Published in final edited form as:

Cell Calcium. 2013 ; 53(0): 307–314. doi:10.1016/j.ceca.2013.01.003.

Characterization of a flatworm inositol (1,4,5) trisphosphate receptor (IP₃R) reveals a role in reproductive physiology

Dan Zhang^{a,1}, Xiaolong Liu^a, John D. Chan^a, and Jonathan S. Marchant^{a,b,*}

^aDepartment of Pharmacology, University of Minnesota Medical School, MN 55455, USA

^bThe Stem Cell Institute, University of Minnesota Medical School, MN 55455, USA

Abstract

Inositol 1,4,5-trisphosphate receptors (IP₃Rs) are intracellular Ca²⁺ channels that elevate cytoplasmic Ca²⁺ in response to the second messenger IP₃. Here, we describe the identification and *in vivo* functional characterization of the planarian IP₃R, the first intracellular Ca²⁺ channel to be defined in flatworms. A single *IP₃R* gene in *Dugesia japonica* encoded a 2666 amino acid protein (*Dj*.IP₃R) that shared well conserved structural features with vertebrate IP₃R counterparts. Expression of an NH₂-terminal *Dj*.IP₃R region (amino acid residues 223–585) recovered high affinity ³H-IP₃ binding (0.9 ± 0.1 nM) which was abolished by a single point mutation of an arginine residue (R495L) important for IP₃ coordination. *In situ* hybridization revealed that *Dj*.IP₃R mRNA was most strongly expressed in the pharynx and optical nerve system as well as the reproductive system in sexualized planarians. Consistent with this observed tissue distribution, *in vivo* RNAi of *Dj*.IP₃R resulted in a decreased egg-laying behavior suggesting *Dj*.IP₃R plays an upstream role in planarian reproductive physiology.

Keywords

Ca²⁺ signaling; Intracellular Ca²⁺ channel; IP₃; Invertebrate; Platyhelminth; Planarian

1. Introduction

Inositol (1,4,5) trisphosphate receptors (IP₃Rs) are Ca²⁺ release channels localized within the endoplasmic reticulum. Their activation by agonists that generate IP₃ increases cytoplasmic Ca²⁺ concentration and thereby controls the execution of many cellular processes [1]. Vertebrates possess three genes encoding discrete IP₃R isoforms (IP₃R1–3, [1–5]), invertebrate species characterized to date have a single IP₃R gene [6–10], while the ciliated protozoan *Paramecium* possesses numerous IP₃R-like genes [11]. The role played by these intracellular Ca²⁺ channels in organismal physiology has been investigated in knockout mouse models [4,5] and genetically tractable invertebrate systems [12–14]. Collectively, such studies have demonstrated a broad range of events in developmental and adult physiology that depend on appropriate IP₃R functionality and that these different outcomes are differentially sensitive to changes in IP₃R properties and or expression. As

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*Corresponding author at: 6-120 Jackson Hall, 321 Church St SE, Minneapolis, MN 55455, USA. Tel.: +1 612 624 4664. march029@umn.edu (J.S. Marchant).

¹Present address: Cancer Institute, Penn State Hershey Medical Center and Penn State College of Medicine, 500 University Drive, Hershey, PA 17033, USA.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ceca.2013.01.003>.

methods are optimized to permit functional genetic approaches in a broader swathe of emerging model systems, including the Lophotrochozoa [15], it is becoming possible to dissect how other organisms customize IP₃-evoked Ca²⁺ signaling to support unique physiological outputs and behaviors.

In invertebrate models, full length IP₃Rs have been well characterized in *Caenorhabditis elegans* and *Drosophila melanogaster* [12–14], but also described in an echinoderm [6], a mollusk [9] and another arthropod [10]. Bioinformatic prospecting of available genomic/transcriptomic data has provided further insight into the sequence diversity of various invertebrate IP₃Rs but biological verification of annotated sequence information and functional genetic insight is lacking. One grouping that is frequently overlooked (even from *in silico* studies) are the flatworms. The lophotrochozoan phylum *Platyhelminthes* ('flatworms') represents a diverse grouping of unsegmented, bilaterally symmetrical, triploblastic worm species that hold significance for both basic and medical science (Fig. 1A). The free-living platyhelminths include planarian flatworms, which have long used as a model system for studying regenerative biology [16–19]. Planarian worms can be cut into small pieces, and each of these pieces retains an intrinsic ability to regenerate the original body plan. This remarkable behavior is driven by stem cells called 'neoblasts', which are the only mitotically active cells in the adult animal and therefore generate all differentiated cell types. Understanding the behavior and regulation of neoblasts is a key driver of the renaissance of planarians as a model organism for interrogating *in vivo* stem cell biology and the molecular basis of *de novo* tissue formation. In terms of medical outcomes, the majority of platyhelminth species are parasitic (Fig. 1A) and associated infections have clinical and veterinary significance. For example, over 200 million people worldwide harbor schistosome infections (*Schistosoma mansoni*, *S. japonicum* and *S. haematobium*) and the burden of this neglected tropical disease, manifest through gastrointestinal and liver pathology, anemia, undernutrition, growth retardation, genitourinary disease (*S. haematobium*) and the increased prevalence of co-morbidities, is arguably second only to HIV/AIDS in impact [20,21]. Cestode ('tapeworm') infections of humans and livestock also have important clinical and economic consequences [22].

Recent data have underscored the importance of understanding the molecular basis of Ca²⁺ signaling in these different platyhelminth systems. Drugs that impact Ca²⁺ homeostasis have been shown to modify regenerative polarity in planarians [23–25] revealing a novel effect of Ca²⁺ signaling on *in vivo* stem cell differentiation and regenerative outcomes. Drugs targeting Ca²⁺ channels have also been revealed to possess antischistocidal activity in drug screening assays against various stages of the schistosome life cycle [26–28]. Validation of the underlying targets that underpin these pharmacological effects in either system necessitates molecular characterization of the flatworm Ca²⁺ signaling 'toolkit'. This is becoming easier within the availability of genomic sequencing data and notable flatworm representatives include the planarian *Schmidtea mediterranea* [29], clinically relevant flukes (*Schistosoma* species [30], *Clonorchis sinensis*) and several animal cestodes (*Taenia sp.*, *Echinococcus sp.* and *Hymenolepis sp.*). To supplement bioinformatic resources, we report here a biological characterization of an IP₃ receptor from the planarian *Dugesia japonica*, the first intracellular Ca²⁺ channel to be described in flatworms.

2. Materials and methods

2.1. Isolation and analysis of Dj.IP₃R

Sexual and asexual planarians (*D. japonica*, Gifu, Iruma river strain) were maintained at room temperature (20–23 °C) and fed with strained beef and chicken liver puree once a week [23,31]. Worms were starved for 5 days before most experiments, and for at least 10 days before *in situ* hybridization. Starving is performed in order to decrease background

staining from residual gut contents. Total RNA was extracted from 20 worms using TRIzol® (Invitrogen) and treated with DNase (Ambion) to digest residual genomic DNA. 5 µg of total RNA was then used for first-strand cDNA synthesis (SuperScript III First-Strand Synthesis System, Invitrogen). PCR amplification was performed using degenerative primers (Supplementary Table 1) targeting the evolutionary conserved regions, and amplification products were cloned into pGEM-T Easy vector (Promega). After sequencing, primers were iteratively designed until the whole coding sequence of *Dj.IP₃R* was obtained (Supplementary Figure 1). To obtain the 5' and 3' UTR, 5' and 3' RACE was performed. Briefly, total mRNA was isolated and purified using Oligotex® mRNA mini Kits (Qiagen) and used to synthesize cDNA (5'/3' RACE Kit, Roche) with gene-specific primers (Supplementary Table 1) or Oligo-dT. Nested PCR was performed to amplify 5' and 3' UTR sequence of IP₃R. The PCR products were then gel purified (High Pure PCR Product Purification Kit, Roche) and ligated into pGEM-Teasy vector for sequencing. The final sequence assembled by DNASTar represented at least two-fold coverage. IP₃R sequences were aligned using ClustalW2 (BLOSUM62) and alignments scored by amino acid identity, unless otherwise noted. For structural analysis, the amino acid sequence of the planarian IP₃R IBC was superimposed on the crystal structure of the mouse IP₃R1 IBC (Protein Data Bank: IN4K, [32]) using UCSF Chimera 1.6.2 and Modeller 9.11 platforms [33].

2.2. Expression of *Dj.IP₃R* fragments for ³H-IP₃ binding assays

The NH₂-terminal region of the planarian IP₃R (amino acids 1–705) was codon optimized in pUC57 by a commercial service (Genscript), and used as a template for mutagenesis. The region corresponding to the IP₃ binding core (*Dj.IP₃R* amino acids 223–585, equivalent to 224–604 in mouse IP₃R1) was subsequently subcloned into a GST tagging vector (pGEX-6p2) for bacterial expression. Fragments were expressed using a two-step culture protocol. A pre-culture, grown for 19 h (27 °C), was used to inoculate the main culture (300 ml) which was grown until the optical density reached ~1.3 (~7 h at 22 °C). Expression was induced by IPTG inoculation (0.5 mM final concentration) for 20 hrs (14 °C). After induction, the culture was centrifuged (5000 × *g* for 10 mins) and the pellet resuspended in PBS (15 ml) supplemented with a protease inhibitor cocktail (Roche, cOmplete® tablet) and β-mercaptoethanol (1 mM, final concentration). Soluble protein was harvested following addition of lysozyme (150 µg/mL) over multiple freeze-thaw cycles. Deoxyribonuclease I (~50 units/ml final concentration, Roche) was added in the final wash step prior to incubation on ice (20 mins). Finally, samples were sonicated twice and then centrifuged (30,000 × *g* for 1 h) prior to storage of the supernatant. For enrichment of the GST-tagged product, a stock of glutathione sepharose beads (GE Healthcare) was washed multiple times in PBS prior to the addition of soluble protein. The mixture was incubated on a shaker (2 h, 4 °C), then centrifuged and the supernatant removed. Following further washing of the beads (PBS), elution buffer supplemented with the protease inhibitor cocktail was added and the mixture incubated with shaking (20 mins, 4 °C). The mixture was centrifuged (500 × *g*, 5 mins) and the supernatant containing the GST-tagged fusion protein saved for experiments. Rat IP₃R binding domain constructs, used as controls, were a generous gift from Prof. Colin Taylor (Cambridge, UK).

Indirect binding assays were performed by incubating protein samples with ³H-IP₃ (1.6 nM, Perkin-Elmer) for 15 mins in TE (500 µl, 1 mM EDTA, 50 mM Tris-HCl, pH 8.3 at 4 °C) with appropriate concentrations of unlabeled IP₃. Binding reactions were terminated by addition of PEG (30%) and IgG (0.5 mg/ml final concentration) followed by centrifugation (20,000 × *g*, 5 min at 4 °C). Pellets were washed (15% PEG in TE) and recentrifuged. Finally, pellets were solubilized in TE containing 2% Triton X-100 prior to liquid scintillation counting. Non-specific binding was determined by addition of 10 µM IP₃ (Calbiochem) and was typically ~10% of total ³H-IP₃ binding. Competition curves were

fitted to Hill equations (Origin 8.5, OriginLab) to calculate IC_{50} and thereby K_D values. Data are expressed as mean $\pm$ SEM for n independent experiments using different bacterial samples.

2.3. In situ hybridization

To generate probes for *in situ* hybridization, the *Dj.IP₃R*-coding region was cloned into pGEM-T easy vector. After verification by sequencing, the recombinant-plasmid was linearized (*ApaI*) and DIG-labeled antisense riboprobes (targeting both NH₂ and COOH terminal *Dj.IP₃R* regions, Supplementary Figure 2) were synthesized by *in vitro* transcription. Samples were first treated with 2% HCl (5 mins on ice) and then fixed (Carnoy's fixative, 2–3 h on ice). Samples were then rinsed (100% methanol) and bleached (6% H₂O₂ in methanol for 16–24 h at room temperature). Samples were subsequently rehydrated in a graded series of methanol/PBS solutions and then blocked (0.3% Triton X-100, PBS). Whole-mount *in situ* hybridization was carried out at 55 °C in hybridization solution (50% formamide, 5× SSC, 100 mg/ml yeast tRNA, 100 mg/ml heparin sodium salt, 0.1% Tween-20, 10 mM DTT, 5% dextran sulfate sodium salt) incorporating digoxigenin (DIG)-labeled antisense riboprobe (40 ng/ml) denatured at 72 °C for 15 mins prior to use. Due to the larger size of sexualized worms, samples were usually treated with Proteinase K prior to hybridization (12 µg/mL for 12–20 mins at 37 °C). A standard mixture of BCIP/NBT in chromogenic reaction solution was used for color development, followed by 4% paraformaldehyde fixation. Samples were captured using a Leica MZ16F stereomicroscope and a QiCAM 12-bit cooled color CCD camera. *In situ* probe regions, and accession numbers for related gene products, were: *Opsin* (1–475 bp, AJ421264), *Dj.IP₃R* (320–1528nt and 5070–7399nt, KC249981).

2.4. In vivo RNAi

Dj.IP₃R coding region (324–2471nt) was amplified using gene specific primers incorporating a Kozak sequence (GCCACCATGG) and cloned into the IPTG-inducible vector pDONRd7. The negative control (*Sm.Six1*) and positive control (*Dj.Six1*, which causes an eyeless phenotype) were described previously [23,24]. dsRNA for *in vivo* RNAi was generated by induction of *E. coli* HT115[DE3] containing the recombinant plasmids with IPTG (1 mM, 2 h at 37 °C). *In vivo* RNAi was performed as described previously [23,24,31]. Briefly, worms were fed with a mixture of chicken liver and bovine red blood cells and the transformed bacteria HT115 [DE3] expressing dsRNA over several cycles. To assess the extent of RNAi knockdown, either semi-quantitative RT-PCR or quantitative real-time PCR (qPCR) was performed using SYBR GreenER qPCR SuperMix Universal (Invitrogen) on an ABI 7500 real-time PCR thermocycler (Applied Biosystems). mRNA levels of specific genes were compared with controls using planarian β -actin to normalize cDNA input. All data are presented as mean $\pm$ standard error of the mean for the indicated number of experiments. Primers used were: *Dj.IP₃R*: 5'-TTTCAAATGGAACCCGAATTTTGGGA (forward), 5'-GCAAAATATCACCAATACCACCTCCA (reverse); β -actin: 5'-GGT AATGAACGATTTAGATGTCCAGAAG (forward), and 5'-TCTGCATACGATCAGCAATACCTGGAT (reverse). *In situ* and *in vivo* RNAi probe locations are shown in Supplementary Figure 2.

2.5. Western blotting

Worms were homogenized in 500 µl of 20 mM HEPES buffer supplemented with Complete™ protease inhibitor tablet (Roche) and centrifuged at $5000 \times g$ for 3 mins at 4 °C. The supernatant was subsequently collected and centrifuged at $70,000 \times g$ for 1 h at 4 °C to yield a microsomal pellet, which was then resuspended in NP40 lysis buffer (20 mM HEPES, 1% NP40). Samples of planarian membranes (50 µg/lane), rat cerebellar

membranes (30 µg/lane) and whole cell lysate (50 µg/lane) of HEK293 transiently expressing a NH₂-region (residues 1–705) encompassing the *Dj.IP₃R* binding domain were heated (94 °C, 8 mins under reducing conditions) prior to SDS-PAGE gel electrophoresis (4–12% Tris–Bis Gel, Invitrogen). The peptide antibody to planarian IP₃R was raised in rabbit (epitope ELEKTQNDIEHKLVG, Open Biosystems) and used at a dilution of 1:200. For analysis of HEK293 cell expression of *Dj.IP₃R*(1–705)-mCherry fusion protein, a cross-reacting DsRed antibody was used (sc-33353, Santa Cruz).

3. Results

3.1. Identification of the planarian IP₃R

Although IP₃ has been shown to be generated in cestodes and trematodes [34,35], there are no reports of IP₃ efficacy or characterization of the molecular targets of IP₃ across the entire platyhelminth phylum (Fig. 1A). *D. japonica* is a planarian species widely used in regenerative bioassays [16], and as such components of the organismal Ca²⁺ signaling toolkit, have previously been cloned in this system [23–25]. Using degenerate PCR methods informed by fragmented genomic data from another planarian species (*Schmidtea mediterranea*, [29]), we isolated the entire coding sequence of *D. japonica* IP₃R (*Dj.IP₃R*). The full length *Dj.IP₃R* protein was encoded as a ~8.3 kb mRNA, comprising 75nt of 5' UTR, a 7998nt protein coding region and ~300nt of 3' UTR (Supplementary Figure 1). Sequencing data evidenced the existence of only a single IP₃R gene in planarians, a conclusion supported by homology mining of the *Schmidtea mediterranea* genomic database (mk4.000868.02/4/6).

The *Dj.IP₃R* protein (2666 amino acids) showed ~45–50% overall identity with IP₃R isoforms previously characterized from both vertebrates and invertebrates (with the exception of a greater sequence divergence from *C. elegans*, ~34% identity). This level of identity was considerably higher than observed with ryanodine receptors characterized in some of the same species (<10%). The extent of homology was even higher within key functional domains of the IP₃R protein: NH₂ terminal regions that mediate high affinity IP₃ binding and the COOH terminal transmembrane spanning regions that harbor the Ca²⁺ channel pore (Fig. 1C, 65% amino acid identity). These NH₂ and COOH terminal regions are known to physically interact to mediate IP₃ activation of Ca²⁺ release [36–39]. This high level of identity encompassed conservation of critical amino acid residues within the NH₂ terminal region important for determining IP₃ binding. For example, IP₃ binding is preserved with nanomolar affinity to solely the IP₃ binding core (IBC) region of the IP₃R (residues 224–604 in mouse IP₃R1, [32]) and there is complete conservation of eleven residues known to be important for IP₃ coordination within the planarian IBC (Fig. 1C). Seven residues in the NH₂-terminal suppression domain (SD, residues 1–224 in mouse IP₃R1, [40]) attenuate the high affinity of the IBC and again these residues are identical in *Dj.IP₃R*. Eleven other residues within the SD determine the overall, isoform-specific affinity for IP₃ of the three vertebrate IP₃Rs [41] and here there was expected variation within the *Dj.IP₃R* sequence (Fig. 1C). However, the tyrosine residue (Tyr-167 in mouse IP₃R1, [38,39]) important for functional coupling between the NH₂ ligand-binding and COOH terminal Ca²⁺ channel domain was conserved (Tyr-166 in *Dj.IP₃R*). Hydropathy analyses predict six transmembrane spanning regions within the COOH terminus of *Dj.IP₃R*, and overall amino acid identity within the channel forming domain to mouse IP₃R1 was high. The selectivity filter motif between TM5 and TM6 conformed to the consensus ('GGIGD') sequence identified in all invertebrate IP₃Rs to date [6,9,10,12–14] characteristically divergent from the 'GGVGD' sequence diagnostic of vertebrate IP₃R isoforms. Therefore, from sequence analysis there is strong bioinformatic support for IP₃ binding and Ca²⁺ channel activities of the reported *Dj.IP₃R* sequence.

Finally, characterization of the planarian IP₃R allowed enabled a more targeted prospecting of other platyhelminth (*e.g.* the parasites *Schistosoma mansoni* and *Clonorchis sinensis*) and lophotrochozoan genomes (*Capitella teleta*, *Lottia gigantea*) for IP₃R sequence. With the caveat that several of these sequences represent only gene predictions, confidence derives from unambiguous conservations within sequence diagnostic of IP₃Rs, for example within the COOH terminal selectivity filter and pore-lining transmembrane domain (Fig. 1D). Here, all the lophotrochozoan channels harbor the 'GGIGD' selectivity filter motif known to increase K⁺ conductance relative to the vertebrate IP₃Rs [42], as well as sequence variants diagnostic of platyhelminth IP₃Rs (Fig. 1D). In the NH₂ terminal region, there was good conservation of IP₃ coordinating residues in the IBC of the different flatworm species, and invariant presence of the suppressor domain tyrosine residue implicated in functional coupling of IP₃ binding to channel gating [38,39].

3.2. Biochemical characterization of Dj.IP₃R

Does IP₃ bind to Dj.IP₃R? This was assessed by evaluating ³H-IP₃ binding to the NH₂ region of Dj.IP₃R, as expression of the IBC region alone has proved sufficient to recapitulate IP₃ binding of high affinity and selectivity [32,43]. Expression of the NH₂ terminal sequence (residues 1–705) of the Dj.IP₃R tagged with mCherry (predicted *M_r* ~ 109 kDa) yielded a cytosolic expression profile in mammalian cells, but levels were insufficient to resolve adequate ³H-IP₃ binding despite demonstration of expression of the fusion protein using an antibody designed against a NH₂-terminal region of Dj.IP₃R. Therefore, we resorted to previous optimized bacterially expression methods [43,44] employing the shorter IBC domain (residues 223–585). Induction of the GST-tagged Dj.IP₃R[223–585] sequence (predicted *M_r* ~ 68 kDa), followed by glutathione-sepharose bead-based purification, resulted in the recovery of a protein product of expected size as observed by Coomassie staining (Fig. 2A) or immunoreactivity on a Western blot (GST antibody, Fig. 2B). Indirect binding assays performed with these samples revealed high affinity ³H-IP₃ binding (*K_d* = 0.91 ± 0.11 nM, *n* = 3, Fig. 2C) to the planarian IBC.

To confirm the specificity of ³H-IP₃ binding, we mutagenized a single residue within the IBC (R495L, Fig. 1C and Supplementary Figure 1) predicted to impact ³H-IP₃ coordination. This residue is conserved in all IP₃Rs isoforms detailed to date. The equivalent arginine residue in the mouse IBC (R511) forms a hydrogen bond with the 5-phosphate of I(1,4,5)P₃ [32] and mutation of this residue (R511A) causes a complete loss of ³H-IP₃ binding activity [43,45]. Whereas the R495L mutation in the planarian IBC (Dj.IP₃[R223–585.(R273L)]) did not impact protein production in *E. coli* (Fig. 2A and B), no specific binding activity could be resolved with this construct (Fig. 2C). These data suggest a high degree of structural conservation between the IBCs across the evolutionary spectrum. Indeed, predictive homology modeling of the planarian IBC sequence onto the mouse IBC crystal structure revealed the close extent of structural homology (Fig. 2D).

3.3. Tissue distribution of Dj.IP₃R

The localization of mRNA was examined by whole mount *in situ* hybridization. In asexual planarians, staining was strongest in the pharynx and optical nerve regions (Fig. 3A), although background levels were generally higher than antisense controls implying a pervasive distribution of Dj.IP₃R. Higher magnification images of the planarian eyespots are shown in Fig. 3B to highlight the staining in the optic nerve compared with sense controls and the distribution of opsin.

Planarians exist in both asexual (multiply by fission) and sexual forms (crossfertilizing hermaphrodites). Sexualization is a seasonal and a highly plastic phenomenon: it occurs through *de novo* generation of germ cells from neoblasts (*i.e.* not from an embryonically

segregated germ line, [17,46,47]). This process occurs after the animal has reached a certain size and leads to the addition of ovaries, testes and the copulatory apparatus as schematically shown in Fig. 3C, together with a size comparison of the asexualized and sexualized form (Fig. 3D). Examination of IP₃R distribution in sexualized form of *D. japonica* revealed expression as two bilateral lines of patches on the dorsal side, which are regions of the testes (Fig. 3E). There was also pronounced staining in the ovaries localized anteroventrally as well as components of the copulatory apparatus (Fig. 3F) including the copulatory bursa, bursa canal, penis papilla and gonopore. As with asexualized forms, *Dj*.IP₃R staining was also seen in the optical nerve (Fig. 3E) but no equivalent signal was observed with the sense probe. Attempts to examine the protein distribution of *Dj*.IP₃R using a polyclonal antibody reactive against the NH₂ terminal region of overexpressed *Dj*.IP₃R fragments were not successful owing to high background activity. Indeed, while Western blotting approaches in planarian homogenates revealed immunoreactivity of a ~235 kDa band (Supplementary Figure 2), this staining was not unique with lower molecular weight bands possibly representing IP₃R degradation products or cross reactions within the planarian proteome.

3.4. In vivo RNAi of *Dj*.IP₃R

On the basis of the distribution of *Dj*.IP₃R mRNA in reproductive tissues, we considered *Dj*.IP₃R may play a role in reproductive physiology. The impact of *Dj*.IP₃R knockdown was therefore examined by *in vivo* RNAi. Worm cohorts were fed RNAi constructs targeting either *Dj*.IP₃R or a control construct (*Sm*.Six1) that was not associated with any obvious phenotype [23,24]. To examine the specificity and effectiveness of *Dj*.IP₃R RNAi, quantitative real-time PCR was performed using cDNA from worms in different RNAi cohorts prepared 3 weeks after first RNAi construct feeding. Worms subjected to *Dj*.IP₃R RNAi displayed a significant decrease in *Dj*.IP₃R transcript levels (reduction of $83 \pm 7\%$) relative to worms fed the *Sm*.Six1 RNAi control. An example of an RT-PCR gel performed in parallel with qPCR measurements is shown in Fig. 4A, and the results from both different approaches concur. Following the period of RNAi feeding (~4 weeks), it was noted that the number of eggs appearing in the culture dishes of the *Dj*.IP₃R RNAi worms was low. Developing planarian embryos eggs are laid within egg capsules which are easily visible in the external media compared to within the body (Fig. 4B). Quantification of this difference revealed a much higher degree of egg production in *Sm*.Six1 RNAi worms compared with *Dj*.IP₃R RNAi worms (Fig. 4C). Examination of IP₃R mRNA levels confirmed successful knockdown of levels in these same *Dj*.IP₃R RNAi worms but not the control cohort. This effect was also examined over a longer-term feeding experiment (3 months). Again, a similar phenotype was observed. *Sm*.Six1 RNAi worms laid 30 eggs/10 worms while *Dj*.IP₃R RNAi worms only laid 1 egg/10 worms. These effects of *Dj*.IP₃R knockdown were not a result of an overall defect in growth as both control and *Dj*.IP₃R RNAi animals grew to similar sizes over this time period. Collectively, these data suggest that *Dj*.IP₃R plays an important role in planarian reproductive physiology to impair egg-laying.

4. Discussion

The second messenger IP₃ has been shown to be generated in cestode and trematode parasites [34,35] but no characterization of downstream effects or targets have been reported. This situation typifies the general lack of molecular information regarding Ca²⁺ channels in many emerging flatworm models. Beyond the recent characterization of voltage-operated Ca²⁺ channels in planarians [23–25], little molecular characterization of Ca²⁺ channels or transporters in platyhelminths has been performed. Progress is important not solely for understanding the role of Ca²⁺ fluxes in flatworm biology, but because the molecular divergence of flatworm Ca²⁺ channels from vertebrate counterparts holds promise for exploitation by novel antiparasitic agents [25,48]. Indeed, the key pharmacotherapy for

large scale treatment of schistosomiasis is praziquantel, a drug proposed to mediate parasite clearance via stimulation of Ca^{2+} entry [25].

Like other invertebrates, flatworms possess only a single *IP₃R* gene which encodes a protein displaying strong architectural conservation with vertebrate *IP₃R*s especially within the NH_2 -terminal ligand binding domain and COOH-terminal pore forming regions (Fig. 1). The observed affinity of the planarian IBC ($K_d \sim 0.9$ nM, Fig. 2C) corresponds with values reported for rodent IBCs (range ~ 0.2 – 2 nM, [40,41,43,49]). This represents a higher affinity, as expected, than longer NH_2 terminal fragments containing the suppressor domain (for example, 7 nM for residues 1–705 of the *C. elegans* *IP₃R*, [7]). *In situ* hybridization revealed expression of *Dj.IP₃R* mRNA in pharyngeal, nervous (optical nerve) and gonadal tissue (Fig. 3). This distribution parallels that observed by antibody staining in *C. elegans*, where *IP₃R* expression was resolved in the pharynx, nerve ring, gonad as well as within excretory cells and the intestine [7]. One might expect a more ubiquitous staining pattern given the pervasive expression of mammalian *IP₃R1*, but it is important to remember low levels of *IP₃R* expression would not be readily demonstrable by *in situ* hybridization approaches, so a broader role for *IP₃R*s cannot be excluded from localization data alone. The expression of *Dj.IP₃R* mRNA in photoreceptor neurons was also evident from a recent transcriptomic study [50] in a different planarian species (*Schmidtea mediterranea*). Both, the eye-specific RNAseq dataset and subsequent hybridization studies revealed an enrichment of a *Smed-ip3r* in photoreceptor neurons.

The contribution of *Dj.IP₃R* to physiological outcomes was assessed by *in vivo* RNAi. In light of the current lack of transgenic methods in planarians, *in vivo* RNAi is the approach of choice for graded attenuation of gene function. This method is useful for revealing processes at the organismal level that are most sensitive to decreased *IP₃R* levels, while avoiding the high level of lethality resulting from knockout of the single invertebrate *IP₃R* gene [51,52]. On the downside, phenotypes can be less evident through RNAi (owing to residual mRNA) than from knockout methods. For example, although *Dj.IP₃R* was expressed in the optical nerve, no crude visual defect (e.g. in light avoidance responses) was observed following *IP₃R* RNAi (data not shown). This result could be attributed to strong reserve capacity in *IP₃R*-evoked signaling, or exceptional protein perdurance of the *IP₃R* in the face of RNAi (despite $>80\%$ decrease in *IP₃R* mRNA levels). Alternatively we note the irrelevance of the *Drosophila* *IP₃R* to phototransduction despite expression in the fly eye [51]. The safest conclusion to draw is that in all invertebrates examined to date, *IP₃R*s contribute to a subset of sensory processes, spanning chemosensation [9], mechanosensation [53] and olfactory processing [10,54] in different organisms.

In the tractable *C. elegans* system, functional genetic analyses have correlated the observed *IP₃R* tissue distribution with a requirement for *IP₃R* activity in feeding, embryogenesis and reproductive physiology [55], amongst other physiological events [12,13]. Notably, loss of *C. elegans* *IP₃R* function causes sterility, an outcome resulting from a multiplicity of deficits in ovulation [56,57], oocyte maturation [58], male mating and sperm transfer [59]. Given the widespread distribution of *Dj.IP₃R* mRNA in planarian sexualized tissues (Fig. 3), the observed deficit in egg laying could result from similar defects. Possibilities could span from immaturity of the ovaries and testes yielding defects in germ cell production to defective oocyte maturation, fertilization and early embryogenesis. All are *IP₃*-dependent events in other systems [59,60]. As peptide hormones maintain mature reproductive organs and differentiated germ cells in planarians [61], it is not unreasonable to suggest phosphoinositide signaling may act as the downstream effector of some neuropeptide receptors. One of the first *IP₃R* dependent processes to be defined in *C. elegans* was as a downstream transducer from an epidermal growth factor receptor homolog (LET-23, [56]). LET-23 function was needed for spermathecal dilation, ovulation and hermaphrodite fertility

[56]. Deciphering the downstream coupling of planarian cell surface receptors that regulate reproductive development and function will likely reveal a similar requirement for phosphoinositide signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Work in the laboratory was supported by the NIH (GM088790 to J.S.M.) and NSF (MCB 0919933). J.D.C. was supported by a Stem Cell Biology Training Grant (T32HD060536).

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Fig. 1. Identification of an IP₃R from the planarian *Dugesia japonica*. (A) Schematic phylogeny to illustrate the placement of the phylum *Platyhelminthes* in the protostome-deuterostome classification. Groupings highlighted with an open box indicate a prior biological characterization of an IP₃R within that particular grouping (e.g. the *Aplysia californica* IP₃R is the molluscan representative, [9]). This now includes the description in this study of the *Dugesia japonica* IP₃R (*Dj*.IP₃R, right). Deuterostome representatives are not shown for simplicity, but see Fig. 4 in [2]. (B) Schematic representation of IP₃R sequence highlighting conservation of regions and residues within the planarian IP₃R (left) and mouse IP₃R1 (right; accession number P11811). Sequence is depicted circularly from the NH₂ to COOH

terminus to facilitate comparison, and is representative of known physical interactions between the IP₃R termini. Highlighted domains are the suppressor domain (red), IBC domains (yellow & green) and the six transmembrane domain spanning region (TMD, purple) at the COOH terminus. Splice sites (S1-S3) are positioned in the mouse IP₃R1 sequence. (C) Conservation of key residues between NH₂ and COOH terminal domains of the planarian and mouse IP₃R. Residues in close proximity within the primary amino acid sequence are shown as linked. Eleven residues essential for high affinity IP₃ binding are identical between the IBC domains (green, yellow). The arginine residue mutagenized in binding studies is highlighted (*). Seven residues in the SD (solid red, top row) shown to mediate physiological suppression of IP₃ binding affinity are also identical between species, as well as the tyrosine residue (black box) implicated in contacting the TM4-TM5 region in the COOH terminus. The COOH terminal selectivity filter motif is shown in purple. (D) Alignment of pore forming regions of IP₃Rs from various lophotrochozoans. Residues conserved in only platyhelminths (blue), only parasitic platyhelminths ('P', light blue) and identical in all species examined (gray) are highlighted. FL, free-living. The selectivity filter isoleucine is shown in brown. Accession Numbers are: *Cs* (GAA48211.1), *Sm* (XP_002576842/3), *Ct* (51964^{JGI}), *Lg* (81367/91259^{JGI}), *Ac* (DQ397517).

Fig. 2.

Expression and modeling analysis of the planarian IBC. (A) Coomassie-stained 4–12% Bis–Tris SDS–PAGE gel loaded (2 μg of protein) with soluble (lanes 1,2) and GST-enriched samples (lanes 3,4) isolated from bacteria expressing *Dj*.IP₃R[223–585] (lanes 1 & 3) or *Dj*.IP₃R[223–585(R495L)] (lanes 2 & 4). (B) Western blot of GST-enriched samples (same lane loading pattern as ‘A’), using an anti-GST antibody for detection. Arrows indicate expected size of proteins (68 kDa). (C) Competitive $^3\text{H-IP}_3$ binding experiment using bacterially expressed *Dj*.IP₃R[223–585] (solid squares) and *Dj*.IP₃R[223–585(R495L)] GST fusion proteins (open square). Non-specific binding was ~10% of total binding ($B_{\text{max}} < 1$ nmol/mg protein). Data represent the mean from three independent inductions and

purifications. (D) Structure of mouse IP₃R IBC in complex with IP₃ (brown, from [32]) used as a template for predictive superimposition of the planarian IP₃R IBC (blue). The β-domain (left) and α-domain (right) are shown together with IP₃ at the top interface, and the location of the mutagenized arginine residue (R495, ~R511 in mouse sequence) critical for ³H-IP₃ binding is highlighted (green). S1, residues corresponding to the mammalian S1 splice site are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Tissue distribution of *Dj.IP3R* mRNA. (A) Whole mount *in situ* hybridization staining for *Dj.IP3R* mRNA in an asexual worm. (B) Enlarged views of eyespot regions to show staining patterns using the antisense probe for *Dj.IP3R* mRNA (left), the sense control (middle), and staining with an antisense probe against *opsin* (right). (C) Cartoon depicting distribution of planarian sexualized organs. Right, an enlarged view to show seminal vesicles (SV), copulatory bursa (CB), bursa canal (BC), penis papilla (PP), gonopore (GP), cement glands (G). (D) Side by side comparison of size of asexual (left) and sexualized (right) planarians typically used for *in situ* hybridization experiments. Scale bar, 1 cm. (E) Dorsal view of sexualized planarian showing *Dj.IP3R* mRNA distribution, with areas of detail enlarged. Regions of testes are arrowed. (F) Ventral view of sexualized planarian showing *Dj.IP3R* mRNA distribution. Enlarged regions show ovarian tissue (left) and parts of the copulatory apparatus (right).

Fig. 4. Effect of *in vivo* RNAi of *Dj.IP3R* on egg laying. (A) RT-PCR gel examining IP3R mRNA abundance (lanes 3 & 4) in sexualized (top) and asexual worms (bottom). β -actin levels (lanes 1 & 2) were used as a control for normalization of loading of experimental samples. L, DNA ladder. (B) Bright field images of a planarian egg *ex corpore* (top) *in corpore* (bottom). Scale bar is 500 μ m. (C) Quantification of eggs laid over a 1 month period in negative control (*Sm.six1*), positive control (*Dj.six1*), and experimental samples (*Dj.IP3R*). Each symbol represents an independent experiment, mean is shown by horizontal bar. Inset, RT-PCR gel showing knockdown of *Dj.IP3R* only in cohort feed RNAi construct targeting *Dj.IP3R*.