Characterization of Ca^{2+} signaling in the external yolk syncytial layer during the late blastula and early gastrula periods of zebrafish development ☆

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A B S T R A C T

Preferential loading of the complementary bioluminescent (*f*-aequorin) and fluorescent (Calcium Green-1 dextran) Ca^{2+} reporters into the yolk syncytial layer (YSL) of zebrafish embryos, revealed the generation of stochastic patterns of fast, short-range, and slow, long-range Ca^{2+} waves that propagate exclusively through the external YSL (E-YSL). Starting abruptly just after doming (~4.5 h post-fertilization: hpf), and ending at the shield stage (~6.0 hpf) these distinct classes of waves propagated at mean velocities of ~50 and ~4 μm/s, respectively. Although the number and pattern of these waves varied between embryos, their initiation site and arcs of propagation displayed a distinct dorsal bias, suggesting an association with the formation and maintenance of the nascent dorsal-ventral axis. Wave initiation coincided with a characteristic clustering of YSL nuclei (YSN), and their associated perinuclear ER, in the E-YSL. Furthermore, the inter-YSN distance (IND) appeared to be critical such that Ca^{2+} wave propagation occurred only when this was <~8 μm; an IND >~8 μm was coincidental with wave termination at shield stage. Treatment with the IP_3R antagonist, 2-APB, the Ca^{2+} buffer, 5,5′-dibromo BAPTA, and the SERCA-pump inhibitor, thapsigargin, resulted in a significant disruption of the E-YSL Ca^{2+} waves, whereas exposure to the RyR antagonists, ryanodine and dantrolene, had no significant effect. These findings led us to propose that the E-YSL Ca^{2+} waves are generated mainly via Ca^{2+} release from IP_3Rs located in the perinuclear ER, and that the clustering of the YSN is an essential step in providing a CICR pathway required for wave propagation. This article is part of a Special Issue entitled: 12th European Symposium on Calcium.

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1. Introduction

Substantial evidence is beginning to accumulate to suggest that in developing zebrafish and other teleosts, extra-embryonic domains such as the enveloping layer (EVL), the yolk cell (YC), and the yolk syncytial layer (YSL), may play a significant role in generating signals that serve to pattern the early embryonic domain (i.e., the deep cells) during the blastula and gastrula periods, as well as throughout later developmental stages [1–12]. Here, we report the visualization of stochastic, dorsally-biased Ca^{2+} waves propagating exclusively within the external YSL (E-YSL); propose a mechanism for their generation and propagation; and from wave disruption experiments, make suggestions as to their possible developmental function(s).

The occurrence and possible roles of Ca^{2+} signalling during embryonic development have been reviewed by several authors [13–18], as well as that specifically during zebrafish embryogenesis [19–24]. Furthermore, Ca^{2+} signalling during the embryonic time window when the YSL forms (and then features prominently), i.e., the mid-blastula and gastrula periods, respectively, has also been the subject of a recent review [25].

Detailed descriptions of the formation of the YSL in a variety of teleost species have been reported: For example, in zebrafish [26,27]; *Fundulus* [28]; and medaka [29], and this essential teleostean developmental event has also been the subject of excellent reviews [8,10]. Several authors have reported visualizing Ca^{2+} transients in a region of a normally developing zebrafish embryo that may well have encompassed the YSL [14,19,20,30,31]. However, from the way that the data were collected and presented, it is sometimes not clear whether the Ca^{2+} transients were being generated exclusively in the YSL itself or in the marginal cells of the blastoderm that lie immediately above the YSL, or indeed in a combination of both these domains. These reports do, however, suggest that Ca^{2+} signalling in this extra-embryonic domain may have

a profound effect on patterning the overlying embryonic domain, and in particular on establishing and/or maintaining the dorsal-ventral axis.

For example, Westfall et al. [32] reported that manipulation of Wnt-5 activity by either gain-of-function or loss-of-function approaches resulted in changes in endogenous Ca^{2+} signalling activity. They described a reduction in Ca^{2+} release in zebrafish Wnt-5/*pipetail* mutant embryos and reported that the embryonic region displaying the greatest reduction of Ca^{2+} activity includes the YSL. Embryos genetically depleted of both maternal and zygotic Wnt-5 displayed hyperdorsalization and axis duplication phenotypes. They suggested that the dorsalized phenotypes resulted from increased β-catenin accumulation and activation of downstream genes, and they proposed that the Wnt-5 loss-of-function defect is consistent with Ca^{2+} modulation via the non-canonical Wnt/Ca^{2+} signalling pathway having an antagonistic interaction with canonical Wnt/β-catenin signalling [15,17,32,33].

However, in spite of the growing number of reports where Ca^{2+} transients have been described (and manipulated either by genetic or molecular means) during the late blastula/early gastrula period from a region of the embryo suggested to be the YSL, to date none has indisputably demonstrated such transients to be generated exclusively in the YSL, or to have any dorsal-ventral asymmetry. This is in contrast to such a dorsal bias being reported in the EVL of the blastoderm following the mid-blastula transition at ~2.75 h post-fertilization (hpf) [34]. We suggest that this failure to establish beyond a doubt that the Ca^{2+} transients are indeed generated specifically in the YSL, or to detect any signalling asymmetry in the YSL-generated Ca^{2+} signals, may be due (to a substantial degree) to a combination of the technique used to load the Ca^{2+} reporter into the embryo as well as the Ca^{2+} imaging technique employed. We thus undertook an extensive re-evaluation of YSL Ca^{2+} signalling using both aequorin-based luminescence imaging and complementary fluorescence-based imaging. We suggest that the former is best suited to provide a near-continuous recording of global Ca^{2+} signalling events in a large pan-embryonic domain such as the YSL, while the latter is essential for confirming that the Ca^{2+} transients are being generated exclusively within the YSL. Furthermore, in the case of aequorin-based imaging, we examined entire embryos from an animal pole as well as a lateral view, as the former is the best orientation from which to observe any asymmetry with regards to the spatial distribution of the Ca^{2+} transients in the YSL, with respect to the emerging dorsal-ventral axis. We also investigated the distribution of nuclei and the perinuclear ER in the YSL at various stages during epiboly and demonstrated that Ca^{2+} waves propagated exclusively through regions of the E-YSL but only at times when the inter-nuclear distance (IND) was less than ~8 μm. Furthermore, embryos were treated with antagonists of IP_3Rs and RyRs, in order to determine if either, or both, receptors were involved in YSL Ca^{2+} wave generation, as well as the Ca^{2+} buffer, 5,5′-dibromo BAPTA (DBB), and the sarco/endoplasmic reticulum Ca^{2+} ATPase- (SERCA-) pump inhibitor, thapsigargin (Tg). Treatment with 2-APB and DBB resulted in a near-complete knockdown of the E-YSL Ca^{2+} signals and disrupted subsequent development of the embryo, whereas treatment with ryanodine and dantrolene had no significant effect either on these signals or on development. On the other hand, treatment with Tg resulted in a rapid and large increase in Ca^{2+} in the YSL, which also severely disrupted the endogenous E-YSL signals, and resulted in later developmental events being affected.

Our new data thus confirm that the E-YSL is indeed another extra-embryonic domain (i.e., in addition to the EVL and the YC), that generates regionalized Ca^{2+} signals, and that disrupting these signals has a profound effect on the subsequent development of the embryo proper.

2. Materials and methods

2.1. Embryo collection

Wild-type (AB strain) zebrafish (*Danio rerio*) were maintained on a 14-hour light/10-hour dark cycle to stimulate spawning [35]. Fertilized eggs were collected within ~5 min of spawning, as described previously [36]. Embryos were maintained in Danieau's solution (17.4 mM NaCl, 0.21 mM KCl, 0.18 mM $Ca(NO_3)_2$, 0.12 mM $MgSO_4 \cdot 7H_2O$, 1.5 mM HEPES, pH 7.2) at ~28.5 °C during the course of all experiments.

2.2. Microinjection techniques

Intact embryos were injected through the chorion at either the 1-cell stage (i.e., ~0.5 hpf) or the 128-cell stage (i.e., ~2.25 hpf) into the top of the yolk close to the yolk/blastodisc or yolk/blastoderm margin, respectively. Injected embryos were incubated at ~28.5 °C in the dark and then dechorionated manually with two 21-gauge needles (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) just prior to imaging to improve optics. Embryos were then immobilized in small grooves made in agarose in the microwells of P35G-1.5-14-C glass-bottom culture dishes (MatTek, Ashland, MA, USA). These were used as imaging chambers, and embryos were oriented with a pair of watchmaker's forceps to the desired orientation to facilitate imaging of a side or animal pole (AP) view. Further details regarding microelectrode preparation, microinjection techniques, and embryo positioning and immobilization methods are described by Webb et al. [36].

2.3. Confocal and multi-photon excitation microscopy techniques

Unless otherwise specified, fluorescence was visualized using a Leica TCS SP5 II laser scanning confocal system mounted on a Leica DMI 6000 inverted microscope (Leica Microsystems, Wetzlar, Germany) equipped with multi-photon excitation capability. Images were collected using either a Leica HC PL APO 20×/0.7NA dry, or HCX PL APO 63×/1.4-0.6 NA oil-immersion, objective lens. In experiments where embryos were injected with FITC-aequorin or Calcium Green-1 dextran alone, fluorescence was observed via multi-photon excitation microscopy using 800 nm excitation and 475–575 nm detection. However, when embryos were dual-labelled with FITC-aequorin or Calcium Green-1 dextran and Hoechst 33258, the FITC or Calcium Green-1 fluorescence was then observed via confocal microscopy using 488 nm excitation/ 500–550 nm detection and Hoechst 33258 fluorescence was observed via multi-photon excitation microscopy using 790 nm excitation/ 410–510 nm detection. The multiphoton wavelengths were optimized for each fluorophore used. Rhodamine B fluorescence was observed via confocal microscopy using 561 nm excitation/570–700 nm detection wavelengths while SYTOX Green and Alexa Fluor 488 fluorescence was observed using 488 nm excitation/500–550 nm detection wavelengths. GFP fluorescence was visualized via confocal microscopy using 488 nm excitation/500–606 nm detection.

2.4. Microinjection of FITC-aequorin and Hoechst 33258

Embryos were injected with ~2 nl of fluorescein isothiocyanate-tagged aequorin (FITC-aequorin; custom-made by Molecular Probes, Eugene, OR, USA) at the 1- or 128-cell stages. The embryos were then visualized at sphere stage (i.e., ~4 hpf) using the Leica TCS SP5 II system and excitation/detection wavelengths described in Section 2.3. In some experiments, ~1.6 mM Hoechst 33258 (Invitrogen, Carlsbad, CA, USA) was co-injected with FITC-aequorin at ~2.25 hpf (i.e., the ~128-cell stage) in order to label the nuclei. Embryos were visualized at 30% epiboly again using the confocal and multi-photon excitation imaging methods described in Section 2.3.

2.5. Aequorin-based Ca^{2+} imaging and recording

Embryos were injected with approximately 2.3 nl of *f*-aequorin (supplied by Dr Osamu Shimomura, the Photoprotein Laboratory, Falmouth, MA, USA; at ~0.5–1% in 100 mM KCl, 5 mM MOPS, 50 μM EDTA) at the 128-cell stage and then placed in imaging chambers as described in Section 2.2. Aequorin-generated luminescence images