The Steroid Hormone 20-Hydroxyecdysone via Nongenomic Pathway Activates Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase II to Regulate Gene Expression*

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Background: 20E triggers calcium signaling to regulate 20E response gene expression.

Results: 20E induces CaMKII phosphorylation and nuclear translocation, which maintains USP1 lysine acetylation by regulating HDAC3 phosphorylation and nuclear export.

Conclusion: CaMKII transmits the 20E signal from cell membrane to nucleus.

Significance: This study reveals that a steroid hormone, via GPCR activation and calcium signaling, regulates USP1 lysine acetylation for gene transcription.

The steroid hormone 20-hydroxyecdysone (20E) triggers calcium signaling pathway to regulate 20E response gene expression, but the mechanism underlying this process remains unclear. We propose that the 20E-induced phosphorylation of Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) serves an important function in 20E response gene transcription in the lepidopteran insect Helicoverpa armigera. CaMKII showed increased expression and phosphorylation during metamorphosis. 20E elevated CaMKII phosphorylation. However, the G protein-coupled receptor (GPCR) and ryanodine receptor inhibitor suramin, the phospholipase C inhibitor U73122, and the inositol 1,4,5-triphosphate receptor inhibitor xestospongin C suppressed 20E-induced CaMKII phosphorylation. Two ecdysone-responsive GPCRs and G\(\alpha_q\) protein were involved in 20E-induced CaMKII phosphorylation by RNA interference analysis. 20E regulated CaMKII threonine phosphorylation at amino acid 290, thereby inducing CaMKII nuclear translocation. CaMKII knockdown by dsCaMKII injection into the larvae prevented the occurrence of larval-pupal transition and suppressed 20E response gene expression. CaMKII phosphorylation and nuclear translocation maintained USP1 lysine acetylation at amino acid 303 by inducing histone deacetylase 3 phosphorylation and nuclear export. The lysine acetylation of USP1 was necessary for the interaction of USP1 with EcR\(B1\) and their binding to the ecdysone response element. Results suggest that 20E (via GPCR activation and calcium signaling) activates CaMKII phosphorylation and nuclear translocation, which regulate USP1 lysine acetylation to form an EcR\(B1\)-USP1 complex for 20E response gene transcription.

Steroid hormones can diffuse fairly freely into the nucleus of target cells through the cell membrane because they are fat-soluble molecules; then these hormones bind to nuclear steroid hormone receptors to greatly affect gene expression (1). However, increasing evidence suggests that the steroid hormones could induce nongenomic signaling through the plasma membrane. For instance, estrogen triggers a signaling cascade through activating the GPR30 (G protein-coupled receptor 30) in the endoplasmic reticulum (2). GPR30 may be translocated to the plasma membrane and mediate CAMP production (3). Estrogen also induces calcium influx, which leads to the activation of cytokines and growth factors (4, 5). In the lepidopteran insect Helicoverpa armigera, the steroid hormone 20-hydroxyecdysone (20E) regulates calponin phosphorylation and its nuclear translocation (6). 20E triggers an intracellular calcium ion increase through an ecdysone-responsive GPCR (ErGPRC, here renamed ErGPRC1 to distinguish from ErGPRC2) (7). 20E, via G\(\alpha_q\) protein activation, modulates an increase of intracellular Ca\(^{2+}\) (8). 20E regulates gene transcription via the ErGPRC, G\(\alpha_q\) phospholipase C\(\gamma\)1, calcium, and PKC nongenomic pathway (9). In addition, another ecdysone-responsive GPCR, designated as ErGPRC2, also serves a function in 20E-triggered nongenomic biological processes in H. armigera. Previous studies showed that animal steroids initiate cellular responses rapidly via a nongenomic pathway. However, information on the underlying mechanism of the steroid hormone-triggered nongenomic pathway is lacking, especially information on calcium signaling and the key molecules involved in the pathway.

Compared with the nongenomic pathway, the 20E genomic pathway is well studied; this pathway starts from the binding of

- **The abbreviations used are:** 20E, 20-hydroxyecdysone; EcR, ecdysone nuclear receptor; USP, ultraspiracle protein; dsRNA, double-stranded RNA; GPCR, G protein-coupled receptor; ErGPRC, ecdysone-responsive GPCR; CaMKII, Ca\(^{2+}\)/calmodulin-dependent protein kinase II; HDAC, histone deacetylase; qRT-PCR, quantitative real time RT-PCR; NTA, nitrilotriacetic acid; PLC, phospholipase C.

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20E up to the nuclear ecdysone receptor (EcR), after which a heterodimeric transcriptional EcR-USP complex with the ultraspiracle protein (USP) is formed (10). The formation of transcriptional complex EcRB1-USP1 is regulated by 20E via nongenomic signaling (11, 12). The EcR-USP complex can bind to ecdysone response elements (EcRE), which are located at the 5′ promoter region of 20E response genes for initiating gene transcription (12, 13). These 20E response genes include the transcription factor Broad (Br) in Bombyx mori (13), also known as Br-C (broad complex) (14), and the transcription factor HR3 (hormone receptor 3) in H. armigera (12). Br initiates metamorphosis in Manduca sexta and Drosophila melanogaster (15). Broad Z7 (BrZ7) promotes larval-pupal transition in H. armigera (16). HR3 in M. sexta is the early delay gene in the 20E genomic pathway (17) and is recognized as a central regulator in 20E-driven developmental switches during insect development and metamorphosis (18). HR3 could also mediate the expressions of EcRB1 and USP1 in Aedes aegypti (19). The EcRE of H. armigera HR3 and the red fluorescence protein (RFP) are used to construct the 20E response reporter plasmid, which can be used to detect 20E-induced EcRB1-USP1-dependent gene transcription in the genomic pathway (12). These studies provide a basis for further study of the mechanism underlying the nongenomic pathway and the connection between genomic and nongenomic pathways.

The Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), a serine/threonine kinase, serves an important function in calcium signaling (20). CaMKII can be activated by Ca²⁺ and calmodulin, and activation leads to the autophosphorylation of CaMKII at amino acid threonine 287 (or 286 in different isoforms) in mammalian cells (21). CaMKII, which may be located in the cytosol, cytoskeleton (22), and nucleus (23), responds to the elevation of intracellular calcium ion concentration (24) and mediates a variety of biological processes, including neurotransmitter synthesis (25), neurotransmitter exocytosis (26), and ion channel regulation in mammalian (27) and insect cells (28). CaMKII induces histone deacetylase 4 (HDAC4) phosphorylation and nuclear export, which keep the target protein acetylation regulated by histone acetyltransferases (29). The acetylation of histone catalyzed by histone acetyltransferases results in loose nucleosomes structure and promotes gene activation (30). By contrast, the deacetylation of histone catalyzed by HDACs leads to chromatin condensation and transcriptional repression (31). In addition, HDACs can regulate a variety of cellular processes by regulating a variety of non-histone protein deacetylations, some of which are transcription factors and co-regulators, e.g. nuclear receptor corepressor SMRT (silencing mediator of retinoid and thyroid hormone receptors) (32) and MEF2 (myocyte enhancer factor 2) (33). Therefore, CaMKII can be used as a target in studies on the nongenomic pathway and those on the connection between the genomic and nongenomic pathways of the steroid hormone.

We examined the CaMKII expression profile and hormonal regulation on the CaMKII expression level, nuclear translocation, and phosphorylation. We also studied the mechanism by which CaMKII regulated the 20E response gene expression. 20E promoted CaMKII phosphorylation via GPCR, Gαq, phospholipase C (PLC), and calcium signaling. The phosphorylated CaMKII transferred into the nucleus to induce HDAC3 phosphorylation and translocation from the nucleus to the cytosol, which maintained USP1 lysine acetylation. The acetylation of USP1 was necessary for the formation of the 20E-induced EcRB1-USP1 transcription complex. Our results suggest that 20E regulates CaMKII phosphorylation via a nongenomic pathway for gene transcription in the genomic pathway.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**The following reagents were purchased for analyses: pET-32a vector system (Promega Corporation, Madison, WI), pEx-4–His vector system (containing a His tag) (provided by Dr. Marek Jindra, Biology Center, Academy of Sciences of the Czech Republic), restriction enzymes (Thermo Fisher Scientific, Lithuania), DNA polymerase (TransGen Biotech, Beijing, China), Unizol reagent (Bioray, Shanghai, China), protein A resin (GenScript, Piscataway, NJ), first strand cDNA synthesis kit (BioTeke Corporation, Beijing, China), 20E (Sigma), PCR primers (Sangon Biotech, Shanghai, China), gene sequencing primer kit (BGI, Shenzhen, China), and UltraSYBR Mixture (With ROX) (Beijing ComWin Biotech Co. Ltd., Beijing, China). Other chemicals were of analytical reagent grade and were purchased in China.

**Insect—**The cotton bollworms (H. armigera) were fed on an artificial diet at 27 ± 1 °C and exposed to a light/dark photoperiod of 14 h/10 h, as described by Zhao et al. (34). The cotton bollworms were obtained from the Wuhan Institute of Virology of the Chinese Academy of Sciences (Wuhan, China).

**Cell Culture—**The HaEpi cell line, a Helicoverpa epidermal cell line, was obtained from the H. armigera integument and has been well characterized previously. This cell line has been used as a platform to investigate hormonal regulation during lepidopteran insect development. HaEpi cells were developed as a loosely attached monolayer and were maintained at 27 ± 1 °C with Grace’s medium containing 10% FBS (Invitrogen) (35).

**Bioinformatics Analysis—**CaMKII was obtained by transcriptome sequencing of the HaEpi cells cDNA library, which was established in our laboratory (GenBank™ accession no. KJ650044). Protein translation and prediction were achieved using Expasy software. cDNA and encoded protein were analyzed by performing a BLAST search in the NCBI database.

**Preparation of Antiserum against CaMKII—**By using the corresponding primers (Table 1), the cDNA fragment encoding a part of the CaMKII was amplified from H. armigera and was inserted into the expression vector pET-32a (+). The recombinant plasmid was transformed into Escherichia coli DH5α cells and then isolated and transformed into E. coli Rosetta host cells. Isopropyl-β-D-thiogalactopyranoside (0.5 mM) was used to induce the production of target proteins by the host cells in the LB medium (containing 1% tryptone, 0.5% yeast extract, and 1% NaCl). The recombinant CaMKII protein was purified using a Ni²⁺-NTA affinity column (GE Healthcare) and used as antigen for antiserum preparation. The antiserum specificity was examined by immunoblotting analysis.

**Western Blot—**The total protein of cells or larvae tissue was extracted using TBS, which contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride.
The protein was centrifuged at 10,000 × g at 4 °C for 10 min. The supernatant was collected, and the protein concentration was measured according to Bradford’s method. The 20 μg of total protein of each sample was loaded on 7.5% to 12.5% SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. The membrane was incubated with a blocking buffer (2% skim milk in TBS) for 1 h at room temperature, after which primary antibodies were diluted with blocking buffer for 2–4 h. The membrane was washed thrice with TBST (0.02% Tween in TBS) for 10 min each time, and the second antibody of alkaline phosphatase-conjugated AffiniPure horse anti-rabbit/anti-mouse IgG was diluted to 1:10,000 with the same blocking buffer. The membrane was washed with TBST thrice (for 10 min each time) and subsequently washed with TBS thrice (for 5

### TABLE 1
Oligonucleotide sequences of PCR primers

| Primer name | Oligonucleotide sequence (5′ → 3′) |
|-------------|----------------------------------|
| CaMKII-RNA F | 5′-gccatatattgagatgaggtcctggtc-3′ |
| CaMKII-RNA R | 5′-gcagcagctgcgcgctatttt-3′ |
| ErGPCR1-RNA F | 5′-gcacgctgcttggcgagggggc-3′ |
| ErGPCR1-RNA R | 5′-ctgtgtgctgggggtaagc-3′ |
| ErGPCR2-RNA F | 5′-gcgctgatcagcagctgcggg-3′ |
| ErGPCR2-RNA R | 5′-gctgtgtgcttggtgagc-3′ |
| GFP-RNA F | 5′-gcagcagctgattggcgagggggg-3′ |
| GFP-RNA R | 5′-ctgtgtgctgtggtgagc-3′ |
| Gaβ-RNA F | 5′-gcagcagctgattggcgagggggg-3′ |
| Gaβ-RNA R | 5′-ctgtgtgctgtggtgagc-3′ |
| HDAC3-RNA F | 5′-gcagcagctgattggcgagggggg-3′ |
| HDAC3-RNA R | 5′-ctgtgtgctgtggtgagc-3′ |
| HDAC4-RNA F | 5′-gcagcagctgattggcgagggggg-3′ |
| HDAC4-RNA R | 5′-ctgtgtgctgtggtgagc-3′ |
| HDAC6-RNA F | 5′-gcagcagctgattggcgagggggg-3′ |
| HDAC6-RNA R | 5′-ctgtgtgctgtggtgagc-3′ |
| Overexpression | 5′-gccatatattgagatgaggtcctggtc-3′ |
| CaMKII-OE F | 5′-gcagcagctgcgcgctatttt-3′ |
| CaMKII-OE R | 5′-ctgtgtgctgggggtaagc-3′ |
| EcRB1-OE F | 5′-gcagcagctgattggcgagggggg-3′ |
| EcRB1-OE R | 5′-ctgtgtgctgtggtgagc-3′ |
| HDAC3-OE F | 5′-gccatatattgagatgaggtcctggtc-3′ |
| HDAC3-OE R | 5′-gcagcagctgcgcgctatttt-3′ |
| USP1-OE F | 5′-gcagcagctgattggcgagggggg-3′ |
| USP1-OE R | 5′-ctgtgtgctgtggtgagc-3′ |
| qRT-PCR | 5′-gccatatattgagatgaggtcctggtc-3′ |
| BrZ7-qRT F | 5′-gcagcagctgcgcgctatttt-3′ |
| BrZ7-qRT R | 5′-ctgtgtgctgggggtaagc-3′ |
| CaMKII-qRT F | 5′-gcagcagctgattggcgagggggg-3′ |
| CaMKII-qRT R | 5′-ctgtgtgctgtggtgagc-3′ |
| EcRB1-qRT F | 5′-gcagcagctgattggcgagggggg-3′ |
| EcRB1-qRT R | 5′-ctgtgtgctgtggtgagc-3′ |
| ErGPCR1-qRT F | 5′-gcagcagctgattggcgagggggg-3′ |
| ErGPCR1-qRT R | 5′-ctgtgtgctgtggtgagc-3′ |
| ErGPCR2-qRT F | 5′-gcagcagctgattggcgagggggg-3′ |
| ErGPCR2-qRT R | 5′-ctgtgtgctgtggtgagc-3′ |
| USP1-qRT F | 5′-gccatatattgagatgaggtcctggtc-3′ |
| USP1-qRT R | 5′-gcagcagctgcgcgctatttt-3′ |

#### ChIP assay

| Primer name | Oligonucleotide sequence (5′ → 3′) |
|-------------|----------------------------------|
| EcRB1-P F | 5′-gccatatattgagatgaggtcctggtc-3′ |
| EcRB1-P R | 5′-gcagcagctgcgcgctatttt-3′ |

#### Prokaryotic expression

| Primer name | Oligonucleotide sequence (5′ → 3′) |
|-------------|----------------------------------|
| CaMKII-exp F | 5′-gccatatattgagatgaggtcctggtc-3′ |
| CaMKII-exp R | 5′-gcagcagctgcgcgctatttt-3′ |

#### Site-directed mutagenesis

| Primer name | Oligonucleotide sequence (5′ → 3′) |
|-------------|----------------------------------|
| CaMKII T290A F | 5′-gccatatattgagatgaggtcctggtc-3′ |
| CaMKII T290A R | 5′-gcagcagctgcgcgctatttt-3′ |
| USP1 K58R F | 5′-gccatatattgagatgaggtcctggtc-3′ |
| USP1 K58R R | 5′-gcagcagctgcgcgctatttt-3′ |
| USP1 K71R F | 5′-gccatatattgagatgaggtcctggtc-3′ |
| USP1 K71R R | 5′-gcagcagctgcgcgctatttt-3′ |
| USP1 K303R F | 5′-gccatatattgagatgaggtcctggtc-3′ |
| USP1 K303R R | 5′-gcagcagctgcgcgctatttt-3′ |
using corresponding primers (Table 1) and then inserted into the cells for 6–12 h with the help of the DNAfectin transfection reagent. Subsequently, the cells were replenished with a complete medium. After being cultured in full nutrient culture medium for 24 h, 20E was added to the cells at a final concentration of 2 μM, according to the method used in our previous work. The control cells received an equivalent volume of DMSO, which was used as a solvent for 20E. The pEx4-RFP-His/pEx4-His vector was transfected and served as the negative control. The α-phosphatase (Milipore, Temecula, CA) treatment was conducted using protein extracts from HaEpi cells treated with 2 μM 20E for 0.5 h. The gel concentration of SDS-PAGE was 7.5%. β-Actin was used as the protein control using antiserum against β-actin in H. armigera.

**Co-immunoprecipitation (Co-IP)—** The ORFs of EcRB1 (GenBank accessions EU526831 and USP1 (EU526832) were amplified from H. armigera using corresponding primers (Table 1) and were then inserted into the pIEx-4-RFP-His and pIEx4-His vectors, respectively. The reconstructed plasmids were transfected into the cells, as described above. The antibody against RFP (1 μl) and PBS (400 μl) was added to the resin-antibody complex and incubated for 2–4 h with gentle shaking at 4 °C. The supernatant was added to protein A resin to eliminate nonspecific binding and harvested by centrifugation. The supernatant was washed with 500 μl of PBS thrice. HaEpi cells were transfected with dsCaMKII for RNA interference. The dsGFP was used as the negative control, as previously shown. The cells were treated with 20E for 1 h, and DMSO was used as the control. The protein was extracted from cells using radioimmunoprecipitation assay buffer containing 0.1 M Tris-HCl buffer (pH 8.0), 150 mM NaCl, and 1% Nonidet P-40. The supernatant was harvested at centrifugation at 12,000 × g for 10 min (4 °C). The supernatant was added to protein A resin to eliminate nonspecific binding and harvested by centrifugation. The supernatant was added to the resin-antibody complex and incubated for 2–4 h with gentle shaking at 4 °C. The supernatant was harvested using antiserum against RFP (Zhongshan, Beijing, China) and His (Zhongshan, Beijing, China) against EcRB1-RFP and USP-His, respectively.

**RNAi in the HaEpi Cell Line—** The MEGAscript RNAi kit (Ambion, Austin, TX) was used to generate dsRNA. The dsRNA was transcribed from PCR templates of the CaMKII (or other genes) at 37 °C for 4 h (primers are shown in Table 1), according to the manufacturer’s instructions. DNsase I was used to remove DNA from the dsRNA solution. dsGFP was transcribed utilizing GFP DNA as a template and used as a nonspecific RNAi control. The dsRNA concentration was determined by spectrophotometrical analysis at 260 nm. For the transfection of dsRNA into the cell line, HaEpi cells were seeded in 6-well plates with 5 × 10^5 cells/well. The RNAfectin transfection reagent (Tiangen, Beijing, China) was used for dsRNA transfection according to the manufacturer’s instructions. The final dsRNA concentration was 1 μg/ml in the medium without FBS. After incubation at 27 °C for 6–12 h, the cells were replenished with a complete medium and were used for experiments.

**RNAi in H. armigera Larvae—** The H. armigera larvae were selected for dsCaMKII injection (dsGFP injection as control) at 6 h after the appearance of the sixth instar. The larvae were randomly separated into two groups with 30 larvae/group, and three independent experiments were performed. Each larva in the experimental group was injected with dsCaMKII (1 μg), whereas each larva in the negative control group was injected with dsGFP. The statistical data of the larval-pupal transition phenotypes of each group were obtained. mRNA was extracted from the sixth instar larvae at 120 h for qRT-PCR analysis.

**Overexpression and Phosphorylation Analysis of CaMKII—**

The CaMKII ORF or mutation (obtained by site-directed mutagenesis of CaMKII in vitro) was amplified from H. armigera using corresponding primers (Table 1) and then inserted into the pIEx-4-His vector or pIEx4-RFP-His vector (pIEx4-His vector fusing with RFP). HaEpi cells were maintained at 80% confluence under normal growth conditions, as previously described by Shao et al. (35). The reconstructed plasmids were transfected into the cells for 6–12 h with the help of the DNAfectin transfection reagent. Subsequently, the cells were replenished with a complete medium. After being cultured in full nutrient culture medium for 24 h, 20E was added to the cells at a final concentration of 2 μM, according to the method used in our previous work. The control cells received an equivalent volume of DMSO, which was used as a solvent for 20E. The pEx4-RFP-His/pIEx4-His vector was transfected and served as the negative control. The α-phosphatase (Milipore, Temecula, CA) treatment was conducted using protein extracts from HaEpi cells treated with 2 μM 20E for 0.5 h. The gel concentration of SDS-PAGE was 7.5%. β-Actin was used as the protein control using antiserum against β-actin in H. armigera.

Hormonal regulation in H. armigera larvae—20E was initially dissolved in DMSO to prepare a 10 mg/ml solution and subsequently diluted with sterile PBS (140 mM NaCl and 10 mM sodium phosphate, pH 7.4) at 1:100. The sixth instar larvae at 6 h were injected with 20E at 500 ng/larva. The controls were treated with an equivalent amount of DMSO at the same stage. The total protein was isolated from the integument, midgut, and fat body of larvae (3 to 5 larvae) after the insects were treated with 20E for 0.25, 0.5, 1, 3, or 6 h. The proteins were used for Western blot analysis.

The gel concentration of SDS-PAGE was 7.5%. β-Actin was used as the protein control using antiserum against β-actin in H. armigera.
20E Regulates Gene Expression through CaMKII Signaling

HDAC3 Phosphorylation Levels Detection—HDAC3-RFP-His was overexpressed in HaEpi cells, and the cells were transfected with dsCaMKII (1 μg/ml in the medium) for 12 h. The dsMock was the nonspecific dsRNA control. Finally, the cells were treated with 20E at 2 μM for 0.5 h. Equal volumes of DMSO were used for the negative control. HDAC3-RFP-His was purified by Ni²⁺-NTA affinity column for detecting phosphorylation levels. The number of moles of phosphorus per mole of HDAC3-RFP-His was determined using the Phospho-protein phosphate estimation assay kit (Sangon Biotech, Shanghai, China) based on the alkaline hydrolysis of phosphate from seryl and threonyl residues in phosphoproteins. The released phosphate was quantified in a 96-well microplate according to the manufacturer’s instructions.

Chromatin Immunoprecipitation Assay—The USP1-His, USP1-K303R-His, or pIEx-4-RFP-His (as negative control) plasmids were transfected into the cells. Subsequently, the cells were treated with 20E for 3 h. DMSO treatment was used as the control. Formaldehyde (37%) was added to the cells at a final concentration of 0.5% for cross-linking at 37 °C for 10 min. Glycine was subsequently added at a final concentration of 125 mM at room temperature for 10 min. The cells were washed twice with ice-cold PBS, harvested by centrifugation, and then suspended in 200 μl of SDS lysis buffer containing 1% SDS, 10 mM EDTA, and 50 mM Tris-HCl (pH 8.1). Ultrasonication was performed to break up the genomic DNA into 200–1,000-bp fragments. After centrifugation, the supernatant was pre-cleared with protein A resin at 4 °C for 1 h. After centrifugation, 20 μl of supernatant was used as an input sample for qRT-PCR (negative control sample). The remaining supernatant (180 μl) was incubated with anti-His antibody for 12 h. The protein A resin was added to collect the protein and DNA complex at 4 °C. The complex was washed as follows: once with low salt buffer containing 200 mM Tris-HCl (pH 8.0), 2 mM EDTA, 150 mM NaCl, 0.1% SDS, and 1.0% Triton X-100; once with high salt wash buffer containing 20 mM Tris-HCl (pH 8.0), 250 mM LiCl, 1 mM EDTA, 1% Nonidet P-40, and 1% deoxycholate; and twice with Tris-EDTA buffer containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The proteins were eluted with buffer (1% SDS and 100 mM NaHCO₃). The DNA-protein cross-links were reversed at 65 °C overnight and treated with RNase A and proteinase K at 56 °C for 2 h. DNA was purified and analyzed by qRT-PCR to detect the EcRB1-USP1-binding element in the upper band at the metamorphic stage. CaMKII protein was increased at the fifth instar molting and sixth instar metamorphosis stages of the different tissues, i.e. integument, midgut, and fat body. The results of qRT-PCR showed that the mRNA levels of CaMKII also increased at fifth instar molting and sixth instar metamorphic stages (Fig. 1A). The abovementioned results showed that the expression profile of CaMKII is in agreement with the 20E titer in Lepidoptera (10), thereby implying that CaMKII plays a role in metamorphosis.

To test whether the upper band was post-translationally modified after induction by 20E, the sixth instar larvae at 6 h were injected with 20E or DMSO (solvent control). The expression levels of the upper band were significantly up-regulated in the three tissues (compared with the DMSO control) by 20E induction for 15 min (Fig. 1B). In addition, the molecular weight of the upper band was degraded to the molecular weight of the lower band by lambda phosphatase treatment (Fig. 1C), thereby indicating that the upper band is the phosphorylated CaMKII. These results suggest that CaMKII expression and phosphorylation levels are up-regulated by 20E in the metamorphic stage.

To address the pathway of 20E-regulating CaMKII phosphorylation, the intracellular calcium concentration increase in HaEpi cells was blocked by inhibitors, including the GPCRs and rydnone receptor inhibitor suramin, receptor tyrosine kinase inhibitor SU6668, PLC inhibitor U73122, and inositol 1,4,5-triphosphate receptor inhibitor xestospongin C (XeC). To exclude the effects of gene transcription induced by 20E, the time of 20E induction was limited to 30 min. Compared with the DMSO treatment, 20E increased the phosphorylation level of CaMKII. Suramin, U73122, and XeC significantly inhibited 20E-increased CaMKII phosphorylation, but inhibition was not observed under the SU6668 treatment. Suramin blocked both GPCR (36) and rydnone receptor signaling (37). Therefore, the two ecdysone-responsible GPCRs (ErGPCR1 and ErGPCR2) and Go₉ protein were knocked down by RNAi in HaEpi cells to address the involvement of GPCR in 20E-induced CaMKII phosphorylation. The knockdown of ErGPCR1, ErGPCR2, and Go₉ suppressed 20E-induced CaMKII phosphorylation (Fig. 2A). The efficacy of RNAi was confirmed by semiquantitative RT-PCR analysis (Fig. 2A, panel a). These results suggest that 20E regulates CaMKII phosphorylation through the GPCR, Go₉, PLC, and calcium signaling.

The Thr²⁸⁷ is a key phosphorylation site for its activation in mammalian CaMKII (21), and the Thr²⁹⁰ in H. armigera CaMKII is the conserved threonine with mammalian Thr²⁸⁷ by homologous analysis. To understand the consequence of the CaMKII phosphorylation, CaMKII and its mutant CaMKII-T290A (threonine was mutated to alanine at 290 site) were overexpressed by pIEx-4-RFP-His vector in the HaEpi cells. In the DMSO control, CaMKII-RFP-His was mainly distributed in the cytoplasm and partially translocated into the nucleus by 20E induction in 0.5 h. However, when threonine was mutated to alanine at the 290 site, CaMKII-T290A-RFP-His was not translocated into the nucleus by 20E induction. The overexpression of RFP alone by pIEx-4-RFP-His plasmid did not change subcellular location under 20E (Fig. 2B). Western blot showed that the nuclear located CaMKII was phosphorylated, whereas the cytoplasmatic located CaMKII was not phosphorylated at Thr²⁹⁰ (Fig. 2C). The 20E-induced phosphorylation site of CaMKII at Thr²⁹⁰ was confirmed with the overexpression of CaMKII-RFP-His and CaMKII-T290A-RFP-His in HaEpi cells, as shown by the results of Western blot analysis (Fig. 2D). The overexpression of
RFP alone by plEx-4-RFP-His plasmid showed no phosphorylation by 20E induction (Fig. 2D, panel d). These results suggest that 20E induces CaMKII phosphorylation in the 20E pathway. CaMKII Knockdown Represses 20E Response Gene Expression and Pupation—To understand the role of CaMKII in the 20E pathway, CaMKII was knocked down by injecting dsCaMKII into the sixth instar larvae at 6 h and transfecting dsCaMKII into the HaEpi cells. Compared with the dsGFP injection control, 31.8% of larvae did not shed off the old cuticle, formed larval-pupal chimera, and failed to pupate after CaMKII was knocked down (Fig. 3, A and B). The growth time from sixth instar larva to pupa after CaMKII knockdown was delayed (data not shown). The expression levels of the 20E pathway genes, including 20E nuclear receptor EcR/B, heterodimeric protein USP1, and transcription factors HR3 and BrZ7, decreased after CaMKII knockdown (Fig. 3C). Similar results were found in the HaEpi cells after CaMKII knockdown (Fig. 3D). Therefore, CaMKII serves an important function in metamorphosis and 20E-regulated gene expression.

CaMKII Regulates USP1 Lysine Acetylation by Mediating HDAC3 Nuclear Export in 20E Induction—To reveal how CaMKII regulates gene expression in the nucleus in the 20E pathway, the post-transcriptional modification of transcriptional factor USP1 was investigated using Western blot. We first examined the possibility of USP1 phosphorylation regulated by CaMKII. However, the CaMKII knockdown failed to affect the 20E-induced USP1 phosphorylation (data not shown). Because CaMKII could regulate the transcription factor lysine acetylation (29) and USP1 was predicted to be acetylated at Lys58, Lys71, and Lys303, the possibility of USP1 lysine acetylation regulation by CaMKII was examined by overexpressing USP1-His (USP1 was cloned into plEx-4-His vector containing a His tag) in the HaEpi cells. In the input, USP1-His was equally detected in various treated cells, including dsCaMKII-treated cells. The USP1-His lysine acetylation was detected in the 20E induction or 20E + dsGFP treatment by Western blot using the anti-acetylation lysine antibody. However, when CaMKII was knocked down by dsCaMKII treatment in the HaEpi cells, 20E failed to induce USP1-His lysine acetylation (Fig. 4A). The plEx-4-His vector was overexpressed separately to confirm that lysine acetylation occurred in USP1, but not in His tag (Fig. 4A, panel a). Therefore, CaMKII regulates USP1 lysine acetylation in 20E induction.

To determine the 20E-induced lysine acetylation site in USP1, three predicted lysine acetylation sites at amino acids 58,
71, and 303 of USP1 were mutated from lysine to arginine, namely, K58R, K71R, and K303R. In the input, the USP1-His, USP1-K58R-His, USP1-K71R-His, and USP1-K303R-His were equally overexpressed in the HaEpi cells, respectively. The 20E-induced lysine acetylation level was not reduced by USP1- K58R-His and USP1-K71R-His mutations but was reduced by the USP1-K303R-His mutation (Fig. 4B). Therefore, 20E regulates USP1 lysine acetylation at Lys303.

CaMKII regulates transcriptional factor acetylation by inducing HDACs phosphorylation and nuclear export (29). Depletion of either DHDAC1 or DHDAC3 could promote edcsyne-induced *eip74ef* gene transcription in *D. melanogaster* (38). We obtained three HDAC cDNA sequences (HDAC3, HDAC4, and HDAC6) from HaEpi cells cDNA library and detected the transcript levels of 20E response gene *HR3* after silencing the three genes in HaEpi cells. Therefore, 20E induces HDAC3 nuclear export by inducing CaMKII phosphorylation and nuclear export via acetylation (39), the effect of HDAC3 on USP1 lysine acetylation was studied by co-overexpressing HDAC3-RFP-His in the HaEpi cells, combined with USP1-His in the HaEpi cells, and USP1 knockdown (Fig. 5). Thus, HDAC3 (GenBank™ accession no. KM983338) is suggested to repress 20E-induced gene expression.

HDAC3 was overexpressed with pIEx-4-RFP-His vector in HaEpi cells to study the mechanism underlying HDAC3 repression of 20E-induced gene expression. Compared with the DMSO control and *dsGFP*, 20E could induce HDAC3 nuclear export obviously after 0.5 h of induction. However, when *CaMKII* was knocked down, 20E could not induce HDAC3 nuclear export (Fig. 6A). To address the mechanism of HDAC3 nuclear export induced by 20E, HDAC3 phosphorylation levels were detected using the phosphoprotein phosphate estimation assay kit. Compared with the DMSO and *dsGFP* controls, the number of moles of phosphorus per mole of HDAC3-RFP-His increased under 20E inducs for 0.5 h but was suppressed after *CaMKII* knockdown (Fig. 6B). These results suggest that 20E induces HDAC3 phosphorylation and nuclear export via activating CaMKII.

Because HDAC3 can catalyze transcriptional factor deacetylation in the nucleus (39), the effect of HDAC3 on USP1 lysine acetylation was studied by co-overexpressing HDAC3-RFP-His and USP1-His in the HaEpi cells, combined with *CaMKII* knockdown to block HDAC3-RFP-His translocation from nucleus to cytoplasm. Compared with the pIEx-4-RFP-His overexpressing control, the overexpression of HDAC3-RFP-His showed obvious inhibitory effect on USP1 lysine acetylation under 20E induction (Fig. 6C). These results suggest that
CaMKII regulates USP1 lysine acetylation by inducing HDAC3 nuclear export during 20E induction.

**USP1 Acetylation Determines the Interaction between USP1 and EcRB1 and Their Binding to EcRE—**To address the mechanism of USP1-K303 acetylation serving in the 20E response gene transcription, the protein interaction between EcRB1 and USP1 was examined. In the input, EcRB1-RFP-His and USP1-His were simultaneously and equally overexpressed in the HaEpi cells, including cells with CaMKII knockdown. In the co-immunoprecipitation (Co-IP) using an antibody against RFP to precipitate EcRB1-RFP-His, USP1-His band was detected increasingly in the 20E-induced cells compared with the DMSO control. However, when CaMKII was knocked down, USP1-His band was lighter than that in the dsGFP control during 20E induction (Fig. 7A). pEx-4-RFP-His and pEx-4-His overexpressions were controlled to exclude the possibility of protein interaction caused by His or RFP tag (Fig. 7A, panel a). These results suggest that 20E regulates the interaction between EcRB1-RFP-His and USP1-His through activating CaMKII.

To confirm the involvement of the 20E-induced USP1-His lysine acetylation in the interaction between EcRB1-RFP-His and USP1-His, USP1-His and USP1-K303R-His were separately overexpressed in the HaEpi cells, as shown in the input. USP1-His interacting with EcRB1-RFP-His was detected in Co-IP using an antibody against RFP, whereas USP1-K303R-His failed to interact with EcRB1 under 20E induction (Fig. 7B). Furthermore, USP1-His could bind to the EcRE under 20E induction by ChIP analysis. However, USP1-K303R-His reduced the ability of binding to EcRE. pEx-4-His was overexpressed as a control to exclude the possibility of His tag binding to EcRE (Fig. 7C). Therefore, USP1-K303 acetylation is required for the formation of EcRB1-USP1 transcriptional complex and increases the ability of the complex to bind to the EcRE in the 20E pathway.

**DISCUSSION**

20E Induces a Rapid Increase of Ca$^{2+}$ Ion Concentration in the Cytoplasm of the HaEpi Cell Line through ErGPCR Signaling (7). However, the function and mechanism of the increase of intracellular calcium triggered by 20E nongenomic signaling pathway are still unclear. It is well known that USP1 can be phosphorylated by PKC through ErGPCR and PLC signaling pathways (9, 40). However, information on 20E-induced lysine acetylation of USP1 has been limited. The present study showed that 20E regulates CaMKII phosphorylation at the Thr$^{290}$ site for nuclear translocation via ErGPCR, Go$_{q}$, PLC, and calcium signaling. Subsequently, the phosphorylated CaMKII regulates USP1 lysine acetylation at the Lys$^{303}$ site through inducing HDAC3 phosphorylation and nuclear export, which is necessary for the interaction between USP1 and EcRB1 and their binding to EcRE. Therefore, nongenomic pathway is
20E Regulates Gene Expression through CaMKII Signaling

The catalytic domain and regulatory region are tightly associated at basal resting state, thereby resulting in autoinhibition of the kinase activity of CaMKII (41). The C-terminal subunit association domain is responsible for the assembly of subunits into large multimers (8–14 subunits) that can comprise one or several different isoforms (42). The Thr287 in the regulatory region of mammalian CaMKII can be rapidly autophosphorylated by the binding of calcium and calmodulin (Ca\(^{2+}/\mathrm{CaM}\)), followed by the phosphorylation of the secondary sites (Thr\(^{305}\) and Thr\(^{306}\)), thus CaMKII is sustainably activated (21). CaMKII in H. armigera also had an N-terminal serine-threonine kinase catalytic domain (amino acids 17–275), a regulatory region (amino acids 281–318), and a C-terminus (amino acids 388–510). Thr\(^{290}\) was conserved with mammalian CaMKII autophosphorylation site Thr287, and Thr\(^{305}\) and Thr\(^{310}\) were conserved with mammalian CaMKII Ca\(^{2+}/\mathrm{CaM}\) binding sites Thr\(^{305}\) and Thr\(^{306}\) in the regulatory region. Previous studies have proved that 20E triggers the rapid increase of intracellular calcium ion concentration through ErGPCR and PLC signaling pathways (7, 9), and such a phenomenon is one of the important features of nongenomic pathway. In the present study, we further demonstrated that 20E induced CaMKII phosphorylation at the Thr287 site in 30 min via ErGPCRs, G\(\alpha_q\), PLC, and calcium signaling pathways, thereby illustrating the output of the increase of intracellular calcium ion under 20E induction.

CaMKII is localized in specific tissues and subcellular compartments with different functions (21). CaMKII contained a nuclear localization signal in many species (43). However, CaMKII in D. melanogaster was detected without canonical nuclear localization signal in nuclear extracts (21). Similarly, nuclear localization signal was not detected in the amino acid sequence of H. armigera CaMKII. Nonetheless, CaMKII-RFP-His could still be partially transported into the nucleus in a phosphorylated form during 20E induction. Results suggest that CaMKII nuclear translocation is dependent on its phosphorylation in 20E-induced signaling, which is confirmed by site-directed mutant CaMKII-T290A.

20E Regulates USP1 Lysine Acetylation for the Gene Transcription Initiation via CaMKII—CaMKII regulates gene transcription initiation via directly phosphorylating transcription factors, such as CAMP response element binding protein (44) and serum response factor (45). However, in the present study, the direct phosphorylation of USP1 mediated by CaMKII was not detected. CaMKII, as a serine/threonine kinase in nucleus, can also phosphorylate HDAC4 and promote its outflux from the nucleus (44). In the nucleus, HDAC4 binds to transcription factors, such as serum response factor, and induces histone deacetylation. Histone deacetylation promotes chromatin condensation and favors transcriptional repression (29), whereas histone acetylation relays the nucleosome structure and favors gene activation (30). In addition, as a coressor, HDAC4 can regulate deacetylation of transcription factors, such as transcription factor forkhead box protein O (46). In Drosophila S2 cells, the RNAi-mediated depletion of the various HDACs (DHDAC1, DHDAC2, DHDAC3, DHDAC4, and DHDACX) revealed that only the depletion of HDAC1 or HDAC3 affected transcription, and depletion of HDAC3 caused the up-regulation of 29 genes, including the ecysodine-induced eip74af gene

involved in 20E-induced ErRBl1-USP1-dependent transcription initiation in genomic pathway triggered by 20E.

20E Induces CaMKII Phosphorylation and Nuclear Translocation via Nongenomic Pathway—CaMKII is a multimeric protein, and each monomer has three domains, i.e., N-terminal catalytic domain, regulatory region, and C-terminal subunit association domain. The catalytic domain and regulatory

region are tightly associated at basal resting state, thereby resulting in autoinhibition of the kinase activity of CaMKII (41). The C-terminal subunit association domain is responsible for the assembly of subunits into large multimers (8–14 subunits) that can comprise one or several different isoforms (42). The Thr\(^{287}\) in the regulatory region of mammalian CaMKII can be rapidly autophosphorylated by the binding of calcium and calmodulin (Ca\(^{2+}/\mathrm{CaM}\)), followed by the phosphorylation of the secondary sites (Thr\(^{305}\) and Thr\(^{306}\)), thus CaMKII is sustainably activated (21). CaMKII in H. armigera also had an N-terminal serine-threonine kinase catalytic domain (amino acids 17–275), a regulatory region (amino acids 281–318), and a C-terminus (amino acids 388–510). Thr\(^{290}\) was conserved with mammalian CaMKII autophosphorylation site Thr287, and Thr\(^{305}\) and Thr\(^{310}\) were conserved with mammalian CaMKII Ca\(^{2+}/\mathrm{CaM}\) binding sites Thr\(^{305}\) and Thr\(^{306}\) in the regulatory region. Previous studies have proved that 20E triggers the rapid increase of intracellular calcium ion concentration through ErGPCR and PLC signaling pathways (7, 9), and such a phenomenon is one of the important features of nongenomic pathway. In the present study, we further demonstrated that 20E induced CaMKII phosphorylation at the Thr287 site in 30 min via ErGPCRs, G\(\alpha_q\), PLC, and calcium signaling pathways, thereby illustrating the output of the increase of intracellular calcium ion under 20E induction.

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20E Induces CaMKII Phosphorylation and Nuclear Translocation via Nongenomic Pathway—CaMKII is a multimeric protein, and each monomer has three domains, i.e., N-terminal catalytic domain, regulatory region, and C-terminal subunit association domain. The catalytic domain and regulatory
Similar to HDAC4, HDAC3 can also function for many sequence-specific transcription factors, including NF-κB (47), SMAD7 (mothers against decapentaplegic homolog 7) (48), and c-Jun (49). HDAC3 belongs to a multimolecular complex that contains nuclear receptor corepressor and SMRT protein subunit, which are required for many nuclear hormone receptors involved in physiological action (50, 51). In Drosophila, SMRT-related and ecdysone receptor interacting factor, which shares a similar function and regional homology to the vertebrate nuclear corepressors SMRT and nuclear receptor corepressor, can interact with EcR and can mediate transcription repression by interacting with Sin3A, a repressor known to

FIGURE 5. 20E induced HR3 transcription through HDAC3. Cells were transfected with dsHDAC3, dsHDAC4, or dsHDAC6 (1 μg/ml in the medium) for 12 h and subsequently treated with 20E (2 μM) for 6 h. An equivalent volume of DMSO was applied to cells as solvent control for 20E, and dsGFP was used as nonspecific dsRNA. Total mRNA was isolated for qRT-PCR analysis using the 2−ΔΔCT method. The bars indicate the means ± S.D. of three independent biological experiments. The asterisks denote significant differences as determined by Student’s t test: *, p < 0.05; **, p < 0.01.

FIGURE 6. 20E induced HDAC3 phosphorylation and nuclear export through CaMKII to maintain USP1 lysine acetylation. A, 20E regulated HDAC3 subcellular location through activating CaMKII in HaEpi cells. The HDAC3-RFP-His or pIEx-4-RFP-His (negative control) was overexpressed in HaEpi cells, and the cells were transfected with dsCaMKII or dsGFP (1 μg/ml in the medium) for 12 h. dsGFP was used as the nonspecific dsRNA control. The cells were treated with 20E (2 μM) for 0.5 h for immunocytochemical localization analysis. An equivalent volume of DMSO was applied to cells as solvent control for 20E. Red fluorescence indicated HDAC3-RFP-His or pIEx-4-RFP-His. The yellow bars denote 20 μm at 40× magnification. Blue fluorescence distinguished the cell nucleus stained by DAPI. The fluorescence was observed using an Olympus BX51 fluorescence microscope. B, 20E induced HDAC3 phosphorylation, which could be depressed by dsCaMKII treatment in HaEpi cells. The number of moles of phosphorus per mole of HDAC3-RFP-His was determined using a phosphoprotein phosphate estimation assay kit after HDAC3-RFP-His was purified by Ni2+-NTA affinity column. HDAC3-RFP-His was overexpressed in HaEpi cells, after which the cells were treated with dsCaMKII and treated with 2 μM 20E or an equivalent amount of DMSO for 0.5 h. pIEx-4-RFP-His was overexpressed as the control. Significant difference was determined by Student’s t test based on three independent biological experiments: *, p < 0.05. C, 20E-induced USP1 lysine acetylation was depressed by HDAC3 in the nucleus. USP1-His and HDAC3-RFP-His were co-overexpressed in HaEpi cells, after which the cells were treated with dsCaMKII and treated with 2 μM 20E or an equivalent amount of DMSO for 0.5 h. pIEx-4-RFP-His was co-overexpressed, and dsGFP were used as the negative controls. USP1-His was isolated by Ni2+-NTA affinity column and detected by Western blot using antibody anti-His and anti-Ac-Lys. Input, protein expression levels of CaMKII, HDAC3-RFP-His, pIEx-4-RFP-His, and -actin in HaEpi cells were detected by Western blot using antibody anti-CaMKII, anti-RFP, anti-His, and anti-β-actin, respectively. Gel concentration of SDS-PAGE was 10%. Density statistical analyses of Western blot bands of Ac-Lys-USP1-His/USP1-His were acquired by Quantity One software based on three independent biological experiments. The bars indicate the means ± S.D. of three independent biological experiments. The asterisks denotes significant differences as determined by Student’s t test: *, p < 0.05.
form a complex with the histone deacetylase Rpd3/HDAC (52). We observed that 20E regulated USP1 lysine acetylation to form EcRB1 and USP1 heterodimer by activating CaMKII. Meanwhile, 20E could also induce HDAC3 phosphorylation and nuclear export, and CaMKII silencing reduced HDAC3 phosphorylation and translocation from nucleus to cytoplasm in HaEpi cells. In addition, HDAC3 overexpression reduced USP1 lysine acetylation in HaEpi cells under 20E induction, and HDAC3 knockdown could enhance HR3 transcript increase induced by 20E. HDAC3 may be involved in the EcR-mediated transcription repression via the depression of USP1 lysine acetylation and can be regulated by CaMKII under 20E induction.

20E regulates the formation of heterodimer EcRB1-USP1 for gene transcription initiation (10). A previous study showed that USP1 was phosphorylated by PKC, and this modification was necessary for USP1 to bind to EcRB1 in HaEpi cells under 20E induction (9). The CaMKII knockdown had no effect on the 20E-induced USP1 phosphorylation but down-regulated USP1 lysine acetylation at the Lys303 site and the formation of EcRB1-USP1 transcriptional complex. USP1 phosphorylation and lysine acetylation are two separate processes that contribute to the gene transcription regulation in the 20E pathway.

Conclusion—20E induces CaMKII phosphorylation through ErGPCRs, Goαq, PLC, and calcium signaling pathways. The phosphorylated CaMKII is partially translocated into the
nucleus. CaMKII regulates USP1 lysine acetylation via induction of HDAC3 phosphorylation and nuclear export. USP1 lysine acetylation is necessary in the formation of EcRB1-USP1 transcriptional complex, which binds with EcRE to initiate gene transcription in the 20E pathway.

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