Visualization of Ca^{2+} Signaling During Embryonic Skeletal Muscle Formation in Vertebrates

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Dynamic changes in cytosolic and nuclear Ca^{2+} concentration are reported to play a critical regulatory role in different aspects of skeletal muscle development and differentiation. Here we review our current knowledge of the spatial dynamics of Ca^{2+} signals generated during muscle development in mouse, rat, and *Xenopus* myocytes in culture, in the exposed myotome of dissected *Xenopus* embryos, and in intact normally developing zebrafish. It is becoming clear that subcellular domains, either membrane-bound or otherwise, may have their own Ca^{2+} signaling signatures. Thus, to understand the roles played by myogenic Ca^{2+} signaling, we must consider: (1) the triggers and targets within these signaling domains; (2) interdomain signaling, and (3) how these Ca^{2+} signals integrate with other signaling networks involved in myogenesis. Imaging techniques that are currently available to provide direct visualization of these Ca^{2+} signals are also described.

The recognition of Ca^{2+} as a key regulator of muscle contraction dates back to Sydney Ringer's seminal observations in the latter part of the 19th Century (Ringer 1883; Ringer 1886; Ringer and Buxton 1887; see reviews by Martonosi 2000; Szent-Györgyi 2004). More recently, evidence is steadily accumulating to support the proposition that Ca^{2+} also plays a necessary and essential role in regulating embryonic muscle development and differentiation (Flucher and Andrews 1993; Ferrari et al. 1996; Lorenzon et al. 1997; Ferrari and Spitzer 1998, 1999; Wu et al. 2000; Powell et al. 2001; Jaimovich and Carrasco 2002; Li et al. 2004;

Brennan et al. 2005; Harris et al. 2005; Campbell et al. 2006; Terry et al. 2006; Fujita et al. 2007; and see reviews by Berchtold et al. 2000; Ferrari et al. 2006; Al-Shanti and Stewart 2009). What is currently lacking, however, is extensive direct visualization of the spatial dynamics of the Ca^{2+} signals generated by developing and differentiating muscle cells. This is especially so concerning in situ studies. The object of this article, therefore, is to review and report the current state of our understanding concerning the spatial nature of Ca^{2+} signaling during embryonic muscle development, especially from an in vivo perspective, and to suggest

Editors: Martin D. Bootman, Michael J. Berridge, James W. Putney, and H. Llewelyn Roderick
Additional Perspectives on Calcium Signaling available at www.cshperspectives.org

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Cite this article as *Cold Spring Harb Perspect Biol* 2011;3:a004325

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possible directions for future research. The focus of our article is embryonic skeletal muscle development because of this being an area of significant current interest. Several of the basic observations reported, however, may also be common to cardiac muscle development and in some cases to smooth muscle development. What the recent development of reliable imaging techniques has most certainly done, is to add an extra dimension of complexity to understanding the roles played by Ca^{2+} signaling in skeletal muscle development. For example, it is clear that membrane-bound subcellular compartments, such as the nucleus (Jaimovich and Carrasco 2002), may have endogenous Ca^{2+} signaling activities, as do specific cytoplasmic domains, such as the subsarcolemmal space (Campbell et al. 2006). How these Ca^{2+} signals interact with specific down-stream targets within their particular domain, and how they might serve to communicate information among domains, will most certainly be one of the future challenges in elucidating the Ca^{2+} -mediated regulation of muscle development.

Any methodology used to study the properties of biological molecules and how they interact during development should ideally provide spatial information, because researchers increasingly need to integrate data about the interactions that underlie a biological process (such as differentiation) with information regarding the precise location within cells or an embryo where these interactions take place. Current Ca^{2+} imaging techniques are beginning to provide us with this spatial information, and are thus opening up exciting new avenues of investigation in our quest to understand the signaling pathways that regulate muscle development (Table 1).

Ca^{2+} IMAGING METHODOLOGIES USED IN BOTH IN VIVO AND IN VITRO STUDIES

Ca^{2+} signals have been visualized during muscle formation using both fluorescent and luminescent Ca^{2+} -sensitive reporters. Fluorescent reporters can be incorporated into cells via microinjection or infusion from whole-cell patch pipettes. Alternatively, many fluorescent

Ca^{2+} probes are also available as acetoxymethyl (AM) esters. In this form, the probes are membrane permeable and insensitive to Ca^{2+} . Thus, cells and tissues can simply be incubated with the probe, which is dissolved in normal bathing medium. Once inside the cell, intracellular esterases then hydrolyse the AM moiety, and thus reveal the active Ca^{2+} -sensitive reporter (Tsien 1981). Unfortunately, using these methods, it is not possible to target the reporters to particular organelles, cells, or tissues in vivo. However, a few years ago a number of genetically encoded fluorescent Ca^{2+} reporters that are based on GFP or its spectral variants, were designed. The first family of genetic Ca^{2+} indicators, the cameleons, were based on the Ca^{2+} -sensitive protein, calmodulin, and relied on a fluorescence resonance energy transfer to occur between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) in the presence of Ca^{2+} (Miyawaki et al. 1997; Persechini et al. 1997). More recently, several other genetically encoded fluorescent Ca^{2+} reporters, such as camgaroo-2, inverse pericam and DRIP (DsRed2-referenced Inverse Pericam), have been engineered (Baird et al. 1999; Hasan et al. 2004; Shimozono et al. 2004). Most of the groups who have imaged Ca^{2+} signals during skeletal muscle formation in cells, or in dissected or intact animals, used single-wavelength fluorescent Ca^{2+} reporters that are excited by visible light and either incubated the cells/tissues in the AM ester form, e.g. fluo-3 AM (Ferrari and Spitzer 1999; Jaimovich et al. 2000; Carrasco et al. 2003) or else microinjected individual cells, e.g., with oregon green 488 BAPTA-1 dextran (10 kDa), fluo-4 dextran (10 kDa), or calcium green-1 dextran (Brennan et al. 2005; Cheung et al. 2010). Although a transgenic line of *Caenorhabditis elegans* that expresses cameleon specifically in the pharyngeal muscle has been reported (Kerr et al. 2000), Ca^{2+} transients were only imaged in the adult worms during feeding behavior and not during the formation of the muscle.

The majority of reports that describe fluorescence Ca^{2+} imaging of developing muscle cells, exposed myotome and intact animals use confocal laser scanning microscopy to visualize

Ca²⁺ Signaling and Skeletal Muscle Formation**Table 1.** Examples of developing muscle systems in which Ca²⁺ signals have been directly visualized and spatial information is provided.

Animal	Intact animals/Cells in culture	Ca ²⁺ reporter	Reporter Loading Protocol	Reference
Rat	1° cultures prepared from hind limb muscle of neonatal rat pups	Fluo 3-AM	Cells incubated in 5.4 μM reporter for 30 min at 25°C.	Jaimovich et al. 2000
Mouse	Myotubes grown from C2C12 subclone of the C2 mouse muscle cell line	Fluo 3-AM	Incubated in 5 μM reporter plus 0.1% pluronic F-127 for 1 h at r.t.	Flucher and Andrews 1993
	Myotubes isolated from the intercostal muscles of E18 wild-type and RyR type 3-null mice.	Fluo 3-AM	Cells incubated with 4 μM for 30 min at r.t.	Conklin et al. 1999b
	Myotubes in culture prepared from newborn mice.	Fluo 3-AM	Cells incubated in 10 μM for 20 min.	Shirokova et al. 1999
	1° cultures prepared from hind limb muscle from newborn mice.	Fluo 3-AM	Cells incubated in 5.4 μM reporter for 30 min at 25°C.	Powell et al. 2001
Chick	Embryonic day 18 (E18) isolated diaphragm muscle fibers	Fluo 4-AM	Incubated in 10 μM reporter for 30 min.	Chun et al. 2003
	Myotubes prepared from leg or breast of 11-day chick embryos	Fluo 3-AM	Incubated in 5 μM reporter plus 0.1% pluronic F-127 for 1 h at r.t.	Flucher and Andrews 1993
	Myoblasts isolated from thigh muscle of E12 embryos.	Fluo 3-AM	1 mM stock was diluted 1:200 with 0.2% pluronic F-127. Cells were incubated for 60 min at r.t. in the dark.	Tabata et al. 2006
Xenopus	Exposed myotome in dissected embryo	Fluo-3 AM	Incubated dissected tissue in 10 μM reporter for 30–60 min.	Ferrari and Spitzer 1999
	1° myocyte cultures prepared from stage 15 <i>Xenopus</i> embryos.	Fluo-4 AM	Cells incubated in 2 μM reporter plus 0.01% pluronic F-127 for 60 min.	Campbell et al. 2006
Zebrafish	Intact animals	Calcium green-1 dextran (10S)	Reporter at 20 mM was injected into a single blastomere between the 32- and 128-cell stage.	Zimprich et al. 1998
	Intact animals	Oregon Green 488 BAPTA dextran	Single blastomeres from 32-cell stage embryos injected with reporter (i.c. 100 μM) and tetramethylrhodamine dextran (i.c. 40 μM).	Ashworth et al. 2001
	Intact animals	Oregon Green 488 BAPTA dextran	Microinjected with rhodamine dextran to give an intracellular concentration of ~40 μM.	Ashworth 2004
	Intact animals	Aequorin	^a Embryos injected with 700 pg aeq-mRNA at the 1-cell stage and then incubated with 50 μM f-coelenterazine from the 64-cell stage.	Cheung et al. 2006
	Intact animals	Aequorin	Transgenic fish that express apoequorin in the skeletal muscles were incubated with 50 μM f-coelenterazine from the 8-cell stage.	Cheung et al. 2010

^aExpression of aequorin was ubiquitous but it was suggested that the Ca²⁺ signals visualized in the trunk at the approximately 8–20-somite stage and at ~47 hpf might play a role in muscle development.

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the Ca^{2+} signals (Shirokova et al. 1999; Conklin et al. 1999a, 1999b; Ferrari and Spitzer 1999; Jaimovich et al. 2000; Ashworth et al. 2001; Powell et al. 2001; Chun et al. 2003). In the past, this imaging method tended to be somewhat incompatible with long-duration Ca^{2+} imaging because of photobleaching of reporters and phototoxicity caused by the excitation light, which led to a disruption of development. The development of Ca^{2+} reporters that can be excited with less damaging, longer wavelengths of light, as well as the application of experimental protocols that minimize the exposure of the reporter to excitation, have greatly helped to offset these constraints. However, for short duration imaging experiments, fluorescent confocal imaging has excellent spatial resolution and so can be used to accurately determine the organelles, cells or tissues responsible for generating particular Ca^{2+} transients.

Although fluorescent Ca^{2+} reporters require light excitation, luminescent Ca^{2+} reporters do not. The most common luminescent Ca^{2+} reporter used is aequorin, which consists of a 21-kDa protein, apoaequorin, in combination with a hydrophobic prosthetic group, coelenterazine, and oxygen. Although so-called “native” aequorin was originally extracted and purified from *Aequoria* spp. jellyfish (Shimomura et al. 1962), the successful production of the apoaequorin protein component by molecular cloning techniques (Inouye et al. 1985; Prasher et al. 1987), in addition to the development of synthetic analogues of coelenterazine (Shimomura et al. 1988, 1989, 1990), meant that a variety of recombinant semi-synthetic aequorins, with improved sensitivity to Ca^{2+} ions, are now available. Some of the first applications of native aequorin were in the study of Ca^{2+} transients in isolated crab (*Maia squinado*) muscle fibers (Ashley 1969) and the classical study of isolated barnacle (*Balanus nubilus*) muscle fibers (Ashley and Ridgeway 1970). The main advantages of aequorins are that they are nontoxic; do not buffer Ca^{2+} to any great extent; are available at a variety of different sensitivities; involve ultra-low background signal (i.e., there is no autoluminescence, analogous to autofluorescence); show inherent

contrast enhancement (i.e., the light increases as the approximate second power of the Ca^{2+} concentration); have very long life times in the cytosol; and have a very wide dynamic range (from 0.01–10 μM in vivo; Miller et al. 1994). Thus, aequorin imaging is an excellent technique to use when imaging Ca^{2+} signals over periods of hours, rather than minutes. This is a particular advantage when studying developmental Ca^{2+} signaling over a period of days. The advantages of aequorin, however, are offset by that fact that the images obtained have a low spatial resolution, especially in the z-axis. In addition, the Ca^{2+} signals generated in embryos at the later stages of development (for example, when the muscles are forming) are difficult to image because recombinant aequorin injected into embryos at the one-cell stage is almost used up by approximately 24 hours postfertilization (hpf; Webb and Miller 2000). It is also clearly impractical to microinject the aequorin into thousands of individual cells in a later-stage embryo. One way to overcome this restriction is to express the protein component of the aequorin complex (i.e., apoaequorin), via genetic means, and then to reconstitute the active aequorin protein complex in vivo by loading the aequorin complex cofactor, coelenterazine, into the embryos separately.

In 2006, we developed a technique in which we transiently and ubiquitously expressed apoaequorin in zebrafish embryos by microinjecting an apoaequorin-mRNA (*aeq*-mRNA) into 1-cell stage embryos (Cheung et al. 2006). Although this transient aequorin expression approach successfully extended the aequorin-based Ca^{2+} imaging window by an additional 24 hours (to ~48 hpf), the *aeq*-mRNA was gradually degraded in the injected embryos resulting in a steady decline in the production of the apoaequorin (Cheung et al. 2006). Furthermore, as the expression of aequorin was ubiquitous, it was impossible to identify the precise groups of cells, tissues or organ anlage that were generating a particular signal in more complex, later-stage embryos (Cheung et al. 2006). Thus, most recently, we have successfully generated a line of transgenic zebrafish that stably expresses apoaequorin specifically in the

musculature using a muscle-specific α -actin promoter (Cheung et al. 2010). In this transgenic line, an EGFP marker was also coexpressed with the apoaequorin from a bicistronic mRNA using the internal ribosome entry site (IRES) sequence. Thus, the expression level and distribution of EGFP reflected the expression level and distribution of apoaequorin in the embryos. Using this transgenic α -actin *aeq* line of fish, it has been possible to continuously image Ca^{2+} signals specifically from the developing musculature in zebrafish embryos up to ~ 52 hpf (Cheung et al. 2010).

Currently, several different types of equipment are commercially available that can be used to detect or visualize aequorin-generated luminescence. Several companies supply relatively inexpensive equipment that provides simple temporal Ca^{2+} information via a photomultiplier tube (PMT). However, only a small number of very specialist companies build the sort of custom-designed imaging systems that provide both temporal and spatial luminescent information, as well as bright-field and fluorescence images, thus enabling the correlation of Ca^{2+} signaling events with morphological features and other cellular changes. All of these imaging systems are built around some form of position-sensitive photon detector. We have two such systems in our laboratory, which were both designed by Mr. Eric Karplus of Science Wares, Inc. (Falmouth, Massachusetts); the first we have had for approximately 15 years and is based around a resistive anode imaging photon detector (RA-IPD; Photek Inc.; Miller et al. 1994; Webb et al. 1997), whereas the other, which has just been commissioned, is based around an electron multiplying charge coupled device (EMCCD; Andor Inc.; Webb et al. 2010). Figure 1 illustrates a block diagram of the RA-IPD-based imaging system, which was used to acquire Ca^{2+} signaling information from the developing skeletal musculature of the intact zebrafish embryo shown in Figure 2Bi.

Most recently, we have developed a complementary luminescent and fluorescent Ca^{2+} imaging approach, in which we use aequorin imaging initially, to provide a two-dimensional, low resolution imaging map of Ca^{2+} signaling

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over extended time-periods. From the information acquired from aequorin-based imaging, we then use a fluorescent Ca^{2+} reporter in conjunction with confocal imaging, to explore the specific characteristics of the Ca^{2+} transients at much higher spatial resolution. Aequorin-based imaging tells us “where” and “when” to look, and then we investigate any interesting Ca^{2+} signals observed in further detail with fluorescent confocal imaging. This approach is especially useful when imaging Ca^{2+} signaling events in the relatively large, three-dimensional zebrafish embryos.

VISUALIZATION OF Ca^{2+} SIGNALS IN DIFFERENT ANIMAL SYSTEMS

The regulation of $[\text{Ca}^{2+}]_i$ has been reported to play a critical role in many aspects of muscle development (myogenesis) and function. Although the majority of Ca^{2+} -muscle studies has been conducted in vitro, with cells in culture, a number of more recent studies have been conducted in vivo, i.e., using intact, normally developing animals. In the following sections we will describe some of seminal Ca^{2+} imaging studies performed using mouse and rat myotubes in culture, in intact normally developing zebrafish embryos, and in the developing myotome of *Xenopus*.

Ca^{2+} Transients Visualized in Mouse and Rat Myotubes

Flucher and Andrews (1993) were one of the first to image Ca^{2+} transients in developing myotubes. They used the C2C12 mouse muscle cell-line as well as preparing primary cultures of both chicken embryo and rat fetal thigh muscle, thus giving a variety of myotubes that differentiated at different rates. Using the fluorescent Ca^{2+} reporter, fluo-3, they showed that in well-differentiated myotubes, spontaneous and electrically-induced contractions were accompanied by transient increases in $[\text{Ca}^{2+}]_i$ that were generated synchronously throughout the myotube (Flucher and Andrews 1993). In young myotubes, however, a different type of spontaneous Ca^{2+} release was observed. These

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Figure 1. Schematic representation of our luminescence imaging system. This system can be used to acquire bright-field and fluorescence imaging information (via a CCD camera) as well as collect bioluminescence data (via a resistive-anode Imaging Photon Detector; RA-IPD). A high level of automation for the microscope makes it possible to rapidly switch among the various imaging modes, and makes it possible to have the computer run automated acquisition sequences over extended periods, for example overnight. The motorized focus allows the computer to acquire image stacks in any imaging mode for three-dimensional reconstructions. This system was designed and built by Science Wares Inc., Falmouth, Massachusetts.

were brief (i.e., ~50–200 ms) Ca^{2+} transients that were localized to small regions (i.e., ~20–50 μm) within the myotubes. These so-called “fast-localized Ca^{2+} transients” were not associated with contractions, were insensitive to electrical stimulation, and occurred in consistent locations and in the absence of extracellular Ca^{2+} . Flucher and Andrews (1993) also showed that in noncontracting myotubes, a low concentration of caffeine stimulated the generation of Ca^{2+} waves, and these were accompanied by a localized contraction. These authors suggested that the different types of Ca^{2+} transients might reflect the stage of

differentiation of the myotubes, with the fast-localized Ca^{2+} transients being a temporary event occurring in the younger cells before the functional differentiation of excitation-contraction (E-C) coupling is accomplished.

Lorenzon et al. (1997) also imaged Ca^{2+} transients in differentiating C2C12 mouse cells. Using fura-2 AM as the Ca^{2+} reporter, they showed that distinct patterns of Ca^{2+} transients could again be visualized at different times during myogenesis, and that they could be induced following a change from growth medium to fusion medium. They described these transients as spontaneous $[\text{Ca}^{2+}]_i$ oscillations, which

developed in microdomains of 5–10 μm in length along the myotube and then propagated within the myotubes as waves with a velocity $\sim 20 \mu\text{m/s}$. They also described localized $[\text{Ca}^{2+}]_i$ spikes, some of which were restricted to a length of just 2–3 μm , whereas others expanded to lengths of up to 20 μm along the myotube; and global $[\text{Ca}^{2+}]_i$ spikes, which were generated in the entire myotube and occurred mainly in mature myotubes. The smaller Ca^{2+} spikes (i.e., those restricted to lengths of $\sim 2\text{--}3 \mu\text{m}$) were suggested to resemble the Ca^{2+} sparks previously described in heart muscle (Cheng et al. 1993). Lorenzon et al. (1997) also showed that at the start of differentiation, i.e., before the voltage-operated Ca^{2+} channels (VOCCs) and ryanodine receptors (RyRs) were coupled, the transients were caused by the release of Ca^{2+} from an intracellular source, but when the VOCCs and RyRs were coupled later in myogenesis there was a switch to a membrane oscillatory mechanism, with spontaneous depolarizations of the membrane causing the observed global Ca^{2+} transients. Conklin et al. (1999a) and Shirokova et al. (1999) also described Ca^{2+} sparks in myotube cultures prepared from wild-type and ryanodine receptor type 3 (RyR3) knockout embryonic and newborn mice, respectively that had been loaded with fluo-3 AM.

It has been shown that in embryonic mammalian skeletal muscle, RyR3s are coexpressed with RyR1s at high levels; however, during the postnatal period the expression of RyR3 declines to very low levels (Flucher et al. 1999). In addition, although RyR1s are coupled to dihydropyridine receptors (DHPRs) to provide a voltage-dependent SR Ca^{2+} release mechanism, which is independent of Ca^{2+} flux across the sarcolemma, RyR3s are activated by voltage-independent mechanisms, for example via Ca^{2+} induced Ca^{2+} release, rather than being coupled to DHPRs (Fessenden et al. 2000; Protasi et al. 2000). Conklin et al. (1999a) showed that when the RyR3 channels are absent then RyR1 channels play a role in generating the Ca^{2+} sparks, however in wild-type embryonic muscle both RyR1s and RyR3s contribute to the Ca^{2+} release for a particular Ca^{2+} spark.

Conklin et al. (1999b) also investigated the kinetics of the Ca^{2+} sparks generated in myotubes prepared from mouse embryos lacking either the β_{1a} subunit or the α_{1S} subunit of the DHPR. They showed that E-C coupling is not essential for the generation of Ca^{2+} sparks in these cells, as sparks were still observed when either the β_{1a} or α_{1S} subunits of the DHPR were absent. As the properties of the Ca^{2+} sparks generated in the DHPR β_{1a} or α_{1S} deficient cells were very different, however, Conklin et al. (1999b) suggested that the subunits might regulate the RyRs independently when the DHPR voltage sensor is nonfunctional. Furthermore, Pisaniello et al. (2003) showed that the expression of both RyR1s and RyR3s corresponds to that of the muscle-specific differentiation marker, sarcomeric myosin. They also showed that Ca^{2+} release via RyRs seems to be required for the differentiation of fetal myoblasts as treatment with ryanodine inhibited the formation of myotubes and the expression of myosin in the fetal muscle cells.

Jaimovich et al. (2000) and Powell et al. (2001) analyzed the Ca^{2+} waves propagating through primary cultures of newborn rat and mouse myotubes loaded with fluo 3-AM. They showed that there are both fast and slow propagating Ca^{2+} waves both of which are triggered by high K^+ and are independent of external Ca^{2+} . They also showed that the former is associated with RyRs and E-C coupling, whereas the latter resulted in an increase in nucleoplasmic Ca^{2+} but not in contraction. An example of a slow Ca^{2+} wave propagating through a rat myotube from nucleus to nucleus is shown in Figure 2A. They showed that the treatment of myotubes with nifedipine or 2-APB completely blocked the slow nucleoplasmic Ca^{2+} wave, which suggests that DHPRs are the main membrane voltage sensors for this Ca^{2+} signal, and that the signal is generated mainly by Ca^{2+} release from internal stores via IP_3Rs (Powell et al. 2001; Jaimovich and Carrasco 2002; Araya et al. 2003). Furthermore, this slow IP_3R -mediated Ca^{2+} transient was reported to be linked to developmental gene expression, in which K^+ -induced depolarization of mouse myotubes resulted in the rapid (i.e., within 30 s –10 min)

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Figure 2. Examples of the spatial patterns of Ca^{2+} signals observed in rat myotubes, in intact normally developing zebrafish embryos and following photoactivation of caged IP_3 in exposed myotome in dissected *Xenopus* embryos. (A) A slow Ca^{2+} wave propagating in a rat myotube that had been preloaded with fluo 3-AM. Series of fluorescence images taken at the times indicated after the addition of 47 mM K^+ to the incubation medium. High- K^+ medium was added after two previous waves were elicited, and the corresponding high- K^+ solutions were replaced by normal medium. The relatively low level of fluorescence elicited by this slow wave shows that the Ca^{2+} signal is located mainly in the cell nuclei and that it is propagated in sequence, from one nucleus to another. Red arrowheads indicate the elevated Ca^{2+} in the nuclei. Scale bar is 50 μm . (B) The Ca^{2+} transients observed in the developing trunk muscles of intact zebrafish embryos, visualized by (Bi) luminescence and (Bii–Biv) fluorescence imaging methodologies (modified from Cheung et al. 2010). (See facing page for legend.)

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).

Ca²⁺ Signaling During the Formation of Muscle in Zebrafish Embryos

Zebrafish embryos provide an excellent model system for studying embryogenesis in vivo. A combination of their optical transparency, large size, and relatively rapid development makes the embryos especially useful for Ca²⁺ imaging.

In zebrafish, as with other vertebrates, the skeletal muscles contain two main types of muscle fibers. Slow muscle fibers generate low-force but long duration contractions and they are localized largely in the lateral periphery of the myotome in a wedge, which is centered on the horizontal myoseptum. On the other hand, fast muscle fibers generate high-force, short duration contractions and are located in the deep part of the myotome. Located between the slow and fast muscle fibers there are

intermediate fibers which produce both high-force and long duration contractions (van Raamsdonk et al. 1978; Devoto et al. 1996). A timeline of the key events that occur during the development and innervation of the skeletal muscle in zebrafish is shown in Figure 3, starting with the localization of the slow and fast muscle cell precursors in the marginal zone of the blastoderm during the gastrula period at ~6 hpf and ending with the onset of swimming at 26–27 hpf.

The slow muscle cells develop from adaxial cells, a population of muscle precursor cells that lie adjacent to the notochord before segmentation begins (Devoto et al. 1996; Barresi et al. 2001). The adaxial cells are formed from the paraxial mesoderm when the notochord precursors secrete signaling molecules such as Hedgehog proteins (Currie and Ingham 1996; Blagden et al. 1997; Stickney et al. 2000). Shortly after somite formation the adaxial cells, which are initially cuboidal in shape, elongate to extend across the anterior-posterior axis of the somite and then the majority migrate medially to laterally through the somite to form a superficial monolayer of approximately 20 cells at the

Figure 2. (Continued) (Bi) A representative example of the spatial pattern of Ca²⁺ transients generated in the trunk of an apoaequorin expressing transgenic embryo at ~18.5 hpf. This image represents 10 seconds of accumulated light superimposed on to the appropriate bright-field image. Color scale indicates luminescent flux in photons/pixel. Scale bar is 200 μ m. (Bii–Biv) Representative examples of Ca²⁺ signals generated in the trunk of embryos at (Bii) ~17.5 hpf (~17-somite stage), (Biii) ~18.5 hpf (~18-somite stage), and (Biv) ~24 hpf (~prim-5 stage) as visualized by confocal microscopy using calcium green-1 dextran. Red arrowheads in panel Bii indicate elevated Ca²⁺ in the nuclei. The color scale represents the level of [Ca²⁺]_i, where red indicates a high level and blue indicates a low level. Ant. and Pos. are anterior and posterior, respectively. S7, S8, S9, and S10 are somites 7–10. Scale bars are 50 μ m. Panels Bii'–Biv' are schematics to show the location in the trunk where the single confocal sections were acquired. (C) Developmental expression of functional IP₃Rs in the anterior somites (AS) from *Xenopus* embryos at (Ci–Ciii) stage 26 and (Civ–Cvi) stage 33. Panels Ci and Civ show fluo-3 fluorescence prior to IP₃ uncaging; panels Cii and Cv show the Ca²⁺ elevations induced after illumination with UV light for 5 seconds to uncage the IP₃; and panels Ciii and Cvi show the relative difference in fluorescence pre- and post-IP₃ photolysis in inverted format. Red arrowheads in panel Cii indicate elevated Ca²⁺ in the nuclei. Scale bar is 250 μ m. Panels Cvii and Cviii show schematics of intact *Xenopus* embryos at stage 26 and 33, respectively. Ant. and Pos. are anterior and posterior, respectively. (A, Reprinted, with permission, from Jaimovich et al. [2000], American Physiological Society; C, Reprinted, with permission, from Ferrari and Spitzer 1999, Elsevier.)

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Figure 3. A timeline of muscle development in zebrafish. This illustrates the main events that have been reported to occur during mesodermal patterning, somitogenesis, myogenesis and the onset of muscle function from ~5 hpf to ~27 hpf. Included with this developmental timeline are the patterns of Ca²⁺ signals observed in the forming trunk muscles. Ca²⁺ signaling periods 1 and 2 (SP1 and SP2) and the Ca²⁺ signaling quiet period (QP) observed in slow muscle cells during development (Cheung et al. 2010) are labeled.

periphery of the myotome (Devoto et al. 1996; Du et al. 1997). Devoto et al. (1996) showed that in the rostral trunk this process takes approximately 5 hours (i.e., from ~17 hpf to ~23 hpf or the 16- to 28-somite stages), during which time the adaxial cells differentiate into slow muscle cells. A subgroup of adaxial cells do not migrate but remain in a medial location within the somite. These are called muscle pioneer cells and they form the horizontal myoseptum, which separates the dorsal and ventral regions of the myotome (Halpern et al. 1993). The other, nonadaxial, cells in the myotome (called the lateral presomitic cells) also do not migrate and these differentiate into the fast muscle cells via a process induced by the medial-to-lateral migration of the slow muscle cells through the somite (Devoto et al. 1996; Wolff et al. 2003; Henry and Amacher 2004).

The first spontaneous contractions occur in the trunk muscle at ~17 hpf (i.e., 16-somite stage) (Liu and Westerfield 1992; Saint-Amant and Drapeau 1998; Drapeau et al. 2002), when the individual myotome blocks are innervated by axons of the first of the developing primary motoneurons, the caudal primary motoneurons (Eisen et al. 1986; Myers et al. 1986; Westerfield et al. 1986; Brennan et al. 2005). These initial spontaneous muscle contractions are then followed sequentially by touch-evoked rapid coils (starting at ~21 hpf) and then organized swimming behavior (starting at ~30 hpf) (Saint-Amant and Drapeau 1998).

Use of Aequorin to Image Ca²⁺ Transients in the Developing Zebrafish Myotome

Créton et al. (1998) was one of the first groups to report the visualization of Ca²⁺ signals in the developing trunk of zebrafish embryos. They loaded embryos with aequorin at the single-cell stage so that it was partitioned into all the subsequent progeny, and in this way, they were able to image Ca²⁺ signals continuously during early development. At the 18–22 somite stages (i.e., ~18–20 hpf), Créton et al. (1998) visualized regions of high Ca²⁺ in the trunk and suggested that these signals might arise from the early muscle movements of the

embryos. Webb and Miller (2003) also used aequorin imaging and described Ca²⁺ signals in the developing trunk of embryos at the 18-somite stage (~18 hpf). In this report, by using a relatively low luminescence accumulation time (i.e., just 60 s), the dynamic nature of the trunk Ca²⁺ signals was clearly shown.

As aequorin is largely used up by approximately 24 hours when it is injected into embryos at the single-cell stage, the level of luminescence generated during the latter stages of muscle differentiation tends to be relatively low, and continues to fall to barely detectable levels. However, by injecting apoaequorin mRNA into embryos at the single-cell stage to express the protein component of aequorin transiently in embryos, and then reconstituting the active aequorin in vivo by incubating embryos in the coelenterazine cofactor, Cheung et al. (2006) were able to extend the aequorin-based Ca²⁺ imaging window by approximately 24 hours and they described several different types of Ca²⁺ signals in the developing trunk, for example during the Segmentation Period at ~12 hpf (i.e., the 6-somite stage) and at ~18–19 hpf (i.e., the 18–20-somite stage), as well as during the Pharyngula Period at ~27–36 hpf. Cheung et al. (2006) described the first signal as being a highly localized Ca²⁺ transient with a duration of approximately 60–120 seconds, and it was suggested that it might correlate with somite formation. They described the second signal as being a relatively widespread and long lasting (i.e., ~10 min) Ca²⁺ transient generated by the approximately 12–14 anterior-most somites but not by the approximately 6 posterior somites. They suggested that this transient might play a role in some aspect of muscle development but with the long duration, it was unlikely to be involved in E-C coupling. They described the third type of signal as being a series of rapid Ca²⁺ transients, each lasting in the order of just a few seconds and suggested that they may result from the more rapid contractions that have been reported to occur in the older embryos (Saint-Amant and Drapeau 1998).

Although the transient aequorin expression approach successfully extends the aequorin-based Ca²⁺ imaging window to ~48 hpf, the

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aeq-mRNA is gradually degraded in the injected embryos, which results in a steady decline in the production of apoaequorin (Cheung et al. 2006). Furthermore, as the expression of aequorin is ubiquitous in mRNA-injected embryos, it is difficult to identify the groups of cells, tissues or organ anlage that are generating a particular signal in the more complex, later stage embryos (Cheung et al. 2006). Thus, most recently, we developed a transgenic line of zebrafish, in which a muscle-specific α -actin promoter was used to target the apoaequorin specifically to the musculature (Cheung et al. 2010). Imaging intact embryos revealed two distinct periods of long-range Ca^{2+} signaling in the developing trunk musculature: between ~ 17.5 and 19.5 hpf and after ~ 23 hpf. Figure 2B shows a representative example of a Ca^{2+} signal generated at ~ 18.5 hpf, i.e., in the first Ca^{2+} signaling period. These signals were only generated between somites 2 and 12, i.e., they were never observed to arise in the posterior 6 somites. This is somewhat similar to the Ca^{2+} signals generated at 18–19 hpf that were reported for embryos in which apoaequorin was expressed transiently (Cheung et al. 2006).

Ashworth and Brennan (2005) presented preliminary data from intact zebrafish embryos describing the injection at the 16-cell stage of an expression plasmid encoding GFP-aequorin under the control of the immediate early cytomegalovirus (CMV) promoter. This was followed by incubation with coelenterazine for an hour at the end of the Gastrula Period to reconstitute active aequorin. At the 28-somite stage (~ 24 hpf), using a PMT they were then able to record some Ca^{2+} activity and identify the expression of GFP-aequorin in some muscle cells via subsequent fluorescent microscopy.

The Use of Fluorescent Ca^{2+} Reporters to Image Ca^{2+} Transients in the Developing Myotome of Zebrafish

Fluorescent Ca^{2+} reporters have also been used to visualize Ca^{2+} transients in the developing musculature of zebrafish embryos. Ashworth et al. (2001) visualized Ca^{2+} transients in spontaneously contracting muscle cells within intact

embryos at 22 hpf using the fluorescent Ca^{2+} reporter, Oregon green 488 BAPTA dextran. They also showed that in the presence of the Ca^{2+} buffer, BAPTA dextran, the amplitude of the Ca^{2+} transients was significantly reduced but not completely inhibited during muscle contraction. Ashworth (2004) also showed the changes in intracellular Ca^{2+} during the contraction of a single muscle fiber within an intact zebrafish embryo, this time at 24 hpf. In this report, cells were loaded with both Oregon green 488 BAPTA dextran and rhodamine dextran, with the latter reporter being insensitive to changes in Ca^{2+} but revealing changes in fluorescence that occur because of other factors, for example, because of cell movement during muscle contraction. This methodology resulted in a more accurate measure of the Ca^{2+} changes that take place during muscle cell contraction because of the elimination of fluorescence artifacts (Ashworth 2004).

Most recently, Brennan et al. (2005) injected embryos with either Oregon green 488 BAPTA dextran, which is considered to be a high-affinity Ca^{2+} indicator, or Fluo-4 dextran, a low affinity Ca^{2+} reporter, in conjunction with tetramethylrhodamine dextran, a Ca^{2+} insensitive fluorescent reporter similar to rhodamine dextran. They then recorded Ca^{2+} signals in the developing slow muscle cells at a time when the first neuromuscular contacts are known to form (i.e., from 16–22 hpf). Brennan et al. (2005) reported that the first Ca^{2+} signals were detected in the Oregon green 488 BAPTA dextran-loaded embryos at ~ 17.25 hpf and these corresponded to the first contractions of these muscle cells in the embryo. Ca^{2+} transients were also detected in the Fluo-4 dextran-labeled muscle cells but these transients were not observed until 18–19 hpf. Brennan et al. (2005) also characterized the developmental distribution of RyRs in the somites and myotome using in situ hybridization and immunohistochemistry. They showed that at the 10-somite stage, the *RyR* gene is expressed in the muscle pioneer cells and adaxial cells of the newly formed somites. By 20 hpf to 24 hpf, bands of RyR protein were clearly observed in the developing slow muscle cells. Brennan et al. (2005)

treated embryos at ~20–20.5 hpf with the L-type Ca²⁺ channel blocker, nifedipine, and with the RyR blockers, dantrolene and ryanodine, and showed that all three drugs resulted in a decrease in the number of contracting embryos. They also showed that these spontaneous contractions were inhibited, and Ca²⁺ transients were blocked, by treatment with the acetylcholine receptor inhibitor, α -bungarotoxin. In addition, ryanodine and α -bungarotoxin treatment also disrupted the organization of the myofibrils in the slow muscle cells. Brennan et al. (2005) thus proposed a critical role for nerve-mediated Ca²⁺ signals in the formation of physiologically functional slow muscle during zebrafish development.

Representative examples of the Ca²⁺ signals visualized in the developing slow muscle cells of intact zebrafish embryos are shown in Figure 2Bii–2Biv (modified from Cheung et al. 2010). These embryos were loaded at the one-cell stage with calcium green-1 dextran and then visualized by confocal microscopy at ~17.5 hpf, ~18.5 hpf, and ~24 hpf. At ~17.5 hpf and ~18.5 hpf the Ca²⁺ signals appeared to be generated predominantly in the nucleus and peri-nuclear region of each individual muscle cell, with less signal being generated in the cytoplasm (Fig. 2Bii, 2Biii). At ~24 hpf, however, the Ca²⁺ signals appear to be largely cytosolic (Fig. 2Biv).

Ca²⁺ TRANSIENTS VISUALIZED IN CULTURED *XENOPUS* MYOCYTES AND IN EXPOSED MYOTOME

Ferrari et al. (1996) reported that spontaneous Ca²⁺ signals are generated during the first 15 hours in culture in primary cultures of *Xenopus* embryonic myocytes. They loaded young cultures (i.e., within 4–6 h of culture) with the AM ester forms of fura-2 and fluo-3 to measure resting Ca²⁺ and Ca²⁺ transients, respectively. In mixed myocyte/neuron cultures, young myocytes (i.e., at 0–4 h in culture) were shown to generate regular Ca²⁺ transients at a frequency of 7–10 per hour for approximately the first 2 h, after which transients were produced less regularly at a rate of approximately 6 per hour.

In addition, myocytes only generated spontaneous transient elevations of Ca²⁺ for the first 15 hours of culture. Furthermore, cultures containing both myocytes and neurons had more transient-generating cells than myocyte cultures cultured without neurons. The Ca²⁺ signals were shown to be generated by Ca²⁺ release from intracellular stores via RyRs as treatment with caffeine, a RyR agonist, was shown to stimulate the generation of Ca²⁺ transients. In addition, transients were abolished following treatment with an inhibitory concentration (100 μ M) of ryanodine. Furthermore, application of the Ca²⁺ chelator, BAPTA-AM significantly disrupted myofibril organization and sarcomere assembly. Ferrari et al. (1998) subsequently confirmed that the release of Ca²⁺ from RyR stores is necessary for myofibrillogenesis as treatment with 100 μ M ryanodine also disrupted the formation of the myofibrils and reduced the number of sarcomeres by disrupting the assembly of the myosin thick filament. Ferrari et al. (1998) showed that the inhibition of Ca²⁺/calmodulin-dependent myosin light chain kinase (MLCK) resulted in a disruption of sarcomeric organization, and they suggested that RyRs and MLCK might play key roles in the formation of the contractile apparatus during myocyte development. It was subsequently shown that the spontaneous Ca²⁺ signals generated in cultured *Xenopus* myocytes also play a role in the organization of titin and capZ during the assembly of the actin thin filaments at early stages of sarcomere assembly (Li et al. 2004; Harris et al. 2005) but are not involved in the association between titin and myosin (Harris et al. 2005). Li et al. (2004) also proposed a model highlighting just some of the steps in sarcomere assembly that might be regulated by Ca²⁺. For thin filament assembly, these included Ca²⁺ activation of titin kinase in the formation of the Z-discs, of troponin/tropomyosin in thin filament capping, and of MLCK for thick filament assembly.

In addition to their visualization of Ca²⁺ transients in differentiating myocytes in culture, Ferrari and Spitzer (1999) also showed that similar Ca²⁺ dynamics occur in embryos in which the ectoderm and dermatome had been

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Figure 4. Schematic diagram of the proposed signaling cascade for the generation of the 17–18 hpf Ca^{2+} signals in slow muscle cells during myofibrillogenesis in zebrafish embryos. The time when the myotome Ca^{2+} transients are first observed (i.e., at ~17.5 hpf) corresponds to the time when the myotomes are first innervated by the caudal primary (CaP) axons (Eisen et al. 1986; Myers et al. 1986; Liu and Westerfield 1992). (1) Acetylcholine (ACh), released from the axons, binds to nicotinic acetylcholine receptors (nAChRs) on the surface of the slow muscle cells (SMCs), which (2) induces depolarization of the sarcolemma (Behra et al. 2002; Brennan et al. 2005). This depolarization activates dihydropyridine receptors (DHPRs) (3), that in turn stimulate the release of Ca^{2+} (see red circles) from intracellular stores. The Ca^{2+} signals are generated by Ca^{2+} release via both IP_3 R and RyRs (Ferrari and Spitzer 1999; Jaimovich et al. 2000). It has been proposed that DHPRs can activate the production of IP_3 from phospholipase C (PLC) and then induce Ca^{2+} release via IP_3 R from the sarcoplasmic reticulum (4) and the perinuclear cisternae (5) (Jaimovich et al. 2000; Powell et al. 2001; Jaimovich and Carrasco 2002; Araya et al. 2003). (See facing page for legend.)

removed on one side to expose the somites. The dissected embryos, which were at stage 23/24 (i.e., with 12–15 formed somites) were loaded with fluo-3 AM prior to time-lapse confocal imaging. Three main types of spontaneous Ca²⁺ signals were observed: those arising in single cells, those arising in groups of cells and those propagating as waves in the ventrolateral region of the myotome. They reported that these correlated with both somite formation and with myocyte maturation (Ferrari and Spitzer 1999). They also showed the functional distribution of both RyR and IP₃R stores in intact myotome by inducing increases in Ca²⁺ in response to incubation with caffeine and photo-activation of caged IP₃, respectively. An example of the Ca²⁺ signals elicited in the myotome of stage 26 and stage 33 embryos following photo-activation of caged IP₃ is shown in Figure 2C. At stage 26, functional IP₃ receptors were expressed in the muscle cells of anterior somites (AS), with the most prominent expression occurring in the nuclei. By stage 33, however, these more mature muscle cells no longer responded to the generation of IP₃ although Ca²⁺ elevations were observed in nonmuscle cells located along the somite borders. It was suggested that although immature myocytes have both RyR- and IP₃R-activated Ca²⁺ stores, there is a down-regulation of the IP₃R as these cells differentiate. This down-regulation of IP₃R activity and up-regulation of RyR activity as skeletal muscle cells mature has also been reported from intact zebrafish embryos (Cheung et al. 2010). A somewhat similar situation has been reported during the development of cardiac myocytes, where it has been shown that IP₃R expression occurs before the appearance of RyRs in early

embryos (Kockskämper et al. 2008). In mature cardiac myocytes on the other hand, IP₃R are expressed at approximately 100-fold lower levels than RyRs. This suggests a similar switch in importance of the Ca²⁺ release mechanism as the cardiac myocytes mature (Llewellyn Roderick and Bootman 2007). In addition, it has been suggested that Ca²⁺ release via IP₃R may initiate Ca²⁺-induced Ca²⁺ release from the RyR-activated store, as the generation of propagating waves of Ca²⁺ in the *Xenopus* myotome ceases coincidentally with the down-regulation of IP₃R expression (Ferrari and Spitzer 1999).

Most recently, a new class of spontaneous Ca²⁺ transients has been described in cultured *Xenopus* myocytes in addition to those previously described (Ferrari et al. 1996; 1998; Ferrari and Spitzer 1999). Using high-speed confocal imaging in line scanning mode, Campbell et al. (2006) described a series of short duration transients, or SDTs (i.e., with a mean duration of ~2 s), which propagate in the subsarcolemmal space and are generated from RyR stores located in this restricted cytoplasmic domain. Campbell et al. (2006) thus called the previously described Ca²⁺ transients, which had a duration of approximately 80-second, long duration transients, or LDTs. Although other Ca²⁺ transients with short durations have been previously reported, i.e., Ca²⁺ sparks and fast localized transients (FLTts) generated in embryonic mouse skeletal muscle (Conklin et al. 1999a; Chun et al. 2003) and frog skeletal muscle fibers (Hollingworth et al. 2001; Lacampagne et al. 2003), it was proposed that SDTs represent a separate, new class of Ca²⁺ transients (Campbell et al. 2006). In addition, although SDTs arise before myofibrillogenesis

Figure 4. (Continued) Moreover, it has been shown that elevations of nuclear Ca²⁺ can activate gene expression in skeletal muscle cells (6) (Carrasco et al. 2003; Cárdenas et al. 2005). In addition, it has been proposed that direct coupling of DHPRs with juxtaposed RyRs, which are localized along the interface of the sarcoplasmic reticulum (SR) and T-tubule, at the terminal cisternae of the SR (Ward and Lederer 2005), can trigger Ca²⁺ release through RyRs (7) (Schneider and Chandler 1973; Flucher et al. 1999). Ca²⁺ released via RyRs is reported to play a role in various aspects of sarcomere assembly as well as regulating muscle contraction (Li et al. 2004; Brennan et al. 2005; Harris et al. 2005).

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and persist during the time of sarcomere assembly (i.e., similar to the Ca^{2+} sparks and FLTs), they do not appear to be necessary for the assembly process. On the other hand, LDTs are generated throughout the whole cytosol of myocytes rather than being restricted to the subsarcolemmal space. Like SDTs, LDTs arise prior to myofibrillogenesis but they stop before the onset of sarcomere assembly. Furthermore, Campbell et al. (2006) showed that sarcomere assembly could be blocked by extending the period of LDT generation via treatment with caffeine and they suggested that LDTs might play a role in activating sarcoplasmic regulatory pathways and the regulation of gene expression.

CONCLUSIONS

From processes as diverse as developmental gene activation (Wu et al. 2000; McKinsey et al. 2000; Berridge et al. 2003; Carrasco et al. 2003; Kubis et al. 2003; and reviewed by Berchtold et al. 2000; Al-Shanti and Stewart 2009) to sarcomeric protein assembly (Ferrari and Spitzer 1998, 1999; Li et al. 2004; Brennan et al. 2005; Harris et al. 2005; Campbell et al. 2006; Fujita et al. 2007; and reviewed by Ferrari et al. 2006), Ca^{2+} transients have been correlated with specific events and processes during muscle development and thus assigned numerous possible regulatory roles (Fig. 4). It is encouraging that many aspects reported from imaging isolated muscle cells have also been observed in intact, or near-intact, systems. This feature has been nicely illustrated by data reported from *Xenopus*, in which Ca^{2+} signals from near-intact embryos (where the ectoderm and dermatome were removed to expose the underlying somites; Ferrari and Spitzer, 1999) closely resembled those from cultured embryonic myocytes (Ferrari et al. 1996). Furthermore, the generation of distinct subcellular Ca^{2+} domains in cultured skeletal muscle cells that are either membrane-bound or otherwise (Jaimovich et al. 2000; Cárdenas et al. 2005) has also been shown to occur in slow muscle cells of intact zebrafish embryos (Cheung et al. 2010). We suggest, therefore, that the use of both in vitro and in vivo models will continue to serve as

complementary systems; in which the former is a relatively simple experimental set-up that is easy to image, whereas the latter can be used to confirm in vitro results as well as to extend the investigation of Ca^{2+} signaling in muscle cell development into the normal in situ environment. For example, a recent study using intact zebrafish embryos has shown that the attachment of slow muscle cells to the vertical myosepta is dependent on RyR-generated Ca^{2+} transients (Cheung et al. 2010). Furthermore, maintaining the in situ environment will also allow the exploration of the relationship between Ca^{2+} dynamics, endogenous biophysical forces, and the differentiation of skeletal muscle cells. One of the key challenges during the next decade will be to identify the precise upstream triggers and down-stream targets of the multitude of Ca^{2+} signaling events that occur during the differentiation of a variety of different types of muscle cells. A second challenge will be to understand how the Ca^{2+} -mediated muscle-assembly instructions are integrated into multiple signaling networks during muscle development (Ochi and Westerfield 2007): For example with IP_3 signaling pathways (Kockskämper et al. 2008; Berridge 2009); with Wnt signaling pathways (Cossu and Borello 1999; Ling et al. 2009); and with MAP kinase signaling pathways (Gotoh and Nishida 1995; Lluís et al. 2006), to name but a few. Furthermore, we suggest that temporal data, although being helpful and supportive, will not, however, tell the whole story. Four-dimensional Ca^{2+} imaging information, i.e., the three spatial dimensions plus time, will be an essential component in extending our understanding of muscle development and differentiation.

ACKNOWLEDGMENTS

We would like to thank Enrique Jaimovich, Michael B. Ferrari, and Nicholas C. Spitzer who kindly gave us permission to use their previously published work. Special thanks also to Osamu Shimomura for his generous support of aequorin-based imaging over the years. We acknowledge financial support from Hong Kong Research

Grants Council GRF awards: HKUST-6416/06M, HKUST-661707 and HKUST-662109, and from Hong Kong University Grants Committee award: SEG_HKUST01.

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