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Characterization of calcium transients during early embryogenesis in ascidians *Ciona robusta* (*Ciona intestinalis* type A) and *Ciona savignyi*

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ABSTRACT

The calcium ion (Ca^{2+}) is an important second messenger, and a rapid increase in Ca^{2+} level (Ca^{2+} transient) is involved in various aspects of embryogenesis. Although Ca^{2+} transients play an important role in early developmental stages, little is known about their dynamics throughout embryogenesis. Here, Ca^{2+} transients were characterized by visualizing Ca^{2+} dynamics in developing chordate embryos using a fluorescent protein-based Ca^{2+} indicator, GCaMP6s in combination with finely tuned microscopy. Ca^{2+} transients were detected in precursors of muscle cells in the late gastrula stage. In the neurula stage, repetitive Ca^{2+} transients were observed in left and right neurogenic cells, including visceral ganglion (VG) precursors, and the duration of Ca^{2+} transients was 39 ± 4 s. In the early tailbud stage, Ca^{2+} transients were observed in differentiating precursors of nerve cord neurons. A small population of VG precursors showed rhythmical Ca^{2+} transients with a duration of 22 ± 4 s, suggesting a central pattern generator (CPG) origin. At the mid tailbud stage, Ca^{2+} transients were observed in a wide area of epidermal cells and named CTECs. The number and frequency of CTECs increased drastically in late tailbud stages, and the timing of the increase coincided with that of the relaxation of the tail bending. The experiment using Ca^{2+} chelator showed that the CTECs were largely depending on the extracellular Ca^{2+}. The waveform analysis of Ca^{2+} transients revealed different features according to duration and frequency. The comprehensive characterization of Ca^{2+} transients during early ascidian embryogenesis will help our understanding of the role of Ca^{2+} signaling in chordate embryogenesis.

1. Introduction

The calcium ion (Ca^{2+}) is a versatile and universal second messenger involved in the regulation of embryogenesis (Christodoulou and Skourides, 2015; Markova and Lenne, 2012; Shindo et al., 2010; Slusarski and Pelegri, 2007). A rapid Ca^{2+} increase (Ca^{2+} transient) plays a pivotal role in a wide range of developmental processes from fertilization to organ formation (Chen et al., 2017; Webb et al., 2011; Berridge et al., 2003). Recent advances in Ca^{2+} imaging techniques facilitated the study of Ca^{2+} transients in developing vertebrates (Muto et al., 2011). In particular, examining Ca^{2+} transients in ascidians, the closest relatives of vertebrates, is important to understand the roles of Ca^{2+} signaling during development in chordates. Ca^{2+} transients are involved in several processes in ascidian embryogenesis, including oocyte meiosis (McDougall and Levasseur, 1998; McDougall and Sardet, 1995) and myofibril arrangement in ascidian larval muscles (Ohtsuka and Okamura, 2007). Two reports using fluorescence imaging of Ca^{2+} transients during ascidian development demonstrated their involvement in the differentiation of anterior neural plate and neural tube closure (Abdul-Wajid et al., 2015; Hackley et al., 2013) respectively.

Despite the involvement of Ca^{2+} signaling in various aspects of ascidian embryogenesis, comprehensive observation is lacking, and there are no reports of long-time tracing of Ca^{2+} transients in whole-mount ascidian embryos. In this study, GCaMP6s-encoding mRNA was injected into eggs prior to fertilization, and versatile Ca^{2+} transients were observed in different tissues and stages using fluorescence microscopy and confocal laser scanning microscopy with long time-lapse imaging. High time-resolution imaging enabled single waveform analysis of Ca^{2+} transients in different cell types. We successfully characterized Ca^{2+} transients in a wide range of developmental stages ranging from early gastrula to late tailbud before the hatching of larva. The possible biological processes mediated by these newly found Ca^{2+} transients related to the function of the central pattern generator (CPG) are discussed.

2. Materials and methods

2.1. Samples

Ciona robusta (*Ciona intestinalis* type A) adults were obtained from Maizuru Fisheries Research Station (Kyoto University), Onagawa

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Field Center (Tohoku University), and Misaki Marine Biological Station (The University of Tokyo) through the National Bio-Resource Project (NBRP), Japan. *Ciona savignyi* adults were obtained from Onagawa Field Center (Tohoku University). Eggs were collected by dissection of gonoducts. GCaMP6s-encoding mRNA was injected into eggs before fertilization. After artificial insemination, fertilized eggs were incubated at 18 °C until observation.

Although it was technically difficult to observe Ca^{2+} transients in late tailbud stages because of sample motility, we could observe spontaneous Ca^{2+} transients in late tailbud stages by physically fixing the sample's tail.

2.2. Preparation of reporter constructs and microinjection

GCaMP6s-encoding mRNA was obtained from pGP-cmv-GCaMP6s provided by Addgene (USA) (Chen et al., 2013). The ORF of GCaMP6s was PCR-amplified from pGP-cmv-GCaMP6s (forward primer GAAGGCCTATGGGTTCTCATCATCAT; reverse primer CTAGTC-TAGAGTCGCGGCCGCT) and subcloned into the expression vector pSPE3 (Roure et al., 2007) between *Stu*I and *Xba*I sites. The pSPE3-GCaMP6s plasmid was linearized using *Sfi*I or *Dra*III, and GCaMP6s mRNA was produced and precipitated using the mMESSAGE mMACHINE T3 kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. GCaMP6s mRNA was injected into dechorionated eggs at 0.5 μg/μl. The same method was used to produce m-Cherry-encoding mRNA, which was subcloned from pcDNA3.1-m-Cherry, provided by Dr. Y. Shindo in our lab. In some embryos, m-Cherry-encoding mRNA was co-injected with GCaMP6s mRNA into dechorionated eggs at 0.5 μg/μl for counterstaining.

2.3. Pharmacological and chelation treatment

To increase the cellular concentration of Ca^{2+} artificially, 100 μM ionomycin was added to seawater (final concentration, 10 μM) containing GCaMP6s-expressing embryos and monitored under a microscope. EGTA-containing artificial seawater was prepared according to the previous protocol ("Calcium- and magnesium-free artificial seawater (CMF-ASW)", 2009). The late tailbud embryos were transferred into CMF-ASW and observed for 10 min. After observation, embryos were retransferred to natural seawater.

2.4. Microscopy

Early embryos were observed by fluorescence microscopy with a 3CCD camera and confocal laser scanning microscopy (CLSM).

A Nikon inverted microscope (Nikon eclipse, IX71) with a 20× objective lens (LUCPlanFLN) was used for fluorescence imaging with the U-MWBV2 mirror unit (Olympus). The LED light SOLA (Lumencor) was used as the light source, and fluorescence images were captured with a 3CCD camera (C7800-20, Hamamatsu Photonics) and AQUACOSMOS software (Hamamatsu Photonics). This fine-tuned microscope system minimizes invasiveness in analyzing ascidian development and enables longer recording times and higher time-resolution. The time interval was set to 1–2 s per frame. The time-lapse movie was shot over a period of 1–7.5 h at 20 °C, corresponding to 3600 to 22,000 frames. To avoid samples that were out of frame or focus, the time-lapse imaging was restarted after every rearrangement of the sample. Therefore, the recorded time stamp displayed in movies was divided (SupplMov. 1).

For CLSM imaging, an Olympus fv1000 microscope was used. In some experiments, FM4-64 (Invitrogen) was used to visualize plasma membrane simultaneously. The final concentration of FM4-64 was 10 μM in seawater. Excitation lasers of 488 nm and 559 nm were used to visualize the signals of GCaMP6s and FM4-64, respectively. An Olympus ×20 or ×40-oil immersion lens was used.

2.5. Image analysis

The intensity of imaging data was analyzed by AQUACOSMOS software. Numerical data were exported to Microsoft Office Excel 2016 (Microsoft, Redmond, WA, USA) for statistical analyses and graph plotting. To identify the region of Ca^{2+} transients, 3D-reconstructed anatomical images of embryos stained with Alexa phalloidin 488 (Invitrogen) were used for comparison.

The number of Ca^{2+} transients was manually counted. The tail bending was calculated using ImageJ (National Institutes of Health, Bethsesda, MD, USA) as follows: the anterior-most, middle, and posterior-most points of the tail midline were determined from the outline of the embryo. Then, the radius of the circle (r) in contact with these three points was calculated, and tail-bending curvature was defined as 1/r.

3. Results

3.1. Detection of Ca^{2+} transients by the Ca^{2+} sensor GCaMP6s

To confirm the detection of Ca^{2+} increase by the Ca^{2+} indicator, GCaMP6s and its distribution in cells, the fluorescence intensity of GCaMP6s was visualized before and after treatment with the calcium ionophore, ionomycin, at the early gastrula (Fig. S1a, stage 11) and late tailbud III (Fig. S1b, stage 25) stages (Hotta et al., 2007), which corresponded to our observed developmental time span. After applying of the ionophore, the fluorescence intensity increased significantly by 2–2.5-fold in the whole-embryo at both early gastrula and late tailbud stages. These results indicated that the GCaMP6s protein was expressed in each cell of the embryo and that it detected the Ca^{2+} increase at least from early gastrula to late tailbud stage III (SupplMov. 1). Before treatment with ionomycin, multiple spot-like signals were observed in the tail and trunk regions of the late tailbud embryo (SupplMov. 2). To confirm whether these spots were transient Ca^{2+} increases rather than biased distribution of the GCaMP6s protein in different cells, the distribution of GCaMP6s and m-Cherry (as counterstaining control) was compared using a short time course (Fig. S1c). The m-Cherry signal did not change, whereas the GCaMP6s signal clearly changed in the trunk and tail regions after 17 s (Fig. S1c, compare the location of arrows and arrowheads). These results suggested that GCaMP6s detected the spontaneous change of Ca^{2+} and could therefore be used to characterize Ca^{2+} transients at least from early gastrula to late tailbud stages.

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3.2. Gastrula stage

In the early gastrula stage, blastomeres localize to the surface of the embryo, and Ca^{2+} increases can be detected in all cells. The number and frequency of Ca^{2+} transients correlates with cell division in zebrafish (Créton et al., 1998) and *Xenopus* (Muto et al., 1996; Kubota et al., 1993). Moreover, a recent report on *Xenopus* neurulation showed that Ca^{2+} transients correlate with the apical constriction of invaginating endodermal cells (Christodoulou and Skourides, 2015). As ascidian endodermal cells also show apical constriction at the gastrula stage (Sherrard et al., 2010), the Ca^{2+} increase was expected to occur in cleaving cells or endoderm cells. However, transient Ca^{2+} increases were not observed until the late gastrula stage (SupplMov. 1; Fig. 1a, 1a', N= 6/6). Ca^{2+} transients were first observed in muscle-lineage cells in the late gastrula embryos (stage 13, 6 hpf) (SupplMov. 1; Fig. 1b, 1b', N = 2/6). Several Ca^{2+} transients were observed in muscle lineage cells and continued to the early neurula stage. These Ca^{2+} transients were considered to be related to muscle cell arrangement and cell elongation occurring from gastrulation to the tailbud stage (Ohtsuka and Okamura, 2007). The spontaneous Ca^{2+} transients during myogenesis