34]. Many cells express all the three isoforms in varying degrees. Certain splice variants of the type I $InsP_3R$ are also expressed in a tissue-specific manner. For example, the SII splice variant of the type I $InsP_3R$ predominates in most

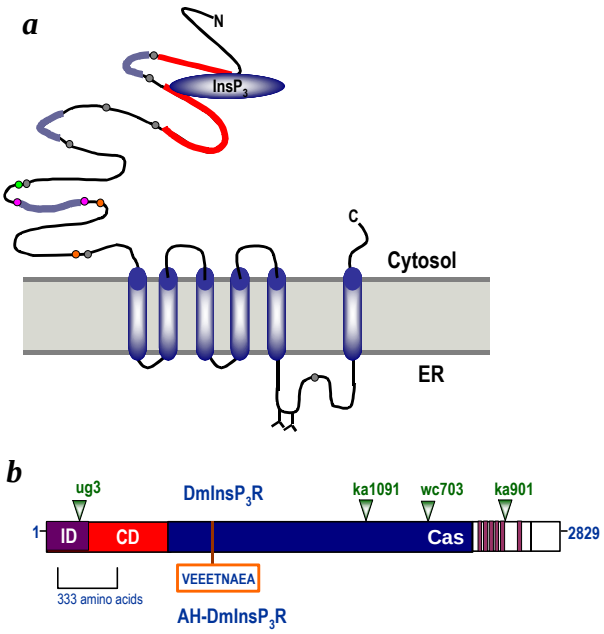


**Figure 2.** ***a***, Schematic of the structure of mammalian $InsP_3R$ type I showing the huge N-terminal cytoplasmic domain with six transmembrane domains and the C-terminal tail protruding out into the cytosol. The N-terminal region consists of $InsP_3$-binding sequence (red region bound to $InsP_3$), various splice sites (SI, SII and SIII) and modulatory sites like ATP-binding sites (orange circles), cytosolic and luminal $Ca^{2+}$ binding sites (grey circles), protein kinase A phosphorylation sites (pink circles) and FK-506 binding protein-(FKBP) binding site (green circle). Also shown are N-glycosylation sites (人) in the luminal region. ***b***, Schematic of the *Drosophila* $InsP_3R$ showing the splice sequence present in the neuronal isoform and location of mutations in the fly $InsP_3R$ conserved across all the known $InsP_3Rs$. Abbreviations; ID, inhibitory domain; CD, core $InsP_3$ binding domain; CaS, calcium sensor domain; ER, endoplasmic reticulum. See text for details.

brain regions (except the cerebellum) while the short form predominates in peripheral tissues[30]. The sequence of the longer form in humans appears to create an additional consensus protein kinase C phosphorylation site.

The endoplasmic reticulum is the organelle in which the $InsP_3Rs$ are most abundantly expressed[35]. However, $InsP_3$-mediated $Ca^{2+}$ release has been reported to occur from the nuclear envelope[36,37], Golgi apparatus[38,39] and plasma membrane of some cells[40,41]. Though there is abundant evidence of specific targeting of the $InsP_3Rs$ to different intracellular locations, there is very little evidence to relate such targeting to $InsP_3R$ subtypes. The ER retention signals in the type I $InsP_3R$ are also conserved within the other subtypes. The finer details of the mechanisms responsible for specific intracellular targeting of $InsP_3Rs$ remain unexplored.

## Functional properties of $InsP_3Rs$

Study of wild-type $InsP_3Rs$ from crude microsomal preparations[42] and permeabilized cell systems[43] measured properties like ligand-binding affinity of the receptor and identified modulators of the activity of $InsP_3R$ like protein kinase A[42], calcium[44] and ATP[45]. Interestingly, $Ca^{2+}$ was found to modulate the activity of $InsP_3R$ in a biphasic manner[44]. In smooth muscle fibres of guinea pig, the rate of $InsP_3$ induced $Ca^{2+}$ release was enhanced by free $Ca^{2+}$ between 0 and 300 nanomolar, but further increase in the $Ca^{2+}$ concentration exerted an inhibitory effect[44]. Much of the earlier understanding of how structure affects function of the $InsP_3Rs$ was based on properties observed for type I mammalian $InsP_3R$, since it is the only subtype to be abundantly expressed as a homotetramer in cerebellar Purkinje cells[46]. However, it is important to note that most cell types express more than one isoform of $InsP_3R$, and $InsP_3Rs$ can also form heterotetramers[47] that are likely to exhibit properties different from those exhibited by homo-tetramers of a single isoform. Additionally, the presence of various splice variants for the type I $InsP_3R$ is likely to exhibit different $Ca^{2+}$ release properties and thus generate further heterogeneity in $Ca^{2+}$ signalling. It is known that the excision of the SII insert changes the PKA phosphorylation pattern of the $InsP_3RI$[29,48] and creates additional ATP[49] and calmodulin[50,51]-binding sites in the $InsP_3RI$ sequence. To understand the functional differences between the three isoforms and splice variants, techniques like single channel recordings of over-expressed or purified native or recombinant channels in artificial lipid bilayers and patch clamp recordings of native type I $InsP_3Rs$ and over expressed type II and III receptors from nuclei of *Xenopus* oocytes have been used[52–54].

Type I mammalian $InsP_3Rs$ have been studied from their native environment, namely cerebellum and *Xenopus* nuclei, using artificial lipid bilayers and patch clamp studies respectively[52,53]. From these studies the structural determinants responsible for $InsP_3R1$ conductance and gating properties[55,56] and modulation by $Ca^{2+}$ (refs 57–59), ATP[59] and phosphorylation[60] have been uncovered. Mammalian $InsP_3RI$ activity is potentiated in the presence of ATP[61] while $Ca^{2+}$ modulates the opening of $InsP_3Rs$ by binding to non-canonical $Ca^{2+}$ binding sites on the $InsP_3RI$ in a biphasic manner. The open probability of the type I rat $InsP_3R$ is very low in presence of low amounts of free $Ca^{2+}$ (sub nM levels) while the open probability is maximal at ~200 nM concentrations of free $Ca^{2+}$. Further increasing the amounts of free $Ca^{2+}$ to μM levels drastically reduce the open probability. This biphasic regulation by $Ca^{2+}$ may thus lead to inhibition of $InsP_3Rs$ after enough $Ca^{2+}$ has been released from the stores. Interestingly the type II rat $InsP_3R$ differs from the type I in both the $Ca^{2+}$ and $InsP_3$ sensitivity[62]. While the type II rat $InsP_3R$ has a higher affinity for $InsP_3$ than the type I $InsP_3R$, its $Ca^{2+}$ dependence is much broader and it stays active till mM amounts of free $Ca^{2+}$ in the cytosolic side[62]. The $Ca^{2+}$ dependence of type III rat $InsP_3R$ remains controversial. A single channel study using artificial lipid bilayers shows only positive modulation of the predominantly expressed native type III $InsP_3R$ from RINm–5F cells even in the

presence of hundreds of μM free $Ca^{2+}$ in the cytosolic side[58]. Another single channel study using expression of recombinant rat type III $InsP_3R$ in Xenopus oocytes and patch clamp studies, shows a bell-shaped curve for $Ca^{2+}$ dependence of the type III $InsP_3R$[63]. $Ca^{2+}$ efflux experiments using permeabilized RINm–5F cells also showed a biphasic $Ca^{2+}$ dependence for the type III $InsP_3R$[64]. Careful analysis of the distribution of the types of $InsP_3Rs$ in RINm–5F cells showed that 77% of the $InsP_3R$ population comprised of type III $InsP_3Rs$ while 17% comprised of type I $InsP_3R$[64]. It is possible that the single channels observed by Hagar *et al.*[58] are homotetramers of type III $InsP_3Rs$ while the presence of low levels of type I $InsP_3R$ in permeabilized RINm–5F cells imparts biphasic $Ca^{2+}$ dependence to the global response of the cells. Future experiments using heterologous expression of only the rat type III $InsP_3R$ and artificial lipid bilayer analysis will help resolve this issue. Interestingly, the $InsP_3R$ isoforms also differ in their sensitivity to $InsP_3$. In single channel studies and labelled $InsP_3$-binding studies, the affinity of $InsP_3Rs$ for $InsP_3$ varies as follows: type II > type I > type III[65–67]. Both native and recombinant type II $InsP_3Rs$[65,62] have been studied and the type II $InsP_3R$ has higher $InsP_3$ affinity ($EC_{50}$ of 58 and 122 nM for native and recombinant receptors respectively) than the native type I or type III $InsP_3Rs$ ($EC_{50}$ of 500 and 3200 nM respectively[66]). Thus the $InsP_3R$ isoforms in a cell are likely to contribute to and define the spatial and temporal nature of local $Ca^{2+}$ signalling events. They also lead to the segregation of parallel $InsP_3$ signalling cascades in mammalian cells.

It has been suggested that the inhibitory effect mediated by high amounts of $Ca^{2+}$ on the $InsP_3R$ (discussed below) is mediated by calmodulin (CaM), a ubiquitous $Ca^{2+}$-binding protein that binds to and regulates functions of many proteins. A calmodulin-binding site on $InsP_3R$ was identified in earlier experiments[25,68] and addition of purified CaM to $InsP_3Rs$ in bilayers was found to inhibit the activity of $InsP_3R$[68]. However, other studies in purified lipid bilayers, patch clamping of *Xenopus* oocytes and co-immunoprecipitation experiments have not observed a detectable effect of purified CaM on $InsP_3R$ wild type or mutant channels with the CaM-binding site mutated[69–71]. Together these recent studies suggest that CaM does not have an effect on the gating of $InsP_3R$ channels. It is possible that in earlier experiments where inhibition with CaM was observed, contaminating amounts of an as-yet unidentified protein, that inhibits $InsP_3R$ mediated $Ca^{2+}$ release in the presence of high amounts of cytosolic $Ca^{2+}$, may have been present along with CaM.

## Structure of the $InsP_3Rs$

The large size of $InsP_3Rs$ (> 2600 amino acids) has so far hindered any crystallographic studies on this channel protein. Initially, the three-dimensional structure of another intracellular $Ca^{2+}$ channel, the RyRI tetramer was reconstructed at a resolution of 25–30 Å, using image-processing techniques on electron micrographs of frozen-hydrated individual isolated channel proteins[72,73]. Overall, the RyR resembles a mushroom with a 'cap' composed of the cytoplasmic domains of the component monomers and the 'stalk' made up of the four putative membrane-spanning domains (Figure 3 *a*). Initially crude structures of the $InsP_3R$ were obtained by negative staining electron microscopy of $InsP_3R$ purified from bovine aorta. These data showed a pinwheel-like appearance[74], while electron micrographs of $InsP_3R$ purified from mouse cerebellum resembled a square shape[75,76] broadly indicative of structural organization similar to that of the RyR. Crystal structure information of the much smaller (~ 350 amino acids) bacterial cation ($K^+$) channel, KcsA, has allowed a reasonable hypothesis to be formulated about the structure and localization of the pore region of intracellular $Ca^{2+}$ channels, due to remarkable similarity of the selectivity filter region of the $K^+$ channel pore with both $InsP_3Rs$ and RyRs[3,77]. The ion-conduction mechanism of the $K^+$ channels, which also function as tetramers, involves the presence of three ions at a time in the pore, one is found in the cavity and two in the narrow selectivity filter region of the protein[79]. The high conductance and ion discrimination property of the $K^+$ channels is attributed to the presence of multiple ions in the pore region. It is proposed that $InsP_3Rs$ and RyRs that have conductance higher than $K^+$ channels, are likely to incorporate similar or more number of $Ca^{2+}$ ions in its pore region[3].

In spite of the similarity in the selectivity filter regions of the $InsP_3Rs$, RyRs and K channels, the former two have

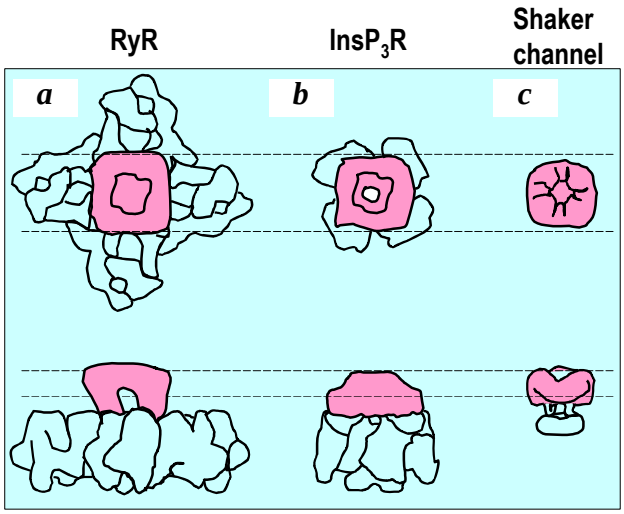


**Figure 3.** Cartoon representations of the 3D structures of the $InsP_3R$ with other cation channels with known 3D structures. ***a***, Cartoon of the type I RyR[113]; ***b***, Cartoon of the type I $InsP_3R$[82]; ***c***, Cartoon of the shaker channel[114] viewing the channel from the lumen of the endoplasmic reticulum (top) and parallel to the membrane plane (lower). The sizes of the channels are proportionately depicted.

at least 6–8-fold higher selectivity for divalents as opposed to monovalents[3]. Mutations in residues in the selectivity filter region of the $InsP_3R$ have shed light on ion selectivity properties of the $InsP_3Rs$. Mutation of Val (V in GVGD) of the rat type I $InsP_3R$ to Ile results in channels which exhibit a higher $K^+$ conductance than wild type while mutation of the Asp residue (D in GVGD) to a Glu residue makes the channels nonselective for divalents over monovalents[55]. Thus the same residues as in $K^+$ channel, function as selectors for divalents over monovalents in the $InsP_3Rs$.

Recent studies using negative-stained electron cryomicrographs[78–80] and cryo-electron micrographs[81] of purified type I $InsP_3R$ show that the tetrameric structure of $InsP_3R$ resembles that of the RyR with regard to its large size and four-fold symmetry but differs from the latter in appearance and protomer size. While there are differences observed in the structures of $InsP_3R$ purified using different detergents and observed with different methods, some common themes do emerge. Broadly, the structure of the $InsP_3R$ resembles that of a pinwheel[78] (Figure 3 *b*) or a flower[80] or an uneven dumbbell[81]. It exhibits a four-fold symmetry with dimensions ranging from $18 \times 18 \times 18$ nm to $25 \times 25 \times 25$ nm. Overall, the protein structure comprises of a large domain that consists of almost three-fourth of the protein and a small stalk that comprises the remaining one fourth. From the domain structure of the $InsP_3R$ described above it is known that there is a huge cytoplasmic and a rather short membrane anchored C-terminus. Thus, it is almost certain that the large domain observed in electron micrographs comprises the cytoplasmic domain and the smaller one the transmembrane region. Significantly, these structural studies show that in the tetramer, the N-terminal ligand-binding domain comes in close proximity to the C-terminal channel domain, thus providing an answer to the long-standing question of how ligand-binding at the extreme N-terminus of the protein leads to opening of the channel domain in the extreme C-terminus of the protein. Interestingly, in negatively stained electron micrographs of type I $InsP_3R$ purified from mouse cerebellum and reconstituted in a buffer with either 200 μM free $Ca^{2+}$ or 1 mM EGTA, a transition in structure from a windmill form (in the presence of $Ca^{2+}$) to a square-shaped one (in the presence of EGTA) is observed. These data indicate that a $Ca^{2+}$ dependent change occurs in the structure of the tetramer[79]. Figure 3 shows a cartoon representation of the three-dimensional structure of $InsP_3R$ compared to the type I RyR and Shaker channel. Recently, the crystal structure of the core ligand-binding domain of the mouse type I $InsP_3R$ (amino acid residues 224–604), in complex with the ligand $InsP_3$ has been made available at a resolution of 2.2 Å. The structure comprises two distinct domains, one N-terminal *b*-trefoil-like domain and the other C-terminal *a*-helical domain and the junction of the two domains forms a cleft, exposing the previously identified Lys and Arg residues[27] that co-ordinate the three phosphoryl groups of the ligand[82] $InsP_3$. Additionally, this structure identifies two putative $Ca^{2+}$ binding sites in the ligand-binding domain.

## Mammalian mutants of $InsP_3Rs$

In spite of the plethora of information available regarding the domain organization, structure and functional properties of individual isoforms and splice variants of the mammalian $InsP_3Rs$, not much is known about how these differential properties affect the physiology of the organism. Such studies have been hindered by the expression of multiple isoforms in individual cell types. Genetic approaches that allow for knockouts of isoforms in a tissue-specific manner have not been attempted so far. The only available mammalian knockout of the $InsP_3R$ is a mouse in which the type I $InsP_3R$ gene has been knocked out in all the tissues[83]. This mutant shows a high rate of *in utero* death and neuronal phenotype of ataxia and epileptic seizures in live animals, which die during their weaning period[83]. Immunohistochemical and electrophysiological examination of the brain and Purkinje cells revealed no morphological abnormality possibly due to partial compensation by other isoforms of the $InsP_3R$[83]. The type I $InsP_3R$ in mammals is highly concentrated in cerebellar Purkinje cells and in some areas of the hippocampus and cerebral cortex[83]. Electrophysiological studies on cerebellar slices of the $InsP_3RI$ knockout mouse showed a lack of long-term depression (LTD[8]) whereas in the hippocampus, these mice showed facilitation of long-term potentiation (LTP), induction and attenuation of depotentiation[84]. These data indicate that the $InsP_3R$ could play an important role in maintaining synaptic plasticity in certain regions of the brain. Examination of electrophysiological properties of gastric smooth muscles of type I $InsP_3R$ knockout mice showed absence of slow waves, that in turn initiate spike potentials. Thus $InsP_3RI$ was found to be essential in generation of slow waves, but not in generation of action potentials[85]. In addition, there occurs a spontaneous mutation of the mouse $InsP_3RI$ called *opisthotonos* (*opt*), which has a deletion of two exons of the $InsP_3RI$ mRNA in the modulatory domain, that does not affect the translational reading frame. The altered protein is predicted to have lost several modulatory sites and is present at significantly lower levels in *opt* homozygous mice[86]. These mice show a phenotype of ataxia and convulsions, similar to knockouts of the type I $InsP_3Rs$. Single channel properties of *opt* mutant $InsP_3RI$ protein show decreased conductance, ~20 fold lower affinity for ATP and a narrow bell-shaped $Ca^{2+}$ dependence, as compared to wild-type[59]. So far neither mutants nor genetic knockouts for the mammalian type II and type III $InsP_3Rs$ are available. From these studies it is hard to correlate the functional properties of $InsP_3Rs$ with intracellular $Ca^{2+}$ dynamics in a physiological context. For such an analy-

sis, studies in simpler model organisms could prove informative.

## $InsP_3R$ genes in other genetically amenable model systems

$InsP_3Rs$ and RyRs have also been identified and cloned from eukaryotes like *Caenorhabditis elegans*[87] and *D. melanogaster*[88,89]. Interestingly, the genome of the unicellular eukaryote-yeast, does not encode a gene for the $InsP_3R$[90], suggesting that modulation of cellular function by intracellular $Ca^{2+}$ release as a signalling mechanism, probably evolved in multicellular systems. The genomes of *C. elegans* and *D. melanogaster* each code for a single gene for the $InsP_3R$, as opposed to three different genes in mammals. The worm $InsP_3R$ is less homologous to the type I rat isoform (~ 40% identity) compared to the fly $InsP_3R$ (57% identity). The homology of the worm $InsP_3R$ with the human type II $InsP_3R$ in the three domains is as follows: 38% in the ligand-binding domain, 35% in the modulatory domain and 44% in the channel domain[91]. The homology of the fly $InsP_3R$ is highest with the type I rat $InsP_3R$. It is 57% in the N-terminal ligand-binding domain, 50% in the modulatory domain and 58% in the channel domain (Figure 4).

## Splice variants of the *Drosophila* $InsP_3R$

So far, no splice variants of the *C. elegans* $InsP_3R$ have been reported. However, a splice variant specifically expressed in embryonic[92] and larval tissues ($DmInsP_3R$[93]) has been identified for the fly $InsP_3R$. This splice form is shorter than the previously cloned adult head cDNA (AH-$DmInsP_3R$[88]) by nine amino acids, namely VEEETNAEA, in the modulatory domain of the protein. In addition, the AH-$DmInsP_3R$ has also been isolated from larval brains, which express both the spliced forms[93], thus imparting it neuronal tissue-specific function. The nine extra amino acids in AH-$DmInsP_3R$ encode a putative casein kinase II phosphorylation site (as judged by Prosite scan analysis). The embryonic and the adult head isoforms may thus be differentially modulated by phosphorylation. Interestingly, the SIII splice variant of the rat and human type I $InsP_3R$ is also spliced in a region homologous to the *Drosophila* adult head isoform and is similarly enriched in brain tissues, except cerebellum[30]. This SIII splice variant also encodes nine additional amino acids, which show a putative protein kinase C phosphorylation site. Thus, a splice variant in this region of the modulatory domain seems to be an evolutionarily conserved feature. The insertion of nine amino acids in the mammalian SIII and *Drosophila* neuronal isoforms occurs near a putative $Ca^{2+}$ binding motif as examined by binding of radioactive $Ca^{2+}$ to GST fusion proteins of parts of the cytoplasmic domain of $InsP_3R$[94]. The inserted sequence in *Drosophila* contains four negatively charged glutamate residues[92] and might interact with the $Ca^{2+}$-binding motif to affect the $Ca^{2+}$ dependence of this isoform. It would thus be of interest to look at the single channel properties of these neuronal isoforms and compare them with the other isoforms.

## $InsP_3R$ mutants in *C. elegans* and *D. melanogaster*

Single point mutants of the worm $InsP_3R$ were first isolated in a suppressor screen to search for components of the epidermal growth factor receptor (LET-23) mediated fertility pathway[95]. By using mutation and over-expression of $InsP_3R$ cDNA, Clandinin *et al.* showed that $InsP_3R$ has a role in spermathecal dilation, leading to defects in ovulation[95]. Another study reported a physiological role for $InsP_3R$ as a regulator of the defecation rhythm frequency of *C. elegans*[91]. The worm shows a ~50s interval between two defecation cycles. Increasing the expression of $InsP_3R$ in the intestinal cells of the worm, decreases the time interval between two defecation cycles, whereas mutations in $InsP_3R$, that presumably decrease the functional activity of the $InsP_3R$ protein, increase the time interval between two defecation cycles[91]. A recent study in *C. elegans* shows the involvement of $InsP_3R$ along with RyR and a $Ca^{2+}$ binding protein, calreticulin, in regulating the process of necrotic cell death[96]. Over-expression of the ligand-binding domain of the *C. elegans* $InsP_3R$ showed the involvement of $InsP_3R$ in up-regulation of pharyngeal pumping in response to food mediated most likely by interaction with myosin II[97] and in the process of cytokinesis and gastrulation during embryogenesis[98].

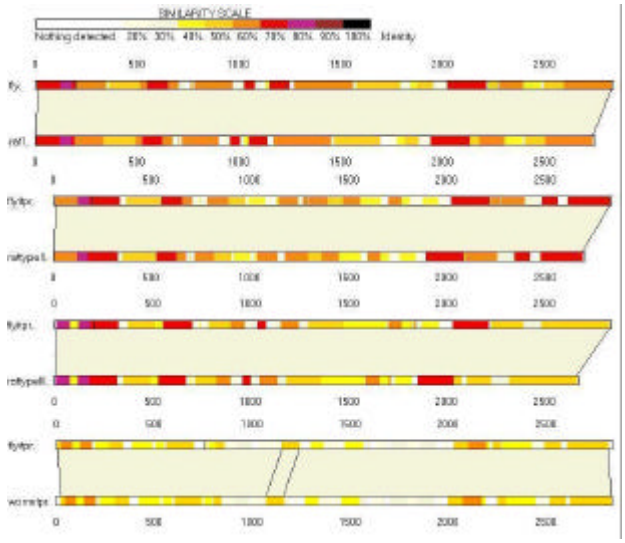

**Figure 4.** Sequence alignment of the *Drosophila* $InsP_3R$ with the type I, type II and type III rat $InsP_3Rs$, and with the *C. elegans* $InsP_3R$. The regions of homology are colour-coded as indicated in the scale bar. The alignment was generated using Lalnview program available at www.expasy.ch.

Genetic studies have also been performed in *Drosophila*, where again, the presence of a single gene makes it a favourable system for the study of $InsP_3R$ function. A complete deletion of the *Drosophila* $InsP_3R$ is larval lethal[99], indicating the importance of the $InsP_3R$ in the development of the organism. Apart from this, a series of mutants for the *Drosophila* $InsP_3R$ gene have been generated, ranging from insertions[99] to single point mutations in the gene[100]. All the hypomorphs and single point mutants show delays in moulting (the process by which an embryo or larva proceeds in its life cycle from one stage of growth to the next[99,100]), followed by lethality. The focus of lethality lies in larval aminergic cells[100] while moulting delays originate from cells of the prothoracic gland (R. Joshi, unpublished data). Mutants of the *Drosophila* $InsP_3R$ also show a faster recovery from olfactory adaptation[101], a phenomenon that is known to be regulated by $Ca^{2+}$ (refs 104, 105). The enhanced recovery from olfactory adaptation has been partially rescued in one of the single point mutants (*itpr*$^{ka901}$) by over-expression of $DmInsP_3R$ cDNA transgene in the domain of olfactory neurons (D. Wagh, unpublished results). Genetic methods have thus allowed for the identification of specific cell types that require $InsP_3R$ function in *Drosophila*. Of interest now is to understand how the $InsP_3R$ affects $Ca^{2+}$ signalling in these cells. The use of single point mutants, whose functional properties have been determined, at a single channel level, should prove an invaluable tool in this analysis.

## A genetic approach to $InsP_3R$ structure–function studies using single point mutants of the *D. melanogaster* $InsP_3R$

Some of the point mutants of the *Drosophila* $InsP_3R$ have been sequenced[100] and four of these mutations, namely, *ka901*, *wc703*, *ka1091* and *ug3*, lie in stretches of amino acids conserved across all known $InsP_3Rs$ (Figure 5). The mutation *ka901* is a Gly → Ser change in a region homologous to the selectivity filter region of mammalian $InsP_3Rs$. The pore-forming regions of $InsP_3R$ and RyR families are highly conserved and contain a putative pore-forming motif GXRXGGGI/VGD (the G2630 residue of ka901 is underlined). Additionally, heterozygotes of *itpr*$^{ka901}$ and an *itpr* null allele (*itpr*$^{90B0}$) show the same extent of lethality as seen in homozygotes of the null allele for *itpr*, indicating that mutation of the ka901 residue results in a non-functional channel[100]. Mutation of the homologous Gly as in ka901, to Cys in RyR shows non-functional homomeric channels[104]. Co-infection of the Gly → Cys RyR with the wild-type RyR shows presence of a single type of hybrid functional channel, with an increased single channel conductance[104]. Mutations in this region of the RyR have been implicated in severe central core disease in which an Ile residue (which lies next to Gly2630 at position 2631 in the fly $InsP_3R$) has been mutated to Thr (Figure 5). The homologous residue is Ile4898Thr in RyR[105]. $Ca^{2+}$ photometry experiments in HEK 293 cells transfected with RyR I4898T showed no $Ca^{2+}$ release in the presence of agonists, indicating that homomeric channels are non-functional[105]. Heteromeric co-infections with wild-type channels showed higher resting $Ca^{2+}$ concentrations in the cytoplasm, indicating that the heterotetramers of mutant and wild-type channels are leaky[105]. Overall, these results highlight the importance of the putative pore-forming motif for ion conductance by $InsP_3R$ and RyR channels. These studies also highlight the importance of the side chain of the amino acid in the selectivity filter region. Gly, which has only –H as a side chain, forms wild type functional channels, whereas the mutation to Cys with a $–CH_2–SH$ side chain (as seen in RyR, discussed above) results in non-functional homomeric channels. Mutation of the neighbouring Ile residue with a $–CH(CH_3)CH_2CH_3$ side chain (hydrophobic) to Thr with a $CH(OH)CH_3$ side chain (shorter with a charged –OH group) also results in non-functional homomeric channels, implying that the size and charged groups present on the side chain affect the ion conductance properties of the RyRs. It would thus be of interest to study the conductance and $Ca^{2+}$ release properties of ka901 channels. This would need to be done in a heterologous system, since homozygous animals die as early second instar larvae. Such a study would help in correlating the physiological phenotypes of larval lethality and olfactory adaptation in ka901 mutants with the single channel properties of ka901.

Biochemical and single channel experiments have led to identification of a putative $Ca^{2+}$ sensor domain in the $InsP_3R$[94,106]. The wc703 mutation of the *Drosophila* $InsP_3R$ (Figure 2 *b*) results in mutation of a Gly to Glu within a

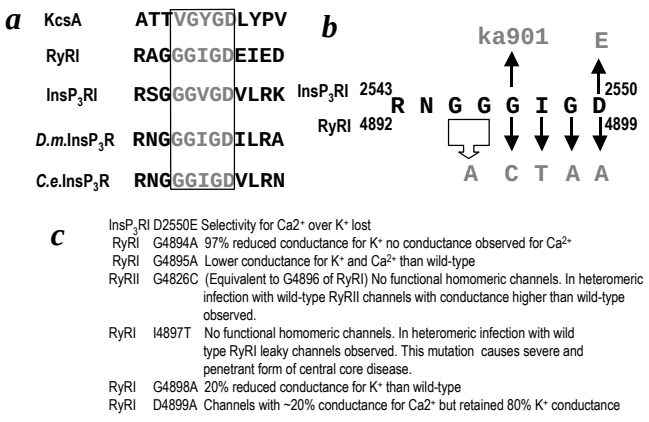


**Figure 5.** ***a***, Alignment of the residues forming the selectivity filter pore in K-channel (KcsA) with homologous regions in Ryanodine receptorI (RyRI) and InsP3R type I from mammals (InsP3RI), *Drosophila melanogaster* (D.m.$InsP_3R$) and *Caenorhabditis elegans* (C.e.$InsP_3R$); ***b***, Mutations in the selectivity filter region of the Ryanodine receptors (written below the selectivity filter sequence) and those in the $InsP_3R$ (written above the selectivity filter sequence) generated and studied. ***c***, Altered properties of the point mutant channels of the selectivity filter region mentioned in B in the InsP3Rs and the RyRs as observed in single channel analysis.

putative $Ca^{2+}$ sensor domain identified by biochemical and single channel experiments for the rat type I $InsP_3R$[106]. While the mutations in rat type I $InsP_3R$ led to removal of a negative charge (mutation of Glu resides) and observation of a broader $Ca^{2+}$ dependence in single channel studies[106], the addition of a negative charge in wc703, may be expected to narrow the $Ca^{2+}$ dependence curve. Sequence comparison with mammalian $InsP_3Rs$ shows that the wc703 residue resides in a well-conserved putative non-canonical $Ca^{2+}$-binding motif of the $InsP_3R$[94]. Hence it is likely to affect $Ca^{2+}$-mediated modulation of the channel. Interestingly, heterozygotes of *wc703* also show an enhanced recovery from olfactory adaptation[101]. Heterozygotes of *wc703* with one copy of *90B0* survive longer than that of *ka901* with *90B0*, indicating that wc703 probably does not affect the function of the channel as severely as ka901. A comparison of the single channel properties of ka901 with wc703 channels might help understand how two mutations in different regions of the channel lead to a similar phenotype of faster recovery from olfactory adaptation[101] (D. Wagh, unpublished results).

Functional studies on the ligand-binding domains of the $InsP_3R$ have divided the ligand-binding domain (~ first 650 amino acids) into two sub-domains, consisting of a core-binding domain (amino acid residues ~ 225–650) and an inhibitory domain[107] (first 224 amino acids, Figure 1). Studies have shown that the ligand-binding domain can be split into two separate domains by trypsin digestion, but the two domains can come together to form a functional ligand-binding site[108]. Bacterial expression and ligand-binding studies of first 604 amino acid residues of the rat type I $InsP_3R$ showed an affinity of 45 nM for $InsP_3$, similar to that observed with full length mouse $InsP_3R$ (85 nM), while the deletion of first 224 amino acid residues increased the affinity to ~ 100 pM[107]. Surprisingly, expression of full length $InsP_3R$ with the first 223 amino acid deletion resulted in non-functional channels[109]. These results were observed in DT40 cells (null for all the three isoforms of $InsP_3Rs$) over-expressing this N-terminal deleted channel by monitoring agonist mediated intracellular $Ca^{2+}$ release[109]. The expressed protein surprisingly retained its pM affinity for $InsP_3$ binding[109], indicating the importance of the most N-terminal region of the channel in gating. This idea is further supported by trypsin digestion followed by cross-linking and co-immunoprecipitation of peptide fragments[110]. These data suggest an inter-subunit N- and C-terminal interaction in both homo- and hetero-tetramers of $InsP_3Rs$[110]. The mutation, ug3, interestingly lies at the interface of the two sub-domains of the ligand-binding domain. Understanding the single channel properties of this mutant should help in judging the role of the N-terminal region of the channel in gating. u*g3*/*90B0* heterozygotes survive as late third instars, implying that the effect of ug3 on channel function is milder than both, ka901 and wc703. Due to the conservation of all the three residues, ka901, wc703 and ug3 across all known $InsP_3Rs$, results obtained with these mutants could be extrapolated towards understanding channel function in mammalian $InsP_3Rs$.

Single channel characterization of randomly generated mutant forms of $InsP_3Rs$ from a genetic model organism, the fly, could thus identify through an unbiased strategy, novel residues that affect the structure and function of the $InsP_3R$. Such an approach is unlike targeted mutagenesis which is presently widely used, where residues are identified based upon homology with other channels[57,55]. Given the high identity of the isoforms of $InsP_3Rs$ of mammals and invertebrates and the large size of $InsP_3R$, a random mutagenesis approach by isolating mutants of $InsP_3R$ based on a phenotype is likely to provide novel insights in identifying conserved and non-conserved residues involved in regulating the structure and/or function of the protein.


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