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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