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Delayed pharyngeal repolarization promotes abnormal calcium buildup in aging muscle

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ABSTRACT

In the pharynx of *Caenorhabditis elegans*, the accessory subunit MPS-4, homolog to human KCNE1, forms a complex with K^+ channel EXP-2 that terminates the action potential. An aspartate residue critical for KCNE1 function, asp76, is conserved in MPS-4 (asp74). Here, we studied the effects of D74N-MPS-4 on the aging pharynx. Electrophysiological studies showed that D74N delays pharyngeal repolarization. Pharynxes of transgenic worms expressing D74N exhibited higher levels of intracellular calcium compared to normal pharynxes. Accordingly, loss of pharyngeal function was accelerated in aging D74N worms. The pharyngeal action potential resembles the action potential that controls the mechanical activity of human left ventricle. Hence, these findings argue that the hearts of patients affected by delayed repolarization, a condition known as long QT syndrome, may experience dysregulated calcium homeostasis.

1. Introduction

The pharynx of *Caenorhabditis elegans*, is a peristaltic pump that sucks, grinds and transfers bacteria to the gut. Its mechanical activity is controlled by a plateau action potential that resembles the action potential in human left ventricle [1]. In both signals, the plateau phase is sustained by highly conserved L-type calcium channels [2,3]. The action potentials are terminated by K^+ currents conducted by channels, HERG and KCNQ1 in left ventricle and EXP-2 and KQT-3 in the pharynx, that share similar gating mechanisms even though they can be distantly related [4–10]. Moreover all these channels form complexes with accessory subunits of the KCNE family. KCNQ1 and HERG assemble with KCNE1 and KCNE2 whereas EXP-2 form a complex with MPS-4 the homolog of KCNE1 (KQT-3-MPS-4 interactions have not yet been demonstrated) [9–13]. These and other similarities have led to the hypothesis that the pharynx belongs to the evolutionary lineage of the human heart but this notion is controversial [14]. Nonetheless, whether the pharynx is an ancestor of human heart or they are the result of convergent evolution, the fact that they exhibit similar electrical activity argues that the pharynx can provide a system to study long-term effects associated with abnormalities in action potential. In fact, the short lifespan of *C. elegans* makes this worm a particularly useful system for studying aging.

In a previous study we found that a conserved mutation in MPS-4 (D74N), alters the conducting properties of EXP-2 in a fashion consistent with delayed pharyngeal repolarization [13]. Genetic mutations in the ion channels that orchestrate the activity of the left ventricle, as well as in their KCNE1 accessory subunits-including conserved D76N in KCNE1-cause delayed repolarization [11,15–17]. This condition is known as Long QT syndrome (LQTs) and predisposes to syncopes and ventricular tachyarrhythmias [15,16]. However, the effects of chronic delayed repolarization in aging cardiac muscle are not known.

In this study we investigated the effects of delayed repolarization on aging pharynx. We found that D74N acts to delay pharyngeal repolarization in a fashion that mimics the LQTs phenotype. D74N pharynxes exhibit age-dependent increase in intracellular calcium that correlates with accelerated loss of mechanical function. We conclude that in the pharynx of *C. elegans* a condition of chronically delayed repolarization causes progressive loss of function.

2. Methods

2.1. Genotyping

The cDNAs encoding wild type MPS-4 or D74N-MPS-4 were subcloned in the pPD118.33 Fire vector using Xma 1 restriction

Abbreviations: N2, bristol strain; MPS-4, MiRP K^+ channel accessory subunit 4; EXP-2, expulsion defective defecation 2; HERG, human ether-a-go-go-related-gene; LQTs, long QT syndrome; DAD, delayed afterdepolarization; <APd>, mean action potential duration; <APi>, mean action potential interval.

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sites, for selective expression in the pharynx under the *myo*-2 promoter ($P_{myo\text{-}2}$). We constructed the following strains: FDX(ses152): tm2596($P_{myo\text{-}2}$::MPS-4)(*rol-6*) or WT worm. FDX(ses153): tm2596($P_{myo\text{-}2}$::D74N)(*rol-6*) or D74N worm.

The constructs were injected into the syncytial gonads of adult tm2596 hermaphrodites. Transformant lines were stabilized by a mutagenesis-induced integration into a chromosome by irradiating 40 animals with γ-ray with 4000 rads for 40 min. The progeny were checked for 100% transmission of the marker and also for the presence of the transgene by PCR amplification. Integrated lines were crossed 4 times.

2.2. Age-synchronization

Nematodes were grown in standard 10 cm NGM plates + OP50 *Escherichia coli* until a large population of gravid adults was reached (3–5 days). The animals were collected in 50 ml Falcon tubes, washed in M9 buffer (22 mM KH_2PO_4, 22 mM NaH_2PO_4, 85 mM NaCl, 1 mM $MgSO_4$), and treated with 10 volumes of basic hypochlorite solution (0.25 M NaOH, 1% hypochlorite freshly mixed). Worms were incubated at room temperature for 10 min, then the eggs (and carcasses) collected by centrifugation at 400*g* for 5 min at 4 °C, incubated overnight in M9 buffer and seeded on standard NMG plates.

2.3. Electrophysiology

Data were recorded with an Axopatch 200B (Axon) a PC (Dell) and Clampex software (Axon) and filtered at f_c = 1 kHz and sampled at 2.5 kHz. Agar bridges were used throughout this study. The heads of the animals were chopped from age-synchronized worms using a 25 gauge needle and transferred to the recording chamber in the electrophysiological set up, using a Pasteur pipette and held in place with a suction electrode. The pharynx was continuously perfused with a solution containing: 6 mM KCl, 140 mM NaCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES pH = 7.5 with NaOH and 5 μM 5-HT to stimulate autonomic pharyngeal activity. A second intracellular electrode filled with 2 M KCl was used to record the electrical activity of the pharynx in current-clamp.

Continuous recordings of pharyngeal electrical activity were analyzed using the half-threshold method [18]. Histograms of the time between two consecutive action potentials and action potential duration were computed using Clampfit 9.2 software (Axon) and fitted to a single Gaussian distribution:

$$A\exp\left[-\left(\frac{t-t_o}{\sigma}\right)^2\right] \quad (1)$$

where A is a constant, σ is the variance and t_0 the time at which the Gaussian is maximal.

2.4. Fura-2-AM emission measures

Pharynxes were dissected from age-synchronized worms as described and transferred to a 5 mL test tube containing M9 buffer and centrifuged at 5000 rpm for 1 min. The supernatant was gently aspired and replaced with fresh M9 buffer and this cycle was repeated 2 times. 30 μM Fura 2-AM + Pluronic F-127 (1:1 ratio) was added to the test tube and incubated at 37 °C for 1 h and then left at room temperature for 15 min. Pharynxes were centrifuged at 7000 rpm for 1 min and washed in M9 buffer three times. Pharynxes were immobilized on a 2% agarose pad on a glass slide and quickly mounted on the stage of an inverted microscope (Nikon TE 200). Fura-2 emissions were measured with a dual-wavelength spectrofluorometer (Photon Technology International, Inc.) with excitation wavelengths at 350 nm and 390 nm and emission at 510 nm.

2.5. Pumping measurement

Worms were observed under a stereomicroscope and their pharyngeal activity was monitored by eye. Experiments were performed without knowledge of the worms' genotype.

2.6. Statistics

Data are indicated as mean ± S.E. The number of determinations is indicated by *n*. Student's *t*-test and coefficients of correlation were calculated using Excel routines. A level of confidence $P \leqslant 0.05$ was assumed as statistically significant.

3. Results

Electrophysiological studies of EXP-2-D74N channel complexes expressed in mammalian cells showed that D74N shifts the half-maximal voltage of activation ($V_{1/2}$) by ~20 mV to the right and slows down inactivation kinetics [13]. This argues that D74N may impair pharyngeal repolarization by (1) reducing the number of EXP-2 channels primed to conduct at the end of the plateau phase and (2) prolonging the refractory period. To test this idea we recorded the electrical activity in pharynxes of transgenic worms expressing wild type or D74N in a *mps-4* KO background (for simplicity, we refer to these worms or pharynxes simply as WT and D74N). Representative electrical recordings from dissected pharynxes of 4 day old worms are shown in Fig. 1A. We calculated the mean amplitude, mean duration of the action potential (<APd>) and mean duration of the interval separating two action potentials (<APi>) using the half-threshold method as done before [13,18]. <APd> and <APi> histograms calculated from the recordings in Fig. 1A are shown in panel B of the figure (histograms were calculated over the entire traces, 100 and 400 s, respectively). Relevant statistical quantities of this analysis are listed in Table 1. Thus, action potentials were 20% broader in D74N than wild type pharynxes. Firing frequency was 10% lower in the D74N pharynx whereas the amplitude of the signal was not altered. Duration, frequency and amplitude of the action potential were comparable in wild type and parental (N2) pharynxes [13]. This indicated that the rescue of wild type MPS-4 in the *mps-4* KO background restored normal electrical activity in the pharynx. In a previous study we showed that genetic ablation of *mps-4* impairs EXP-2 trafficking to the plasma membrane [13]. The reduced availability of EXP-2 channels in the *mps-4* KO pharynx causes irregular rhythm, action potentials of variable lengths and delayed after depolarizations (DADs) which are reflected in larger <APd> and <APi> values [13]. Therefore, the *mps-4* KO pharynx was used as positive control throughout this study. The fact that the action potential is broader in D74N and *mps-4* KO pharynxes, argues that more calcium than normal goes in these muscles during each excitatory period. To test this idea we assessed the levels of intracellular calcium in pharynxes of the various genotypes using Fura-2-AM fluorescence. Typical measurements in pharynxes of 8 day-old worms are shown in Fig. 2A. Results of measurements in 4, 8 and 12 day old pharynxes are summarized in Fig. 2B. Notably, the levels of intracellular calcium progressively increased in aging pharynxes irrespective of their genotypes (coefficients of correlation were in the 0.99–0.95 range). However, the absolute amounts of calcium were larger in D74N and *mps-4* KO pharynxes even in young worms (+24% and +41% compared to WT, in 4 day-old worms, for D74N and *mps-4* KO ($P < 0.034$) respectively, Fig. 2B). Calcium is critical for muscle contractility. This implies that the high concentrations of calcium

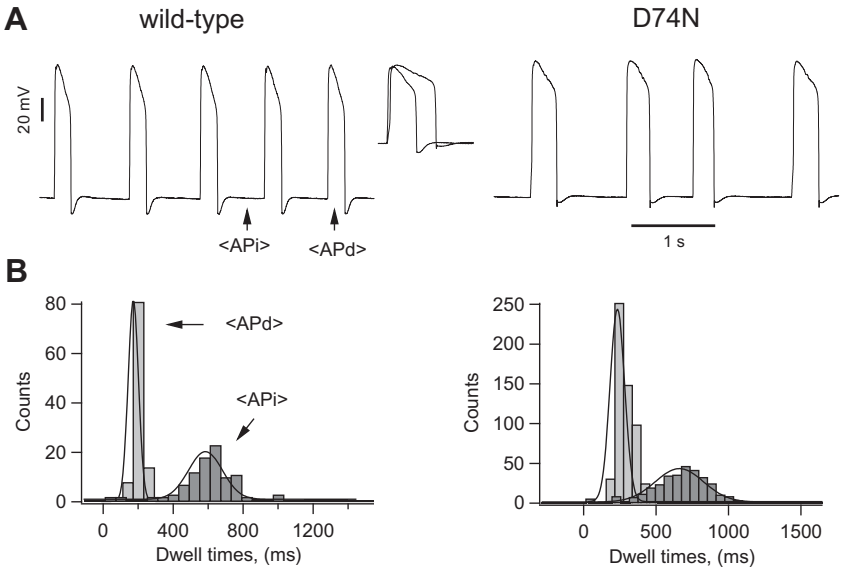

Fig. 1. Electrophysiological characteristics of the D74N action potential. (A) Representative electrical activities of WT and D74N pharynxes. Typical WT and D74N action potentials are superimposed in the *inset*. (B) Dwell time histograms of the traces shown in A (computed over the entire traces, 100 and 400 s respectively). Traces were idealized with the half-threshold algorithm and histograms were fitted to a single Gaussian function Eq. (1).

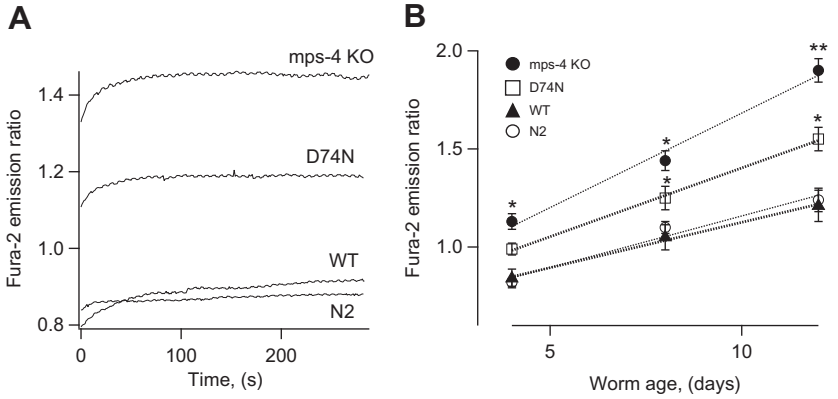

Fig. 2. Intracellular calcium levels in aging pharynxes. (A) Representative Fura-2 emission ratio measurements in the pharynxes of the indicated genotypes. Pharynxes were dissected from 8 day-old animals. (B) Increase in intracellular calcium in the pharynxes of the indicated genotypes at the indicated time points. Linear fit of the data gave slopes of: 0.056 ± 0.01, 0.55 ± 0.01, 0.064 ± 0.07 and 0.1 ± 0.01 Fura-2 emission ratio/day, in, respectively, N2, WT, D74N and *mps*-4 KO pharynxes. These differences were not statistically significative (coefficients of correlation varying from 0.99 to 0.95). $n \geqslant 5$ pharynxes/genotype/time point. Pharynxes were dissected from age-synchronized animals and loaded with Fura 2-AM. Intracellular calcium was expressed as the ratio of the Fura-2 emissions at 350 nm and 390 nm excitation wavelengths [21]. Statistically significant differences from control were calculated with Student's *t*-test and are indicated by $^{*}P < 0.05$ or $^{**}P < 0.001$.

in D74N and *mps-4* KO pharynxes may cause loss of function. To test this hypothesis we calculated the number of pharyngeal contractions per minute (pumping rate), which is a standard measure of pharyngeal function. Pumping rate declined in all genotypes during aging, a result largely expected (Fig. 3). However, D74N and *mps-4* KO pharynxes exhibited pumping rates that were significantly lower compared to control, irrespective of animal's age. (See Table 1).

4. Discussion

To gain insights into the long-term effects that abnormalities in repolarization exert on muscle function, we studied the pharynx of a transgenic *C. elegans* worm expressing a conserved aspartate to asparagine replacement (D74N) in the accessory subunit MPS-4. We found that D74N pharynxes exhibit delayed repolarization, abnormal levels of intracellular calcium and impaired mechanical function.

In all genotypes tested in this study, the levels of intracellular calcium increased at a constant rate during aging. Neither replacement of wild type with D74N, nor genetic ablation of the *mps-4* gene, significantly accelerated calcium buildup. We conclude that pharyngeal muscle is subject to a natural process of calcium accumulation during aging. On the other hand, calcium is crucial for muscle contractility in both vertebrates and invertebrates and it is therefore not coincidental that calcium buildup correlated with progressive loss of mechanical function. Evidence suggests that also in aging mammalian skeletal muscle, changes in ion content, especially Ca^{2+}, may be important (reviewed in Ref. [19]). Our observations corroborate the idea that calcium homeostasis may constitute a key factor for the physiology of aging muscle but also challenge current thinking that predicts that calcium decreases during aging. Pharyngeal depolarization is entirely sustained by calcium currents conducted by T-type (upstroke) and L-type (plateau) calcium channels [20]. The genes that encode these channels are not mutated in the D74N pharynx, nor there is evidence that

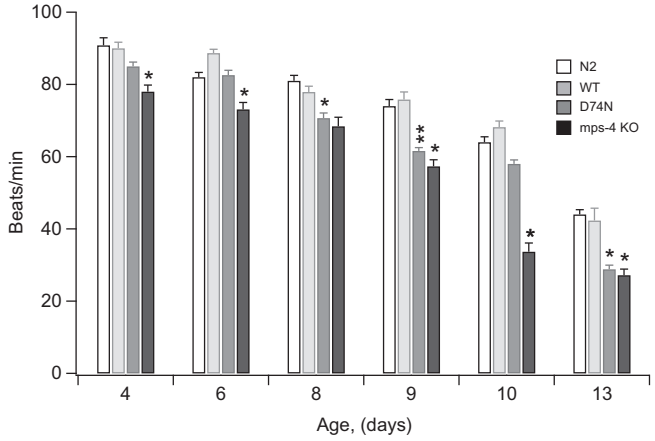

Fig. 3. Loss of mechanical function in aging pharynxes. Normalized mean number of pharyngeal contractions/min in N2, WT, D74N and *mps*-4 KO animals during aging. Statistically significant differences from control were calculated with Student's *t*-test and are indicated by $^{*}P < 0.05$ or $^{**}P < 0.001$.

Table 1
Action potential characteristics.

	Wild type	D74N
<A>, (mV)	82 ± 4	81 ± 4
<APd>, (ms)	179 ± 17	214 ± 19 ($P < 0.033$)
<APi>, (ms)	559 ± 39	619 ± 47
n	5	5

The mean amplitude (<A>), action potential duration (<APd>) and mean interval between two consecutive action potentials (<APi>) were calculated using the half-threshold method [18]. Statistically significant differences from WT genotype were calculated with the Student's *t*-test and are indicated.

MPS-4 assembles with their products [13]. Therefore by hampering K^+ current that repolarizes the pharynx and indirectly, augmenting calcium influx during the plateau phase, D74N acted to increase calcium buildup in the pharynx. Accordingly, impairment of mechanical function was faster in the D74N pharynx. A question that remains open is whether L-type current is also the main source of calcium in physiological buildup. While this possibility cannot be ruled out it appears unlikely. It is more probable that D74N acted on top of physiological mechanisms to build up calcium in pharyngeal muscle. In summary, a condition of prolonged depolarization promoted excess accumulation of calcium ions in the pharyngeal muscle thereby accelerating the loss of mechanical function.

The electrical phenotype of the D74N pharynx mimics the LQTs phenotype in human left ventricle. This naturally raises the question of whether and to which extent, the pharynx may provide a system to understand cardiac muscle. Since in both organs the plateau phase of the action potential is sustained by calcium currents conducted by conserved L-type calcium channels (the homology between Cav1.2 and EGL-19 is 87%), also the heart of patients with long QT syndrome must be subject to dysregulated calcium homeostasis. Therefore the question that these results pose is how cardiac muscle in LQTs, responds to chronically dysregulated calcium homeostasis. Little is known about how calcium levels change in hearts of patients affected by LQTs, nor it has been ascertained if cardiac contractility declines faster in these patients. Understanding these mechanisms might unveil new therapeutic insights.

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