**unc-68 Encodes a Ryanodine Receptor Involved in Regulating C. elegans Body-Wall Muscle Contraction**

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*Abstract.* Striated muscle contraction is elicited by the release of stored calcium ions through ryanodine receptor channels in the sarcoplasmic reticulum. ryr-1 is a *C. elegans* ryanodine receptor homologue that is expressed in body-wall muscle cells used for locomotion. Using genetic methods, we show that ryr-1 is the previously identified locus unc-68. First, transposon-induced deletions within ryr-1 are alleles of unc-68. Second, transformation of unc-68 mutants with ryr-1 genomic DNA results in rescue of the Unc phenotype. *unc-68* mutants move poorly, exhibiting an incomplete flaccid paralysis, yet have normal muscle ultrastructure. The mutants are insensitive to the paralytic effects of ryanodine, and lack detectable ryanodine-binding activity. The Unc-68 phenotype suggests that ryanodine receptors are not essential for excitation-contraction coupling in nematodes, but act to amplify a (calcium) signal that is sufficient for contraction.

Intracellular calcium (Ca\(^{2+}\)) channels play a key role in regulating muscle contraction. During excitation-contraction coupling (E-C coupling) in striated muscle, two classes of Ca\(^{2+}\) channels act sequentially to cause the release of Ca\(^{2+}\) ions stored within the sarcoplasmic reticulum (SR)\(^1\) (Caterall, 1991). Cholinergic excitation opens voltage-gated Ca\(^{2+}\) channels in the plasma membrane, which in turn cause voltage-insensitive channels in the SR to flood the cell with Ca\(^{2+}\) ions, thus eliciting sarcocmere contraction (Coronado et al., 1994; McPherson and Campbell, 1993a; Meissner, 1994). These high conductance calcium release channels in the SR are termed ryanodine receptors (RyRs) because of their specific, high affinity interaction with the plant alkaloid ryanodine (Fill and Coronado, 1988). Ryanodine locks the channels open in a submaximal conductance state, causing a steady leak of Ca\(^{2+}\) into the myoplasm and inducing hypercontractive paralysis in skeletal muscle fibers (Fill and Coronado, 1988).

Different striated muscle types express different RyR isoforms, and the mechanism of E-C coupling varies in these tissues. In cardiac muscle, external Ca\(^{2+}\) admitted through voltage-gated channels in the plasma membrane triggers Ca\(^{2+}\) release from the SR by binding to regulatory sites on RyRs. This mechanism is called "calcium-induced calcium release" (CICR) (Fabiato, 1983). In skeletal muscle, the voltage-gated channels and RyRs appear to be mechanically coupled. A conformational change in the plasma membrane channels associated with gating-charge movement directly evokes Ca\(^{2+}\) release from RyRs by protein-protein interaction (Rios et al., 1991; Yano et al., 1995). In some vertebrate skeletal muscle more than one RyR isoform (and both E-C coupling mechanisms) are present in the same cells (Airey et al., 1990; Giannini et al., 1995; Jacquemond et al., 1991; O'Brien et al., 1995; Oyamada et al., 1994).

RyRs belong to a family of intracellular Ca\(^{2+}\) channels that includes RyRs and inositol trisphosphate receptors (Berridge, 1993). Functional RyR channels are homotetramers composed of subunits of ~565 kD. The channel pore resides in the carboxyl 10–20% of the molecule (Callaway et al., 1994; Meissner et al., 1989), while the bulk of the protein extends into the cytoplasm from the SR membrane (Marty et al., 1994; Radermacher et al., 1994). RyRs have been cloned from a variety of vertebrate and invertebrate species, and are found in a number of tissues in addition to muscle (Giannini et al., 1995; Hakamata et al., 1992; Otsu et al., 1990; Oyamada et al., 1994; Takeshima et al., 1994a, 1989). Sequence analysis of the cloned genes (each about 5,000 amino acids) shows extensive sequence and structural homology, particularly in predicted transmembrane-spanning domains in the carboxyl ends of the proteins (Coronado et al., 1994; McPherson and Campbell, 1993a; Meissner, 1994).

We are investigating the role of RyRs in E-C coupling in *C. elegans* striated muscle. Nematodes have relatively simple body-wall musculature used for locomotion, consisting of rows of longitudinal striated muscle cells on dorsal and ventral sides of the animal (Wood, 1988). Opposing dorsal and ventral muscle cells are coordinately contracted and relaxed, producing sinusoidal waves that propel the animal.

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1. Abbreviations used in this paper: E-C coupling, excitation-contraction coupling; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

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forward or backward (Nicholas, 1984). Electrophysiologi-
ical experiments in Ascaris lumbricoides have established
that the contractile state of body-wall muscle is controlled
by membrane potential (Weisblat et al., 1976; Johnson
and Stretton, 1980), which itself is controlled by excitatory
and inhibitory inputs from motor neurons (Walrond et al.,
1985). Excitatory cholinergic and inhibitory GABAergic
neuromuscular synapses have been characterized in As-
caris and C. elegans (Johnson and Stretton, 1980; Lewis
et al., 1987; McIntire et al., 1993), but nothing is known
about E-C coupling mechanisms that directly control cyto-
plasmic Ca\textsuperscript{2+} levels (and therefore contraction) in nema-
todes. The location and nature of the nematode SR is not
certain; it is assumed to consist of vesicular structures that
surround dense bodies, the attachment sites for thin fil-
aments in nematode sarcosomes (Nicholas, 1984; Wood, 1988).

C. elegans is known to have RyRs. We characterized a
[3H]ryanodine-binding activity in C. elegans membranes
that copurifies with high conductance ion channels. The
channels are cation and Ca\textsuperscript{2+} selective, and are gated by
ryanodine (Kim et al., 1992). Addition of ryanodine to the
growth medium induces an incomplete hypercontractive
paralysis of C. elegans, suggesting an effect on body-wall
muscle (Kim et al., 1992). Sakube and Kagawa have
cloned, sequenced, and characterized a RyR gene in C. el-
egans. This gene, called ryr-1, is predicted to encode a pro-
tein of 5,017 amino acids that is ~40% identical to cloned
RyRs from vertebrates. Ten membrane-spanning domains
are predicted in the carboxyl end of ryr-1, the region of
greatest homology to mammalian RyRs (Sakube et al.,
1993). ryr-1 promoter-β-galactosidase fusion constructs
are expressed in adult body-wall muscle and pharyngeal
muscle cells (Kagawa, H., personal communication). To
better understand the role of RyRs in E-C coupling in nematodes, we undertook a genetic analysis of ryr-1 in C. elegans.

Materials and Methods

Strains and Handling of C. elegans

Handling of C. elegans was done using established methods (Wood, 1988). The strains and relevant mutations used were N2 (wild type), MT3126
(mut-2(r459)), BC1283 (stf50), BC1285 (ndf23), BC1999 (ndf18), unc
68 (e540), unc-70 (e524), and dpy-11 (e224). unc-68 alleles isolated in this
study were r1151:Tcl, r1152:Tcl, r1158, r1160, r1161, r1162, r1167, r1207,
1208, 1209, 1210, 1211, 1212, 1213, and r122. The strain TR2267 is ho-
mozygous for r1161, and contains a stable extrachromosomal array
(rEx95) containing wild-type ryr-1 sequences (see below).

Molecular Methods

Preparation of nematode genomic DNA was as previously described (Rushforth et al., 1993). Standard methods were used for preparation of
probes for DNA sequencing, and for hybridization analysis. Screening for
Tcl insertions and deletions was done as described (Rushforth et al., 1993; Zwaal et al., 1993) with some modifications. Populations founded with
about 30 MT3126 animals were grown until F2 generation eggs were being
laid, and then screened in two-dimensional matrices. Each population was
divided into four parts: two parts were used for the matrix, one part was
saved for subdivision, and one part was saved to verify a suspected inser-
tion or deletion. For example, each of 49 populations were assigned a row
and a column in a 7 × 7 matrix. Worms from seven populations were
pooled for each row or column. DNA prepared from pooled animals in
rows and columns was screened with PCR in duplicate. A population hav-
ing the same polymorphism in both its row and column was further tested
by PCR with a third portion of the animals. If positive, the remaining ani-
mals were subdivided into smaller populations and rescreened to ulti-
mately isolate individual animals with insertion or deletion. Single animal
PCR was done as described (Williams et al., 1992).

Wild-type ryr-1 sequences used for transformation rescue were ob-
tained by amplifying three 10.3–10.7-kb fragments from N2 genomic DNA
with the Expand” long template PCR system (Boehringer Mannheim
Corp., Indianapolis, IN). The three ryr-1 primer pair sequence coordi-
nates (5' base and length of primer) are 1,520 (32-mer, sense direction)
and 11,870 (32-mer, antisense); 11,440 (39-mer, sense), and 21,770 (35-
mer, antisense); 21,361 (25-mer, sense) and 32,145 (35-mer, antisense).
The PCR fragments were purified on spin columns, mixed 1:25 with linear
DNA fragments (BRL-1 kb ladder), and injected into unc-68(e1161) adult
hermaphrodites as described (Mello and Fire, 1995) at a total DNA con-
centration of 100 μg/ml. Transformsants were identified by screening the
F2 progeny of injected (Unc) animals for animals with wild-type motility.

Isolation of unc-68(r1158)

unc-68 (e1158) was obtained by screening populations of r1151::Tcl with
flanking primers as described (Zwaal et al., 1993). A 5.4-kb deletion of
ryr-1, contained in strain TR2125, was identified in this screen. This
deletion, r1158, removes bps 24,530 to 29,902 of ryr-1. TR2125 is phenotypi-
cally wild type, but proved to contain a duplication of ryr-1. In one copy of
ryr-1, TR2125 is homozygous for r1158. In a second copy, TR2125 is ho-
mozygous for r1151::Tcl. Both the deletion-containing and insertion-contain-
ing alleles of ryr-1 are tightly linked to unc-70 (data not shown, see
Fig. 1). During these tests of linkage, LGV from TR2125 was placed in
trans to unc-70(e524). From one such heterozygote, animals having an
Unc phenotype distinct from that of unc-70 segregated unexpectedly.
Southern analysis and PCR tests demonstrated that these novel Unc ani-
mals (strain TR2126) were homozygous for r1158 but no longer contained
r1151::Tcl. We have not characterized further the duplication/deletion
arrangement of TR2125, but we presume that the simple deletion r1158 in
TR2128 represents resolution of the duplication via homologous recombi-
nation.

Noncomplementation Screens

In the screen for new spontaneous alleles, wild-type males were crossed to
r1151::Tcl or r1152::Tcl hermaphrodites. The resulting ryr-1::Tcl/+ males
were then crossed to dpy-11(e222) unc-68(e1158) homozygotes. The F1
progeny were then screened for Unc non-Dpy hermaphrodites. Putative
new unc-68 alleles were outcrossed, tested for complementation with unc
68 (e540), and checked for the absence of the r1158 deletion with PCR.
The new alleles were characterized with PCR and Southern analysis. In the
screen for EMS-induced alleles, wild-type males were mutagenized with
EMS (Anderson, 1995) and crossed with dpy-11(e224) unc-68(e1158) her-

maphrodites as above. Putative new EMS-induced alleles were out-
crossed, tested for complementation with unc-68 (e540), and checked for
the absence of the r1158 deletion with PCR. Six new alleles were desig-
nated r1207-r1212.

Binding Assays and Membrane Purification

Whole C. elegans were collected, floated on 35% sucrose (Wood, 1988)
and washed in H2O. Washed worms were resuspended in 20 mM MOPS,
pH 7.2, in a protease inhibitor cocktail previously described (Kim et al.,
1992). Worms were homogenized by passing twice through a french pres-
sure cell at 10,000 psi. To purify the microsomal fraction, the crude homo-
genates were spun at 2,400 g for 30 min in corex tubes. The supernatant
was layered on an equal volume of 30% sucrose (in 0.4 M KC1, 20 mM
MOPS, pH 7.2) and spun at 90,000 g for 45 min. The pellets were
resuspended in 1.0 M KC1, 20 mM Tris, pH 8.0, plus 4.3. Binding assays
were done in resuspension buffer at 37°C with indicated concentrations of
[3H]ryanodine (Dupont-New England Nuclear, Boston, MA). Assays
were in triplicate, with 70 μg microsomal protein per sample. Bound
[3H]ryanodine was collected on glass fiber filters and counted in scintilla-
tion cocktail as described (Kim et al., 1992). Specific counts for each ryan-
odine concentration were determined by subtracting counts bound in the
presence of 100 μM unlabeled ryanodine (Agrisystems International,
Wind Gap PA) from counts in the presence of labeled ryanodine only.

Electron Microscopy

Animals were fixed for 1 h on ice in a solution composed of 0.67% fresh
glutaraldehyde and 0.67% osmium tetroxide in 0.1 M cacodylate buffer,
pH 7.4 (C buffer). The heads were cut off, the animals washed three times in C buffer, and then incubated at 4°C overnight in 2% osmium tetroxide in C buffer. Animals were washed again, dehydrated, and embedded in Spurr's resin. Adult hermaphrodites were sectioned perpendicular to the long axis of the body posterior to the terminal bulb of the pharynx. The sections were viewed in a JEOL (Peabody, MA) 100 electron microscope at a magnification 19,000.

**Results**

**Genetic and Physical Location of ryr-1**

A *C. elegans* ryanodine receptor homologue cDNA (cm16C2) was identified in a screen of expressed genes (Waterston et al., 1992). The sequence of cm16C2, a 1,232-bp partial cDNA of *ryr-1*, is homologous over its entire length to several mammalian ryanodine receptors in the databases (~30% identical). cm16C2 hybridized to three overlapping YACs on linkage group V (see Fig. 1). These YACs span the interval between *snb-1* and *odr-2*, two genes whose positions are known on both the physical and genetic maps (see Fig. 1). We located cm16C2 more precisely on the genetic map using several overlapping deficiencies affecting this region. Deficiency homozygote embryos were tested with PCR for cm16C2 sequences, cm16C2 hybridized to three overlapping YACs on linkage group V (see Fig. 1). These YACs are deleted in *nDf32* and *sDf20*, and eight complementation groups defined by recessive lethal mutations (Johnson and Baillie, 1991).

**Site-selected Tc1 Insertions within ryr-1**

Y. Sakube and H. Kagawa independently identified *ryr-1*, and determined the complete sequence of both genomic and cDNA clones. *ryr-1* is composed of 46 exons spanning over 30-kb of genomic DNA (see Fig. 2). With genomic sequence information generously provided by Sakube and Kagawa, we screened for animals having Tcl transposable element insertions within the 3’ third of *ryr-1*. Using a PCR/sib-selection method previously described (Rushforth et al., 1993), two site-selected Tcl insertions were obtained (see Fig. 2). Sequencing of PCR fragments spanning the insertion junctions revealed that *r1151* contained an insertion within intron 37 at TA dinucleotide 27,147–27,148 (coordinates of Sakube et al., Genbank accession number D54899) and *r1152* contained an insertion within the 5’ splice donor of intron 43 (TA dinucleotide 29,618–29,619). Southern hybridization of DNA from insertion homozygotes confirmed the size and location of the Tcl insertions in *r1151* and *r1152* (Fig. 3, lanes 2 and 9, respectively). Both *r1151* and *r1152* homozygotes are wild-type in phenotype.

**Isolation of ryr-1 Deletions**

Because Tcl sequences are spliced from most, if not all Tcl-containing pre-mRNAs (Rushforth and Anderson, 1996), we suspected that the wild-type phenotypes of *r1151* and *r1152* did not represent the *ryr-1* null phenotype. Using previously described methods (Zwaal et al., 1993), we isolated *r1158*, an excision derivative of *r1151*: Tcl, in which 5.4 kb of *ryr-1* are deleted (see Fig. 2 and 3, lane 3). Sequencing of the *r1158* deletion junction (generated by PCR) demonstrated that *ryr-1* coordinates 24,531 to 29,901 (inclusive) are deleted in *r1158*. *r1158* homozygotes are uncoordinated and exhibit a phenotype that is identical to that of *unc-68(e540)*, the *unc-68* reference allele. Furthermore, *r1158* fails to complement *unc-68(e540)*.

To confirm that the Unc-68 phenotype of *r1158* was caused by the *ryr-1* deletion, a noncomplementation screen for new alleles of *unc-68* was performed, using *r1151::Tcl* and *r1152::Tcl* as a source of new mutations. Our expectation was that imprecise excision of Tcl in a mutator background would yield additional deletion alleles of *ryr-1* at relatively high frequency (Zwaal et al., 1993). If the Unc-68 phenotype of *r1158* were due to a mutation in a gene other than *ryr-1*, then the frequency of new *unc-68* alleles would be very low, or the deletions obtained from Tcl excisions would always extend beyond *ryr-1* and include nearby genes. Six new spontaneous alleles of *unc-68* were isolated from 2,330 *r1151::Tcl* and *r1152::Tcl* chromosomes screened (see Materials and Methods). Three of these alleles (*r1160*, *r1161*, and *r1162*) delete the Tcl element used to generate them as well as 2.4–7.2 kb of adjacent *ryr-1* sequences (see Fig. 2, also see Fig. 3, lanes 4, 6, and 9). Two alleles (*rDf1* and *rDf2*) contain deletions that, based on PCR tests, extend beyond *ryr-1* coding sequences. Both *rDf1* and *rDf2* are lethal as homozygotes.

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**Figure 1.** Physical and genetic maps of LGV surrounding *ryr-1*. The partial *ryr-1* cDNA cm16c2 hybridized to three (bold type) of four overlapping YACs shown in the expanded area. *ryr-1* is ordered on the YAC contig with respect to *snb-1* and *odr-2*, two genes whose location is known on both genetic and physical maps. Cosmids having all or a portion of *snb-1*, *odr-2*, or *ryr-1* are shown with gene names below the contig. *snb-1* and *odr-2* are also ordered on the genetic map above with respect to *dpy-11*, *unc-70*, *unc-68*, and three deficiencies: *nDf18*, *nDf32*, and *sDf30*. The shaded area shows the interval to which the cm16C2 clone mapped. The relative order of *snb-1* and *unc-70* has recently been established (Nonet, M., and E. Jorgensen, personal communication).
rescue of unc-68 with ryr-1 Genomic DNA
determine the nature of its new mutation.

presumably because they delete other genes in addition to
ryr-1. A sixth allele (r1167, see Fig. 3, lane 10) retains the
r1152::TcI insertion. It is being further characterized to
determine the nature of its new mutation.

Rescue of unc-68 with ryr-1 Genomic DNA
As further proof that unc-68 and ryr-1 are synonymous,
ryr-1 genomic DNA was used for transformation rescue of
unc-68 mutants (Mello and Fire, 1995). The cosmid
M04C11 (see Fig. 1) contains the entire ryr-1 coding se-
quence, but M04C11 DNA failed to rescue the Unc-68
phenotype. Y. Sakube and H. Kagawa have shown that
body-wall muscle expression of ryr-1 requires noncoding
upstream regulatory sequences not present in M04C11
(Kagawa, H., personal communication). We therefore
used PCR to amplify ryr-1 and 3 kb of upstream sequence from N2 genomic DNA in three 10.3-10.7-kb fragments,
which together span ryr-1 sequence coordinates 1,520-
32,145 (see Materials and Methods). The central fragment
overlapped the 5' and 3' fragments by 300-350 bp to allow
homologous recombination in vivo (see Mello and Fire,
1995).

The three PCR fragments were injected into unc-
68(r1161) hermaphrodites. F2 progeny of the injected Unc
animals included wild-type hermaphrodites. One such ani-
mal established the strain TR2267, which carries an extra-
chromosomal array (rEx95) that includes unc-68 (+). TR2267
wild-type hermaphrodites segregate wild-type and Unc
progeny. PCR of TR2267 wild-type animals showed that
both the r1161 deletion and wild-type alleles of ryr-1 were
present, while TR2267 Unc animals contained only the
r1161 deletion allele. rEx95 was also able to rescue other
unc-68 alleles, including e540, as shown by crossing wild-
type TR2267 males to unc-68 homozygotes and recovering
wild-type F1 progeny. These experiments demonstrate
that unc-68 and ryr-1 are the same gene. Hereafter, we refer
to this gene simply as unc-68.

unc-68 Null Phenotype
Three lines of evidence indicate that the viable, uncoor-
dinated phenotype of the unc-68 deletion homozygotes rep-
resents the unc-68 null phenotype. First, the four deletions
remove substantial (and differing) portions of the unc-68
coding sequence, yet exhibit the same Unc-68 phenotypes.
In particular, r1162 deletes all of the predicted membra-
ne-spanning domains (Sakube et al., 1993) (see Fig. 2). Since
homologous domains in mammalian RyRs appear to con-
tain the channel pore (Callaway et al., 1994, Meissner et al.,
1989) r1162 is certainly a "channel null" allele, regardless
of whether it is a protein null allele. Second, the deletion
mutations are fully recessive, and their Unc-68 phenotype
is not more severe when any of them is placed in trans
to a large deficiency that removes all of unc-68 (sDf20, see Fig.
1). Third, new alleles of unc-68 that are indistinguishable
from those described above arise at high frequency follow-
ing EMS mutagenesis. From 2871 EMS-mutagenized chro-
mosomes, we isolated six new unc-68 alleles (see Materials
and Methods). Such frequencies of isolation suggest that
the Unc-68 phenotype is due to loss of function alleles
(Anderson, 1995).

Effects of unc-68 Mutations on Muscle Function
Wild-type C. elegans moves by propagating sinusoidal con-
traction waves in strips of striated muscle cells attached to
the cuticle along the body length (Croll, 1975; Nicholas,
1984). Contraction waves of unc-68 homozygotes propa-
gate more slowly than those of wild type. unc-68 mutants
move slowly, and exhibit a languid, incomplete flaccid pa-
ralysis. The animals travel for shorter average distances

Figure 2. TcI site-selected insertions and TcI-induced dele-
tions. Exons 20-46 of ryr-1 showing the location of two
TcI insertions and four dele-
tions. r1158 was isolated from r1151::TcI using the
primer pair shown (see Ma-
terials and Methods). Three
allesles isolated in a noncom-
plementation screen (r1160,
\textit{r1161}, r1162, see Materials
and Methods) are deletions
within ryr-1 that extended
unidirectionally from the TcI
insertion from which they
were derived. r1161 and
r1162 were derived from
r1151, while r1160 was de-

r1151::TcI

r1152::TcI

r1158 5.4 kb

r1160 2.4 kb

r1161 7.2 kb

r1162 3.7 kb

1 kb

I = predicted transmembrane domain

= PCR primers

mated from Southern analysis and from the size of PCR fragments amplified from primers flanking the deletion. Ten predicted trans-
membrane-spanning domains are shown as vertical black bars (Sakube et al., 1993).
than wild type, are immobile for more extended periods of time, and assume unusual extended or curled body postures. In a motility assay performed in liquid media, \textit{unc-68} mutants propagated 25–30\% of the number of contraction waves observed in wild-type animals (see Table I). As shown in Table I, the motility of \textit{unc-68(r1161)} was restored to wild-type level by rescue with the \textit{unc-68(+) array} (see \textit{r1161;rEx95}).

\textit{unc-68} mutants do not exhibit defects of anal depressor or sex muscles, although pharyngeal muscle function may be affected. Defecation is grossly normal (Reiner, D., and J. Thomas, personal communication), and \textit{unc-68} mutants are proficient in egg laying. While \textit{unc-68} males mate poorly, this is most likely a consequence of their impaired motility. Pharyngeal pumping is somewhat weaker in \textit{unc-68} mutants than in wild-type, although electrical activity during pharyngeal muscle contraction is normal in \textit{unc-68} mutants (Lee, R., and L. Avery, personal communication). The mutants grow more slowly than their wild-type counterparts, perhaps due to weaker pumping (feeding) during larval development.

\subsection*{Body-Wall Muscle Ultrastructure Is Normal in \textit{unc-68} Mutants}

The motility defects of \textit{unc-68} mutants could arise from structural abnormalities of the muscle contractile apparatus, or from defective regulation of contraction. We compared wild-type and \textit{unc-68} body-wall muscle using electron microscopy. \textit{unc-68} sarcomeres exhibit nearly wild-type ultrastructure, as seen in transverse sections (see Fig. 4). The distribution of thick and thin filaments, and the number and location of dense bodies and M-lines (Wood, 1988) were essentially normal in \textit{unc-68} animals. We observe two subtle irregularities in \textit{unc-68} body-wall muscles. First, the depth of the spindle region (but not its myofilament organization) is more heterogeneous. Thus, many \textit{unc-68} sarcomeres are deeper than their wild-type counterparts. Second, dense bodies are more often irregular in shape than wild type (e.g., lower left image in Fig. 4). We conclude from this analysis that \textit{unc-68} body-wall muscle ultrastructure is nearly normal, and that motility defects are due to defective regulation of contraction.

\subsection*{\textit{unc-68} Mutants Are Insensitive to Ryanodine}

Contraction of body-wall muscle is impaired, but not eliminated in \textit{unc-68} mutants. Because many species express

\section*{Table I. Motility Assay for Wild-type and \textit{unc-68} Mutant Animals}

| \textbf{unc-68 allele} | W.T. | e540 | r1158 | r1162 | r1161 | r1161;rEx95 |
|------------------------|------|------|-------|-------|-------|-------------|
| \textbf{– Ryanodine}   |      |      |       |       |       |             |
| exp. 1                 | 107 ± 8 | 29 ± 4.6 | 29 ± 5.5 | 27 ± 4.4 | 22 ± 4.0 | 25 ± 4.5 | 103 ± 8.1 |
| exp. 2                 | 114 ± 18 | 28 ± 3.8 | 28 ± 5.0 | 27 ± 4.1 | 22 ± 5.0 | 24 ± 4.4 | 104 ± 8.6 |
| \textbf{+ Ryanodine}   |      |      |       |       |       |             |
| exp. 1                 | 12 ± 1.9 | 30 ± 4.6 | 29 ± 5.5 | 28 ± 7.5 | 23 ± 4.9 | 25 ± 4.7 | 8 ± 1.6  |
| exp. 2                 | 12 ± 2.6 | 28 ± 6.3 | 28 ± 3.5 | 27 ± 5.3 | 23 ± 5.3 | 23 ± 3.7 | 9 ± 1.6  |

The relative motility of wild-type and \textit{unc-68} mutants was measured by counting the waveforms (Croll, 1975) propagated by individual animals in one minute. Each value is the average number of waveforms counted (± SD) in ten animals. Before counting, adult hermaphrodites were placed in 24-well plates in 0.5 ml S-media (Wood, 1988) with or without 2 μM ryanodine. The plates were incubated at room temperature, while shaking, until wild-type animals were fully contracted by ryanodine (at least 30 min). A waveform was defined as the propagation of a contraction wave from head-to-tail, from tail-to-head, or the curling and uncurling of the body. Results from two independent experiments are shown.
Figure 4. Transverse sections of wild-type (N2) and unc-68 body-wall muscle. Wild type or unc-68 (r1162) animals were prepared for EM as described in Materials and Methods. Transverse sections from adult hermaphrodites taken posterior to the terminal bulb of the pharynx are shown. Magnification, 19,000. db, dense bodies; m, M line (Nicholas, 1984; Wood, 1988) unc-68 (r1162) exhibits nearly normal muscle ultrastructure. Bar, 1.0 μM.

two RyR isoforms in the same muscle cell type (Airey et al., 1990; Giannini et al., 1995; O'Brien et al., 1993; Oyamada et al., 1994), we tested unc-68 mutants for sensitivity to contraction by ryanodine, which induces incomplete hypercontractive paralysis of C. elegans (Kim et al., 1992). After exposure to ryanodine, wild-type animals become shorter and thicker, indicating that the body-wall muscles are hypercontracted. Wild-type animals in 2 mM ryanodine move very slowly compared to animals without drug, as shown in a motility assay (see Table 1), and by the length of tracks left in bacterial lawns (Fig. 5). In contrast, unc-68 mutants are completely resistant to ryanodine.
Both the unc-68 deletion mutants described above and the unc-68 reference allele e540 are unaffected by 2 mM ryanodine, a concentration 20 times higher than the minimum required to paralyze wild type (see Table I and Fig. 5). Rescue of unc-68(r161) with the wild-type unc-68 gene restored sensitivity to ryanodine (see r161; rEx95, Table I). These experiments show that unc-68-encoded RyRs alone are responsible for the effects of ryanodine on body-wall muscle function.

unc-68 Mutants Lack Ryanodine-binding Activity

Microsomes prepared from C. elegans homogenates contain a saturable, high affinity [3H]ryanodine-binding activity. Although the amount of binding activity is low in comparison to rabbit skeletal muscle, the binding affinity (Kd 26 nM) is similar to that measured for vertebrate RyRs (Kim et al., 1992). If unc-68 encodes the only RyR in C. elegans (as defined by ryanodine binding), then unc-68 mutants should lack high affinity ryanodine-binding activity. We measured [3H]ryanodine binding in microsomes prepared in parallel from wild-type or unc-68 mutants. One such experiment is shown in Fig. 6. We were unable to detect specific [3H]ryanodine binding in unc-68 microsomes, while those from wild-type animals contained saturable, high affinity [3H]ryanodine-binding sites. These data, together with the ryanodine resistance of unc-68 mutants, suggest that unc-68 encodes the only RyR in C. elegans.

Discussion

We provide five lines of evidence demonstrating that unc-68 encodes a ryanodine receptor (RyR) involved in regulating C. elegans body-wall muscle contraction. First, the cloned RyR gene ryr-1 (Sakube et al., 1993) maps to a genetic interval that includes unc-68, and in-frame deletions of ryr-1 isolated with reverse genetic methods and noncomplementation screens are alleles of unc-68. Second, transformation of unc-68 mutants with ryr-1 sequences fully rescues the Unc-68 phenotype. Third, unc-68 mutants exhibit motility defects (incomplete paralysis), but their body-wall muscle ultrastructure is essentially normal. Fourth, unc-68 mutants are insensitive to the paralyzing effects of ryanodine. Fifth, microsomal fractions of unc-68 mutants are devoid of the high affinity binding activity that characterizes wild-type microsomes.

Sakube and Kagawa have shown that unc-68 is expressed in body-wall muscle cells (Kagawa, H., personal communication). ryr-1 promoter fusions to lacZ are expressed in body-wall muscle cells, and are variably expressed in pharyngeal muscle and in egg-laying muscles. We and others have not observed severe behavioral defects of unc-68 mutants in pharyngeal muscle and in egg-laying muscles. We and others have not observed severe behavioral defects of unc-68 mutants in pharyngeal pumping or egg laying, although the weaker pumping (Lee, R., and L. Avery, personal communication) and slow larval growth (Maryon, E., unpublished results) of unc-68 mutants suggest a role of unc-68 in the regulation of pharyngeal muscle contraction.

Maryon et al. unc-68 Encodes a C. elegans Ryanodine Receptor
RyRs play an important, but nonessential role in E-C coupling in *C. elegans* body-wall muscle. *unc-68* appears to encode the only RyR expressed in *C. elegans*, since *unc-68* mutants are resistant to the paralytic effects of ryanodine and lack detectable *[^3H]ryanodine*-binding activity. Based on these observations, RyRs are not necessary for contraction of body-wall muscle because *unc-68* mutants propagate attenuated sinusoidal contraction waves up or down the body length (see Table I and Fig. 5). Furthermore, in wild-type animals, ryanodine induces an incomplete paralysis. At high drug concentration, the hypercontracted animals still move with slow, sinusoidal motion, as shown by the tracks left in bacterial lawns (see Fig. 5). Thus, coordinated contraction and relaxation of body-wall muscle cells still occur with either a (presumed) deficit of RyR-supplied Ca\(^{2+}\) in *unc-68* mutants or an excess of Ca\(^{2+}\) in the presence of ryanodine. Wild-type RyR function greatly enhances motility (see Table I and Fig. 5), but is not necessary for E-C coupling per se.

The RyR-independent contraction observed in *unc-68* body-wall muscle cells could be initiated by Ca\(^{2+}\) from an external or internal source. In many invertebrates, including nematodes, muscle plasma membrane depolarization emanates from neuromuscular junctions as a graded Ca\(^{2+}\) spike (Hagiwara and Naka, 1964; Weisblat et al., 1976). In *C. elegans*, Ca\(^{2+}\) entering through plasma membrane voltage-gated channels might suffice to initiate contraction, unlike the modest Ca\(^{2+}\) flux through analogous channels in cardiac myocytes (Varro et al., 1993). The small size of body-wall muscle cells and close proximity of sarcomeres to the plasma membrane could reduce the need for internal Ca\(^{2+}\) release. On the other hand, an internal Ca\(^{2+}\) source cannot be ruled out. For example, cardiac myocytes and smooth muscle cells contain inositol trisphosphate-gated Ca\(^{2+}\) stores (Otani et al., 1988; Somlyo and Somlyo, 1994). Regardless of the source of Ca\(^{2+}\) that evokes RyR-independent contraction in *unc-68* mutants, RyRs appear to amplify the signal in body-wall muscle cells.

Certain RyR isoforms in vertebrate skeletal muscle also appear to amplify calcium transients. The major skeletal-type RyRs, termed RYR-1 in mammals or α-RyR in other vertebrates (Airey et al., 1990; McPherson and Campbell, 1993a), are directly gated by membrane potential (Rios et al., 1991). A second skeletal isoform, termed RYR-3 in mammals or β-RyR in other vertebrates, does not respond directly to depolarization (Airey et al., 1990, McPherson and Campbell, 1993a; Oyamada et al., 1994). Mutations that specifically eliminate RYR-1 channels in mice (skrrm1) or α-RyRs in chickens (en) have recessive lethal phenotypes, resulting in perinatal death (Ivanenko et al., 1995; Takeshima et al., 1994a). Muscle fibers from mutant embryos lack normal Ca\(^{2+}\) transients or contractile responses upon electrical stimulation, but exhibit these responses when exposed to caffeine or treatments that increase Ca\(^{2+}\) entry through plasma membrane channels (Ivanenko et al., 1995; Takeshima et al., 1994a). Further experiments suggest that the responses of mutant fibers are due to CICR through RyR-3 or β-RyR-type channels (Percival et al., 1994; Takeshima et al., 1995). These and other experiments have provoked a two receptor model for Ca\(^{2+}\) release in skeletal muscle, in which β-type RyRs amplify the Ca\(^{2+}\) released by α-type RyRs by CICR (Ivanenko et al., 1995; Jacquemond et al., 1991; O’Brien et al., 1995).

The amplification of Ca\(^{2+}\) signals by CICR is probably the more ancient and most common role of RyRs in E-C coupling. The major skeletal isoforms (and "mechanical" E-C coupling) appear to have evolved from β-RyR-type channels to serve the needs of fast twitch muscle types (O’Brien et al., 1993). Other RyR isoforms in cardiac muscle, in smooth muscle, in crustaceans, and in the CNS are known (or believed) to use a CICR mechanism (Fabiato, 1983; Györke and Palade, 1992; Lynn and Gillespie, 1995; McPherson and Campbell, 1993b). We infer from the *Unc-68* phenotype that RyRs in nematodes, one of the more primitive metazoan phyla, also amplify Ca\(^{2+}\) signals. While there is no direct evidence of a CICR mechanism for nematode RyRs, it is notable that *UNC-68* is more similar to cardiac RyRs than to the major skeletal isoforms (Sakube et al., 1993).

Our experiments lead us to conclude that the role of RyRs in *C. elegans* body-wall muscle is to enhance contraction by amplifying a depolarization-coupled Ca\(^{2+}\) transient. We consider it less likely that RyRs are involved in relaxation (e.g., repolarization of the muscle membrane), as proposed for RyRs in arterial smooth muscle cells (Nelson et al., 1995), because the absence of RyR function in *unc-68* mutants results in languard, flaccid paralysis, while ryanodine, known to lock the channels open, causes hypercontraction of wild-type animals. Further studies of *unc-68*-encoded RyR channels will help to uncover the molecular mechanisms regulating nematode body-wall muscle contraction, and should also be useful in understanding the evolution of RyRs and E-C coupling in more complex organisms.
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