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

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Summary

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].

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Conflict of Interest

The authors have no conflict of interest to report.

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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 re-enter 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 hours post amputation. Blockade of spontaneous Ca^{2+} transients impairs muscle regeneration 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 μ M 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, 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 hours 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 μ M 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 μ M ryanodine (Tocris, pre-incubated for 30 min). We performed a paired comparison of Ca^{2+} activity before and after addition of any agent.

2.3. Blockade of Ca^{2+} -mediated activity in amputated tadpoles during regeneration

Amputated tadpoles were incubated in vehicle only (0.5% DMSO or Ethanol) or 5–50 μ M Ryanodine. Compounds were dissolved in 10% MMR and applied immediately after amputation. Following treatment, tadpoles were fixed at 24, 48, and 72 hpa in MEMFA (in mM: 100 MOPS, 2 EGTA, 1 MgSO_4 , and 3.7% formaldehyde).

2.4. Wholemount immunostaining

Samples were fixed in MEMFA at 4°C overnight and washed with phosphate buffer. To clear pigmented cells for enhanced visualization of wholemount immunostaining, samples were incubated in 1:2 Dent's fixative/ H_2O_2 at 23°C overnight. Following this procedure, samples were washed, permeabilized with 0.5% Triton X100 and incubated with 1:200 anti-12101 (Developmental Studies Hybridoma Bank) for labeling skeletal muscle, followed by secondary antibody overnight at 4°C.

2.5. Quantitative assessment of muscle regeneration

The extent of muscle regeneration was determined by measuring the surface area of the regenerated muscle labeled with anti-12101 in whole-mount immunostained samples. The area was estimated by thresholding the labeled image and by using particle analysis from Image J (NIH software) [28]. Although 12101 labels both preexisting myotomes anterior to the amputation site and differentiated muscle cells in the regenerating tail we only measure the area covered by regenerated muscle by tracing a region of interest (ROI) posterior to the amputation using the limits of the most posterior organized myotome (not present in regenerating tissue, [29]) as the anterior limit of the polygonal ROI and the tip of the tail as the posterior limit (see Fig. 3B).

2.6. Immunostaining of tissue sections

As previously described [16, 26–28]. Samples were fixed with 4% PFA overnight at 4°C, washed, dehydrated through a graded ethanol series and embedded in paraffin. 10 μ m-sections were rehydrated and antigen retrieval was performed by immersing sections in 10

mM sodium citrate buffer and boiling them for 8 min in a standard microwave. Sections were cooled for 30 min before incubation with primary and secondary antibodies overnight at 4°C and for 2 h at 23°C, respectively. Primary antibodies used: Pax7 1:75 (R&D Systems), MyoD 1:20 (Santa Cruz Biotechnology), PCNA 1:300 (Sigma). Secondary antibodies used were Alexa Fluor 488, 568 and 647 (Life Technologies, Inc.). Samples were imaged using an epifluorescence, relieve contrast Olympus microscope equipped with a Xenon lamp and appropriate filters for the fluorophores mentioned above. Negative controls were done by incubating secondary antibodies only. Staining profile was comparable to that obtained by imaging the immunostained samples in a point laser Nikon A1 confocal microscope. All these controls support specific and reliable immunostaining with each of the antibodies used in this study.

2.7. Data collection and Statistics

At least 5 samples were analyzed for each group from at least 3 independent clutches of embryos. Statistical significance was determined with paired or unpaired t-test or ANOVA, differences were considered significant when $p < 0.05$. Traces of Ca^{2+} dynamics were obtained using NIS Elements time-lapse measurement (Nikon, Inc.).

3. Results

The developing axial musculature of the *Xenopus laevis* exhibits spontaneous Ca^{2+} transients during the transitory period from muscle cell progenitor to differentiated muscle fiber [30]. The maturation of the axial musculature follows a rostro-caudal progression which is accompanied by the progressive disappearance of spontaneous Ca^{2+} transients [20]. These studies recorded Ca^{2+} transients from embryos at the beginning of somitogenesis (stage 23) throughout a 1-day period (stage 33), when spontaneous Ca^{2+} transients cease [20].

We then assessed whether injury elicits Ca^{2+} transients in the regenerating tissue. Cells in the regenerating tail of amputated stage 37–39 tadpoles, exhibit Ca^{2+} transients with frequencies of $1\text{--}56\text{ h}^{-1}$ (Fig. 1). These Ca^{2+} transients are apparent as early as 2 h post-amputation (hpa) and cease after 24 hpa (Fig. 1C–D). Immunostaining of Ca^{2+} -imaged cells for the skeletal muscle marker 12101 reveals that most of the cells exhibiting activity during the first 10 hpa are immature muscle cells because they are immunopositive for the muscle marker but they do not have mature muscle cell morphology (Fig. 2A–B). The transients in muscle cells are still apparent when extracellular Ca^{2+} is eliminated and are completely abolished by blockade of ryanodine receptors (Fig. 2C) suggesting that these spontaneous transients are solely mediated by Ca^{2+} release from intracellular stores.

Skeletal muscle regeneration consisting of newly differentiated muscle cells becomes apparent 72 hpa (Fig. 3). We incubated amputated tadpoles right after amputation with ryanodine and compared tail and muscle regeneration in control and treated tadpoles. Results show that blockade of ryanodine receptors impairs tail and muscle regeneration without affecting the integrity and gross morphology of the preexisting and intact mature muscle tissue (Fig. 3).

The regeneration of skeletal muscle depends on the activation of muscle satellite cells, their proliferation and subsequent differentiation to replenish the missing myotomes. Immunostaining of coronal sections of amputated tadpoles 24 and 48 hpa shows that this period coincides with active cell proliferation (PCNA-immunolabeling) in the regenerating tail, including that of muscle satellite (Pax7 immunopositive) and muscle progenitor (MyoD immunopositive) cells (Fig. 4). In contrast, in tadpoles incubated with ryanodine the overall presence of proliferating cells in the regenerating tail is reduced, evidenced by the significant decrease in PCNA+ cells. In particular, the proliferation of muscle precursor cells, Pax7+/PCNA+, Pax7+/PCNA+/MyoD+ and MyoD+/PCNA+, is inhibited in ryanodine-treated amputated tadpoles (Figs. 5 and 6). These results suggest that spontaneous Ca^{2+} transients in the regenerating tissues are necessary for the activation and proliferation of muscle satellite cells during the early stages of muscle regeneration (Fig. 6).

4. Discussion

In this study, we show that Ca^{2+} transients manifest in cells of regenerating tissues and specifically the regenerating muscle and demonstrate that these spontaneous Ca^{2+} dynamics are mediated by Ca^{2+} release from stores. Furthermore, this study indicates that Ca^{2+} release from ryanodine receptors is necessary for the activation and proliferation of muscle satellite cells and muscle cell precursors, which give rise to regenerated muscle tissue (Fig. 6C). Our findings reveal an important function for Ca^{2+} -mediated activity during regeneration, particularly in the early phases of muscle repair.

Immature muscle cells exhibit Ca^{2+} transients during the embryonic development and these transients cease once muscle cells differentiate and mature. Abolishing these Ca^{2+} dynamics disrupts muscle development [20, 30, 31] and extending artificially the presence of these transients also impairs sarcomere assembly and muscle cell maturation [32]. Our findings support a similar mechanism for Ca^{2+} -mediated activity during muscle regeneration. The frequency of these Ca^{2+} transients is comparable to the one exhibited by embryonic muscle cells, they are solely dependent on intracellular Ca^{2+} stores, and their appearance is also transitory. The spontaneous Ca^{2+} dynamics in the regenerating tail are largely apparent within the first 24 hpa, a time window when the damaged tissue is cleared up and progenitor cells are activated to replenish the missing tissues [24, 25, 33, 34] but the regenerative process itself is far from finished. This suggests that Ca^{2+} -mediated activity is important for the initial cellular processes of muscle regeneration. More specifically, we show that these injury-elicited Ca^{2+} transients are critical for the activation and proliferation of muscle satellite cells and muscle cell progenitors. This coincides with previous findings that show that disruption of Ca^{2+} dynamics decreases proliferation of radial glial neuronal precursors in the cortical ventricular zone [21] and proliferation of cerebellar granule cell progenitors [35, 36]. The failed proliferation of muscle satellite cells in the initial phases of regeneration subsequently results in reduced number of differentiated muscle cells in the regenerating tail, leading to a significant decrease in regenerated muscle at 72 hpa.

Blockade of ryanodine receptors affects overall cell proliferation in the regenerating tail. This could be due to the dependence on Ca^{2+} dynamics for the proliferation of cell precursors of all regenerating tissues in the tail. Indeed, skin repair after wound healing in

Xenopus embryos and skin homeostasis in human cells and mice depend on Ca^{2+} stores for the enhancement of skin cell proliferation [37, 38]. Similarly, neural cell precursor proliferation is regulated by Ca^{2+} dynamics [21, 35, 36]. Alternatively, inhibiting Ca^{2+} transients in certain tissues such as the regenerating muscle may impair muscle precursor cell proliferation and muscle regeneration and this, in turn, may have a negative impact on the cell proliferation and regeneration of other tissues. Supporting the idea of partnership among regenerating tissues, it has been shown that successful tail regeneration depends on the presence of the spinal cord [39].

An essential feature of muscle regeneration is the activation of muscle satellite cells. Our study reveals that Ca^{2+} release from ryanodine-operated stores is required for the activation and proliferation of muscle satellite cells during regeneration. Other molecular mechanisms involved in muscle satellite cell activation include Cripto, a member of the EGF-CFC GPI-anchored/extracellular family of proteins, that regulates early embryogenesis [40] and also regulates muscle regeneration by promoting myogenic cell proliferation [41]. It appears that several signaling pathways involved in regulating muscle satellite cells and muscle regeneration are shared with early developmental events [41–44]. This reinforces the notion that molecular mechanisms triggered by injury and involved in regeneration may be recapitulated from embryonic morphogenesis. Furthermore, the described pathways may potentially interplay with one another to promote muscle satellite cell activation. The ras/raf/MEK/MAPK pathway plays a critical role in myogenic differentiation and muscle regeneration [45] and activators of this pathway include membrane depolarization and Ca^{2+} influx [46]. Intriguingly, Cripto has been shown to activate intracellular components in the ras/raf/MEK/MAPK pathway [47]. Ca^{2+} dynamics may potentially crosstalk with Cripto to trigger the activation of the ras/raf/MEK/MAPK pathway to regulate gene expression during muscle regeneration. The precise signaling pathways triggered by Ca^{2+} dynamics as well as the potential pathways that activity may interact with during regeneration, remain largely unknown and warrant further investigation. Increased levels of reactive oxygen species shown to trigger Wnt signaling, and necessary for increasing cell proliferation soon after amputation in the tadpole regenerating tail [48] are good candidates for inducing Ca^{2+} dynamics and the activation of muscle satellite cells.

Our results may help to understand the beneficial effects of functional electrical stimulation for the regeneration of atrophic muscle in humans [49].

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Fig. 1.

The regenerating tail exhibits Ca²⁺ transients during the first day post amputation. Regenerating tail from amputated tadpole was dissociated and cells were cultured, loaded with Fluo4-AM and Ca²⁺ imaged for 1 h at 0.2 Hz acquisition rate. (A) Image shows a representative Ca²⁺-imaged field of view from a 6-h post amputation (hpa) tadpole. Cells showing Ca²⁺ 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–E) Data show mean $\pm$ SEM percent of active cells (D) and Ca²⁺ transient frequency (E) during 1 h recording, after different hours post amputation. * $p < 0.05$, $n = 5$ per time bin.

Fig. 2.

Regenerating muscle cells exhibit Ca^{2+} transients during the first hours 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 followed by immunostaining against the muscle marker 12101. (A) Image shows a representative Ca^{2+} -imaged and then immunostained region of field of view. Cells showing Ca^{2+} transients during 1 h recording are outlined in red. Green indicates 12101-immunopositive cells. (B) Data show mean $\pm$ SEM percent of active muscle cells during the first 10 hpa. (C) Ca^{2+} -imaged cells for 30 min were perfused with Ca^{2+} -free saline, 5–50 μM ryanodine or vehicle only and imaged for another 30 min followed by immunostaining with the muscle marker 12101. Results show mean $\pm$ SEM percent of active muscle cells compared to the 30 min before addition of treatment. * $p < 0.05$, $n = 5$ per group.

Fig. 3. Blockade of Ca^{2+} transients impairs tail regeneration and muscle replenishment. (A–C) Amputated tadpoles were incubated in vehicle or 50 μM ryanodine for 72 hpa, fixed and photomicrographed (A) followed by whole-mount immunostaining against the muscle marker 12101 (B–C). (B) Maximum intensity projections of the whole-mount 12101-immunostained samples (left) were threshold (right) and a region of interest comprising longitudinally, the tip of the tail on one end, the most posterior organized myotome on the other end, and the full width of the dorsoventral axial musculature, was used to measure the labeled area of the regenerated and differentiated muscle. (C) Maximum intensity projections of whole-mount 12101-immunostained control and treated samples. Values are

mean $\pm$ SEM percent regenerated muscle compared to control. In A–C, amputation site is indicated with arrowheads. * $p < 0.001$, $n = 5$ per group.

Fig. 4.

Active proliferation of muscle cell precursors is apparent during the first days post amputation. Amputated tadpoles were fixed, sectioned and processed for immunostaining after 24 and 48 hpa. Shown is a representative example of a triple-immunolabeled coronal section of the tail region with anti-Pax7, muscle satellite cell marker in red, anti-MyoD, muscle cell precursor marker in blue and anti-PCNA, proliferative marker in green. Nuclei are labeled with DAPI, in grayscale. A: anterior, P: posterior. Arrowheads indicate amputation site.

Fig. 5.

Proliferation of muscle cell precursors is impaired in ryanodine-treated tadpoles. Ryanodine-treated amputated tadpoles were fixed, sectioned and processed for immunostaining after 24 and 48 hpa. Shown is a representative example of a triple-immunolabeled coronal section of the tail region with anti-Pax7, muscle satellite cell marker in red, anti-MyoD, muscle cell precursor marker in blue and anti-PCNA, proliferative marker in green. Nuclei are labeled with DAPI, in grayscale. A: anterior, P: posterior. Arrowheads indicate amputation site.

Fig. 6.

Ca^{2+} transients are necessary for muscle satellite cell activation and muscle cell precursor proliferation in the regenerating tail. Amputated tadpoles were fixed, coronally sectioned and processed for immunostaining after 24 and 48 hpa with anti-Pax7, muscle satellite cell marker, anti-MyoD, muscle cell precursor marker and anti-PCNA, proliferative marker. (A) Mean $\pm$ SEM immunopositive cells per 440 μ m thick regenerating tail in control and ryanodine-treated tadpoles at 24 (left) and 48 (right) hpa. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, $n = 9$ per group. (B) Model of Ca^{2+} transient participation in the progression of muscle cell generation and differentiation in the regenerating tail. RyR: ryanodine receptor.