**Protein kinase C modulates transcriptional activation by the juvenile hormone
receptor Methoprene-tolerant**

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Abbreviations:

AAEL, all VectorBase gene identifiers for *Aedes aegypti* begin with the 4 letters AAEL;
JHRE, juvenile hormone response element; PKC, protein kinase C; PLC, phospholipase
C; CaMKII, calcium/calmodulin-dependent protein kinase II.

ABSTRACT

Juvenile hormone (JH) controls many biological events in insects by triggering dramatic changes in gene expression in target cells. The Methoprene-tolerant (MET) protein, an intracellular JH receptor, acts as a transcriptional regulator and binds to the promoters of tissue- and stage-specific JH target genes when JH is present. Our recent study has demonstrated that the transcriptional activation by MET is modulated by a membrane-initiated JH signaling pathway, involving phospholipase C (PLC) and calcium/calmodulin-dependent protein kinase II (CaMKII). Here we report that protein kinase C (PKC) is another essential intermediate of this pathway. PKC was activated by JH and this action was PLC-dependent. Inhibition of the PKC activity substantially weakened the JH-induced gene expression in mosquito cells. RNAi experiments indicated that several PKC isoforms were involved in the JH action during the post-emergence development of adult female mosquitoes. JH treatment considerably increased the binding of MET to the promoters of JH response genes in cultured mosquito abdomens that were collected from newly emerged female adults. The JH-induced DNA binding of MET was hindered when the abdomens were treated with a PKC inhibitor and JH. Therefore, the results suggest that PKC modulates the transactivation activity of MET by enhancing the binding of MET to JH response elements in the JH target genes. This mechanism may allow for variable and stage- and tissue-specific genomic responses to JH.

Keywords: Juvenile hormone; Protein kinase C; Phospholipase C; Hormone receptor; Gene regulation

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43 **1. Introduction**

44 Juvenile hormones (JH) are a group of acyclic sesquiterpenoids secreted in insects by
45 the corpora allata, a pair of endocrine glands located on the posterior side of the brain
46 (Tobe and Stay, 1985). JH is responsible for the regulation of insect metamorphosis and
47 reproduction (Goodman and Cusson, 2012; Nijhout, 1994; Riddiford, 1994). In addition,
48 this pleiotropic master hormone governs many other aspects of insect life such as caste
49 differentiation, migratory behavior, diapause and longevity (Flatt et al., 2005; Nijhout,
50 1994; Wyatt and Davey, 1996). Several signaling pathways have been postulated so far to
51 be involved in the action of JH, but the mechanistic details largely remain to be
52 elucidated (Jindra et al., 2013; Wheeler and Nijhout, 2003).

53 Many effects of JH rely on its intracellular receptor MET, the product of the
54 *Methoprene-tolerant (Met)* gene (Ashok et al., 1998; Jindra et al., 2013). MET contains
55 an N-terminal basic helix-loop-helix (bHLH) DNA recognition motif, followed by two
56 spaced Per- ARNT-Sim (PAS) domains (PAS-A and PAS-B) (Ashok et al., 1998). In the
57 presence of JH, MET binds to another bHLH-PAS domain protein called Taiman (TAI)
58 (also known previously as FISC in *Aedes aegypti* and SRC in *Tribolium castaneum*)
59 (Charles et al., 2011; Li et al., 2011; Zhang et al., 2011). TAI acts as the obligatory DNA
60 binding partner of MET; the MET-TAI complex recognizes an E-box like sequence (5'-
61 GCACGTG-3') in the regulatory regions of JH target genes, leading to their
62 transcriptional activation (Kayukawa et al., 2012; Li et al., 2014). In adult female *Ae.*
63 *aegypti* mosquitoes, expression of the early trypsin (AaET) gene and the Krüppel
64 homolog 1 (AaKr-h1) gene is induced by JH via the MET-TAI complex (Li et al., 2014;

Li et al., 2011; Zhu et al., 2010). Shortly after adult emergence, JH activates transcription of *AaET* in the midgut, but the transcripts will not be translated until the mosquitoes take a blood meal (Noriega et al., 1996; Noriega et al., 1997). Kr-h1 is a zinc finger transcription factor that mediates the anti-metamorphic action of JH in phylogenetically distant insect orders (Konopova et al., 2011; Lozano and Belles, 2011; Minakuchi et al., 2009; Minakuchi et al., 2008).

In addition to binding to an intracellular JH receptor, several lines of evidence have suggested that JH action involves certain form of transmembrane signaling (Davey, 2000). In the ovary of *Rhodnius prolixus* and *Locusta migratoria*, JH causes the follicle cells to shrink and to create large intercellular spaces, thereby facilitating the transport of yolk proteins into the developing oocytes (Davey and Huebner, 1974; Davey et al., 1993). This rapid hormonal response is initiated by the binding of JH to a plasma membrane protein and involves activation of a Na^+/K^+ ATPase via a Protein kinase C (PKC)-dependent pathway (Ilenchuk and Davey, 1987; Sevala and Davey, 1989; Sevala et al., 1995). PKC has also been implicated in the JH-stimulated protein synthesis in male accessory glands of *Drosophila melanogaster*. Yamamoto et al. showed that addition of JH caused an increase in protein synthesis in cultured accessory glands from the wild-type flies, but not from flies with mutation in PKC (Yamamoto et al., 1988).

We have recently reported that JH activates the phospholipase C (PLC) pathway in *Ae. aegypti* mosquitoes and triggers an increase in the concentration of diacylglycerol (DAG), inositol 1,4,5-triphosphate (IP_3) and intracellular calcium (Ca^{2+}), which in turn activates calcium/calmodulin-dependent protein kinase II (CaMKII) (Liu et al., 2015). This membrane-initiated JH signaling modulates phosphorylation of MET and TAI, and

substantially enhances DNA binding of the MET-TAI complex to juvenile hormone response element (JHRE). Besides CaMKII, PKC is another intracellular mediator normally associated with the PLC pathway. Here we provide the first evidence that PKC is also activated by JH in a PLC-dependent manner in *Ae. aegypti*. PKC plays a critical role in transcriptional activation of JH target genes. Activation of PKC by JH is essential for potent binding of MET-TAI to JHRE. This study significantly advances our understanding of the function of PKC in the action of JH.

2. Materials and Methods

2.1. Chemicals

JH-III was purchased from Sigma Aldrich and was dissolved in ethanol. In all inhibition experiments, cells were pre-incubated with inhibitors for 1 h before the addition of JH-III. Final concentrations of inhibitors in all cell-culture studies were as follows: calphostin C (Santa Cruz Biotechnology), 5 μ M; RO31-8220 (Santa Cruz Biotechnology), 10 μ M; Gö6983 (Santa Cruz Biotechnology), 10 μ M; KT 5720 (Santa Cruz Biotechnology), 10 μ M; U73122 (EMD Millipore), 1 μ M. Phorbol-12-myristate-13-acetate (PMA, Sigma Aldrich) was used at a final concentration of 10 μ M.

2.2. Cell culture

Ae. aegypti Aag-2 cells (Lan and Fallon, 1990; Peleg, 1968) were maintained at 28°C in Schneider's *Drosophila* media (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals). Passages of cells were conducted every 4 days with a 1:5 dilution of cells.

2.3. Mosquito rearing and tissue culture

The *Ae. aegypti* Liverpool strain was maintained in an insectary at 28°C and 60–70% humidity, with a 14/10 h day/night light cycle. Larvae were fed with pulverized fish food (TetraMin Tropical Flakes) and adults were maintained on a 10% sucrose solution. Female mosquitoes (7 days post eclosion) were fed on defibrinated sheep blood using an artificial membrane feeder to produce eggs. All tissue dissections were performed in *Aedes* physiological saline (APS) (Hagedorn et al., 1977). Tissues were collected from female mosquitoes within 30 min after eclosion. *In vitro* tissue culture was carried out as previously described (Deitsch et al., 1995; Raikhel, 1997). The mosquito abdomens were cut open and placed on top of the fat body culture medium. Fat bodies attached to the inner wall of cuticles were in the medium and the outside surface of cuticle was exposed to air. Three groups of five mosquitoes were used for each treatment. When inhibitors were used, dissected tissues were pre-incubated with the inhibitors for 1 h before the addition of JH-III.

2.4. Real-time PCR

Total RNA was extracted from Aag-2 cells or mosquitoes using TRIzol reagent (Life technology), according to the manufacturer's instruction. RNA was primed with oligo (dT) primer and reverse-transcribed using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative PCR was performed in triplicate on an ABI 7300 system (Applied Biosystem) using the GoTaq qPCR Master Mix (Promega) and gene-specific primers (Table S1). Transcript abundance was normalized to that of *RpS7*.

2.5. RNA sequencing (RNA-seq) and data analysis

mRNAs were purified using NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). RNA integrity of all samples was assessed by Agilent 2100 Bioanalyzer. RNA-seq libraries were prepared using the NEBNext mRNA Library Prep Reagent Set (New England Biolabs), and were loaded onto flow cell channels of the Illumina MiSeq platform. Paired-end reads (100 nt $\times$ 2) were aligned to the *Ae. aegypti* reference genome (AaegL3, Vectorbase) using TopHat (Trapnell et al., 2009). The mRNA annotation file (AaegL3.3) from Vectorbase was provided to TopHat during the runs. The normalized mRNA abundance was calculated as FPKM values (Fragments Per Kilobase of transcript per Million mapped reads) using Cufflinks (Trapnell et al., 2012). We used a FPKM cutoff of 1 for transcripts to be considered reliable for further analysis. All the data have been submitted to NCBI SRA database (accession number: SRS1128317).

2.6. Double-stranded RNA (dsRNA)-induced gene silencing (RNAi)

DsRNAs were synthesized by *in vitro* transcription of PCR-generated DNA templates (Table S2) and injected into *Ae. aegypti* mosquitoes as described previously (Zhu et al., 2003). Briefly, female mosquitoes were injected with 0.5 μ g of dsRNA within 1 h post-eclosion. DsRNA for green fluorescent protein (GFP) was used as a negative control. Three days after dsRNA injection, RNA was extracted for real-time PCR analysis.

For RNAi in the cell line, Aag-2 cells were cultured in a 6-well plate and incubated overnight to allow cells to adhere to the plate. Complete media was replaced with FBS- and antibiotic-free media containing 5 μ g dsRNA per well. Cells were then incubated for

3 days before adding JH-III (5 μ M) and incubating for an additional hour, followed by RNA extraction and real-time PCR analysis.

2.7. Luciferase reporter assay

pCMA-GAL4 and UAS $\times$ 4-188-cc-Luc were from Dr. Lucy Cherbas (Hu et al., 2003). Construction of pCMA-AaMET, pCMA-AaTAI and 4 $\times$ JHRE1-luc has been explained previously (Li et al., 2014; Li et al., 2011). Aag-2 cells were cultured in 48-well plates and were transfected with pCMA-AaMET (0.2 μ g per well), pCMA-AaTAI (0.2 μ g per well), 4 $\times$ JHRE1-luc reporter plasmid (0.2 μ g per well). Cellfectin II reagent (Life technology) was used for transfection according to the manufacturer's instruction. The Renilla luciferase construct pRL-CMV (0.1 μ g per well) was used as an internal control of transfection efficiency. At 24 h after transfection, cells were treated with JH-III and various inhibitors for 4 h. Luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was determined as the ratio of firefly luciferase activity to Renilla luciferase activity. Data are presented as the mean $\pm$ S.D. of three independent experiments.

2.8. Western blot analysis

Whole cell lysates were extracted from Aag-2 cells or cultured mosquito tissues using a modified RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1 mM sodium fluoride). Protein concentrations were measured using the bicinchoninic acid (BCA) assay. An equal amount of protein was

loaded in each lane and separated on a SDS-PAGE gel in all experiments. Production of polyclonal antibodies against AaMET and AaTAI has been reported previously (Zhu et al., 2010; Zhu et al., 2006). Anti-Phospho-(ser) PKC substrate antibody was purchased from Cell Signaling Technology.

2.9. Chromatin immunoprecipitation assay (ChIP)

The ChIP assay was performed using SimpleChIP Plus Enzymatic Chromatin Immunoprecipitation Kit (Cell signaling Technology). After Aag-2 cells were treated with ethanol, JH-III, or JH-III together with a PKC inhibitor, cells were washed and resuspended in PBS. Formaldehyde was added to a final concentration of 1%, and crosslinking was performed for 10 minutes at 37°C. Crosslinking was stopped by adding 1.25 M glycine to reach a final concentration of 125 mM. After nuclei preparation, chromatin digestion was performed using 0.25 µl of Micrococcal nuclease for 5 min at 37°C. Immunoprecipitation was carried out using the anti-AaMET antibody or anti-AaTAI antibody. Non-specific goat IgG (ImmunoReagents Inc) was used for mock immunoprecipitation. DNA was purified from the pellets according to the manufacturer's protocol and analyzed by real-time PCR. For cultured mosquito abdomens, the tissues were homogenized in PBS on ice and then incubated at 37°C with 1% formaldehyde for 10 min. The ChIP assay was then performed as described above for the Aag-2 cells. Primers used in the ChIP assays are listed in Table S3.

3. Results

3.1. PKC is required for the JH-induced expression of AaKr-h1

A previous study has shown that JH rapidly induces *AaKr-h1* expression in mosquito Aag-2 cells (Cui et al., 2014). To examine the involvement of PKC in the JH signaling pathway, we inactivated PKC in Aag-2 cells using specific inhibitors and then measured the expression level of *AaKr-h1* after JH treatment. RO31-8220, Gö6983 and calphostin C are non-isoform-selective PKC inhibitors (Beltman et al., 1996; Gschwendt et al., 1996; Kobayashi et al., 1989). Incubation of Aag-2 cells with 5 μ M JH-III for 1 h resulted in a 70-fold increase in the *AaKr-h1* expression (Fig. 1A). Pre-incubation of Aag-2 cells with RO31-8220, Gö6983 or calphostin C all substantially abolished the stimulatory effect of JH on *AaKr-h1* expression, indicating an involvement of PKC in this JH response. In contrast, the protein kinase A (PKA) inhibitor KT5720 did not markedly affect the JH-induced expression of *AaKr-h1* (Fig. 1A). The role of PKC in JH signaling was also investigated using *in vitro*-maintained fat bodies of newly emerged adult female mosquitoes, in which JH has been shown to considerably upregulate the expression of *AaKr-h1* and *AAEL002576* (another JH target gene) via the PLC pathway (Liu et al., 2015). The JH-induced expression of both genes was decreased by more than 70% when the fat bodies were pre-incubated with RO31-8220 or U73122, a PLC inhibitor. Conversely, inactivation of PKA by KT5720 did not show a marked inhibitory effect in this experiment (Fig. 1B). Therefore, the results suggest that functional PKC is required in the JH signaling pathway.

To test whether the PKC inhibitors used in this study affect global gene expression in mosquito cells, we performed transcriptome analyses in Aag-2 cells treated with JH or with JH and RO31-8220. mRNA levels of the majority of genes were not altered by the addition of RO31-8220 to the medium (Fig. 2), suggesting that under our experimental

conditions the inhibitors did not cause adverse effect on global gene expression and that PKC plays a specific role in the JH-regulated gene expression. To assess the role of PKC in the JH signaling pathway, we treated Aag-2 cells with the PKC activator PMA (Phorbol-12-Myristate-13-Acetate). In the absence of JH-III, PMA was not able to cause any increase in the amount of *AaKr-h1* mRNA (Fig. 3), suggesting that the activation of PKC alone was not sufficient to induce the expression of JH target genes. When the cells were treated simultaneously with PMA and JH-III, PMA did not further enhance the JH-induced expression of *AaKr-h1* (Fig. 3). This observation implied that PKC was almost fully activated after the cells were exposed to JH.

3.2. JH induces PKC activity in Aag-2 cells and in mosquito fat bodies

After demonstrating that PKC plays an essential role in the JH-regulated gene expression, we examined whether the kinase activity of PKC was increased by JH in mosquito cells. Phosphorylation of mosquito proteins by PKC was detected by Western blot analysis using a specific antibody that recognizes the phosphorylated serine residues in conserved PKC phosphorylation sites. Although identities of the detected protein bands were not known, the overall intensity of signal on the blot was proportional to the activity of PKC. Compared with ethanol, JH-III substantially increased the phosphorylation of a group of proteins in Aag-2 cells within 30 minutes after the hormone treatment (Fig. 4A). The kinase activity was stronger at 2 h after the hormone treatment. Interestingly, when PLC was inactivated by its inhibitor (U73122), phosphorylation of those proteins after the JH treatment was not detected in the Western blot. When fat bodies of newly emerged female mosquitoes were maintained *in vitro* and exposed to JH-III, the PKC activity was also considerably enhanced in a PLC-dependent

fashion (Fig. 4B). Based on these results, we conclude that PKC is activated in the presence of JH and that PKC acts downstream of PLC in the JH signaling pathway.

3.3. Roles of individual PKC isoforms in the regulation of JH-inducible genes

PKC is a family of serine/threonine protein kinases. Based on homology with the PKC isoforms in *D. melanogaster* (Shieh et al., 2002), we identified five putative PKC isoforms in *Ae. aegypti*: a conventional PKC (AaPKC1), two novel PKCs (AaPKC2 and AaPKC3), an atypical PKC (AaPKC4) and a PKC-related kinase (AaPKC5) (Table S1). Five isoform-specific dsRNAs were synthesized and dsRNA against GFP was used as control. Knockdown of each PKC isoform in Aag-2 cells displayed minor effect on the JH-regulated expression of *AaKr-h1* (Fig. 5A and Fig. S1). However, when a combination of dsRNAs for all five PKC isoforms was applied to the cells at the same time, the JH-induced expression of *AaKr-h1* decreased by 59% compared with the dsGFP-treated cells, implying potential functional redundancy of the PKC isoforms in the JH signaling.

The role of PKC was also investigated in newly emerged adult female mosquitoes. The depletion of AaPKC3, AaPKC4 and AaPKC5 showed the most dramatic effect and reduced the expression of *AaKr-h1* by 74%, 62% and 73%, respectively (Fig. 5B and Fig. S1). Depletion of AaPKC1 and AaPKC2 caused a mild decrease (by 38% and 23%, respectively) in *AaKr-h1* expression. In contrast, the mosquitoes injected with dsRNA for GFP exhibited similar levels of *AaKr-h1* mRNA transcripts to the uninjected controls. The influence of PKC was not exclusive to *AaKr-h1* as the RNAi knockdown of PKCs had similar impact on the expression of *AaET*, another well-characterized JH target gene (Fig. 5B). The involvement of PKC isoforms from different subfamilies implies

synergistic action of PKC isoforms in cellular response to JH.

3.4. Inactivation of PKC decreases the transcriptional activation by MET in response to JH

The JH-induced expression of *AaET* and *AaKr-hI* has been shown to be mediated by the AaMET-AaTAI complex (Li et al., 2011). To investigate the connection between PKC and the intracellular JH receptor AaMET, we performed a reporter assay using a luciferase gene that was placed under the control of a minimal promoter and the JHRE identified in *AaET*. When AaMET and AaTAI were over-expressed in Aag-2 cells, JH-III increased the reporter activity by 92-fold over treatment with ethanol (Fig. 6A). Pre-incubation with PKC inhibitors (RO31-8220, Gö6983 and calphostin C) either completely abolished or substantially weakened the JH-induced expression of the luciferase reporter, indicating that the PKC activity is required for proper function of the AaMET-AaTAI complex on JHRE. In the control experiment, we explored the role of PKC in activation of the UAS-luc reporter gene by GAL4. Inactivation of PKC by its inhibitors under similar experimental conditions showed no remarkable effect on the GAL4-activated luciferase expression, suggesting that the JH-induced PKC activity specifically modulates function of the AaMET-AaTAI complex on JHRE (Fig. 6B).

3.5. PKC activity is essential for binding of the MET-TAI complex to JHRE

To test the possibility that inactivation of PKC causes a decrease in binding of MET to JHRE, chromatin immunoprecipitation assays (ChIP assay) were performed. We have recently shown that AaTAI is the obligatory DNA-binding partner of AaMET in

regulating JH response genes (Li et al., 2014). Therefore, binding of AaMET to JHRE *in vivo* is expected to be accompanied with the DNA binding of AaTAI. In Aag-2 cells at 30 minutes after addition of JH-III to the culture medium, the binding of AaMET and AaTAI to the proximal promoter of *AaKr-h1* increased by 20.4- and 26.7-fold, respectively, compared with the cells exposed to ethanol (Fig. 7A and B). Binding of AaMET and AaTAI to the 3' UTR of *AaKr-h1* remained at the background level regardless of the presence of JH. Interestingly, inhibition of PKC by RO31-8220 decreased the JH-induced DNA binding of AaMET and AaTAI by 69% and 73%, respectively (Fig. 7A and B). Adding RO31-8220 to the cell culture did not markedly change the nuclear protein levels of AaMET and AaTAI in Aag-2 cells (Fig. S2), suggesting that PKC regulates the transactivation activity of AaMET and AaTAI by modulating DNA binding property of the AaMET-AaTAI heterodimer.

The role of PKC in the DNA binding of AaMET and AaTAI was also assessed in the mosquito abdomens that were dissected from newly emerged adult female animals and cultured *in vitro*. The ChIP results indicated that binding of AaMET and AaTAI to the *AaKr-h1* promoter in the JH-treated abdomens was 8.2- and 6.1-fold stronger, respectively, than the ethanol-treated abdomens (Fig. 7C and D). However, this JH-induced specific DNA binding was dramatically repressed when PKC was inhibited by RO31-8220 in the cultured abdomens. Likewise, inhibition of the PKC activity adversely affected the recruitment of AaMET-AaTAI to the JHRE of *AaET* (Fig. 7E and F), suggesting that the activation of PKC by JH is a general mechanism to modulate the DNA binding activity of AaMET and AaTAI.

4. Discussion

Accumulating evidence has indicated that JH regulates insect metamorphosis, reproduction and other biological processes via a MET-dependent pathway (Jindra et al., 2013). Our recent study has demonstrated that DNA binding of the MET-TAI complex is governed by a membrane-initiated PLC pathway. Activation of this PLC pathway considerably increases the intracellular levels of IP₃, DAG and Ca²⁺, leading to phosphorylation of MET and TAI which is presumably a requisite for the binding of MET and TAI to JHRE (Liu et al., 2015). CaMKII is a component of the PLC pathway. It is activated by the JH-triggered elevation of intracellular Ca²⁺; its activity is essential for the proper binding of MET and TAI to JHRE (Liu et al., 2015). In current study, we demonstrate that PKC is another component of the JH-activated PLC pathway. The PKC activity is also critically important for the DNA binding of MET and TAI.

The membrane-initiated JH signaling was discovered in the study of the development of ovarian patency in *R. prolixus*. JH causes the ovarian follicle cells to shrink within minutes after application of JH (Davey and Huebner, 1974). This process is not affected by inhibitors of transcription, indicating that it is a non-genomic hormone response (Abu-Hakima and Davey, 1977). *In vitro* studies have suggested that JH binds to an unknown membrane protein on *R. prolixus* follicle cells (Ilenchuk and Davey, 1985) and activates PKC, which then phosphorylates the alpha subunit of Na⁺/K⁺ ATPase, leading to the decrease in cell volume (Davey, 1996). In *Heliothis virescens*, JH-I regulates the patency development primarily via a G-protein coupled receptor (GPCR) and a cyclic adenosine monophosphate (cAMP)-dependent pathway (Pszczolkowski et al., 2005), while JH-II and JH-III presumably evoke patency by stimulation of the PLC-PKC pathway

(Pszczolkowski et al., 2008). In *Ae. aegypti*, current evidence indicates that JH activates the PLC-PKC pathway via a receptor tyrosine kinase on the cell membrane to modulate gene expression during post-emergence development in adult mosquitoes (Liu et al., 2015). These membrane-initiated signal transduction events bear some resemblance.

PKC has been previously reported to curb DNA binding of several transcription factors involved in the JH-controlled gene expression (Kethidi et al., 2006; Zhou et al., 2002). A DNA-binding protein in *Locusta migratoria* was implicated in the JH-activated synthesis of yolk proteins in the fat body (Zhou et al., 2002). *In vitro* assays indicated that PKC activity present in the fat body nuclear extract inhibited binding of this locust protein to a JHRE. Similarly, the PKC activity in *D. melanogaster* L57 cells was shown to repress binding of a *Drosophila* nuclear protein (or a protein complex) to a JHRE identified from the JH esterase gene of *Choristoneura fumiferana* (Kethidi et al., 2006). In both cases, the JHREs do not resemble the E-box sequence that is recognized by MET-TAI, suggesting that the nuclear proteins bound to those JHREs are transcription factors other than MET. Although the regulation of PKC activity by JH has not been fully elucidated in *L. migratoria* and *D. melanogaster*, these examples imply that PKC may either increase or decrease the DNA binding of different transcription factors involved in the JH signaling. The JH-activated PLC-PKC pathway thus may alter the amplitude and dynamics of gene expression to facilitate stage- and tissue-specific biological responses to JH.

Integration of extranuclear and intranuclear signaling is common in steroid responses in mammals (Hammes and Davis, 2015). The biological effects of steroids are primarily mediated by binding to their classical intranuclear receptors (Evans and Mangelsdorf,

2014). The liganded receptors act as transcription factors and together with coregulators bind to specific DNA response elements located in the promoters of target genes, causing activation or repression of transcription (Tsai and O'Malley, 1994). However, a vast body of work indicates that cellular responses to steroids may be mediated by a small population of classical steroid receptors located at the plasma membrane, which interact with G proteins, signaling kinases and other G protein-coupled receptors, to mediate rapid effects of the hormones (androgen, estrogen, progesterone, etc.) (Mani et al., 2012). The nongenomic steroid activity typically involves the rapid activation of second messenger systems and activation of PKC, PI3K and mitogen-activated protein kinase (Hammes and Levin, 2007; Pietras and Szego, 1975; Szego and Davis, 1967). This rapid action initiates at the cell surface and can be readily induced by cell-impermeable hormone conjugates (Zhao et al., 2005; Zheng et al., 1996).

Recent reports have demonstrated in mammals that normal function of both extranuclear and nuclear steroid signaling is required for many hormonal responses in important biological processes (Pedram et al., 2009; Pedram et al., 2014; Roforth et al., 2014). The membrane-initiated signaling promotes phosphorylation of the nuclear steroid receptors and their co-regulators, modulates recruitment of these transcription factors to target promoters, and imposes an additional control in the regulation of transcription by the nuclear receptors (Marino et al., 2006). Therefore, the genomic and non-genomic pathways are inextricably linked. The rapid, non-classical hormonal responses are often associated with and likely required for more slow-acting (but long-lasting) genomic hormonal effects. The integration is believed to provide plasticity for cell response to steroids (Marino et al., 2006). Steroids could activate discrete signaling pathways via

their membrane receptors and associated proteins, depending on the cellular type and on the physiological status of the cell. This should lead to different gene expression in various types of target cells and different biological outcomes in response to the same hormone (Hammes and Davis, 2015). Similar mechanism may be used by insects in response to JH, although this hypothesis needs to be tested in additional tissue- and stage-specific JH actions.

In our study, the activation of PKC by JH led to enhanced binding of the AaMET-AaTAI complex to JHRE. The phosphorylation targets of PKC in the JH signaling pathway are yet unknown. We have shown that JH induces phosphorylation of both AaMET and AaTAI, and that this JH action is mediated by the PLC pathway (Liu et al., 2015). Whether the phosphorylation of both proteins relies on the function of PKC awaits further investigation. A bioinformatic analysis with the GPS 2.1 software (Xue et al., 2011) revealed dozens of putative PKC phosphorylation sites in AaMET and AaTAI. While it is tempting to speculate that AaMET and AaTAI are direct phosphorylation targets of PKC, there is no evidence so far to substantiate this hypothesis. AaMET purified by immunoprecipitation from the JH-treated Aag-2 cells was not detectable by a phospho-(Ser) PKC substrate antibody (Fig. S3). Although we could not rule out the possibility that AaMET is phosphorylated by PKCs on threonine or serine residues that could not be recognized by this antibody, it is conceivable that PKC uses another kinase as an intermediate to regulate the phosphorylation of AaMET. Mass spectrometry analysis of AaMET and AaTAI from Aag-2 cells that are treated with ethanol, JH alone, or JH together with a PKC inhibitor is expected to reveal the PKC-dependent phosphorylation sites. This information will enable us to examine whether the JH-

induced phosphorylation of AaMET affects its binding to JH, and determine whether phosphorylation of both proteins affects their dimerization and their binding to JHRE. It is important to note that phosphorylation of AaMET and AaTAI may require the synergistic action of PKC, CaMKII and other unidentified kinases. This may explain why the PKC activator was not able to enhance the expression of AaKr-h1 in Aag2 cells (Fig. 2).

PKC sits at the crossroads of divergent intracellular signaling pathways. Several studies have indicated that PKC plays a critical role in response to the steroid hormone 20-hydroxyecdysone (20E) in insects. The nuclear receptor complex for 20E is composed of the ecdysone receptor (EcR) and ultraspiracle protein (USP). In *D. melanogaster*, phosphorylation of USP is regulated by 20E in the salivary glands during early third instar (Song et al., 2003). *In vitro* studies have indicated that the *Drosophila* USP is phosphorylated by PKC on Ser35 (Wang et al., 2012). Inactivation of PKC in the salivary glands *in vivo* leads to a decrease in USP phosphorylation and attenuates 20E signaling in *D. melanogaster* (Wang et al., 2012). In the lepidopteran insect *Helicoverpa armigera*, 20E activates the PLC-PKC pathway via a G protein-coupled 20E receptor (Wang et al., 2015). The 20E-induced phosphorylation of USP promotes binding of the EcR-USP complex to ecdysone-response elements and is essential for the 20E-induced gene expression (Liu et al., 2014). The fact that PKC is capable of modifying DNA binding of the ecdysteroid receptor complex and the JH nuclear receptor complex obviously raises several questions: (1) Are the putative membrane receptors for JH and 20E present simultaneously in the same cell or tissue? (2) Is phosphorylation of USP and MET mediated by the same PKC isoforms? (3) Is USP phosphorylated by PKC when cells are

exposed to JH instead of 20E? (4) Does the phosphorylation of MET and USP affect formation of the MET-TAI and EcR-USP complexes, respectively? Addressing these questions will significantly advance our understanding of the crosstalk between 20E and JH signaling pathways, and the anti-metamorphic function of JH.

Acknowledgements

We thank Dr. Lucy Cherbas (Indiana University, Bloomington, IN) for providing the pCMA-GAL4 and UAS $\times$ 4-188-cc-Luc plasmids. This work was supported by NIH grant R01 AI099250 to JZ. Funding for this work was provided in part, by the Virginia Agricultural Experiment Station and the Hatch Program of the National Institute of Food and Agriculture, U.S. Department of Agriculture.

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Figure Legends

Figure 1. PKC activity is required for the JH-induced gene expression in mosquito cells.

(A) Aag-2 cells were pretreated with the three PKC inhibitors (RO31-8220, Gö6983 and calphostin C) or the PKA inhibitor KT5720 for 1 h. Ethanol was used as a negative control. JH-III was added to the culture medium at a final concentration of 5 μ M and cells were incubated for an additional hour. The expression level of *AaKr-h1* was measured using real-time PCR. Results are the mean $\pm$ S.D. of three independent experiments. Statistical analysis was conducted by paired t-test (*, $p < 0.05$; **, $p < 0.01$; ns, not significant, if $p > 0.05$). (B) Fat bodies from newly emerged *Ae. aegypti* mosquitoes

were cultured *in vitro* and incubated with the indicated inhibitors for 1 h. After JH-III (5 μ M) was added to the culture medium, the fat bodies were cultured for another hour and collected for RNA extraction. The expression levels of *AaKr-h1* and *AAEL002576* were measured by real-time PCR. Results are the mean $\pm$ S.D. of three independent experiments.

Figure 2. Inhibition of PKC does not alter global gene expression in mosquito cells. Aag-2 cells were cultured in the presence of ethanol (a negative control), JH-III (5 μ M), or JH-III together with the PKC inhibitor RO31-8220 (10 μ M) for one hour. RNA sequencing was performed to compare transcriptomes of the cells after different treatments. The scatter plot shows normalized read counts for each gene between the treatment of JH and the treatment of JH with RO31-8220. FPKM - fragments per kilobase of exon per million fragments mapped.

Figure 3. Activation of PKC alone is not sufficient to induce *AaKr-h1* expression. Aag-2 cells were treated with ethanol, JH-III (5 μ M), PKC activator PMA (10 μ M), or a combination of PMA and JH-III. Total RNA was extracted from the cells one hour after the treatment to measure abundance of the *AaKr-h1* mRNA using real-time PCR. Data represent mean $\pm$ S.D. of three independent experiments. Statistical analysis was conducted by paired t-test. ns, not significant, $p>0.05$.

Figure 4. JH treatment activates the kinase activity of PKC. (A) Whole cell lysate was extracted from Aag-2 cells treated with ethanol, JH-III (5 μ M), and the PLC inhibitor

U73122 (1 μ M) together with JH-III. Equal amount of proteins (20 μ g/lane) was loaded into the wells of the SDS-PAGE gel. Western blot analysis was conducted using a phospho-(Ser) PKC substrate antibody. (B) Fat bodies from newly emerged mosquitoes were cultured *in vitro* with ethanol, JH-III (5 μ M) and U73122 (1 μ M) for the indicated time periods. To inactivate PLC, fat bodies were pre-incubated with U73122 for 1 h before JH-III was added to the culture. Protein extracts were subjected to Western blot analysis using the phospho-(Ser) PKC substrate antibody.

Figure 5. RNAi analysis of the *Ae. aegypti* PKC genes in the genomic response to JH. (A) Aag-2 cells in a 6-well plate were treated with dsRNAs (5 μ g per well) for individual PKC isoforms (AaPKC 1 to 5) or a mix of dsRNAs (a total of 5 μ g per well) for all five PKC isoforms. DsRNA for GFP was used as negative control. After incubating with the dsRNAs for 72 h, cells were treated with JH-III (5 μ M) for 1 h. Total RNA was then extracted from the cells and the expression of *AaKr-h1* was measured using real-time PCR. For comparison, expression in the cells treated with the dsRNA for GFP was set as 1. Data represent mean $\pm$ S.D. of three independent experiments. Statistical analysis was conducted by paired t-test (*, $p < 0.05$; **, $p < 0.01$; ns, not significant, $p > 0.05$). (B) Newly emerged female mosquitoes were injected with dsRNAs for individual PKC isoforms within 1 h after eclosion. At 72 h after injection, total RNA was extracted from the mosquitoes. The amount of *AaKr-h1* or *AaET* transcripts in the uninjected control mosquitoes was set as 1.

Figure 6. Inhibition of PKC lessens JH-induced gene activation by the MET-TAI

complex. (A) Aag-2 cells were transfected with the following plasmids: pCMA-AaMET, pCMA-AaTAI, 4×JHRE-luc firefly luciferase reporter construct, and a constitutively expressed Renilla luciferase plasmid (pRL-CMV, Promega). At 24 h after transfection, cells were treated with the inhibitors for 1 h before JH-III (5 μM) was added to the medium; the culture was extended for additional 4 h. Firefly luciferase activity was normalized to the Renilla luciferase activity of each sample. Results represent mean ± S.D. for three independent experiments. Statistical analysis was conducted by paired t-test (**, $p < 0.01$; ns, not significant, $p > 0.05$). (B) Aag-2 cells were transfected with pCMA-GAL4, UAS×4-188-cc-Luc and pRL-CMV. Transfected cells were treated with JH-III and the PKC inhibitors under the same conditions as described in (A).

Figure 7. The JH-induced PKC activity is required for MET to bind to JHRE. ChIP assays were performed using antibodies against AaMET (A, C and E) and AaTAI (B, D and F). (A and B) Aag-2 cells were treated with ethanol, JH-III and the PKC inhibitor RO31-8220 as indicated. (C-F) Abdomens of newly emerged mosquitoes were cultured *in vitro* and treated with ethanol, JH III and RO31-8220 as indicated. After chromatin immunoprecipitation, the precipitated DNA was analyzed using real-time PCR. For each JH target gene, two pairs of primers were designed to amplify the proximal promoter (Pro) region and a control (Con) region. Results are shown as a percentage of input chromatin and represent mean value ± S.D. of three independent experiments. Statistical analysis was conducted by paired t-test (*, $p < 0.05$; **, $p < 0.01$).

Figure 1

Figure 2

Figure 3

Figure 4

Aag-2 cells

cultured fat bodies

Figure 5

Figure 6

Figure 7