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Stage-specific changes in calcium concentration in crustacean (*Callinectes sapidus*) Y-organs during a natural molting cycle, and their relation to the hemolymphatic ecdysteroid titer

Hsiang-Yin Chen [a], Richard M. Dillaman [b], Robert D. Roer [b], R. Douglas Watson [a,*]

[a] Department of Biology, University of Alabama at Birmingham, Birmingham, AL 35294, USA
[b] Department of Biology and Marine Biology, University of North Carolina at Wilmington, Wilmington, NC 28403, USA

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ABSTRACT

Secretion of ecdysteroid molting hormones by crustacean Y-organs is suppressed by molt-inhibiting hormone (MIH). The suppressive effect of MIH on ecdysteroidogenesis is mediated by one or more cyclic nucleotide second messengers. In addition, existing data indicate that ecdysteroidogenesis is positively regulated (stimulated) by intracellular Ca^{++}. Despite the apparent critical role of calcium in regulating ecdysteroidogenesis, the level of Ca^{++} in Y-organ cells has not been previously measured during a natural molting cycle for any crustacean species. In studies reported here, a fluorescent calcium indicator (Fluo-4) was used to measure Ca^{++} levels in Y-organs during a molting cycle of the blue crab, *Callinectes sapidus*. Mean calcium fluorescence increased 5.8-fold between intermolt (C4) and stage D3 of premolt, and then dropped abruptly, reaching a level in postmolt (A) that was not significantly different from that in intermolt ($P>0.05$). The level of ecdysteroids in hemolymph of Y-organ donor crabs (measured by radioimmunoassay) showed an overall pattern similar to that observed for calcium fluorescence, rising from 2.9 ng/mL in intermolt to 357.1 ng/mL in D3 ($P<0.05$), and then dropping to 55.3 ng/mL in D4 ($P<0.05$). The combined results are consistent with the hypothesis that ecdysteroidogenesis is stimulated by an increase in intracellular Ca^{++}.

1. Introduction

Calcium signaling is involved in the regulation of multiple developmental and physiological processes, including fertilization, embryonic patterning, cell-cycle progression, muscle contraction, and neurotransmitter release (Kahl and Means, 2003; Whitaker, 2006; Clapham, 2007; Slusarski and Pelegri, 2007). Results reported here are consistent with the hypothesis that calcium signaling is also involved in the regulation of steroid hormone secretion by crustacean molting glands (Y-organs).

Crustacean Y-organs, paired endocrine glands located in the anterior cephalothorax, secrete C-27 steroid hormones (ecdysteroids) (Lachaise et al., 1993). Ecdysteroids elicit the molecular events in target cells that lead to molting and incremental growth (Riddiford et al., 2000). Secretion of ecdysteroids by Y-organs is regulated (suppressed) by molt-inhibiting hormone (MIH), a polypeptide neurohormone produced in a cluster of eyestalk neurosecretory soma (the X-organ), and released from their associated axon terminals in the neurohemal sinus gland (Lachaise et al., 1993; Nakatsuji et al., 2009). Thus, surgical removal of the eyestalks leads to enhanced ecdysteroid secretion by Y-organs, an increase in the ecdysteroid titer, and precocious molting (Keller and Schmid, 1979; Chang and Bruce, 1980; Hopkins, 1983), while replacement therapy using eyestalk extract or synthetic MIH lowers the ecdysteroid titer and delays molting (Bruce and Chang, 1984; Chang et al., 1987; Nakatsuji and Sonobe, 2004). Based on these and related findings, a long-held model proposes that MIH from the X-organ/sinus gland complex inhibits Y-organs during much of the molting cycle (principally intermolt), and that a drop in MIH secretion permits the surge in ecdysterogenesis that leads to molting (Skinner, 1985). Although recent studies indicate that the model is incomplete (Nakatsuji and Sonobe, 2004; Chung and Webster, 2005), it remains useful as a base from which testable hypotheses can be formed.

Existing data indicate that MIH acts directly on Y-organs to suppress synthesis of ecdysteroids (Soumoff and O'Connor, 1982; Mattson and Spaziani, 1985a; Watson and Spaziani, 1985a) and uptake of lipoprotein-bound cholesterol, the biosynthetic precursor of ecdysteroids (Watson and Spaziani, 1985a,b; Kang and Spaziani, 1995a,b), and that MIH action is mediated through cellular signaling pathways involving cAMP, cGMP, or both (Covi et al., 2009; Nakatsuji et al., 2009). Cell signaling molecules other than cyclic nucleotides have also been implicated in MIH action (Spaziani et al., 2001). Among these, calcium appears to play a critical role. Mattson and Spaziani (1986) preloaded dispersed Y-organ cells with $^{45}Ca^{++}$, and found that addition of eyestalk extract (containing MIH activity) elicited a statistically significant efflux of $^{45}Ca^{++}$ into incubation medium. The

* Corresponding author at: Department of Biology, University of Alabama at Birmingham, Birmingham, AL 35244, USA. Tel.: +1 205 934 2031; fax: +1 205 975 6097.
E-mail address: rdwatson@uab.edu (R.D. Watson).

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authors hypothesized that MIH may suppress ecdysteroidgenesis, at least in part, by promoting Ca^{++} efflux. In companion experiments, treatment of crab (*Cancer antennarius*) Y-organs with the calcium ionophore A23187 (which increases intracellular Ca^{++}) stimulated ecdysteroid production in a dose-dependent manner (Mattson and Spaziani, 1986). These and related findings led to the hypothesis that an increase in intracellular calcium may promote the surge in ecdysteroidogenesis that leads to molting (Mattson and Spaziani, 1986).

Consistent with this hypothesis, we previously reported that activation of Y-organs by eyestalk ablation produced an increase in free Ca^{++} in Y-organ cells, and that the increase in intracellular free Ca^{++} was associated with an increase in the hemolymphatic ecdysteroid titer (Chen and Watson, 2011). However, although eyestalk ablation has historically been used as an experimental tool to activate Y-organs, the procedure removes the source of neurohormones other than MIH, and biochemical and physiological parameters of an experimentally accelerated (induced) molting cycle differ from those of a natural molting cycle in multiple respects (Spaziani et al., 1982). Therefore, in studies described here, we measured Ca^{++} levels in Y-organ cells and determined the level of ecdysteroids in hemolymph during various stages of a natural molting cycle of the blue crab, *Callinectes sapidus*.

2. Materials and methods

2.1. Experimental animals

Blue crabs (*C. sapidus*) were purchased from local fishers in Wilmington, NC, USA, and staged according to accepted criteria (Drach and Tchernigovtzeff, 1967; Stevenson, 1972).

2.2. Preparation of Y-organ cells and measurement of intracellular fluorescence

The level of intracellular Ca^{++} in Y-organ cells was determined using a fluorescent calcium indicator, Fluo-4. Fluo-4 binds freely diffusible Ca^{++}. In response to Ca^{++} binding, there occurs a large (>100-fold) increase in fluorescence intensity (Gee et al., 2000). Cells were loaded with a cell-permeant acetoxymethyl (AM) ester derivative, Fluo-4 AM (Molecular Probes). Fluo-4 AM is capable of passively diffusing across cell membrane; once inside the cell, intrinsic esterases cleave the AM group from the probe leaving the cell-impermeant Fluo-4 indicator.

Dissociated Y-organ cells were prepared as previously described (Chen and Watson, 2011). Briefly, Y-organs were dissected from crabs during selected stages of the molting cycle, rinsed with Pantin's saline (Pantin, 1934), cut into pieces, transferred to Pantin's saline containing EDTA (1 mM) and 0.25% collagenase (Sigma), and then swirled gently on an orbital shaker (120 rpm) at 28 °C for 1 h. The medium containing dissociated tissue was then transferred to a centrifuge tube and filtered through 200 micrometer nylon mesh by brief centrifugation (100*g*). The pellet of dispersed Y-organ cells was washed once in Pantin's saline containing EDTA (1 mM), resuspended and incubated for 30 min in Pantin's saline containing EDTA (1 mM), Fluo-4 AM (10 M), and Pluronic F-127 (20%) (Molecular Probes). After incubation, Y-organ cells were collected by brief centrifugation (100*g*), washed twice, and then re-suspended in 50 μL Pantin's saline containing EDTA (1 mM). A 10-μL aliquot of the cell suspension was used for determination of intracellular fluorescence.

The fluorescent signal from Y-organ cells was visualized using an Olympus confocal microscope fitted with a high-energy argon laser at 488 nm. Images were collected for each stage, and the intensity of total intracellular fluorescence was determined using MetaMorph 7.5 software (Universal Imaging) (Pradhan et al., 2008; Xu et al., 2008; Chen and Watson, 2011). In addition, the software was used to determine the intensity of fluorescence along a transect drawn through representative cells of each stage.

2.3. Determination of the hemolymphatic ecdysteroid titer

The hemolymphatic ecdysteroid titer was determined by radioimmunoassay as previously described (Lee et al., 1998).

2.4. Statistical analyses

The statistical significance of differences among means was determined by one-way analysis of variance followed by Tukey's HSD test using SAS 9.1 software (SAS Institute Inc.).

3. Results

In studies reported here, a fluorescent calcium indicator (Fluo-4) was used to determine the level of Ca^{++} in Y-organ cells during a natural molting cycle of *C. sapidus*. As shown in Fig. 1A, there occurred stage-specific changes in total intracellular calcium fluorescence in Y-organ cells during the molting cycle. Mean calcium fluorescence increased by 5.8-fold between intermolt (C4) and stage D3 of premolt ($P<0.05$), and then dropped abruptly, reaching a level in postmolt (A) that was not significantly different from that in intermolt ($P>0.05$). Measurement of fluorescence along a transect drawn through representative cells from various stages of the molting cycle indicated that the intensity of calcium fluorescence was generally greater in cytoplasm than in nuclei (Fig. 1B). Although the imaging methods used here provide an indirect (rather than direct) measure of intracellular Ca^{++}, the most straightforward interpretation of the results is that there occur stage-specific changes in the level of intracellular Ca^{++} in Y-organ cells during the molting cycle of *C. sapidus*.

The level of ecdysteroids in hemolymph of Y-organ donor crabs was determined by radioimmunoassay. The hemolymphatic ecdysteroid titer rose from 2.9 ng/mL in intermolt to 357.1 ng/mL in D3 ($P<0.05$), and then dropped to 55.3 ng/mL in D4 ($P<0.05$) (Fig. 2).

4. Discussion

Results shown in Fig. 1 constitute the first report of stage-specific changes in Ca^{++} levels in Y-organ cells during a natural molting cycle for any crustacean species. The pattern of change in hemolyphatic ecdysteroid levels (Fig. 2) is temporally and quantitatively similar to that previously reported for *C. sapidus* (Lee et al., 1998), and is consistent with the several existing reports of ecdysteroid titers in other decapod crustaceans (Skinner, 1985). In general, the stage-specific changes in Ca^{++} levels in *C. sapidus* Y-organs appear to be associated with changes in the hemolymphatic ecdysteroid titer. This interpretation is consistent with our previous report that activation of Y-organs by eyestalk ablation produced an increase in intracellular Ca^{++} in Y-organs, and that the increase in intracellular Ca^{++} was associated with an increase in the ecdysteroid titer (Chen and Watson, 2011). The combined results are consistent with the hypothesis that ecdysteroidogenesis is stimulated by an increase in intracellular Ca^{++}.

Similarly consistent with that hypothesis are results from experiments in which various pharmacological agents were used to experimentally manipulate the level of Ca^{++} in Y-organs. Mattson and Spaziani (1986) observed that calcium antagonists (lanthanum or ruthenium red), an intracellular calcium chelator (TMB-8), a calmodulin inhibitor (trifluorperazine), or calcium channel blockers (verapamil, nifedipine, or nicardipine) individually suppressed basal ecdysteroid production by crab (*C. antennarius*) Y-organs *in vitro*, or enhanced the suppressive effect of MIH, or both (Spaziani et al., 2001). Similarly, Sedlmeier and Seinsche (1998) found that calcium channel blockers (pimozide, nimodipine, or flunarizide) individually suppressed basal ecdysteroid production by crayfish (*Orconectes limosus*) Y-organs *in*