The L-type voltage-dependent Ca\textsuperscript{2+} channel EGL-19 controls body wall muscle function in Caenorhabditis elegans

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Caenorhabditis elegans is a powerful model system widely used to investigate the relationships between genes and complex behaviors like locomotion. However, physiological studies at the cellular level have been restricted by the difficulty to dissect this microscopic animal. Thus, little is known about the properties of body wall muscle cells used for locomotion. Using in situ patch clamp technique, we show that body wall muscle cells generate spontaneous spike potentials and develop graded action potentials in response to injection of positive current of increasing amplitude. In the presence of \(K^+\) channel blockers, membrane depolarization elicited Ca\textsuperscript{2+} currents inhibited by nifedipine and exhibiting Ca\textsuperscript{2+}-dependent inactivation. Our results give evidence that the Ca\textsuperscript{2+} channel involved belongs to the L-type class and corresponds to EGL-19, a putative Ca\textsuperscript{2+} channel originally thought to be a member of this class on the basis of genomic data. Using Ca\textsuperscript{2+} fluorescence imaging on patch-clamped muscle cells, we demonstrate that the Ca\textsuperscript{2+} transients elicited by membrane depolarization are under the control of Ca\textsuperscript{2+} entry through L-type Ca\textsuperscript{2+} channels. In reduction of function egl-19 mutant muscle cells, Ca\textsuperscript{2+} currents displayed slower activation kinetics and provided a significantly smaller Ca\textsuperscript{2+} entry, whereas the threshold for Ca\textsuperscript{2+} transients was shifted toward positive membrane potentials.

Introduction

The nematode Caenorhabditis elegans has become a preparation of prime interest to investigate the relationships between genes and physiological processes and behaviors. The main advantages of this model system include the fully sequenced genome, the short generation time, and the ability to perform extensive genetic maneuvers. However, to precisely determine how the product of a gene influences a cell function requires measurements of its effect on cell activity. In situ physiological studies have been nevertheless greatly restricted in this model system by the difficulty to dissect this microscopic animal and to expose the cells of interest. Thus, although large screens of mutants have led to the identification of genes involved in a variety of functions, very few of these mutants have been characterized at the cellular level.

\textit{egl-19} has been postulated to encode the \(\alpha 1\) subunit of a pharyngeal voltage-activated L-type Ca\textsuperscript{2+} channel in \textit{C. elegans} (Lee et al., 1997). \textit{egl-19} was also found to be expressed in body wall muscle cells used for locomotion by the animal (Lee et al., 1997). Null mutants of \textit{egl-19} are lethal, whereas reduction of function causes feeble contraction, suggestive of an important role played by these channels in body wall muscle function. Using in situ patch clamp techniques on split worms, high voltage-activated Ca\textsuperscript{2+} currents were first recorded by Richmond and Jorgensen (1999) in body wall muscles from \textit{C. elegans}, but the properties and the role of these channels in nematode body wall muscle function are unknown. A primary \textit{C. elegans} cell culture developed recently also appeared promising for electrophysiological approach (Christensen et al., 2002). However, whole cell Ca\textsuperscript{2+} currents were not measured in cultured muscle cells.

In this paper, using the whole cell configuration of the patch clamp technique on acutely dissected worms we give a detailed description of the properties of voltage-activated Ca\textsuperscript{2+} currents in body wall muscle cells from \textit{C. elegans} and provide experimental evidence that the Ca\textsuperscript{2+} channels involved belong to the L-type class. Furthermore, we succeeded in coupling a Ca\textsuperscript{2+} imaging system and the patch clamp technique on muscle cells and demonstrate that these channels play a pivotal role in \textit{C. elegans} muscle activation. Finally, we show...
that partial loss of function egl-19 mutant muscle cells have strongly altered Ca\(^{2+}\) currents and require stronger depolarizations to induce intracellular Ca\(^{2+}\) rise, most likely responsible for the flaccid phenotype observed in these worms.

**Results**

Voltage responses and membrane currents in standard saline

Using the whole cell configuration of the patch clamp technique, we first investigated the electrical excitability of body wall muscle cells. In the presence of standard external medium in the bath and a K\(^{-}\)-rich solution in the pipette, the average resting membrane potential of body wall muscle cells was –19.7 ± 1.8 mV (n = 12). In two of eight muscle cells tested, the resting potential was interrupted by spontaneous abortive or overshooting spikes whose amplitude varied from one to another (Fig. 1 A). Under current clamp conditions, the injection of a hyperpolarizing current bringing the membrane potential close to –30 mV totally blocked this spike activity likely because the threshold for the production of these spontaneous responses could not be reached. Fig. 1 B shows one of the spontaneous spikes on an expanded scale. It can be seen that the potential overshot to +15 mV and was followed by a transient hyperpolarization whose amplitude apparently depended upon the height of the preceding spike. To test the possibility to evoke active responses, depolarizing currents of increasing amplitude were then injected into muscle cells from a membrane potential held at –70 mV by applying constant negative current. Measurement of the amplitude of the voltage change induced by injection of 500 ms current pulse of 20 pA amplitude indicated a mean input resistance of 1 ± 0.08 G\(\Omega\) (n = 10). Out of the 14 cells tested, injection of depolarizing currents never induced voltage responses which could be unambiguously identified as all or none action potentials. However, graded humps were observed for injection of current higher than 300 pA superimposed on the electrotonic potentials (Fig. 1 C). An inflection point visible during the rising phase of the potential responses likely indicated that the graded humps represent active electrical response, resulting from the development of a net inward current activating upon depolarization. In support of this hypothesis, net inward currents were indeed recorded on the same cell under voltage clamp conditions in response to depolarizing voltage pulses. Fig. 1 D shows membrane currents elicited by 20 ms voltage pulses of increasing amplitude from a holding potential of –70 mV. Depolarizing steps to 0, +10, +20, and +30 mV evoked net transient inward currents followed by an outward current, which stabilized to an apparent plateau level. The use of the pharmacological compounds, 4-aminopyridine (4-AP)* (3 mM) and tetraethylammonium (TEA) (20 mM) indicated that the total outward current resulted from the presence of two main voltage-dependent K\(^{+}\) conductances, a fast transient 4-AP–sensitive and a delayed outward rectifier TEA-sensitive component as first described by Richmond and Jorgensen (1999) (Jospin et al., 2002). Together, these results strongly suggest that the successive activation of inward and outward currents upon depolarization underlie the spontaneous and evoked graded action potentials generated by *C. elegans* muscle cells.

Voltage-activated inward Ca\(^{2+}\) currents

To reveal inward currents during depolarizing steps, opposing outward going potassium currents needed to be blocked by replacing external sodium by TEA, adding the potassium blocker 4-AP and substituting equimolarly Cs\(^{+}\) to K\(^{+}\) in the pipette. Under these conditions, muscle cells were depolarized by steps of 200 ms duration from a holding potential of –70 mV. Fig. 2 A shows that an inward current maintained throughout the depolarizing step developed, and its amplitude increased with depolarization to reach a maximum around +20 mV. It was also systematically observed that at –10, 0, +10, and +20 mV the inward cur-

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*Abbreviations used in this paper: 4-AP, 4-aminopyridine; DHP, dihydro-}

 pyridine; TEA, tetraethylammonium.
current displayed an early transient peak lasting for \( \sim 10 \) ms before stabilizing to a plateau level. Fig. 2 B presents the mean current-voltage relationships established for the peak and the steady state currents. For peak and maintained components, threshold of activation was \(-20\) and \(-30\) mV, respectively. Currents peaked at \(+10\) and \(+20\) mV and reversed at \(+50\) \pm \(2\) mV and \(+59\) \pm \(2\) mV for the peak and the maintained components, respectively. For each cell, the current-voltage relationship was fitted using Eq. 1 (see Experimental procedures). Mean values for \( \tilde{G}_{\text{max}} \), \( V_{\text{rev}} \), \( V_{0.5} \), and \( k \) were \(199 \pm 26\) S/F, \(+50 \pm 2\) mV, \(+0.6 \pm 1.3\) mV, and \(4.7 \pm 0.5\) mV for the peak and of \(127 \pm 20\) S/F, \(+59 \pm 2\) mV, \(+6.4 \pm 1\) mV, and \(7.9 \pm 0.5\) mV for the steady state current, respectively.

Fig. 3 A shows that the inward current elicited by depolarization was totally suppressed in the presence of \(500\) \(\mu\)M \(\text{Cd}^{2+}\) in the external medium (\(n = 5\)). Together, these observations strongly suggest that the two components of currents activated by depolarizing pulses correspond to voltage-dependent \(\text{Ca}^{2+}\) currents.

Long duration \(\text{Ca}^{2+}\) spikes

A series of current clamp experiments was then performed to determine if the inward \(\text{Ca}^{2+}\) current, which develops in the presence of \(\text{K}^+\) channel blockers in the external medium, was able to elicit a regenerative all or none response. As described in Fig. 1 C, in the presence of a standard control medium injection of depolarizing currents of increasing amplitude induced graded responses (Fig. 3 B). When \(3\) mM 4-AP plus \(20\) mM TEA were added to the bath solution, a current injection of \(400\) pA amplitude led to the firing of a long lasting all or none action potential that overshot at \(+42\) mV. Subsequent addition of \(500\) \(\mu\)M \(\text{Cd}^{2+}\) to the external solution abolished the regenerative response, indicating that the inward \(\text{Ca}^{2+}\) current was responsible for the action potential firing.

Dihydropyridine sensitivity and \(\text{Ca}^{2+}\)-dependent inactivation of \(\text{Ca}^{2+}\) currents

In the presence of \(1\) \(\mu\)M nifedipine, a selective blocker of L-type voltage-dependent \(\text{Ca}^{2+}\) channels, partial inhibition of the peak, and the maintained component was observed (Fig. 4 A). In five cells, the average percentage of inhibition of the peak and of the steady state currents by \( \text{nifedipine} \) was \(56 \pm 5\% \) and \(47 \pm 6\% \), respectively. In two cells tested, the agonist dihydropyridine Bay K 8644, up to \(1\) \(\mu\)M, was unable to potentiate current amplitude (unpublished data). When \(\text{Ba}^{2+}\) was substituted equimolarly to external \(\text{Ca}^{2+}\), the amplitude of the maintained component was not altered, whereas the peak component reversibly disappeared (Fig. 4 B).

The transient aspect of the early peak component of the \(\text{Ca}^{2+}\) current strongly suggests that this current undergoes
Ca\textsuperscript{2+} transients in voltage-clamped cells

To investigate the physiological role of the voltage-gated Ca\textsuperscript{2+} current in *C. elegans* muscle cells, intracellular Ca\textsuperscript{2+} was measured simultaneously with the Ca\textsuperscript{2+} current. Fig. 6 A shows the muscle cell under study with the sealed recording pipette under transmitted light (Fig. 6 A, left) and a fluorescence view of the same field before (Fig. 6 A, middle) and during a depolarizing pulse to +10 mV, evoking a Ca\textsuperscript{2+} transient (Fig. 6 A, right). Fig. 6 B illustrates the membrane currents and the Ca\textsuperscript{2+} transients obtained from this voltage-clamped body wall muscle cell stimulated with 500 ms duration voltage steps of increasing amplitude. As illustrated in the above experiments, the Ca\textsuperscript{2+} current appeared at −30 mV, reached a maximum at +10 mV, and then decreased in amplitude for stronger depolarizations. A residual outward voltage-activated K\textsuperscript{−} current was present in the recordings likely because in this series of experiments the external solution corresponded to an *Ascaris* medium containing only 20 mM TEA and 3 mM 4-AP. It is clearly shown that the amplitude of the Ca\textsuperscript{2+} transient followed the one of the Ca\textsuperscript{2+} current; the Ca\textsuperscript{2+} transient appeared at −30 mV became maximal at −10 and +10 mV and decreased in amplitude thereafter. This decrease in the amplitude of the Ca\textsuperscript{2+} transient for high membrane potentials did not result from a run down process, since a maximal Ca\textsuperscript{2+} transient could again be elicited in response to voltage steps inducing a large inward Ca\textsuperscript{2+} current (unpublished data).
intracellular Ca$^{2+}$ increase and the Ca$^{2+}$ influx through the voltage-gated Ca$^{2+}$ channels.

**Voltage responses and Ca$^{2+}$ currents in egl-19 mutants**

We then investigated the electrophysiological properties of egl-19 mutants. Among the different mutant alleles of the egl-19 gene, we selected one reduction of function allele, n582, a flaccid mutation class causing a feeble muscle contraction phenotype originally described by Trent et al. (1983). Under current clamp conditions, spontaneous spike potentials could also be recorded in egl-19 mutant muscle cells. However, as shown in Fig. 7 A, where a spontaneous spike from a wild-type and an egl-19 muscle cell are superimposed, the rising phase of the voltage response was considerably slowed in the egl-19 cell. The mean maximal slope (dV/dt) of the rising phase of the spikes was 1.38 ± 0.11 V/s in wild-type cells (n = 6) and significantly lower (Mann-Whitney test, P = 0.0022), 0.63 ± 0.015 V/s, in egl-19 mutant cells (n = 6). A series of voltage clamp experiments on egl-19 mutant muscle cells did not reveal...
any apparent alteration of voltage-dependent K+ currents (unpublished data). On the other hand, using the same experimental conditions as the one used previously for Ca2+ current recordings, we found that egl-19 mutant muscle cells displayed strongly altered voltage-activated Ca2+ currents. Fig. 7 B shows that Ca2+ currents activated in response to depolarizing pulses in egl-19 cells did not display the fast transient peak observed in wild-type worm muscle cells. In the 13 egl-19 muscle cells tested, this component was never observed. Current traces also clearly indicated that the sole maintained component activated much slower than in the wild-type worm muscle (Fig. 7 C, inset). An exponential function was fitted to the rising phase of the current at –10, 0, +10, +20, and +30 mV, and the mean time constant of activation was plotted against membrane potential in wild-type and mutant cells (Fig. 7 C, inset). At –10 and +30 mV, $\tau$ was 0.95 ± 0.06 ms and 0.54 ± 0.06 ms in wild-type muscle (n = 11), whereas it was 3.4 ± 0.34 and 4.14 ± 0.37 ms for corresponding potentials in egl-19 mutant cells (n = 13); the time constants of activation of the Ca2+ current were significantly higher in mutant cells (Mann-Whitney test, $P = 0.0002$ for –10 mV and $P < 0.0001$ for other potentials). Moreover, activation became faster with increasing depolarization in wild-type muscle, whereas in egl-19 muscle activation slightly slowed with depolarization, suggesting that the voltage dependence of the time course of activation was lost. Fitting the steady state current-voltage relationship in egl-19 mutant muscle cells indicated mean values for $G_{\text{Max}}, V_{\text{rev}}, V_{0.5}$, and $k$ of 93 ± 9 S/F, $-57 ± 2$ mV, $+14.4 ± 1.3$ mV, and $8.9 ± 0.4$ mV, respectively (Fig. 7 D). Statistical analysis indicated that the significant effect observed in egl-19 mutants was a shift of 8 mV of $V_{0.5}$ toward positive potentials (Student’s unpaired $t$ test, $P = 0.0002$). This voltage shift is clearly shown in Fig. 7 E where the mean normalized conductance voltage relationships in wild-type and egl-19 mutant cells are presented.

Ca2+ currents in egl-19 mutant muscle cells were also found to be completely blocked by 500 μM Cd2+ and reduced to the same extent as in wild-type muscle by 1 μM nifedipine (unpublished data). Although substitution of Ba2+ for Ca2+ suppressed the peak component of the Ca2+ current in wild-type muscle, this maneuver had no marked effect on egl-19 Ca2+ currents, as expected, given the absence of such a component in egl-19 currents.

For voltage pulses eliciting a Ca2+ current of maximal amplitude, i.e., +10 and +20 mV in wild-type and mutant cells, respectively, the integral of the Ca2+ current was calculated. The mean value of the integral of the current was 0.816 ± 0.087 and 0.554 ± 0.071 C/F in wild-type and mutant cells, respectively. Thus, the loss of the early component of Ca2+ influx was accompanied by a significant lower amount of Ca2+ entering muscle cells in mutant compared with wild-type muscle (Student’s unpaired $t$ test, $P = 0.029$).

**Ca2+ transients in voltage-clamped egl-19 mutant cells**

Intracellular Ca2+ was also measured simultaneously with Ca2+ currents in voltage-clamped egl-19 mutant cells. Fig. 8 A illustrates the membrane currents and the Ca2+ transients obtained from an egl-19 mutant cell stimulated with 500 ms duration voltage steps of increasing amplitude. As observed in wild-type muscle cells, a close relationship was found between the Ca2+ transient and the Ca2+ current. The inset, displaying a Ca2+ current and a Ca2+ transient on the same time scale, also clearly shows that intracellular Ca2+ kept increasing, whereas the Ca2+ current developed and then stabilized upon termination of the voltage pulse. A marked discrepancy found in egl-19 muscle cells was that the threshold for Ca2+ current and Ca2+ transient was shifted toward positive membrane potentials with respect to wild-type cells. This result is clearly illustrated in Fig. 8 B where the mean normalized amplitude of the Ca2+ transient and the mean normalized value of the integral of the Ca2+ current are plotted as a function of membrane potential in wild-type and egl-19 mutant cells. Ca2+ influx and Ca2+...
transient both appeared at –30 mV in wild-type muscle cells, whereas neither Ca\(^{2+}\) influx nor Ca\(^{2+}\) transient could be detected at this membrane potential in egl-19 mutant cells (Fig. 8 A). For stronger depolarizations, the relationship between Ca\(^{2+}\) transient and membrane potential mirrored the relationship between Ca\(^{2+}\) influx and membrane potential in both cell types. The mean maximal amplitude of the Ca\(^{2+}\) transient in wild-type (ΔF/F = 1.34 ± 0.49 [n = 4]) and in egl-19 muscle cells (ΔF/F = 2.2 ± 0.64 [n = 5]) were found to be not significantly different (Student’s unpaired t test, P = 0.34). Finally, the sampling period of Ca\(^{2+}\) imaging (20 ms), during which Ca\(^{2+}\) currents fully activated in both cell types did not allow us to detect modification in the rate of rise of the Ca\(^{2+}\) transient in egl-19 mutant cells, possibly caused by the slowing of the activation of the Ca\(^{2+}\) current.

**Discussion**

**Ca\(^{2+}\) currents properties in C. elegans muscle**

In this study, using in situ patch clamp technique on dissected worms we give the first detailed description of voltage-dependent Ca\(^{2+}\) channel properties in body wall striated muscle cells from wild-type and egl-19 mutant nematodes. In wild-type muscle, in the presence of K\(^+\) channel blockers membrane depolarizations above –40 mV elicited Ca\(^{2+}\) inward currents, which peaked around +20 mV and reversed between +50 and +60 mV. These Ca\(^{2+}\) currents were characterized by two kinetically distinct components: a fast transient component lasting ~10 ms followed by a component maintained throughout the 200 ms depolarization step. At first sight, this could indicate the existence of two types of Ca\(^{2+}\) channels. However, we showed that the Ca\(^{2+}\) channel blocker of the dihydropyridine class, nifedipine, reduced to the same extent both peak and maintained component of the current. Our results are thus consistent with only one channel population exhibiting a biphasic time course of activation of the Ca\(^{2+}\) current. Moreover, steady state inactivation experiments demonstrated that the early decay in Ca\(^{2+}\) current was related on an inactivation process resulting from Ca\(^{2+}\) current flow. Conditioning voltage pulses eliciting Ca\(^{2+}\) entry led to an inhibition of the transient peak component of the inward current elicited by a subsequent test depolarization, suggesting that inactivation was due to accumulation of Ca\(^{2+}\) at the inner surface of the membrane. Our experiments performed in the presence of Ba\(^{2+}\) confirm our Ca\(^{2+}\)-dependent inactivation hypothesis. Indeed, in the presence of Ba\(^{2+}\), which is known to be less effective as intracellular Ca\(^{2+}\) in blocking the channel, inactivation was not observed. Additionally, the amplitude of the early peak component was reduced suggesting that Ba\(^{2+}\) does not conduct as well as Ca\(^{2+}\); this reduced permeation might be compensated by the reduced inactivation so that the maintained component remained unaffected in the presence of Ba\(^{2+}\). Ca\(^{2+}\)-dependent inactivation of high voltage threshold Ca\(^{2+}\) channels have also been reported in other invertebrate cells, for example, *Paramecium*, *Amphioxus* myotome, and *Aplysia* neuron or muscle (Brehm and Eckert, 1978; Tillotson, 1979; Benterbusch and Melzer, 1992; Brezina et al., 1994).

**Voltage-activated Ca\(^{2+}\) channels in C. elegans muscle belong to the L-type class**

The Ca\(^{2+}\) currents described in this study share the main characteristics of vertebrate voltage-activated Ca\(^{2+}\) currents belonging to the L-type class, i.e., high voltage threshold of activation, the presence of a long lasting phase of current, total blockade by Cd\(^{2+}\), and sensitivity to dihydropyridines (DHPs). However, Ca\(^{2+}\) currents in *C. elegans* muscle exhibit much faster activation and inactivation kinetics than in vertebrate preparations. Such rapid kinetics in the millisecond range seem nevertheless to be common features in invertebrate tissues and have been observed for example in *Amphioxus* myotome and *Aplysia* muscle (Benterbusch and Melzer, 1992; Brezina et al., 1994). We also found that Ca\(^{2+}\) currents in *C. elegans* muscle were less sensitive to the DHP antagonist nifedipine than mammalian L-type channels and insensitive to the DHP agonist BayK 8644. Such discrepancies in the pharmacological properties with vertebrate L-type channels were also found in *Amphioxus* myotome and *Aplysia* muscle (Benterbusch et al., 1992; Brezina et al., 1994), and more generally it is known that invertebrate channels classified as L-type channels on the basis of molecular and biophysical criteria may display marked pharmacological differences with vertebrate L-type channels (Kits and Mansvelder, 1996; Skeer et al., 1996; Jeziorski et al., 2000). Along this line, by investigating mutant *C. elegans* strains Lee et al. (1997) have cloned a gene, egl-19, that encodes a protein which exhibits structural similarity to the pore-forming α1 subunit of vertebrate muscle L-type voltage-activated Ca\(^{2+}\) channel. egl-19 was found to be expressed in body wall, pharyngeal, egg-laying, and enteric muscles. Our electrophysiological data strongly suggest that the voltage-gated Ca\(^{2+}\) channel displaying L-type properties here described corresponds to EGL-19, although the possible contributions of other calcium channels whose genes are known to be present in *C. elegans* cannot be excluded (Bargmann, 1998; Jeziorski et al., 2000).

**Mutation in the S4 segment in egl-19 mutants alters Ca\(^{2+}\) currents**

Null mutation in *egl-19* is lethal and was demonstrated to induce an embryonic arrest phenotype due to block of body wall muscle contraction (Williams and Waterston, 1994). Among viable egl-19 alleles, one loss of function mutation, *n582*, led to a flaccid phenotype characterized by feeble body wall muscle contraction (Trent et al., 1983; Lee et al., 1997). Interestingly, intracellular measurement of action potentials from pharyngeal terminal bulb muscle revealed that the rate of depolarization was reduced in *n582* animals (Lee et al., 1997). Our results obtained on patch-clamped body wall muscle cells demonstrate that voltage-activated Ca\(^{2+}\) currents are also strongly altered in *n582* mutants. First, the rate of current activation was dramatically reduced in mutants, and the voltage dependence of the time constant of activation was disrupted; this has to be paralleled to the reduced rate of depolarization observed in the body wall muscle spontaneous spikes but also in pharyngeal muscle action potentials (Lee et al., 1997). Second, the midpoint voltage (*V_{0.5}* of the conductance-voltage relationship was...
shifted by 8 mV in the depolarized direction. In n582 mutation, arginine 899 is changed to a histidine in the S4 transmembrane segment of the third repeat in the α1 subunit (Lee et al., 1997). The S4 transmembrane segment has been shown to function as a voltage sensor in voltage-gated L-type Ca\(^{2+}\) channels (Tanabe et al., 1987; Garcia et al., 1997; Yamaguchi et al., 1999). Significant shift to the right of the midpoint potential and lengthening of the time constant of activation were produced by mutations of S4 arginine to neutral or negative residues principally in the first and third repeats (Garcia et al., 1997). Similar perturbations in channel gating and loss of the voltage dependence of the activation time course were also observed with a mutation of an arginine to a histidine in the S4 segment of the fourth domain of the skeletal muscle L-type Ca\(^{2+}\) channel identified in families suffering from hypokalaemic periodic paralysis (Morrill and Cannon, 1999). Our data thus reinforces the notion that charged residues in the S4 segment play a fundamental role in Ca\(^{2+}\) channel activation by setting the voltage dependence of the channel.

**Physiological role of voltage-gated Ca\(^{2+}\) channels in *C. elegans* muscle**

No voltage-activated Na\(^+\) channel has been found in the *C. elegans* genome (The *C. elegans* Sequencing Consortium, 1998), which is consistent with the absence of such an activity in our voltage clamp recordings. In spite of the absence of voltage-activated Na\(^+\) channels, we found that *C. elegans* body wall muscle cells were able to generate spontaneous spike potentials in standard external saline. Such spontaneous activity has been recorded early on in somatic muscle spike potentials in standard external saline. Such spontaneous voltage-activated Na\(^+\) currents may have not only influence on *C. elegans* muscle excitability but also likely, itself, play a pivotal role in *C. elegans* body wall muscle function. Body wall muscle cells are polyinnervated both by excitatory acetylcholine and inhibitory γ-aminobutyric acid motor neurons (Lewis et al., 1980; McIntire et al., 1993a,b; Richmond and Jorgensen, 1999). As described in *Ascaris* muscle cells, it is plausible that opening of cholinergic receptors initiates graded action potentials at the level of the syncitium where synaptic inputs from motor neurons converged and graded action potentials next propagate electrotonically to the sarcomere-containing compartment of the muscle. Given the close proximity of sarcomeres to the plasma membrane, the resulting activation of voltage-gated Ca\(^{2+}\) channels may provide a Ca\(^{2+}\) influx sufficient to directly initiate contraction as described in many invertebrate muscle cells (Hagiwara and Byerly, 1981; Brezina et al., 1994; Kits and Mansvelder, 1996). Kerr et al. (2000) have imaged calcium transients in intact transgenic motor neurons that gates Ca\(^{2+}\) transients accompanied body wall muscle contractions. Here, for the first time, we used a Ca\(^{2+}\)-sensitive dye in *C. elegans* muscle and successfully recorded Ca\(^{2+}\) transients in voltage-controlled muscle cells. Our fluorescence experiments show that the amplitude of the Ca\(^{2+}\) transient depended on the magnitude of the inward current: Ca\(^{2+}\) transient appears at the voltage threshold of the Ca\(^{2+}\) current, is maximal at voltages inducing the largest currents, and becomes smaller with the reduced Ca\(^{2+}\) current at further depolarizations. Alternatively, Ca\(^{2+}\) entering through voltage-gated Ca\(^{2+}\) channels may also trigger a Ca\(^{2+}\)-induced release of Ca\(^{2+}\) from internal stores as well as defined in vertebrate cardiac cells and also already described in invertebrate crayfish muscle cells (Fabiato, 1983; Gýörke and Palade, 1992). Sarcoplasmic reticulum has indeed been identified in *C. elegans* body wall muscle cells as a network of vesicles that surround dense bodies, the site of attachment for thin myofilaments (Wood, 1988; Maryon et al., 1998). The ryanodine receptor, the ionic channel that gates Ca\(^{2+}\) release from the sarcoplasmic reticulum, has been characterized in *C. elegans* (Kim et al., 1992). This receptor is encoded by the unc-68 gene (Maryon et al., 1996; Sakube et al., 1997), and UNC-68 has been localized in body wall muscle cells to the vesicular structures assumed to represent the sarcoplasmic reticulum (Maryon et al., 1998). *unc-68* null mutants are defective in locomotion (Brenner, 1974) but exhibit only incomplete flaccid paralysis, suggesting that ryanodine receptor function enhances motility, but is not necessary in excitation-contraction coupling (Maryon et al., 1996). Consistent with our data, ryanodine receptors can then be thought to only act to amplify the Ca\(^{2+}\) signal that is...
mainly activated directly by the Ca\(^{2+}\) ions entering through the voltage-gated Ca\(^{2+}\) channels characterized in this study.

Finally, consistent with the n582 mutation Ca\(^{2+}\) imaging experiments on voltage-clamped egl-19 muscle cells indicated that the threshold for intracellular Ca\(^{2+}\) rise was shifted toward positive membrane potentials with respect to wild-type cells. The amount of Ca\(^{2+}\) entering egl-19 muscle cells was also found to be reduced by 30%. However, in terms of absolute peak-free Ca\(^{2+}\) concentration the consequence of a 30% reduction in total Ca\(^{2+}\) influx was expected to be hardly detectable under the present conditions due to limitations related both to the dye and to the complexity of the relationship between the rate of Ca\(^{2+}\) entry and the actual size of the free Ca\(^{2+}\) transient. Because of technical limitations, we were also unable to determine whether or not the rate of rise of the Ca\(^{2+}\) transient was decreased in egl-19 mutant cells. Nevertheless, the shift of the relationship between Ca\(^{2+}\) transient and membrane potential toward positive voltages may alone account for the flaccid phenotype that characterizes egl-19 mutants. We indeed demonstrated that C. elegans muscle cells develop spontaneous spike potentials of various amplitudes; depending on the potential size of the free Ca\(^{2+}\) entering the cell according to Attwell and Wilson (1980) is as follows in Eq. 2:

\[
\text{I}(V) = \frac{G_{\text{max}}(V - V_{rev})}{1 + e^{V_{th} - V}} + I_{\text{leak}}(V)
\]

where \(I(V)\) is the density of the current measured, \(V\) is the test pulse, \(G_{\text{max}}\) is the maximum conductance, \(V_{\text{th}}\) is the apparent reversal potential, \(V_{\text{leak}}\) is the half-activation voltage, and \(k\) is a steepness factor. Individual curves of the voltage dependence of the normalized conductance were obtained by dividing \(I(V)\) by \(G_{\text{max}}\), and \(V_{\text{leak}}\). The time constant of activation of the Ca\(^{2+}\) current was obtained by fitting a single exponential function to the current from the point where the charge of capacitance was completed to the peak of the current.

Voltage uniformity

Utrastructural studies suggest that body wall muscle cells are coupled via gap junctions (White et al., 1976, 1986). However, our Ca\(^{2+}\) fluorescence experiments indicated that only the cells patched and dialysed with fluo-3, which is known to cross gap junctions (Carter et al., 1996), emitted fluorescence even after 20 min of dialysis. The fact that fluo-3 never diffused to adjacent cells strongly suggests that muscle cells are not coupled under our experimental conditions.

Recordings have been made on ventromedial muscle cells adjacent to the ventral nerve cord that project short muscle arms, which minimizes space clamp problems. Nevertheless, an estimate of the spatial uniformity of the membrane voltage was made with the following assumptions: cells of 100 μm in length and 10 μm in diameter were considered to be analogous to a short cable with an internal resistance of 100 Ωcm. In the worst case, i.e., when a maximal K\(^{+}\) current of 5 nA amplitude developed, the input resistance drops to 20 MΩ. In that case, \(R_{\text{m}}\) was calculated to be 628 Ωcm\(^2\) and the space constant (λ) 560 μm. From linear cable theory, if a current is injected into one end of a cable of length \(L\) and space constant \(\lambda\), the other end being sealed, then the fractional variation of voltage along the cell according to Attwell and Wilson (1980) is as follows in Eq. 2:

\[
\Delta V/V = \frac{(1 + e^{\lambda/L} - 2e^{-\lambda/L})}{(1 + e^{\lambda/L})}
\]

Using the above values for \(L\) and \(\lambda\), we found that ΔV/V is 0.016, so the voltage can be considered as uniform and the cell isopotential.

Fluorescence measurements

For these experiments, muscle cells were not labeled with GFP. Cells were loaded with fluo-3 through the patch pipette, the dye being added at a concentration of 300 μM to the intrapietute solution. After whole cell voltage-clamp was established, fluo-3 was allowed to diffuse for 2 min before voltage steps were applied. Experiments were performed on an inverted microscope (Olympus IMT2) equipped for epifluorescence. Cells were imaged using a ×20 objective. Fluo-3 fluorescence was produced by excitation from a 100 W mercury-vapor lamp and an appropriate filter set (excitation, 450–480 nm; emission, above 515 nm, dichroic mirror 500 nm). Images from a region of interest (280 μm × 280 pixels) centered on the cell under study were captured with a Cool Snap charge-coupled device camera (Roper Scientific) at a frequency of 50 Hz. In some cases (as illustrated in Fig. 6 A), 4 × 4 binning was used to increase the image acquisition rate. Image acquisition and processing were performed using the MetaVue im-

Materials and methods

Strains

Experiments were performed on derivatives of the N2 reference strain and of strain MT1212, which carries the egl-19(n582) mutation. To identify body wall muscle cells more easily after dissection, both strains were marked by an extra chromosomal unc-54:gfp transgene, which promotes GFP expression in body wall muscles. In these strains, LS543 and LS716, respectively, most body wall muscles were strongly fluorescent. LS543 was obtained by microinjecting unc-54:gfp DNA in N2 at a concentration of 2 mg/ml of col-lagenase (Type 1; Sigma-Aldrich). Recording pipettes were sealed on ventral GFP-expressing body wall muscle cells except for experiments in Figs. 6 and 8 where muscle cells did not express GFP to avoid interference with Ca\(^{2+}\) fluorescence measurements. All experiments were performed at room temperature (21–24°C).

Electrophysiology

Membrane currents and membrane potentials were recorded in the whole cell configuration using a patch clamp amplifier (model RK 400; Bio-Logic). The resistance of recording pipettes was within 2–3 MΩ. Acquisition, generation of command voltage, and current pulse were done using the Biopatch software (Bio-Logic) driving an A/D, D/A converter (Lab Master Data Systems Inc., Mountain View, CA). Currents and potential differences were analyzed using Microcal Origin software (Microcal Software Inc.). Resistance and capacitance were not compensated. Cell capacitance was determined by integration of a control current trace obtained with a 10-mV depolarizing pulse from the holding potential. This capacitance was used to calculate the density of Ca\(^{2+}\) currents (A/P). Leak currents were subtracted from all recordings using a 10-mV depolarizing pulse from the holding potential supposing a linear evolution of leak current with depolarization. Individual curves of the voltage dependence of the Ca\(^{2+}\) current density were fitted with Eq. 1:
aging workbench (Universal Imaging Corp.). The mean intensity of fluorescence (F) was measured from an area of the cell away from the tip of the patch pipette. Background fluorescence was measured from an area distant from the studied cell and subtracted from the corresponding F. No attempt was made to convert the changes in fluo-3 fluorescence in terms of absolute changes in [Ca\(^{2+}\)]. Fluorescence values were expressed as ΔF/ F being the baseline fluorescence and ΔF the change in fluorescence from baseline.

**Solutions and chemicals**

For potential and current recordings in the whole cell configuration in the presence of standard saline (Fig. 1, Fig. 3 B, and Fig. 7 A), pipettes were filled with an internal solution containing (in mM) 120 KCl, 20 KOH, 4 MgCl\(_2\), 5 MgCl\(_2\), 3 4-AP, nifedipine, and cadmium were diluted to the required concentrations in the mouth of a perfusion tube from which flowed by gravity the rapidly exchanged solutions. Voltages were not corrected for liquid junction potentials, which were calculated to be lower than 5 mV with the presence of standard saline (Fig. 1, Fig. 3 B, and Fig. 7 A), pipettes were filled with an internal solution containing (in mM) 120 KCl, 20 KOH, 4 MgCl\(_2\), 5 MgCl\(_2\), 3 4-AP, and 10 Hepes, pH 7.2; pipettes contained (in mM) 140 NaCl, 5 KCl, 6 CaCl\(_2\), 5 MgCl\(_2\), 11 sucrose, and 5 Hepes, pH 7.2. For inward current recordings without fluorescence measurements, worm dissection was conducted in a modified Ascaris solution containing (in mM) 23 NaCl, 110 NaAc, 5 KCl, 6 CaCl\(_2\), 5 MgCl\(_2\), 11 sucrose, and 5 Hepes, pH 7.2. After dissection, the muscle cells were superfused with solution containing (in mM) 140 TEA-methanesulfonate, 6 CaCl\(_2\), 6 BaCl\(_2\) when mentioned, 5 MgCl\(_2\), 3 4-AP, and 10 Hepes, pH 7.2; pipettes contained (in mM) 140 CsCl, 4 MgATP, 5 TEACl, 5 EGTA, 5 TEA, 5 MES, pH 7.2. For inward current recordings coupled to fluorescence measurements, pipettes contained the Cs\(^+\)-rich solution without EGTA plus 300 μM of the Ca\(^{2+}\) dye fluo-3, and the bath solution contained an Ascaris Ringer plus 20 mM TEA and 3 mM 4-AP. Nifedipine (iCN Biochemicals) was dissolved in DMSO at a concentration of 10 mM. TEACl, 4-AP, nifedipine, and cadmium were diluted to the required concentrations in the bath solution. Cells were exposed to different solutions by placing them in the mouth of a perfusion tube from which flowed by gravity the rapidly exchanged solutions. Voltages were not corrected for liquid junction potentials, which were calculated to be lower than 5 mV with the different solutions used.

**Statistics**

Nonlinear least-squares fits were performed using a Marquardt-Levenberg algorithm routine included in Microcal Origin. Data values are presented as means ± SEM. Data were statistically analyzed using Student’s unpaired t test or Mann-Whitney test. Values were considered significant when P < 0.05.

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