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Spontaneous calcium transients manifest in the regenerating muscle and are necessary for skeletal muscle replenishment

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ABSTRACT

Tissue regeneration entails replenishing of damaged cells, appropriate cell differentiation and inclusion of regenerated cells into functioning tissues. In adult humans, the capacity of the injured spinal cord and muscle to self-repair is limited. In contrast, the amphibian larva can regenerate its tail after amputation with complete recovery of muscle, notochord and spinal cord. The cellular and molecular mechanisms underlying this phenomenon are still unclear. Here we show that upon injury muscle cell precursors exhibit Ca^{2+} transients that depend on Ca^{2+} release from ryanodine receptor-operated stores. Blockade of these transients impairs muscle regeneration. Furthermore, inhibiting Ca^{2+} transients in the regenerating tail prevents the activation and proliferation of muscle satellite cells, which results in deficient muscle replenishment. These findings suggest that Ca^{2+}-mediated activity is critical for the early stages of muscle regeneration, which may lead to developing effective therapies for tissue repair.

1. Introduction

Tissue regeneration relies on replenishing of cells, appropriate cell differentiation and reestablishment of intra- and inter-tissular organization and structure. A powerful model for studying the mechanisms underlying tissue regeneration is the amputated-tail *Xenopus* larva [1]. Following tail amputation, *Xenopus* tadpoles are capable of regenerating the three main axial structures of the tail: spinal cord, notochord and skeletal muscle [2–7].

Muscle satellite cells, a population of undifferentiated mononuclear myogenic cells, are found between the cell membrane and basal membrane of the individual muscle fibers [8]. Satellite cells are present in mammalian, reptilian, and avian skeletal muscle [9–12]. Following amputation, activated satellite cells reenter mitosis and begin to express myogenic transcription factors [13]. Subsequently, the myogenic precursor cells undergo terminal differentiation and replenish the skeletal musculature. Although satellite cells are important in muscle regeneration, the mechanisms that lead to their activation remain unclear.

The cellular processes that take place during tail regeneration resemble early development when neural and muscle cells are being generated and differentiate into functional phenotypes. One developmental cue relevant during early differentiation for both neural and muscle cells are Ca^{2+} transients that occur spontaneously in embryonic cells [14]. Perturbations of this Ca^{2+}-mediated electrical activity lead to changes in neuronal differentiation [15–19]. Developing *Xenopus* muscle cells also exhibit spontaneous Ca^{2+} transients mediated by release from intracellular stores that are important for skeletal muscle cell differentiation and maturation [20]. The necessity for Ca^{2+}-mediated activity during mammalian development has also been shown. A reduction in neuronal proliferation is observed when Ca^{2+} wave propagation through embryonic rat radial glia is disrupted [21]. Also, mouse cortical neuron migration is regulated by GABA-mediated Ca^{2+} signaling [22,23].

Electrical activity may play an active role in tissue regeneration and has been implicated in *Xenopus* tail regeneration. V-ATPase H^+ pump, which is specifically upregulated in existing wound cells shortly after amputation, is necessary for successful tail regeneration. H^+ flux triggers changes in membrane potential, which are required for initiating tail regeneration [24]. Moreover, a voltage-gated channel-mediated Na^+ transport is necessary for successful tail regeneration [25]. Whether the Ca^{2+}-mediated activity observed in early development is recapitulated during this regeneration process has not been studied.

Here we show that Ca^{2+} transients spontaneously manifest in regenerating muscle cells soon after tail amputation. These transients are mediated by ryanodine receptor-operated Ca^{2+} stores and are apparent during the first 20 h post amputation. Blockade of spontaneous Ca^{2+} transients impairs muscle regeneration

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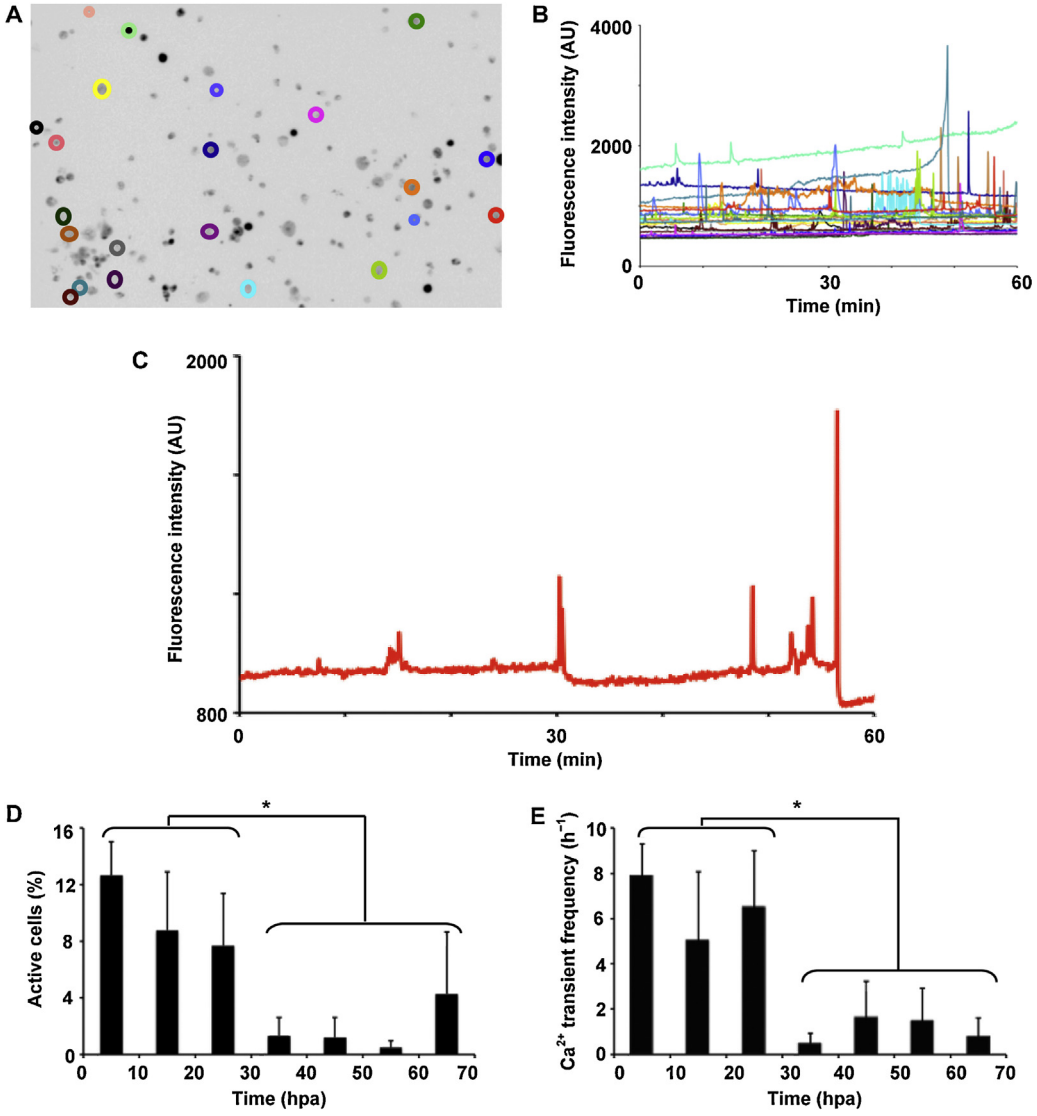

Fig. 1. The regenerating tail exhibits Ca^{2+} transients during the first day post amputation. Regenerating tail from amputated tadpole was dissociated and cells were cultured, loaded with Fluo4-AM and Ca^{2+} imaged for 1 h at 0.2 Hz acquisition rate. (A) Image shows a representative Ca^{2+}-imaged field of view from a 6-h post amputation (hpa) tadpole. Cells showing Ca^{2+} transients during 1 h recording are outlined. (B) Traces of changes in fluorescence intensity for the outlined cells in (A). (C) Representative trace of changes in fluorescence intensity corresponding to one of the active cells outlined in (A). (D and E) Data show mean ± SEM percent of active cells (D) and Ca^{2+} transient frequency (E) during 1 h recording, after different hours post amputation. $^{*}p < 0.05$, $n \geq 5$ per time bin.

of the amputated tadpoles. Activation of muscle satellite cells is inhibited in ryanodine-treated amputated tadpoles suggesting that Ca^{2+} transients in the regenerating musculature are necessary for recruiting muscle satellite cells for tissue repair.

2. Materials and methods

2.1. Amputation procedure

Stage 37–39 (2.2–2.4 days post fertilization) *Xenopus laevis* tadpoles were anesthetized with 0.2% tricaine methanesulfonate and amputated under a dissection stereoscope using a scalpel blade at the point where the tail begins to taper. The anesthetic was washed out and the amputated tadpoles were incubated in 10% Marc's Modified Ringer's (MMR in mM: 10 NaCl, 0.2 KCl, 0.1 $MgSO_4$, 0.2 $CaCl_2$, 0.5 Hepes, 0.01 EDTA; pH to 7.8), with or without 5–50 μM ryanodine, 0.5% DMSO or 0.5% ethanol, at 22 °C for up to 3 days. Tadpoles were cultured at a density of 6 ml^{-1}, sufficient for normal growth and robust regeneration.

2.2. Ca^{2+} imaging

Regenerating tissues of amputated tadpoles were dissociated with 1.5 mg/ml Proteinase K. Cells from the regenerating tissues were collected from amputated tadpoles at various time points during 2–73 h post amputation (hpa) and plated in saline (in mM: 117 NaCl, 0.7 KCl, 1.3 $MgSO_4$, 2 $CaCl_2$, 4.6 Tris, pH 7.8). After 30 min cells were loaded with 1 μM Fluo4-AM (Life Technologies, Inc.) and imaged with a Nikon swept-field confocal microscope at an acquisition rate of 0.2 Hz for 1 h [26,27].

To assess the mechanisms mediating Ca^{2+} transients in regenerating tissues, cells were Ca^{2+}-imaged in the presence of either saline only, vehicle (0.5% DMSO or 0.5% ethanol), Ca^{2+}-free saline or 5–50 μM ryanodine (Tocris, pre-incubated for 30 min). We

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