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Calcium Signaling And The Regulation Of Ecdysteroid Hormone Synthesis By Blue Crab (Callinectes Sapidus) Molting Glands

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CALCIUM SIGNALING AND THE REGULATION OF ECDYSTEROID HORMONE SYNTHESIS BY BLUE CRAB (*CALLINECTES SAPIDUS*) MOLTING GLANDS

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BIOLOGY

ABSTRACT

In crustaceans, cellular events that lead to molting are triggered by steroid molting hormones, ecdysteroids. The synthesis of ecdysteroids is negatively regulated by molt-inhibiting hormone (MIH) mediated by one or more cyclic nucleotide second messengers. Existing data indicate ecdysteroidogenesis is positively regulated by Ca^{2+} through the activation of phosphodiesterases (PDEs). The studies presented in this dissertation composed the first report of intracellular Ca^{2+} measurement in Y-organ cells from any crustacean species. Studies demonstrated that elevated cytosolic Ca^{2+} levels in Y-organ cells were associated with increased hemolymphatic ecdysteroid titer. Measurement of Ca^{2+} levels in Y-organ cells in a natural molting cycle revealed a molting stage-specific change: Ca^{2+} level was low in intermolt (C), increased in premolt (D), and declined to an insignificant level in postmolt (A) stage. Molting-stage specific changes of hemolymphatic ecdysteroid titers showed a similar pattern. These combined data were consistent with the hypothesis that ecdysteroidogenesis was stimulated by an increase in intracellular Ca^{2+}.

In eukaryotic cells intracellular Ca^{2+} is tightly regulated by the interaction of diverse Ca^{2+} regulatory proteins. Among them, plasma membrane Ca^{2+} ATPase (PMCA), which pumps Ca^{2+} outside the cell, certainly plays a crucial role. In the parallel study, a putative PMCA gene (*Cas-PMCA*) was cloned from *Callinectes sapidus* Y-organs. Its deduced amino acid sequence represented all signature domains of an authentic PMCA including phosphorylation, and ATP, and calmodulin (CaM) binding sites. The transcript of *Cas-PMCA* was extensively detected in both neural and nonneural tissues, suggesting an essential role in various cell types. Transcript abundance of *Cas-PMCA* was measured in Y-organs, nonmineralization and mineralization hypodermal tissues in a natural molting cycle. Results showed that expression pattern of *Cas-PMCA* in Y-organs and nonmineralizing hypodermis were similar: it was low in intermolt stage, significantly increased in premolt stage and then back to resting level in postmolt stage. However, it showed an opposite expression pattern in mineralizing hypodermis. It was suggested that the elevated *Cas-PMCA* transcript abundance in Y-organs and nonmineralizing hypodermis might be a systemic response to the increased calcium influx when the exoskeleton is undergoing decalcification.

Keywords: ecdysteroidogenesis, Ca^{2+}, Y-organ, molt-inhibiting hormone, plasma membrane Ca^{2+}-ATPase, crustacean

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LIST OF ABBREVIATIONS

B	Brain
CHH	crustacean hyperglycemic hormone
CaM	Calmodulin
E	Eyestalk
ECaC	epithelial Ca^{2+} channel
FITC	fluorescein isothiocyanate
FSBA	5'-*p*-fluorosulphonylbenzoyladenosine/r-(*N*-2-chloroethyl-*N*-methylamino) benzylamine
H	Hypodermis
Hp	Hepatopancreas
M	Muscle
MIH	molt-inhibiting hormone
MOIH	mandibular organ-inhibiting hormone
NCX	Na^{+}/ Ca^{2+}-exchanger
PDE	Phosphodiesterase
PMCA	plasma membrane Ca^{2+}-ATPase
PKA	protein kinase A

PKC	protein kinase C
PKG	protein kinase G
PO	pericardial organ
RACE	rapid amplification of cDNA ends
rGC	receptor guanylyl cyclase
SCP	sarcoplasmic calcium binding protein
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
sGC	soluble guanylyl cyclase
Tes	Testis
TG	thoracic ganglia
YO	Y-organ

General Introduction

1. Incremental growth and molting in crustaceans

During crustacean growth and postembryonic development, the exoskeleton is periodically shed (molted) and replaced with a larger version capable of accommodating further growth of soft tissues. Based on morphological and physiological criteria, the molting cycle can be divided into the following five stages: premolt (proecdysis, stages D0-D4), molting (ecdysis, stage E), postmolt (metecdysis, stages A1- A2 and B1-B2), and intermolt (anecdysis, stage C1- C4) (Drach, 1939; Lachaise et al., 1993; Skinner, 1962; Skinner, 1985a; Vranckx and Durlita, 1978). Gastrolith formation and regeneration of autotomized limbs begin during early premolt. Subsequently, calcium is reabsorbed from the partially dissolved old exoskeleton, and new epicuticle and exocuticle layers are secreted by underlying epidermal cells (Skinner, 1962). Near the end of premolt, water is taken up through the gills and midgut (Mykles, 1980), producing an increase in hydrostatic pressure that expands the new exoskeleton and separates it from the overlying old exoskeleton. Molting, the shortest stage, lasts only a few minutes. Beginning in postmolt, the newly formed exoskeleton is thickened and hardened as a result of formation of the endocuticle layer, mineralization, and cross-linking of proteins. During intermolt, typically the longest stage, animals resume eating, and there follows soft tissue

growth and storage of organic reserves (O'Brien and Skinner, 1987; Stringfellow and Skinner, 1988).

2. Endocrine regulation of crustacean molting

Crustacean molting is triggered by C-27 steroid hormones termed ecdysteroids. Ecdysteroids are synthesized from cholesterol by paired epithelioid endocrine glands, the Y-organs (Sedlmeier and Seinsche, 1998; Lachaise et al., 1993). The hemolymphatic ecdysteroid titer varies during a molting cycle. The ecdysteroid titer is maintained at a low basal level during intermolt, and then rises during premolt, reaching a peak during late premolt. The level of ecdysteroids in hemolymph declines rapidly just before ecdysis, and remains low during postmolt (Chang, 1989; Steele and Vafapoulou, 1989; Lachaise et al., 1993; Soumoff and Skinner, 1983; Nakatsuji et al., 2000).

Zeleny (1905) was the first to implicate a factor from the eyestalks in the negative regulation (inhibition) of molting. Subsequently, data from multiple sources have shown that ablation of eyestalks leads to increases in the rate of ecdysteroid secretion by Y-organs and the concentration of ecdysteroids in hemolymph, and accelerates molting (Abramowitz and Abramowitz, 1940; Smith, 1940; Watson and Spaziani, 1985a; Chang and O'Connor, 1978; Chang and Bruce, 1980; Keller and Schmid, 1979). Furthermore, the injection of eyestalk extract lowers the level of

ecdysteroids in hemolymph of eyestalk-ablated animals (Keller and O'Connor, 1982; Bruce and Chang, 1984; Hopkins, 1982). Likewise, injection of purified native or recombinant MIH prolongs the interval between molts (Chang et al., 1987; Okumura et al., 2005). The above and related data strongly support Zeleny's hypothesis that a factor from eyestalks negatively regulates molting. The factor has since been purified and named molt-inhibiting hormone (MIH) (Aguilar, et al., 1995; Webster and Keller, 1986; Webster, 1991; Yang et al., 1996).

Amino acid sequence data place MIH in the crustacean hyperglycemic hormone (CHH) family of peptides. The family includes (in addition to CHH and MIH) vitellogenesis-inhibiting hormone (VIH) and mandibular organ-inhibiting hormone (MOIH) (Keller, 1992; Wainwright et al., 1996; Van Herp, 1998). Based on preprohormone structure, the CHH family of peptides can be divided into two groups, one group containing CHH, and the other containing MIH, VIH, and MOIH. Preprohormones of the former group (type I peptides) contain a CHH precursor related peptide (CPRP) that is cleaved out during processing; preprohormones of the latter group (type II peptides) lack the CPRP (Lancome et al., 1999).The specific source of MIH in eyestalks is the X-organ/ sinus gland complex first described by Hanstrom (1931; 1933). The X-organ is a cluster of neurosecretory cell soma located on the surface of an eyestalk neural ganglion (the medulla terminalis); axons extend

distally from the soma and terminate in a neurohemal organ, the sinus gland. MIH and other CHH family peptides are synthesized in the X-organ, and then transported to, stored and released from sinus gland (Andrew, 1983).

Several lines of evidence indicate MIH acts directly on Y-organs to suppress ecdysteroidogenesis. Thus, adding eyestalk extract or sinus gland conditioned medium or purified MIH to incubation medium significantly diminishes the secretion of ecdysteroids by Y-organs *in vitro* (Soumoff and O'Connor, 1982; Mattson and Spaziani, 1985a and b; Toullec and Dauphin-Villemant, 1994; Watson and Spaziani, 1985b; Webster, 1986). Based on the above and related data, a longstanding model has been that MIH suppresses ecdysteroidogenesis during much of the molting cycle, and that initiation of the molting progress is triggered by a decrease in MIH secretion during premolt (Webster, 1998; Lee et al., 1998; Nakatsuji et al., 2000). While the combined data from all sources generally support the above model, several findings suggest the model is incomplete. For example, several studies suggest that ecdysteroid production may be regulated not only by the level of circulating MIH, but also by the responsiveness of Y-organs to MIH (Sefiani et al, 1996; Chung and Webster, 2003; Nakatsuji and Sonobe, 2004). In addition, compounds other than MIH have been implicated, either directly or indirectly, in the regulation of Y-organs (Webster and Keller, 1986; Webster, 1993; Dell et al., 1999; Yu et al., 2002). Foremost among these

alternative regulators is CHH.

CHH, as the member in the same peptide family, shares a certain level of identity with MIH in the amino acid sequence and protein structure (see Soyez, 2003). Research shows that addition of CHH (in high dose) to incubation medium suppresses the production of ecdysteroids by Y-organs *in vitro* (Webster and Keller, 1986; Chang et al., 1990; Yasuda et al., 1994; Yang et al., 1996; Zarubin et al., 2008). In addition, CHH receptors are also found on Y-organ membranes, indicating the potential ability of CHH to regulate Y-organ (Webster, 1993).

3. Cyclic nucleotides cellular signaling pathways linked to MIH action

Existing data indicate that cellular signaling pathways involving cAMP, cGMP, or both play a role in MIH-mediated regulation of ecdysteroidogenesis (Nakatsuji et al., 2009).

Mattson and Spaziani (1985b) showed that incubating crab (*Cancer antennarius*) Y-organs with eyestalk extract caused the dose-dependent accumulation of cAMP. Further, adding to incubation medium db-cAMP (a cAMP analog), IBMX (an inhibitor of phosphodiesterase, PDE) or forskolin (an activator of adenylyl cyclase) dose-dependently suppressed ecdysteroid secretion. The authors concluded that MIH-induced suppression of ecdysteroid production is mediated by a cAMP second messenger. Glandular cGMP accumulation, however, was not measured in this study.

Subsequently, Mattson and Spaziani (1986a) reported that eyestalk extract, a cAMP analog, or agents that increase intracellular cAMP also significantly suppressed incorporation of [^{3}H]leucine into Y-organ proteins *in vitro*. This suppressive effect was reduced by a calcium ionophore (A23187). Mattson and Spaziani (1986a) hypothesized that MIH inhibits ecdysteroidogenesis in part by inhibiting protein synthesis, an effect mediated through cAMP. The observation that the suppressive effect of MIH could be relieved by increasing intracellular Ca^{2+} led the authors to hypothesize that a Ca^{2+}/calmodulin (CaM) -specific PDE may be responsible for controlling the level of cAMP in Y-organ cells (Mattson and Spaziani,1986b).

In contrast to the above results implicating cAMP as the dominant second messenger in MIH action, Sedlmeier and Fenrich (1993) reported that incubating crayfish (*Orconectes limosus*) Y-organs in sinus gland conditioned medium caused a substantial increase in glandular cGMP but not cAMP. In addition, protein kinase G (PKG) activity in Y-organs of *O. limosus* was found to be higher during intermolt than premolt, and addition of sinus gland conditioned medium to Y-organ incubations elicited an increase in PKG activity, but had no effect on protein kinase A (PKA) activity (Von Gliscynski and Sedlmeier, 1993). It was concluded by this group that cGMP is the dominant second messenger in MIH action. In related studies, Baghdassarian et al. (1996) reported that phosphotransferase activity in the cytosolic

fraction of crab (*Carcinus maneas*) Y-organs is predominantly cGMP-dependent.

Our laboratory has studied the link of cyclic nucleotide cell signaling to MIH action in Y-organs of two species, the blue crab, *Callinectes sapidus* (Nakatsuji et al., 2006a), and the crayfish *Procambarus clarkii* (Nakatsuji et al., 2006b). We found that adding recombinant MIH to incubation medium significantly enhanced intracellular cGMP accumulation in Y-organs of *C. sapidus*, but had no effect on intracellular cAMP (Nakatsuji et al., 2006a). In companion experiments, a cGMP analog (8-Br-cGMP) significantly suppressed ecdysteroid production by Y-organs of *C. sapidus*, but neither cAMP analogs (db-cAMP or 8-Br-cAMP) nor an activator of adenylyl cyclase (forskolin) had a detectable effect on ecdysteroidogenesis (Nakatsuji et al., 2006a). Likewise, addition of synthetic MIH to incubation medium, increased cGMP content, but not cAMP content, in Y-organs of crayfish, *P. clarkii* (Nakatsuji et al., 2006b). Thus, results from our laboratory also indicate MIH suppresses ecdysteroidogenesis via a cGMP second messenger.

Consistent with this conclusion, Saïdi et al. (1994) reported that adding purified MIH to incubations of Y-organs from intermolt green crabs (*C. maenas*) produced a large (20-fold) and sustained (≥60 min) increase in intracellular cGMP, but had no effect on cAMP. However, this same group also observed that adding purified MIH to incubations of Y-organs from premolt *C. maenas* produced not only a large

(60-fold) and sustained (≥60 min) increase in intracellular cGMP, but also a smaller (2-fold) and more transient (1-4 min) increase in cAMP. They suggested that cAMP might act cooperatively with cGMP in premolt Y-organs. To summarize, the preponderance of available data favor cGMP as the physiologically relevant second messenger linked to MIH action, but a role for cAMP cannot be completely discounted. Interpretation of the combined results from all sources is hindered by the fact that the matter has been studied in only a few species.

4. The MIH receptor

Little information is available on the MIH receptor. Receptor-binding assays (using [^{125}I]MIH as ligand) showed the existence of high-affinity, specific, and saturable MIH receptors on plasma membranes of *C. maenas* Y-organs (Webster, 1993). Asazuma et al. (2005) subsequently reported the chemical cross-linking of ^{125}I-labeled recombinant MIH to a 70 kDa protein in Y-organs of the prawn, *Marsupenaeus japonicas*, but activation of the protein by MIH was not demonstrated. Thus, while some progress has been made, the MIH receptor has not been fully characterized for any crustacean species.

Studies of cellular signaling in Y-organs can provide insight into the nature of the MIH receptor. As noted above, for the two species studied in our lab (*C. sapidus* and *P. clarkii*), the data strongly favor cGMP as the second messenger of MIH. The

enzymes that regulate synthesis of cGMP, guanylyl cyclases, fall into two major classes: the cytoplasmically localized, soluble guanylyl cyclases (sGC) and the membrane associated receptor guanylyl cyclases (rGC) (Wedel and Garbers, 2001). Both classes have been reported in vertebrate and invertebrate species (Garbers et al., 1994; Schaap, 2004; Pyriochou and Papapetropoulos, 2005). sGC are composed of α- and β- subunits, and under basal conditions, exist as self-associated heterodimers (Lucas et al., 2000; Schaap P, 2004). Atypical sGC isoforms that exist as homodimers have also been reported (Morton and Anderson, 2003). Both α and β subunits of sGC possess three functional domains: an N-terminal heme binding domain, a C-terminal catalytic domain, and a central dimerization domain. Binding of nitric oxide (NO) to the heme binding domain causes a conformational change that activates the catalytic domain and enhances conversion of GTP to cGMP (Lucas et al., 2000; Poulos, 2006). rGC, which exist as homodimers, are integral membrane proteins with five signature domains: an extracellular ligand binding domain (ECD), a hydrophobic transmembrane domain (TM), a protein kinase homology domain (KHD), a dimerization domain (DD) or so-called hinge region, and a guanylyl cyclase catalytic domain (GCD) (the latter three domains are intracellular domains) (Wedel and Garbers, 2001). The binding of a specific ligand to the extracellular domain of the rGC activates the intracellular cyclase catalytic domain. Seven rGCs (GC-A through

GC-G) have been identified from various human tissues. GC-A and GC-B are natriuretic peptides receptors (NPR). GC-C is an endogenous peptide receptor; its ligands included guanylin, uroguanylin, and bacterial heat-stable enterotoxins (STs). The remaining mammalian rGCs (GC-D thtough GC-F) are orphan receptors. The first invertebrate rGC was cloned from a sea urchin (Singh et al., 1988). Subsequently, cDNAs encoding rGC were cloned from crayfish muscle (Liu et al., 2004) and crab Y-organs (Zheng et al., 2006). Additional details on the latter are provided below.

Regarding the MIH receptor, in our hands, a NO donor (sodium nitroprusside) did not suppress ecdysteroid production by blue crab Y-organs *in vitro* (Han and Watson, unpublished). In addition, persuasive data indicate CHH enhances cGMP levels in crustacean muscle by activating an rGC, and has no effect on sGC activity (Goy, 1990). Thus, our working hypothesis is that the MIH receptor is an rGC.

As part of studies to test this hypothesis, our lab has cloned from *C. sapidus* Y-organs a cDNA (*CsGC-YO1*) encoding an rGC (Zheng et al., 2006). Pretreating Y-organs with antipeptide antibodies raised against the extracellular domain of CsGC-YO1 significantly blunts the suppressive effect of MIH on ecdysteroidogenesis (Zheng et al., 2008). In addition, quantitative RT-PCR revealed that expression of the *CsGC-YO1* transcript in Y-organs is elevated during intermolt (a stage when MIH is

thought to actively suppress ecdysteroidogenesis) (Zheng et al., 2008). Results of initial immunocytochemical studies, using anti-CsGC-YO1 as primary antibody, were consistent with the notion that CsGC-YO1 is a membrane-associated protein (Zheng et al., 2008), but additional studies to improve resolution are needed. While the above results generally support the hypothesis that CsGC-YO1 is an MIH receptor, an analysis of the tissue distribution of the *CsGC-YO1* transcript (using end point RT-PCR) revealed the transcript was expressed not only in Y-organs, but also in several other tissues examined, including ventral nerve cord, thoracic ganglion, and brain (Zheng et al., 2006). This pattern of expression was not predicted by current understanding of MIH action (i.e., Y-organs are the only known target tissues for MIH). One explanation is that MIH is not the activating ligand for CsGC-YO1. It is possible, for example, that CsGC-YO1 is a receptor for crustacean hyperglycemic hormone (CHH). Previous studies indicate that Y-organs possess distinct CHH receptors (Webster, 1993). Another interpretation of the observed tissue distribution pattern of the CsGC-YO1 transcript is that MIH affects target tissues other than Y-organs. Pleiotropic effects of polypeptide hormones are widely observed in both invertebrate and vertebrate systems. Recently, a putative MIH binding site has been found on *C. sapidus* hepatopancreas (Zmora et al., 2009). Data suggested MIH exhibited the stage-specific effect on the vitellogenin (VtG) mRNA synthesis (Zmora

et al., 2009). In summary, available data are generally consistent with the hypothesis that CsGC-YO1 is an MIH receptor. However, immunocytochemical studies that more clearly demonstrate the subcellular location of CsGC-YO1 are needed.

5. Cyclic phosphodiesterase determines responsiveness of Y-organs to MIH

It has become increasingly clear that rates of ecdysteroid secretion are influenced not only by MIH, but also by stage-specific changes in the responsiveness of the Y-organs to MIH. Sefiani et al. (1996) observed a marked decline in the sensitivity of shrimp (*Penaeus vannamei*) Y-organs to sinus gland extract during premolt stages D_2 and D_3. A decline in the responsiveness of Y-organs to MIH during middle and late premolt was subsequently reported for crabs (Chung and Webster, 2003) and crayfish (Nakatsuji and Sonobe, 2004). The cellular mechanisms underlying the changes in responsiveness of Y-organs to MIH are not well understood. Radioligand binding assays revealed no change in the number of MIH receptors in Y-organs during a molting cycle in *C. maenas* (Chung and Webster, 2003), indicating that changes in sensitivity to MIH are likely due to events downstream of receptor binding and may involve changes in intracellular signaling pathways. The observation that IBMX suppressed ecdysteroid secretion by Y-organs (Mattson and Spaziani, 1985b) suggests that intracellular PDE activity may be involved in regulation of Y-organ responsiveness.

In mammals, PDEs encoded by 21 PDE genes can be grouped into 11 different families (PDE1-PDE11) based on their structural similarity, sequence homology, and enzymatic properties (including substrate affinity, kinetic properties and sensitivity to endogenous regulators and inhibitors) (Omori and Kotera, 2007). The domains of PDEs that show the greatest sequence variability are generally located at the N terminus. All PDE members have a conserved 270 amino acids core region at the C-terminal catalytic domain with similarity of 35% to 50% among different PDE families (Omori and Kotera, 2007). Among all PDEs, only members in the PDE1 subfamily have a Ca^{2+}/CaM regulatory domain. Binding of Ca^{2+}/CaM to this domain activates PDE1 (Kakkar et al., 1999; Omori and Kotera, 2007).

In recent studies designed to assess the possible involvement of PDE in determining the responsiveness of Y-organs to MIH, Y-organs were removed from crayfish (*P. clarkii*) at various stages of the molting cycle and incubated *in vitro* with MIH, IBMX, or both (Nakatsuji et al., 2006b). The responsiveness of Y-organs to MIH alone was high during the intermolt stage, declined in early premolt, was low during middle and late premolt, and then increased during postmolt, confirming previous reports of stage-specific changes in responsiveness. When IBMX was included in the incubation medium along with MIH, the responsiveness of premolt Y-organs to MIH was restored to a level similar to that observed in intermolt Y-organs.

The results suggested that stage-specific changes in the responsiveness of Y-organs to MIH may be a result of changes in glandular PDE activity. Assays of PDE activity in *P. clarkii* Y-organs revealed enzyme activity was low during intermolt stage, rose to a peak during middle premolt, and then declined again by postmolt. The combined results are consistent with the hypothesis that PDE activity plays a central role in determining the responsiveness of Y-organs to MIH during the molt cycle. PDE activity in Y-organs of *P. clarkii* was strongly inhibited by 8-methylmethoxy-IBMX (a selective inhibitor of PDE1) (Nakatsuji et al., 2006b). The results indicate that the PDE isotype present in Y-organs of *P. clarkii* is PDE1.

Our lab has recently cloned from Y-organs of *P. clarkii* a cDNA (*PcPDE1*) encoding a PDE1 (Nakatsuji and Watson, unpublished). The deduced amino acid sequence showed the putative Ca^{2+}/CaM binding domain as well as the catalytic domain. Real-time PCR showed transcript abundance of *PcPDE1* is elevated during middle premolt, a stage in which the Y-organs are least responsive to MIH.

6. Involvement of Ca^{2+} signaling in ecdysteroidogenesis

Cell signaling molecules other than cyclic nucleotides have also been implicated, directly or indirectly, in MIH action. Among these, Ca^{2+} appears to play a critical role. Mattson and Spaziani (1986b) preloaded dispersed Y-organ cells with $^{45}Ca^{2+}$, and found that addition of eyestalk extract elicited a statistically significant

efflux of radiolabeled Ca^{2+} into incubation medium. Although the cellular mechanisms that link MIH receptor occupancy to Ca^{2+} efflux are not clear, it was reasonably suggested that the drop in intracellular Ca^{2+} might be involved in MIH-induced suppression of ecdysteroidogenesis. Consistent with that possibility are the observations that Ca^{2+} antagonists (lanthanum or ruthenium red), an intracellular Ca^{2+} chelator (TMB-8), a CaM inhibitor (trifluorperazine), or Ca^{2+} channel blockers (verapamil, nifedipine, or nicardipine) individually suppressed basal ecdysteroid production by Y-organs *in vitro*, or enhanced the suppressive effect of MIH, or both (Mattson and Spaziani, 1986b; Spaziani et al. 1999, 2001). Similar results were also observed in crayfish *Orconectes limosus*. Each of Ca^{2+} channel blockers including nimodipine, flunarizine and pimozide significantly reduced ecdysteroidogenesis up to 50% (Sedlmeier and Seinsche, 1998).

In related experiments, treatment of crab (*C. antennarius*) or crayfish (*O. limosus*) Y-organs with the Ca^{2+} ionophore A23187 (which increases intracellular Ca^{2+}) stimulated ecdysteroid production in a dose-dependent manner (Mattson and Spaziani, 1986b; Sedlmeier and Seinsche, 1998). This and other findings led to the hypothesis that an increase in intracellular Ca^{2+} may stimulate the surge in ecdysteroidogenesis that results in the premolt peak in the hemolyphatic ecdysteroid titer (Mattson and Spaziani, 1986b). The cause of the hypothesized increase in intracellular Ca^{2+} is

unknown. One possibility is that it is elicited by a specific ligand; the existence of a positive regulator of ecdysteroidogenesis in crustaceans has been long hypothesized (e.g., see Skinner, 1985b). Consistent with this hypothesis, the application of DAG or IP3 with *O. limosus* Y-organ resulted in amplified ecdysteroid production that pointed to the existence of a stimulatory factor (Sedlmeier and Seinsche, 1998). Another possibility is that the hypothesized increase in intracellular Ca^{2+} is a consequence of the general increase in hemolymphatic Ca^{2+} that occurs during premolt as Ca^{2+} is mobilized from the old exoskeleton (see Greenaway, 1985).

Existing data suggest elevated intracellular Ca^{2+} may be linked to enhanced ecdysteroidogenesis through activation of at least two intracellular enzymes, protein kinase C (PKC) and cyclic nucleotide PDE. Regarding PKC, the results are mixed, depending on species. Adding an activator of PKC (phorbol 12-myristate 13-acetate) to incubations of crab (*C. antennarius*) Y-organs dose-dependently stimulated ecdysteroid production, and the enzyme was Ca^{2+}-sensitive (Mattson and Spaziani, 1987). By contrast, adding phorbol 12-myristate 13-acetate to incubations of crayfish (*Orconectes immunis*) Y-organs suppressed ecdysteroid production (Spaziani et al., 2001). The reason for the difference between species is unknown. Regarding PDE, the results consistently show that Ca^{2+} activates the PDE that lowers intracellular cyclic nucleotide levels and enhances ecdysteroidogenesis. Adding a nonselective PDE

inhibitor (IBMX) to incubations of Y-organs suppressed ecdysteroid secretion (Mattson and Spaziani 1985b, 1986b), while adding a Ca^{2+} ionophore (A23187) stimulated ecdysteroid secretion, an effect that was blocked by IBMX (Mattson and Spaziani, 1986b). Moreover, in cell-free Y-organ preparations, PDE activity was dose-dependently increased by Ca^{2+} (10^{-7}-10^{-4} M) and suppressed by a CaM inhibitor (Mattson and Spaziani, 1986b). Combined data from different lines strongly favor the working hypothesis that Ca^{2+}/CaM-dependent PDE (PDE1) determines the responsiveness of Y-organ to MIH stimulus. Ca^{2+} may be involved in the regulation of responsiveness of Y-organs to MIH through activation of glandular PDE. To summarize, available data suggest MIH may suppress ecdysteroidogenesis, in part, by promoting Ca^{2+} efflux from Y-organs, and that an increase in intracellular Ca^{2+} leads to enhanced ecdysteroidogenesis through activation of PKC, or PDE, or both.

Although Ca^{2+} seems play a critical role in ecdysteroidogenesis, the intracellular Ca^{2+} level in Y-organ cells has not been measured yet. Needed are experiments designed to determine the level of Ca^{2+} in Y-organs during a molting cycle and in response to MIH.

7. Mechanisms regulating cellular Ca^{2+} homeostasis in crustaceans

Ca^{2+} is a ubiquitous molecule involved in regulation of various physiological processes including muscle contraction, embryonic pattern formation, programmed

cell death, fertilization and steroidogenesis (Berridge, et al., 2000; Cooke, 1999; Slusarski and Pelegri, 2007; Webb, 2003; Whitaker, 2006). In order to preserve its function as a signaling messenger, the cytolic Ca^{2+} concentration is maintained at a low basal level by the action of different Ca^{2+} channels, pumps, exchangers, and binding proteins (Berridge, et al., 2000; Carafoli, 1987; Krebs et al., 2003). In crustaceans, Ca^{2+} not only functions as a second messenger, but is essential for hardening (mineralizing) the exoskeleton. During the crustacean molting cycle, exoskeleton goes through the cycles of demineralization (premolt) and remineralization (postmolt). During premolt stage, the old skeleton is partially dissolved with Ca^{2+} being absorbed and transferred to temporary sites of storages. After molting, Ca^{2+} from endogenous and exogenous source is deposited into the newly synthesized flexible exoskeleton to make it rigid again. Animals living in different habitats utalize diverse mechanisms to reserve and uptake Ca^{2+} depending on the environmental Ca^{2+} availability (Greenaway, 1985; Luquet, 2012). Nevertheless, all face the challenge of substantial movement of Ca^{2+} during premolt and postmolt stage regardless of the habitat in which they live. It follows that there must be sophisticated regulatory mechanisms to sustain intracellular Ca^{2+} homeostasis while at the same time carrying out transcellular Ca^{2+} transport. Studies of Ca^{2+} homeostasis and transport in crustaceans have been done mainly using Ca^{2+}-transporting epithelia

such as gill, antennal gland (functionally equivalent to kidney), and hepatopancreas.

In crustaceans, most Ca^{2+}-transporting epithelia display "bidirectional" transportation at different molting stages: during premolt, Ca^{2+} absorbed from the old exoskeleton or taken up from exogenous sources is moved from apical to basolateral membrane (from water/lumen to hemolymph); later, during postmolt, when cuticle is undergoing mineralization, Ca^{2+} is transported from basolateral to apical membrane (from hemolymph to water/lumen) (Wheatly, 1997). *In vitro* experiments using intermolt gill, gut, antennal gland and hepatopancreas from different species have been conducted to identify and locate Ca^{2+} transport proteins expressed on epithelial cell membrane (Ahearn and Franco, 1993; Flik et al., 1994; Flick and Haond, 2000; Zhuang and Ahearn, 1996, 1998; Wheatly et al., 1999, 2002). Experiments done with lobster (*Homarus americanus*) antennal gland and hepatopancreas indicated there existed three independent processes on the apical membrane including 1) simple diffusion via verapamil-sensitive Ca^{2+} channel, 2) amiloride sensitive $2Ca^{2+}/ H^{+}$ (Na^{+}) antipoter, and 3) amiloride insensitive $Ca^{2+}/ 2Na^{+}$ antiporter (Ahearn and Franco, 1993; Zhuang and Ahearn, 1996). Regarding basolateral transportation, studies using gills from crab *Carcinus menaeus* indicated the independent existence of a Ca^{2+}-ATPase and Na^{+} gradient-dependent Ca^{2+} uptake (Flik et al., 1994). Similar findings were recognized in *H. americanus* hepatopancreas (Zhuang and Ahearn,

1998). Results from the freshwater crayfish (*P. clarkii*) were consistent with those found in *C. menaeus* and *H. americanus*. Exepriments employing basolateral membrane vesicle indicated ATP-dependent Ca^{2+} uptake in intermolt gill, antennal gland and hepatopancrea (Wheatly et al., 1999). EGTA suppressed Ca^{2+} uptake by membrane vesicles generated from antennal gland and hepatopancreas vesicles was restored by CaM (Wheatly et al., 1999). Addition of sodium increased Ca^{2+} uptake, which was abolished by pretreatment of ouabain, a Na^{+}/ K^{+} ATPase-specific inhibitor (Wheatly et al., 1999). To summarize, data described above indicate that Ca^{2+}-ATPase, Na^{+}/ Ca^{2+} exchanger (NCX) and Na^{+}/ K^{+} ATPase are involved in moving Ca^{2+} across the basolateral membrane. A working hypothesis was proposed based on these studies to explain vectorial Ca^{2+} transport (from apical-to-basolateral) (Wheatly, 1999; Wheatly et al., 2002). However, this model might not be able to deal with basolateral-to-apical Ca^{2+} transportation due to the lack of Ca^{2+}-ATPase related research in premolt and postmolt stage. Besides, tissues other than Ca^{2+} transporting epithelia, such as Y-organ, might develop strategies different from this model to overcome the calcium influx resulting from the elevated hemolymphatic Ca^{2+} level in premolt and potsmolt stage.

In last decade, research done in *P. clarkii* explored molting-stage specific changes in a group of proteins which were relevant to Ca^{2+} homeostasis. Genes

including epithelia Ca^{2+} channel (ECaC), PMCA, sarco-/endoplasmic reticulum (SERCA), NCX, sarcoplasmic calcium binding protein (SCP), and CaM were cloned (Gao and Wheatly, 2004, 2007; Zhang Z et al., 2000; Gao et al., 2006, 2009; Wheatly et al., 2002; Wheatly et al., 2010). Research showed that all Ca^{2+} transport proteins (ECaC, PMCA, NCX and SERCA) exhibited significantly enhanced expression in mRNA or protein, or both (Wheatly et al., 2010). Ca^{2+} signaling involved in ecdysteroidogenesis has been discussed previously. There exits the possibility that the absorption of Ca^{2+} from old skeleton would result in increased Ca^{2+} level in Y-organ cells that stimulates the ecdysteroid production. Study of regulatory proteins which control Ca^{2+} level in Y-organ cells might provide the knowledgement of ecdysteroidogenesis from different aspects.

8. Summary

Much progress has been made in understanding the endocrine regulation of crustacean growth and molting, but important questions remain unanswered. Ca^{2+} signaling appears to play a key role in regulation of ecdysteroidogenesis, but the level of cytosolic Ca^{2+} in Y-organ cells has never been determined. The main aim of studies reported in this dissertation is to elucidate the role of Ca^{2+} in ecdysteroidogenesis by measuring cytosolic Ca^{2+} levels in Y-organs cells from eyestalk-ablated donor animals and animals selected from different molting stages. Proteins which control the

intracellular Ca^{2+} level in Y-organs might also contribute to the production of ecdysteroids indirectly. As a parallel study, we plan to clone a plasma Ca^{2+} ATPase in Y-organ and measure its transcript abundance by means of real-time PCR during a molting cycle. We anticipate the data from real-time PCR analysis will provide insights into the possible involvement of PMCA in Ca^{2+}-mediated regulation of ecdysteroidogenesis.

CHANGES IN INTRACELLULAR CALCIUM CONCENTRATION IN CRUSTACEAN (*CALLINECTES SAPIDUS*) Y-ORGANS: RELATION TO THE HEMOLYMPHATIC ECDYSTEROID TITER

by

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ABSTRACT

Secretion of ecdysteroid molting hormones by crustacean Y-organs is negatively regulated (inhibited) by molt-inhibiting hormone (MIH), a neuropeptide produced by neurosecretory cells in eyestalk ganglia. The inhibitory effect of MIH is mediated by one or more cyclic nucleotide second messengers. In addition, available data indicate that ecdysteroidogenesis is positively regulated (stimulated) by intracellular calcium. However, despite the apparent critical role of calcium in regulating ecdysteroidogenesis, the level of Ca^{++} in Y-organs cells has not been previously determined. In studies reported here, eyestalks were ablated from blue crabs (*Callinectes sapidus*) to remove the endogenous source of MIH and activate Y-organs. At 0, 3, 6, and 9 days after eyestalk ablation (D0, D3, D6, and D9, respectively), the level of Ca^{++} in Y-organ cells was determined using a fluorescent calcium indicator (Fluo-4), and the hemolymphatic ecdysteroid titer was determined by radioimmunoassay. Calcium fluorescence in D6 Y-organs was 3.5-fold higher than that in D0 controls; calcium fluorescence in D9 Y-organs was 3.9-fold higher than in D0 controls ($P<0.05$). Measurement of fluorescence along a transect drawn through representative cells indicated the calcium fluorescence was localized to cytoplasm and not to nuclei. Associated with the increase in intracellular Ca^{++} was a significant increase in the hemolymphatic ecdysteroid titer: The level of ecdysteroids in hemolymph rose from 5.5 ng/ml on D0 to 49.6 ng/ml on D6 and 87.2 ng/ml on D9

($P<0.05$). The results are consistent with the hypothesis that ecdysteroidogenesis is stimulated by an increase in intracellular Ca^{++}. Our previous studies suggest Ca^{++} promotes ecdysteroidogenesis in Y-organs by activating a Ca^{++}/calmodulin-dependent cyclic nucleotide phosphodiesterase (PDE1).

In crustaceans, incremental growth occurs when the old exoskeleton is shed (molted) and the flexile new exoskeleton, previously formed beneath the old one, is expanded by absorption of water and then hardened by mineralization (Skinner, '85). The cellular events that lead to molting are stimulated by C-27 steroid hormones, ecdysteroids, secreted by paired endocrine glands, the Y-organs (Lachaise et al., '93; Riddiford et al., 2000). Secretion of ecdysteroids is controlled, at least in part, 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., '93; Nakatsuji et al., 2009). Thus, ablation of the eyestalks leads to enhanced ecdysteroid secretion by Y-organs, an increase in the ecdysteroid titer, and precocious molting (Keller and Schmid, '79; Chang and Bruce, '80; Hopkins, '83), while injection of eyestalk extract or synthetic MIH into eyestalk-ablated animals lowers the ecdysteroid titer and delays molting (Bruce and Chang, '84; Chang et al., '87; Nakatsuji and Sonobe, 2004). Based on these and related findings, a long-standing model for molt control in crustaceans suggests 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, '85). While recent studies indicate 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 MIH acts directly on Y-organs to suppress synthesis of ecdysteroids (Soumoff and O'Connor, '82; Watson and Spaziani, '85; Mattson and Spaziani, '85a), and that MIH action is mediated through cellular signaling pathways involving cAMP, cGMP, or both (see Nakatsuji et al., 2009; Covi et al., 2009). For the blue crab (*Callinectes sapidus*), the experimental animal used in studies reported here, existing data strongly favor cGMP as the cyclic nucleotide second messenger directly linked to MIH action (Nakatsuji et al., 2006a).

Cell signaling molecules other than cyclic nucleotides have also been implicated, directly or indirectly, in MIH action (see Spaziani et al., 2001). Among these, calcium appears to play a critical role. Mattson and Spaziani ('86) 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 authors hypothesized that MIH may suppress ecdysteroidgenesis, at least in part, by promoting Ca^{++} efflux.

In related 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, '86).

These and other findings led to the hypothesis that an increase in intracellular calcium may promote the surge in ecdysteroidogenesis that leads to molting (Mattson and Spaziani, '86). The cause of the hypothesized increase in intracellular calcium is unknown. One possibility is that it is elicited by a specific ligand; the existence of a positive regulator of ecdysteroidogenesis in crustaceans has been long hypothesized (see Skinner, '85). Another possibility is that the hypothesized increase in intracellular calcium is a consequence of the general increase in hemolymphatic Ca^{++} that occurs during premolt as Ca^{++} is mobilized from the old exoskeleton (see Greenaway, '85).

Despite the apparent critical role of calcium in regulating ecdysteroidogenesis, the level of Ca^{++} in Y-organs cells has not been previously determined. In studies described here, we report that activation of Y-organs (by eyestalk ablation) produces an increase in intracellular Ca^{++} in Y-organs, and that the increase in intracellular Ca^{++} is associated with an increase in the hemolymphatic ecdysteroid titer.

MATERIALS AND METHODS

Experimental Animals

Blue crabs (*Callinectes sapidus*) were purchased from a local market and maintained in compartmented tanks containing artificial seawater as previously described (Nakatsuji et al., 2006a).

Preparation of Y-Organ Cells

Eyestalks were surgically ablated from crabs to remove the endogenous source of MIH and activate Y-organs. At 0, 3, 6, and 9 days after eyestalk ablation, Y-organs were dissected from crabs, rinsed with Pantin's saline (Pantin, 1934), cut into pieces using a razor blade, 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℃ for 1 h. The medium containing dissociated tissue was then transferred to a centrifuge tube and filtered through 200 micron nylon mesh by brief centrifugation (100g). 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 μM), and Pluronic F-127 (20%) (the latter two from Molecular Probes). After incubation, Y-organ cells were collected by brief centrifugation (100g), washed twice, and re-suspended in 50 μl Pantin's saline containing EDTA (1 mM). An aliquot of the cell suspension was used for determination of intracellular fluorescence.

Measurement of Intracellular Fluorescence

The fluorescent signal from Y-organ cells was visualized in the High Resolution Imaging Facility of the University of Alabama at Birmingham using a Nikon Eclipse optical 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). In addition, the software was used to determine the intensity of fluorescence along a transect drawn through representative cells of each stage.

Determination of the Hemolymphatic Ecdysteroid Titer

The hemolymphatic ecdysteroid titer was determined by radioimmunoassay as previously described (Lee et al., '98).

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 10.0 software (SAS Institute Inc.).

RESULTS AND DISCUSSION

In studies reported here, eyestalks were ablated from blue crabs (*C. sapidus*) to activate Y-organs. At 0, 3, 6, and 9 days after eyestalk ablation (D0, D3, D6, and D9, respectively), the level of Ca^{++} in Y-organ cells was determined using a fluorescent calcium indicator (Fluo-4), and the hemolymphatic ecdysteroid titer was determined by radioimmunoassay. As shown in Figure 1a, total intracellular calcium fluorescence increased with time after eyestalk ablation: Mean calcium fluorescence in D6

Y-organs was 3.5-fold higher than that in D0 controls; mean calcium fluorescence in D9 Y-organs was 3.9-fold higher than in D0 controls ($P<0.05$). Measurement of fluorescence along a transect drawn through representative cells indicated the intensity of calcium fluorescence was generally greater in cytoplasm than in nuclei (Fig. 1b). In addition, there appeared to be foci of calcium fluorescence localized to regions within the cytoplasm (Fig. 1b). Associated with the increase in intracellular Ca^{++} was a significant increase in the hemolymphatic ecdysteroid titer: The level of ecdysteroids in hemolymph rose from 5.5 ng/ml on D0 to 49.6 ng/ml on D6 and 87.2 ng/ml on D9 ($P<0.05$) (Fig. 2). The results are consistent with the hypothesis that ecdysteroidogenesis is stimulated by an increase in intracellular Ca^{++}.

Also consistent with that hypothesis, Mattson and Spaziani ('86) 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 (*Cancer antennarius*) Y-organs *in vitro*, or enhanced the suppressive effect of MIH, or both (see also Spaziani et al. '99, 2001). Similarly, Sedlmeier and Seinsche ('98) found that a Ca^{++} ATPase inhibitor (thapsigargin) or calcium channel blockers (pimozide, nimodipine, or flunarizide) individually suppressed basal ecdysteroid production by crayfish (*Orconectes limosus*) Y-organs *in*

vitro. In addition, incubation of Y-organs from either species with the calcium ionophore A23187 (which increases intracellular Ca^{++}) stimulated ecdysteroid production in a dose-dependent manner (Mattson and Spaziani, '86; Sedlmeier and Seinsche, '98).

Existing data suggest elevated intracellular Ca^{++} may be linked to enhanced ecdysteroidogenesis, at least in part, through activation of cyclic nucleotide phosphodiesterase (PDE). PDE enzymes catalyze degradation (inactivation) of cyclic nucleotide second messengers (Conti and Beavo, 2007), an effect that enhances ecdysteroidogenesis. Thus, Mattson and Spaziani ('86) reported that adding a nonselective PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX), to incubations of crab Y-organs suppressed basal ecdysteroid secretion (Mattson and Spaziani '85b, '86), and blocked the stimulatory effect of A23187 on ecdysteroidogenesis (Mattson and Spaziani, '86). In addition, in cell-free Y-organ preparations, PDE activity was dose-dependently increased by calcium (10^{-7}-10^{-4} M) and suppressed by a calmodulin inhibitor (Mattson and Spaziani, '86). We have recently reported stage-specific changes in PDE activity in Y-organs during a molt cycle of the crayfish, *Procambarus clarkii*: PDE activity was highest during middle premolt (Nakatsuji et al., 2006b), a time when the hemolypmphatic ecdysteroid titer is likewise elevated (Nakatsuji et al., 2000). Further, experiments with selective PDE inhibitors suggested the PDE

isotype present in Y-organs of *P. clarkii* is PDE1 (Nakatsuji et al., 2006b). PDE1 enzymes are Ca^{++}/calmodulin-dependent, and catalyze the hydrolysis of cAMP and cGMP. The combined results are consistent with the hypothesis that calcium promotes ecdysteroidogenesis, at least in part, through activation of glandular PDE.

The finding that an increase in intracellular Ca^{++} in Y-organs is associated with an increase in the hemolymphatic ecdysteroid titer is a key step forward in our understanding of the regulation of ecdysteroidogenesis. However, it should be noted that eyestalk ablation removes the source of hormones other than MIH, and that stages of the experimentally accelerated (induced) molting cycle may not mimic stages of natural molting cycle in all respects (cf. Spaziani et al., '82). Needed are experiments designed to determine the level of Ca^{++} in Y-organ cells during a natural molting cycle.

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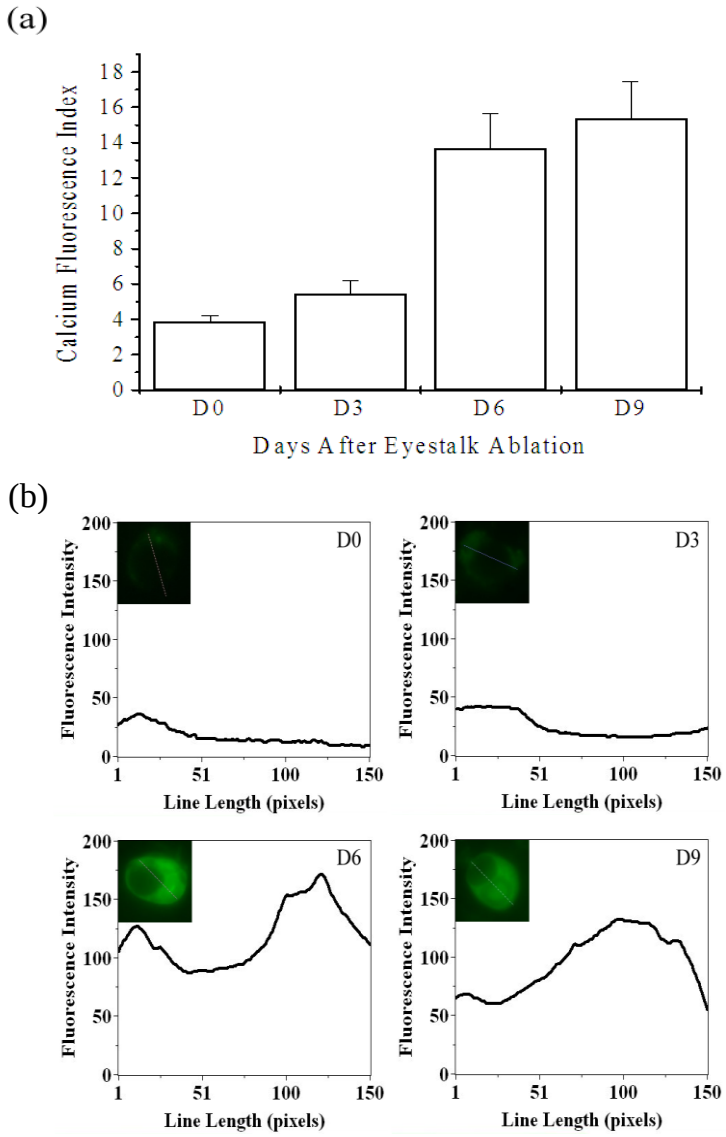

Fig. 1. Calcium-specific fluorescence in Y-organ cells after eyestalk ablation. At 0, 3, 6, and 9 days after eyestalk ablation (D0, D3, D6 and D9, respectively), dispersed Y-organ cells were incubated for 30 min in physiological saline containing Fluo-4 AM (10 μM), and then viewed with a Nikon Eclipse optical microscope fitted with a high energy argon laser at 488 nm. **a)** Total intracellular calcium fluorescence in Y-organ cells was determined using MetaMorph 7.5 software. Bars represent mean calcium fluorescence + SE (N = 25-27 cells). The statistical significance of differences among means was determined by one-way analysis of variance followed by Tukey's HSD test. Tukey's test showed the following pairwise comparisons to be significant at the 0.05 level: D0 – D6, D0 – D9, D3 – D6, D3 – D9. **b)** For representative cells from each stage after eyestalk ablation, calcium fluorescence was measured along a transect (indicated by a line in each inset) drawn through the cells. Calcium fluorescence along the transect was determined using MetaMorph 7.5 software.

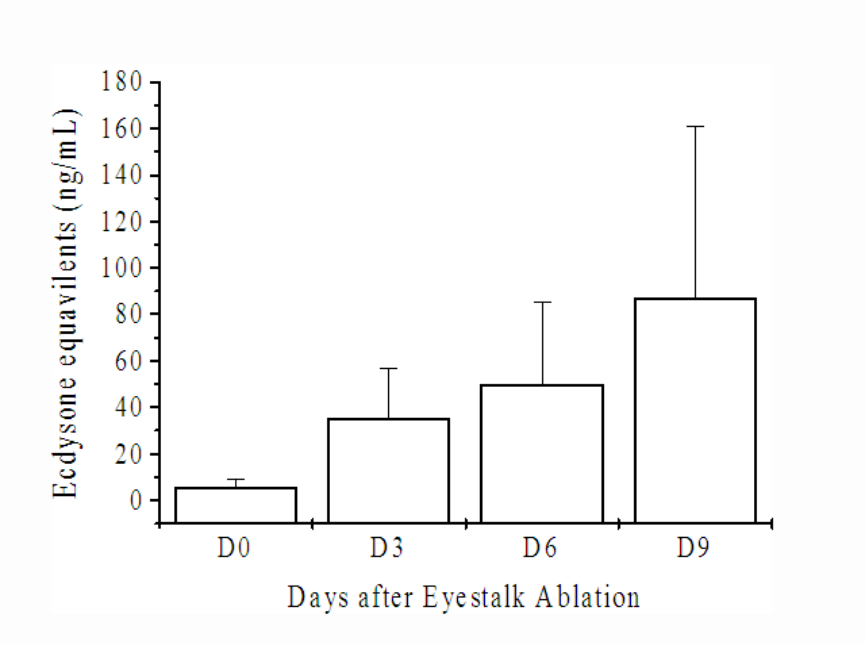

Fig. 2. Hemolymphatic ecdysteroid titer on days after eyestalk ablation. Hemolymph samples were drawn from crabs 0, 3, 6, and 9 days after eyestalk ablation (D0, D3, D6 and D9, respectively) and ecdysteroid content determined by radioimmunoassay. Bars represent mean + SE (N = 9-12). The statistical significance of differences among means was determined by one-way analysis of variance followed by Tukey's HSD test. Tukey's test showed the following pairwise comparisons to be significant at the 0.05 level: D0 – D6, D0 – D9, D3 – D9.

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

by

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Comparative Biochemistry and Physiology

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 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^{++}.

Key words: Ca^{++} signaling, Y-organ, ecdysteroids, molting, crustacean

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 (Clapham, 2007; Kahl and Means, 2003; Slusarski and Pelegri, 2007; Whitaker, 2006). 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 (Chang and Bruce, 1980; Hopkins, 1983; Keller and Schmid, 1979), 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 the model is incomplete (Chung and Webster, 2005; Nakatsuji and Sonobe, 2004), it remains useful as a base from which testable hypotheses can be formed.

Existing data indicate MIH acts directly on Y-organs to suppress synthesis of ecdysteroids (Mattson and Spaziani, 1985a; Soumoff and O'Connor, 1982; Watson and Spaziani, 1985a) and uptake of lipoprotein-bound cholesterol, the biosynthetic precursor of ecdysteroids (Kang and Spaziani, 1995a,b; Watson and Spaziani, 1985a,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 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 (*Callinectes sapidus*) were purchased from local fishermen in Wilmington, NC, 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℃ for 1 h. The medium containing dissociated tissue was then transferred to a centrifuge tube and filtered through 200 micron nylon mesh by brief centrifugation (100g). 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 µM), and Pluronic F-127 (20%) (Molecular Probes). After incubation, Y-organ cells were collected by brief centrifugation (100g), 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) (Chen and Watson, 2011; Pradhan et al., 2008; Xu et al., 2008). 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 Figure 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 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 Figure 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, 1995). 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 (*Cancer 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 vitro*. In addition, incubation of Y-organs from either species with the calcium ionophore A23187 (which increases intracellular Ca^{++}) stimulated ecdysteroid production in a dose-dependent manner (Mattson and Spaziani, 1986; Sedlmeier and Seinsche, 1998).

The current results indicate the Ca^{++} signal in Y-organ cells is global, rather than being spatially restricted or localized to a specific region within the cell. Global increases in intracellular Ca^{++} have been previously reported for other cell types. As examples, Nigam et al. (1992) reported that a global increase in intracellular Ca^{++} promoted formation of cell-cell junctions and establishment of cell polarity during the development of epithelial tissues; and Zemel et al. (2000) found that adding 1,25-dihydroxy-vitamin D_3 to adipocyte incubations elicited a global increase in intracellular Ca^{++} that led to suppression of lipolysis. We are unaware of previous reports linking global increases in intracellular Ca^{++} to stimulation of steroidogenesis. It is worth noting that the methods used in the current studies (imaging of Fluo-4

calcium fluorescence by laser confocal microscopy) provided only a snapshot of Ca^{++} levels in Y-organ cells at specific time points within the molting cycle. Because calcium signaling can be temporally and spatially complex, often involving oscillations and waves that may be highly localized in nature, future experiments designed to permit monitoring of calcium fluorescence over extended time intervals are planned.

The cellular mechanism by which Ca^{++} stimulates ecdysteroidogenesis is an area of active investigation. Existing data indicate MIH suppresses ecdysteroidogenesis through a cyclic nucleotide (cAMP, cGMP, or both) second messenger (Covi et al., 2009; Nakatsuji et al., 2009). Degradation (inactivation) of cyclic nucleotide second messengers is catalyzed by cyclic nucleotide phosphodiesterase (PDE) enzymes (Conti and Beavo, 2007). Several lines of evidence indicate elevated intracellular Ca^{++} may be linked to enhanced ecdysteroidogenesis through activation of PDE. Thus, Mattson and Spaziani (1986) reported that adding a nonselective PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX), to incubations of crab Y-organs suppressed basal ecdysteroid secretion (Mattson and Spaziani, 1985b, 1986), and blocked the stimulatory effect of A23187 on ecdysteroidogenesis (Mattson and Spaziani, 1986). In addition, in cell-free Y-organ preparations, PDE activity was dose-dependently increased by calcium

(10^{-7}-10^{-4} M) and suppressed by a calmodulin inhibitor (Mattson and Spaziani, 1986). We have recently reported the occurrence of stage-specific changes in PDE activity in Y-organs during a molt cycle of the crayfish, *Procambarus clarkii*: PDE activity was highest during middle premolt (Nakatsuji et al., 2006), a time when the hemolymphatic ecdysteroid titer is likewise elevated (Nakatsuji et al., 2000). Further, experiments with selective PDE inhibitors suggested the PDE isotype present in Y-organs of *P. clarkii* is PDE1 (Nakatsuji et al., 2006). PDE1 enzymes are Ca^{++}/calmodulin-dependent, and catalyze the hydrolysis of both cAMP and cGMP. The combined results are consistent with the hypothesis that calcium promotes ecdysteroidogenesis, at least in part, through activation of glandular PDE.

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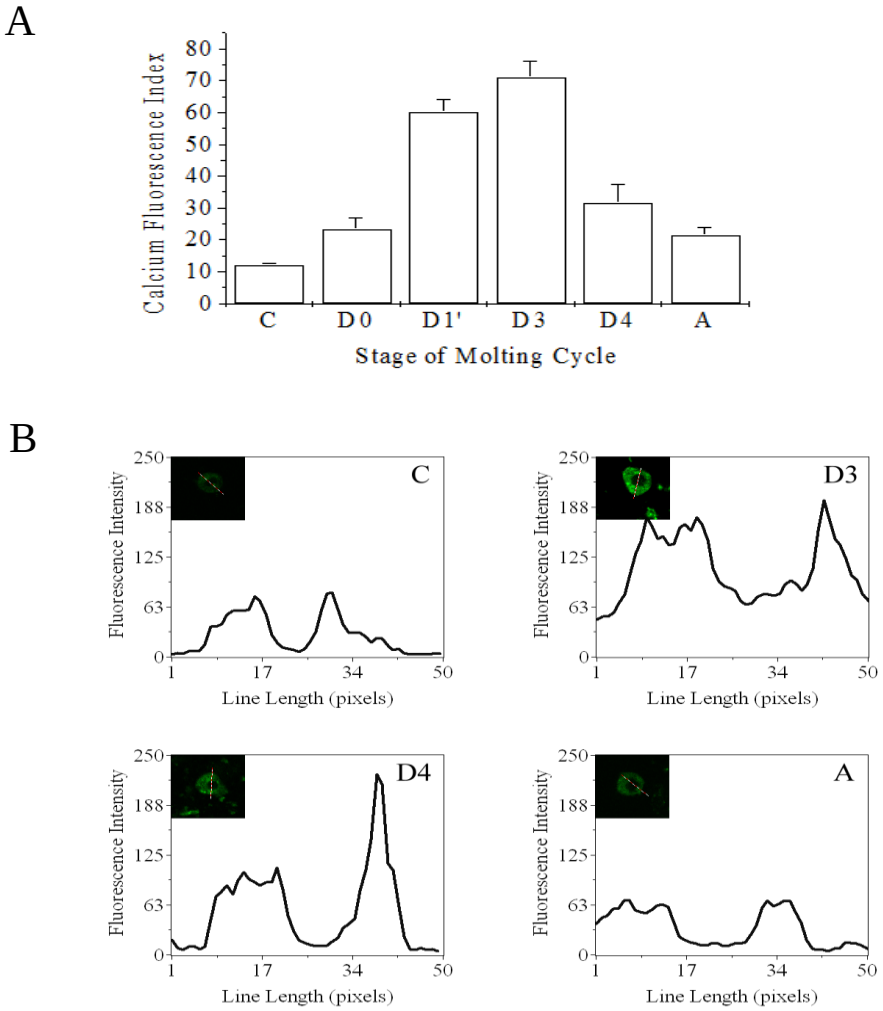

Figure 1. Calcium-specific fluorescence in Y-organ cells during a molting cycle of *C. sapidus*. Y-Organs were dissected from crabs in the following stages of the molting cycle: intermolt (C4), premolt stages D0, D1', D3 and D4, and postmolt (A). Dissociated Y-organ cells were incubated for 30 min in physiological saline containing Fluo-4 AM (10 μM), and then viewed with an Olympus confocal microscope fitted with a high energy argon laser at 488 nm. **(A)** Total intracellular calcium fluorescence in Y-organ cells was determined using MetaMorph 7.5 software. Bars represent mean calcium fluorescence + SE (N = 16 cells per stage). The statistical significance of differences among means was determined by one-way analysis of variance followed by Tukey's HSD test. Tukey's test showed the following pairwise comparisons to be significant at the 0.05 level: C4-D1', C4-D3, C4-D4, D0-D1', D0-D3, D1'-D4, D1'-A, D3-D4, D3-A. **(B)** For representative cells from various stages of the molting cycle, calcium fluorescence was measured along a transect (indicated by a line in each inset) drawn through the cells. Calcium fluorescence along the transect was determined using MetaMorph 7.5 software.

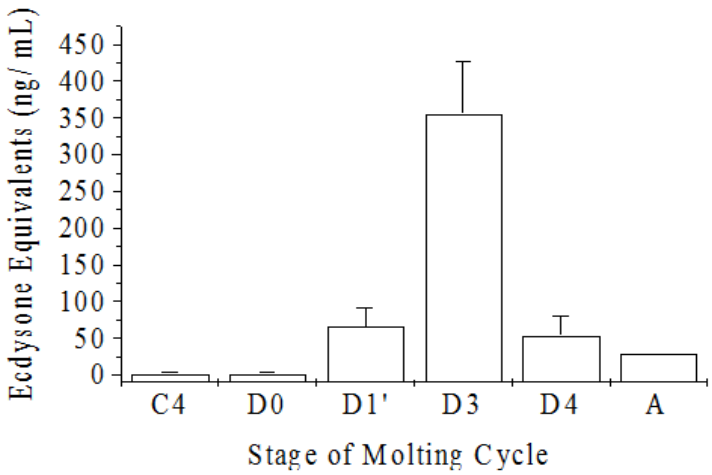

Figure 2. Hemolymphatic ecdysteroid titer during a natural molting cycle of *C. sapidus*. Hemolymph was drawn from crabs during the following stages of the molting cycle: intermolt (C4), premolt stages D0, D1', D3 and D4, and postmolt (A). Ecdysteroid content was determined by radioimmunoassay. Bars represent mean + SE (N = 1-5). The statistical significance of differences among premolt means was determined by one-way analysis of variance followed by Tukey's HSD test; postmolt results were not included in the statistical analyses due to insufficient sample size in that group. Tukey's test showed the following pairwise comparisons to be significant at the 0.05 level: C4-D3, D0-D3, D1'-D3, D3-D4.

MOLECULAR CLONING OF A PLASMA MEMBRANE CALCIUM ATPASE (PMCA) FROM Y-ORGANS OF THE BLUE CRAB (CALLINECTES SAPIDUS), AND DETERMINATION OF SPATIAL AND TEMPORAL PATTERNS OF PMCA GENE EXPRESSION

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SUMMARY

Production of crustacean molting hormone, ecdysteroids, is negatively controlled (inhibited) by molt-inhibiting hormone, a polypeptide neurohormone produced by neurosecretory cells in eyestalk ganglia. Recent studies show that calcium signaling is involved in positive regulation in ecdysteroidogenesis. Increased cytosolic calcium level was observed in experimentally activated and premolt Y-organ cells, and that increase in cytosolic calcium level was associated with elevated hemolymphatic ecdysteroid titer. To investigate the role of calcium signaling in ecdysteroidogenesis in crustacean, a putative plasma membrane Ca^{2+}-ATPase gene, *Cas-PMCA*, was identified in blue crab (*Callinectes sapidus*) Y-organs by means of PCR-based cloning strategy. The complementary DNA sequence of *Cas-PMCA* was 4292-bp in length, containing a 3510-bp open reading frame which conceptually encoded 1170 amino acids with an estimated molecular weight 128.8. The deduced amino acid sequence of *Cas-PMCA* represented all signature domains of an authentic PMCA including phosphorylation, ATP binding, and calmodulin (CaM) binding sites. An analysis of membrane topography predicted Cas-PMCA to have ten transmembrane domains and two large intracellular loops, a pattern consistent with the structure of known PMCA proteins. The transcript of *Cas-PMCA* was extensively detected in both neural and non-neural tissues. Relative transcript abundance of *Cas-PMCA* in Y-organs

significantly increased in premolt (D1 and D2) stage then declined after molting, which exhibited a similar pattern with molting stage-specific change of intracellular Ca^{2+} level. Results suggested the upregulation of *Cas-PMCA* in Y-organs was in response to elevated cytosolic Ca^{2+} level but not related to stimulation of ecdysteroid production during premolt stages.

Key words: calcium signaling, plasma membrane Ca2+ ATPase, PMCA, ecdysteroidogenesis, crustacean molting

INTRODUCTION

Ca^{2+} is a ubiquitous second messenger involved in the regulation of multiple developmental and physiological processes, including fertilization, embryonic pattern formation, programmed cell death, muscle contraction, and neurotransmitter release (Berridge, et al., 2000; Clapham, 2007; Kahl and Means, 2003; Slusarski and Pelegri, 2007; Whitaker, 2006). Recent results are consistent with the hypothesis that calcium signaling is also involved in the regulation of steroid hormone synthesis by crustacean molting glands (Y-organs) (Chen and Watson, 2011; Chen et al., 2012).

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 (Chang and Bruce, 1980; Hopkins, 1983; Keller and Schmid, 1979), 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 the model is incomplete (Chung and Webster, 2003; Nakatsuji and Sonobe, 2004), it remains useful as a base from which testable hypotheses can be formed.

Existing data indicate MIH acts directly on Y-organs to suppress synthesis of ecdysteroids (Mattson and Spaziani, 1985; Soumoff and O'Connor, 1982; Watson and Spaziani, 1985a) and uptake of lipoprotein-bound cholesterol, the biosynthetic precursor of ecdysteroids (Kang and Spaziani, 1995a,b; Watson and Spaziani, 1985a,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 $^{45}Ca^{2+}$, and found that addition of eyestalk extract (containing MIH activity) elicited a statistically significant efflux of $^{45}Ca^{2+}$ into incubation medium. The authors hypothesized that

MIH may suppress ecdysteroidgenesis, at least in part, by promoting Ca^{2+} efflux. In companion experiments, treatment of crab (*Cancer antennarius*) Y-organs with the calcium ionophore A23187 (which increases intracellular Ca^{2+}) 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). In recent studies, we used a fluorescent calcium indicator (Fluo-4) and fluorescence imaging methods to measure free Ca^{2+} in Y-organ cells after experimental activation. We observed that activation of Y-organs (by eyestalk ablation) produced an increase in free Ca^{2+} in Y-organ cells, and that the increase in intracellular free Ca^{2+} was associated with an increase in the hemolymphatic ecdysteroid titer (Chen and Watson, 2011). In follow-up studies, we measured free Ca^{2+} levels in Y-organs during a natural 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. The level of ecdysteroids in hemolymph of Y-organ donor crabs showed an overall pattern similar to that observed for calcium fluorescence (Chen et al., 2012). The combined results strongly support the hypothesis that ecdysteroidogenesis is stimulated by an increase in intracellular

Ca^{2+}.

In eukaryotic cells, the concentration of intracellular free Ca^{2+} is typically ~100 nm, roughly four orders of magnitude less that the concentration of extracellular Ca^{2+} (Kretsinger, 1976). Increases in intracellular Ca^{2+} above this low basal level drive multiple cellular processes. To preserve the functional integrity of the Ca^{2+} signal, and to prevent cellular toxicity, the intracellular Ca^{2+} concentration is tightly regulated. The Ca^{2+} concentration in cytosol is controlled mainly by proteins intrinsic to the plasma membrane and to the membranes of organelles (Berridge et al., 2000; Philipson and Nicoll, 2000; Guerini et al., 2005; Clapham, 2007; Brini and Carafoli, 2010). Several families of proteins are involved, including Ca^{2+} channels, Ca^{2+} pumps (ATPases), and Ca^{2+} exchangers. The influx of Ca^{2+} to cytosol occurs largely via Ca^{2+} channels present in the plasma membrane and in the membranes of organelles. Those present in the plasma membrane may be gated by voltage, by ligands, or by the emptying of intracellular stores. Calcium channels in the membranes of sarco(endo)plasmic reticulum (SR/ER) are gated by second messengers: inositol 1,4,5 triphosphate (IP_3) activates IP_3 receptors; cyclic ADP ribose activates ryanodine receptors. The export of Ca^{2+} from cytosol is accomplished by Ca^{2+} pumps and Ca^{2+} exchangers. Calcium pumps use the energy of ATP to transport Ca^{2+} against its electrochemical gradient. The family of Ca^{2+} pumps includes a plasma membrane

Ca^{2+} ATPase (PMCA), and an SR/ER Ca^{2+} ATPase (SERCA). The former transports Ca^{2+} out of the cell, while the latter transports Ca^{2+} into the SR/ER. In mammals, there are four isoforms of PMCA (PMCA1-4) (Brini and Carafoli, 2010; Strehler and Zacharias, 2001). Each isoform is coded by a different gene, and alternative splicing of transcripts yields different subtypes of each isoform. Similarly, in mammals there are multiple isoforms of SERCA (SERCA1-3) (Strehler and Treiman, 2004), each coded by a different gene, with subtype diversity resulting from alternative splicing of transcripts. Also involved in the control of intracellular Ca^{2+} are exchange proteins. Na^{+}/Ca^{2+} exchangers (NCX) are bidirectional transporters that mediate the exchange of Ca^{2+} for Na^{+} using the energy of the Na^{+} gradient generated by Na^{+}/K^{+} ATPase. In mammals, NCX genes encode three homologous proteins, NCX1, NCX2, and NCX3, with alternative splice variants producing subtypes of each isoform (Philipson and Nicoll, 2000). PMCA proteins have been characterized as high affinity/low capacity Ca^{2+} transporters, while NCX proteins have low Ca^{2+} affinity but high capacity for Ca^{2+} transport (Brini and Carafoli, 2010). As a result of these properties, a housekeeping role in maintaining cytosolic Ca^{2+} has traditionally been ascribed to PMCA, while NCX have been considered to have a more dynamic role in counteracting large increases in cytosolic Ca^{2+}. However, the identification of multiple PMCA isoforms and splice variants suggests that PMCA may influence both

basal and dynamic fluctuations in cytocolic Ca^{2+} (Brini and Carafoli, 2010).

As a step toward understanding Ca^{2+}-mediated regulation of ecdysteroidogenesis, we have begun investigating Ca^{2+} transport proteins in Y-organs. We report here the molecular cloning of a PMCA from Y-organs of the blue crab (*Callinectes sapidus*), and describe PMCA transcript abundance in Y-organs during a molting cycle.

MATERIALS AND METHODS

Experimental animals

Blue crabs (*Callinectes sapidus*) were purchased from local vendors in Birmingham, AL or Wilmington, NC, maintained in compartmented tanks containing artificial seawater as previously described (Nakatsuji et al., 2006), and staged according to established criteria (Drach and Tchernigovtzeff, 1967; Stevenson, 1972).

Isolation of RNA

Tissues were dissected from crabs, transferred to RNAlater (Life Technologies/Ambion, Grand Island, NY, USA), and stored at -20°C. Total RNA was extracted using reagents and a protocol provided in an RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). Briefly, tissue (10-15 mg) was disrupted using a glass tissue grinder (Kimball Chase, Vineland, NJ, USA) in Buffer RLT and the suspension filtered through a QIAshredder spin column (QIAGEN, Valencia CA, USA). The filtered tissue lysates were then centrifuged (16,000g, 3 min) and the supernatant

combined with an equal volume of 70% ethanol. The mixture was transferred to an RNeasy spin column. The column was rinsed (once with Buffer RW1, twice with Buffer RPE), and then eluted with elution buffer. The concentration and purity of RNA in the eluate was determined by u.v. absorption spectrophotometry at 260 and 280 nm. The messenger RNA (mRNA) used for rapid amplification of cDNA ends (RACE) reactions was isolated from total RNA using an mRNA Isolation Kit (Life Technologies/Invitrogen, Grand Island, NY, USA).

Cloning of a cDNA encoding a putative PMCA from Y-organs of *C. sapidus*

For first-strand cDNA synthesis, total RNA (0.5 μg) treated with RQ1 RNase-free DNase was reverse transcribed using random primers (0.5 μg) and M-MLV reverse transcriptase (200 units) (all reagents from Promega, Madison, WI, USA). PCR primers PMCA-F3 (5'-TTGAACCGATGGCGTGTAAT-3') and PMCA-R4 (5'-TGATGTCTGAGGCTTCTTTTG-3') were designed based on a previously published partial cDNA sequence of PMCA from hypodermal tissue of *C. sapidus* (Roer and Towle, 2005). Reactions (total volume 50 μl) included, 2.5 μl first-strand cDNA, 0.2 mM dNTP, 0.2 mM primers, 1% glycerol, 0.3 mM Tris-HCl (pH 8.0), 1.5 mM KCl, 1 μM EDTA, 40 mM Tricine-KOH (pH 8.7), 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 0.005% Tween 20, 0.005% Nonidet-P40, and 1 unit Advantage® 2 Polymerase Mix (Clontech, Mountain View, CA, USA). PCR parameters were as

follows: an initial denaturation (95℃ for 5 min), followed by 30 cycles of 95℃ for 30 s, 56℃ for 30 s, 72℃ for 1 min, and a final extension at 72℃ for 10 min. PCR products were separated by agarose gel electrophoresis and visualized under u.v. light. A 603-bp band was purified, cloned, and sequenced (see below). A search of the NCBI protein database showed its deduced amino acid sequence had high similarity to known PMCAs from other species.

To synthesize first-strand cDNA for RACE reactions, 1 μg mRNA was reverse transcribed using reagents and a protocol provided in a SMARTer^{TM} RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). Gene specific primers used in RACE reactions were designed based on the 603-bp fragment generated by RT-PCR. For 3' RACE, the universal primer mix (UPM) provided with the kit and a gene specific primer (PMCA-3sense2: 5'-CAACCTGACCTGTCTCTGTGTGCTGGGG-3') were used for the initial amplification reaction; the nested universal primer (NUP) and a gene specific primer (PMCA-n3sense2: 5'-ACAGACGAGTCAGAGATTCTACAGG-3') were used to perform nested PCR. PCR parameters for 3'RACE reactions were the same as previously described except the annealing temperature of PMCA-3sense2 and PMCA-n3sense2 were 63℃ and 57℃, respectively, and the final extension was 2 min for both reactions.

In an initial 5'RACE reaction, PMCA-as3 (5'-GCCTGCTCGCTGGCACTTGTGGATTGCA-3') and the UPM were used to amplify the 5’-end of the 603-bp fragment. Sequencing results suggested that a cDNA fragment cloned from this reaction did not reach the start codon. Therefore, PMCA-as4 (5'- GCCTGAACGACAGTCATGCG-3') was selected based on the sequence obtained from the initial 5’RACE reaction. NUP and PMCA-as5 (5'-CAGATGGCTGTTGCATTCCCC-3') were used in the follow-up nested PCR. PCR parameters for the 5'-RACE reactions were the same as those for 3'-RACE reactions except the annealing temperatures for reactions involving PMCA-as3, PMCA-as4 and PMCA-as5 were 61, 58, and 57℃, respectively. Finally, the full-length cDNA sequence of *Cas-PMCA* was amplified using PMCAfull-F (5'-ATGGCCACCATCGACGGACGC-3') and PMCAfull-R (5'-GTCTGTCTCCAGGATAGTGGAGC-3'). PCR parameters and reagents were the same as for 5'-RACE, except the annealing temperature was 60℃ and the final extension was 4 min.

Determination of *Cas-PMCA* transcript abundance during a molting cycle

Y-organs and hypodermal tissues were dissected from crabs in various stages of the molting cycle (intermolt (C4), premolt (stages D1-D3), and postmolt (A)), transferred to RNAlater (Life Technologies/Ambion, Grand Island, NY, USA), and

stored at -20℃. Total RNA was extracted from tissue samples (4 pooled Y-organs or 10-15 mg hypodermal tissue per replicate) as described above. After treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA), total RNA (0.5 μg) was reverse transcribed using random primers and 100 units of SMART^{TM} Scribe Reverse Transcriptase (Clontech, Mountain View, CA, USA). Steady-state level of PMCA transcript was measured by real-time PCR using a Mini Opticon^{TM} Real-Time PCR Thermal Cycler (Bio-Rad, Hercules, CA, USA). Primers PMCAreal-F1 (5'-GCGGGGTTGAGGGAGTAGAG-3') and PMCAreal-R1 (5'-AGGTCTTCGGGGGCTTAGGA-3') were designed to amplify a 171-bp fragment of the *Cas-PMCA* target gene. Primers 18Sreal-F2 (5'-TCAAGTGTCTGCCTTATCAGCT-3') and 18Sreal-R2 (5'-TCGGATGAGTCTCGCATCGT-3') were designed to amplify a 201-bp fragment of the *18S rRNA* reference gene.

To validate the assay, PCR efficiency and specificity were assessed for both of target and reference genes. To generate a standard curve, first strand cDNA samples were reverse transcribed from 0.5 μg total RNA, pooled as stock, serially diluted (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) and amplified using the gene specific primers for each amplicon. The slope of each titration curve (log dilution factor verses threshold cycle number, C_t) was used to calculate the amplification efficiency (Ginzinger, 2002). To ensure

amplification specificity, melting-curve analysis and agarose gel electrophoresis were conducted at the end of each PCR reaction (see below).

Reagents for real-time PCR (SYBR® Advantage® qPCR Premix) were from Clontech (Mountain View, CA, USA). Reaction tubes contained 1 μl cDNA as template, 10 μl SYBR® Advantage® qPCR Premix, forward and reverse primer at the final concentration of 0.2 μM, and nuclease-free water to a total volume 20μl. For both target and reference genes, PCR parameters were as follows: an initial activation at 95 ℃ for 15 s, followed by 40 cycles of amplification (denaturation at 95℃ for 5 s, annealing at 57℃ for 15 s and extension at 72℃ for 10 s). After amplification, a melting curve analysis was conducted at temperatures between 55℃ and 95℃, with the temperature increasing at a rate of 0.2℃/s. PCR specificity was verified by agarose gel electrophoresis. The size of the amplicons was confirmed by comparison to DNA markers. The relative expression level of *Cas-PMCA* in different molting stages was determined by the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$) (Wong and Merdrano, 2005). A representative value from C stage was used as the calibrator and all other quantities were expressed as an n-fold difference relative to calibrator. For each replicate sample, the real-time PCR assay was performed at least 6 times.

Gel purification, cloning, sequencing and phylogenetic analysis

PCR reactions were separated on a 1% agarose gels and visualized with

GelStar® Nucleic Acid Gel Stain (Lonza, Allendale, NJ, USA). PCR products of the expected sizes were excised from gels, purified using a Gel Extraction Kit from QIAGEN (Valencia CA, USA), and cloned into a pGEM® -T sequencing vector (Promega, Madison, WI, USA). Recombinant plasmids were extracted using a Plasmid Mini Kit from QIAGEN (Valencia CA, USA), and sequenced. Sequencing was performed by the UAB DNA Sequencing and Analysis Core (Birmingham, AL, USA). Nucleotide and amino acid sequence analyses were performed using the following software resources: NBCI (http://www.ncbi.nlm.nih.gov/), Workbench (http://workbench.sdsc.edu/), EXPASY molecular biology server (http://expasy.org/), OCTOPUS (http://octopus.cbr.su.se/index.php), and BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

Phylogenetic analyses were performed using deduced amino acid sequences of available PMCA cDNA or RNA sequences. PMCA amino acid sequences were arranged by BioEdit version 7.0.9., optimized using MUSCLE and subjected to phylogenetic analysis by MEGA Version 5.05 (Tamura et al., 2011). A genetic distance tree was built by the Neighbor Joining method. The confidence values of the branching pattern were tested on 1000 bootstrap replicates.

Tissue distribution of *Cas-PMCA* transcript

To assess the spatial distribution of the *Cas-PMCA* transcript, neural (eyestalk,

brain, pericardial organ, and thoracic ganglion) and nonneural (Y-organ, testis, muscle, hypodermis, gill, and hepatopancreas) tissues were dissected from crabs, transferred to RNAlater (Ambion), and stored at -20℃. Total RNA was extracted from tissues as described above. After treatment with RQ1-DNAse (Promega), first strand cDNA was reverse transcribed from 0.5 μg total RNA using random primers and SMARTer^{TM} Scribe Reverse Transcriptase (Clontech). PCR reactions were carried out as described above. The primer pairs used to amplify *Cas-PMCA* were PMCA-F3 & PMCA-R4; those used to amplify *18S rRNA* were 18Sreal-F2 and 18Sreal-R2. PCR parameters were as follows, denaturation at 95℃ for 5 min, followed by 30 cycles of 95℃ for 30 s, 56℃ for 30 s, 72℃ for 1 min, and a final extension at 72℃ for 10 min. Aliquots of PCR reactions were analyzed by agarose gel electrophoresis.

Statistical analysis

The statistical significance of temporal changes in *Cas-PMCA* transcript abundance was determined by one-way analysis of variance followed by Tukey's HSD test using SAS 9.1 software (SAS Institute Inc., Cary, NC, USA). To assess the parallelism of the titration curves for real-time PCR assays, the homogeneity of linear regression lines was determined by analysis of covariance (Sokal and Rohlf, 1969).

RESULTS

Cloning of a cDNA encoding PMCA from Y-organs of *C. sapidus*

Roer and Towle (2005) previously reported the cloning of a partial PMCA cDNA from hypodermal tissue of *C. sapidus*. With those sequence data as a starting point, we used a PCR-based cloning strategy (RT-PCR followed by 3'- and 5'-RACE) to clone a cDNA (*Cas-PMCA*) encoding a putative PMCA from Y-organs of *C. sapidus*. The nucleotide and deduced amino acid sequence are shown in Figure 1. The full length *Cas-PMCA* cDNA (4292 base pairs) is composed of a 274-bp 5' untranslated region (UTR), a 3510-bp open reading frame (ORF), and a 505-bp 3' UTR. The open reading frame encodes an 1170 amino acid protein (Cas-PMCA) having a relative molecular mass of 128.8 daltons. An optimal translational initiation sequence (ACCATGG) is found -3 to +4 nucleotides relative to the start codon (Kozak, 1986). An in-frame stop codon is present at the end of ORF. A predicted polyadenylation signal (AATAAA) is present 126 base pairs upstream of poly(A) region.

Analysis of the deduced amino acid sequence of Cas-PMCA

In general, the deduced amino acid sequence of Cas-PMCA shows high identity to the amino acid sequence of PMCA proteins from other species (64-73% identity to the proteins shown in Fig. 2). Further, the conceptually translated Cas-PMCA shows all signature domains of authentic PMCA proteins, including ten transmembrane (TM) domains, a phosphorylation site, a fluorescein isothiocyanate (FITC) binding site (thought to be part of the ATP binding site), a

5'-*p*-fluorosulphonylbenzoyladenosine/r-(*N*-2-chloroethyl-*N*-methylamino) benzylamine ATP (FSBA) binding site, and a calmodulin (CaM) binding site (Fig. 2). Considered together, the amino acid sequence data strongly support the hypothesis that *Cas-PMCA* encodes an authentic PMCA protein.

It's worth noting that the sequence identity is not uniformly distributed across the primary structure of the PMCA proteins. As examples, there is high sequence identity (80-100%) among TM domains 1, 4, 5, 6 and 8, but lower identity in the N- and C-termini (40% and 17%, respectively).

The predicted membrane topography of Cas-PMCA is as expected for an authentic PMCA protein. That is, like previously identified PMCA proteins, Cas-PMCA is composed of of N- and C- termini, ten TM domains, and two large intracellular (IC) loops (Fig. 3). The bulk of Cas-PMCA is located intracellularly. The first IC loop (between the second and third TM) is hypothesized to be a "transduction domain" involved in coupling ATP hydrolysis to Ca^{2+} transport (Falchetto et al., 1992). The second IC loop (located between fourth and fifth TM) is a critical catalytic core in which the phosphorylation, FITC, and FSBA sites are found (Fig. 2). An aspartate residue (D) proposed to form the acyl-phosphate intermediate during ATP hydrolysis is found within the phosphorylation site (Di Leva et al., 2008; Lee and East, 2001). The C-terminal tail of PMCA, a region which possesses the lowest sequence

homogeneity among PMCA proteins, is another crucial regulatory region. The length of C-terminal tail and amino acid residues right after QTQI varies among different PMCA isoforms and splicing variants (Keeton et al., 1993; Penniston and Enyedi, 1998; Strehler and Zacharias, 2001).

Phylogenetic analysis

A phylogenetic analysis of nonredundant amino acid sequences of PMCA proteins from different species showed that vertebrate PMCA sequences clustered in an isoform-associated pattern (Fig. 4). All arthropod PMCA proteins, excepting crayfish PMCA3, were grouped together in a single clade rather than clustering with corresponding vertebrate isoforms. Nematode PMCAs were clustered in a separate outlier grouping.

Tissue distribution of *Cas-PMCA*

The spatial distribution of the *Cas-PMCA* transcript was assessed by RT-PCR. Separate primers were designed to amplify a 600-bp fragment of *Cas-PMCA* and a 201-bp fragment of *18S rRNA.* The results (Fig. 5) indicate *Cas-PMCA* is broadly expressed in both neural (eyestalk, brain, pericardial organ, and thoracic ganglion) and nonneural (Y-organ, testis, muscle, hypodermis, gill, and hepatopancreas) tissues. An additional 400-bp fragment was amplified in in brain, thoracic ganglion and hepatopancreas (data not shown).

***Cas-PMCA* transcript abundance in Y-organs and control tissues during a molting cycle**

To better understand the possible roles of PMCA in regulating the functional activity of Y-organs, we used real-time PCR to measure the relative abundance of the *Cas-PMCA* transcript in Y-organs and control tissues during a molting cycle of *C. sapidus*. Ginzinger (2002) has recommended that primers used for real-time PCR be designed to span an intron/exon boundary. Based on the sequence of the gene encoding rat PMCA3 (Burk and Shull, 1992), we designed primers (PMCAreal-F1 and PMCAreal-R1) intended to span a hypothesized intron/exon boundary in the gene encoding Cas-PMCA. PCR amplifications using first-strand cDNA or genomic DNA as template produced 171-bp and 400-bp amplicons, respectively (data not shown). The results suggested PMCAreal-F1 and PMCAreal-R1 target regions crossing the intron/ exon boundary.

The steady state level of *Cas-PMCA* in Y-organs and control tissues (hypodermis underlying either carapace or arthrodial membrane) was determined by real-time PCR using *18S rRNA* as a reference. For assay validation, a titration curve (cycle threshold vs log dilution factor) was plotted for each amplicon (Fig. 6), and the slopes used to calculate amplification efficiency as described by Ginzinger (2002). Amplification efficiency was 90% for the *Cas-PMCA* amplicon and 97% for the *18S rRNA* amplicon.

The slopes of the regression lines did not differ significantly ($P > 0.05$) as determined by analysis of covariance. For each primer set, a melting curve analysis showed amplicons having the same melting point (data not shown). Separation of the PCR products by agarose gel electrophoresis revealed the amplicons were of the predicted size (data not shown). The combined results indicate the real time PCR assay is both quantitative and specific.

The level of *Cas-PMCA* transcript in Y-organs was low during intermolt (C), increased ~5-fold to a peak in premolt stage D2, then fell sharply, and remained low during postmolt (A) (Fig. 7A). A generally similar pattern was observed in hypodermis underlying the arthroidial membrane: *Cas-PMCA* transcript abundance was low during intermolt (C), rose abruptly to a peak in stage D2, and then dropped and remained low during postmolt (A) (Fig. 7B). By contrast, the level of *Cas-PMCA* in hypodermis underlying the dorsal carapace was elevated during intermolt (C), dropped to a level in premolt stage D3 that was roughly 1/5 that seen in intermolt, and then rose sharply in postmolt (A) (Fig. 7C).

DISCUSSION

Several lines of evidence indicate that ecdysteroid production by crustacean Y-organs is calcium-dependent (Spaziani et al., 2001). However, despite the apparent critical role of calcium in regulating ecdysteroidogenesis, until recently, the level of

Ca^{2+} in Y-organ cells had not been determined for any crustacean species. We used a fluorescent calcium indicator (Fluo-4) and fluorescence imaging methods to measure the level of Ca^{2+} in blue crab Y-organ cells after experimental activation (Chen and Watson, 2011), and during a natural molting cycle (Chen et al., 2012). The combined results strongly support the hypothesis that ecdysteroidogenesis is stimulated by an increase in intracellular Ca^{2+}. With an ultimate goal of understanding the role of calcium signaling in regulation of ecdysteroidogenesis, we have begun investigating Ca^{2+} transport proteins in Y-organs.

The present paper describes the molecular cloning of a cDNA encoding a putative PMCA from Y-organs of *C. sapidus*. The 4292-bp *Cas-PMCA* contains a 3510-bp ORF that codes for a 1170-residue putative Cas-PMCA protein. The deduced amino acid sequence of Cas-PMCA shows high identity to the amino acid sequence of PMCA proteins from other species, and contains all signature domains of known PMCA proteins, including a CaM binding site, a domain not found in SERCA proteins (Penniston and Enyedi, 1998). Although the current studies have not demonstrated PMCA bioactivity, the most straightforward interpretation of the above results is that *Cas-PMCA* encodes an authentic PMCA protein.

The high amino acid sequence identity observed among PMCAs is not uniformly distributed across the primary structure of the proteins. This may be due, at

least in part, to alternative splicing of transcripts. There are two major hot spots, site A and site C, where alternative splicing takes place (Strehler et al., 2007). Site A is in IC 1 between TM2 and TM3; site C is in the CaM binding site. The amino acid sequence within site A and the number of amino acid residues after site C vary among splice variants (Keeton et al., 1993; Strehler et al., 1989; Strehler and Zacharias, 2001). It is hypothesized that alternative splicing within sites A and C yields PMCA variants that differ in regulatory properties and functional specificity (Strehler et al., 2007).

The C-terminus of PMCA is a crucial regulatory domain. Several regulatory molecules, including CaM, protein kinase C, protein kinase A, and proteases (calpain, caspase-1, caspase-3) have been shown to interact with C-terminus to affect PMCA activity (Brini, 2009; Ortega et al., 2007). Among these important regulators, CaM is most well studied. The CaM binding site (also termed the autoinhibitory domain) interacts with IC 1 and IC 2, and thus prevents the ATP hydrolysis and protein phosphorylation required for activation of PMCA. Binding of Ca^{2+}/CaM to the autoinhibitory domain releases the C-terminal tail from IC 1 and IC 2, thus allowing PMCA to become functionally active (Ortega, et al., 2007; Strehler and Zacharias, 2001). A so-called "IQ motif" has been previously identified within the CaM binding site; the consensus amino acid sequence of this motif is IQXXXRGXXXR (Cheney and Mooseker, 1992). In Cas-PMCA, amino acid residues 1117-1126 form a modified

IQ motif having I and Q transposed, invariant R and G residues, and a terminal R appearing one residue earlier than seen in the consensus sequence. An identical modified IQ motif is found in human PMCA1b (Penniston and Enyedi, 1998). Since site C is in the CaM binding site, alternative splicing in this domain has the potential to generate variants that differ in CaM binding properties. For example, the CaM binding region in human PMCA4a is longer than that human PMCA4b, and associated with this structural change, human PMCA4a has a lower affinity for CaM and a lower effective affinity for Ca^{2+} than does PMCA4b (Enyedi, et al., 1994). It's worth noting that during the cloning studies reported here, we also cloned a partial PMCA cDNA having a longer C-terminus than *Cas-PMCA*. The primary structure of the partial cDNA differed from that of *Cas-PMCA* downstream from the site C hot spot. This result raises the possibility that more than one PMCA isoform may be expressed in *C. sapidus* Y-organs.

It has been previously noted that the percent sequence identity among different PMCA isoforms within a given species is low as compared to the percent sequence identity of corresponding isoforms across species (Strehler, 1991; Takeyasu et al., 2001; Gao and Wheatly, 2004). Our search of the NCBI protein database showed that Cas-PMCA shared the highest sequence identity with PMCA isoform 3 from other species (73% identity with a type 3 isoform from *D. melanogaster*, 68% identity with

a type 3 isoform from *P. clarkii*, and 64% identity with a type 3 isoform from *H. sapiens* and *R. norvegicus*). This suggested to us that Cas-PMCA might be a type 3 isoform. However, a phylogenetic analysis showed Cas-PMCA, unlike *P. clarkii* PMCA3, did not cluster in the same grouping as vertebrate PMCA3 isoforms. Instead, Cas-PMCA clustered with insect PMCA proteins (Fig. 4). The result remained the same when insect sequences were excluded, in which Cas-PMCA by itself formed a branch of the phylogenetic tree (data not shown). It has been previously noted that invertebrate P-type ATPase isoforms do not correspond well to equivalent isoforms in vertebrates (Takeyasu et al., 2001; Wang and Takeyasu, 1997); this is consistent with the observation that Cas-PMCA did not cluster with mammalian PMCA3 isoforms. It is unclear to us why *P. clarkii* PMCA3 clusters with vertebrate PMCA3 isoforms rather than with invertebrate PMCAs.

Previous reports suggest PMCA isoforms are expressed in a tissue-specific manner (Carafoli and Stauffer, 1994). For example, human *PMCA1* and human *PMCA4* appear to be expressed in all tissues, whereas expression of human *PMCA2* and human *PMCA3* is restricted to brain (Carafoli and Stauffer, 1994; Strehler and Zacharias, 2001). That *Cas-PMCA* was expressed in all tissues tested (Fig. 5) suggests it may have a more generalized role in regulating cellular calcium homeostasis.

Quantitative real-time PCR showed the level of the *Cas-PMCA* transcript in

Y-organs was low during intermolt (C), increased significantly to a peak in premolt stage D2, then fell sharply, and remained low during postmolt (A) (Fig. 7A). The overall pattern of *Cas-PMCA* transcript abundance is generally similar to the pattern of stage-specific changes in intracellular free Ca^{2+} seen in Y-organ cells (Chen et al., 2012). The results indicate that stage-specific changes in Ca^{2+} in Y-organs cells are not likely a consequence of downregulation of PMCA activity. Rather, it appears that the increase in *Cas-PMCA* expression in Y-organs during premolt stages D1 and D2 may be a cellular response designed to maintain cytosolic Ca^{2+} homeostasis. This interpretation is consistent with previous reports involving multiple cell types that indicate increases in cytosolic Ca^{2+} are associated with upregulation of PMCA (Gao and Wheatly, 2004; Kuo, et al., 1997; Marian et al., 2007; Usachev et al., 2001). In fact, upregulation of PMCA may be part of a larger coordinated upregulation of the suite of Ca^{2+} transport proteins involved in regulation of intracellular Ca^{2+}. Thus, Marian et al. (2007) reported that adding thapsigargin to cultured human lens epithelial cells (HLE B-3), a treatment expected to increase cytosolic Ca^{2+}, caused upregulation of both PMCA1 and SERCA3 (at the level of mRNA and protein). And Ziegler et al. (2002) observed upregulation of both PMCA and NCX during periods of elevated transcellular Ca^{2+} flux in hypodermis of the isopod *Porcellio scaber*.

For purposes of comparison, *Cas-PMCA* transcript abundance was also

determined for hypodermal tissue underlying the arthroidial membrane (Figure 7B), and separately, for hypodermal tissue underlying the dorsal carapace (Fig. 7C). Because the arthroidial membrane is noncalcified, the underlying hypodermis is thought not to be involved in cuticular mineralization; by contrast, transport proteins located in hypodermal tissue underlying the dorsal carapace are involved in generating the transcellular Ca^{2+} flux associated with demineralization of the exoskeleton during premolt and mineralization of the exoskeleton during postmolt (Greenaway et al., 1995; Roer, 1980; Roer and Towle, 2005). The pattern of change in *Cas-PMCA* transcript abundance in arthroidial hypodermis was similar to that seen in Y-organs. It is plausible that elevation of *Cas-PMCA* in both tissues is a consequence of the increase in hemolymphatic Ca^{2+} concentration observed during premolt stage (Greenaway, 1985). By contrast, the pattern of change in *Cas-PMCA* transcript abundance seen in Y-organs and arthroidial hypodermis differed from that seen in hypodermis underlying the dorsal carapace. A difference in *Cas-PMCA* expression pattern between the two hypodermal tissues was not unexpected given the functional difference in the two tissues with respect to cuticular mineralization.

However, the observed pattern of change in expression of *Cas-PMCA* in hypodermis underlying carapace differs from that expected based on the results of previous studies. Greenaway et al. (1995) used ultrahistochmical methods to assess

quercitin-sensitive ATPase activity (thought to represent PMCA) in hypodermis underlying the exoskeleton (dorso-lateral branchiostegite) during a molting cyle of *C. sapidus*. Staining representative of ATPase activity was observed mainly along basolateral membranes during premolt (D2), and mainly along apical membranes during postmolt (A2); staining was not detectable during intermolt (C4). The results were interpreted to represent transport of Ca^{2+} from cuticle to hemolymph during premolt, and from hemolymph to cuticle during postmolt. Likewise, Gao and Wheatly (2004) used Northern and Western blotting to assess PMCA3 transcript abundance and immunoreactivity, respectively, in transport epithelia during a molting cycle of *P. clarkii*. In general, they found PMCA3 transcript abundance and immunoreactivity to be significantly elevated above intermolt levels during premolt and postmolt stages.

To summarize, in studies reported here we describe the cloning of a cDNA (*Cas-PMCA*) encoding an apparent PMCA from Y-organs of the blue crab. A phylogenetic analysis suggests Cas-PMCA is most closely related to PMCA proteins from insects. The *Cas-PMCA* transcript was widely distributed in both neural and nonneural tissues. Studies using quantitative real-time PCR revealed stage-specific changes in *Cas-PMCA* abundance during the molting cycle, with peak expression occurring during premolt stage D2, a pattern consistent with the hypothesis that Cas-PMCA functions to maintain cellular Ca^{2+} homeostasis in Y-organs. The pattern

of *Cas-PMCA* expression was similar to that observed in hypodermal tissue underlying the arthroidial membrane, but differed from that underlying the dorsal carapace.

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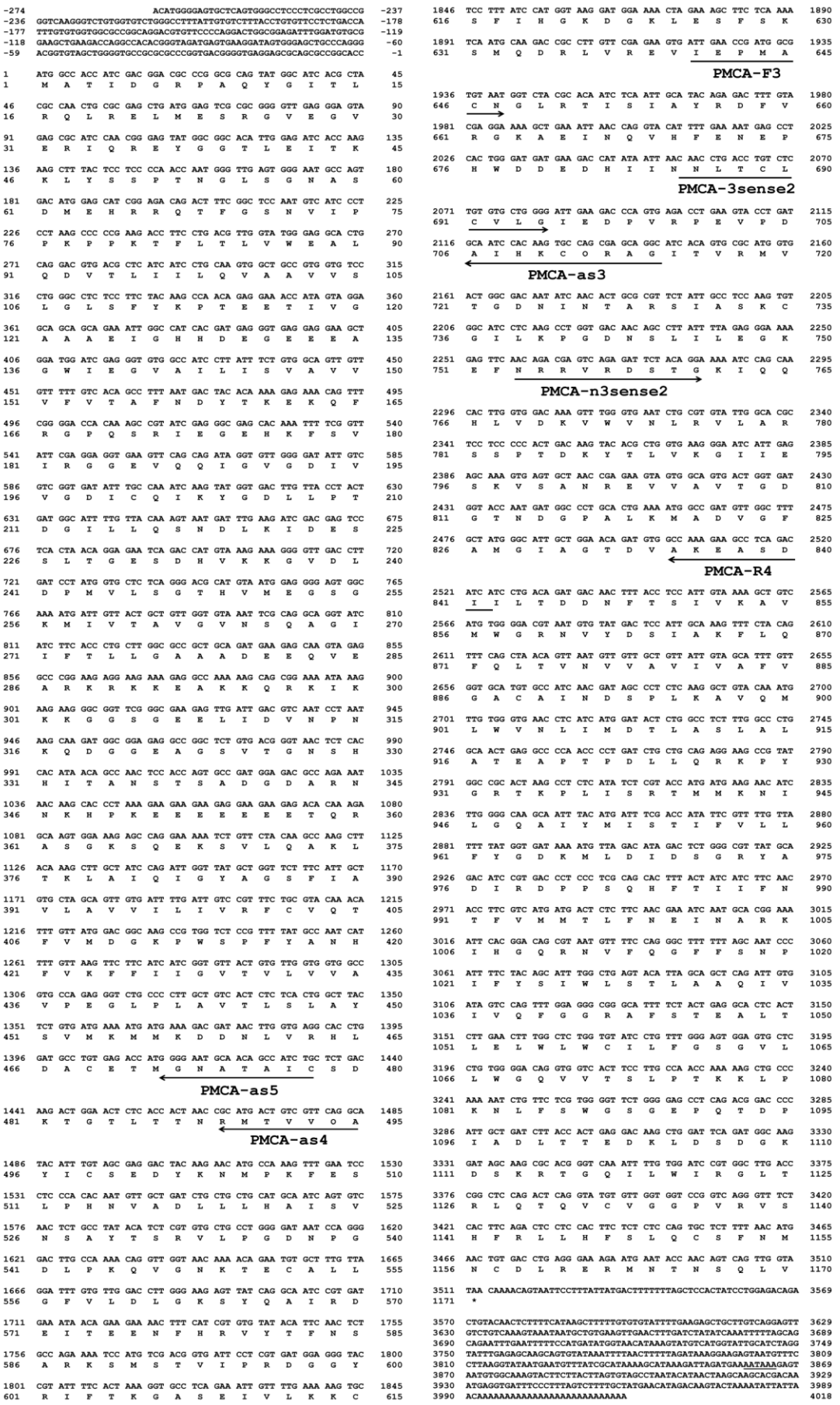
PMCA-as5
PMCA-as4
PMCA-F3
PMCA-3sense2
PMCA-as3
PMCA-n3sense2
PMCA-R4

Fig. 1. The nucleotide and deduced amino acid sequences of a plasma membrane Ca2+ ATPase (Cas-PMCA) from Y-organs of the blue crab *C. sapidus*. The cDNA sequence is numbered from the first nucleotide. The deduced amino acid sequence of the open reading frame is numbered and shown in single-letter code below the relevant nucleotide sequence. Gene specific primers used to conduct 3'- and 5'-RACE reactions are labeled under the corresponding nucleotide sequence. The stop codon is indicated with an asterisk (*). A predicted polyadenylation signal is underlined. The nucleotide sequence data have been submitted to GenBank under the accession number JQ434471.

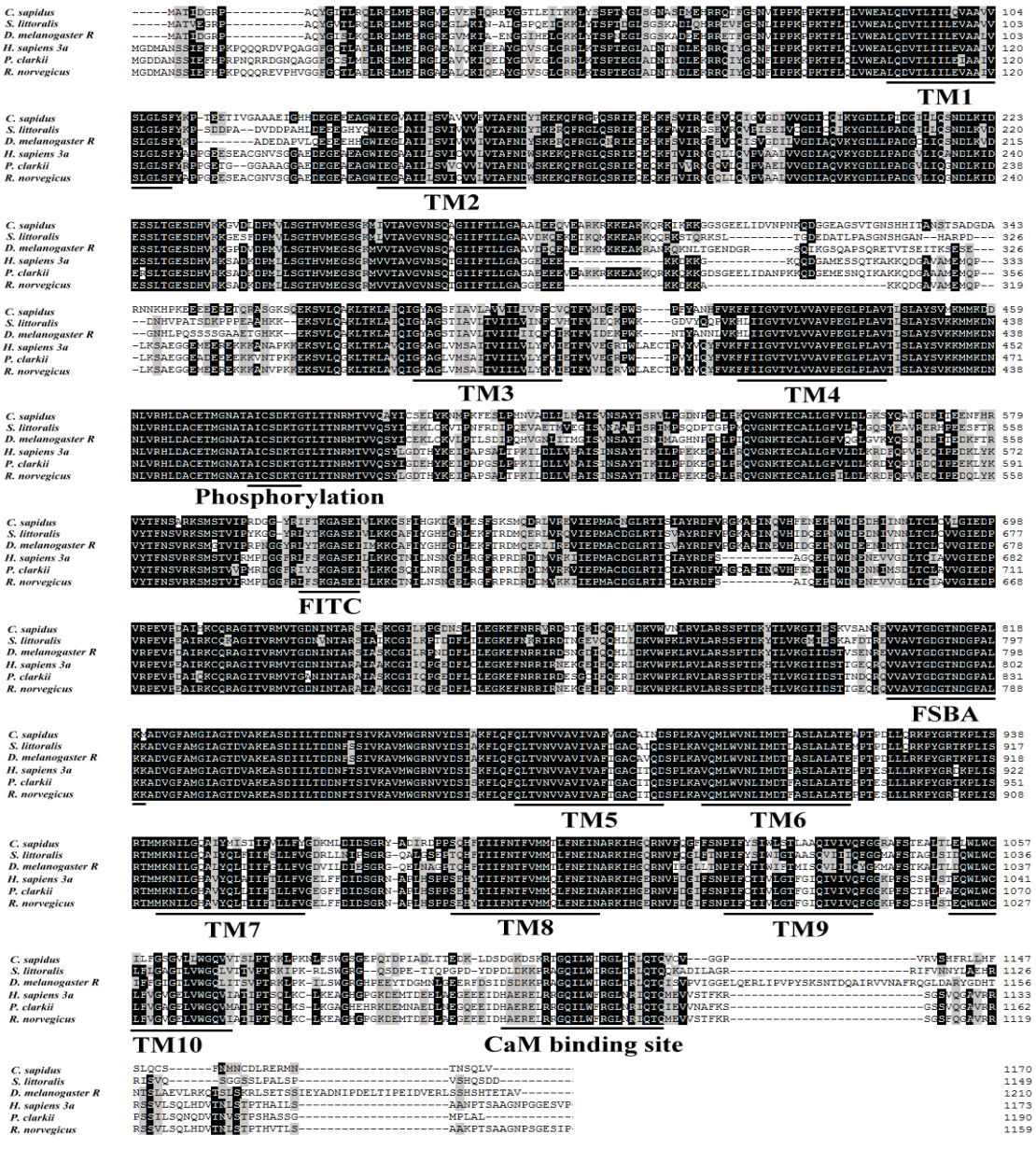

Figure 2. Alignment of the deduced amino acid sequences of multiple plasma membrane Ca^{2+} ATPase proteins. Amino acid sequences of PMCA from different species were arranged and aligned using BioEdit/ClustalW Multiple alignment. Sequences and accession numbers are shown for the following: blue crab (*Callinectes sapidus*, JQ434471), cotton leaf worm *(Spodoptera littoralis*, JQ070811), fruit fly (*Drosophila melanogaster*, NP001188517), human (*Homo sapiens*, NP068768), crayfish (*Procambarus clarkii*, AY455931), and rat (*Rattus norvegicus*, AAA69667). Underlined and labeled are ten predicted transmembrane domains (TM1-TM10), a phosphorylation site, a fluorescein isothiocyanate (FITC) binding site, a 5'-*p*-fluorosulphonylbenzoyladenosine/r-(*N*-2-chloroethyl-*N*-methylamino) benzylamine ATP (FSBA) binding site, and a calmodulin (CaM) binding site.

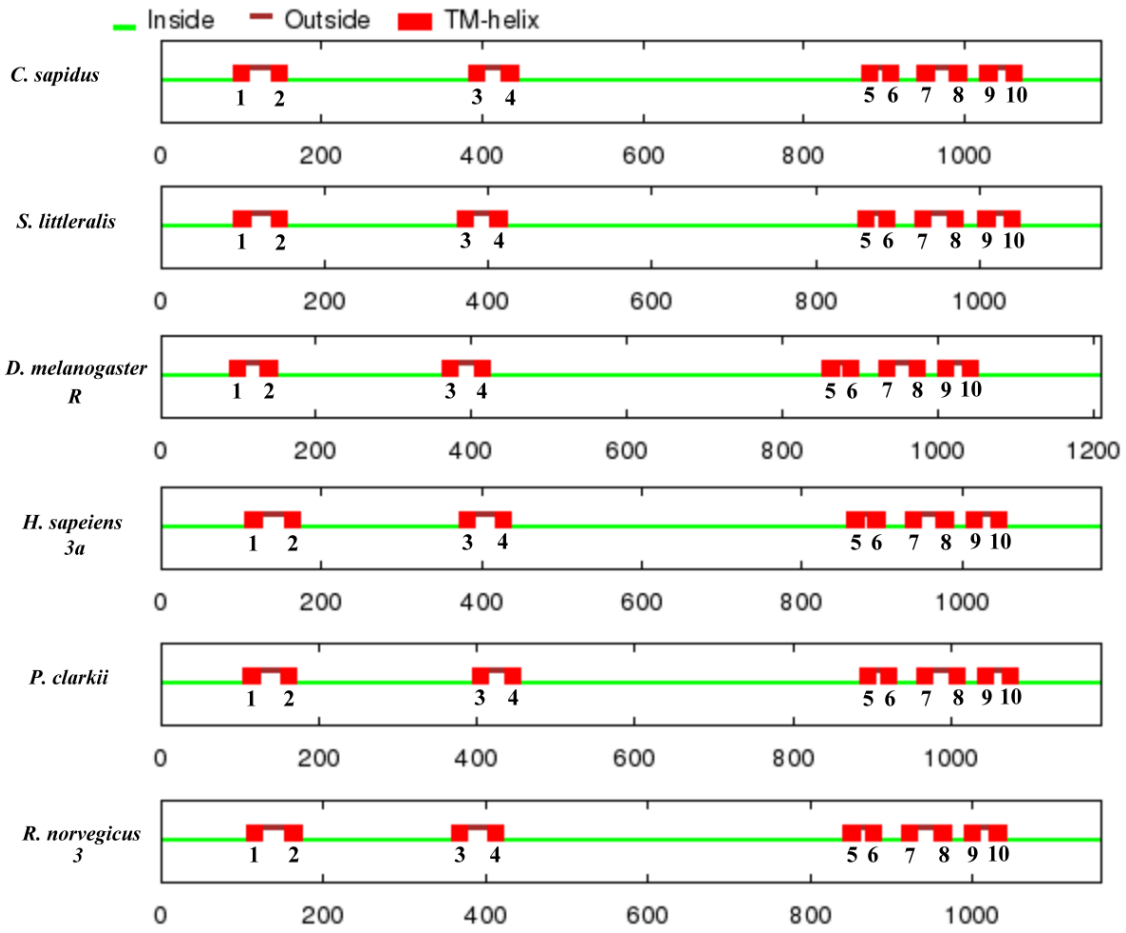

Fig. 3. Membrane topography of selected PMCA proteins. OCTOPUS, software combining artificial neural networks (ANN)-based preference score and HMM (hidden Markov model)-based global prediction algorithm, was used for the prediction of membrane topography (http://octopus.cbr.su.se) (Viklund and Elofsson, 2008) for PMCA proteins from blue crab *Callinectes sapidus* (JQ434471), *Spodoptera littoralis* (JQ070811), *Drosophila melanogaster isoform R* (NP001188517), *Homo sapiens 3a* (NP068768), *Procambarus clarkii isoform 3* (AY455931), and *Rattus norvegicus* (AAA69667). Transmembrane domains are numbered 1-10. Intracellular and extracellular regions are shown in green and brown, respectively.

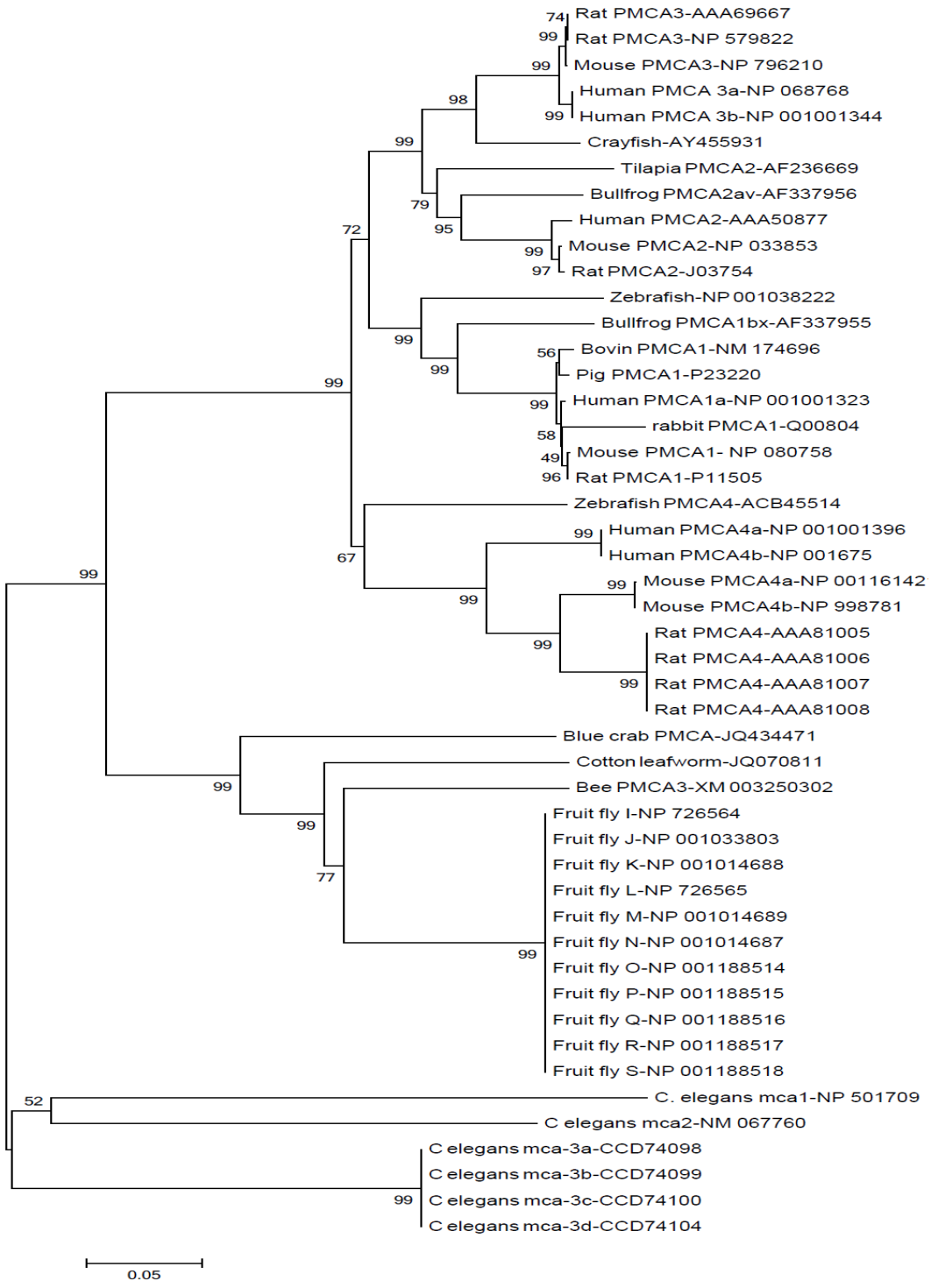

Fig. 4. Phylogenetic analysis of the amino acid sequence of PMCA. Nonredundant amino acid sequences of PMCA from various species were collected from NCBI protein database, aligned by BioEdit and optimized by MUSCLE. The phylogenetic tree was generated using MEGA5. The genetic distance was built by the Neighbor Joining method and confidence values of the branching pattern were tested on 1000 bootstrap replicates. Accession numbers are as shown.

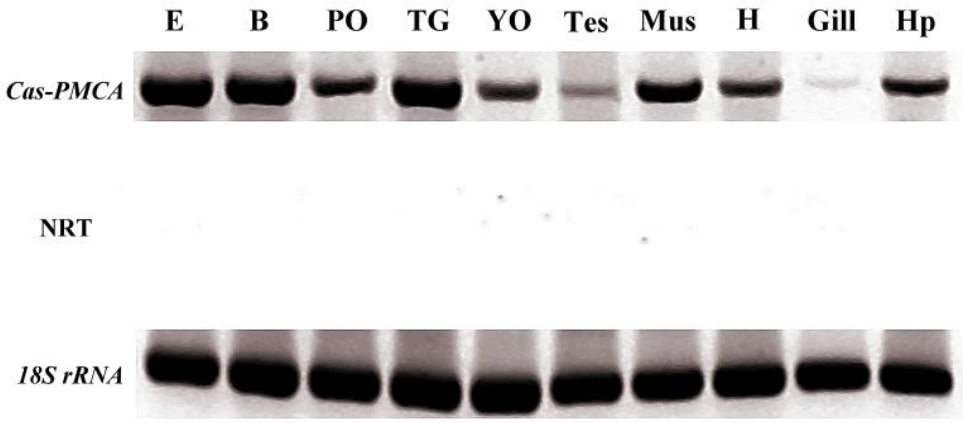

Fig. 5. Distribution of the *Cas-PMCA* transcripts in intermolt tissues of *C. sapidus*. Total RNA was extracted from eyestalk (E), brain (B), pericardial organ (PO), thoracic ganglia (TG)), Y-organ (YO), testis (Tes), muscle (Mus), hypodermis (H), gill, and hepatopancreas (Hp) were reverse transcribed, amplified by PCR using gene specific primers designed to amplify a 600-bp amplicon of *Cas-PMCA* or a 201-bp fragment of *18S rRNA*, respectively. Aliquots of PCR reactions were separated on a 1% agarose gels and visualized with GelStar® Nucleic Acid Gel Stain. PCR reactions with RQ1 DNase-treated total RNA as template were performed as a negative control (NRT).

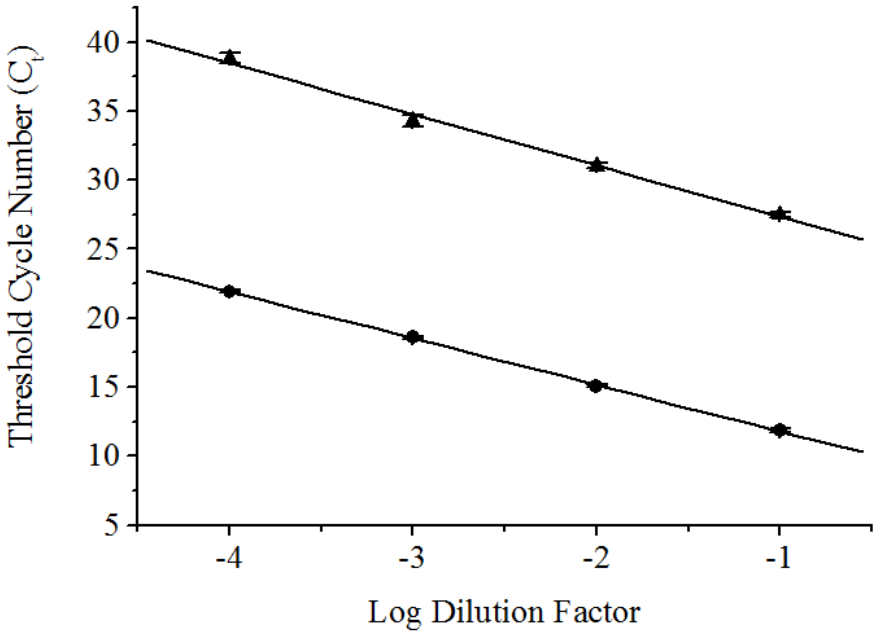

Fig. 6. Sample titration curves (threshold cycle number vs. log dilution factor) for real-time PCR assay of *Cas-PMCA* transcript abundance. Pooled cDNA samples from Y-organ were serially diluted and subjected to real-time PCR using primers designed to amplify a 171-bp fragment of *Cas-PMCA* (▲), or a 201-bp fragment of *18S rRNA* (●). The slopes for *Cas-PMCA* and *18S rRNA* titration curves are -3.58± 0.13 and -3.38± 0.01, respectively. The slopes did not differ significantly as determined by analysis of covariance (P>0.05). Each point is a mean ± SE of six measurements.

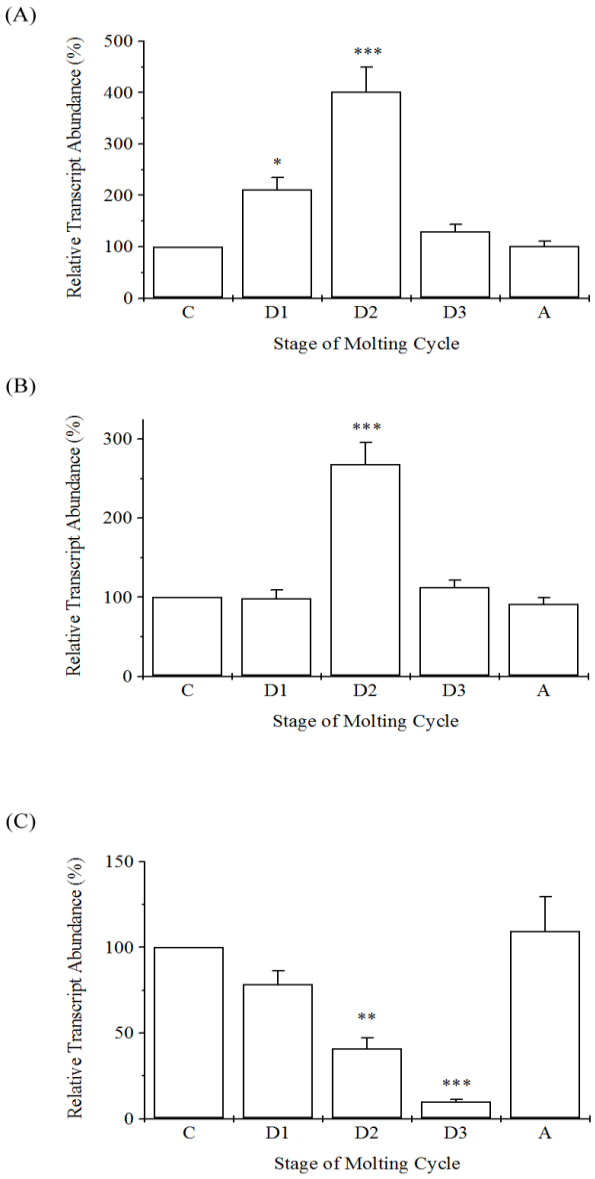

Fig. 7. Relative abundance of the *Cas-PMCA* transcript in Y-organs and control hypodermal tissues during a molting cycle of *C. sapidus*. At various stages of the molting cycle, total RNA was extracted from (**A**) Y-organs, (**B**) hypodermal tissue underlying the arthrodial membrane, or (**C**) hypodermal tissue underlying the dorsal carapace. Relative transcript abundance was determined by quantitative real-time PCR; a replicate from postmolt stage was used as calibrator. Bars represent mean + SD. Molt stages are intermolt (C), premolt (D1–D3), and postmolt (A). Tukey's test results showed the following pairwise comparisons to be significant at the 0.05 level. For Y-organs (panel **A**): C-D1, C-D2, D1-D2, D1-A, D2-D3, D2-A. For hypodermis underlying arthroidial membrane (panel **B**): C-D2, D1-D2, D2-D3, D2-A. For hypodermis underlying dorsal carapace (panel **C**): C-D2, C-D3, D1-D3, D2-A, D3-A.

General Conclusion

Multiple lines of evidence indicate calcium signaling plays a critical role in regulation of ecdysteroidogensis by crustacean Y-organs. In general, treatment of Y-organs *in vitro* with pharmacological agents that increase intracellular Ca^{2+} leads to an increase in ecdysteroidogensis, while agents that lower intracellular Ca^{2+} lower ecdysteroidogenesis (reviews: Spaziani et al., 1999; Spaziani et al., 2001; Nakatsuji et al. 2009). The combined results from all sources are consistent with the hypothesis that an increase in intracellular Ca^{2+} may stimulate the surge in ecdysteroidogenesis that leads to molting. However, despite the apparent crucial role of calcium in regulating ecdysteroidogenesis, until the studies reported in this dissertation, the level of Ca^{2+} in Y-organ cells had not been determined for any crustacean species. As described herein, our laboratory used a fluorescent calcium indicator (Fluo-4) and fluorescence imaging methods to measure the level of Ca^{2+} in crab (*C. sapidus*) Y-organ cells after experimental activation by eyestalk ablation (Chen and Watson, 2011). We found that eyestalk ablation produced an increase in free Ca^{2+} in Y-organ cells, and that the increase in intracellular free Ca^{2+} was associated with an increase in the hemolymphatic ecdysteroid titer. 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 follow-up studies, 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 *C. sapidus* (Chen et al., 2012). Mean calcium fluorescence increased by 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. Measurement of fluorescence along a transect drawn through representative cells from various stages of the molting cycle indicated the intensity of calcium fluorescence was generally greater in cytoplasm than in nuclei, and suggested the Ca^{2+} signal in Y-organ cells is global, rather than being spatially restricted or localized to a specific region within the cell. The pattern of changes in the hemolymphatic ecdysteroid titer of donor crabs mirrored the pattern of changes in intracellular Ca^{2+}. The now published results contained in the two papers summarized above constitute the first reports of Ca^{2+} levels in Y-organ cells for any crustacean species, and strongly support the hypothesis that ecdysteroidogenesis is stimulated by an increase in intracellular Ca^{2+}.

Although the findings are a key contribution to our understanding of the involvement of calcium signaling in regulation of ecdysteroidogenesis, the methods used (imaging of Fluo-4 calcium fluorescence by laser confocal microscopy) provide

only a snapshot of Ca^{2+} levels in Y-organs at specific time points within the molting cycle. Because calcium signaling is temporally and spatially complex, often involving oscillations and waves that can be highly localized in nature, additional studies of the calcium signal in Y-organ cells are needed.

It should also be pointed out that the cause of the increase in intracellular calcium is unknown. One possibility is that it is elicited by a specific ligand; the existence of a positive regulator of ecdysteroidogenesis in crustaceans has been long hypothesized (Skinner, 1985). Another possibility is that the hypothesized increase in intracellular calcium is a consequence of the general increase in hemolymphatic Ca^{2+} that occurs during premolt as Ca^{2+} is mobilized from the old exoskeleton (Greenaway, 1985). Additional studies are needed to resolve this matter.

Finally, additional studies are needed to resolve the cellular mechanism by which an increase in intracellular Ca^{2+} stimulates ecdysteroidogenesis. Our laboratory hypothesizes that Ca^{2+} stimulates ecdysteroidogenesis by activating cyclic nucleotide phosphodiesterase (PDE) and thus enhancing degradation of the cyclic nucleotide second messengers that suppress ecdysteroid production by Y-organs. Results from multiple labs have shown that adding a non-selective inhibitor of PDE (3-isobutyl-1-methylxanthine, IBMX) to incubation medium, a treatment designed to promote accumulation of cyclic nucleotide second messengers, has the effect of

suppressing basal ecdysteroid production by Y-organs and potentiating the inhibitory effect of MIH (Nakatsuji et al., 2009). These results suggest that stage-specific changes in PDE activity in Y-organs may be involved in regulation of ecdysteroidogensis during the molting cycle. Consequently, our laboratory measured PDE activity in Y-organs during a molting cycle of *P. clarkii*. PDE activity was lowest during intermolt and postmolt (stages when ecdysteroid production by Y-organs is likewise low), and higher during premolt (when ecdysteroid production is elevated) (Nakatsuji et al., 2006). The most straightforward interpretation is that enhanced degradation of cyclic nucleotide second messengers by PDE promotes ecdysteroidogenesis. Needed are experiments designed to test the effect of Ca^{++} on glandular PDE activity and cGMP levels.

In all eukaryotic cells, the cytosolic free Ca^{2+} level is tightly regulated by a suite of Ca^{2+} regulatory proteins including Ca^{2+} channels, pumps, exchangers, and Ca^{2+} binding proteins (Berridge, et al., 2000; Carafoli, 1987; Krebs et al., 2003). As a step toward understanding the regulatory mechanism that controls Ca^{2+} levels in Y-organ cells, an additional aim of my dissertation research was to clone from Y-organs a cDNA encoding a plasma membrane calcium ATPase (PMCA), and measure its expression during a molting cycle.Using a PCR based cloning strategy, we cloned from blue crab Y-organs a cDNA (*Cas-PMCA*) encoding a putative Cas-PMCA

protein. The DNA contained a 3510-bp open reading frame which conceptually encoded 1170 amino acids with an estimated molecular weight 128.8. The deduced amino acid sequence of *Cas-PMCA* contained all signature domains of an authentic PMCA including phosphorylation, ATP binding, and CaM binding sites. An analysis of membrane topography predicted Cas-PMCA possessing a similar structure with other known PMCA proteins. The *Cas-PMCA* transcript was widely detected in both neural and non-neural tissues, suggesting it plays an essential role in various types of cells. A real-time PCR assay was developed to quantify the transcript abundance of *Cas-PMCA* in Y-organs during a molting cycle. Results showed that the expression of *Cas-PMCA* in Y-organs significantly increased in early (D1) and middle premolt (D2) stage. A similar change of *Cas-PMCA* transcript abundance was observed in nonmineralizing hypodermis (arthrodial membrane) and it was distinct from that observed in mineralizing hypodermis (carapace). The transcript abundance of *Cas-PMCA* in hypodermis underlying carapace was high in intermolt stage, declined to a significant level in middle (D2) and late premolt (D3) stage then rose up to an insignificant level after molting (stage A). Results from real-time PCR of three tissues suggested that changes in *Cas-PMCA* expression in Y-organs and nonmineralizing hypodermis were in response to the increased intracellular Ca^{2+}. Additional studies are needed to solve the apparent discrepancy between the level of *Cas-PMCA* in

hypodermis underlying carapace and the results of previous studies of PMCA transcript and protein levels in transport hypodermis during the molting cycle.

To directly assess the possible involvement of Cas-PMCA in regulating ecdysteroidogenesis, needed are experiments designed to measure its physiological effect on ecdysteroid production using a PMCA-specific agonist, antagonist, or both. However, so far there is no PMCA-specific agonist or antagonist commercially available. Previously, novel PMCA inhibitors, caloxins, were designed specifically binding on the allosteric sites to block PMCA activity (Szewczyk et al., 2008). Considering the multiplicity of PMCA, each caloxin exhibited variant specificity that enhanced the difficulties to commercialize the production of caloxins. Customized Cas-PMCA-specific caloxins might be needed to conduct a functional analysis of the protein in Y-organs.

To summarize, results presented in this dissertation are the first report t of Ca^{2+} levels in Y-organ cells for any crustacean species, and are entirely consistent with the hypothesis that an increase in intracellular Ca^{2+} promotes the surge in ecdysteroidogenesis that leads to molting. Further, our findings suggest that PMCA plays a critical role in regulation of Ca^{2+} homeostasis in Y-organs during the molting cycle.

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APPENDIX A

TISSUE DISTRIBUTION OF *CSCHH-1* AND *CSCHH-2* IN EYESTALK AND PERICARDIAL ORGAN

1. Introduction

Crustacean hyperglycemic hormone (CHH), a polypeptide neurohormone synthesized from neurosecretory cells in eyestalk ganglia, is a key regulator in crustacean carbonate metabolism which functions to increase hemolymphatic glucose levels (Santos and Keller, 1993). While CHH was originally discovered and purified from eyestalk, increasingly data show that it is also expressed in extra-eyestalk neural (including brain, thoracic ganglia, abdominal ganglia, pericardio organ and sub-esophageal ganglia) and nonneural tissues (including gut, antennal gland, and gill) Those extra-eyestalk CHH isoforms share a certain level of similarity with eyestalk derived CHH however they do not possess hyperglycemic activity (Fanjul-Moles, 2006; Soyez, 2003).

A long-term interest in our lab has been to identify eyestalk-derived CHH family members and their functions as it relates to crustacean endocrine regulation in the blue crab *Callinectes sapidus*. As part of related research conducted in our laboratory, I have assesssed the spatial distribution of *CsCHH-2*, an extra-eyestalk derived CHH isoform that is structurally similar to the eyestalk-derived CHH (*CsCHH-1*).

2. Materials and Methods

2.1. Animal material

Blue crabs *Callinectes sapidus* were purchased from local seafood store and maintained in compartmented tanks with filtered circulatory sea water as previously described (Nakatsuji et al., 2006a).

2.2 Tissue preparation, RNA extraction, and first strand cDNA synthesis

Eyestalk ganglia and pericardial organ were dissected, rinsed with Pantin's saline once and stored in RNAlater (Ambion) immediately at -20 ℃. Total RAN was extracted from both tissues with RNeasy Mini kit (Quiagen) following the instructions of the manufacturor. After treatment with RQ1-DNAse (Promega), 0.5 μg total RNA was reverse transcribed with 200 units M-MLV Reverse Transcriptase and 0.5 μg random primer (both from Promega).

2.3 Analysis of the spatial distribution of CsCHH-2 mRNA by RT-PCR

PCR reaction was conducted in 25-μl reaction containing 1 μl of first strand cDNA (from either eyestalk or pericardial organ), gene specific primers FullCHH-F (5'-CATGCAATCCATCAAAACCGTG-3') and FullCHH-R (5'-TACTTCTTGCCGACAACCTGTA-3') at the final concentration of 0.2 μM, and 12.5 μl GoTaq Green Master Mix (Promega). PCR reaction was conducted at 95 ℃ for 5 min followed by 30 cycles of 95℃ for 30s, 53℃ for 30sec, 72℃ for 1 min and a final extension at 72℃ for 5 min. PCR reaction products were visualized with UV-light

3. Results

The tissue distribution of *CsCHH-2* transcript was assessed in eyestalk and pericardial organ. Result showed that an amplicon about 420-bp was observed in eyestalk. In addition to a 420-bp fragment, another slightly larger amplicon (529-bp in size) was also observed in pericardial organ.

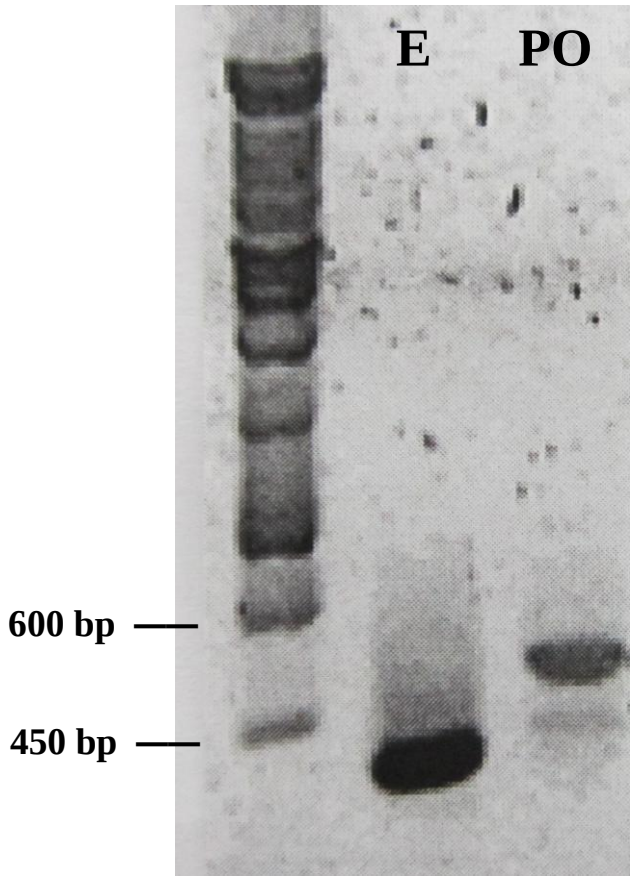

Fig.1. Tissue distribution of *CsCHH-1* and *CsCHH-2*. Total RNA was extracted from eyestalk (E) and pericardial organ (PO), reverse transcribed as described in section 2.2 and subjected to PCR reaction with gene specific primers FullCHH-F and FullCHH-R to amplify a 420-bp amplicon of *CsCHH-1* and a 529-bp amplicon of *CsCHH-2*. Aliquots of PCR products were analyzed by means of gel electrophoresis.

APPENDIX B

IMMUNOCYTOCHEMICAL LOCATION OF CSGC-YO1

1. Introduction

Even though receptor-binding assays had demonstrated the existence of high-affinity, specific, and saturable MIH receptors on plasma membranes of *Carcinus maenas* Y-organs (Webster, 1993), the nature of the MIH receptor has not been identified yet. According to the studies of cellular signaling in *Procambarus clarkii* and *Callinectes sapidus* Y-organs (Nakatsuji et al., 2006a, b), we hypothesized that the MIH receptor is a membrane associated guanylyl cyclase (rGC). A putative receptor guanylyl cyclase gene, *CsGC-YO1* was cloned from *C. sapidus* Y-organ. An anti-CsGC-YO1 antibody was generated to conduct initial immunocytochemical studies. Results were consistent with the notion that CsGC-YO1 is a membrane-associated protein (Zheng et al., 2008), however the resolution of immunocytochemical location of CsGC-YO1 needed to be improved.

2. Materials and Methods

Method for thin section immunecytochemistry Y-organs was adapted from those previously described (Zheng et al., 2008). In brief, Y-organs dissected from donor animals were fixed in Bouin's fixative solution for 24hr. The fixed tissue was dehydrated in a graded ethanol series, cleared, and embedded in paraffin according to conventional methods. Serial sections (10 μM) were placed on coated slides and allowed to dry at 40℃ overnight. Mounted sections were heated at 60℃ for 2 hr

followed by removal of paraffin by xylene and then was rehydrated to 0.01 M PBS (phosphate buffer, 0.14 M NaCl, 0.01M KCl, pH 7.4), containing 0.3% Triton X-100, 0.1% sodium azide (PTA). Rehydrated slides were transferred into glass Coplin jars filled with 0.01Mcitrate, pH 6.0, containing 0.1% (v/v) Tween 20. The Coplin jars were placed in a microwave oven and the solution was boiled for 5 min. After heating, the solution was replenished with distilled water and the heating cycle was repeated 3 times. Sections were rinsed three times (10 min each) in PTA solution (0.01M PBS, pH 7.4, containing 0.3% triton X-100 and 0.1% sodium azide) and incubated in 0.1% Trypsin solution with 0.1% $CaCl_2$ at room temperature for 10 min followed by being rinsed three times (10 min each) in PTA solution. Sections were then blocked in 2% BSA in PTA for 2 hr, rinsed once (5 min), and then incubated at 4℃ overnight in PTA containing primary antibody (1:20). After being rinsed three times (10 min each) in PTA, sections were then incubated for 2 h at room temperature in secondary antibody (goat anti-rabbit Alexa Fluor^{TM} 488) (Invitrogen) diluted 1:850 in PTA. Sections were rinsed three times (10 min each) in PTA and once in PBS (5 min). Sections were then counterstained with Hoest 33258 solution (2 μg/ml in PBS) (Invitrogen). Without rinsing, slides were mounted in VECTASHIELD mounting medium (Vector Laboratories, Inc.) and viewed using a Nikon Eclipse optical microscope.

3. Results

The cellular distribution of Cs-GC-YO1 was assessed by means of immunocytochemical staining. Tissue sections incubated with anti-CsGC-YO1 primary antibody appeared widely distributed immunoreactivity (green fluorescence) (Fig. 1A, 1C). No immunoreactivity was observed in sections probed with control antibody (preimmune) (Fig. 1B, 1D). Under higher magnification power, thin tissue section showed that the immunoreactivity of CsGC-YO1 was located primarily at the peripheral margins of the cell, most likely corresponding to cell membrane (Fig. 1C).

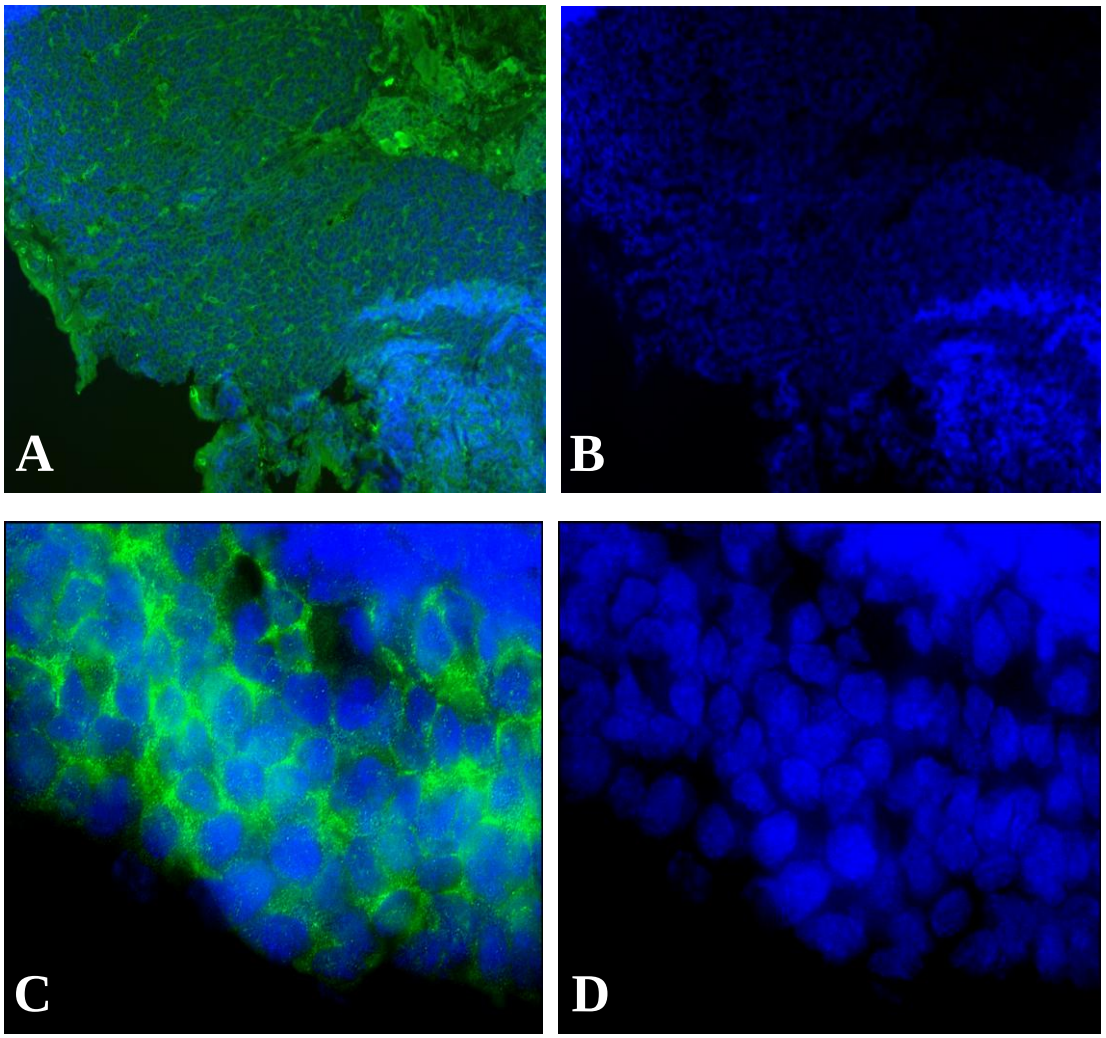

Fig. 1. Immunocytochemical location of CsGC-YO1 in *Callinectes sapidus* Y-organs. Fluorescent images were viewed and taken using a Nikon Eclipse optical microscope fitted with a chilled CCD camera. Magnification power is 60x for panel A, B and 1000x for panel C, D. Panel A, C: positive, sections were probed with purified primary antibody from immune serum followed by incubation of secondary antibody (goat-anti-rabbit Alexa Fluor^{TM} 488). Panel B, D: negative, sections were probed with control antibody purified from preimmune serum followed by incubation of secondary

antibody (goat-anti-rabbit Alexa Fluor™ 488). The nuclei in all tissue sections were stained with Hoechst 33258 solution shown in blue fluorescence.

APPENDIX C

IACUC APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)
NOTICE OF APPROVAL

DATE: June 8, 2010

TO: Watson, R. Douglas
CH-271 1170
934-2031

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: The Crustacean Molt-Inhibiting Hormone Receptor and Induction of Molting in Blue Crabs
Sponsor: Mississippi-Alabama Sea Grant Consortium
Animal Project Number: 100408398

On June 8, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Crabs	A	150
Crayfish	A	150

Animal use is scheduled for review one year from April 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 100408398 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.