Mutations in the α1 subunit of an L-type voltage-activated Ca\textsuperscript{2+} channel cause myotonia in Caenorhabditis elegans

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The control of excitable cell action potentials is central to animal behavior. We show that the egl-19 gene plays a pivotal role in regulating muscle excitation and contraction in the nematode Caenorhabditis elegans and encodes the α1 subunit of a homologue of vertebrate L-type voltage-activated Ca\textsuperscript{2+} channels. Semi-dominant, gain-of-function mutations in egl-19 cause myotonia: mutant muscle action potentials are prolonged and the relaxation delayed. Partial loss-of-function mutations cause slow muscle depolarization and feeble contraction. The most severe loss-of-function mutants lack muscle contraction and die as embryos. We localized two myotonic mutations in the sixth membrane-spanning domain of the first repeat (IS6) region, which has been shown to be responsible for voltage-dependent inactivation. A third myotonic mutation implicates IIIIS4, a region involved in sensing plasma-membrane voltage change, in the inactivation process.

Keywords: animal behavior/Caenorhabditis elegans/muscle excitation/myotonia/voltage-activated calcium channel

Introduction

Regulation of action potential duration is important for excitable cell function. For example, in neurons, the duration of action potentials at the synaptic terminal can affect the amount of transmitter released (Hochner et al., 1986; Spencer et al., 1989), whereas in vertebrate cardiac and gastrointestinal smooth muscles the duration of action potentials modulates the duration and strength of contractions (Noble, 1979; Huizinga, 1991). The physiological importance of regulating the duration of action potentials is exemplified by the human cardiac Long QT Syndrome (LQTS), in which the prolongation of the QT interval on electrocardiograms reflects a delay in the repolarization of ventricular myocytes. LQTS can manifest itself in ventricular fibrillation and syncopal episodes, ultimately leading to the death of young, otherwise healthy individuals (Schwartz et al., 1995).

In ventricular myocytes there are at least three major ionic currents contributing to the action potential: a regenerative Na\textsuperscript{+} current is responsible for fast depolarization, a Ca\textsuperscript{2+} current for the plateau phase and a K\textsuperscript{+} current for repolarization of the membrane. The activation and inactivation kinetics of each of these currents can affect the duration of the action potential. Indeed, mutations in a cardiac Na\textsuperscript{+} channel gene SCN5A and in a K\textsuperscript{+} channel gene HERG have been shown to be the causes of two distinct forms of congenital LQTS (Curran et al., 1995; Wang et al., 1995). Although many pharmacological and physiological studies have shown the importance of Ca\textsuperscript{2+} channels in shaping the cardiac action potential (Noble, 1979; Kass, 1995), mutations have not yet been found.

In general, the mechanisms regulating action potential duration are well conserved in metazoa. In recent years, it has been found that this mechanistic similarity extends to the molecular level. This conservation of molecular mechanisms has allowed physiologists to take advantage of the simplicity of invertebrates and their amenability to genetic approaches to help identify new molecules that are important for vertebrate excitable cell function. For example, the human HERG gene was cloned by its sequence homology to the Drosophila ether-à-go-go (eag) gene (Warmke and Ganetzky, 1994). The eag gene was identified because of its effect on fruit fly neural and muscle functions when mutated (Zhong and Wu, 1991).

We are studying the control of muscle action potential duration at the molecular level by analyzing a simple neuromuscular pump, the pharynx of the nematode C.elegans. The pharynx, consisting of 20 myoepithelial muscle cells, 20 neurons and 22 other structural and secretory cells, is the feeding organ of the nematode. The muscles are arranged radially around a lumen, so that contraction opens the lumen and relaxation closes it. In the anterior pharynx, a terminal bulb muscle contraction rotates a grooved cuticular structure called the grinder that grinds bacteria and passes debris towards the intestine. Relaxation returns the grinder to its resting position. A pump is a cycle of nearly synchronous contraction and relaxation of the corpus and the terminal bulb (Albertson and Thomson, 1976; Avery and Horvitz, 1989).

The pharyngeal muscles are the most experimentally accessible excitable tissue in C.elegans. Pharyngeal electrical activity can be monitored in living animals by a simple extracellular recording called the electropharyngeogram (EPG; Raizen and Avery, 1994). The pharynx can
be dissected from the rest of the animal, allowing pharmacological manipulation and direct measurement of muscle action potentials by intracellular recording (Davis et al., 1995). Similar to vertebrate cardiac and some smooth muscles, pharyngeal muscles can have myogenic activity (Avery and Horvitz, 1989). Contraction and relaxation are tightly correlated with the depolarization and repolarization phases of muscle action potentials. An action potential normally lasts about 150 ms (Raizen and Avery, 1994; Davis et al., 1995; Starich et al., 1996).

We have analyzed mutations that affect the duration of the pharyngeal muscle action potential. We find that egl-19 encodes the α1 subunit of a putative voltage-activated Ca\(^{2+}\) channel that is probably of the L type. Gain-of-function mutations in egl-19 cause prolonged muscle action potentials and contractions (myotonic class), reduction-of-function mutations cause a reduced rate of depolarization and feeble contractions (flaccid class), and severe loss-of-function mutations lead to a complete loss of muscle contraction and thus lethality (lethal class). We have localized two myotonic mutations in the IS6 (the sixth membrane-spanning domain in the first repeat) region and flanking residues. This region has been shown to control the rate of the voltage-dependent inactivation between different types of Ca\(^{2+}\) channels (Zhang et al., 1994). We also found one myotonic mutation in IIS4 (the fourth membrane-spanning domain in the third repeat), a region that is a voltage sensor for channel activation (Catterall, 1995). Our results demonstrate the importance of a voltage-activated Ca\(^{2+}\) channel in regulating the duration of action potentials. Furthermore, our mutant analysis suggests that the IS6 and IIS4 regions play a role in L-type Ca\(^{2+}\) channel inactivation.

**Results**

**Three classes of egl-19 mutations affect muscle contraction and excitation**

There are at least 26 mutant alleles of the egl-19 gene (Table I, see Materials and methods for mutant isolation). These mutant alleles can be classified into three groups based on their genetic and phenotypic characteristics. The myotonic class of mutations causes a semi-dominant excessive muscle contraction phenotype, which is a result of increased or misregulated gene activity. The flaccid class of mutations causes a recessive feeble muscle contraction phenotype which results from a partial reduction of gene function. The lethal mutations are recessive and cause a near-complete block of embryonic muscle contraction as a consequence of a severely reduced or absent gene function. The inability of embryonic body muscles to contract leads to a distinctive embryonic lethal phenotype referred to as the Pat (Paralyzed, Arrested elongation at Two-fold) phenotype (Williams and Waterston, 1994).

**Myotonic mutations**

Three mutations, n2368sd, ad695sd and ad952, are in the myotonic class. In an ad695sd mutant pharynx, the terminal bulb muscles, but not those of the corpus, often showed dramatically delayed relaxation (Figure 1A). To see whether the delay in relaxation was caused by delayed repolarization of muscle action potentials, we measured the electrical activities of the pharyngeal muscles by recording EPGs from intact worms. EPGs are analogous, in principle, to electrocardiograms or electroencephalograms used on humans. The EPG method provides a measurement of the capacitative current flows associated with changes in transmembrane potentials of pharyngeal muscle cells (Raizen and Avery, 1994). Thus, during a pump, fast depolarization of corpus and terminal bulb muscles in near-synchrony together causes a group (usually a pair) of upward transients in EPG. Repolarization of the corpus produces a large downward transient followed by a smaller downward transient caused by the repolarization of the terminal bulb (Figure 1B; Raizen and Avery, 1994). EPGs showed that n2368sd mutant worms had terminal bulb muscle action potentials that were dramatically prolonged (one example is shown in Figure 1B). Delayed relaxation of the terminal bulb in n2368sd mutants is consistently associated with a delay in terminal bulb muscle repolarization (data not shown). This correlation between muscle relaxation and repolarization defects suggests that the muscle relaxation defect is caused at least in part by the repolarization defect. Consistent with their normal corpus muscle contractions, EPGs did not reveal a defect in corpus electrical activities in n2368sd mutant animals (Figure 1B). Nevertheless, n2368sd mutant animals also showed hypercontraction of several other muscles. They had a short and dumpy morphology (Figure 2A), possibly caused by excessive body muscle tone, and they were egg-laying constitutive (Figure 2F), apparently because of excessive contraction of the egg-laying muscles, as the frequency and duration of vulval muscle contractions were increased (data not shown). The n2368sd mutation is semi-dominant. n2368sd/+ heterozygous animals had similar but weaker myotonic defects than those seen in homozygous mutant animals (data not shown).

ad695sd, like n2368sd, causes a semi-dominant myotonic phenotype, although its defects are generally weaker than those of n2368sd animals (Table 1; Avery, 1993). Intracellular measurement of ad695sd terminal bulb muscle action potentials from two animals confirmed that ad695sd causes prolonged plateau phases (Figure 1C), as had been concluded earlier on the basis of EPGs (Raizen and Avery, 1994).

The ad952 mutation was isolated in a genetic screen for dominant suppressors of egl-19(n582) (see Materials and methods). ad952 is tightly linked to n582 (within one map unit, see Materials and methods) and was shown by sequencing (see below) to be in egl-19. n582 ad952 double mutant animals were essentially wild-type in phenotype except that they were slightly dumpy, and the pharyngeal terminal bulb occasionally showed delayed relaxation and repolarization (Table 1 and data not shown). These phenotypes are similar to but much weaker than those seen in either n2368sd or ad695sd animals. n582 ad952/+ heterozygotes are wild-type, unlike n2368sd/+ and ad695sd/+ heterozygotes. These results suggest that egl-19(n582 ad952) is a weak gain-of-function mutant.

**Flaccid mutations**

egl-19 Flaccid mutants have feeble muscle contractions of both the corpus and the terminal bulb in the pharynx (Figure 1A). Their EPGs were normal with respect to the timing and amplitude of major signal peaks (Figure 1B). However, intracellular measurement of terminal bulb
Table I. Summary of egl-19 mutations

<table>
<thead>
<tr>
<th>Class</th>
<th>Mutations</th>
<th>Pharyngeal</th>
<th>Body</th>
<th>Egg-laying</th>
<th>Male mating</th>
<th>Embryonic</th>
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<td>Myotonic</td>
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<td></td>
<td>n2368sd</td>
<td>terminal bulb relaxation-defective</td>
<td>slightly dumpy, slightly jerky movement</td>
<td>constitutive, suppresses HSN</td>
<td>protruding spicules</td>
<td>cold-sensitive Pat</td>
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<td></td>
<td>ad695sd</td>
<td>terminal bulb relaxation-defective</td>
<td>slightly dumpy</td>
<td>weakly constitutive</td>
<td>protruding spicules</td>
<td>normal</td>
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<tr>
<td></td>
<td>n582 ad952</td>
<td>slight terminal bulb relaxation-defective</td>
<td>slightly dumpy</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
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<td>Flaccid</td>
<td>n582e</td>
<td>feeble pumping</td>
<td>long and thin, slow and floppy</td>
<td>defective</td>
<td>unable to protrude spicules</td>
<td>normal</td>
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<tr>
<td></td>
<td>ad1006, ad1013, ad1015, ad995, ad1025</td>
<td>feeble pumping leading to larval lethality</td>
<td>long and thin, slow and floppy</td>
<td>defective</td>
<td>nd</td>
<td>normal</td>
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<tr>
<td></td>
<td></td>
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<td>slightly slow</td>
<td>nd</td>
<td>nd</td>
<td>normal</td>
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<tr>
<td>Lethal</td>
<td>ad980, ad991, ad993, ad1004, ad1008, ad1009, ad1017, st533b, st556, st569, st571, st576, st577, n2368sd ad1023, ad695sd ad1000, ad695sd ad1002, ad695sd ad1021</td>
<td>feeble pumping</td>
<td>variably bulged</td>
<td>defective</td>
<td>nd</td>
<td>occasional Pat</td>
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nd: not determined.

aDuring mating, the male copulatory spicules are inserted into the vulva of the hermaphrodite by means of the protractor muscles (Hodgkin, 1988).
bPat (paralyzed, arrested elongation at 2-fold) phenotype refers to a specific embryonic lethal phenotype shared by many mutants defective in muscle genes, described by Williams and Waterston (1994). n2368sd mutants showed embryonic phenotype only at low (<=15°C) temperatures (see text).
cShort, contracted morphology.
dPreviously described by Avery (1993) as eat-12.
ePreviously described by Trent et al. (1983).
fK.S.Liu, personal communication.
gThis phenotype is apparently a less expressive Pat-like phenotype.
hThe st alleles were previously described by Williams and Waterston (1994) as pat-5.

Muscle action potentials in n582 (eight individuals, one typical action potential shown in Figure 1C) and ad995 (not shown) animals revealed that the rate of depolarization was reduced, suggesting that egl-19 has a role in bringing about fast depolarization. In addition to the pharyngeal phenotype, flaccid mutants showed feeble body wall and egg-laying muscle contractions. They tend to be long and thin, slow in movement and are egg-laying defective (Figure 2C and H; Trent et al., 1983).

Lethal mutations

The lethal class of egl-19 mutants has the Pat phenotype (Figure 2D and E). There was a dramatic reduction in the extent and frequency of embryonic body muscle contractions, and the most severe mutants did not contract at all (Williams and Waterston, 1994; data not shown). Embryonic body wall muscle contractions are apparently myogenic (Hall and Hedgecock, 1991; L.Avery, unpublished observations). Mutations in many genes important for muscle structure or function (for instance, those that encode myosin heavy chain A, vinculin, tropomyosin or troponin C) have the Pat phenotype (Williams and Waterston, 1994, and references therein), but no mutations in genes that affect only nervous system function have been found to have a Pat phenotype. The fact that the strongest alleles of egl-19 eliminated embryonic muscle contraction suggests that egl-19 affects embryonic muscle function directly rather than through the nervous system.

We conclude, based on mutant phenotypes, that the normal activity of egl-19 is necessary for muscle depolarization and for regulating the duration of muscle action potentials.

Cloning of a C.elegans homolog of α1 subunits of vertebrate L-type voltage-activated Ca2+ channels

Genetic mapping data (Trent et al., 1983; Avery, 1993; Williams and Waterston, 1994; and Materials and methods) positioned egl-19 in a small genetic interval between deb-1 and dif-1 on linkage group (LG) IV. This interval corresponds to a physical distance of approximately 150 kb in which we found a putative Ca2+ channel gene in independent experiments.

Specifically, by degenerate PCR (polymerase chain reaction), we cloned from C.elegans a cDNA fragment corresponding to the IIIIS6–IVS6 region (from the sixth membrane-spanning segment of the third repeat to the sixth membrane-spanning segment of the fourth repeat) of known vertebrate voltage-activated Ca2+ channel α1 subunits. We extended the sequence of the putative Ca2+ channel gene by screening a C.elegans cDNA library.
Ca\textsuperscript{2+} channel myotonia in \textit{C.elegans}

**Fig. 1.** Pharyngeal muscle contraction and electrical phenotypes of wild type and \textit{egl-19} mutants. (A) Timing diagrams schematically represent the pumping motion. Each of the three panels represents the pumping motion of a single animal analyzed by observation of video records frame-by-frame. The horizontal (time) scale has a resolution of 1/60 s, whereas the vertical scale (extent of contraction) is qualitative (Avery, 1993). The pumping motions shown are representative of animals of each of the genotypes, respectively. In the \textit{n2368sd} mutant, terminal bulb muscle contractions last longer than those in the wild type. In the \textit{ad995} mutant, both corpus and terminal bulb muscle contractions are often feeble and incomplete. The wild-type diagram is reproduced with permission from Avery (1993). (B) EPG recordings of pharyngeal muscle action potentials. For each genotype, an ~1200 ms stretch of a typical EPG recording is shown. The upward arrowheads mark the timing of peaks representing terminal bulb muscle depolarizations and the downward arrowheads mark the timing of repolarization transients. The duration of a terminal bulb muscle action potential is represented by a dashed line. The terminal bulb muscle depolarization peaks usually coincide with those of the corpus, often as a doublet (Raizen and Avery, 1994). The terminal bulb repolarization transients are the small ones following the larger transients generated by the corpus muscles. (C) Intracellular recordings from terminal bulb muscles. \textit{ad695sd} mutant muscles have occasional action potentials with significantly prolonged plateau phases. One such action potential is shown here. This phenotype agrees with that observed in \textit{ad695sd} EPG measurements (Raizen and Avery, 1994). Terminal bulb muscle action potentials in \textit{n582} mutants routinely show slow kinetics during the depolarization phase. One such action potential is shown with an asterisk marking the slow phase.

and by multiple steps of reverse transcriptase-coupled polymerase chain reaction (RT–PCR; see Materials and methods). We analyzed the presumptive ORF (open-reading frame) of this putative Ca\textsuperscript{2+} channel gene (Figure 3A) by comparing its sequence to available protein sequences in databases and found that it is significantly more similar to known \textit{\alpha}1 subunits of L-type channels than to other types of voltage-activated Ca\textsuperscript{2+} channels (Figure 3B). Furthermore, this putative Ca\textsuperscript{2+} channel has most (19 out of 23) of the otherwise absolutely conserved amino acid residues found in domains IIII-S5 and IVS5-S6 of \textit{\alpha}1 subunits of known L-type Ca\textsuperscript{2+} channels (Figure 3C). IIII-S5 and IVS5-S6 are two regions implicated in mediating channel sensitivity to 1,4-dihydropyridines (Grabner et al., 1996). Dihydropyridine sensitivity is the defining characteristic of L-type Ca\textsuperscript{2+} channels. In fact, pharyngeal muscles are sensitive to nifedipine, a dihydropyridine: nifedipine-treated dissected pharynx showed feeble muscle contraction with an extended action potential (J.A.Dent, personal communication). Other muscles in \textit{C.elegans}, for example body muscles, are also sensitive to L-type Ca\textsuperscript{2+} channel blockers (L.Lobel and H.R.Horvitz, unpublished observations). Because of the high degree of sequence similarity, we believe that we have cloned the \textit{\alpha}1 subunit of a \textit{C.elegans} L-type Ca\textsuperscript{2+} channel.

We mapped the physical location of this Ca\textsuperscript{2+} channel gene to cosmid C48A7 by hybridizing the cloned cDNA fragment to a YAC grid, and subsequently to cosmids covered by the positive YAC clones (see Materials and methods). By comparing its sequence to the recently released C48A7 cosmid sequence (for a description of the \textit{C.elegans} genomic project, see Wilson et al., 1994), we found that the Ca\textsuperscript{2+} channel gene lies completely within C48A7 (Figure 4).

**\textit{egl-19 encodes a voltage-activated Ca}\textsuperscript{2+} channel**

C48A7 is between the genes \textit{deb-1} and \textit{dif-1} on LGIV, consistent with the location of the \textit{egl-19} gene. We considered the possibility that \textit{egl-19} is the Ca\textsuperscript{2+} channel gene, since \textit{egl-19} mutant phenotypes are suggestive of defective muscle Ca\textsuperscript{2+} channels.

By germline transformation rescue experiments, we
found that transgenic egl-19(n582) mutant animals bearing the C48A7 transgene showed a nearly wild-type phenotype with respect to pharyngeal, body and egg-laying muscle contractions, suggesting that C48A7 contains sequences necessary for egl-19 gene activity. By mapping the extent of the egl-19 rescuing activity within C48A7, we found that the rescuing activity is co-extensive with the Ca\(^{2+}\) channel gene (Figure 4).

To confirm that egl-19 encodes the Ca\(^{2+}\) channel and to identify the molecular lesions in egl-19 mutants, we determined the sequences of the entire coding regions of the Ca\(^{2+}\) channel in three egl-19 mutants: the two myotonic mutants ad695sd and n2368sd, and the double mutant n582 ad952, which carries a flaccid mutation n582 and a myotonic mutation ad952. For each of these mutations we found a corresponding single-base G:C→A:T transition in the coding sequence (Figure 3A). The ad695sd mutation changes alanine 906 in IIIS4 to a valine. In n2368sd, glycine 365 near the cytoplasmic end of IS6 is changed to an arginine. Two mutations were found in the n582 ad952 double mutant. Since the n582 ad952 double mutant was derived directly from an n582 single-mutant animal, we were able to identify which of the two mutations found in the double mutant was n582 by determining the sequences of the two mutation sites in DNA isolated from n582 single-mutant animals. The n582 mutation is in IIIS4 and changes arginine 899 to a histidine. The ad952 mutation changes serine 372 to a leucine close to the predicted intracellular end of IS6. The fact that there is a mutation in Ca\(^{2+}\) channel coding sequence corresponding to each of the four identified egl-19 mutations, and that the egl-19 rescuing activity is co-extensive with the Ca\(^{2+}\) channel gene in the genomic sequence, argues strongly that the Ca\(^{2+}\) channel and egl-19 are the same gene.

### egl-19 is expressed and functions in muscle cells

Although it is clear from mutant phenotypes that egl-19 affects excitation and contraction of muscles, egl-19 could nevertheless be influencing muscles indirectly, e.g. through the nervous system. To address the question of how egl-19 affects muscle excitation, we localized its expression by assaying the expression of an egl-19::GFP (green fluorescent protein, Chalfie et al., 1994) reporter (Figure 4). In transgenic animals carrying the fusion gene, an egl-19::GFP fluorescent signal was first detected in body wall muscles in 1–2-fold embryos (Figure 5A and B), before the onset of embryonic muscle contraction. This result is consistent with the Pat phenotype seen in lethal mutants, suggesting a cell-autonomous muscle defect. By the time of hatching, GFP fluorescence was found in pharyngeal muscles pm3, pm4, pm5 and pm7 (Figure 5C), in body wall muscles (Figure 5F) and in the anal depressor muscle (Figure 5D and E). The muscle expression pattern is again consistent with a muscle cell-autonomous defect caused by mutations. We also found expression in the nervous system, including the pharyngeal neuron M4 and several neurons in the head, the ventral nerve cord and the pre-anal ganglion (Figure 5C and E, and data not shown). This expression pattern suggests that egl-19 may also function in neurons (see Discussion).

In the wild type, the HSN neurons are required for normal egg-laying muscle contraction (Trent et al., 1983). We found that egg-laying muscles in egl-19(n2368sd) myotonic mutants contract even in the absence of HSN motor neurons (see Materials and methods). This observation suggests that egl-19 acts in egg-laying muscles to promote contraction. However, we could not detect GFP expression in egg-laying muscles. The promoter fragment we used probably lacks elements for egg-laying muscle expression (see Figure 4 and Materials and methods).

### Discussion

We have identified three classes of egl-19 mutations. Mutations that belong to each of the classes have been isolated previously. We now know that egl-19(n582), isolated by Trent et al. (1983), is a flaccid, partial loss-of-function allele, and eat-12(ad695sd), isolated by Avery (1993), is a myotonic, gain-of-function allele. Six alleles of pat-5 were isolated by Williams and Waterston (1994). These alleles are egl-19 lethal, severe loss-of-function or null mutations (B. Williams, personal communication; see also Materials and methods). The phenotypes of these
Ca\textsuperscript{2+} channel myotonia in \textit{C.elegans}

Fig. 3. EGL-19 Ca\textsuperscript{2+} channel sequence and comparisons with known Ca\textsuperscript{2+} channels. (A) The largest ORF predicted from the Ca\textsuperscript{2+} channel cDNA sequence is shown. This polypeptide of 1783 amino acids has all the hallmarks of \(\alpha\)l subunits of voltage-activated Ca\textsuperscript{2+} channels (Catterall, 1995). Marked features in the sequence are based on sequence alignment analysis to known L-type Ca\textsuperscript{2+} channel genes (referenced in part B). There are four imperfect internal repeats (I–IV), each with six potential membrane-spanning helices (S1–S6, marked on top of the segments). Each repeat has a segment that is thought to line the channel pore (marked as SS1-SS2). Within each SS1-SS2 segment, there is a glutamate residue (marked by a \)'), involved in coordinating the Ca\textsuperscript{2+} ion in the pore (Catterall, 1995). An EF-hand (consensus Ca\textsuperscript{2+}-binding site marked by a line above) is found in the predicted cytoplasmic tail, as in known Ca\textsuperscript{2+} channels (de Leon \textit{et al}., 1995). Bent arrows and double underlines mark the predicted amino acid substitutions found in four \textit{egl-19} mutant alleles (see text). (B) Sequence comparisons of EGL-19 to six different classes of vertebrate Ca\textsuperscript{2+} channel \(\alpha\)1 subunits: \(\alpha\)1A (Mori \textit{et al}., 1991), \(\alpha\)1B (Williams \textit{et al}., 1992a), \(\alpha\)1C (Mikami, \textit{et al}., 1989), \(\alpha\)1D (Williams \textit{et al}., 1992b), \(\alpha\)1E (Niidome \textit{et al}., 1992) and \(\alpha\)1S (Grabner \textit{et al}., 1991). (C) Sequence comparison of EGL-19 to an L-type and a non-L-type Ca\textsuperscript{2+} channel within the regions IIIS5–IIIS6 and IVS5–IVS6, responsible for dihydropyridine sensitivity. In these regions, 23 residues (highlighted in EGL-19 sequence) are identical among all previously known L-type channels and among all known non-L-type channels, but different between L- and non-L-types (Grabner \textit{et al}., 1996). Of these 23 residues, EGL-19 has 19 identical to L-type and only one identical to non-L-type. The sequence of \textit{egl-19} has been deposited in GenBank under the accession No. AF023602.

Fig. 4. Molecular localization of \textit{egl-19}. This figure summarizes our strategy and results in defining the minimal genomic region that contains \textit{egl-19} (+) and its relationship to the Ca\textsuperscript{2+} channel gene. Inserts of the genomic \(\lambda\) phage clones were mapped with respect to the cosmid C48A7. The ability of each genomic clone to rescue \textit{egl-19}(n582) mutants was tested by germline transformation (see Materials and methods for detail). The E2 clone can rescue both pharyngeal and the body muscle defects but not the egg-laying defect. The last line shows the extent of the genomic fragment used to construct the \textit{egl-19}::GFP fusion. *Although most transgenic animals carrying the F2 clone were rescued to an essentially wild-type phenotype, some acquired a new pharyngeal defect not seen in the controls (see Materials and methods).
three classes of mutants considered together speak to the pivotal role that \textit{egl-19} plays in muscle excitation and contraction.

Nematode pharyngeal muscles are similar to vertebrate cardiac and mammalian gastrointestinal smooth muscles that have slow-wave-type action potentials in several respects. For example, these types of muscles depolarize and contract cyclically; their action potentials have a long plateau phase; and the timing of contraction and relaxation is correlated to the depolarization and repolarization of their action potentials, respectively (Noble, 1979; Huizenga, 1991; Raizen and Avery, 1994; Davis \textit{et al}., 1995). In vertebrates, L-type voltage-activated Ca$^{2+}$ channels are known to be important in regulating the excitation and contraction of cardiac myocytes (Bean, 1989). Similarly, L-type channels have been implicated in the slow-wave-type action potentials in mammalian gastrointestinal smooth muscles (Huizenga, 1991). Our results show that a putative L-type voltage-activated Ca$^{2+}$ channel encoded by the \textit{egl-19} gene serves a similar function in \textit{C.elegans} pharyngeal muscles. These observations suggest that the nematode pharynx may be a good model system in which to study how varying Ca$^{2+}$ channel activity may affect the physiology of muscles that have long action potentials.

\textbf{ EGL-19 encodes the major Ca$^{2+}$ channel in \textit{C.elegans} muscles}

Most \textit{C.elegans} muscles are sensitive to \textit{egl-19} gene activity, including embryonic and post-embryonic body wall muscle, pharyngeal muscle, egg-laying muscle and possibly anal depressor and male spicule protractor muscle (Table 1; Figure 5). Therefore, EGL-19 is the \(\alpha 1\) subunit of a major muscle voltage-activated Ca$^{2+}$ channel in \textit{C.elegans}. To date, only one other voltage-activated Ca$^{2+}$ channel \(\alpha 1\) subunit has been genetically characterized (Schafer and Kenyon, 1995). In contrast to \textit{egl-19}, \textit{unc-2} encodes an \(\alpha 1\) subunit more similar to non-L than L-type voltage-activated Ca$^{2+}$ channels. Reduction of \textit{unc-2} function leads to an egg-laying constitutive phenotype, opposite to that of \textit{egl-19} reduction-of-function. Furthermore, \textit{unc-2} primarily affects neural function (Schafer and Kenyon, 1995; Schafer \textit{et al}., 1996). \textit{unc-2}, however, does have a functional role in body muscles. \textit{unc-2} mutants are sluggish in movement, and based on mosaic studies, Schafer and Kenyon (1995) concluded that this phenotype is caused by the loss of \textit{unc-2} function in body muscles. Thus, although our data suggest that \textit{egl-19} is functionally the major voltage-activated Ca$^{2+}$ channel in muscles, other Ca$^{2+}$ channels may also contribute to muscle excitation and contraction.

The expression pattern of an \textit{egl-19}:\textit{GFP} reporter indicates that \textit{egl-19} is also expressed in many neurons. We do not know the functional significance of the nervous system expression, since most of the defects of \textit{egl-19} mutants are consistent with defects in muscle function exclusively. However, muscle defects would mask most nervous system defects. One exception is that \textit{ad695sd} and \textit{n2368sd} animals have a dauver-formation defect (Daf-d). A dauer larva is an enduring, dispersal form of larva that is of a developmental stage equivalent to an L3. Although some dauers did develop from starved \textit{ad695sd} or \textit{n2368sd} mutant L2 larvae, many of these dauer animals appeared to be incompletely modified, as they were less resistant to SDS treatment than wild-type dauers (data not shown). Normal dauers have modified cuticles which render them resistant to harsh environmental conditions (Riddle, 1988). Since the formation of dauers is largely dependent on neural function (Riddle, 1988), and no mutations affecting only muscle function have been reported to cause a dauer-formation defect, this Daf-d phenotype suggests that \textit{egl-19} may also function in the nervous system.

\textbf{Control of pharyngeal muscle repolarization}

Although the EGL-19 channel clearly affects the action potentials of both the corpus and terminal bulb muscles, based on the expression pattern and the loss-of-function phenotype, gain-of-function mutations cause a myotonic phenotype only in the terminal bulb. This observation suggests that different mechanisms are involved in the control of action potential duration in these two parts of the pharynx. This notion is also supported by the fact that the corpus always repolarizes before the terminal bulb. It seems possible that the corpus has an intrinsic ability to repolarize earlier than the terminal bulb. To achieve a well-regulated and fast repolarization, other ion channels must participate in the repolarization process. For instance, mutations in a glutamate-gated Cl$^{-}$ channel, encoded by the \textit{avr-15} gene, cause a delay in muscle repolarization.

\textbf{Fig. 5.} Expression of an \textit{egl-19}:\textit{GFP} fusion construct in transgenic animals. The fusion gene contains a nuclear localization signal so that the fluorescent signal is most concentrated in nuclei, although a less intense signal can be seen in the cytoplasm. (A) Differential interference contrast (DIC) and (B) fluorescent views of a 1–1/2 fold embryo. The bright dots in (B) are two rows of body wall muscle nuclei expressing GFP. Scale bar = 20 μm. (C) GFP fluorescent signal is found in pharyngeal muscles pm3, pm4, pm5 and pm7. The signal is also found in a number of neurons. For example, M4 in the pharynx is marked by a plain arrow and two neurons in the anterior nerve ring are marked by barbed arrows. (D) DIC and (E) fluorescent views of the tail of an adult hermaphrodite. Expression is found in anal depressor muscles (mu anal); barbed arrows mark the nuclei of two neurons in the pre-anal ganglion. (F) Fluorescent signal from three adult body wall muscle cells. The nuclei are marked by arrows.
AVR-15 is apparently part of the pharyngeal muscle receptor for the neurotransmission from M3 inhibitory motor neurons, which directly affect the repolarization of the corpus but not the terminal bulb (Dent et al., 1997). Therefore, M3 activity could preferentially hasten corpus repolarization. However, M3 transmission alone can not entirely account for the difference, since the terminal bulb still repolarizes later than the corpus when M3 transmission is blocked (Raizen and Avery, 1994). Another channel likely to play a role in the repolarization process is the nematode negative spike K⁺ channel, originally characterized by Byerly and Masuda (1979) in the Ascaris pharynx (see also Davis et al., 1995). A difference in the expression or regulation of activity of this and perhaps other channels could account for the different repolarization timing between parts of the pharynx.

Myotonic mutations in egl-19

The myotonic phenotype of egl-19 mutants is well explained by slower inactivation kinetics of the muscle Ca²⁺ current, since that Ca²⁺ current is likely to be the major inward current during muscle depolarization. When inactivation of the EGL-19 channel is retarded, the muscle membrane potential would be held depolarized, and the resulting sustained Ca²⁺ influx would prolong muscle contraction. L-type voltage-activated Ca²⁺ channels are known to inactivate by two mechanisms, voltage- and calcium-dependent. These two means of inactivation are thought to operate independently (Hadley and Lederer, 1991).

By assaying the inactivation kinetics of chimeric channels (made between an α₁A and a marine ray homolog of the α₁E class) in Xenopus oocytes, Zhang et al. (1994) found that a region of the proteins that includes IS6 and flanking residues is responsible for the different rates of voltage-dependent inactivation in voltage-activated Ca²⁺ channels. These authors also suggested that this type of inactivation is reminiscent of the C-type (slow) inactivation observed in voltage-activated K⁺ channels, revealed after the removal of N-type (fast) inactivation by deletion of residues that form the tethered plug to the channel pore (Hoshi et al., 1991). Recently, a mutation (N434A) in IS6 of the rat μ1 Na⁺ channel protein has also been found to affect the slow inactivation of the channel (Wang and Wang, 1997). Two of egl-19 myotonic mutations, n2368sd and ad952, are in the IS6 region. We believe that the myotonic phenotype observed in these mutants is caused by a defective voltage-dependent inactivation of the EGL-19 channel. Thus our data extend the importance of the IS6 region in controlling channel inactivation to L-type channels.

The third myotonic mutation, ad695sd, is in IIIS4. S4 segments are thought to be the voltage sensors for voltage-sensitive channels (Catterall, 1995). Indeed, an R→H mutation in IIIS4 causes the reduced rate of depolarization observed in n582 mutants, as would be expected if this mutation affected the voltage sensor of the channel. It is possible that the A→V mutation in IIIS4 found in ad695sd animals also affects the activation of the channel. However, we have not observed significantly slowed-down depolarization kinetics in ad695sd, in contrast to the dramatically prolonged plateau phases in mutant muscle action potentials. Although the kinetics of channel activation can affect the kinetics of inactivation, it seems unlikely that ad695sd mutant channels inactivate slowly simply as a consequence of slow activation kinetics. Thus, the mutation apparently uncouples the role of IIIS4 in channel activation from its role in inactivation. A similar phenomenon has been reported for mutations in the IVS4 segment in vertebrate skeletal muscle Na⁺ channels. R1448C and R1448H mutations in the α subunit of the human skeletal muscle voltage-activated Na⁺ channel gene SCN4A cause paramyotonia congenita. When the biophysical properties of the channels were assayed in a cell line, it was found that the mutations have only a small effect on activation but dramatically slow inactivation (Chahine et al., 1994).

The two myotonic mutations in the IS6 region of the EGL-19 channel may also affect the activation phase of muscle action potentials. First, n2368sd has a cold-sensitive Pat phenotype (Table I and Materials and methods). This phenotype is apparently caused by a reduction in gene function, since it is recessive to the wild-type allele and is not complemented by other Pat alleles. How the n2368sd mutation imparts this phenotype is not clear. A possibility is that the G→R mutation in IS6 alters the structure of the protein only slightly at high temperatures (~20°C) to affect channel inactivation, whereas the mutation destabilizes the protein dramatically at low temperatures (~12°C) to impair channel formation or function. Second, we have noticed in intracellular records that n582 ad952 double mutants have consistently faster depolarization kinetics than n582 single mutants (unpublished observations). Since we do not have the ad952 mutation in isolation, we do not know if the suppression between n582 and ad952 is mutual. It should be interesting to assay the detailed biophysical properties of each of these mutant channel proteins in a simpler and more electrophysiologically accessible system, e.g. Xenopus oocytes.

In conclusion, our analysis of myotonic mutants in C.elegans has implicated a voltage-activated Ca²⁺ channel in regulating the duration of muscle action potentials. Our in vivo analysis suggests that mutations in IS6 and IIIS4 regions of the α1 subunit can dramatically affect Ca²⁺ channel inactivation. We envisage that continued genetic analysis of C.elegans pharyngeal excitation will further our understanding of the molecular mechanisms that underlie the control of action potential duration in excitable cells.

Materials and methods

General methods

Worm culture, handling and genetic manipulation followed the methods described by Sulston and Hodgkin (1988). Except for cold-sensitivity experiments, all worms were maintained at 20°C. The wild type was the N2 strain of the Bristol variety of C.elegans. Methods used for analyzing pharyngeal behavior and electrical activity have been described elsewhere: timing diagrams by Avery (1993); EPGs by Raizen and Avery (1994); and intracellular recordings by Davis et al. (1995).

Mutant isolation and genetic characterization

All mutagenesis was carried out with ethyl methanesulfonate (EMS), which causes primarily G→A/T transitions (Anderson, 1995). Mutants isolated were backcrossed at least twice against N2 or bli-6(0;XO) unc-2(€1358am) animals. n2368sd. Mutagenized egl-1/H986fdn V males were crossed with unc-79(e1068) ced-4(n1162) III; rol-4(sc16) unc-76(e911) V; lon-2(e678) rol-
Among 15,000 haploid genomes, ad952 was tightly linked to n582 and mapped between bli-6 and unc-24, which are ~1 map unit apart.

**Tests for cold sensitivity**

Gravid hermaphrodites were kept on petri plates in a box placed in a circulating cold water bath in order to maintain a tightly controlled temperature for the embryos. n2368sd embryos were nearly 100% Pat at 12°C. Only a small fraction were affected when grown at 15°C. We found no cold sensitivity for ad98sd, n582 or N2 animals.

**cDNA cloning and sequencing**

General cloning methods followed those described by Sambrook et al. (1989). For sequencing, we used an ABI 377 automatic sequencer. For sequence management, we used the GCG Wisconsin Package.

Degenerate PCR primers were designed based on the conserved sequences in the III and IV domains of the N1 subunits of vertebrate L-type voltage-activated Ca²⁺ channel genes (primer sequences available upon request). A 953 bp PCR product was amplified from worm cDNA and cloned (pCAC1-953). The cDNA was localized in the genome by hybridizing CAC1–953 to a YAC poly-gad grid, poly 1 (kindly provided by R. Waterston; Coulson et al., 1988). Only two overlapping YAC clones, Y51C8 and Y49F12, showed positive hybridization. Three cosmid clones, C4A87, B0496, and K11C12 (kindly provided by Alan Coulson), span the overlap of the YACs. By Southern hybridization, we found that CAC1–953 is contained entirely in C4A87, partially in B0496, and not in K11C12. C4A87 has been placed between two genes deb-1 and dif-1 on the physical map (Coulson et al., 1995).

Using the CAC1–953 library, we sequenced a mixed-stage worm cDNA library (Barstead and Waterston, 1989) to isolate a 2.8 kb cDNA clone (pCE12A). We then designed PCR primers based on the sequences in pCE12A (TATTCTTGAATGACAGCAAGCAGAACCT) and in the genomic sequence of cosmid C4A87 (provided by the C.elegans sequence consortium) corresponding to the first predicted transmembrane domain (IS6) of the Ca²⁺ channel gene (CTTTCGCCCTTCTGACTATCCTCATCGG) to RT–PCR more 5’ cDNA sequence from a cDNA mix made from mixed-stage poly-A selected total RNA. A 3 kb product (e12-R1) was cloned and sequenced. To obtain further 5’ sequence, we used the Genefinder program (PGreen, unpublished; cited in Eckmann and Durbin, 1995) to help us identify the possible initiating ATG from the genomic sequence. We used a primer corresponding to the sequence between the 5’ and 3’ ATGs to PCR-amplify a 0.7 kb product from a cDNA mix. The sequence of the PCR product was determined by direct sequencing, pCE12A lacked the poly-A signal sequence, suggesting incomplete 3’ extension. A cDNA clone (y535d), isolated and kindly given to us by Viji Kohara, overlaps in sequence with pCE12A and extends 0.5 kb further 3’ to include the AATAA poly-A signal. When assembled together, these four pieces would make one 6.2 kb cDNA. The authenticity of this presumptive transcript was verified by RT–PCR using the most 5’ primer (y535d) and kindly given to us by Viji Kohara, overlaps sequence management, we used the GCG Wisconsin Package.

**Isolation of genomic lambda phage clones and germline transformation**

We used CAC1–953 as a probe to screen a C.elegans genomic lambda FIXII phage library (Stratagene). The sizes and extents of the inserts in phage clones that we isolated were determined by restriction pattern analysis and by direct sequencing of the ends using primers corresponding to the multiple cloning site of the vector. We were able to map these clones precisely, since the sequence of cosmid C4A87 became available during the course of our analysis.

The ability of these phage clones and of C4A87 to rescue egl-19 mutants was determined by germline transformation experiments (Mello et al., 1992). The genomic clone was co-injected into egl-19(n582) animals with pRAK3, which contains a dominant rol-6 marker (Mello et al., 1992; Davis et al., 1995). Transgenic lines stably expressed the marker were established and observed for Egl-19 defects, including abnormal egg-laying, body morphology, movement and pharyngeal pumping. For C4A87, two of eight lines showed rescue; for F2 and F3 co-injection, five of five; for F2 alone, six of eight; for E3, zero of three; for H1, none of nine; for E2, nine of nine.

Some unusual phenomena associated with the F2 and E2 transgenes
are worth noting. We have observed occasional terminal bulb muscle relaxation defects in some transgenic animals carrying the F2 clone, while their sisters (also transgenic) appeared to be wild type. This variability may reflect the fact that F2 contains an egl-19 gene truncature at the 3’ end (see Figure 4). Rescue of egl-19(n582) by E2 transgenes was incomplete. In particular, the egg-laying defective phenotype was only slightly improved in all E2 transgenic animals, whereas pharyngeal and body muscle phenotypes were fully rescued. This result suggests that E2 may lack important regulatory elements for proper expression in egg-laying muscles. This hypothesis could explain why the egl-19::GFP reporter was not expressed in egg-laying muscles, as the egl-19 regulatory sequence in the reporter was from E2 (see below and Figure 4).

One transgene carrying C48A7 also rescued an embryonic lethal allele, st556 (Williams and Waterston, 1994). Males of the genotype egl-19(n582)/c48a7(121) unc-24(ad1388) IV (a gift from B.Williams) were crossed with the transgenic line egl-19(n582) IV; adEx1058[egl-19+ rol-6(dj)] V. Viable and sometimes fully wild-type (except for the Rol phenotype of the co-injected rol-6(dj) gene) animals were segregated of the genotype egl-19(st556) IV; adEx1058[egl-19+] rol-6(dj)].

**Mutation detection**

Genomic DNA was prepared from animals homozygous for the respective mutations. Segments of the egl-19 gene were amplified by PCR. The sequences of the entire coding regions and intron–exon boundaries from ad695sd and n2368sd were determined by direct sequencing of PCR products. The sequences were compared to the genomic sequence of the C48A7 cosmid made available by the C.elegans Genome Consortium. Wherever a suspected mutation was found, the sequencing was repeated at least once more to eliminate potential artifacts. The sequences of the oligonucleotides used for amplification and/or sequencing of the exons are available upon request.

egl-19::GFP fusion

A translational fusion of egl-19 and GFP was made by inserting a 4.7 kb fragment of egl-19 into the multiple cloning site in the pPD95.70 GFP expression vector (A.Fire, J.Ahnn, G.Seydoux and S.Xu, personal communication), which also contains a nuclear localization signal. The egl-19 fragment was prepared from phase E2 (see Figure 4). E2 DNA was digested with BcgI. The open ends were blunted by Klenow enzyme. E2 was then digested again with SalI, which cuts in the vector. The 4.7 kb fragment was gel-purified. The pPD95.70 vector was prepared by digestion with SalI and followed by gel purification. Ligation of these two fragments resulted in fusion of the egl-19 translation frame to that of the GFP. The fusion construct (peut-12::sgFP-NLS) was cloned and checked by restriction digests and used to germline transform lin-15(n765ts) animals along with ADAN735 (a plasmid containing lin-15; Huang et al., 1994) as a co-injection marker. Transferring lines that segregated wild-type animals were established and the expression of GFP was observed in wild-type animals by fluorescence microscopy with an FITC filter set. We had 10 independent lines expressing egl-19::GFP, and they looked essentially the same with respect to where the signal was detected. The strength of the signal, however, varied widely among them. We therefore concentrated our further analysis on the line that had the strongest expression. We present (in Figure 5) a view of the pharynx in a dauer animal, because the expression pattern of egl-19::GFP in the pharynx is most easily photographed in dauer, as the pharynx is flat and compact.

**Acknowledgements**

We thank the C.elegans Genome Consortium for cosmid sequences, A.Coulson for cosmids, Y.Kohara for a partial cDNA clone of egl-19, A.Fire for GFP expression vectors, R.Waterston for YAC filters, B.Williams for pat-5 mutant strain, and K.S.Liu and J.A.Dent for sharing unpublished results. Some strains used in mapping experiments were supplied by the Caenorhabditis Genetics Center, which is supported by the National Institute of Health National Center for Research Resources. We also thank M.W.Davis, J.A.Dent, R.Joho, F.Katz, D.Raizen and R.Zwaal for their constructive comments on the manuscript. This research was supported by United States Public Health Service research grants HL46154 to A.Lavery and GM24663 to H.R.Horvitz. H.R.H. is an Investigator at the Howard Hughes Medical Institute.

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Received on April 1, 1997; revised on July 18, 1997