Regulated Disruption of Inositol 1,4,5-Trisphosphate Signaling in *Caenorhabditis elegans* Reveals New Functions in Feeding and Embryogenesis

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Submitted August 24, 2001; Revised January 7, 2002; Accepted January 18, 2002

Monitoring Editor: Judith Kimble

Inositol 1,4,5-trisphosphate (IP₃) is an important second messenger in animal cells and is central to a wide range of cellular responses. The major intracellular activity of IP₃ is to regulate release of Ca²⁺ from intracellular stores through IP₃ receptors (IP₃Rs). We describe a system for the transient disruption of IP₃ signaling in the model organism *Caenorhabditis elegans*. The IP₃ binding domain of the *C. elegans* IP₃R, ITR-1, was expressed from heat shock-induced promoters in live animals. This results in a dominant-negative effect caused by the overexpressed IP₃ binding domain acting as an IP₃ “sponge.” Disruption of IP₃ signaling resulted in disrupted defecation, a phenotype predicted by previous genetic studies. This approach also identified two new IP₃-mediated processes. First, the up-regulation of pharyngeal pumping in response to food is dependent on IP₃ signaling. RNA-mediated interference studies and analysis of *itr-1* mutants show that this process is also IP₃R dependent. Second, the tissue-specific expression of the dominant-negative construct enabled us to circumvent the sterility associated with loss of IP₃ signaling through the IP₃R and thus determine that IP₃-mediated signaling is required for multiple steps in embryogenesis, including cytokinesis and gastrulation.

INTRODUCTION

Inositol 1,4,5-trisphosphate (IP₃) is an important and widely used second messenger in animal cells. IP₃ is produced by the hydrolysis of phosphatidylinositol 4,5-bisphosphate after the activation of phospholipase C (PLC). The best understood routes of PLC activation are through the stimulation of G protein-coupled receptors or tyrosine kinase-linked receptors at the cell surface, although the presence of additional isoforms of PLC suggests other possible routes (Rhee and Bae, 1997; Katan, 1998). The only known action of IP₃ is to induce Ca²⁺ release from intracellular stores through activation of IP₃ receptors (IP₃Rs) (Berridge, 1993). This pathway and the Ca²⁺ signals derived from it are central to a wide range of cellular responses (Berridge, 1993, 1997; Clapham 1995).

Two challenges in the study of this signaling network are to unravel the different functions played by IP₃ signaling in animals and to develop methods for manipulating these processes and thus advance our understanding of how the specificity of IP₃ signaling is achieved. Recent genetic approaches have provided new insights into IP₃ function in animals. The central role played by IP₃Rs in a wide range of cellular responses is reflected by the high level of lethality resulting from gene knockout observed in both *Drosophila* (Acharya *et al.*, 1997; Venkatesh and Hasan, 1997) and mice (Matsumoto *et al.*, 1996). Nevertheless, careful analysis has enabled, for example, the functions of IP₃Rs in cerebellum in mice (Inoue *et al.*, 1998) and phototransduction in *Drosophila* to be studied (Acharya *et al.*, 1997; Raghu *et al.*, 2000). *Caenorhabditis elegans* is also proving to be a powerful system for understanding IP₃ signaling function. IP₃Rs in *C. elegans* are encoded by a single gene, *itr-1*, and are widely expressed (Baylis *et al.*, 1999; Dal Santo *et al.*, 1999). A genetic screen for genes that act downstream of *let-23* (epidermal growth factor receptor) identified *itr-1* as a component of a signaling pathway regulating ovulation (Clandinin *et al.*, 1998). Analysis of *itr-1* loss-of-function mutants (Dal Santo *et al.*, 1999) demonstrated that *itr-1* is a component of a rhythm generator that controls the defecation motor program. Thus, *C.*

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.01-08-0422. Article and publication date are at www.molbiol-cell.org/cgi/doi/10.1091/mbc.01-08-0422.

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C. elegans is an accessible system for dissecting IP₃ signaling in a whole animal.

To complement and extend these studies it is desirable to develop methods for achieving three goals. First, to be able to ablate IP₃ signaling in particular cells and/or at particular points in development. Second, to be able to distinguish between IP₃ signaling and its downstream effect through IP₃Rs. Third, to develop systems that are sufficiently malleable that they enable detection of a broad range of phenotypes without the severe reduction in viability that results from compounding several such phenotypes. To achieve this we have taken the approach of disrupting IP₃ signaling by induction of high-level expression of the IP₃ binding domain of ITR-1. This domain has IP₃ binding activity (Baylis *et al.*, 1999) and should therefore act as an IP₃ "sponge," competing with endogenous targets of IP₃ such as IP₃Rs. We demonstrate that this approach is successful and identify previously unidentified roles for IP₃ in the regulation of pharyngeal pumping and in embryogenesis.

MATERIALS AND METHODS

Production of Transgenic Animals Expressing IP₃ Sponge Constructs

The region of *itr-1* cDNA corresponding to residues 1–705 was amplified by polymerase chain reaction with Expand High Fidelity (Roche Applied Sciences, Indianapolis, IN), and ligated into pPD49.78 (Mello and Fire, 1995) to give the plasmid referred to as sponge. Derivatives of this plasmid, containing the mutations K579Q and R582Q ("control-sponge") or R511C ("super-sponge"), were constructed by single-round amplification of the entire plasmid with Expand High Fidelity and mutagenic primers, and *DpnI* digestion of the parental strand. Restriction fragments carrying the mutations were then cloned into the corresponding region of the wild-type sponge plasmid. Constructs were microinjected into *C. elegans* (Mello and Fire, 1995) with the *rol-6* gene marker plasmid pRF4.

In Vitro Binding Assays

The *itr-1* cDNA encoding the wild-type and mutant binding domains described above were cloned into pGEX2TK (Amersham Biosciences, Piscataway, NJ), and expressed in *Escherichia coli* BLR (Stratagene, La Jolla, CA). Fusion protein expression and total protein lysates preparation were as described previously (Yoshikawa *et al.*, 1996; Baylis *et al.*, 1999). Glutathione S-transferase (GST) fusion proteins were purified as in De Waard *et al.* (1995), except that Triton X-100 was omitted and proteins were dialyzed against binding buffer (50 mM Tris-HCl pH 8, 1 mM 2-mercaptoethanol, 1 mM EDTA). IP₃ binding assays (Yoshikawa *et al.*, 1996) used 500 nM purified fusion protein with 5.3 nM [³H]IP₃ in binding buffer containing the appropriate concentration of unlabeled IP₃. Reactions were incubated at 4°C for 10 min then GST fusion proteins were collected using glutathione-agarose beads, which were washed three times with binding buffer before scintillation counting.

C. elegans Culture and Phenotypic Assays

C. elegans strains were routinely cultured at 20°C on *E. coli* OP50 grown on NGM plates (Brenner, 1974). Expression from the *hsp16-2* promoter was induced by placing worms at 33°C for 2 h (Dixon *et al.*, 1990). In all phenotypic assays, semisynchronized populations were produced by allowing a small number of worms to lay for 12 h then removing them from the plate. The resulting offspring were heat shocked once immediately before egg laying commenced, i.e., as young adults. For brood size and other egg laying experiments,

the heat shock was repeated every 24 h for the duration of the laying period. All assays were carried out at 20°C.

Brood sizes were quantified by placing single worms on individual plates, moving them every 24 h, and counting the eggs laid. Defecation cycles were analyzed by observation with a stereomicroscope, measuring the time interval from one posterior body contraction to the next. For each genotype, young adults were heat shocked as described above and 12 animals (six each at 6 and 18 h after heat shock, or the equivalent stage for wild type) were followed for 10 continuous defecation cycles. Pharyngeal pumping was analyzed by counting pharyngeal contractions, as visualized under a stereomicroscope (Avery and Horvitz, 1989). For each genotype, young adults were heat shocked as described above and 12 animals (six each at 6 and 18 h after heat shock) were followed for five periods of 30 s. In both defecation and pharyngeal pumping experiments, no significant differences were observed between 6 and 18 h. The effects of serotonin were determined after incubation of worms on NGM plus 7.5 mM hydroxytryptamine in the absence of food (Waggoner *et al.*, 1998).

Embryos were dissected from adults 24 h after the second heat shock, i.e., at the start of the third day of egg laying, mounted in embryo culture medium (Zwaal *et al.*, 1996), and analyzed by four-dimensional video recording under Nomarski optics (Zwaal *et al.*, 1996). Terminal phenotypes were observed after incubation of mounted embryos for 18 h at 15°C. Development was also observed in utero. The percentage of embryos corresponding to each of the main phenotypes was quantified from observation of terminal phenotypes. ELT-2 expression was characterized by establishing transgenic animals carrying the relevant IP₃ sponge construct in the strain JM73, which carries an integrated *elt-2::gfp* fusion (Fukushige *et al.*, 1998). Green fluorescent protein (GFP) was visualized using a Leica SP confocal microscope. The percentage of embryos expressing ELT-2 was quantified by observing animals ~24 h after the second heat shock, scoring only embryos that had developed beyond the 2E cell (or equivalent) stage.

Immunofluorescence

Semisynchronized hermaphrodites were heat shocked every 24 h from the onset of laying, removed to fresh plates on day 2, and harvested in M9 on day 3. Permeabilization by freeze cracking, fixation with methanol and acetone, and antibody staining were performed as described previously (Baylis *et al.*, 1999), by using a rabbit anti-PGL-1 polyclonal antibody (Kawasaki *et al.*, 1998). The secondary antibody was goat anti-rabbit IgG-Alexa 488 conjugate (Molecular Probes, Eugene, OR). Propidium iodide (0.2 µg/ml) was included to stain nuclei. Images were collected using a Leica SP confocal microscope.

RNA-mediated Interference (RNAi) of *itr-1*

Clones of two regions of *itr-1* cDNA, base pairs 136–1056 (pNG019) and 6308–7266 (pNG030), were generated in the vector pPD129.36 that contains two T7 RNA polymerase promoters flanking the insert site (Timmons *et al.*, 2001). As a control we used pPD128.110, a derivative of pPD129.36 with a GFP cDNA insert (Timmons *et al.*, 2001). Plasmids were transformed into *E. coli* HT115 (DE3) and these strains used to perform RNAi feeding experiments (Timmons *et al.*, 2001). Five L4 hermaphrodites were moved onto each feeding plate and incubated at 20°C for 36 h then moved onto separate feeding plates and allowed to lay for 24 h at 20°C. These progeny were scored.

RESULTS

Establishing an Inducible IP₃ Dominant-Negative System to Identify IP₃-dependent Signaling Processes in *C. elegans*

To determine the functional consequences of disrupting IP₃ signaling in *C. elegans*, we overexpressed the IP₃ binding

Figure 1. IP₃ sponge constructs used in this study. Site-directed mutagenesis was used to abolish and to increase the affinity of IP₃ binding. (A) Schematic representation of an IP₃R subunit, showing domain organization. (B) In vitro binding to [³H]IP₃ of the following fusion proteins: GST-BD, GST fusion protein expressing the wild-type IP₃ binding domain; and GST-BD R511C, derivative of GST-BD with the mutation indicated (i.e., super-sponge). Binding was measured by the inhibition of specific [³H]IP₃ binding to purified fusion protein by various concentrations of IP₃. Specific binding was calculated by subtraction of nonspecific binding and normalized by expression as a percentage of maximal binding. (C) IP₃ binding to GST fusion proteins in vitro. GST-BD K579Q, R582Q, derivative of GST-BD with the mutations indicated (i.e., control-sponge). Error bars are SD.

domain of the *C. elegans* IP₃R, ITR-1, by using an inducible promoter. The IP₃ binding domain of ITR-1 was placed under the control of the *hsp16-2* promoter (Figure 1A) and this construct, referred to as the sponge plasmid, used to generate stable, transgenic animals. The *hsp16-2* promoter, although never directing constitutive expression, directs heat shock-induced expression in a wide range of tissues, including the intestine, nerves, muscle (body wall, pharyngeal, anal, and vulval), and hypodermis, the main exception being the germ line (Stringham *et al.*, 1992), a general feature of *C. elegans* transgenes. Thus, heat shocking of worms could be used to induce high-level, widespread expression of the IP₃ binding domain.

The IP₃ binding domain is substantial in size (705 residues), so to control for effects due to properties other than the IP₃ binding activity of the IP₃ sponge (or effects due to the experimental protocol itself), we made a construct, referred to as "control-sponge" in which two residues, each of which was identified as being essential for IP₃ binding in mouse IP₃R-1 (Yoshikawa *et al.*, 1996), were substituted (K579Q, R582Q). These residues have no other known function.

The efficacy of the dominant-negative sponge will reflect a balance between the levels of sponge, native receptors, and IP₃ in a given location. We therefore made a third construct "super-sponge" that is predicted to have an increased affinity

Figure 2. Disruption of IP₃ signaling results in increased defecation cycle length and increased variation in cycle length. Defecation was observed on food at 20°C, ~6 h (worms 1–6) or 18 h (worms 7–12) after onset of heat shock. Worms were recorded for 10 cycles. Squares are mean; bars are minimum-maximum, for individual worms. Mean ± SD is shown above each graph. *, worms failed to defecate in >10 min and are not included in the mean.

ity for IP₃ as a result of the substitution R511C. Substitution of the equivalent residue in the mouse type 1 receptor, R441, by glutamine results in a twofold increase in binding (Yoshikawa *et al.*, 1996), whereas conversion to cysteine produces a fivefold increase in affinity (Hirota, personal communication). This substitution is identical to that found in the gain-of-function allele, *itr-1* (*sy290*) (Clandinin *et al.*, 1998).

We confirmed that the IP₃ sponge constructs showed the predicted binding to IP₃ in vitro, by using GST fusion proteins of these binding domains. As Figure 1B shows, the wild-type sponge exhibits properties identical to those reported previously for the ITR-1 IP₃ binding domain (Baylis *et al.*, 1999), whereas the super-sponge construct exhibits an approximately twofold increase in affinity. Figure 1C shows that the control-sponge does not bind IP₃.

Induction of IP₃ Sponge Disrupts Defecation, a Known IP₃R-mediated Process

If the IP₃ sponge functions to reduce IP₃ signaling then at least some of its consequences should be similar to those caused by disrupting IP₃R function. Defecation in *C. elegans* is a coordinated and regular motor program, which occurs every 45 s (Croll and Smith, 1978; Liu and Thomas, 1994). Loss-of-function mutations in *itr-1* (*dec-4*) increase the period of, or eliminate, the defecation cycle (Dal santo *et al.*, 1999), indicating that the cycle is regulated by the IP₃R. We

therefore investigated whether expression of an IP₃ sponge conferred a similar effect. As Figure 2 shows, induction of either the sponge or super-sponge results in a substantial increase in mean cycle length, and also an increased variability (i.e., a decrease in rhythmicity). Some of the super-sponge worms did not defecate at all in 10-min periods of observation. These results provide evidence for the efficacy of the sponge as a device for disruption of IP₃ signaling.

Expression of an IP₃ Sponge Has No Effect on Ovulation

Genetic analysis implicates *itr-1* in ovulation (Clandinin *et al.*, 1998) and *itr-1* is clearly expressed in the spermatheca and uterine sheath cells (Baylis *et al.*, 1999; Dal Santo *et al.*, 1999; Gower *et al.*, 2001). Furthermore, depletion of ITR-1 by RNAi results in the predicted endomitotic (Emo) phenotype (Gower and Baylis, unpublished data). Disruption of IP₃ signaling is therefore predicted to result in defective ovulation. However, induction of the sponge and super-sponge under the control of the *hsp16-2* promoter did not result in an Emo phenotype, and sheath cell contraction and spermathecal dilation were normal (our unpublished data). This effect may be the result of poor expression from the *hsp16-2* promoter in the relevant somatic gonad cells. However, expression of the same sponge constructs under the control of the *hsp16-41* promoter (in pPD49.83; Mello and Fine, 1995), which has been used to disrupt ovulation via overexpression of LFE-2 (Clandinin *et al.*, 1998), also had no effect. In addition, inducing expression earlier in development, by commencing heat shock at the first larval stage, had no effect on brood size or ovulation (our unpublished data).

Disruption of IP₃ Signaling Interferes with Modulation of Pharyngeal Pumping

Because ITR-1 is highly expressed in the terminal bulb and isthmus of the pharynx (Baylis *et al.*, 1999; Dal Santo *et al.*, 1999), we tested the hypothesis that IP₃ signaling has a role in pharyngeal pumping. The pharynx is a rhythmic pump that forces food into the intestine. As Figure 3A shows, animals expressing the sponge or super-sponge exhibit a dramatic decrease in the rate of pharyngeal pumping in the presence of food, as well as an increase in the variability of pumping rate. When we compared pharyngeal pumping rates in the absence of food, no significant difference was observed between the wild-type and transgenic animals expressing either active or control IP₃ sponge constructs. Thus, interfering with IP₃-mediated signaling does not interfere with the basal rate of pharyngeal pumping, but severely disrupts the increase in pumping rate in response to the presence of food. To determine whether this effect was due to an inability of the pharyngeal muscle to pump at the higher frequency we exposed the worms to serotonin, which induces higher rate pharyngeal pumping (Croll and Smith, 1978; Horvitz *et al.*, 1982). This ability is unimpaired in any of the sponge-treated animals. Thus, the pharynx is able to pump at the higher rate and at least one mechanism by which serotonin induces this remains unperturbed.

To ascertain whether the effect of IP₃ on stimulation of pharyngeal pumping was mediated through the IP₃R we analyzed the weak loss of function allele *itr-1* (*sa73*) (Dal Santo *et al.*, 1999). As Figure 3A shows, the rate of pumping

Figure 3. Disruption of IP₃ signaling or IP₃R expression results in slower and more erratic pharyngeal pumping. (A) Pharyngeal pumping in transgenic strains expressing IP₃ sponge constructs was observed on food or in the absence of food at 20°C, ~6 or 18 h after heat shock [wild-type and *dec-4* (*sa73*) animals were not heat shocked]. The effects of serotonin were assayed after 1–2 h on NGM, 7.5 mM 5-hydroxytryptamine in the absence of food. Mean calculated from five observations for 12 worms. Error bars are SD. (B) Pharyngeal pumping in RNAi-treated animals. Assays were carried out as described above; pumping on food was assayed after 1–2 h on OP50. Mean calculated from five observations for 10 worms.

in the presence of food is significantly reduced ($p < 0.001$) in these animals compared with the wild-type, although this effect is less severe than for the IP₃ sponge constructs. Again, restoration of full pumping rate was observed in the presence of serotonin. To extend this observation we performed RNAi on wild-type animals to knock down *itr-1*. We observed the same results with two different parts of the *itr-1* cDNA. Figure 3B shows that knock down of *itr-1*, like ablation of IP₃ signaling, reduced pumping in the presence of food. Pumping rate is increased in response to serotonin, but is not fully restored to wild-type levels. These animals also display other phenotypic consequences of *itr-1* knock down and are generally rather sick, which most likely explains this discrepancy. We conclude that signaling through IP₃ and IP₃R is required for the up-regulation of pharyngeal pumping in response to food.

Disruption of IP₃ Signaling Results in Embryonic Arrest

As discussed above knocking down IP₃R in *C. elegans* results in sterility, at least in part due to a failure in ovulation.

Figure 4. Disruption of IP₃ signaling results in embryonic arrest. (A) Percentage of eggs laid that were arrested. (B–D) Early cell divisions generally proceed as normal. Typical embryo from an adult expressing the super-sponge. Arrow indicates an example of the large vesicle-like structures. (E) In some cases, defects are seen in cytokinesis. One-cell embryo from an adult expressing the super-sponge, showing failure of the pronuclei (arrows) to separate fully before formation of the cleavage furrow (c). (F) Typical embryo from a control-sponge transgenic adult. (G) Immunofluorescence labeling with anti-PGL-1 polyclonal antibody (green), and propidium iodide staining of DNA (red) of an embryo illustrating the most common arrest phenotype of embryos from adults expressing the super-sponge. The most commonly observed terminal phenotype (H and I) and another terminal phenotype of embryos from adults expressing the super-sponge (J and K). Nomarski images, except H and J, which are fluorescence images (showing rhabditin granules). ELT-2::GFP fluorescence (L) and corresponding transmission image of typical adult expressing the super-sponge (M). ELT-2::GFP fluorescence (N) and corresponding transmission image of typical adult expressing the control-sponge (O).

We exploited the fact that expression of the IP₃ sponge from the *hsp16-2* promoter does not disrupt ovulation to study the effect of disrupting IP₃ signaling on embryonic development. Many of the eggs laid by animals expressing the sponge and especially the super-sponge are abnormal and fail to develop into larvae (Figure 4A). Indeed, the results shown underestimate this effect as the number of dead eggs increases through time. The effect is stronger in the super-sponge worms. This increase in embryonic arrest is not a side effect of starvation (resulting from the pharyngeal defects; our unpublished data). Clearly, disruption of IP₃ signaling results in substantial embryonic lethality.

To determine the basis of the embryonic arrest we began by using three techniques for the observation of embryonic development. Using Nomarski microscopy, we 1) observed

embryos in utero, 2) analyzed early cell divisions by using a four-dimensional video recording system to record embryos in multiple focal planes (Zwaal *et al.*, 1996), and 3) characterized the terminal phenotypes of mounted embryos. This led us to identify three classes of arrested embryos.

In general, early cell divisions in embryos from adults expressing the sponge or super-sponge proceed normally (Figure 4, B–D), with no evidence of defects in the asymmetric nature of these divisions or establishment of polarity. A small proportion of embryos appears misshapen and swollen and displays difficulties in cytokinesis. The spindle appears weak and poorly anchored, and in some cases the cleavage furrow forms before the pronuclei are sufficiently separated (Figure 4E). These embryos usually arrest at the one-cell stage (9.1% of embryos observed, *n* = 154). This

phenotype was observed despite mounting embryos in osmotically balanced medium, and was also observed in utero, so it seems unlikely that it is an indirect effect of osmotic sensitivity. Expression of the sponge or super-sponge results in the presence of large granule-free zones in the embryos produced by these transgenic worms (Figure 4, B–E). The nature of these is unknown, although they are reminiscent of the large endocytotic vesicles observed in *pod-1* mutants (Rappleye *et al.*, 1999). These are not observed in embryos from worms expressing the control-sponge (Figure 4F).

To further characterize the arrest phenotype, mounted embryos were incubated overnight at 15°C and terminal phenotypes analyzed. By far the most common phenotype (33.8%) consisted of a mass of <100 undifferentiated cells (Figure 4, H and I), with no evidence for rhabditin granules (a marker for gut differentiation; Babu, 1974; Laufer *et al.*, 1980). This phenotype was investigated more fully, as described below. A less frequently observed phenotype (10.4%) consisted of a disorganized mass of partially differentiated cells, with evidence for pharyngeal and muscle tissue and rhabditin granules (Figure 4, J and K). The partial differentiation, and clustering of gut granules close to the posterior surface, seen in this third phenotype is similar to that observed in maternal effect mutants such as *gad-1* (Knight and Wood, 1998) and *emb-5* (Nishiwaki *et al.*, 1993), where the E cells divide aberrantly and fail to migrate inwards, so that cell movements associated with gastrulation do not occur.

We further characterized the most common phenotype (Figure 4, H and I), examining whether gastrulation had occurred in these embryos. Gastrulation initiates when the two gut precursors, Ea and Ep, start to move from the ventral surface into the blastocoel. After this, as cell divisions continue, the mesodermal and germline (P_4) precursors also ingress and then divide to form the three germ layers of the embryo (Sulston *et al.*, 1983). We investigated the fate of the P lineage, via immunofluorescence labeling of P granules. As Figure 4G shows, these embryos exhibit staining in a single cell, P_4 , which remains at the ventral surface and consequently fails to divide to form the germ line cells Z2 and Z3. This is similar to the arrest phenotype that results from *ama-1* antisense (Powell-Coffman *et al.*, 1996).

A failure in E cell differentiation might explain the failure in gastrulation. To further define when in E-cell development the differentiation process goes awry we investigated the fate of the E lineage by using an *elt-2::gfp* transgene (Fukushige *et al.*, 1999). *ELT-2* is a gut cell-specific GATA family transcription factor that is first expressed at the 2E cells stage (Fukushige *et al.*, 1998). Overall, expression of *ELT-2::GFP* was observed in only 35.1% of embryos in super-sponge worms, compared with 95.9% for the control-sponge (Figure 5, L–O). In agreement with our other observations, the effect is maternal, in that although some animals are indistinguishable from the wild type, others produce a very high proportion of arrested, unlabeled embryos. Thus, it appears that E-cell differentiation fails very early in the E-cell lineage. E is derived by the division of the cell EMS; in the absence of the necessary inductive signal (from P2) both derivatives of EMS take on an MS-like fate (Goldstein, 1995). We therefore tested for extra MS descendants by staining with the anti-pharyngeal muscle antibody 3NB12 (Priess

and Thomson, 1987). In agreement with our visual characterization of terminal phenotypes, these embryos showed no evidence for differentiation of pharyngeal tissue.

DISCUSSION

We describe the successful application of a system for regulated, transient disruption of IP_3 signaling in a whole animal. When the IP_3 binding domain of the IP_3R is overexpressed under the control of an inducible promoter in *C. elegans*, defecation, a previously identified putative IP_3 -mediated process, is disrupted and new roles for IP_3 signaling are identified.

In contrast to the analysis of mutations in IP_3Rs the IP_3 sponge has the potential to disrupt IP_3 signaling through any target, and thus to detect IP_3 -dependent processes that are not mediated through IP_3Rs . Other proteins are known to bind IP_3 , for example, phospholipase $C\delta$ and p130 (Yoshida *et al.*, 1994), although functions for the IP_3 binding of such proteins remain unknown. Of the IP_3 -dependent phenotypes analyzed in this article the evidence that the regulation of defecation and pharyngeal pumping also involve IP_3Rs is strong (Dal Santo *et al.*, 1999; this study). The evidence is less clear-cut for the roles of IP_3 in embryogenesis, because disruption of IP_3R expression results in sterility.

The efficiency with which the sponge reduces IP_3 signaling may be influenced by a number of variables. The concentration of the sponge proteins themselves will depend on the efficiency of expression, which may vary depending on the particular transgenic line, cell or subcellular locale, or developmental stage. Furthermore, the efficacy of the sponge may depend on its binding affinity and also the concentration of IP_3 . Thus, the current application of this technique is not quantitative and cannot be regarded as a method for ablating all IP_3 signaling; nevertheless, its utility is demonstrated by the novel phenotypes identified.

IP_3 and Defecation Rhythm

We used the previously identified role of IP_3Rs in the rhythm generator underlying the defecation cycle to assess the effectiveness of the IP_3 sponge as a means of ablating IP_3 function. Ablating IP_3 signaling with the sponge clearly disrupts this process, supporting the model (Dal Santo *et al.*, 1999) that IP_3 plays a role in regulating this process. Comparison of the effects of the sponge and loss-of-function alleles of *itr-1* (Dal Santo *et al.*, 1999) allows some insight into the effectiveness of the sponge in the cells regulating this process. *itr-1* (*n2559*) is a severe allele and animals are sterile and do not defecate, whereas *itr-1* (*sa73*) is a weak allele with relatively normal fertility, disrupted but functional defecation (Dal Santo *et al.*, 1999), and moderately disrupted pharyngeal pumping (this study). In terms of defecation, expression of the sponge or super-sponge from the *hsp16-2* promoter has an effect that is more disruptive than *sa73* and in some cases as extensive as *n2559*. Overall, however, sponge disruption is not as extreme as the severe allele. Thus, a range of effectiveness, presumably reflecting the variables discussed above, is observed.

IP_3 and Ovulation

Clandinin *et al.* (1998) demonstrated that *itr-1* lies downstream of *let-23* in the regulation of ovulation. Gain-of-func-

tion alleles of *itr-1* suppress *lin-3* sterility. It is therefore surprising that IP₃ sponge expression (from *hsp16-2* or *hsp16-41* promoters) does not result in sterility. One possible explanation is inadequate expression in the cells responsible, although Clandinin and colleagues used the *hsp16-41* promoter to successfully disrupt ovulation by overexpression of *lfe-2*. Another possibility is that this mechanism is mediated through IP₃Rs but not IP₃. Several lines of evidence suggest this may not be true. First, Clandinin *et al.* (1998) clearly showed a role for *lfe-2*, IP₃ 3-kinase, which converts IP₃ to inositol 1,3,4,5-tetrakisphosphate, IP₄. The ability to alter ovulation by altering *lfe-2* may result from alterations in IP₃ levels. However, IP₄ is also an active signaling molecule (Irvine and Schell, 2001) and it cannot be ruled out that changes in *lfe-2* function through IP₄. We have performed RNAi on phospholipase C γ , the predicted mediator of IP₃ production by *let-23*. These animals have an Emo phenotype, suggesting that PLC γ and presumably IP₃ are required for ovulation (our unpublished data). We believe the most likely explanation is that the local sponge concentration is insufficient. Sponge levels in these cells may be lower than elsewhere; IP₃ levels may be too high to disrupt; or IP₃ signals and associated signaling components may be localized, with the sponge lacking the information required for localization such that its local concentration is insufficient.

Role of IP₃ Signaling in Pharynx

The pharynx of *C. elegans* is a good model for understanding behavioral control of a rhythmic physiological process. Results from analysis of sponge expression, RNAi, and the *itr-1* (*sa73*) mutant demonstrate that the IP₃ signaling pathway is required for the up-regulation of pharyngeal pumping in response to food. Both sponge expression and RNAi severely reduce this effect. This is without any significant effect on either basal rates of pharyngeal pumping or the ability of the pharynx to pump when stimulated by exogenous serotonin (although RNAi worms do show some reduction in the effect of serotonin).

IP₃Rs are expressed both in the muscle cells of the terminal bulb and isthmus and in the nervous system (Baylis *et al.*, 1999). That expression of an IP₃ sponge does not interfere with basal pharyngeal pumping is perhaps not surprising, given that this is thought to be largely intrinsic to the muscle, where the IP₃R would not be expected to respond sufficiently rapidly to play a central role. Although pumping per se is not dependent on function of the pharyngeal nervous system these neurons are required for the proper control of feeding behavior. Specifically, the neuron MC is central to the regulation of pumping rate in response to food (Avery and Horvitz, 1989; Raizen *et al.*, 1995). Based on the role that we have demonstrated in increasing pumping rate in response to food, it appears that IP₃R signaling plays a role in the behavioral modulation of pumping rate. The observation that the effect of serotonin is not altered is interesting. Serotonin stimulates pumping rate via at least two mechanisms (Avery and Thomas, 1997), one through stimulation of MC, the other through a direct action on the pharyngeal muscle. Our results suggest that at least one of these is not affected by ablating IP₃ signaling but that nevertheless the normal stimulation of pharyngeal pumping in response to food is disrupted. One explanation is that the direct effect of serotonin on the pharynx remains intact.

Alternatively, the mechanism ablated in IP₃ sponge worms may be an additional, serotonin-independent mechanism. We are currently using sponge expression from cell-specific promoters (Gower *et al.*, 2001) to dissect this process further.

Role of IP₃ Signaling in Embryogenesis

Our results demonstrate that disruption of IP₃ signaling interferes with multiple events during embryogenesis. We observe three classes of arrested embryos: 1) embryos in which cytokinesis is defective and which usually arrest at the one cell stage; 2) embryos that form a ball of <100 cells, with no apparent differentiation of cell types and no signs of gastrulation; and 3) embryos that have undergone some, disorganized differentiation. Using the IP₃ sponge has enabled us to circumvent the sterility conferred by ablating IP₃ receptors. However, interpretation of the data is made complex by the fact that the sponge levels are unlikely to be constant over time, between animals or between embryos. This may partially explain why we see three different phenotypes. First, our results indicate roles for IP₃ signaling in cytokinesis or events required for it, this seems to be true only in the first cell division, suggesting a specific role for this signal in these very early events. Parallels can be drawn with other systems. In zebrafish and humans, the distribution of both Ca²⁺ and IP₃Rs is highly dynamic during this period, but IP₃Rs are particularly highly localized to cleavage furrows, polar bodies, and points of contact between blastomeres (Creton *et al.*, 1998; Goud *et al.*, 1999). Lithium inhibition of the phosphoinositide pathway in sea urchin embryos results in arrest at cytokinesis (Becchetti and Whitaker, 1997).

The major phenotype observed is that described in 2 above. Our results suggest that IP₃ signaling plays a role in early E-cell differentiation. The failure of the E cells to differentiate inevitably leads to a failure in gastrulation. The failure in E-cell differentiation is probably part of a more widespread failure in cell differentiation as suggested by the lack of observable differentiation and the lack of MS cell derivatives. Perhaps the most similar phenotype to this is that which results from blocking embryonic transcription by *ama-1* antisense (Powell-Coffman *et al.*, 1996). One possibility is therefore that an IP₃-mediated calcium signal is required to permit embryonic transcription. Alternatively, IP₃ signaling may play a fundamental role in the signals required for cell determination. The third class of embryos develop to a later stage and the defect in these embryos remains unclear, although by analogy to *gad-1* (Knight and Wood, 1998) and *emb-5* (Nishiwaki *et al.*, 1993) mutants this may suggest a failure in cell migrations associated with gastrulation, as a result of aberrant E-cell division.

It is unclear whether these functions are mediated through IP₃Rs. *itr-1* (*n2559*) and RNAi-treated animals are sterile. During RNAi treatment, a few viable and affected worms are produced. We have not observed dead embryos in these escapees (regardless of whether RNAi treatment is by feeding or microinjection; Gower and Baylis, unpublished data). Nor have we observed any disruption of embryogenesis in the *sa73* weak loss-of-function allele of *itr-1*. Thus, it appears that by definition any fertile animal has substantial IP₃R function; therefore, in both the *itr-1* (*sa73*) and RNAi fertile offspring, the lack of effect may merely reflect limited disruption of ITR-1 function. Using the currently available

tools, we are therefore unable to test the hypothesis that IP₃ functions in embryogenesis in an ITR-1-independent manner. We are currently attempting to generate systems in which this will be possible.

Use of the IP₃ sponge has enabled the identification of new IP₃-mediated processes in *C. elegans*. Despite this, others may well remain to be identified. In particular, IP₃Rs are expressed in the excretory cell and neurons and no known functional correlates have yet been found for these sites of expression. The application of the IP₃ sponge leads to new tools for dissecting these processes in *C. elegans* and other animals.

ACKNOWLEDGMENTS

We are grateful to P. Zipperlen and J. Ahringer for help in analyzing embryos and for antibodies. The IP₃ sponge was so-named by K. Mikoshiba. We thank A. Fire for heat-shock and RNAi feeding vectors; J. McGhee for JM73; S. Strome for the anti-PGL-1 antibody; the *Caenorhabditis* Genetic Center for *C. elegans* strains; and K. Mikoshiba, T. Furuichi, F. Yoshikawa, and J. Hirota for helpful discussions and for communicating unpublished data. This work was supported by the Medical Research Council (United Kingdom) (to H.A.B., S.L., and D.S.W.) and the Biotechnology and Biological Sciences Research Council (United Kingdom) (to N.J.D.G.). H.A.B. is a Medical Research Council Senior Fellow.

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