Molecular and Functional Characterization of Inositol Trisphosphate Receptors during Early Zebrafish Development

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Fluctuations in cytosolic Ca\(^{2+}\) are crucial for a variety of cellular processes including many aspects of development. Mobilization of intracellular Ca\(^{2+}\) stores via the production of inositol trisphosphate (IP\(_3\)) and the consequent activation of IP\(_3\)-sensitive Ca\(^{2+}\) channels is a ubiquitous means by which diverse stimuli mediate their cellular effects. Although IP\(_3\) receptors have been well studied at fertilization, information regarding their possible involvement during subsequent development is scant. In the present study we examined the role of IP\(_3\) receptors in early development of the zebrafish. We report the first molecular analysis of zebrafish IP\(_3\) receptors which indicates that, like mammals, the zebrafish genome contains three distinct IP\(_3\) receptor genes. mRNA for all isoforms was detectable at differing levels by the 64 cell stage, and IP\(_3\)-induced Ca\(^{2+}\) transients could be readily generated (by flash photolysis) in a controlled fashion throughout the cleavage period in vivo. Furthermore, we show that early blastula formation was disrupted by pharmacological blockade of IP\(_3\) receptors or phospholipase C, by molecular inhibition of the former by injection of IRBIT (IP\(_3\) receptor-binding protein released with IP\(_3\)) and by depletion of thapsigargin-sensitive Ca\(^{2+}\) stores after completion of the second cell cycle. Inhibition of Ca\(^{2+}\) entry or ryanodine receptors, however, had little effect. Our work defines the importance of IP\(_3\) receptors during early development of a genetically and optically tractable model vertebrate organism.

Cytosolic Ca\(^{2+}\) regulates a whole host of cellular processes (1). Ca\(^{2+}\) is involved in all stages of development of an organism from fertilization and cell division to cell proliferation and differentiation and, ultimately, cell death (1). How the same signaling ion can control such a vast array of diverse processes is perplexing. The exact form that the Ca\(^{2+}\) signal takes is likely a key factor. Complexities in the Ca\(^{2+}\) signaling ion can control such a vast array of diverse processes is perplexing. The exact form that the Ca\(^{2+}\) signal takes is likely a key factor. Complexities in the Ca\(^{2+}\) signals with respect to time and space are apparent and appear to be decoded by the cell to mediate a given response (1).

Ca\(^{2+}\) signals often derive from the mobilization of intracellular Ca\(^{2+}\) stores. Inositol 1,4,5-trisphosphate (IP\(_3\)), which is produced by the enzyme phospholipase C in response to a range of extracellular cues, activates intracellular Ca\(^{2+}\) channels located predominantly in the membrane of the endoplasmic reticulum (2). Three isoforms of the IP\(_3\) receptor have been described with distinct properties (3–5). IP\(_3\)-sensitive Ca\(^{2+}\) channels are regulated by interactions with accessory proteins (6) including protein kinases (7–13), calmodulin (14–16), homer (17), and IRBIT (18–20). They are also regulated by smaller ligands such as Ca\(^{2+}\) itself (21, 22). The latter is particularly important for the generation of complex Ca\(^{2+}\) signals, perhaps the most striking example of which are those generated at fertilization (23). In nearly all species studied, a large transient increase in cytosolic Ca\(^{2+}\) concentration is observed that originates from the point of sperm binding and traverses the egg in a regenerative manner (24). In mammals, IP\(_3\) receptors are critical for this increase (25), whereas in echinoderms, such as sea urchins, ryanodine receptors (a related family of Ca\(^{2+}\) channels) (26) and distinct Ca\(^{2+}\) channels regulated by NAADP (27, 28) also appear to be involved in sperm-mediated Ca\(^{2+}\) signals (29–31).

Although intracellular Ca\(^{2+}\) mobilizing messengers have been extensively studied at fertilization, what role they play in the early developing embryo is less clear (23). The zebrafish is a well studied, genetically tractable vertebrate model system for the study of early embryonic development. Fertilization triggers a cascade of events beginning with the first zygotic cell cycle, which gives rise to a two-cell embryo within ~40 min. The following cleavage period (2–64 cell stage) comprises a series of rapid and synchronous meroblastic cell divisions occurring at 15-min intervals. Metasynchronous cell cleavage begins during the early blastula period (128–256 cell stage) and after the midblastula transition (512 cell stage), asynchronous
cell cycle lengthening is observed (32). Various spatial and temporal patterns of cytosolic Ca\(^{2+}\) signals occur from fertilization through to the midblastula period (33). Localized Ca\(^{2+}\) signals are associated with identified cytokinetic steps during the first two cell cycles (34–36). Furthermore, Ca\(^{2+}\) signals deriving from IP\(_3\)-sensitive Ca\(^{2+}\) stores appear critical for progression from the zygotic to cleavage stage (35, 36). However, despite Ca\(^{2+}\) signals being evident in the embryo up to and including the tenth zygotic cell cycle, there is currently a paucity in information regarding the underlying mechanisms involved in their generation at this key stage of development (37, 38).

In the present study we establish the role of IP\(_3\) receptors in zebrafish development from the cleavage period up to the midblastula transition. We provide molecular evidence that three subtypes of the IP\(_3\) receptor are expressed at the 64 cell stage, that Ca\(^{2+}\) increases in response to IP\(_3\) are demonstrable after the first two cell divisions, and that blockade of IP\(_3\) signaling at this stage disrupts development of the embryo. Our data highlight the importance of IP\(_3\)-mediated Ca\(^{2+}\) signaling during early development of a model vertebrate system.

**EXPERIMENTAL PROCEDURES**

**Fish Maintenance—Zebrafish (Danio rerio)** were purchased from a local supplier and maintained as described previously (39, 40). Embryos were collected at the beginning of the light cycle and washed in embryo medium comprising 13.7 mM NaCl, 0.54 mM KCl, 25.2 mM Na\(_2\)HPO\(_4\), 44.1 mM KH\(_2\)PO\(_4\), 1.3 mM CaCl\(_2\), 1 mM MgSO\(_4\), 10 mM HEPES, 4–5 drops/liter methylene blue, pH 7.2. Staging of the embryos was performed as described by Kimmel and co-workers (32).

**In Silico Cloning of Zebrafish IP\(_3\) Receptors—**A BLAST search of the zebrafish genomic data bases with the protein sequence of the human type 1 IP\(_3\) receptor (BAA05065) identified many annotated partial sequences for all three isoforms (supplemental Fig. 1). In most of the cases, two or more sequences mapped to the same genomic region; however, they were not always in proximity. For example, for the type 1 IP\(_3\) receptor, three sequences were identified on chromosome 11 (supplemental Fig. 1). Although annotated as distinct genes, the two C-terminal sequences were adjacent on the chromosome and, thus, likely corresponded to the same gene (depicted by a loop). The gene corresponding to the N terminus, however, was located some 1 million bp upstream and on the opposite strand. The type 2 IP\(_3\) receptor sequences on chromosomes 11 and 18 were also in clearly different genomic contexts. A long contiguous match was obtained for the type 3 IP\(_3\) receptor; however, the 5’ region of this gene was annotated as DNA photolyase. Note that although this sequence and the other two type 3 IP\(_3\) receptor sequences were on contigs that have yet to be assigned a chromosomal location, they were unlikely to be overlapping based on inspection of the flanking genes. In summary, the above analysis is consistent with the presence of distinct, possibility duplicated IP\(_3\) receptor genes in the zebrafish genome. Whether some of the sequences correspond to pseudogenes and/or errors in the assembly is not clear at present.

The initial BLAST search did, however, lead to the identification of two large un-annotated genomic clones containing related sequences that spanned the entire length of type 1 and type 3 IP\(_3\) receptor. No clones corresponding to the full-length type 2 IP\(_3\) receptor were found. Putative type 1 and type 3 IP\(_3\) receptor genes were predicted from the identified genomic sequences using the FGENESH+ program, a hidden-Markov model-based algorithm for finding genes with protein similarity, accessible at the Softberry web site.

For the type 1 zebrafish IP\(_3\) receptor, we used the genomic clone BX548005 and the following mammalian type 1 source sequences (accession numbers in parentheses): human (BAA05065), mouse (P11881) rat (P29994), and cow (AAF00613). The different models generated were identical except in six small regions. Supplemental Fig. 2 shows a ClustalW alignment of the four source sequences and the corresponding predictions for the zebrafish homologue in the regions that differed. The deviation beginning at residue 2409 (numbering for the prediction based on the human type 1 IP\(_3\) receptor sequence) corresponds to the variable region of luminal loop 3 (supplemental Fig. 2A). This region was deleted in all of the predictions except the human one. The apparent discrepancies at positions 318 (supplemental Fig. 2B) and 1672 (supplemental Fig. 2C) correspond to the SI and SII splice sites, respectively. These differences are, thus, likely because of the particular splice variants used as the source sequence since the human sequence lacked the SI splice site, and both the human and cow source sequences lacked the SII site. Minor discrepancies were noted in the predictions at positions 895, 1137, and 1021 (supplemental Fig. 2, D–F). A dipeptide (KG) at position 895, which is conserved in all the known mammalian receptors, was missing in the zebrafish predictions based on the rat and cow sequences (supplemental Fig. 2D). The latter model also lacked a pentapeptide (SSRSN) at position 1137 that was present in the other predictions (supplemental Fig. 2E). In light of the above comparisons, the prediction based on the human sequence was likely the more accurate of the models and, thus, was used for subsequent analysis. However, this model (and the model based on the mouse sequence) did contain a serine residue at position 1021 that was not present in any of the other sequences (supplemental Fig. 2F). A BLAST search of the putative type 1 zebrafish IP\(_3\) receptor sequence against EST databases resulted in the identification of two clones (accession numbers EB771432 and EE318107) that confirmed the prediction at the regions depicted in Fig. 1B.

A similar analysis was performed to obtain the sequence for the type 3 zebrafish IP\(_3\) receptor using the genomic clone BX784023 and the following mammalian type 3 sequences (accession numbers in parentheses): human (BAA05385), mouse (P70227), rat (AAA41446), and cow (AAL39078). All four models generated were again largely consistent with only three deviations in sequence at positions 313, 2159, and 2334 (numbering of the prediction based on the human sequence). The sequence flanking the first divergent region was experimentally determined by sequencing of RT-PCR products (see below). ESTs confirmed the human sequence-based prediction at the two remaining divergent regions and also the putative N and C termini (see Fig. 1B). We also identified an additional EST (accession number AL906759) corresponding to residues 78–237. Although all of the models converged in this region, the sequence of the EST indicated the presence of an insert
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(supplemental Fig. 3A) that was conserved in IP$_3$ receptors from human and mouse but not rat and cow (supplemental Fig. 3B). Because this sequence and a second insert (identified by direct sequencing; see below) were also present in the source genomic contig used for the predictions, they were incorporated in to the human-based zebrafish model.

RT-PCR—Poly(A)$^+$ mRNA was isolated from ~100 embryos at the 64-cell stage, and cDNA was synthesized after priming with random oligonucleotides using the Micro-FastTrack mRNA isolation kit and cloned AMV First-Strand cDNA synthesis kit (both from Invitrogen).

For end point PCR (Fig. 1A), the following three sets of isoform-specific oligonucleotide primers (spanning at least 1 intron) were designed based on the putative open reading frames: type 1 IP$_3$ receptor, zIPTR1_1F, 5'-GAGGAGGTCTGGGTGTTCTG-3', and zIPTR1_1R, 5'-GCAGACAACATCCCTCAGGT-3'; type 2 IP$_3$ receptor, zIPTR2_1F, 5'-ACATGAGGAGGCAAGGAG-3', and zIPTR2_1R, 5'-CAAAAAGAGGACCGGAGGTT-3'; type 3 IP$_3$ receptor, zIPTR3_1F, 5'-AGGAGGCGGACATCGGAACA-3', and zIPTR3_1R, 5'-GGGATTGCTACATTTAGG-3'. Amplification was performed using the Expand High Fidelity PCR system (Roche Applied Science) with an annealing temperature of 52 °C and an extension time of 1 min. Both strands of the amplified products were sequenced (MWG Biotech). The sequences obtained for type 1 and 2 zebrafish IP$_3$ receptors confirmed the predicted sequences (data not shown). An additional insert, however, was identified for the type 3 sequence (supplemental Fig. 3C) that was partly conserved in other mammalian type 3 IP$_3$ receptors (supplemental Fig. 3D) and which corresponded to an additional exon in the source genomic contig (data not shown).

For quantitative PCR (Table 2), the following primer pairs were designed using the Applied Biosystems Primer Express software: type 1 IP$_3$ receptor, zIPTR1_2F, 5'-GGGATTGCTACATTTAGG-3', and which corresponded to an additional exon in the source genomic contig (data not shown). Primer efficiencies were calculated using a 2000-fold dilution series of cDNA. The values obtained for amplification of type 1, 2, and 3 IP$_3$ receptors were 1.93 (R$^2 = 0.982$), 1.82 (R$^2 = 0.991$), and 1.89 (R$^2 = 0.995$), respectively. Data obtained were normalized to the expression of $\beta$-actin.

Phylogenetic Analysis—IP$_3$ receptor sequences were aligned using ClustalW and distance matrices generated using the Phylip program, Protdist. Phylogeny relationships were built using the distance program, FastME.

Microinjection of Embryos—Zebrafish embryos were pressure-injected with caged IP$_3$ (NPE-caged inositol 1,4,5-trisphosphate, Molecular Probes) and the low affinity calcium indicator Fluo-5F (K$_d$ for Ca$^{2+}$ ~ 2.3 μM, Molecular Probes) or rhodamine-dextran (Molecular Probes) according to the methods described previously (40, 41). Briefly, de-chorionated embryos were injected at the 2–512 cell stage using an injection solution composed of 130 mM KCl and either ~800 μM caged IP$_3$ and ~1 μM Fluo-5F or 10 μM rhodamine-dextran. The embryos were visually checked to ensure that the injection solution had been retained and that the cell had survived.

Digital Imaging Fluorescence Microscopy and Flash Photolysis—Individual injected embryos were transferred to Petri-perm dishes (Sartorius) lined with agarose (1% w/v) within which small wells had been manually etched. The dish was placed on the stage of an inverted epifluorescence microscope (Olympus IX71), and embryos were viewed using a 10× objective. Fluo-5F fluorescence (emission >500 nm) was captured using a cooled CCD camera after periodic illumination at 488 nm provided by a mono chromator light source (T.I.L.L. Photonics). A Xenon flash lamp (T.I.L.L. Photonics) was used to deliver brief high intensity pulses of UV light (360 nm, 0.5–5 ms duration) to effect photo-release of IP$_3$. Both light sources were coupled by fiber optic cables to a double epifluorescence condenser fitted with a 410-nm cut-off dichromatic mirror. Rhodamine fluorescence was captured using a 570 nm cut-off dichromatic mirror after excitation at 540 nm using an independently coupled fiber optic cable.

Purification of Recombinant IRBIT from Sf9 Cells—The fragment corresponding to IRBIT-OneSTrEP from pEXPR-IBA103-IRBIT (described in Devogelaere et al. (19)) was subcloned in the His$_6$ fusion vector pET21b (+) to generate a C-terminal double-tagged construct, IRBIT-OneSTrEP-His$_6$. The corresponding cDNA was subcloned in pFASTBAC1TM, resulting in pFASTBAC1TM-IRBIT-OneSTrEP-His$_6$. The latter was used to generate the recombinant IRBIT-OneSTrEP-His$_6$ baculovirus according to the manufacturer’s protocols (Bac-to-Bac® Baculovirus Expression System, Invitrogen). This baculovirus enabled purification of the IRBIT-OneSTrEP-His$_6$ fusion protein using Ni$^{2+}$-nitritoliacetic acid-agarose according to the manufacturer’s protocol (Invitrogen).

Embryo Treatments—Individual de-chorionated embryos at the 4-cell stage were transferred to chambers mounted on a glass slide lined with agarose (1% w/v). Embryos were then treated with 2-aminoethoxydiphenylborane (2-APB) (Sigma), thapsigargin (Sigma), ryanodine (Calbiochem), U73122 (Calbiochem), U73343 (Calbiochem), or vehicle (Me$_2$SO, 0.1% v/v) and transferred to an incubator at 26 °C for the indicated times before capture of transmitted light images. Recombinant IRBIT was pressure-injected into a single cell of embryos at the four-cell stage through the chorions (39).

RESULTS

Molecular Characterization of Zebrafish IP$_3$ Receptors—We searched the zebrafish genomic databases for IP$_3$ receptor-like sequences. Although many highly homologous annotated
sequences were found for all three isoforms, none was full-length (supplemental Fig. 1; see “Experimental Procedures”). We did, however, identify two large un-annotated genomic clones that contained homologous sequences spanning the entire coding regions of the type 1 and type 3 IP3 receptor. We used a gene prediction with protein similarity algorithm to obtain the structure of these putative genes (see “Experimental Procedures”). Table 1 compares some properties of the predicted zebrafish IP3 receptor genes and protein products with that of their human counterparts. This analysis reveals that the type 1 and type 3 IP3 receptors from zebrafish and humans are very similar. One clear exception is the size of the type 1 IP3 receptor gene, which appears almost six times more compact in the zebrafish than human.

To provide additional evidence for the presence of multiple IP3 receptors in the zebrafish embryo, we performed RT-PCR analysis using oligonucleotide primers specific for the putative type 1 and type 3 IP3 receptor isoforms. We also used primers specific to the longest of the annotated type 2 IP3 receptor sequences (accession number XP_691057). RT-PCR analysis using these primers and mRNA isolated from the 64 cell embryo resulted in amplification of products of the expected size (type 1, 468 bp; type 2, 1072 bp; type 3, 999 bp; Fig. 1A).

The predicted full-length protein sequences for the type 1 and type 3 are schematically depicted in Fig. 1B together with the partial predicted sequence of the type 2 IP3 receptor. Direct sequencing of the products amplified by RT-PCR (gray boxes) and several ESTs (white boxes) confirmed the putative open-reading frames of our models with the exception of two small inserts not present in the predicted type 3 IP3 receptor (see “Experimental Procedures” and supplemental Fig. 3). A ClustalW alignment of the three zebrafish IP3 receptors is presented in supplemental Fig. 4.

We inspected the predicted zebrafish IP3 receptors for regulatory sites previously identified for known homologues. Our analysis was restricted to regions in which the zebrafish sequences were experimentally determined and/or verified by EST data. IP3 receptors bind Homer through a PPXXF motif in the N terminus (17), possess a consensus tyrosine phosphorylation site at tyrosine 482 (43) and are phosphorylated at serine 436 by mitogen-activated protein kinase/extracellular signal-regulated kinase 2 (13) and at serine 421 and threonine 799 by Cdc2-cyclin B1 (12). Threonine 799 is found in both in type 1 and type 3 mammalian IP3 receptors, whereas the remaining sites are present only in the type 1 isoform. This isoform-specific phosphorylation signature is absolutely conserved in the zebrafish IP3 receptors (Fig. 1B, supplemental Fig. 4). Moreover, a short stretch of amino acids in the suppressor domain that differs markedly between mammalian type 1 and 3 IP3 receptors (a known determinant of isoform-specific IP3 affinity and sensitivity to apoptosis (44)) is also divergent in the zebrafish (Fig. 1B, supplemental Fig. 4). Indeed, the sequence of each IP3 receptor subtype within this region is conserved between the zebrafish and mouse (data not shown). This analysis validates the subtype assignment of the zebrafish IP3 receptor sequences. Finally, the confirmed sequence of the type 1 zebrafish IP3 receptor encompasses two regions (residues 741–849 and 2124–2136; black boxes in Fig. 1B) containing clusters of negatively charged amino acids that were previously identified as sites in the mouse type 1 IP3 receptor that bind Ca2+ in vitro (45, 46). Of the aspartate and glutamate residues found in these regions (which are probably important in binding Ca2+), all but one (in the N-terminal site) were conserved in the zebrafish (data not shown).

A ClustalW alignment of protein sequences for known IP3 receptors, the full-length type 1 and type 3 zebrafish IP3 receptors, and the partial type 2 IP3 receptor was performed, and a cladogram was generated (Fig. 1C). As shown, all three zebrafish isoforms were assigned to their anticipated group. Phylogeny analysis also showed that zebrafish IP3 receptors are the most divergent members within their group and clearly different from the invertebrate IP3 receptors. Both the type 1 and type 3 zebrafish IP3 receptor (for which full-length sequences were available) were found to be most similar to the mammalian isoforms displaying 86–88 and 83% similarity at the amino acid level, respectively.

We quantified the relative levels of IP3 receptor transcripts by real-time RT-PCR. An independent set of isoform-specific primers (different to those used for end-point PCR) were designed to all three IP3 receptors and amplification of products compared with β-actin. This analysis revealed that at the 64-cell stage, the type 2 and 3 IP3 receptor transcripts were present at similar levels. Transcripts for the type 1 IP3 receptor, however, were ~50-fold less abundant (Table 2). Taken together, the above results indicate that the zebrafish genome contains three distinct IP3 receptor genes and that mRNAs encoding all three isoforms are differentially expressed in the early zebrafish embryo.

**IP3-induced Ca2+ Release during Early Zebrafish Development**—To determine whether IP3 receptors are functionally expressed in the early zebrafish embryo, the effect of IP3 on cytosolic Ca2+ concentration was examined. Individual blastomeres of the embryo were injected with a fluorescent Ca2+-

**TABLE 1**

| Properties of predicted zebrafish IP3 receptor genes and translation products |
|---|
| Data are compared to the human type 1 and type 3 IP3 receptor genes (parentheses). |
| aa, amino acids. |
| zITPR1 | zITPR3 |
|---|---|
| Number of coding exons | 56 (59) | 57 (58) |
| Length of coding region | 56 kb (330 kb) | 68 kb (75 kb) |
| Length of predicted peptide | 2654 aa (2743 aa) | 2624 aa (2671 aa) |
| Calculated molecular mass of peptide | 303 kDa (312 kDa) | 301 kDa (304 kDa) |
| Similarity of peptide to human isoform | 88% | 83% |

*Includes additional exon identified by RT-PCR analysis (see “Experimental Procedures” for details).
sensitive indicator together with caged IP$_3$. Before the 32-cell stage, the blastomeres are interconnected by cytoplasmic bridges allowing diffusion of the dye from the injected cell throughout the embryo. A representative example is shown in Fig. 2C where all four cells of the embryo were labeled after injection of a single blastomere at the two-cell stage. After the 32-cell stage, cell bridges are lost such that the dye is confined to the injected cell and daughter cells after division. As can been seen in Fig. 2D, only 2 of 64 cells of an embryo injected at the 32 cell stage are fluorescent. Photolysis of caged IP$_3$ by brief delivery of high intensity UV light resulted in a rapid transient increase in Fluo-5F fluorescence, whereas no response was observed in embryos injected with dye alone (Fig. 2E). These data are consistent with the expression of functional IP$_3$ receptors within the embryo.

We compared the effects of IP$_3$ on cytosolic Ca$^{2+}$ concentration at different developmental stages (Fig. 3). IP$_3$-induced Ca$^{2+}$ signals (in response to a maximal flash duration; see below) were readily demonstrable from the 4–64-cell stage. The peak fluorescence increase upon photolysis from all embryos analyzed is shown in Fig. 3A. From these data it is apparent that there is some variability in the responses to IP$_3$ between embryos at the same developmental stage (Fig. 3A). We, therefore, examined the effect of varying the duration of the flash period on the resulting fluorescence increases within a single blastomere of a developing embryo (Fig. 3, B–C). Peak fluorescence responses after progressively longer photolysis periods (Fig. 3B) were fit well by a sigmoid relationship (Fig. 3C). Similar results were obtained in two other embryos. Thus, concentration-effect relationships for IP$_3$-induced Ca$^{2+}$ mobilization can be readily established within a single cell in vivo.

IP$_3$-induced Ca$^{2+}$ Release Is Required for Early Embryonic Development—In the next set of experiments we examined the requirement for IP$_3$ receptors in early development of the embryo. Time-lapse microscopy of control embryos from the 4-cell stage (1 h) onward revealed regular cleavage at an interval

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**TABLE 2**

Quantitative determination of zebrafish IP$_3$ receptor mRNA transcripts

|                         | Relative to $\beta$-actin | Relative to zITPR1 |
|-------------------------|---------------------------|--------------------|
| zITPR1                  | 0.02 ± 0.002              | 1                  |
| zITPR2                  | 1.1 ± 0.04                | 55                 |
| zITPR3                  | 0.9 ± 0.03                | 41                 |

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of ~15 min. (Fig. 4A). In contrast, the addition of the IP₃ receptor antagonist 2-APB (10 μM) (47) at this stage resulted in abortive cleavage and retraction of boundaries formed following meroblastic division Fig. 4B).

After more protracted incubations, embryos bathed in control medium and medium containing vehicle went on to develop into blastulae (Fig. 5A). Cells that had divided but were not in the focal plane were evident (arrows). The proportion of embryos displaying this phenotype was 89% (of 28 embryos examined, n = 7) in control medium and 94% (of 31 embryos examined, n = 9) in media supplemented with Me₂SO. In the continued presence of 2-APB, however, blastula formation was clearly disrupted in nearly all (94%) of the 31 embryos examined, and no cell division was observed (Fig. 5A, n = 9). A lower concentration of 2-APB (1 μM) had little effect on development. At this concentration all 14 embryos appeared normal (n = 5).

To determine whether the inhibitory effects of 2-APB on cell division were reversible, embryos were treated with Me₂SO or 2-APB for 15 min at the 4-cell stage (1 h) and then transferred to

**FIGURE 2.** Flash photolysis of IP₃ in early zebrafish embryos. Shown are brightfield (A and B) and fluorescence (C and D) images of embryos at the 4 (A and C) and 64 (B and D) cell stage. Single blastomeres were injected with caged IP₃ and Fluo-5F at the 2- and 32-cell stage, respectively. In the early embryo the blastomeres remain interconnected by cytoplasmic bridges such that the fluorescent indicator diffuses to all cells (C). At later stages fluorescence is confined to the injected cell and its daughters (D). E, time course showing changes in cytoplasmic Fluo-5F fluorescence (F) relative to resting fluorescence (F₀) after UV exposure (5 ms) of an embryo at the 64-cell stage injected with (closed circles) or without (open circles) caged IP₃.

**FIGURE 3.** Evoked IP₃-induced Ca²⁺ transients during early development. A, embryos were injected as in Fig. 2, and the peak responses to maximal UV exposure were compared at the indicated cell stage. Each point is data from an individual embryo. B, time course comparing the effect of increasing the duration of UV exposure on cytosolic calcium levels of an embryo at the four-cell stage. Peak responses are plotted in C. Similar results were obtained in two other embryos.
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![Figure 4. Cell cleavage is disrupted in the presence of an IP$_3$ receptor antagonist.](image)

**Figure 4.** Cell cleavage is disrupted in the presence of an IP$_3$ receptor antagonist. Embryos at the 4-cell stage were incubated with either Me$_2$SO (DMSO) (0.1% v/v, A) or 2-APB (10 μM, B). Transmitted light images were then captured at the times (post-fertilization) indicated. Results are representative of three experiments.

![Figure 5. IP$_3$ receptors are required for blastula formation.](image)

**Figure 5.** IP$_3$ receptors are required for blastula formation. A, embryos were maintained in embryo medium with either Me$_2$SO (0.1% v/v) or 2-APB (10 μM) added at the 4-cell stage. Images were taken at the sphere stage (4 h). Dividing cells not in the focal plane are highlighted by arrows. B, embryos were incubated with Me$_2$SO (DMSO) or 2-APB as in A and then transferred to control embryo medium after 15 min of incubation. C, images of embryos 4 h post-fertilization after either injection with IRBIT or transfer to nominally Ca$^{2+}$-free medium containing 1 μM thapsigargin (TG) at the 4-cell stage.

![Figure 6. Phospholipase C requirement for development.](image)

**Figure 6.** Phospholipase C requirement for development. Embryos at the 4-cell stage (1 h) were incubated with the phospholipase C inhibitor U73122 (A, 10 μM) or the less active analogue U73334 (B, 10 μM). Images were captured at the 128 cell (2.25 h, left) and sphere (4 h, right) stage.

control medium (lacking the drug) for a further 2 h. Under these conditions 13 of 14 Me$_2$SO-treated embryos (93%) and 5 of 11 2-APB-treated embryos (45%) exhibited normal morphology (Fig. 5B, n = 2).

To achieve more specific inhibition of IP$_3$ receptors, we examined the effect of IRBIT injection on embryo development. IRBIT at an intracellular concentration of ~1 μM disrupted blastula formation in 24% of 41 embryos (n = 3; Fig. 5C). The observed phenotype (Fig. 5C) was reminiscent of that obtained with 2-APB (Fig. 5A). A higher concentration of IRBIT (~3 μM) also affected development in 43% of 21 embryos in two experiments; however, in a third we did not observe any effect of IRBIT. Consistent with the effects of 2-APB and IRBIT, depletion of endoplasmic reticulum Ca$^{2+}$ stores with thapsigargin (1 μM) in Ca$^{2+}$-free medium disrupted cell division (Fig. 5C). In these experiments, all 12 embryos showed developmental abnormalities. Indeed, like 2-APB (Fig. 5A), breakdown of cell boundaries by thapsigargin was clearly evident (Fig. 5C); however, the effect of the latter was less marked and appeared restricted to the embryo boundaries.

To provide further evidence for activation of the phosphoinositide cycle during early zebrafish development, we examined the effects of U73122, an inhibitor of phospholipase C (48). U73122 (10 μM) when added at the 4-cell stage (1 h) disrupted cell division in all 36 embryos examined (Fig. 6A, n = 8). In contrast, the less active analogue U73334 (10 μM) had little effect on embryo morphology (Fig. 6B). In these experiments, 28 of 29 (n = 7) embryos appeared normal. At 2.25 h post-fertilization (128 cell stage), the U73122 phenotype (Fig. 6A, left) was similar to that of 2-APB and was characterized by a loss of cell boundaries. At the sphere stage (4 h), the phenotypes differed somewhat between the two drugs (compare Fig. 6A, right, and Fig. 5A) although it was clear that both prevented normal cell division.

To better visualize blastomeres, we injected embryos with rhodamine-dextran at the 128–512-cell stage after prior treatment with inhibitors of IP$_3$ signaling (Fig. 7). In control embryos, the dye was retained by the injected cell as expected. In contrast, the dye was evenly distributed throughout embryos exposed to either 2-APB or U73122 but not U73334. These data confirm the results from our morphological analyses, suggesting that inhibition of IP$_3$ signaling disrupts cell boundary formation.

Finally, we probed the role of other potential sources of Ca$^{2+}$ in early development. In nominally Ca$^{2+}$-free medium, 83% of the 12 embryos examined developed normally (n = 3), indicating that Ca$^{2+}$ influx is not required for blastula formation (Fig. 8). Ryanodine (100 μM) also did not affect cell division and embryo development in any of the 12 treated embryos (Fig. 8, n = 2).
Zebrafish IP₃ Receptors

IP₃ receptors are ubiquitously expressed proteins (49). Upon activation, these channels mediate complex changes in Ca²⁺ that in turn influence a whole variety of, if not all cellular processes (1). Little, however, is known concerning the effects of IP₃ in the zebrafish despite the clear advantages of this preparation for the study of both Ca²⁺ signaling (due to its optical transparency) and development (which is rapid) (41). In the present study we provide both molecular and functional evidence that IP₃ receptors are expressed and active in the early zebrafish embryo.

We report for the first time characterization of zebrafish IP₃ receptors at the molecular level. Although the zebrafish genome has been sequenced, the assembly and annotation of the IP₃ receptor is far from complete (supplemental Fig. 1). We, therefore, performed our own inspection of genomic data for IP₃ receptor-like sequences. To our surprise, it appears that like mammals, three isoforms of the IP₃ receptor have evolved in the zebrafish. These findings were confirmed by RT-PCR (Fig. 1). Using a gene prediction with similarity algorithm, we were able to derive full-length sequences for the type 1 and type 3 isoforms. On the whole, our data were verified by EST data and by sequencing of RT-PCR products (Fig. 1). Our subtype assignment, based initially on overall sequence similarity, is supported by conservation of several isoform-specific regulatory sites (Fig. 1B). To our knowledge, this is the first report of multiple verified IP₃ receptor sequences outside of mammals. Indeed, it notable that such multiplicity contrasts that of Xeno- pus, another commonly used vertebrate model for development, for which only one IP₃ receptor gene has been reported to date (50). However, this organism, too, has likely evolved additional IP₃ receptors.⁵ These findings underscore the usefulness of fish (and frogs) for developmental studies of IP₃ receptors in relation to mammalian physiology. In this respect, it is notable that each zebrafish IP₃ receptor isoform is most similar to a mammalian counterpart.

Interestingly, results from our quantitative PCR experiments suggest that the major IP₃ receptor transcripts present at the 64-cell stage are those for the type 2 and type 3 isoforms (Table 2). Although we exercise caution in extrapolating quantification of mRNA levels to that of protein (51), it is possible that the type 1 receptor plays a lesser role in generating IP₃-dependent calcium signals at this developmental stage given that type 1 IP₃ receptor protein levels have been reported to decrease after fertilization (51, 52).

The role of IP₃ receptors in the events leading up to and immediately after fertilization is relatively well characterized. Maturation of mouse oocytes is associated with an increase in the sensitivity of intracellular Ca²⁺ stores to IP₃ (51, 53, 54). IP₃ receptor sensitivity decreases after fertilization (51, 52) at the time of pronuclear breakdown and then increases at mitosis (55). Thus, changes in the sensitivity of IP₃-mediated Ca²⁺ release due to changes in IP₃ receptor levels (51, 52, 56), redistribution (57), or phosphorylation (13) might well determine the temporal nature of the Ca²⁺ signal during development. Strikingly little information is available regarding the effects of IP₃ on cytosolic calcium at later stages. Lee et al. (36) demonstrated that microinjection of IP₃ into zebrafish embryos before the first cell division induces an increase in cytosolic Ca²⁺. Our data extend these findings significantly. First, we show that IP₃ is capable of inducing cytosolic Ca²⁺ increases throughout the cleavage period. Second, these increases are readily resolved using a low affinity Ca²⁺ dye, indicating that they likely peak well in to the micromolar range, and third, by using flash photolysis we define for the first time in vivo concentration-effect relationships for IP₃-mediated calcium release (Fig. 3). The latter should prove more than useful in defining the sensitivity of IP₃ receptors during early development particularly in a rapidly dividing embryo.

Several studies have indicated a requirement for IP₃ receptors during the first two cell divisions (35, 58, 59). In contrast, the role of intracellular messengers in the control of subsequent early development is less clear. We show here that the addition of an IP₃ receptor antagonist at the 4-cell stage (1 h) disrupts early blastula formation and that the effects of the drug are partially reversible (Figs. 4 and 5). These data demonstrate that IP₃ receptors are activated and indeed required for normal development after the initial two cell divisions. Although 2-APB (the IP₃ receptor antagonist used in the present study) also inhibits store-operated Ca²⁺ channels on the plasma membrane (60), the finding that zebrafish embryos readily divide in the total absence of extracellular Ca²⁺ (Fig. 8) (34)

FIGURE 7. Disruption of cell boundaries by inhibitors of phosphoinositide signaling. Embryos were maintained in medium containing vehicle (Me₂SO (DMSO)) or medium supplemented with 2-APB, U73122, or U73334 (10 μM) added at the 4-cell stage. At the 128–512 cell stage (2.25–2.75 h), the embryos were injected with rhodamine dextran, and fluorescence images were captured.

FIGURE 8. Neither Ca²⁺ influx nor ryanodine receptors are required for blastula formation. Effect on development of transferring embryos at the 4-cell stage to control medium (CTRL), nominally Ca²⁺-free medium (−Ca²⁺), or control medium containing ryanodine (RY, 100 μM).

DISCUSSION

⁵ J. S. Marchant, personal communication.
suggestions that the effects of 2-APB reported here are likely due to inhibition of Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores rather than Ca\(^{2+}\) influx. Indeed, inhibition of IP\(_3\) receptors by IRBIT also produced a similar phenotype to 2-APB (Fig. 5C) in a significant proportion of injected embryos. Disruption of cell division by IRBIT, however, was not observed in all embryos. Of note is that the inhibitory effect of IRBIT on IP\(_3\) receptors is dependent on phosphorylation of the former (20). Possible dephosphorylation of IRBIT upon injection might, therefore, comprise its ability to regulate IP\(_3\) receptors. Furthermore, we have previously shown that interaction of IP\(_3\) receptors and IRBIT is disrupted upon cleavage of IRBIT within an identified PEST motif, a process that occurs in vivo (19). Thus, a combination of conditions that promote dephosphorylation and/or proteolysis within the embryo could explain why disruption cell division by IRBIT occurred in only a fraction of the embryos.

Inhibition of phospholipase C was also found to interfere with development providing further evidence for IP\(_3\) signaling at the developmental stage studied (Fig. 6). IP\(_3\) levels in both Xenopus (61, 62) and zebrafish (37) embryos, however, appear not to increase during this period. It should be noted that possible increases in IP\(_3\) levels may well occur but be localized and, thus, not readily detectable from measurements using whole embryos. This is all the more plausible given the large size (1 mm) of the embryo. Thus, we suggest that the phosphoinositide cycle is probably activated much earlier than previously thought. Consistent with the lack of effect of cyclic ADP-ribose on cytosolic Ca\(^{2+}\) concentration (36), ryanodine had little effect on embryo development (Fig. 8), suggesting that IP\(_3\) receptors are likely the major intracellular Ca\(^{2+}\) release channels involved in cell division.

Our data implicating IP\(_3\) receptors in cytokinesis is strikingly similar to recent results obtained in two independent studies (published in tandem while this work was in progress) using Drosophila spermatocytes (63) and several mammalian cell lines (64). Our results also corroborate those of Miller and coworkers (65) using 1–2-cell stage zebrafish embryos. In all of the aforementioned reports, cytokinesis was inhibited by the same pharmacological agents used here, namely 2-APB and/or U73122.

That IP\(_3\) receptors are activated at fertilization and during subsequent cell divisions raises the question as to what are the signals that raise IP\(_3\) levels during these events. Recent data suggest that sperm-induced changes in IP\(_3\) levels at fertilization are due to introduction in to the egg of a novel sperm-specific phospholipase C isoform (PLC\(_{\gamma}\)) (66). Phospholipase C activation may also occur later on in development via the actions of a certain members of the Wnt family of secreted glycoproteins on target (probably G-protein coupled) transmembrane receptors (67). It is intriguing that this Wnt-Ca\(^{2+}\) pathway (68) negatively modulates canonical Wnt signaling in Xenopus (69) since the latter pathway is implicated in the specification of dorsal structures. Thus, dorsal phenotypes induced by inhibition of the phosphoinositide cycle with lithium (70) and suppression of ventral development in Xenopus embryos by monoclonal antibodies to the type I IP\(_3\) receptor (71) may result due to unregulated actions of canonical Wnts. Indeed, expression of dorsal markers and B-catenin (which is normally stabilized by activation of the canonical Wnt cascade) is increased in the zebrafish by a variety of maneuvers that inhibit release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores (72, 73). Moreover, dorsal phenotypes have recently been reported in zebrafish upon overexpression of both zebrafish and human IRBIT (74), effects again consistent with inhibition of IP\(_3\) receptors (19, 20). Indeed it is intriguing that zebrafish IRBIT expression is developmentally regulated (74). Modulation of IP\(_3\) receptor action by changes in IRBIT levels may, thus, influence dorso-ventral patterning in vivo.

In summary, we show that receptors for the ubiquitous Ca\(^{2+}\)-mobilizing messenger IP\(_3\) are important during early development of the zebrafish. We provide the first molecular description of zebrafish IP\(_3\) receptors and demonstrate that these Ca\(^{2+}\) channels can be exogenously activated in vivo. The zebrafish embryo may, thus, prove a suitable experimental platform for further defining the role of IP\(_3\)-sensitive Ca\(^{2+}\) channels during development in a highly versatile experimentally tractable setting.

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