Inositol 1,4,5-Trisphosphate Signaling Regulates Mating Behavior in *Caenorhabditis elegans* Males[□]

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Complex behavior requires the coordinated action of the nervous system and nonneuronal targets. Male mating in *Caenorhabditis elegans* consists of a series of defined behavioral steps that lead to the physiological outcomes required for successful impregnation. We demonstrate that signaling mediated by inositol 1,4,5-trisphosphate (IP₃) is required at several points during mating. Disruption of IP₃ receptor (*itr-1*) function results in dramatic loss of male fertility, due to defects in turning behavior (during vulva location), spicule insertion and sperm transfer. To elucidate the signaling pathways responsible, we knocked down the six *C. elegans* genes encoding phospholipase C (PLC) family members. *egl-8*, which encodes PLC- β , functions in spicule insertion and sperm transfer. *itr-1* and *egl-8* are widely expressed in the male reproductive system. An *itr-1* gain-of-function mutation rescues infertility caused by *egl-8* RNA interference, indicating that *egl-8* and *itr-1* function together as central components of the signaling events controlling sperm transfer.

INTRODUCTION

Complex behaviors in animals require not only processing and production of information by the nervous system, but also coordinated changes in the physiology of the target organs required for the execution of that behavior. Male mating behavior in *Caenorhabditis elegans* is an example. Male mating relies on specialized sex-specific structures and is one of the most complex behavioral and physiological processes exhibited by *C. elegans*. Mating begins with the posterior of the male making contact with the hermaphrodite; the male places his tail against the hermaphrodite and moves along its body. When the head or tail of the hermaphrodite is reached a turn is made via a deep ventral flexion, this behavior continues until the vulva is located. The spicules, a pair of copulatory structures, are then inserted into the vulva and sperm is transferred through the vas deferens, to the hermaphrodite uterus (Sulston *et al.*, 1980; Loer and Kenyon, 1993; Liu and Sternberg, 1995). This behavior thus requires integration of sensory information, coordinated body movements, and coordinated muscle activity to bring about spicule insertion and the correctly timed release of sperm.

The sequence of mating activities that culminates in sperm transfer requires many levels of regulation, and their coordination is clearly based on many inter- and intracellular signaling events. The second messenger inositol 1,4,5-trisphosphate (IP₃) is a prime candidate for playing a signaling role at the interface of neuronal and target organ physiology. IP₃ signaling transduces signals from a wide range of extracellular stimuli into intracellular Ca²⁺ signals that regulate cell physiology. IP₃, produced by the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) by activated PLC, diffuses through the cytosol and causes Ca²⁺ release from intracellular stores through the activation of IP₃Rs (Berridge, 1993).

IP₃Rs in *C. elegans* are encoded by a single gene, *itr-1* and are widely expressed throughout the animal (Baylis *et al.*, 1999; Dal Santo *et al.*, 1999; Gower *et al.*, 2001). Genetic and transgenic approaches in *C. elegans* have led to the characterization of roles for IP₃ signaling. A genetic screen for genes that act downstream of *let-23* (epidermal growth factor [EGF] receptor) identified *itr-1* as a component of a signaling pathway regulating ovulation (Clandinin *et al.*, 1998). Part of this requirement stems from the importance of IP₃ signaling in the regulation of gonadal sheath cell contraction (Yin *et al.*, 2004). Analysis of *itr-1* loss-of-function mutants led to the identification of a central role in the regulation of the defecation motor program (Dal Santo *et al.*, 1999). We have demonstrated, using *itr-1* mutants, RNA-mediated interference (RNAi) and an inducible dominant-negative construct (IP₃ sponge), that IP₃R and IP₃ signaling are involved in the up-regulation of pharyngeal pumping in response to food and in multiple stages of embryogenesis (Walker *et al.*, 2002).

Ca²⁺ release is a key integratory intracellular signaling event and is central to a wide range of cellular responses (Berridge, 1993, 1997; Clapham, 1995). The spatial and temporal structure of Ca²⁺ signals is regulated through components of the Ca²⁺ signaling toolkit (Berridge *et al.*, 2000). Previous studies have identified components of the Ca²⁺ signaling network in the regulation of male mating behavior. The *unc-68*-encoded ryanodine receptor sarcoplasmic

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Abbreviations used: IP₃, inositol 1,4,5-trisphosphate; IP₃R, inositol 1,4,5-trisphosphate receptor; PLC, phospholipase C; GFP, green fluorescent protein; ACh, acetylcholine; RNAi, RNA-mediated interference.

reticulum Ca²⁺ channel and the *egl-19*-encoded L-type voltage-gated Ca²⁺ channel α_1 subunit play a role in spicule insertion through their action in protractor muscles (Garcia *et al.*, 2001; Maryon *et al.*, 1998).

To further dissect the Ca²⁺ signaling networks that modulate this complex series of behaviors, we have determined the role of IP₃-mediated signaling in male mating behavior. We describe results that implicate IP₃R-mediated Ca²⁺ release as being important for correct execution of turning and spicule insertion. We demonstrate that IP₃ signaling is fundamental to the process of sperm transfer in *C. elegans* males and provide evidence that EGL-8 (PLC- β) is the specific PLC that functions in the generation of this IP₃ signal.

MATERIALS AND METHODS

C. elegans Strains and Culture

C. elegans were maintained using standard culture methods (Lewis and Fleming, 1995). The following strains were used: Bristol N2 wild-type; JT73 (*itr-1* (*sa73*) IV, Iwasaki *et al.*, 1995); CB1091 (*unc-13* (*e1091*) I, Waterston and Brenner, 1978); BA1 (*fer-1* (*hc1*) I, Ward and Miwa, 1978); CB1467 (*him-5* (*e1467*) V, Hodgkin *et al.*, 1979); CB1489 (*him-8* (*e1489*) IV, Hodgkin *et al.*, 1979); and CB138 (*unc-24* (*e138*) IV, Brenner, 1974). HB100 (*itr-1* (*sa73*); *him-5* (*e1467*)) was constructed by mating CB1467 males and JT73 hermaphrodites. HB101 and HB102 (*itr-1* (*sy290*) *unc-24* (*e138*) IV; *him-5* (*e1467*) V *juwEx101* [*unc-24* (+), *mec-7::gfp*]) were constructed by first crossing PS2582 hermaphrodites with CB1467 males and then rescuing *unc-24* (*e138*) by transgenic expression of wild-type *unc-24*. A genomic DNA fragment from ~1 kb upstream of the *unc-24* start codon to 600 base pairs downstream (bp 11573–15174 of cosmid F57H12) was amplified by PCR and ligated into pGEM-T (Promega, Madison, WI) to give pNG135. PS2582 was injected with pNG135 and pPD117.01 (a *mec-7::gfp* reporter construct that expresses in mechanosensory neurons) as a marker. HB103 (*unc-24* (*e138*) IV; *him-5* (*e1467*) V *juwEx101* [*unc-24* (+), *mec-7::gfp*]) was constructed in a similar way, except that CB138 was used instead of PS2582. The resulting strains display normal movement.

RNA-mediated Interference

RNAi of *itr-1* was carried out using *Escherichia coli* HT115 carrying derivatives of the vector pPD129.36 (Timmons *et al.*, 2001), which contains two flanking T7 RNA polymerase promoters. For RNAi of *itr-1* we used pNG030 (Walker *et al.*, 2002), which carries a 1-kb region of the *itr-1* cDNA. For RNAi of PLC genes we constructed derivatives of pPD129.36, carrying ~1 kb of the cDNA of each PLC gene; these are pHAB301 (*egl-8*), pHAB302 (*plc-1*) pHAB303 (*plc-3*) pHAB304 (*plc-4*), pHAB305 (*plc-2*), and pHAB306 (*pll-1*). As a control we used pPD128.110, a derivative of pPD129.36 with a *gfp* cDNA insert (Timmons *et al.*, 2001). Five L4 *him-8* (*e1489*) hermaphrodites were placed onto each feeding plate and incubated at 25°C for 24 h and then transferred onto separate feeding plates and allowed to lay eggs for 24 h at 25°C. L4 male offspring were then removed for functional analysis.

Fertility Assays

To assay male fertility we used *fer-1* (*hc1*) “females” (Ward and Miwa, 1978) as recipients. Both 1:1 and 12:4 male to female crosses were set up and scored in the same way: 30-mm NGM plates (2.5% agar, to prevent burrowing) were poured, seeded the following day with a 5-mm diameter *E. coli* lawn, and used for fertility assays the following day. *Fer-1* (*hc1*) animals were maintained at the permissive temperature of 15°C, at which they are fertile. Adult hermaphrodites were then transferred to the restrictive temperature of 25°C and allowed to lay eggs for 24 h, and the offspring were allowed to develop at 25°C. Either 1 or 4 L4 larvae were then transferred to the *E. coli* spot on the cross plate, with either 1 or 12 RNAi-treated or mutant males. Crosses were performed at 25°C and offspring were counted up to a maximum of 100. Fertility was compared with *fer-1* (*hc1*) only (i.e., no males) plates. To remove the background fertility we subtracted the highest *fer-1* (*hc1*) only control plate value from the number of progeny on each test plate. A plate was deemed to be fertile if the number of offspring exceeded the highest number observed on a *fer-1* (*hc1*) only plate.

Scoring Male Behaviors

Male mating behaviors were assayed by placing a single young adult male at *t* = 0 in the center of a plate containing 30, evenly distributed, young adult *unc-13* (*e1091*) hermaphrodites. *Unc-13* mutant hermaphrodites were used because greater mating efficiencies are observed when the hermaphrodites are unable to move (Hodgkin, 1983). To control for variation of mating activity due to age of plate (Liu and Sternberg, 1995), 30-mm agar plates were poured one day, seeded with a 5-mm lawn of *E. coli* OP50 the next day, and used for mating behavior assays the following day. Male mating behavior (Loer and

Kenyon, 1993; Liu and Sternberg, 1995) was assessed during a 20-min observation period using the following criteria: hermaphrodite body contact, backing with tail contacting the hermaphrodite, turning of the tail around the circumference or an end of the hermaphrodite, forward movement of tail in contact with hermaphrodite, general vulva location movements, fine tuning vulva location movements, attachment of the tail to the vulva, spicule prodding, and spicule insertion. All observations of the cross plates were made at 400 $\times$ on a Zeiss Axiovert S100 inverted microscope (Thornwood, NY; 23–25°C).

To score turning ability crosses were set up as described above but only turns were scored in the 20-min period. A turn or attempted turn was scored when the male backed up to the end (head or tail) of a hermaphrodite and attempted to curl his tail to contact the opposite surface of the mate. Each such turn was categorized (Loer and Kenyon, 1993) as follows: “good” (tail remained in contact with the hermaphrodite throughout the turn and continued backing on the opposite side after the turn was complete), “sloppy” (tail temporarily lost contact, but quickly regained contact on the opposite side because of a favorable trajectory), or “missed” (tail sailed off the end of the hermaphrodite, completely losing contact).

Construction of Transgenic Animals Carrying GFP Fusions and Constructs

Three *itr-1::gfp* constructs had previously been constructed (Gower *et al.*, 2001); pNG007 contains the 650-base pair sequence upstream of exon 1; pGT001 contains the 2.4-kb sequence that separates exon 1 and exon 2, and pGT002 contains the 4.4-kb sequence that separates exon 2 and the first common exon, 4. *plc::gfp* fusions were made using a PCR fusion-based approach (Hobert, 2002). Genomic fragments were amplified for each member of the family; *egl-8* was amplified from 4 kb upstream to exon 6; *plc-1* was amplified from 4 kb upstream to exon 2; *plc-2* was amplified from 1.8 kb upstream to exon 14; *plc-3* was amplified 3.4 kb upstream to exon 8; *plc-4* was amplified from 4.2 kb upstream to just upstream of the start site; and *pll-1* was amplified from 4 kb upstream to exon 3. PCR products were the fused in-frame to the *gfp* gene. DNA for microinjection was prepared using Qiaprep Spin Miniprep kit (Qiagen, Valencia, CA) and further purified by precipitation with ethanol in the presence of 0.1 M potassium acetate, before microinjection into *him-8* (*e1489*) hermaphrodites (Hodgkin *et al.*, 1979; Mello and Fire, 1995). Images were recorded using a Leica SP confocal microscope (Bannockburn, IL). Images are maximum projections of a Z-series.

Fluorescent Sperm Tracking

Sperm tracking assays used the method of Hill and L'Hernault (2001). L4 virgin males were picked from the RNAi feeding plates to a freshly seeded plate and grown overnight in the absence of hermaphrodites. Males were incubated in 70 μ M SYTO17 dye (Molecular Probes, Eugene, OR) at 25°C for 3 h, transferred to an NGM plate and allowed to recover, and then picked for mating experiments. Twelve males and 4 *fer-1* (*hc1*) females were picked onto a cross plate and allowed to mate at 25°C for 24 h. Recipient females were mounted onto 2% agar pads and examined by differential interference contrast (DIC) and fluorescence microscopy to locate male-derived (fluorescent) sperm. The level of sperm transferred was classified as good (>10 sperm visible in recipient), weak (between 1 and 10 sperm visible), and none.

Small Scale Sperm Isolation

Three males were placed in a 7- μ l drop of SM (L'Hernault and Roberts, 1995) containing 1 mg/ml bovine serum albumin and 200 μ g/ml pronase. Sperm were then released by cutting just in front of the tail with a razor blade (L'Hernault and Roberts, 1995). Spermatids were released into the solution and 80–95% became activated to form spermatozoa within 5 min. Sperm were examined using DIC optics on a Zeiss Axiovert S100 inverted microscope.

Spicule Protraction

Spicule protraction in response to levamisole was assayed as described by Garcia *et al.* (2001). Twenty to 40 animals were tested for each concentration. EC₅₀ values were calculated by fitting sigmoidal curves to log-transformed data using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

Statistical Analysis

All statistical analysis was carried out using Chi-squared tests.

RESULTS

itr-1 Function Is Essential for *C. elegans* Male Fertility

The ability of males to mate successfully is dependent on functions endowed by a range of male-specific structures. Therefore defects in the function or development of any of these structures will result in reduced male fertility. To test

whether *itr-1* is required for fertility, we performed mating assays between *itr-1* RNAi males and *fer-1* (*hc1*) “females” (Ward and Miwa, 1978). We performed RNAi on *him-8* (*e1489*) (Hodgkin *et al.*, 1979), which produces a high incidence of males. *Fer-1* (*hc1*) is a temperature-sensitive allele that results in sterility at 25°C, due to an inability to produce active sperm (Ward and Miwa, 1978). At the restrictive temperature, *fer-1* (*hc1*) animals therefore require the contribution of functional sperm from males for successful fertilization. We performed crosses between single *fer-1* (*hc1*) hermaphrodites and test males, i.e., 1:1 crosses. In this situation, even in the presence of wild-type males, not all plates yield progeny. These assays clearly demonstrated that males in which *itr-1* is knocked down using RNAi have drastically reduced fertility when compared with control males (Figure 1A). Because *fer-1* (*hc1*) animals have a low level of background fertility, this was subtracted from the number of offspring produced in each cross, before calculating the percentage of fertile crosses (see *Materials and Methods*). Using this assay, 52% of control crosses were fertile compared with only 4% of crosses using *itr-1* RNAi males.

As a more stringent test of fertility, in which all the control crosses were likely to produce offspring, we performed 12:4 male to female mating assays. In addition to testing *him-8* (*e1489*) *itr-1* RNAi males we also tested males from HB100, a strain carrying the *him-5* (*e1467*) allele (Hodgkin *et al.*, 1979) and the *itr-1* mild loss-of-function allele *itr-1* (*sa73*) (Iwasaki *et al.*, 1995). In the 12:4 crosses 100% of the control RNAi and untreated *him-5* (*e1467*) crosses were fertile, compared with 7.7% for *itr-1* RNAi animals and 20% for *itr-1* (*sa73*); *him-5* (*e1467*) males (Table 1). In these assays only one successful mating has the potential to produce a “fertile” plate. Thus we conclude that males in which *itr-1* function is disrupted have a severe fertility defect.

itr-1 Functions in Male Mating Behavior

The mating behavior of males follows a set sequence of steps culminating in sperm transfer (Loer and Kenyon, 1993; Liu and Sternberg, 1995). The key stages are; hermaphrodite recognition, backing, turning, vulval location, and copulation. Each of these steps can be further divided into subcategories. In particular, copulation is divided into spicule prodding (i.e., repeated contraction and relaxation of spicule protractor muscles), spicule insertion (i.e., prolonged spicule protractor contraction), and sperm transfer. To determine whether reduction in *itr-1* function disrupted any of these steps, we scored *him-8* (*e1489*) *itr-1* RNAi animals for their ability to execute each of these behaviors properly (Figure 1B). *itr-1* RNAi males responded to the hermaphrodites by backing and performed turning, backing, and vulval location with a frequency similar to that of the control males. *itr-1* RNAi males showed a very slight reduction in spicule prodding, whereas spicule insertion occurred at <25% of the frequency observed for control males ($p < 0.05$).

Although *itr-1* RNAi males performed turns with a similar frequency to control animals, these turns were often poorly executed. A normal turn consists of a sharp ventral arch of the tail, performed at the proper time (Liu and Sternberg, 1995). Loer and Kenyon (1993) have produced a classification for turns, in which they are deemed to be “good,” “sloppy,” or “missed” (see *Materials and Methods*). *itr-1* RNAi males (Figure 1C) perform significantly fewer good turns than the control males ($p < 0.001$), the number of sloppy turns is substantially increased, and the number of missed turns is also increased. Thus *itr-1* males fail to coordinate turns properly. These results demonstrate that ITR-1 has a role in regulating male turning and prolonged spicule inser-

Figure 1. *itr-1* functions in male fertility. (A) Fifty 1:1 crosses between *fer-1* (*hc1*) “females,” which are unable to produce active sperm and therefore require male-derived sperm for fertilization to occur, and males generated by feeding dsRNA specific for *gfp* (i) or *itr-1* (ii), compared with *fer-1* (*hc1*) only mock crosses (iii). For simplicity, crosses that produced 100 or more offspring are classed as having produced 100. (B) The role of *itr-1* in male mating behavior. A single male was placed at the center of a seeded NGM plate with 30 *unc-13* (*e1091*) hermaphrodites and observed for 20 min. Graph shows the percentage of males that performed the behavior at least once during the observation period. Performance of a given step implies successful performance of preceding steps; $n = 17$ (*gfp* RNAi) and 16 (*itr-1* RNAi). (C) Turning ability of *itr-1* and *gfp* RNAi males was determined by observing males as in B, but for 10 min. Turns were classified as missed, sloppy, or good (see *Materials and Methods*); $n = 23$ for each treatment.

tion. Nevertheless, these results do not explain the extent of sterility observed, as discussed later.

itr-1 Is Expressed in Many Male-specific Structures

To identify cells and tissues that might correlate with the functional requirements for *itr-1*, we determined the expression pattern of *itr-1* in males. The expression pattern of *itr-1*

Table 1. *itr-1* and *egl-8* function in male fertilityMales crossed with *fer-1* (*hc1*) "females"

Strain	RNAi	% Crosses fertile	% Crosses > 100 offspring	n
<i>him-5</i> (<i>e1467</i>)	—	100	100	10
<i>him-5</i> (<i>e1467</i>) <i>itr-1</i> (<i>sa73</i>)	—	20	0	10
<i>him-8</i> (<i>e1489</i>)	—	100	93.9	33
<i>him-8</i> (<i>e1489</i>)	<i>gfp</i>	100	100	47
<i>him-8</i> (<i>e1489</i>)	<i>itr-1</i>	7.7	3.8	26
<i>him-8</i> (<i>e1489</i>)	<i>egl-8</i> (PLC- β)	0	0	21
<i>him-8</i> (<i>e1489</i>)	<i>plc-1</i> (PLC- ϵ)	100	100	8
<i>him-8</i> (<i>e1489</i>)	<i>plc-2</i> (PLC- β -like)	100	100	8
<i>him-8</i> (<i>e1489</i>)	<i>plc-3</i> (PLC- γ)	100	87.5	8
<i>him-8</i> (<i>e1489</i>)	<i>plc-4</i> (PLC- δ)	100	62.5	8
<i>him-8</i> (<i>e1489</i>)	<i>pll-1</i> (PLC-like)	100	100	8

12:4 male to *fer-1* (*hc1*) "female" crosses. A cross was termed fertile if it produced more offspring than the highest *fer-1* (*hc1*) only mock cross.

has been well characterized in hermaphrodites using transgenic animals carrying *itr-1::gfp* reporter constructs and antibody staining (Baylis *et al.*, 1999; Dal Santo *et al.*, 1999; Gower *et al.*, 2001). The *itr-1* gene contains three promoters, each of which drives tissue-specific expression of an alternative *itr-1* mRNA (Gower *et al.*, 2001). Therefore, we made transgenic *him-8* (*e1489*) lines carrying *itr-1::gfp* reporter constructs for each promoter and determined their expression pattern in males.

Expression in male-specific structures is directed by all three promoters. Promoter pA directs expression in the spicule protractor muscles of the proctodeum and in a single male-specific neuron that is likely to be CP8 or CP9 (Figure 2A). The spicule protractor muscles are required for the prodding of the spicule before vulval insertion and for the maintenance of spicule insertion while sperm is transferred (Garcia *et al.*, 2001). Promoter pB directs expression in the spicule retractor muscles, gubernaculum retractor muscles, posterior oblique muscles, diagonal muscles, and the vas deferens (Figure 2B). The spicule retractors and gubernaculum retractors are required for spicule retraction after sperm transfer (Garcia *et al.*, 2001). The posterior oblique muscles and diagonal muscles contribute to the ventral flexure of the male tail and ablation of the diagonal muscles results in animals that never make normal turns (Loer and Kenyon, 1993). The vas deferens connects the seminal vesicle to the proctoderm and is involved in sperm transfer (Emmons and Sternberg, 1997). Promoter pC also directs expression in the vas deferens, as well as the seminal vesicle and the valve that separates the seminal vesicle and vas deferens (Figure 2C). Thus *itr-1* is expressed widely in the sex-associated muscles and somatic gonad of males.

itr-1 Mutants Fail to Transfer Sperm to Hermaphrodites

Despite the observed defects in turning and in spicule insertion we would still expect to see crosses producing offspring in the 12:4 mating experiments. Even given that the overall spicule insertion rate (which includes the effect of the turning defect) was reduced to 13% compared with 59% in normal animals, we would still expect 1 in 4 matings to

Figure 2. *itr-1* is expressed in male-specific structures. (A) Schematic diagram showing the male-specific structures discussed in this report. Spic Retr, spicule retractor muscles; Spic Pro, spicule protractor muscles; Gub Retr, gubernaculum retractor; Ant Ob, anterior oblique; Gub Erec, gubernaculum erector; PostOb, posterior oblique; *Vas def*, vas deferens; Diag, diagonal muscles. GFP reporter analysis of the three *itr-1* promoters. (B) Promoter pA directs expression in the spicule protractor muscles and in a male-specific neuron. (C) Promoter pB directs expression in the vas deferens, diagonal muscles, gubernaculum retractor, posterior oblique muscles, and spicule retractor muscles. (D) Promoter pC directs expression in the vas deferens, valve cell, and seminal vesicle (SEM Ves).

result in spicule insertion. In a situation where there are 12 males and 4 hermaphrodites, which may mate many times, we would thus expect most of the plates to produce progeny. Indeed the control animals only achieved 59% spicule insertion in assayed matings but the control 12:4 crosses demonstrated 100% fertility. The dramatic loss of fertility observed in these mating assays therefore suggests another severe defect. Because *itr-1* is expressed in the components of the male gonad involved in sperm transfer, we decided to monitor sperm transfer during mating.

A live fluorescent sperm-tracking assay, to assess the insemination, activation, and spermathecal targeting of male-derived sperm, has previously been described (Hill and L'Hernault, 2001). *him-8* (*e1489*) *itr-1* RNAi animals and *itr-1* (*sa73*) *him-5* (*e1467*) males were treated with the fluorescent dye SYTO 17 (Figure 3A; see *Materials and Methods*) to label their sperm and were then used in 12:4 fertility assays as described previously. Knockdown of *itr-1* resulted in a significant ($p < 0.001$) reduction in sperm transfer, with the majority of 12:4 crosses showing a complete lack of transfer. Similarly, *itr-1* (*sa73*) males showed a significant ($p < 0.001$) decrease in the efficiency of sperm transfer, compared with controls (Figure 3B). This sperm transfer defect goes further to explain the fertility defect observed and is sufficient to account for the loss of fertility observed.

itr-1 Mutant Males Produce Sperm That Are Able to Undergo Normal Spermiogenesis

Sperm development (spermiogenesis), in *C. elegans* is a complex process in which spermatocytes develop into spermatids that are stored, and then following ejaculation the spermatids develop into spermatozoa (Kimble and Ward, 1988). To identify whether spermatids are produced normally in

Figure 3. *itr-1* mutant males are defective in sperm transfer, but produce normal sperm. Males were stained with SYTO 17 (Ai) and mated with *fer-1* (*hc1*) “females” in a 12:4 ratio. Twenty-four hours later recipient “females” were analyzed for labeled sperm in their spermatheca (Aii, arrow). Levels of transferred sperm were classified as none, weak, or good (see Materials and Methods); *n* = 40. (B) After pronase treatment, isolated *itr-1* RNAi male-derived spermatids develop (C, i–iv) in the same way as *gfp* RNAi male-derived spermatids (D, i–iv).

itr-1 mutant males and to determine if they develop correctly, sperm were isolated and activated in vitro (Ward *et al.*, 1983). The *itr-1* RNAi males that had failed to produce offspring in 12:4 cross situations were removed from the cross plates after 48 h and dissected to release any spermatids in their gonads. Many hundreds of spermatids were released upon dissection and the numbers were similar to *him-8* (*e1489*) males that had been maintained in the absence of hermaphrodites. Spermatids from *itr-1* RNAi males (Figure 3Ci) and those from control RNAi males (Figure 3Di) are morphologically identical, undergo spermiogenesis upon pronase treatment at similar rates, and develop into morphologically identical spermatozoa (Figure 3C, ii–iv, and D, ii–iv). This demonstrates that *itr-1* mutants contain high levels of sperm that are capable of being activated and therefore are likely to be functional gametes. Together with the low sperm transfer data, this all points to a role for *itr-1* in either sperm transfer or in the signal that triggers transfer. Thus *itr-1* function is required for turning, spicule insertion, and sperm transfer.

Phospholipase-C β Functions in Male Fertility

IP₃ production is triggered by the rapid hydrolysis of PIP₂ by PLC isozymes. In *C. elegans* six PLC-like genes have been

identified in the genome (see Supplementary Data). They correspond to vertebrate PLC- β (Lackner *et al.*, 1999; Miller *et al.*, 1999), PLC- δ , PLC- γ (Yin *et al.*, 2004), PLC- ϵ , PLC-like (Shibatohge *et al.*, 1998), and an unusual protein that is most like PLC- β and is referred to as β -like. To determine which PLCs function upstream of the IP₃R in the regulation of male-specific behaviors, we used RNAi to assay their contribution to male fertility. Using RNAi allowed us to test all six genes (most have no known mutants) and to avoid the locomotion defects associated with *egl-8* (PLC- β) mutants. Males treated with gene-specific dsRNA were tested in the 12:4 mating assay for fertility (Table 1). *egl-8* RNAi males showed a dramatic loss of fertility, whereas none of the remaining five PLC gene knockdowns produced fertility defects. This implicates *egl-8* as a possible upstream component of one or more of the mating functions that have been identified for *itr-1*.

Members of the *plc* Gene Family Are Expressed in Male-specific Structures

The expression pattern of the six PLC genes was determined in males by establishing transgenic *him-8* (*e1489*) animals carrying green fluorescent protein (GFP) reporter fusions. The fusions contained up to 4-kb genomic sequence upstream of the start site and *gfp* was fused in frame to the PLC gene (see Materials and Methods for details). *egl-8* is expressed in the vas deferens, spicule protractor muscles, diagonal muscles and a male-specific neuron that is probably CP8 or CP9 (Figure 4A, i and ii) and is also widely expressed in the nervous system, as expected from previous work (Lackner *et al.*, 1999; Miller *et al.*, 1999). *plc-3* (encoding PLC- γ) is expressed in the vas deferens and in the valve cell that separates the seminal vesicle and vas deferens (Figure 4Aiii). *plc-4* (encoding PLC- δ) is expressed in the vas deferens (Figure 4Aiv). GFP reporters for *plc-1*, *plc-2*, and *pll-1* are not expressed in male-specific structures. Thus three different PLC subtypes are expressed in the somatic gonad of the male, suggesting that complex signaling pathways may exist in the vas deferens; however, in the context of male fertility it seems that only *egl-8* is essential.

egl-8 Functions in the Regulation of Spicule Insertion

egl-8 is an important regulator of neuronal and muscular function in *C. elegans* (Lackner *et al.*, 1999; Miller *et al.*, 1999). *egl-8* mutants are defective in locomotion, making them unsuitable for studies investigating the contribution of *egl-8* to male fertility. We therefore used RNAi to knock down *egl-8* expression. *egl-8* males have previously been reported to have defects in male tail development (Chisholm, personal communication); however, we did not see this phenotype after RNAi. The *egl-8* RNAi males developed at a rate similar to that of control animals with normal locomotion. To assess the extent to which *egl-8* RNAi was knocking down *egl-8* expression in males, we performed RNAi on the *egl-8::gfp* fusion-carrying worms. We found that expression was knocked down in all male-specific muscles except the diagonal muscles, whereas expression in the nervous system was unaffected (unpublished data). *egl-8* functions in locomotion through its role in regulating synaptic transmission in the ventral nerve cord, so the lack of knockdown in the nervous system explains why the *egl-8* RNAi males are fully coordinated.

To identify the fertility defect associated with *egl-8* RNAi males, we performed behavioral assays on them (Figure 4B). Contact, turning, backing, vulva location, and spicule prodding were all performed at levels similar to that of the control animals. The percentage of animals that successfully

Figure 4. The roles of *C. elegans* phospholipase C genes in male fertility. (A) Expression patterns of the phospholipase C family in males were determined using GFP reporter constructs. *egl-8::gfp* (A, i and ii) is expressed in the diagonal muscles, spicule retractors, and vas deferens and a male-specific neuron; *plc-3::gfp* (Aiii), is expressed in the valve cell and in the vas deferens, and *plc-4::gfp* (Aiv) is expressed in the vas deferens. *plc-1*, *plc-2*, and *pll-1* reporters were not expressed in male-specific structures. (B) The role of *egl-8* in male mating behavior was assessed as for Figure 1B; *n* = 20 for each treatment. (C) Turning ability of *egl-8* RNAi males was scored as for Figure 1C.

inserted their spicules during the timed period was, however, reduced from 35 to 15% (although this is not statistically significant, *p* > 0.05). This level of reduction is similar to that observed on *itr-1* RNAi, although the absolute levels for the control were lower in the *egl-8* experiments, presumably reflecting environmental variability. This indicates that *egl-8* may function upstream of the IP₃ signal, which we have shown to be involved in regulating spicule insertion.

Table 2. *itr-1* and *egl-8* function in levamisole-induced spicule protraction

RNAi	EC ₅₀
None	320 nM
<i>gfp</i>	520 nM
<i>itr-1</i>	>1 mM
<i>egl-8</i>	3.4 μM

him-8 (*e1489*) animals were subjected to RNAi by feeding, and then male offspring were tested for spicule protraction in six concentrations of levamisole ranging from 100 nM to 1 mM.

Given that *egl-8* is the only *plc* expressed in the diagonal muscles and that *itr-1* seems to play a role in male turning, we scored *egl-8* knockdown males for their turning ability (Figure 4C). We found that *egl-8* knockdown males are capable of performing correct turns at the same frequency as the controls (*p* > 0.05). However, because *egl-8* expression may not be effectively knocked down in these muscles and the nervous system that controls them, it is not possible to discount *egl-8* from a function in turning.

itr-1 and *egl-8* Function in Levamisole-induced Spicule Protraction

Spicule protraction is dependent on acetylcholine (ACh)-mediated signaling, and *egl-30* (encoding Gα_q, which would be predicted to act upstream of *egl-8* and *itr-1*) is also required for this process (Garcia *et al.*, 2001). We therefore tested whether, like *egl-30*, *itr-1* and *egl-8* are required for the induction of spicule protraction by the nicotinic ACh receptor agonist levamisole. As Table 2 shows, knockdown of *itr-1* results in a very substantial increase in the EC₅₀ of levamisole, whereas knockdown of *egl-8* results in a more subtle, but nevertheless more than sixfold increase in EC₅₀. Thus both *itr-1* and *egl-8* function downstream of nicotinic ACh receptors in spicule protraction.

egl-8 RNAi Males Produce Active Sperm but Fail to Transfer Sperm to Hermaphrodites

As for *itr-1*, the observed reduction in mating efficiency does not explain the almost complete sterility of *egl-8* RNAi animals so we investigated the effect on sperm transfer (Figure 5A). *egl-8* RNAi males were also defective for sperm transfer (*p* < 0.001); of 31 recipient hermaphrodites tested only 1 contained a single transferred sperm. We found that *egl-8* RNAi males produced normal numbers of spermatids, which were capable of undergoing spermiogenesis upon pronase treatment (Figure 5B, i–iii) in a manner that was identical to that observed for control RNAi males (Figure 5C, i–iii). These results demonstrate that *egl-8* RNAi animals produce apparently normal sperm, and in support of this, both *egl-8* mutant and *egl-8* RNAi hermaphrodites produce fertilized eggs, demonstrating that hermaphrodites at least produce active sperm. Thus knock down of either *egl-8* or *itr-1* results in almost complete loss of sperm transfer, and furthermore, both genes are expressed in the vas deferens. These results implicate *egl-8* as an upstream component in the *itr-1*-mediated pathway that regulates sperm transfer, and it seems plausible that the vas deferens is the site of function.

egl-8 Functions in Male Fertility through the Production of an IP₃ Signal

Because PLC-β catalyzes the production of two second messengers, IP₃ and diacyl glycerol, we wished to demonstrate

Figure 5. *egl-8* mutant males are defective in sperm transfer, but produce wild-type sperm. (A) After SYTO 17 labeling, *egl-8* RNAi males and control RNAi males were assessed for sperm transfer as for Figure 3B; $n = 31$. (B and C) Sperm were isolated from *gfp* and *itr-1* RNAi males and activated in vitro by treatment with pronase. *itr-1* RNAi male-derived spermatids developed (B, i–iii) in the same way as *gfp* RNAi male-derived spermatid (C, i–iii).

that it is via the generation of an IP_3 signal that *egl-8* regulates sperm transfer. To this end, we investigated whether *itr-1* (*sy290*), a gain-of-function allele (Clandinin *et al.*, 1998), could rescue the sterility resulting from *egl-8* RNAi. *itr-1* (*sy290*) has a mutation, R582Q, in the IP_3 binding site (Clandinin *et al.*, 1998), which results in a twofold increase in IP_3 binding affinity (Walker *et al.*, 2002). In the strain PS2582 (Clandinin *et al.*, 1998) the *itr-1* (*sy290*) mutation is quite closely linked to an *unc-24* (*e138*) mutation, resulting in a locomotion (weak kinker) phenotype, which would presumably itself interfere with mating efficiency. Therefore, having first crossed PS2582 with *him-5* (*e1467*) animals, we rescued the *unc-24* deficiency by transgenic expression of a genomic fragment containing the wild-type *unc-24* gene under the control of its own promoter (see *Materials and Methods*). As a control, we rescued *unc-24* (*e138*) *him-5* (*e1467*) animals in

the same way. We then examined the effects of *egl-8* RNAi on male fertility by performing 12:4 crosses with *fer-1* (*hc1*) hermaphrodites. As Table 3 shows, *egl-8* RNAi results in a dramatic loss of fertility in *unc-24* (*e138*) *him-5* (*e1467*) *jwEx101* [*unc-24* (+), *mec-7::gfp*] males, in agreement with the results for *him-5* (*e1467*) males. *egl-8* RNAi of *itr-1* (*sy290*) *unc-24* (*e138*) *him-5* (*e1467*) *jwEx101* [*unc-24* (+), *mec-7::gfp*] males failed to disrupt male fertility to the same extent, with 66.6% of crosses showing some degree of fertility (compared with 8.3% for *unc-24* (*e138*) *him-5* (*e1467*) *jwEx101* [*unc-24* (+), *mec-7::gfp*] controls). Thus an *itr-1* mutation that increases the affinity for IP_3 partially rescues the male infertility that results from knockdown of *egl-8*.

DISCUSSION

itr-1 and *egl-8* Function in Male Fertility

Male mating is a complex behavior comprising a series of steps that require correct processing of information by the nervous system and appropriate changes in physiology of the target organs. We have shown that the IP_3 R is essential for the coordination of these events in *C. elegans* and that in the absence of IP_3 R function males are sterile. We have demonstrated that the IP_3 R functions in distinct events during male mating, each of which are required to varying degrees to enable a male to mate in the correct manner. Knockdown of *egl-8*, that encodes the *C. elegans* PLC- β , also results in male sterility, which can be partially rescued by a gain of function mutation in *itr-1*. This study demonstrates the versatility of signaling through the IP_3 R and reveals insight into how complex behaviors such as male mating are regulated.

egl-8 and *itr-1* Function in Spicule Insertion

Spicule insertion occurs once the cloaca makes contact with the hermaphrodite vulva. Initially the spicule protractors contract and relax repeatedly, causing the spicules to prod the vulva. When the spicules penetrate the vulva, the protractors shorten while the retractor muscles lengthen, allowing the spicules to extend fully into the vulva (Garcia *et al.*, 2001). Knockdown of either *itr-1* or *egl-8* resulted in a substantial reduction in spicule insertion. *itr-1* is expressed in the spicule protractor and retractor muscles, whereas *egl-8* seems only to be expressed in the protractors.

Garcia *et al.* (2001) have shown that the PCB, PCC, and SPC neurons induce protraction through the secretion of ACh. ACh receptors transduce this signal to either the ryanodine receptor (UNC-68), to promote prodding via peri-

Table 3. An *itr-1* gain-of-function mutation can partially rescue the male fertility defect resulting from *egl-8* knockdown

Males crossed with *fer-1* (*hc1*) “females”

Strain	RNAi	% Crosses fertile	% Crosses > 100 offspring	n
<i>him-5</i> (<i>e1467</i>)	<i>gfp</i>	100	100	16
<i>him-5</i> (<i>e1467</i>)	<i>egl-8</i>	21.4	7.1	14
<i>unc-24</i> (<i>e138</i>) <i>him-5</i> (<i>e1467</i>) <i>jwEx101</i> [<i>unc-24</i> (+), <i>mec-7::gfp</i>]	<i>gfp</i>	100	100	12
<i>unc-24</i> (<i>e138</i>) <i>him-5</i> (<i>e1467</i>) <i>jwEx101</i> [<i>unc-24</i> (+), <i>mec-7::gfp</i>]	<i>egl-8</i>	8.3	0	12
<i>itr-1</i> (<i>sy290</i>) <i>unc-24</i> (<i>e138</i>) <i>him-5</i> (<i>e1467</i>) <i>jwEx101</i> [<i>unc-24</i> (+), <i>mec-7::gfp</i>]	<i>gfp</i>	100	90.9	11
<i>itr-1</i> (<i>sy290</i>) <i>unc-24</i> (<i>e138</i>) <i>him-5</i> (<i>e1467</i>) <i>jwEx101</i> [<i>unc-24</i> (+), <i>mec-7::gfp</i>]	<i>egl-8</i>	66.6	8.3	12

12:4 male to *fer-1* (*hc1*) “female” crosses. A cross was termed fertile if it produced more offspring than the highest *fer-1* (*hc1*) only mock cross.

odic protractor muscle contraction through intracellular Ca²⁺ release, or to EGL-19, an L-type voltage-gated Ca²⁺ channel α_1 subunit, to promote prolonged protractor muscle contractions through Ca²⁺ influx. EGL-30, the *C. elegans* G α_q protein, is implicated in the signal pathway downstream of the ACh response in the protractor muscles. ACh released from the PCB, PCC, and SPC sensory neurons induces muscle contraction through the activation of a number of ACh receptors. Garcia *et al.* (2001) have shown that agonists for both muscarinic and nicotinic ACh receptors activate multiple pathways in the protractor muscles and that mutations in *egl-30* alter the muscle response to these agonists. These pathways differentially regulate *unc-68* and *egl-19* function and hence alter spicule prodding and prolonged spicule protraction differentially. EGL-30, like its vertebrate homologues, can stimulate PLC activity (Brundage *et al.*, 1996), and *egl-8* (PLC- β) is known to function downstream of *egl-30* in other muscle types (Lackner *et al.*, 1999; Bastiani *et al.*, 2003). It therefore seems likely that ITR-1 is involved in the Ca²⁺ signals required for protractor muscle contraction and that EGL-8, by catalyzing the production of IP₃, functions to couple EGL-30 activation with this Ca²⁺ release. This is supported by our observation that, like *egl-30*-deficient animals (Garcia *et al.*, 2001), animals in which *itr-1* or *egl-8* have been knocked down are defective in spicule protraction induced by the nicotinic ACh receptor agonist levamisole.

The expression of *itr-1* in the gubernaculum retractors suggests that *itr-1* could also function in the initial movement of the spicules. Before insertion, the gubernaculum is thought to be responsible for moving the spicules to a more transverse position (Sulston *et al.*, 1980). Therefore even if the spicule protractors are capable of inserting the spicules, they may be unable to if the spicules have not have been positioned correctly by the gubernaculum. Thus *itr-1* may play multiple roles in spicule insertion; however, in this case activation of the IP₃R may not be mediated by *egl-8*, because it does not appear to be expressed in the gubernaculum.

EGL-8 Regulates Sperm Transfer through Activation of IP₃Rs

We have demonstrated that disruption of either *itr-1* or *egl-8* results in a dramatic defect in sperm transfer. Because the defects in mating behavior observed would be expected to have only very subtle effects on the results of 12:4 crosses, and because the spermatids produced appear to be normal, the defect in sperm transfer is likely to be the dominant factor contributing to the infertility observed. We have also demonstrated that an *itr-1* mutation that increases affinity for IP₃ can partially rescue the sterility effect of knocking down *egl-8* expression. This suggests that EGL-8 (PLC- β), which is predicted to catalyze the production of IP₃, functions upstream of the IP₃R in the control of sperm transfer. Although there is evidence that diacyl glycerol can act as an effector for EGL-8 (Lackner *et al.*, 1999) and that ITR-1 can act downstream of PLC-3 (Yin *et al.*, 2004), this is the first demonstration that ITR-1 can also act as an effector for EGL-8.

We propose that the site of action for this IP₃-mediated pathway is the smooth muscles of the somatic gonad. More precisely, expression data for both *itr-1* and *egl-8* indicate that the sites of function are the vas deferens, seminal vesicle, and the valve that separates the two. The vas deferens has not been fully characterized but it is a complex structure of 30 cells, many of which appear to be secretory. After spicule insertion, in which the SPC motor neurons function by inducing the switch from periodic to prolonged spicule protraction (Garcia *et al.*, 2001), a signal originating from the

hermaphrodite uterus is required to induce the valve between the vas deferens and seminal vesicle to open, releasing spermatids (reviewed by Emmons and Sternberg, 1997). The SPV sensory neurons appear to inhibit transfer, so the signal presumably acts to inhibit the action of SPV. The SPD sensory neurons, on the other hand, play a positive role, perhaps to signal that spicule insertion has occurred (Liu and Sternberg, 1995). The male wiring project (www.wormatlas.org.) has recently shown that the SPCs, as well as the PCB and PCC sensory-motor neurons, innervate the gonad. It is interesting in this respect that all three of these cholinergic neurons also function in spicule insertion (Garcia *et al.*, 2001), making them prime candidates for a role in the coordination of spicule insertion and ejaculation. The vas deferens of higher eukaryotes convey sperm from the testis to the exterior and are composed of smooth muscle. The smooth muscle of the rat vas deferens has a complex endomembrane system that contains high levels of IP₃Rs (Villa *et al.*, 1993), which appear to function both in the modulation of Ca²⁺ sparks, and as a leak pathway to limit the Ca²⁺ content of stores (White and McGeown, 2003). ITR-1 may play similar functions in regulating the contraction of the vas deferens through the activation of EGL-8, but it is also possible that they play a role in the upstream neuronal signals that trigger contraction.

Two of the three N-terminal ITR-1 variants are expressed in the vas deferens, this is under the control of promoter pB and pC. Given that the spermatheca and the vas deferens are the only two tissues to express these same two N-terminal ITR-1 variants, it is interesting to note the similarity in structure between the two. Rhodamine-phalloidin staining of the spermatheca reveals circumferentially arranged actin microfilaments (McCarter *et al.*, 1997) that undergo peristaltic constrictive and dilatory behavior like epithelial smooth muscle (McCarter *et al.*, 1999). *itr-1* functions downstream of *let-23* (encoding an EGF receptor) in the regulation of spermathecal dilations (Clandinin *et al.*, 1998). Given that two ITR-1 variants are coexpressed in the spermatheca it would seem likely that their combined effect is required during spermathecal dilation, and the same combination is presumably required for sperm transfer in the vas deferens. It will be very interesting to investigate whether *itr-1* has parallel functions in the spermatheca and vas deferens. Until the structure of the vas deferens is further characterized, this question will be difficult to answer, although genetic analysis of males using genes known to function in the spermathecal pathway may provide some information.

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