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Integrative cytological analysis of the effects of Ca^{2+} and vitamin D_3 on extracellular Ca^{2+} flux and intracellular Ca^{2+} reserves in the mantle of the pearl oyster (*Hyriopsis cumingii Lea*)

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ABSTRACT

To examine Ca^{2+} absorption and transportation in the freshwater pearl oyster, *Hyriopsis cumingii Lea*, we studied the effects of different levels of either extracellular Ca^{2+} or $1,25(OH)_2D_3$ on extracellular Ca^{2+} flux and intracellular Ca^{2+} concentrations in mantle cells using the non-invasive micro-test technique and laser scanning confocal microscopy. The inner and outer mantle (IM and OM) cells from mussels were cultured and then treated with different concentrations of Ca^{2+} and $1,25(OH)_2D_3$. Extracellular Ca^{2+} flux and intracellular Ca^{2+} reserves were analyzed. The results showed that both extracellular Ca^{2+} and $1,25(OH)_2D_3$ had significant effects on Ca^{2+} flux and reserves in mantle cells, especially in IM cells ($P < .05$). The increase in extracellular Ca^{2+} concentrations resulted in the conversion of extracellular Ca^{2+} flux into influx with an increase in flow rate ($P < .05$). The calcium ion fluorescence intensity of OM cells was higher than that of IM cells ($P < .05$). $1,25(OH)_2D_3$ addition also significantly increased the influx rate of extracellular Ca^{2+}, especially in IM cells, which were more sensitive to $1,25(OH)_2D_3$ addition and had significantly higher Ca^{2+} influx rates than did OM cells ($P < .05$). Fluorescence intensities of intracellular Ca^{2+} first increased and then decreased with increasing $1,25(OH)_2D_3$ levels. The study showed that IM cells play an important role in absorbing Ca^{2+} from the environment, while OM cells mainly function in the temporary storage and transportation of Ca^{2+} in the body. The current results suggested that high levels of extracellular Ca^{2+} (1.25 mM) or $1,25(OH)_2D_3$ (over 100 IU/L) were favorable for Ca^{2+} uptake and maintenance in the body.

1. Introduction

In mollusks, the shell and pearl are ultimate products of biomineralization and comprise 95%–99% $CaCO_3$ and < 5% organic matrix (Kobayashi and Samata, 2006; Bjarnmark et al., 2016). Studies have shown that the mantle tissues, comprising an inner membrane and an outer membrane as well as the connective tissue between them, play a vital role in pearl and shell growth (Sillanpaa et al., 2016; Yarra et al., 2016). Calcium metabolism in the mantle cells and mantle cavity fluid eventually result in $CaCO_3$ deposition in mantle tissues (Xiang et al., 2014; Kong et al., 2015).

Studies have suggested that Ca^{2+} can associate with binding proteins in the mantle cells of *Mercenaria mercenaria* and are transported via endocytosis or exocytosis (Yao et al., 2009) due to the existence of calcium channels in the outer membrane epithelium. Given the low calcium content in freshwater-farming environments, shellfish use tissues or organs that directly contacting water, such as the mantle, as an important way to absorb environmental calcium (Kobayashi and Samata, 2006; Bach, 2015). Environmental Ca^{2+} levels were considered a major factor in controlling $CaCO_3$ deposition in pearl oysters (Qian, 2006; Diaz de Barboza et al. 2015). Vitamin D_3 and its derivatives have attracted extensive attention due to their roles in promoting calcium uptake and deposition in higher organisms (Zanatta et al., 2012; Lee et al., 2015). Previous studies have demonstrated that vitamin D_3 significantly affected mineral deposition in the abalone (Zhou and Mai, 2004).

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As one of the major freshwater mussel species, *Hyriopsis cumingii Lea* (*H. cumingii*) are very important for the aquaculture business (Li et al., 2017a, 2017b). It is a kind of freshwater bivalve mollusk and is widely cultured in the south of China for the production of pearls (Bai et al., 2013). Currently, the annual production of freshwater pearls has reached 1800 metric tons in China, accounting for > 90% of the total output in the world (Wang et al., 2015). However, relatively little is known about calcium metabolism in *H. cumingii*, especially for calcium absorption, transport, and deposition, in inner and outer mantle tissues (IM and OM), which limits our understanding of the mechanisms involved in shell and pearl formations.

The non-invasive micro-test technique is a technology used for non-invasively obtaining dynamic information on specific ionic/molecular activities on material surfaces (Smith et al., 1999). The non-invasive micro-test technique include scanning ion selective electrode technology (SIET), which mainly measures information such as the flux rate and flow direction of small molecules or ions. To date, Ca^{2+}, H^+, K^+, Cl^-, and O^{2-} have been detected as sensors for ionic/molecular species (Mclamore et al., 2009; Porterfield et al., 2009; Yang et al., 2012; Song et al., 2015). Furthermore, Laser Scanning Confocal Microscopy (LSCM) analysis can be used to observe the intracellular accumulation of ions (Li et al., 1997; Zhang et al., 2000; Wiesner et al., 2003). Therefore, SIET combined with LSCM is a good method for investigating ion metabolism.

In this study, the IM and OM cells of *H. cumingii* were collected and placed in primary cell cultures. The effects of varying concentrations of Ca^{2+} and $1,25(OH)_2D_3$ on intracellular and extracellular calcium metabolism were measured using SIET and LSCM techniques. This research will provide a theoretical basis for the physical research of calcium metabolism in freshwater pearl oysters and a biological basis for further research on pearl formation and growth.

2. Materials and methods

2.1. Materials

Two-year-old *H. cumingii* (5–6 cm shell length) were obtained from the experimental pearl farm in Zhejiang Province, China, and maintained in oxygenated fresh water at 1.25 mM Ca^{2+} ($CaCl_2$; Sigma, USA), with pH 7.2 and 26 °C conditions, for 2 weeks in the laboratory.

2.2. Cell and tissue cultivation

The IM and OM cells were obtained according to the striping method (Zhang, 2005). Under aseptic conditions, the IM and OM cells were taken and sterilized for 20 min in an antibiotic solution containing 1 mg/mL streptomycin, 500 IU/mL penicillin, 100 IU/mL gentamicin, and 2 μg/mL nystatin in Hanks balanced salt solution without Ca^{2+} (Sigma, USA). All cell culture reagents were supplied by Gibco (USA). Suspension cultures of IM and OM cells were obtained and cultured at 26 °C using the methods of Gong et al. (pH 7.2, Gong et al., 2008). During the culturing process, the cells were observed under an inverted microscope (Olympus, Japan). After 12 h of culturing, cell suspensions were centrifuged at 1500 rpm for 10 min, and the resulting cell pellets were resuspended in HBSS (with 1.25 mM Ca^{2+}) ($CaCl_2$, Sigma, USA) to cell densities of 5×10^5 to 1×10^6 cells/mL. An aliquot (1 mL) of the cell suspension was dispensed into nontreated petri dishes and confocal petri dishes (Gibco, USA) that were pretreated with Poly-L-Lysine (MW > 300,000, Invitrogen, USA). Confocal petri dishes were incubated for 2 h in 5% CO_2 at 26 °C in a thermostatic incubator (SANYO, Japan) to allow cells to adhere to the bottom of the dish.

2.3. Analysis of extracellular Ca^{2+} fluxes using SIET

IM and OM cells obtained from nontreated petri dishes (12 replicates per sample) were washed three times with Ca^{2+}-free HBSS solution and incubated in HBSS solutions supplemented with different concentrations of $CaCl_2$ (0.5, 1.25, and 3 mM, Sigma, USA) for 30 min at 26 °C. Extracellular Ca^{2+} fluxes were measured using SIET (BIO-001A, Younger USA Sci. & Tech. Co., Amherst, MA, USA) at a constant temperature of 26 °C (Mclamore et al., 2009). Different concentrations of $1,25(OH)_2D_3$ (0, 50, 100, 200 and 1000 IU/L; Sigma, USA) were loaded at a distance of ~5 μm away from cells treated with $CaCl_2$, and the Ca^{2+} influxes were continuously recorded for approximately 5 min (Li et al., 2017a, 2017b). To construct the microelectrodes, borosilicate micropipettes (2–4 μm aperture, XYPG120–2) were silanized with tributylchlorosilane, and the tips were filled with Calcium Ionophore I - Cocktail A. An Ag/AgCl wire electrode holder (XYEH01–1) was inserted in the back of the electrode to make electrical contact with the electrolyte solution (100 mM $CaCl_2$). Only electrodes with Nernstian slopes > 25 mV were used. Ca^{2+} fluxes were calculated by Fick's law of diffusion: $J_o = -[D \times (dC/dX)]$ (Zhang et al., 2009; Lu et al., 2013), where D is the self-diffusion coefficient for Ca^{2+} (in cm^2/s), dC is the difference value of Ca^{2+} concentrations between the two positions, and dX is the 10 μm excursion over which the electrode moved in our experiments. Each cycle of microelectrode movement and voltage measurement was completed in 5 s. The Ca^{2+} transfer was represented by the number of moles of Ca^{2+} passed per second per cm^2 ($pmol/cm^2 \cdot s$). Positive values represented Ca^{2+} effluxes, whereas negative values represented influxes.

2.4. Analysis of intracellular Ca^{2+} concentrations using LSCM

IM and OM cells in confocal petri dishes (24 replicates per sample) were washed three times with Ca^{2+}-free HBSS solution and incubated in HBSS solutions supplemented with 8 μM Fluo-3/AM, 0.02% Pluronic F-127 as well as different concentrations of $CaCl_2$ (0, 0.5, 1.25 and 3 mM) (Sigma, USA), for 30 min at 37 °C. After 3 washes with Ca^{2+}-free HBSS, the cells were incubated in HBSS solutions supplemented with 1.25 mM Ca^{2+}, 8 μM Fluo-3/AM, 0.02% Pluronic F-127, and different concentrations of $1,25(OH)_2D_3$ (50, 100, 200 and 1000 IU/L) for another 30 min at 37 °C. The intracellular fluorescence intensity of the treated cells was detected by using the green fluorescence detector of LSCM (Leica TCS SP5, Germany) after the cells were washed with Ca^{2+}-free HBSS (Shen et al., 2012). The LSCM parameters were set up as follows: 40× magnification, 488 nm excitation, 525 nm emission, and 20 scans (Zhang et al., 2000). The intracellular calcium imaging was performed as described previously (Yuan et al., 2010). The fluorescence intensity in individual cells was measured using LSM 5 Image software. All tests were conducted at 26 °C, 30 cells were measured for each treatment, and the mean fluorescence intensity per cell was calculated.

2.5. Statistical analysis

All data were analyzed by a one-way analysis of variance (ANOVA) using a factorial arrangement of treatments (Bewick et al., 2004). Data were expressed as the mean ± SE. The differences between the treatment means were analyzed by either Duncan's multiple range tests or *t*-tests, at a significance level of 0.05. A value of $P < .05$ was considered significant.

3. Results

3.1. Mantle cell cultivation and Fluo-3/AM loading

IM and OM cells were typically ovoid in shape, and the cell diameters were significantly different between the cells (6.75 ± 0.27 μm vs. 11.92 ± 1.28 μm; $P < .05$). After 12 h of culturing, both IM and OM cells had intact cytomembranes and had adhered to the bottom of the dishes. Strong levels of Fluo-3/AM fluorescence intensities indicated that cells were highly active during the whole assay (Fig. 1). The outline of the mantle cells, the particulate matter, and the