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

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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.

1. Introduction

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

Many effects of JH rely on its intracellular receptor MET, the product of the *Methoprene-tolerant* (*Met*) gene (Ashok et al., 1998; Jindra et al., 2013). MET contains an N-terminal basic helix-loop-helix (bHLH) DNA recognition motif, followed by two spaced Per-ARNT-Sim (PAS) domains (PAS-A and PAS-B) (Ashok et al., 1998). In the presence of JH, MET binds to another bHLH-PAS domain protein called Taiman (TAI) (also known previously as FISC in *Aedes aegypti* and SRC in *Tribolium castaneum*) (Charles et al., 2011; Li et al., 2011; Zhang et al., 2011). TAI acts as the obligatory DNA binding partner of MET; the MET-TAI complex recognizes an E-box like sequence (5′-GCACGTG-3′) in the regulatory regions of JH target genes, leading to their transcriptional activation (Kayukawa et al., 2012; Li et al., 2014). In adult female *A. aegypti* mosquitoes, expression of the early trypsin (*AaET*) gene and the Krüppel homolog 1 (*AaKr-h1*) gene is induced by JH via the MET-TAI complex (Li et al., 2014, 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, 1997). Kr-h1 is a zinc finger transcription factor that

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.

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mediates the anti-metamorphic action of JH in phylogenetically distant insect orders (Konopova et al., 2011; Lozano and Belles, 2011; Minakuchi et al., 2009, 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 *A. 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 *A. 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 μM; RO31-8220 (Santa Cruz Biotechnology), 10 μM; Gö6983 (Santa Cruz Biotechnology), 10 μM; KT5720 (Santa Cruz Biotechnology), 10 μM; U73122 (EMD Millipore), 1 μM. Phorbol-12-myristate-13-acetate (PMA, Sigma Aldrich) was used at a final concentration of 10 μM.

2.2. Cell culture

A. 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 *A. 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 × 2) were aligned to the *A. 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 *A. aegypti* mosquitoes as described previously (Zhu et al., 2003). Briefly, female mosquitoes were injected with 0.5 μg 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 μg dsRNA per well. Cells were then incubated for 3 days before adding JH-III (5 μM) and incubating for an additional hour, followed by RNA extraction and real-time PCR analysis.

2.7. Luciferase reporter assay

pCMA-GAL4 and UAS × 4-188-cc-luc were from DrLucy Cherbas (Hu et al., 2003). Construction of pCMA-AaMET, pCMA-AaTAI and 4 × JHRE1-luc has been explained previously (Li et al., 2014, 2011). Aag-2 cells were cultured in 48-well plates and were transfected with pCMA-AaMET (0.2 μg per well), pCMA-AaTAI (0.2 μg per well),