Visualization, characterization and modulation of calcium signaling during the development of slow muscle cells in intact zebrafish embryos

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ABSTRACT Intact zebrafish embryos were used as an in vivo animal model to investigate the role of Ca²⁺ signaling during the differentiation of slow muscle cells (SMCs) within forming skeletal muscle. Transgenic zebrafish were generated using an α-actin promoter that targeted apoaequorin expression specifically to muscle cells. Two distinct Ca²⁺ signaling periods (CSPs) were visualized in the developing SMCs: between ~17.5-19.5 hours post-fertilization (hpf) and after ~23 hpf, separated by a ~3.5 h Ca²⁺ signaling quiet period. Further spatial characterization of these Ca²⁺ signals using confocal fluorescent microscopy and calcium green-1 dextran as a reporter, indicated that the earlier CSP displayed distinct nuclear and cytoplasmic components, whereas the later CSP was predominantly cytoplasmic. Both CSPs consisted of a series of oscillating Ca²⁺ waves generated at distinct frequencies, while the earlier CSP also displayed a slow rise then fall in the Ca²⁺ baseline-level. Imaging of cyclopamine- and forskolin-treated wild-type, or smo⁻/⁻ mutant embryos, where SMCs do not form, confirmed the specific cell population generating the signals. Treating embryos with antagonists indicated that both IP₃Rs and RyRs are responsible for generating the temporal characteristics of the Ca²⁺ signaling signature, and that the latter plays a necessary role in SMC differentiation and subsequent myotome patterning. Together, these data support and extend the proposition that specific spatiotemporal patterns of spontaneous Ca²⁺ signals might be used for different as well as combinatorial regulation of both nuclear and cytosolic signal transduction cascades, resulting in myofibrillogenesis in SMCs as well as myotome patterning.

KEY WORDS: Ca²⁺ imaging, IP₃R, RyR, slow muscle cell, transgenic zebrafish

Introduction

The regulation of intracellular Ca²⁺ concentration, [Ca²⁺]ᵢ, has been reported to play a critical role in many aspects of muscle development and function (Ferrari et al., 1996; Brennan et al., 2005; Campbell et al., 2006). While the majority of Ca²⁺-muscle studies has been conducted in vitro, with cells or explants in culture, relatively few have been conducted in vivo, i.e., using intact, normally developing animals. For example, using the C2C12 mouse muscle cell line, it has been shown that three different patterns of Ca²⁺ signals are generated during in vitro myogenesis (Lorenzon et al., 1997) and that the frequency of the Ca²⁺ transients determines the time required for de novo sarcomere assembly (Fujita et al., 2007). In isolated chick skeletal myoblasts, David et al. (1981) showed that a net movement of Ca²⁺ is essential for myoblast fusion. Furthermore, in primary cultures of Xenopus embryonic myocytes, Ferrari et al. (1996)

Abbreviations used in this paper: CSP, calcium signaling period; DMSO, dimethyl sulphoxide; E-CC, excitation-contraction coupling; FMCs, fast muscle cells; hpf, hours post-fertilization; IP₃Rs, inositol 1,4,5-trisphosphate receptors; QP, calcium signaling quiet period; RLU, relative light units; RyRs, ryanodine receptors; SMCs, slow muscle cells; SP1, calcium signaling period 1.

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reported that spontaneous Ca\textsuperscript{2+} signals are generated during just the first 15 hours in culture. These Ca\textsuperscript{2+} signals were shown to be generated by Ca\textsuperscript{2+} release from intracellular stores via ryanodine receptors (RYRs) and it was proposed that the signals play a role in the assembly of the myosin thick filaments (Ferrari et al., 1996; 1998). Ferrari and Spitzer, (1999) subsequently showed that similar Ca\textsuperscript{2+} dynamics also occur in the exposed myotome of dissected embryos and that these Ca\textsuperscript{2+} events seem to be correlated with somite formation and myocyte maturation. Li et al. (2004) then reported that spontaneous Ca\textsuperscript{2+} signals appear to regulate patterned actin assembly during myofibrillogenesis in cultured Xenopus myocytes, where blocking these signals disrupted the assembly of the actin thin filaments and the actin-associated z-disc affiliated proteins, titin and capZ. More recently, Brennan et al. (2005) reported that in intact zebrafish embryos, the release of intracellular Ca\textsuperscript{2+} from RYRs drives the initial contraction of slow muscle cells (SMCs) and that these contractions are responsible for the embryonic movements that occur at ~17 - 22 hpf. They also reported that this process is regulated by acetylcholine and results from spontaneous neuronal activity. Furthermore, blocking Ca\textsuperscript{2+} release with antagonists of either the acetylcholine receptor (AChR) or RyR disrupted the organization of the myofibrils without affecting the number of SMCs, or their elongation or migration. They thus proposed a critical role for nerve-mediated Ca\textsuperscript{2+} signals in the formation of physiologically functional slow muscle during zebrafish development (Brennan et al., 2005). Most recently, Campbell et al. (2006) reported ryanodine receptor (RYR)-mediated generation of both localized (i.e., in the subsarcolemma space), short duration Ca\textsuperscript{2+} transients (SDTs; lasting ~2 secs), as well as spontaneous global Ca\textsuperscript{2+} transients of long duration (LDTs; lasting ~80 sec) in embryonic myocytes prepared from neural plate (stage 15) Xenopus embryos. They proposed that the distinct spatiotemporal patterns of SDTs vs LDTs may be used for differential regulation of cytosolic signaling cascades, control of nuclear gene expression, and localized activation of assembly events at the sarcolemma (Campbell et al., 2006).

In this current study, we used intact zebrafish embryos as an in vivo animal model in order to extend our holistic understanding of the role of Ca\textsuperscript{2+} signaling during embryonic muscle development, complementary aequorin-based and fluorescence-based confocal Ca\textsuperscript{2+} imaging techniques were applied. With regard to the former, we developed a new line of transgenic zebrafish that express apoaequorin specifically in the trunk musculature. Active aequorin in these transgenic zebrafish embryos was then reconstituted by incubation in a solution containing the apoaequorin co-factor, coelenterazine (Cheung et al., 2006). While aequorin-based imaging allows for the continuous, non-disturbing visualization of intact zebrafish embryos for long-duration developmental studies (for example, during the entire development of the embryonic trunk musculature), a drawback of this technique is that it only allows for two-dimensional and low resolution Ca\textsuperscript{2+} imaging. Thus, after we applied aequorin-based imaging to establish where and when Ca\textsuperscript{2+} transients arose in the developing musculature, we then examined the signals at higher spatial resolution using the fluorescent Ca\textsuperscript{2+} reporter, calcium green-1 dextran, in conjunction with confocal microscopy. The use of these two imaging techniques allowed us to add new spatial information, from both a rostrocaudal perspective, as well as at a sub-cellular level, to what is known about Ca\textsuperscript{2+} signaling in SMCs in an intact vertebrate system. In addition, it has allowed us to extend our understanding of the various Ca\textsuperscript{2+} release mechanisms responsible for generating the signals as well as to investigate (via their
inhibition) the possible functional roles of these signals during SMC differentiation and myotome development.

Results

Visualizing Ca$^{2+}$ signals from transgenic fish that express apoaequorin in the musculature

Transgenic zebrafish that express the apoaequorin (aeq) gene (Inouye et al., 1989) exclusively in the musculature were generated by microinjecting an aeq construct driven by an α-actin promoter (Higashijima et al., 1997) into embryos at the 1-cell stage (Fig. 1). This plasmid also contained an EGFP marker gene, which was regulated by the IRES-sequence (a short sequence of DNA that initiates the internal translation of RNA) for the subsequent identification of transgenic fish. We have termed this α-actin-apoaequorin-IRES-EGFP transgenic line of fish, α-actin-aeq. Use of the IRES-sequence enables the translation of both aeq and EGFP from a single mRNA, thus the expression level and distribution of EGFP reflects the expression level and distribution of apoaequorin (Jang et al., 1988; Fahrenkrug et al., 1999; Wang et al., 2000).

Using this plasmid, a single transgenic founder fish (F₀) was identified from 85 candidate fish. This F₀ fish was then out-bred with a wild-type fish to establish the F₁ transgenic line. The F₁ embryos that expressed EGFP (Fig. 2A) were identified and raised to adults for the generation of a stable homozygous F₂ line. In this transgenic line, the EGFP and thus the apoaequorin were expressed at low levels at ~12 hpf (i.e., ~6-somite stage; Fig. 2B) and the level of expression increased in an approximately linear manner over the time range measured, i.e., up to ~24 hpf. Fig. 2C shows a transverse cross section through the myotome of a representative (n=5) transgenic zebrafish embryo at ~24 hpf and shows that the EGFP (Fig. 2Ci) and thus apoaequorin were expressed throughout the entire musculature including the slow muscles (the myosin of which was labeled with the F59 antibody; Fig. 2Cii and 2Ciii). Together, these results suggest that this α-actin-aeq fish line can be used to visualize and characterize the Ca$^{2+}$ signals generated in the developing trunk musculature (Fig. 2D) between approximately 16 hpf and 25 hpf, (i.e., the period reported in this study).

Characterizing the trunk Ca$^{2+}$ signals in zebrafish with apoaequorin-based Ca$^{2+}$ imaging

Transgenic embryos (n=6) were incubated in f-coelenterazine solution to reconstitute active aequorin, and the Ca$^{2+}$ signals generated in the developing trunk between ~17 hpf to ~24.5 hpf were visualized using our PIMS (Fig. 3Ai). Using a region of interest (ROI) that covered the entire embryo (Fig. 3Aii), and acquiring the luminescent data in photons/sec, two distinct periods of Ca$^{2+}$ signaling were observed in the developing anterior trunk (Fig. 3Ai). The first period of Ca$^{2+}$ signaling was comprised of two main components: The first to appear was rapid Ca$^{2+}$ spiking, which began suddenly at ~17.5 hpf (i.e., the 17-somite stage), where the maximum amplitude of signaling was detected almost immediately. Spiking activity lasted until ~19.5 hpf (i.e., the 21-somite stage). The spiking frequency was not, however,
constant during this time, but altered in a distinct and highly reproducible manner. Unlike the sudden initiation of the spiking activity, its termination was the result of a steady decline in spiking amplitude (Fig. 3Ai). The second main component of the Ca\(^{2+}\) signaling activity was related to the baseline level of the Ca\(^{2+}\) spiking. This did not remain constant during the spiking period, but displayed a gradual increase, which started at ~18 hpf, reached a maximum at ~18.75 hpf and then returned to its original resting value by ~19.5 hpf. This was concomitant with the cessation of the Ca\(^{2+}\) spiking activity. Thus, from ~18 hpf onwards, the Ca\(^{2+}\) spiking activity was superimposed on top of this baseline modulation. We have termed this second Ca\(^{2+}\) signaling feature the “slow rising component”. We have called the combination of Ca\(^{2+}\) spiking and the slow rising component, “Ca\(^{2+}\) Signaling Period 1” (or SP1).

Between ~19.5 hpf and ~23 hpf, the Ca\(^{2+}\) signaling activity (both the Ca\(^{2+}\) spiking and baseline modulation) returned to pre-SP1 levels. We have called this the “Ca\(^{2+}\) Signaling Quiet Period” (or QP). This was followed at ~23 hpf (i.e., the 28-somite stage), beginning with a gradual build-up (rather than a sudden initiation as was the case with the SP1 Ca\(^{2+}\) signals), of a second phase of Ca\(^{2+}\) signaling activity. This was comprised of a second period of Ca\(^{2+}\) spiking with no slow rising component. We have termed this “Ca\(^{2+}\) signaling period 2” (or SP2). This second phase was ongoing when our observations ended at ~24.5 hpf. The onset of SP2 at ~23 hpf indicated that the earlier fall in Ca\(^{2+}\) signaling activity (which was complete by ~19.5 hpf) was not due to the consumption of active aequorin in the trunk muscle cells.

The SP1 and SP2 Ca\(^{2+}\) spikes were analyzed in further detail (Fig. 3B and 3C, respectively). For this analysis, an ROI restricted

Fig. 3. The changes in [Ca\(^{2+}\)] that occur in \(\alpha\)-actin-aeq transgenic embryos from ~17 hpf to ~24.5 hpf (i.e., the ~16-somite stage to ~Prim-5 stage). (Ai) Representative \((n=6)\) profile of aequorin-generated light from ~17 hpf to 24.5 hpf. Data were plotted every 10 s, each data point representing 10 sec of accumulated luminescence in a region of interest (ROI) of ~1.24 mm\(^2\) (see panel Aii). This trace clearly illustrates two distinct periods of Ca\(^{2+}\) signaling activity. The first Ca\(^{2+}\) signaling period, termed Signaling Period 1 (SP1), occurs from ~17.5 hpf to 19.5 hpf, and the second Ca\(^{2+}\) signaling period, termed Signaling Period 2 (SP2), starts at ~23 hpf and runs at least to the end of the imaging period at 24.5 hpf. Between SP1 and SP2 (i.e., from ~19.5 hpf to ~23 hpf) a period of significantly reduced Ca\(^{2+}\) activity was consistently observed. We have termed this the Ca\(^{2+}\) signaling quiet period (QP).

(Bi) Profile of the SP1 Ca\(^{2+}\) signals alone (i.e., from 17 hpf to 20 hpf). (Bii, Biv, Bvi) Profiles of the Ca\(^{2+}\) signals generated over a period of 100 sec from 17.5-18 hpf, 18-18.5 hpf, 18.5-19 hpf and 19-19.5 hpf, respectively, as indicated in panel (Bi). (Ci) Profile of the Ca\(^{2+}\) signals generated at the end of the QP and start of SP2 (i.e., from 22 hpf to 25 hpf). The shaded regions show bursts of Ca\(^{2+}\) signaling activity. (Ciii) Profile of the Ca\(^{2+}\) signals generated over a period of 100 sec from 24-24.5 hpf, as shown in panel (Ci). For the profiles in B and C, data were plotted every 1

s, each data point representing 1 sec of accumulated luminescence for ROIs located in somite 8 (i.e., ~5000 \(\mu\)m\(^2\); see panels Bi and Cii, respectively). The average frequency (in Hz) of the Ca\(^{2+}\) signals generated in 30-min periods are shown in panels (Bi, Ci).
to somite 8 was used (Fig. 3Bi and 3Cii), and the data were reviewed at higher temporal resolution. For both signaling periods, we made the assumption that rates of luminescence of 2 photons/sec and above were “real” Ca$^{2+}$ signals and anything below this was background noise. During SP1, the frequency of signal generation showed distinct variations (Fig. 3Bi). The lowest frequency of signaling (~0.02 Hz) occurred during the first ~30 minutes of SP1 (i.e., between 17.5 hpf and 18 hpf). The frequency of the signaling then increased ~4-fold to ~0.09 Hz between 18 hpf and 18.5 hpf, and then peaked at ~0.12 Hz between 18.5 hpf and 19 hpf. Between 19 hpf and 19.5 hpf, the frequency of signals then dropped again to ~0.03 Hz, and finally fell to zero, indicating the beginning of the QP. The varying frequencies observed during SP1 are also shown in the higher temporal resolution plots (Fig. 3Bii and 3Cii) and were a conserved feature seen in all embryos examined (Fig. 4A). When signaling resumed at the start of SP2, the frequency of the Ca$^{2+}$ signals was initially just ~0.007 Hz and then as the SP2 signals became established at ~24 hpf, the frequency of the Ca$^{2+}$ signals was not statistically different from those generated in the aequorin-mRNA injected embryos during the respective time periods. In SP1, the frequency of the Ca$^{2+}$ signals was significantly lower (at p<0.01) between 17.5-18 hpf (i.e., ~0.02 Hz) than at any other time in SP1 and SP2. The frequency increased from ~0.02 between 17.5-18 hpf to ~0.09 Hz between 18-18.5 hpf, and reached a maximum of ~0.13 Hz between 18.5-19 hpf. The frequency of the signals then decreased to ~0.06 Hz between 19-19.5 hpf. The frequency of the SP2 Ca$^{2+}$ signals (i.e., ~0.03 Hz) was significantly lower than that generated between 18-18.5 hpf (i.e., ~0.09 Hz) and between 18.5-19 hpf (i.e., ~0.13 Hz), at p<0.01. On the other hand, the frequency of the SP2 signals was not statistically different from the frequency of the SP1 signals at 19-19.5 hpf (i.e., ~0.06 Hz).

The duration of the SP1 and SP2 Ca$^{2+}$ signals for each of the α-actin-aeq transgenic and aequorin-mRNA injected embryos was also compared (Fig. 4B). We followed the protocol described by Brennan et al. (2005), and calculated the duration as the time taken for a signal to decay from the maximum amplitude to half of that value. The durations that were calculated for each of the four periods comprising SP1 (i.e., between 17.5-18 hpf, 18-18.5 hpf, 18.5-19 hpf and 19-19.5 hpf) generated in the α-actin-aeq transgenic embryos were not significantly different from those generated in the aequorin-mRNA injected embryos, during the respective time periods. In SP1, the Ca$^{2+}$ signals between 17.5-18 hpf had the longest duration (i.e., ~0.84 sec). The duration of the signals then decreased through SP1 such that by 19-19.5 hpf, the mean duration of the signals was just ~0.46 sec. The duration of the signals between 18-18.5 hpf was significantly lower than those between 17.5-18 hpf at p<0.01. Likewise, the duration of the signals between 19-19.5 hpf was significantly lower than between 17.5-18 hpf at p<0.01. We conducted a detailed comparison of SP1 and SP2 Ca$^{2+}$ signals during restricted time windows using both α-actin-aeq transgenic embryos and aequorin-mRNA injected embryos (n≥3 for each; see Fig. 4A, B). All statistical analyses were conducted using the student’s t-test for unpaired samples.

Fig. 4A shows that the frequencies of the SP1 Ca$^{2+}$ signals (i.e., between 17.5-18 hpf, 18-18.5 hpf, 18.5-19 hpf and 19-19.5 hpf) generated in the α-actin-aeq transgenic embryos were not statistically different from those generated in the aequorin-mRNA injected embryos between 18-18.5 hpf and 18.5-19 hpf (i.e., ~0.13 Hz). The frequency of the signals then decreased to ~0.06 Hz between 19-19.5 hpf. The frequency of the SP2 Ca$^{2+}$ signals (i.e., ~0.03 Hz) was significantly lower than that generated between 18-18.5 hpf (i.e., ~0.09 Hz) and between 18.5-19 hpf (i.e., ~0.13 Hz), at p<0.01. On the other hand, the frequency of the SP2 signals was not statistically different from the frequency of the SP1 signals at 19-19.5 hpf (i.e., ~0.06 Hz).
than those between 18.5-19 hpf at p<0.01. On the other hand, all the signals between 18-18.5 hpf and 18.5-19 hpf had a similar duration of ~0.7 sec. The durations of the SP2 Ca2+ transients (~0.6 sec) were significantly greater than those of the 19-19.5 hpf SP1 signals (at p<0.01).

Fig. 4 C, D are horizontal bar charts to show the location of the SP1 Ca2+ signals with respect to the formed somites in 4 α-actin-aequorin transgenic embryos and 4 aequorin mRNA injected embryos. In all 8 examples, these signals were generated in a discrete number of somites in the anterior trunk. At the start of the SP1 Ca2+ signaling window (i.e., ~17.5 hpf; the 17-somite stage), the low frequency (i.e., ~0.02 Hz) Ca2+ signals were generated between somites 4 and 10 in all 8 embryos examined at this stage of development (Fig. 4Ac). At ~18.5 hpf (i.e., the 19 somite stage), when the frequency of the Ca2+ signals rose to ~0.12 Hz, these signals were generated between somites 2 and 12. and in this case were never seen to extend into somite 1 in an anterior direction, or beyond somite 12 in a posterior direction (Fig. 4D). At both time points analyzed (i.e., ~17.5 hpf and ~18.5 hpf), there was no difference in the anterior-to-posterior spread of the signals generated, when comparing the transgenic embryos with the mRNA-injected embryos (Fig. 4 C,D).

Further characterization of the trunk Ca2+ signals with calcium green-1 dextran and confocal imaging

The fluorescent Ca2+ reporter, calcium green-1 dextran (10S) in conjunction with confocal microscopy, was used to confirm and support the data collected using aequorin-based imaging. In addition, the higher spatial resolution of this imaging technique enabled us to characterize the spatial aspects of the trunk Ca2+ signals in greater detail.

Fig. 5 shows a representative example (n=3) of the Ca2+ signals generated in individual muscle cells in somite 8 at ~17.5 hpf (i.e., the 17-somite stage). Fig. 5A shows an example of two prominent Ca2+ signals that were visualized from two individual muscle cells located in somite 8, both of which lasted for at least 6 sec. These Ca2+ signals were generated predominantly in the nucleus and peri-nuclear region of each individual muscle cell, but were also seen to spread into the cytoplasmic region of each cell. This is especially obvious in the lower of the two cells shown in this example (see arrowheads in Fig. 5Aii-Aiv). The profiles of two consecutive Ca2+ signals generated in the upper of the two muscle cells in somite 8 are shown in Fig. 5Bi, and these again indicate that the total duration of the Ca2+ signals is in the range of 5 to 10 seconds. The reliability of the calcium green-1 dextran signals was confirmed via the co-injection of and subsequent imaging with an equivalent molecular weight molecule, rhodamine B dextran (Fig. 5Bii). The Ca2+ signals shown in Fig. 5 correlated with a spontaneous contraction in the muscle cells (see Supplemental Movie #1).

Fig. 6 is a representative example (n=3) of the Ca2+ signals generated by individual muscle cells in somites 7-10 at ~18.5 hpf (i.e., the 19-somite stage). Fig. 6A shows a representative Ca2+ signal that was imaged in the trunk, which lasted ~5 sec. The profiles of the Ca2+ signals generated over a period of 100 sec in somites 7-10 (Fig. 6Bi-Biv) show that the signals generated in one somite were in phase with those generated in the other somites (indicated by the red dashed lines). In addition, the Ca2+ signals generated in the dorsal, medial and ventral parts of a somite (i.e., somite 9; Fig. 6C) were also found to be in synchrony. Again, the reliability of the calcium green-1 dextran signals was confirmed via the co-injection and subsequent imaging of rhodamine B dextran (Fig. 6By). Moreover, the Ca2+ signals observed at ~17.5 hpf and ~18.5 hpf via confocal microscopy support and confirm the SP1 Ca2+ signals generated in the α-actin-aequorin transgenic fish at the same time (compare Fig. 5Bi with Fig. 3Biii and Fig. 6Bi-Biv with Fig. 3Bv).

Using calcium green-1 dextran and confocal microscopy, we also investigated Ca2+ signals at the level of a single muscle cell (Fig. 7). Fig. 7 C, D show a representative example of a Ca2+
signal generated in a single cell in somite 10 (Fig. 7B) at ~18.5 hpf. In this case, the Ca\textsuperscript{2+} signal was generated in the nucleus first, after which it spread through the cytoplasm. In addition, the nuclear Ca\textsuperscript{2+} signal remained relatively high even when the Ca\textsuperscript{2+} signal in the cytoplasm had decreased to the resting level again.

Fig. 8 shows a representative example (n=3) of a SP2 Ca\textsuperscript{2+} signal generated in the trunk at ~24 hpf (i.e., the Prim-5 stage). Fig. 8A shows a representative Ca\textsuperscript{2+} signal that was imaged in the ventral portion of somite 8, which lasted for ~1.4 sec. The Ca\textsuperscript{2+} signal was generated in the entire cell when the muscle contracted. However, unlike the Ca\textsuperscript{2+} signals generated at ~17.5 hpf and ~18.5 hpf, where the signal was generated mainly in the nucleus, at ~24 hpf the Ca\textsuperscript{2+} signals appeared to be mainly cytosolic. The profiles of the Ca\textsuperscript{2+} signals and the rhodamine-B dextran generated fluorescence recorded over a period of 100 sec in somite 8 are shown in Fig. 8Bi and 8Bii, respectively.

**Effect of cyclopamine and forskolin on slow muscle development and Ca\textsuperscript{2+} signaling in the trunk**

During confocal imaging, the cells that generated the SP1 Ca\textsuperscript{2+} signals appeared, from their morphology and location within the somite, to be SMCs (see Figs. 5 and 6). To confirm whether this was the case, α-actin-aeq transgenic embryos were treated with either 0.1 mM cyclopamine (Chen et al., 2002) or 0.15 mM forskolin (Barresi et al., 2000). It has previously been reported that these drugs inhibit the development of SMCs by blocking different aspects of sonic hedgehog signaling. Cyclopamine antagonizes the function of the smoothened protein, which is a component of the hedgehog signaling pathway (Chen et al., 2002), while forskolin indirectly inhibits the response of cells to hedgehog signaling by activating cAMP-dependent protein kinase (PKA; Barresi et al., 2000). Embryos treated with these drugs exhibited several gross developmental abnormalities at 24 hpf, compared with the untreated and DMSO controls (Fig. 9 Ai-Di). Both drugs induced abnormalities in the trunk and tail, and forskolin additionally caused abnormal head formation (compare Fig. 9 Ci, Di with Fig. 9 Ai, Bi). Fig. 9Aii-Dii are bright-field images of the trunk at higher magnification. While the somites in the untreated and DMSO-treated embryos displayed a normal chevron-shape, those in the embryos treated with cyclopamine or forskolin were U-shaped (compare Fig. 9 Cii, Dii with Fig. 9 Aii, Bii). In addition, immunohistochemistry of the slow muscle fibers with the F59 antibody shows that cyclopamine and forskolin treatment almost completely blocked slow muscle formation at 24 hpf (compare Fig. 9 Ciii, Diii with Fig. 9 Aiii, Biii).
Fig. 7 (above). A Ca^{2+} signal generated in a single muscle cell in the trunk of an intact embryo at ~18.5 hpf (i.e., the 19-somite stage). Embryos (n=3) were loaded with calcium green-1 dextran (10S) at the 1-cell stage and images were acquired via confocal microscopy. (A) Schematic diagram to show the position of somite 10 (red box) in the zebrafish embryo where the Ca^{2+} signal was acquired. (B) Bright-field image of somite 10 with the position of the imaged cell indicated (see red box). (C) Single confocal sections to show the Ca^{2+} signal generated in the imaged cell over a period of ~8 s. The images were acquired every ~0.58 sec. The nucleus is outlined with a white dashed line in the first panel. The color scale represents the level of [Ca^{2+}]_i, where red indicates a high level and blue indicates a low level. Ant. and Pos. are anterior and posterior, respectively. Scale bars are (B) 25 μm and (C) 10 μm. (D) Temporal profile of the fold change in fluorescence intensity in the nucleus and cytoplasm in this muscle cell for the duration of the Ca^{2+} signal.

or forskolin (Fig. 9Div; n=6). These profiles show that the SP1 Ca^{2+} signals that normally occurred between ~17.5 to 19.5 hpf in the untreated and DMSO-treated controls (Fig. 9 Aiv, Biv), were completely blocked in the cyclopamine- and forskolin-treated embryos (Fig. 9 Civ, Div). These results strongly suggest that the SP1 Ca^{2+} signals are generated exclusively by SMCs.

**Slow muscle development and Ca^{2+} signaling in the trunk of smo^{−/−} mutant embryos**

As well as treating embryos with drugs to inhibit hedgehog signaling and thus the formation of the SMCs, a genetic approach, involving the homozygous smo^{−/−} mutant (Barresi et al., 2000) was also used to confirm whether the SP1 Ca^{2+} signals were generated exclusively from SMCs (Fig. 10). The slow-muscle-omitted (smo) gene encodes the zebrafish smoothened protein, which (as mentioned previously) is a component of the hedgehog signaling pathway and is thus required for the development of the SMCs. In smo^{−/−} embryos, fast muscles develop normally; however, almost 99% of the SMCs are lost and the muscle pioneers are completely absent (Barresi et al., 2000). We found that at 24 hpf, the homozygous smo^{−/−} embryos exhibited a curved trunk and tail, compared to normal control embryos (i.e., either wild-type or heterozygous smo^{+/−} mutants; compare Fig. 10Bi with Fig. 10Ai). In addition, while the somites in the normal control embryos displayed the normal chevron-shape (Fig. 10Aii), those in the smo^{−/−} em-
bryos were U-shaped (Fig. 10Bii). Immunohistochemistry of the slow muscle fibers using the F59 antibody showed that at 24 hpf there was negligible slow muscle formation in the smo-/embryos (compare Fig. 10Biii with the normal control, Fig. 10Aiii). Fig. 10Aiv and 10Biv show the profiles of aequorin-generated light from aeq-mRNA-injected normal control and smo-/embryos, respectively. In these experiments, we used aeq-mRNA injected homozygous smo-/embryos. The profiles of aequorin-generated light show that the SP1 Ca\(^{2+}\) signals that occurred at ~17.5-19.5 hpf in normal control embryos (Fig. 10Aiv; n=5) were completely absent in the smo-/embryos (Fig. 10Biv; n=3). These results support our cyclopamine and forskolin treatment experiments (Fig. 9), which together indicate that the SP1 Ca\(^{2+}\) signals are generated exclusively by SMCs.

Effect of \(\alpha\)-bungarotoxin and nifedipine on the SP1 Ca\(^{2+}\) signals

It has been previously reported that in zebrafish embryos, the trunk muscles start to show spontaneous contractile activity at ~17 hpf and these movements are generated by acetylcholine (ACh) release from motor nerve terminals (Grunwald et al., 1988; Melançon et al., 1997). When acetylcholine binds to nicotinic acetylcholine receptors (nAChRs) expressed by the muscle cells, this binding is reported to activate dihydropyridine receptors (DHPRs) located on the surface membrane or on T-tubules, which in turn triggers the release of Ca\(^{2+}\) from intracellular stores via RyRs, and hence contraction occurs (Schneider and Chandler, 1973).

To investigate whether the nAChRs and DHPRs play a role in generating the SP1 Ca\(^{2+}\) signals, \(\alpha\)-actin-aeq transgenic embryos were incubated with either the nAChR blocker, \(\alpha\)-bungarotoxin-Alexa Fluor 488 (n=3; Fig. 11Bi) or the DHPR antagonist, nifedipine (n=3; Fig. 11Ci) and the luminescence was compared with that generated by untreated controls (Fig. 11Ai). The distal ~100 \(\mu\)m portion of the tail of each embryo was excised with a tungsten needle just prior to incubation with the \(\alpha\)-bungarotoxin-Alexa Fluor 488 or nifedipine solution, in order to help facilitate the diffusion of drugs into trunk tissues containing the SMCs. The Alexa Fluor 488 moiety on \(\alpha\)-bungarotoxin was used to visualize and thus confirm the diffusion of the drug into the trunk via the tail-cut (data not shown). We found that the SP1 Ca\(^{2+}\) signals were completely blocked by treatment with either \(\alpha\)-bungarotoxin or nifedipine right from the earliest time these signals appeared, which suggests that both nAChRs and DHPRs play a role in the initiation and generation of the SP1 Ca\(^{2+}\) signals in the trunk of zebrafish embryos. Moreover, the addition of triton X-100 at the end of each experiment (Fig. 11Aii-Cii) indicates that the inhibition of luminescence observed following the \(\alpha\)-bungarotoxin and nifedipine treatments was due to the drugs inhibiting the SP1 signals, rather than being due to a lack of active aequorin in the embryos.
To investigate the mechanism of generation of the SP1 trunk Ca\textsuperscript{2+} signals, embryos were treated with the IP\textsubscript{3}R antagonist, 2-APB, or the RyR antagonist, ryanodine (Fig. 12). The \(\alpha\)-actin-aeq transgenic embryos were imaged using our PIMS starting at \(-16\) to \(-17\) hpf and then they were treated with either 25 \(\mu\)M 2-APB or 50 \(\mu\)M ryanodine for 3 hr following the first appearance of the SP1 Ca\textsuperscript{2+} signals. Once again, \(-100\) \(\mu\)m of the distal portion of the tail of each embryo was excised just prior to incubation with the 2-APB, or ryanodine solution, in order to ensure that the drugs diffused into trunk tissues containing the SMCs. Fig. 12Ai shows a profile of aequorin-generated light in a representative \((n=9)\) untreated control embryo from \(-17\) hpf to \(-24\) hpf with the SP1 Ca\textsuperscript{2+} signals, the QP, and the SP2 Ca\textsuperscript{2+} signals indicated. A bright-field image of this embryo at \(-20.5\) hpf is shown in Fig. 12Aii. Fig. 12Bi is the profile of aequorin-generated light in a representative \((n=6)\) embryo that was treated with 2-APB, which shows that the SP1 rapid Ca\textsuperscript{2+} spikes were blocked within \(\pm 3\) \(\pm 1.5\) min following the start of the 2-APB treatment but that the SP2 Ca\textsuperscript{2+} spikes were still generated normally at \(-23\) hpf. Treatment with 2-APB also inhibited the slow rising component of SP1. The gross morphology of the 2-APB-treated embryo at \(-20.5\) hpf is shown in Fig. 12Bii, with the location of the tail-cut indicated by a black arrowhead. The aequorin burn-out experiment conducted at \(-24.5\) hpf (Fig. 12Biii) along with the appearance of the SP2 Ca\textsuperscript{2+} signals indicate that the low level of luminescence observed from \(-17.5\) hpf to \(-23\) hpf following 2-APB treatment, was due to the drug inhibiting SP1 Ca\textsuperscript{2+} signals rather than being due to a lack of active aequorin in the trunk muscle cells.

Fig. 12Ci is a profile of aequorin-generated light in a representative embryo \((n=7)\) that was treated with ryanodine, which...
shows that the SP1 Ca\textsuperscript{2+} spikes were not blocked immediately following the start of treatment (i.e., just prior to ~17.5 hpf) but that inhibition only occurred at ~51 ± 7 min following the start of ryanodine treatment. Treatment with ryanodine also inhibited the slow rising component of SP1 that began at ~18 hpf in untreated controls. In addition, even though ryanodine treatment ended just before ~21 hpf, the SP2 Ca\textsuperscript{2+} spikes that were normally generated at ~23 hpf were also significantly reduced. The gross morphology of the ryanodine-treated embryo at ~20.5 hpf is shown in Fig. 12Cii, with the location of the tail-cut once again indicated by a black arrowhead. An aequorin burnout experiment conducted just before 24.5 hpf (Fig. 12Ciii) indicates that the low level of luminescence observed following ryanodine treatment was due to the drug inhibiting the later portion of the SP1 signals and significantly reducing the SP2 Ca\textsuperscript{2+} signals, rather than being due to a lack of active aequorin in the embryo. When embryos were treated with ryanodine before the appearance of the SP1 Ca\textsuperscript{2+} signals (i.e., from ~16.5 hpf onward), once again the SP1 Ca\textsuperscript{2+} signals were only blocked after ~18 hpf (data not shown).

Localization of IP\textsubscript{3}Rs and RyRs in the developing trunk musculature

Wild-type embryos were fixed at 16 hpf, 18 hpf, 20 hpf, 22 hpf and 24 hpf (n=3 per time point) and then the IP\textsubscript{3}Rs were labeled via immunohistochemistry with the IP\textsubscript{3}R type 1 antibody (Fig. 13A). At ~16 hpf, IP\textsubscript{3}Rs were expressed homogeneously throughout the SMCs (Fig. 13Ai). At 18 hpf (i.e., during SP1), the IP\textsubscript{3}Rs began to organize into some clusters in the cytosol of SMCs (see white arrowheads in Fig. 13Aii). At 20 hpf, some IP\textsubscript{3}R banding was observed in the cytoplasmic region of the SMCs and by 24 hpf, very distinct IP\textsubscript{3}R banding was observed along the entire length of the SMCs (Fig. 13 Aiii-Av). Fig. 13C shows representative images of SMCs at 24 hpf that were dual labeled for both IP\textsubscript{3}Rs (Fig. 13Ci) and myosin (labeled with the F59 antibody; Fig. 13Cii). When these images were merged (Fig. 13Ciii), the IP\textsubscript{3}Rs appeared to be expressed in the SR located between the myosin thick filament bands and thus were mainly associated with the sarcomeric I-bands. This labeling pattern is schematically represented in Fig. 13E.

In addition, embryos were fixed at 16 hpf, 18 hpf, 20 hpf, 22 hpf and 24 hpf (n=6 per time point) and the RyRs were labeled via immunohistochemistry with the 34C antibody (Fig. 13B). No RyR expression was detected in the SMCs at 16 hpf (i.e., prior to the start of SP1; Fig. 13Bi). At 18 hpf (i.e., at the beginning of the slow rising phase of SP1), some RyR banding in the cytoplasmic region of the SMCs became apparent (see white arrowheads in Fig. 13Bii). By 20 hpf, however, some RyR banding in the cytoplasmic region of the SMCs became apparent (see white arrowheads in Fig. 13Bii). At 22 hpf and 24 hpf (i.e., during the QP and SP2, respectively), very distinct banding of RyRs was observed along the entire length of the SMCs (Fig. 13 Biii, Biv). When embryos at 24 hpf were dual-labeled with 34C and rhodamine-phalloidin, to label the RyRs (Fig. 13Di) and F-actin (Fig. 13Dii), respectively, the superimposed RyR/F-actin images (Fig. 13Diii) showed that the RyR bands were approximately in the middle of the F-actin bands (see asterisks in Fig. 13 Di, Diii). This suggests that the RyRs were located in the SR located near to the Z-line. This is
schematically represented in Fig. 13F.

Together, the results from Figs. 12 and 13 suggest that both IP₃Rs and RyRs contribute to the SP1 Ca²⁺ signals, with Ca²⁺ release via IP₃Rs being more dominant at the start of this signaling period (i.e., from ~17.5 hpf to ~18 hpf), and this is associated with the initial phase of low frequency SP1 Ca²⁺ signaling. Ca²⁺ release via RyRs becomes more prominent later (i.e., after 18 hpf) and thus perhaps contributes to both the higher frequency SP1 Ca²⁺ signaling as well as the slow rising Ca²⁺ component of SP1. In addition, the change in the pattern of IP₃Rs and RyRs is also reflected in the change in pattern of the Ca²⁺ signals generated by the SMCs. This transition is clearly indicated in Figs. 5, 6 and 8.

**The organization of myosin and F-actin in developing SMCs**

Wild-type embryos were fixed at 16 hpf, 17 hpf, 18 hpf, 20 hpf, 22 hpf and 24 hpf (n=3 per time point), and then myosin and F-actin were labeled with the F59 antibody and rhodamine-phalloidin, respectively (Fig. 14). At 16 hpf, a relatively low level of myosin and actin labeling was observed in the SMCs (Fig. 14A). The low expression of myosin was relatively homogeneous within individual SMCs, with no labeling being observed in the regions of the vertical myoseptae (Fig. 14 Ai, Aiiii). On the other hand, the level of labeling of F-actin was higher in the vertical myoseptae and in the end of the SMCs attached to these boundaries than in the more central regions of the SMCs (Fig. 14 Aii, Aiiii). At ~17 hpf, the level of expression of both myosin and F-actin in the SMCs had increased significantly (i.e., compare Fig. 14 Bi, Bii, with Fig. 14 Ai, Aii), and both contractile proteins were beginning to be expressed along the whole length of a few individual SMCs (Fig. 14 Bi-Biii) with the first hints of F-actin banding being observed (see white arrowhead in Fig. 14Bii). At 18 hpf, a more prominent level of F-actin banding was observed and the first clear signs of myosin banding appeared in the SMCs (Fig. 14Cii and 14Ci, respectively). Furthermore, nearly all the SMCs showed expression of the two proteins. The appearance of these organized contractile proteins coincides with both the beginning of the slow rising phase of SP1, and the high frequency transients superimposed on this phase. From ~20 hpf to 24 hpf, the banding patterns of both F-actin and myosin were clearly well formed along the entire length of each slow muscle cell (Fig. 14 Di-Diii, 14 Ei-Eiii and 14 Fi-Fiii). The organization of myosin and F-actin in a slow muscle cell at 24 hpf is shown at higher magnification in Fig. 14 Gi-Giii and at higher magnification again in Fig. 14Giii* to show specific components of the sarcomere (labeled in the schematic in Fig. 14H).
The effect of 2-APB and ryanodine on the organization of the trunk musculature and the formation of sarcomeres

Embryos were treated with various concentrations of 2-APB or ryanodine from ~17 hpf to 20 hpf (i.e., from the 16- to 22-somite stage), after which they were fixed and then the myosin and F-actin were labeled with the F59 antibody and rhodamine-phalloidin, respectively (n=3 for each treatment; Fig. 15). In the untreated controls (Fig. 15A) and the DMSO solvent controls (Fig. 15B), the trunk musculature and sarcomeres within the SMCs formed normally.

When embryos were treated with 2-APB at 25 μM and 50 μM (Fig. 15C and 15D, respectively), the gross morphology of the trunk musculature did not appear to be very different from that in the control embryos. Furthermore, the morphology of the SMCs themselves was also similar to the untreated and DMSO-treated controls. For example, they remained attached to the vertical myoseptae and spanned the entire myotome. At a sarcomeric level, however, the organization of the myosin thick filaments appeared to be disrupted to a greater extent than that of the actin thin filaments. For example, in Fig. 15Cii* and 15Dii* there is a clear degree of thin filament banding, whereas, in Fig. 15Ci* and 15Di* the banding of the myosin thick filaments appears to be disrupted in a concentration-dependent manner.

When embryos were treated with ryanodine at 10 μM and 50 μM, this caused significant abnormalities to both the myotome morphology and the organization of individual SMCs that increased in severity in a concentration-dependent manner (Fig. 15 E,F). Treatment at both concentrations caused a severe disruption of the vertical myoseptae and as a result also to the SMCs spanning the gap between them. Embryos treated with 10 μM ryanodine still possessed obvious SMCs but these were interspersed with bright aggregates of myosin and F-actin that were largely located near the vertical myoseptae (Fig. 15 Ei-Eiii). This suggests that some of the outermost (with respect to the midline) SMCs had become detached at one end from the vertical myoseptae and had constricted or collapsed, forming brightly-labeled aggregates. At 50 μM, there were far fewer intact SMCs and far more bright aggregates (Fig. 15 Fi-Fiii). In addition, although at 10 μM ryanodine, some sarcomeric banding was still observed in both the F-actin and myosin of the SMCs (Fig. 15 Ei*-Eiii*), at 50 μM, this banding was completely disrupted for both proteins (Fig. 15 Fi*-Fiii*).

In order to examine the effect of 2-APB and ryanodine on the number of SMCs per myotome unit, these were quantified within somite 8 in both untreated and treated embryos. In the
case of ryanodine-treated embryos, a brightly-labeled aggregate was considered to be a collapsed SMC and was thus counted as such. Fig. 16 shows that both 25 μM and 50 μM 2-APB resulted in a significantly lower number of SMCs in this somite at p<0.001, when compared with the controls, but that ryanodine (at both 10 μM and 50 μM) had no significant effect on SMC number.

**Discussion**

**Generation of muscle-specific apoaequorin-expressing transgenic fish**

We previously developed protocols to express apoaequorin, the protein component of aequorin, transiently and ubiquitously in zebrafish embryos by microinjecting an apoaequorin-mRNA ($aeq$-mRNA) into 1-cell stage embryos. Active aequorin was then reconstituted *in vivo* by incubating the embryos from the 64-cell stage with the coelenterazine co-factor (Cheung et al., 2006). Although this transient aequorin expression approach successfully extends the aequorin-based Ca$^{2+}$ imaging window by an additional ~24 hours (to ~48 hpf), the $aeq$-mRNA is gradually degraded in the injected embryos resulting in a steady decline in the production of apoaequorin (Cheung et al., 2006). Furthermore, as the expression of aequorin is ubiquitous, and our current aequorin-based imaging platforms have no resolution in the z-axis, it is difficult to identify specific groups of cells, tissues or organ anlagen that are generating a particular signal in more complex, later stage embryos (Cheung et al., 2006). Thus, to follow-on from this transient, ubiquitous aequorin expression technique, we now report the successful generation of a transgenic zebrafish line that expresses apoaequorin targeted to the musculature using a muscle-specific α-actin promoter.

Our new results show that this targeted apoaequorin is expressed as early as ~12 hpf (i.e., the 6-somite stage; Fig. 2B) and continues to be expressed until at least 48 hpf, allowing visualization of trunk-generated Ca$^{2+}$ transients up to ~52 hpf (data not shown), which enables us to more precisely image Ca$^{2+}$ signaling during trunk muscle development in a regular and reproducible manner. Consequently, we identified two distinct periods of spontaneous Ca$^{2+}$ signaling generated exclusively by SMCs in the trunk musculature, which occur between ~17.5 and 19.5 hpf (SP1) and from ~23 hpf onward (SP2), with a Ca$^{2+}$ signaling quiet period (QP) of around 3.5 hours duration between the two (Fig. 3Ai).

**Temporal characteristics of the SP1 and SP2 Ca$^{2+}$ signals**

From the data acquired via aequorin-based Ca$^{2+}$ imaging (using both transgenic embryos and $aeq$-mRNA injected embryos) we show that the SP1 and SP2 Ca$^{2+}$ signals are generated within a restricted number of anterior somites and at specific times in a regular and reproducible manner. Although the occurrence of trunk Ca$^{2+}$ signals between ~17 hpf and ~24 hpf have been reported previously using...
Ca²⁺ signaling in zebrafish slow muscle cells

From the DMSO control at p < 0.001, as determined by the Student's t-test for unpaired samples.

Figure 16. Bar chart to show the effect of 2-APB and ryanodine on the number of SMCs in somite 8. Data are expressed as means ± SEMs of 3-4 experiments. Asterisks indicate data that are significantly different from the DMSO control at p < 0.001, as determined by the Student's t-test for unpaired samples.

Aequorin as the Ca²⁺ reporter (Créton et al., 1998; Webb and Miller, 2000; Cheung et al., 2006), the detailed characteristics and functions of these signals were not described or explored. Using fluorescent Ca²⁺ indicators, Brennan et al. (2005) subsequently reported and characterized (from a temporal perspective), a series of “nerve-mediated Ca²⁺ signals” that are generated in the SMCs of zebrafish from ~17 to 22 hpf. Our results confirm the generation of these signals, which correspond to what we have termed the SP1 Ca²⁺ signals, to distinguish them from the subsequent post-QP SP2 Ca²⁺ signals. The QP and the SP2 signals represent two new discoveries with respect to the dynamic Ca²⁺ signaling patterns generated during SMC development. Furthermore, the sudden appearance of the SP1 transients initially in somites 4 to 10, follow a period of quiescent Ca²⁺ signaling as far as the zebrafish trunk is concerned. We have previously reported that a period of relatively localized stochastic Ca²⁺ signaling occurs in the formed somites and to a much lesser extent in the pre-somitic mesoderm during the early segmentation period, i.e., during the 1 to 8 somite stages (~10 to 13 hpf; Leung et al., 2009). However, both we and others have reported that prior to the sudden appearance of the pan-somitic SP1 signals at ~17.5 hpf, there is essentially a background resting level of Ca²⁺ within the zebrafish trunk (i.e., from all tissues) for at least 1 hour prior to the onset of SP1 (Figs. 3Ai, 12Bi and 12Ci; Gilland et al., 1999; Leung et al., 2009).

The data reported by Brennan et al. (2005) and our new observations correlate with the first of three different types of movement behavior reported by Saint-Amant and Drapeau (1998). The first of these movements consists of spontaneous, alternating left to right slow contractions of the trunk and tail, which were reported to begin suddenly at ~17 hpf, with an average frequency of ~0.57 Hz. They peaked at ~19 hpf with a frequency of ~0.96 Hz, and then gradually decreased to ~0.1 Hz per side of the embryo, i.e., one complete contraction cycle comprised of two alternating contractions, one on the left side, followed by one on the right side (Saint-Amant and Drapeau, 1998).

The maximum Ca²⁺ signaling frequency we recorded on one side of the embryo at ~19 hpf, however, was ~0.12 Hz, i.e., almost an order of magnitude slower than the contraction frequency reported by Saint-Amant and Drapeau (1998). In both cases embryos were dechorionated as part of the experimental protocol to improve imaging. Saint-Amant and Drapeau (1998), however, immobilized just the head of the embryos with low melting point agarose, thus allowing the trunk and tail to move freely, whereas in our experiments, embryos were immersed in 3% methyl cellulose for the duration of imaging to prevent them moving out of the field of view. This may have restricted the degree of trunk/tail contraction, and thus explain the lower rates of Ca²⁺ signals observed during our imaging experiments. However, the onsets of the trunk/tail contractions reported by Saint-Amant and Drapeau (1998), and the spontaneous Ca²⁺ signaling activities that we visualized (i.e., at ~17 and ~17.5 hpf, respectively), are remarkably similar. Indeed, while our aequorin-based imaging system did not allow us to record whether or not a trunk/tail contraction accompanied a Ca²⁺ signal, we were able to confirm this correlation during our confocal fluorescent Ca²⁺ imaging experiments, (see Supplemental Movie #1).

We also report an additional new observation regarding a slow rising component of SMC Ca²⁺ activity during SP1, that started at ~18 hpf, reached a maximum at ~18.75 hpf and then returned to its original resting value by ~19.5-20 hpf (Fig. 3Ai). At present, we do not understand the significance or developmental function of the slow rising Ca²⁺ component. It could, however, be completely abolished via treatment with either 2-APB or ryanodine (Figs. 12Bi, Ci) suggesting a dual generation mechanism that involves Ca²⁺ release from both receptor types. The appearance of the slow rising component correlates with the beginning of the higher frequency oscillations of SP1 (Fig. 3Ai), the expression of RyRs (Fig. 13Bii) and the appearance of organized sarcomeres in all the SMCs (Fig. 14Cii). A somewhat similar slow rising Ca²⁺ component, with accompanying super-imposed Ca²⁺ spiking, has been reported during the process of neural induction in X. laevis development (Leclerc et al., 2000). It was subsequently shown that these Ca²⁺ signals correlated with the expression pattern of the early neural precursor gene, Zic3 (Leclerc et al., 2003).

SP1 ends at ~19.5 hpf (Fig. 3), as embryos enter into the QP. We are assuming, therefore, that zero Ca²⁺ signaling activity in the slow muscle between 19.5 and 23 hpf equates to no contractile activity during this period. Saint-Amant and Drapeau (1998) do report that in their methyl cellulose-free set-up, the frequency of the spontaneous trunk/tail contractions begins to fall following a peak at ~19 hpf. However, they also describe the onset, at ~21 hpf, of a second type of locomotor behaviour, which they called a “touch-evoked” response. Under our experimental conditions, embryos were never subjected to any touch stimulation. Furthermore, our methyl cellulose embedding protocol may have in some way speeded up the fall in spontaneous contractile activity, leading to the establishment of the QP at ~19.5 hpf.

It is interesting to note, however, that the QP of Ca²⁺ signaling from the SMCs coincides with a period (i.e., from 21 to 22 hpf) of intense spontaneous Ca²⁺ signaling activity within the developing spinal cord of the zebrafish embryo (Ashworth and Bolsover, 2002). We failed to detect these spinal cord transients either due to the tissue-specificity of apoaequorin expression in our aequorin-based imaging experiments, or due to the focal plane selected for the fluorescence-based confocal scanning. Thus, the QP appears to correlate with a transition from spontaneous contractile...
activity that does not require sensory input and motor activity circuitry, to touch-response contractile activity that does. Perhaps it is not surprising, therefore, that developmental Ca\textsuperscript{2+} signaling activity in the embryonic spinal column increases during this period while the required circuitry and contacts are being established, while that in the SMCs themselves, declines.

A second period of spontaneous Ca\textsuperscript{2+} signaling, the SP2, begins at ~23 hpf. The frequency, duration, and spatial signature of the SP2 signals are quite distinct from those of the early SP1 signals. Brennan et al. (2005) only reported signaling activity up to ~22 hpf, thus our new study extends the visualization of trunk muscle Ca\textsuperscript{2+} signaling into the SP2 period. Saint-Amant and Drapeau (1998) report that in older embryos (i.e., >24 hpf) the spontaneous contractions change from being continuous to occurring in bursts of between 3-5 coils, followed by periods of inactivity. These might prove to correlate with the bursts of Ca\textsuperscript{2+} signaling activity followed by periods of inactivity that we observed to start at ~23 hpf (Fig. 3Ci).

The distinct spatiotemporal pattern of our SP1 and SP2 Ca\textsuperscript{2+} signals bare some similarities to the Xenopus primary myocyte-generated LDTs and SDTs, respectively, reported by Campbell et al. (2006). The durations of the SP1 signals and LDTs, i.e., ~5-10 sec and ~80 secs, respectively, and SP2 signals and SDTs, i.e., ~0.6 sec and ~2 sec, respectively are, however, significantly different. This may reflect species differences, but is more likely due to the fact that we were recording the transients in situ, rather than in cell culture, where a number of endogenous in situ feedback mechanisms that are absent in the simplified cell culture system, might restrict the duration of the transients. What is similar is the fact that both the SP1 signals and the LDTs are inactivity in the embryonic spinal column increases during this period while the required circuitry and contacts are being established, while that in the SMCs themselves, declines.

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The distinct spatiotemporal pattern of our SP1 and SP2 Ca\textsuperscript{2+} signals bare some similarities to the Xenopus primary myocyte-generated LDTs and SDTs, respectively, reported by Campbell et al. (2006). The durations of the SP1 signals and LDTs, i.e., ~5-10 sec and ~80 secs, respectively, and SP2 signals and SDTs, i.e., ~0.6 sec and ~2 sec, respectively are, however, significantly different. This may reflect species differences, but is more likely due to the fact that we were recording the transients in situ, rather than in cell culture, where a number of endogenous in situ feedback mechanisms that are absent in the simplified cell culture system, might restrict the duration of the transients. What is similar is the fact that both the SP1 signals and the LDTs are inactivity in the embryonic spinal column increases during this period while the required circuitry and contacts are being established, while that in the SMCs themselves, declines.

Rostrocaudal spatial characteristics of the SP1 Ca\textsuperscript{2+} signals

Our data clearly demonstrate that at ~17.5 hpf, the low frequency early SP1 signals were only generated from S4 to S10. At ~18.5 hpf, the higher frequency SP1 signals were seen to extend rostrally into S2 and S3 (but never S1) and caudally to S12 (Fig. 4C,D). We have evidence from our addition of triton X-100 to aequorin-expressing embryos at various times during slow muscle cell differentiation, to show that luminescence is generated along the entire length of the trunk musculature and not just from S2-S12 (data not shown). Our aequorin burn-out data suggest that even though the normal Ca\textsuperscript{2+} signals are only generated between S2-S12, the stable expression and reconstitution of aequorin does extend to the other somites too. This was found to be the case for both the transgenic embryos (n=6) and where the aequorin is transiently expressed (n=3). In teleosts, the first three somites (i.e., S1-S3) are regarded as giving rise to mainly occipital and other head structures (Ma et al., 2009). Thus, the most rostral somite that gives rise to truly axial structures, including the trunk musculature, has been suggested to be S4 (Holley and Nüsslein-Volhard, 2000; Ma et al., 2009). We propose therefore that a possible reason why the initial low-frequency SP1 Ca\textsuperscript{2+} signals (i.e., at ~17.5 hpf) are not seen in S1 to S3, might be because the precursor cells (including the SMCs) in these somites are destined to have different developmental fates than the more caudal somites. The higher frequency SP1 Ca\textsuperscript{2+} signals that start at ~18.5 hpf do extend into S2 and S3, which again may reflect differences in the type of SMCs derived from these somites. The caudal restriction of the SP1 signals (i.e., S10 and S12 at ~17.5 hpf and ~18.5 hpf, respectively) may reflect the fact that this block of more anterior somites differentiates and matures first. This also fits in well with observations made by Saint-Amant and Drapeau (1998) that only a restricted number of more anterior somites appear to be active in generating spontaneous contractile movements. Furthermore, the anterior trunk somites have been described as being genetically different from the tail somites, although the primary mechanism for somite formation appears to be the same for both anterior trunk and tail somites (Szeto and Kimelman, 2006; Holley, 2006). We suggest that these differences reflect, and/or maybe the result of differences in the embryonic Ca\textsuperscript{2+} signaling signature.

Sub-cellular spatial characteristics of the SP1 and SP2 Ca\textsuperscript{2+} signals

Our new imaging data further revealed that the SP1 signals also had distinct spatial characteristics with regard to where they were generated within a differentiating SMC. At ~17.5 hpf, the low frequency Ca\textsuperscript{2+} signals appeared to be generated mainly in and around the nucleus with relatively little spread out into the cytoplasm (Fig. 5). At ~18.5 hpf, however, it is clear that although nuclear signaling still plays a significant role, the Ca\textsuperscript{2+} signals also propagate out into the cytosolic fraction of the SMC (Figs. 6 and 7). It is also clear that all the muscle cells within a single somite, and the muscle cells in adjacent somites, "fire" their Ca\textsuperscript{2+} signals at approximately the same time (Fig. 6). This indicates synchronized activity and suggests a role for neuronal-based co-ordination of the Ca\textsuperscript{2+} signaling in these somites, thus supporting the observations of Brennan et al. (2005). The synchronized Ca\textsuperscript{2+} activity in S7 to S10 at ~18 hpf is tightly correlated with a spontaneous rostral/caudal contraction of these somites (see Supplemental Movie #1).
primary culture of skeletal muscle (Jaimovich et al., 2000). The Ca²⁺ signal then dies down in the cytoplasmic regions of the cell after ~2 secs, but lingers longer in the nucleus, i.e. in this particular example, for at least 7.5 secs (Fig. 7).

The complex spatial nature of the spontaneous Ca²⁺ signals shown in Fig. 7 also bear a strong similarity to aspects of Ca²⁺ signals induced by elevated K⁺ in cultured rat and mouse myotubes (Jaimovich et al., 2000; Powell et al., 2001). In these cells two types of Ca²⁺ transient, with different kinetics were identified. One was a fast Ca²⁺ transient, which was associated with RyRs and excitation-contraction coupling (E-CC). Following this fast transient was a slower Ca²⁺ transient, which resulted in an increase in nucleoplasmic Ca²⁺ (that lasted in the range of 30-40 secs), but not in contraction. We suggest that something similar is observed in zebrafish SMCs (Fig. 7) where the shorter duration cytoplasmic signals are associated with E-CC and the longer duration nuclear signals serve another function. There would also appear to be clear differences between our new observations in intact zebrafish and those from cultured mouse myotubes. For example, we observe a rise in nuclear Ca²⁺ before the generation of the cytosolic transient (Fig. 7).

In addition, it has been reported that treatment of rat and mouse myotubes with nifedipine or 2-APB completely blocked the slow nucleoplasmic transient, thus suggesting that DHPRs are the main membrane voltage sensors for this Ca²⁺ signal, and that the signal was generated mainly by Ca²⁺ release from internal stores via IP₃Rs (Powell et al., 2001; Jaimovich and Carrasco, 2002). Furthermore, this slow IP₃R-mediated Ca²⁺ transient was reported to be linked to developmental gene expression, where K⁺-induced depolarization of mouse myotubes resulted in the rapid (i.e., within 30 sec - 10 min) phosphorylation of the mitogen activated kinases, ERK 1/2 and the transcription factor, CREB, as well as the expression (within 5 – 15 min) of various early genes (Powell et al., 2001; Jaimovich and Carrasco, 2002; Carrasco et al., 2003; Cárdenas et al., 2005). Thus, we suggest that our early Ca²⁺ oscillations located in and around the nucleus of SMCs at ~17.5 hpf (Fig. 5A), as well as the longer duration nuclear component of the later signals observed at ~18.5 hpf (Figs. 6A, 7C), may play a role in gene expression and the translation of some important proteins during myofibrillogenesis, whereas the rapid cytoplasmic phase of the SMC generated signals might play a role in the spontaneous E-CC along the anterior trunk (Supplemental Movie #1).

In the case of the spontaneous SP2 Ca²⁺ signals, we did not visualize any specific long-duration nuclear component, as the SP2 signals consisted exclusively of short-duration cytoplasmic signals (Fig. 8). The SP2 Ca²⁺ signals were generated only when the banding patterns of F-actin and myosin were clearly formed along the entire length of each SMC (Fig. 14 Fi-Fiil); when the IP₃Rs and RyRs were well organized into distinct bands (Fig. 13Av,Bv); and they were well-correlated with stronger contractions within the SMCs.

**Confirming the specific slow muscle cell location of the SP1 Ca²⁺ signals**

From observations using confocal Ca²⁺ imaging, the SP1 Ca²⁺ signals appeared to be generated exclusively in SMCs. This was tested and confirmed both pharmacologically and genetically. In the case of the former, embryos were treated with cyclopamine or forskolin (Fig. 9), while in the case of the latter, we imaged the Ca²⁺ signaling signature of smo⁻²⁰³ mutant fish (Fig. 10). In all three cases, embryos displayed U-shape somites (Figs. 9Cii,Dii, 10Bii) and the SMCs did not develop (Figs. 9Ci,Di, 10Bi) as previously reported by (Barresi et al., 2000; 2001). As no Ca²⁺ signals were generated from the trunk of these embryos (Figs. 9Ci,Div, 10Bi) we concluded that the normal pattern of Ca²⁺ signals observed in the untreated wild-type embryos are generated exclusively by the SMCs.

We did not, via either aequorin- or fluorescence-based imaging, visualize any Ca²⁺ signals from the fast muscle cells (FMCs). This was in spite of the aequorin being clearly expressed in the FMC precursors (Fig. 2C) and confocal optical sectioning through the entire myotome block of calcium green-1 dextran-loaded embryos (data not shown). This result was somewhat puzzling as FMCs have been reported to differentiate immediately after the medial-to-lateral migration of the SMCs, which begins at ~20 hpf (i.e., the 21-somite stage) in the anterior somites (Blagden et al., 1997). Therefore, our Ca²⁺ signaling data seems to suggest that the spontaneous contractions of the embryonic trunk up to at least 24.5 hpf (i.e., when imaging was terminated) are generated by SMCs alone.

**Exploring the mechanisms of Ca²⁺ release in SMCs**

Our new data showed that when embryos were treated with 25 µM 2-APB as soon as the first SP1 Ca²⁺ signals were observed (i.e., at ~17 - 17.5 hpf), then these signals were blocked within ~3 ± 1.5 min (n = 6) of treatment (Fig. 12Bi). On the other hand, when embryos were treated with 50 µM ryanodine as soon as the first SP1 Ca²⁺ signals were observed, then initially only the amplitude of SP1 Ca²⁺ signaling was reduced. Complete inhibition of the SP1 signals only occurred ~51 min ± 7 min (n = 5) after treatment (Fig. 12Ci), i.e., some 17-fold slower than treatment with 2-APB. These data suggest, therefore, that the initial high amplitude, low frequency SP1 Ca²⁺ signals that were visualized mainly in and around the nucleus at ~17.5 hpf, may be generated primarily via IP₃Rs rather than RyRs. The latter then become more prominent in generating the higher frequency cytoplasmic signals that start after ~18 hpf. We do not propose, however, that either one of these two Ca²⁺ release mechanisms operates to the exclusion of the other during these time periods, but rather that one plays a more prominent role with regard to the method of Ca²⁺ release at a particular developmental time and in a specific cellular location. We suggest that the identification of functional IP₃Rs generating spatially and temporally distinct sub-cellular Ca²⁺ transients during the early stages of SMC differentiation in intact zebrafish is one of the significant new findings presented in this paper.

In addition to treatment with different antagonists of Ca²⁺ release from intracellular stores, we report that the SP1 Ca²⁺ signals could be completely blocked from their very earliest appearance, i.e., from ~17.5 hpf onward, by treatment with either the acetylcholine receptor (AchR) antagonist, α-bungarotoxin (Fig. 11Bi) or the DHPR antagonist, nifedipine (Fig. 11Ci). Brennan et al. (2005) also showed that somewhat later, i.e., between 18-20 hpf, the Ca²⁺ signals and spontaneous muscle contractions could be completely blocked with α-bungarotoxin, and that >21 hpf the muscle contractions were significantly blocked with nifedipine. We felt it most important to establish that treatment with α-bungarotoxin and nifedipine could block the very earliest of
the SP1 transients, thus establishing a clear developmental time-course with respect to the relationship between neuronal innervation of the somites and the generation of Ca\(^{2+}\) transients by the SMCs. In addition, Saint-Amant and Drapeau (1998) reported that lesioning the hindbrain had no effect on the generation of the spontaneous contractions and they suggested that these contractions resulted from activation of a primitive spinal circuit. Indeed, our α-bungarotoxin and nifedipine data support this suggestion, and we propose that acetylcholine induces periodic depolarizations of the sarcolemma of SMCs, which, via the activation of DHPRs, stimulates the release of Ca\(^{2+}\) from intracellular stores initially by IP\(_3\)Rs, then mainly RyRs, and thus generates the periodic spontaneous trunk contractions in zebrafish (Supplemental Fig. S1).

**Localization of IP\(_3\)Rs and RyRs in SMCs**

We show that between ~20 hpf to ~24 hpf there is a progressive organization of IP\(_3\)Rs into distinct bands (Fig. 13 Aiii-Av). Co-labeling with the F59 myosin heavy chain antibody indicates that at ~24 hpf the IP\(_3\)Rs were localized to the region of sarcoplasmic reticulum (SR) adjacent to the sarcomeric I-band, with a higher intensity of labeling in the SR adjacent to the z-line (Fig. 13 C, E). This is similar to what was found in cultured muscle myotubes, where the IP\(_3\)Rs were also reported to be localized in the I-band SR (Powell et al., 2001). We were unable to detect a clear and distinctive pattern of IP\(_3\)R staining in the nuclear region of SMCs in zebrafish (especially during the early SP1), as has been reported in cultured muscle myotubes (Jaimovich et al., 2000; Powell et al., 2001). This localization would have supported our calcium green-1 dextran imaging experiments with untreated embryos (Figs. 5-7) and the aequorin imaging experiments following treatment with 2-APB (Fig. 12). We tried several commercially available (as well as one custom-made zebrafish) anti-IP\(_3\)R antibodies without success. However, we suspect that the IP\(_3\)Rs responsible for generating the nuclear Ca\(^{2+}\) signal are most likely to be localized preferentially in the inner nuclear membrane, as has been reported in cultured rat skeletal muscle cells (Cárdenas et al., 2005). We found that this region of the nucleus is difficult to access successfully and thus label via immunohistochemistry in intact embryos, and we suggest that this might be why we do not see any IP\(_3\)R labeling of the nuclear envelope in SMCs that would correspond to the IP\(_3\)-mediated nuclear Ca\(^{2+}\) signaling during the early SP1.

There is also a progressive organization of RyRs into bands between ~20 hpf to ~24 hpf (Fig. 13 Biii-Bv). Co-labeling of the RyRs and F-actin (with rhodamine-phalloidin) indicated that at 24 hpf the RyRs are also located in the I-band SR, as has been reported in the region of the z-line (Fig. 13 D, F). It has been previously reported that RyRs are localized along the interface of the SR and T-tubule, at the terminal cisternae of the SR (Ward and Lederer, 2005) and that in Xenopus and zebrafish, a single T-tubule is located at the z-line (Davis and Carlson, 1994; Zhang et al., 2009), which supports our RyR immunolabeling data. In addition, Fig. 13C and 13D indicate that as both IP\(_3\)Rs and RyRs are localized at the z-line SR, both types of Ca\(^{2+}\) channels may be able to interact with the DHPRs located in the T-tubule either indirectly (IP\(_3\)Rs) or directly (RyRs). This idea was originally proposed by Araya et al., 2003) who reported that this spatial arrangement was responsible for generating depolarization-evoked, IP\(_3\)-mediated, slow Ca\(^{2+}\) signals in primary cultures of rat skeletal muscle cells. We suggest that a somewhat analogous situation may also be found in zebrafish where IP\(_3\)-mediated Ca\(^{2+}\) release is responsible for the initial, and prolonged, signal from the nucleus, while predominantly RyR-mediated release drives the subsequent faster cytosolic phase of SMC Ca\(^{2+}\) signaling.

**Possible roles of spontaneous Ca\(^{2+}\) signals in slow muscle development**

To explore the possible developmental function of the spontaneous SP1 Ca\(^{2+}\) signals, we examined the effect of the antagonists, 2-APB and ryanodine, on the organization and localization of the key contractile proteins, actin and myosin, as well as on the overall number and gross morphology of the SMCs. Treatment with either 2-APB or ryanodine resulted in a disruption of the normal pattern of actin and myosin banding in a dose dependent fashion (Fig. 15 C-F). Our ryanodine treatments support the observation of Brennan et al. (2005) that AChR activity generates Ca\(^{2+}\) signals via RyRs, and that these play a crucial role in the development of myocardial fiber organization. This role includes regulating both sarcomere and myofiber length, as well as the bundling and alignment of myofilibrils.

Our new data show that both 2-APB and ryanodine-treated embryos have abnormal sarcomere assembly (Fig. 15). It has been previously reported, however, that treatment of tail-cut zebrafish embryos at 17 hpf with 10 μM and 50 μM ryanodine leaves sarcomeric banding relatively intact (Brennan et al., 2005), whereas we report that while this is true to a certain extent for treatment with 10 μM ryanodine, 50 μM ryanodine treatment almost completely abolishes the banding pattern (Fig. 15 Fi", Fi"i and Fi"ii"). This difference might be explained by the amount of the tail excised in order to expose the internal tissues to the receptor antagonists contained in the bathing medium. We attempted to remove the caudal 100 μm of the tail, and found this to be sufficient to expose the anterior somite blocks to the diffusion of solutes from the bathing medium through the interior trunk intercellular space. This diffusion was assessed via tracking the spread of fluorescence through the trunk of embryos that were incubated, following the tail-cut procedure, in α-bungarotoxin-Alexa Fluor 488.

Treatment with ryanodine did not disrupt SMC number (Fig. 16). This supports the observations previously reported by Brennan et al. (2005). On the other hand, treatment with 2-APB had a significant effect on the number of SMCs generated (Fig. 16). This observation supports our suggestion that the IP\(_3\)-mediated signals may act to regulate the expression of a particular set of genes required for the differentiation of SMCs (Jaimovich et al., 2000; Powell et al., 2001; Araya et al., 2003).

Ryanodine treatment also resulted in the detachment of the SMCs from the vertical segment boundaries (especially at the dorsal and ventral regions of the somites; Fig. 15 E, F). The SMCs in the medial region of the somites tend to remain attached to the vertical segment boundaries after treatment, and could thus be seen to extend across the whole A/P length of the somite. This may result from the concentration of ryanodine being greater at the dorsal and ventral edges of the somites than in the medial regions, or that the SMCs are more mature, and thus have developed better connections with the segment boundary (Barresi et al., 2001). This result, along with the overall disruption in
myotome shape caused by ryanodine treatment, suggests that there may be Ca\textsuperscript{2+}-sensitive elements in the complex segment boundary matrix that regulate the interaction between SMCs and the boundaries. These elements may therefore play a key role in maintaining the overall architecture of the chevron-shaped myotome block during myofibrillogenesis (Henry et al., 2004). It is interesting to note that treatment with 2-APB had little effect on the overall architecture of the myotome blocks, whereas ryanodine-treatment resulted in a gross disruption, where the somites assumed a U-shaped morphology. These observations are similar to those made in cyclopamine- or forskolin-treated embryos (Fig. 9) or in the smo\textsuperscript{-/-} mutant fish (Fig. 10), when SMCs were completely absent. The data suggest that normally formed SMCs may play an important role in determining the overall morphology of the trunk myotome blocks.

Our new data thus support and extend the pioneering studies using *Xenopus* embryos, where Ca\textsuperscript{2+} is released predominantly from RyRs, which is intimately involved in the differentiation of skeletal muscle. This differentiation involves cytoskeletal organization during myofibrillogenesis and the formation of sarcomeres in both cultured *Xenopus* myocytes and maturing somites of *Xenopus* embryos (Ferrari et al., 1996; 1998; Ferrari and Spitzer, 1999; Campbell et al., 2006).

**Materials and Methods**

**Embryo collection**

Zebrafish (*Danio rerio*), including the wild-type AB strain, the smo\textsuperscript{-/-} mutant (both from the Zebrafish International Resource Centre; University of Oregon, Eugene, OR, USA) and the \(\alpha\)-actin-apoaequorin-IRESGFP (\(\alpha\)-actin-aeg) transgenic line, were maintained on a 14 hr light/10 hr dark cycle to stimulate spawning (Westerfield, 1994), and their fertilized eggs were collected as described elsewhere (Webb et al., 1997). Embryos were maintained in 30% Danieau’s solution (17.4 mM NaCl, 0.21 mM KCl, 0.18 mM Ca(NO\textsubscript{3})\textsubscript{2}, 0.12 mM MgSO\textsubscript{4}.7H\textsubscript{2}O, 1.5 mM Hepes, pH 7.2) at ~28.5°C throughout development and during all experiments.

**Preparation of the pa-KS-aeg-IRESGFP plasmid**

To prepare the pa-KS-aeg-IRESGFP plasmid (Fig. 1) used for generating the \(\alpha\)-actin-aeg transgenic zebrafish, PCR was carried out to amplify the apoaequorin gene from the pIP-HE plasmid (Inouye et al., 1989), using the following oligonucleotide primers: 5’-accagcaatcagtaacaacaacataactcgtacgctacagcgc-3’ and 5’-accagcactagggagcactcgctagagg-3’, in which two restriction enzyme recognition sites for, EcoRI and SaI, were added to the 5’ and 3’ ends of the apoaequorin gene, respectively. The apoaequorin gene was then cloned into the pBluescriptII-KS+ plasmid (Clontech Laboratories, Inc., Mountain View, CA, USA) using these restriction enzyme sites. The aeg-IRESGFP fragment was then excised with EcoRI and NotI, after which it was cloned into the pa-SK plasmid (Higashijima et al., 1997) to obtain an aeg-IRESGFP fragment with an \(\alpha\)-actin promoter (i.e., \(\alpha\)-aeg-IRESGFP). In parallel, the SV40 late polyadenylation signal (pA) was amplified from the pCMVT\(_{\text{T}}\)x vector (Promega Corp., Madison, WI, USA) using the following oligonucleotide primers:

5’-accacggccgcccagcatagatacattg-3’ and 5’-accagcacttagcagctagctagcctaggttt-3’, in which the NotI recognition sequence was added to the 5’ end and AfeI, XbaI and SacI recognition sequences were added to the 3’ end. The SV40 late polyadenylation signal was then cloned into the pBluescriptII-KS+ plasmid following NotI and SacI double digestion, after which the \(\alpha\)-aeg-IRESGFP fragment was cloned into this plasmid following XhoI and NotI double digestion. All the restriction enzymes used in this study were obtained from New England Biolabs, Inc.

**Generation of transgenic fish**

The \(\alpha\)-actin-aeg transgenic embryos (and, for some experiments, aeg-mRNA injected embryos) were dechorionated manually with watchmaker’s forceps at the 8-cell stage and then incubated in a custom-designed holding/imaging chamber with 50 mM \(\alpha\)-coelenterazine (Molecular Probes, Invitrogen Corp., Carlsbad, CA, USA) in 30% Danieau’s solution to reconstitute the active aequorin. Apoaequorin was transiently expressed in zebrafish embryos by injecting ~1 nl (i.e., ~500 pg/nl, prepared in nuclease-free water) aeg-mRNA into the center of the yolk at the 1-cell stage following protocols described by Cheung et al. (2006). The \(\alpha\)-coelenterazine was prepared as a stock solution of 5 mM in 100% methanol and was diluted in 30% Danieau’s solution just prior to use. Chambers containing embryos bathed in \(\alpha\)-coelenterazine solution were then transferred to a custom-built Photon Imaging Microscope System (PIMS; Science Wares, East Falmouth, MA, USA) for data acquisition from ~16.5 to 24 hpf (i.e., the 15- to 30-somite stage) at ~28.5°C. An outline of the PIMS and the photon acquisition procedure is described by Webb et al. (1997). The imaging software, IpdWin2k, allowed the collected photon data to be subsequently analyzed with any chosen integration time and region of interest (ROI). All data were exported to Microsoft Office Excel 2003 (Microsoft Corp., Redmond, WA, USA), GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA) and Corel Draw X3 (Corel Corp., Ottawa, Canada) for statistical analysis and figure preparation.

**Western blot analysis**

Embryos of the \(\alpha\)-actin-aeg transgenic line were collected at 12, 14, 18, 20, 22 and 24 hpf for the preparation of protein extracts used in Western blot analysis. The preparation of protein extracts and Western blot analysis were conducted as described by Cheung et al. (2006). Extract prepared from 3 embryos was loaded into each lane of the SDS-polyacrylamide gel. 20 pg AquaLight® aequorin (Molecular Probes) was used as an internal control and the extract from wild-type embryos at 24 hpf was used as a negative control.

**Calcium green-1 dextran imaging**

Wild-type embryos were microinjected at the 1-cell stage with 2 nl of a mixture of the fluorescent Ca\textsuperscript{2+}-sensitive indicator, calcium green-1 dextran (10S; 200 µM; Molecular Probes), and the Ca\textsuperscript{2+}-insensitive fluorescent dye, rhodamine B dextran (10S; 200 µM; Sigma-Aldrich Corp). The injected embryos were then maintained at ~28.5°C until the start of the Segmentation Period. A series of time-lapse images was then recorded in somites 7-9 of the injected embryos at ~17.5 hpf (i.e., the 17-somite stage) and ~24 hpf (i.e., ~prim-5 stage) using the Nikon D-Eclipse C1 scanning confocal system, described previously, with a Nikon Fluor 40x/0.8NA water dipping objective lens. Calcium green-1 dextran-generated fluorescence was acquired using a 488 nm excitation laser and a 515/530 nm emission filter. In addition, a series of time-lapse images was recorded in somites 7-10 of injected embryos at ~18.5 hpf (i.e., the 19-
somite stage) using a Nikon D-Eclipse C1si scanning confocal system mounted on a Nikon Eclipse 80I upright microscope with a Nikon Achromat 40x/0.8NA water dipping objective lens. With this system, green fluorescence was acquired using a 488 nm excitation laser and a 525/550 nm (band pass) emission filter, and red fluorescence was acquired using a 561 nm excitation laser and a 605/660 nm (band pass) emission filter. To achieve a significant period of uninterrupted fluorescence-based Ca$$^2$$+ Imaging, ~160 images were acquired over a period of 100 sec, first with 488 nm excitation (for Ca$$^2$$+ imaging), and then with 543 nm (Eclipse C1) or 561 nm (Eclipse C1si) excitation (to monitor Ca$$^{2+}$$-independent changes in fluorescence). All these data were acquired via the Nikon EZ-C1 3.40 software. This software was also used to review and analyze the calcium green-1 dextran and rhodamine dextran-generated fluorescence. At ~17.5 hpf and ~24 hpf, the average fluorescence intensity of a circular sampling ROI covering ~4-5 SMCs in somite 8 was acquired. At ~18.5 hpf, this same ROI was placed in the middle of somites 7, 8, 9 and 10 to compare the Ca$$^2$$+ signals generated in adjoining somites, while for the comparison of Ca$$^2$$+ signals generated in different regions of the same somite, the ROIs were placed in the dorsal, middle and ventral regions of somite 9.

To analyze the Ca$$^2$$+ signals generated from a single muscle cell located in somite 10, the total fluorescence intensities in the nucleus alone and in the entire cell muscle were measured, and then the total fluorescence intensity in the cytoplasm was determined by subtracting the total fluorescence intensity of nucleus from the total fluorescence intensity of the entire muscle cell. The fold-change in fluorescence intensity was then calculated by determining the ratio of the maximum fluorescence intensity in relation to the basal level.

All data were exported to Microsoft Office Excel 2000, GraphPad Prism 5 and Corel Draw X3 for figure preparation and statistical analysis.

**Inhibition of slow muscle development using a pharmacological approach: cyclopamine and forskolin treatment**

To block the development of SMCs, wild-type embryos were incubated either in 0.1 mM cyclopamine (Calbiochem, Gibbstown, NJ, USA; Barresi et al., 2000; Hirsinger et al., 2004) dissolved in 1% DMSO or in 0.15 mM forskolin (Calbiochem; Barresi et al., 2000) dissolved in 2% DMSO in 30% Danieau’s solution, from ~5.5 hpf to ~24 hpf. As a control, some embryos were incubated in 2% DMSO in 30% Danieau’s solution in 30% Danieau’s solution alone. Bright-field images of these embryos were captured at ~24 hpf using a Nikon Dxm 1200F camera mounted on a Zeiss Axioskop microscope with a Zeiss Plan Neofluar 10x/0.3NA objective lens. Fluorescence labeling was then conducted using the F59 myosin heavy chain antibody to confirm the loss of SMCs in the cyclopamine- and forskolin-treated embryos.

In another series of experiments, the α-actin-aeq transgenic embryos were initially incubated in fcoeolenterazine solution to reconstitute active aequorin in vivo at the 8-cell stage and then were incubated in either 0.1 mM cyclopamine or 0.15 mM forskolin in fcoeolenterazine solution starting at ~5.5 hpf. These cyclopamine- and forskolin-treated transgenic embryos were then imaged using the PIMS from ~17 hpf to ~21 hpf.

**Inhibition of slow muscle development using a genetic approach: the smo null mutant**

The slow-muscle-omitted (smo) also known as smut) mutant contains mutations in the smo gene that leads to many developmental processes involving hedgehog signaling being disrupted (e.g., slow muscle formation: Barresi et al., 2000). In this study, a recessive lethal mutant line, smo$$^{de5576857}$, was used. For the Ca$$^2$$+ imaging experiments, normal control (i.e., smo+ or smo+/+) and smo- embryos were initially injected with aeq-mRNA at the 1-cell stage and then were incubated in fcoeolenterazine solution to reconstitute active aequorin in vivo at the ~8-cell stage. These injected embryos were imaged using the PIMS from ~17 hpf to ~21 hpf. As aequorin is expressed throughout the entire embryo in aeq-mRNA-injected embryos, the average luminescent intensity of a circular sampling ROI covering ~8000 μm$$^2$$ on somite 8 was plotted over time. This served to eliminate the possibility of collecting aequorin-generated light from other parts of the embryo.

**Blocking Ca$$^2$$+ signals using a pharmacological approach**

The α-actin-aeq transgenic embryos were incubated in fcoeolenterazine solution to reconstitute active aequorin in vivo. These embryos were imaged using the PIMS and the FB15 luminometer (Zy Lux Corp., Maryville, TN, USA) at ~7 hpf and then they were treated with either 25 μM 2-APB (Sigma-Aldrich Corp.) or 50 μM ryanodine (Calbiochem) for 3 hrs starting at ~17.5 hpf (i.e., when the Ca$$^2$$+ signals started) to block Ca$$^2$$+ release from IP$_3$Rs or RyRs, respectively. The terminal ~100 μm region of the tip of the tail bud was excited (i.e., such that the posterior-most, newly formed somites were not damaged) using a tungsten needle (27G1/2; Becton Dickinson & Co., Franklin Lakes, NJ, USA) just prior to the Ca$$^2$$+ channel antagonist treatments, to ensure that the drugs diffused into the trunk tissues of the later-stage embryos (Liu and Westerfield, 1990). In another series of experiments, tail-tip excised wild-type embryos were treated from ~17 hpf to ~20 hpf with 2-APB (at 25 μM or 50 μM) or with ryanodine (at 10 μM or 50 μM) after which they were fixed immediately prior to dual labeling of myosin and actin.

**α-Bungarotoxin and nifedipine treatment**

The α-actin-aeq transgenic embryos were incubated in fcoeolenterazine solution to reconstitute active aequorin in vivo. The tip of the tail was excised as described previously, after which embryos were treated with either 3 μM α-bungarotoxin-Alexa Fluor 488 (Molecular Probes) or 500 μM nifedipine (Sigma-Aldrich Corp.) in 30% Danieau’s solution at ~16.5 hpf. The former is an inhibitor of nicotinic acetylcholine receptors, nACHRs (Wonnacott, 1986; Pugh and Berg, 1994), while the latter is an organic blocker of the L-type voltage sensitive Ca$$^2$$+ channel, which targets the dihydropyridine binding site on the α-subunit of the L-type Ca$$^{2+}$$ channel (Zamponi, 1997; Fischer and Schäffer, 2002). The Alexa Fluor 488 conjugate of α-bungarotoxin was used so that the distribution of the toxin could be determined within the musculature of the embryonic trunk. The luminescence of these embryos was then detected using the FB15 luminometer from ~17 to ~21 hpf. Triton X-100 was added to “burn-out” all remaining aequorin at the end of the experiment.

**Immunohistochemistry and rhodamine phalloidin labeling**

The F59 IgG1 myosin heavy chain antibody (Crow and Stockdale, 1988) was used to label the slow muscle in the trunk of zebrafish embryos from ~16 hpf to ~24 hpf. Tissue culture supernatant of the F59 antibody (Developmental Studies Hybridsoma Bank, Iowa City, IA, USA) was used at a dilution of 1:10. The 34C monoclonal anti-ryanodine receptor antibody (Sigma-Aldrich Corp., MO, USA; Brennan et al., 2005) recognizes RyR-1 and RyR-2, and was used at a dilution of 1:500. The anti-InsP3R, Type 1 polyclonal IP$_3$R antibody (Upstate, Millipore Corp., Temecula, CA, USA) recognizes the type 1 IP$_3$R channel (Zamponi, 1997; Fischer and Schäffer, 2002). The Alexa Fluor 488 conjugate of α-bungarotoxin was used so that the distribution of the toxin could be determined within the musculature of the embryonic trunk. The luminescence of these embryos was then detected using the FB15 luminometer from ~17 to ~21 hpf. Triton X-100 was added to “burn-out” all remaining aequorin at the end of the experiment.
solution of 1.5 mg/ml; Jackson Immuno Research Laboratories, Inc., PA, USA), was used, while for the anti-InsP3R labeling, an Alexa Fluor 568 goat anti-rabbit secondary antibody (stock solution of 2 mg/ml; Molecular Probes, Invitrogen Corp., Carlsbad, CA, USA) was used. Both of these secondary antibodies were prepared at a dilution of 1:200 in blocking buffer just prior to use. The embryos were incubated with the secondary antibody overnight at 4°C in the dark and they were then washed extensively with PBST/BSA.

The fluorescence images of labeled embryos were acquired with the Nikon D-Eclipse C1 scanning confocal system described previously using a Nikon Fluor 60x/1.0NA water-dipping objective lens. Green fluorescence was captured using a 488 nm excitation wavelength and a 515/530 nm emission filter, while red fluorescence was captured using a 543 nm excitation wavelength and 570 nm (long pass) emission filter.

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References


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