The Steroid Hormone 20-Hydroxyecdysone Enhances Gene Transcription through the cAMP Response Element-binding Protein (CREB) Signaling Pathway*

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Animal steroid hormones regulate gene transcription through genomic pathways by binding to nuclear receptors. These steroid hormones also rapidly increase intracellular calcium and cyclic adenosine monophosphate (cAMP) levels and activate the protein kinase C (PKC) and protein kinase A (PKA) nongenomic pathways. However, the function and mechanism of the nongenomic pathways of the steroid hormones are unclear, and the relationship between the PKC and PKA pathways is also unclear. We propose that the steroid hormone 20-hydroxyecdysone (20E) activates the PKA pathway to enhance 20E-induced gene transcription in the lepidopteran insect Helicoverpa armigera. The expression of the catalytic subunit 1 of PKA (PKAC1) increased during metamorphosis, and PKAC1 knockdown blocked pupation and repressed 20E-responsive gene expression. 20E regulated PKAC1 phosphorylation at threonine 200 and nuclear translocation through an ecdysone-responsive G-protein-coupled receptor 2. PKAC1 induced cAMP response element-binding protein (CREB) phosphorylation at serine 143, which bound to the cAMP response element on DNA to enhance 20E-responsive gene transcription. Through ecdysone-responsive G-protein-coupled receptor 2, 20E increased cAMP levels, which induced CREB PKA phosphorylation and 20E-responsive gene expression. This study demonstrates that the PKA/CREB pathway tightly and critically regulates 20E-induced gene transcription as well as its relationship with the 20E-induced PKC pathway.

Steroid hormones, such as brassinosteroids in plants (1); 20-hydroxyecdysone (20E)2 in insects (2); and glucocorticoids (3), mineralocorticoids (4), androgens (5), estrogens (6), progesterogens, and vitamin D (7) in mammals, regulate various biological processes, including growth and development, inflammation, immunity, and the salt and water balance. Because of their lipid-soluble characteristics, steroid hormones are able to fuse with the cell membrane and regulate gene transcription through a genomic pathway based on nuclear receptors (8, 9). Growing evidence also indicates that steroid hormones transmit signals via activating cell membrane receptors (10–12).

20E is produced in both insects and plants. The plant 20E is produced by various plants, such as Cyanotis vaga, to disrupt the development of insect pests (13). The insect ecdysone is converted from the dietary cholesterol in the prothoracic gland and then processed to 20E in the hemolymph (14). 20E, a hydrophobic steroid hormone, enters cells by freely passing through cellular lipid membrane and then binds to the nuclear ecdysone receptor (EcR) to initiate gene transcription (15). EcR interacts with ultraziprasil (USP) to form a heterodimeric transcription complex (EcR-USP) (16, 17) following 20E induction (18). The EcR-USP complex binds to the ecdysone response element (EcRE) to promote the expression of hormone receptor 3 (HR3; a delayed early gene involved in insect development) in Manduca sexta (19). An EcRE is also found in the 5’ upstream region of Helicoverpa armigera HR3 (HHHR3; GenBank™ accession number AF337637), which can be activated by the binding of the EcR81-USP1 complex associated with eat shock protein 90 and cyclin-dependent protein kinase 10 (20, 21). This is the 20E genomic pathway.

The 20E-induced nongenomic pathway has been reported in the Bombyx mori anterior silk gland; this pathway includes the activation of G-protein-coupled receptors (GPCRs), a rapid increase in calcium, and protein kinase C (PKC) activation (22, 23). Two ecdysone-responsive GPCRs, ErGPCR1 and ErGPCR2, transmit the 20E signal in the cell membrane in the lepidopteran insect cotton bollworm H. armigera (18, 24). The nongenomic GPCR, Gaα, phospholipase C (PLC) γ1, calcium, and protein kinase C (PKC) signaling cascade has been identified in H. armigera, suggesting that the activation of the PKC pathway is necessary for USP phosphorylation and 20E-responsive gene transcription in the 20E genomic pathway (25, 26). 20E triggered the lysine acetylation of USP1 by activating the digoxigenin; CC, chelerythrine chloride; dbcAMP, N6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt; EPAC, exchange protein directly activated by cAMP.
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GPCR, PLC, Ca$^{2+}$, and CaMKII signaling pathway, which is essential for the formation of the EcRB1-USP1 transcription complex and gene transcription (2). In addition to the PKC pathway, 20E binds to the dopamine/ecdysteroid receptor (DmDopEcR), which can mediate an increase of cyclic adenosine monophosphate (cAMP) in *Drosophila melanogaster* (27). A physiological concentration of 20E (1 μM) or ponasterone increases cAMP within 30 s in the anterior silk glands of the silkworm (28). These data suggest that the cAMP-triggered pathway is also activated by 20E. However, the significance of the cAMP-triggered pathway to the 20E-induced gene expression in the genomic pathway is unclear, and the relationship between the PKC and PKA pathways is unclear too.

cAMP is a second messenger produced by adenylyl cyclase, which is located on the inner side of the plasma membrane, following GPCR activation (29). cAMP binds to the regulatory subunits of PKA (PKARs) and dissociates the two regulatory and two catalytic subunits (PKACs) to activate PKAC (30, 31). The activated PKAC phosphorylates the cAMP response element-binding protein (CREB) in the nucleus (32). The activated CREB protein forms a dimer and binds to the cAMP response elements (CREs), which contain the consensus nucleotide sequence 5′-tgaacgca-3′, in the 5′ regions of the target genes to promote or repress gene transcription (33, 34). The PKA/CREB signaling pathway, which is regulated by intracellular cAMP concentrations, is a major intracellular mediator of many hormones. For example, estrogen increases cAMP production via G-protein-coupled receptor 30 (GPR30) and represses mitogen-activated protein kinase (MAPK) signaling through the cAMP/PKA pathway (35, 36). In the mosquito *Aedes aegypti*, 20E regulates AaCREB binding to CRE (5′-atagcctga-3′) in the 5′ regions of yolk protein precursor genes to serve as a potent transcriptional repressor (37). The 20E levels were associated with CREB activation in the formation of long term memory in *D. melanogaster* (38). These data suggest that the cAMP/PKA pathway is involved in 20E signaling.

HR3 is a 20E-responsive transcription factor that plays an important role in the developmental switches during insect development and metamorphosis (39). The expression of *D. melanogaster* HR3 (DHR3) rapidly increases following 20E induction, and it is briefly expressed in the late third instar larval and early prepupa (40). DHR3 is required for the maximal expression of the midprepupal regulatory genes, including EcR, E74B, and βFTZ-1, and is required for the prepupal-pupal transition during *Drosophila* metamorphosis (41). 20E directly induces the expression of *Blattella germanica* HR3 (BgHR3), and BgHR3-silenced larvae are unable to complete metamorphosis (42). The expression of MHR3 in *M. sexta* is induced by 2.5 μM 20E within 2 h in the embryonic cell line (43), and an EcRe is located in its 2.7-kb 5′-flanking region (19). A DNA fragment of *H. armigera* HR3 containing an EcRe in the 5′-flanking region was constructed as a reporter plasmid (pEX-HR3pro-RFP) and used to study 20E-induced gene transcription by expressing the red fluorescent protein (20). A CRE (5′-tagcctga-3′) sequence is also located upstream of the EcRe sequence in the 5′-flanking region of the *H. armigera* HR3 DNA fragment; however, the significance of the CRE sequence to 20E-induced gene transcription is unclear.

To demonstrate the role and mechanism of the cAMP-induced PKA/CREB pathway in 20E signaling and its relationship with the calcium-induced PKC pathway, we studied the roles of PKA and CREB in 20E-induced gene expression in *H. armigera*, a severe agricultural pest in lepidoptera. We found that 20E triggered PKA1 phosphorylation through ErGPCR2. The phosphorylated PKA1 translocated into the nucleus to phosphorylate CREB, which then bound to the CRE sequences in the 5′-flanking region of HHHR3 to enhance 20E-induced gene transcription. This study reveals that 20E acts through the cAMP-induced PKA/CREB pathway to enhance the PKC-mediated EcR-USP1-dependent gene transcription in the genomic pathway.

**Experimental Procedures**

**Insects and Cell Culture**—The cotton bollworms (*H. armigera*) were fed an artificial diet at 27 ± 1 °C with a light:dark photoperiod of 14:10 as described previously (44). The HaEpi cell line was established from the epidermis of *H. armigera* (45). The HaEpi cells grew as a loosely attached monolayer and were maintained in Grace’s insect medium (Invitrogen) containing 10% fetal bovine serum (FBS; Gibco) at 27 ± 1 °C.

**Bioinformatics and Phylogenetic Tree Analyses**—The genes were identified by transcriptome sequencing of the HaEpi cDNA library. The predicted proteins were translated using the ExPASy software. The sequence alignment was conducted on line using ClustalW, and the phylogenetic tree analyses were conducted using MEGA5.1 software. Domain predictions were performed using SMART software (data not shown).

**Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)**—The total RNA was extracted using Unizol reagent (Biostar, Shanghai, China) and then reverse transcribed into cDNAs using the FastQuant RT kit (TIANGEN, Beijing, China). The qRT-PCR was performed at a final volume of 10 μl, which contained 5 μl of the UltraSYBR mixture (with ROX) (CWBio, Beijing, China), 1 μl of the cDNAs, and 2 μl each of the forward and reverse primers (1 μM; Table 1). The qRT-PCR was performed as follows: 95 °C for 15 min for the initial denaturation; 40 cycles of 95 °C for 15 s and 60 °C for 60 s; 78 °C for 2 s for plate reading; and then a melting curve analysis from 65 to 95 °C. β-Actin was used as a control to normalize the gene expression. The experiments were repeated three times, and the data were analyzed using the 2$^{-\Delta\Delta Ct}$ method: 2$^{-\Delta\Delta Ct}$ = 2$^{-(\text{CTtreat of gene} - \text{CTtreat of β-actin}) - (\text{CTcon of gene} - \text{CTcon of β-actin})}$, where CTcon was the CT value of the control group and CTtreat was the CT value of the experimental group.

**Preparation of the Antiserum against PKAC1**—The cDNA fragment (amino acids 94–353) encoding a portion of PKAC1 was amplified from *H. armigera* with specific primers (Table 1) and inserted into the pET-32a(+)-expression vector (Promega, Madison, WI) through double enzyme digestion. The recombinant plasmid was transformed into *Escherichia coli* DH5α cells, and the recombinant plasmid was isolated using the Plasmid Mini kit (Sangon, Shanghai, China). The production of the recombinant protein was induced by isopropyl β-d-thiogalactopyranoside (0.5 mM) in Rosetta cells with LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0), and the protein was purified on a Ni$^{2+}$-NTA affinity column (GE
TABLE 1

Oligonucleotide sequences of PCR primers

| Primer name      | Oligonucleotide sequence (5’ → 3’) |
|------------------|-----------------------------------|
| qRT-PCR          |                                   |
| PKAC1-qRTF       | 5’-gaacctctggcaccatcaact-3’       |
| PKAC1-qRTR       | 5’-tggaggtgctgctggttc-3’          |
| EzR1-qRTF        | 5’-atgttccctgacgtagcta-3’         |
| EzR1-qRTR        | 5’-tgagctctaccatggagaa-3’         |
| USP1-qRTF        | 5’-gtctctgcaacagatctgtg-3’        |
| USP1-qRTR        | 5’-tccagcctgctgtagctgga-3’        |
| HHR3-qRTF        | 5’-tcaagcactacatcggagacgcc-3’     |
| HHR3-qRTR        | 5’-gacctgtctgactctgcgc-3’         |
| CREB-qRTF        | 5’-gaactatacaatcggagatgtg-3’      |
| CREB-qRTR        | 5’-cagacctcttagctatca-3’          |
| β-Actin-qRTF     | 5’-ctgttgaagttggaagagc-3’         |
| β-Actin-qRTR     | 5’-ctgttgaagttggaagagc-3’         |

RNA interference

| PKAC1-RNAi F     | 5’-gctgaatcaatcactatagttgctcaacctcttcttggt-3’ |
| PKAC1-RNAi R     | 5’-gctgaatcaatcactatggttctgtgtgtgcgatgaa-3’  |
| GFP-RNAi F       | 5’-gctgaatcaatcactatggttctgtgtgtgcgatgaa-3’  |
| GFP-RNAi R       | 5’-gctgaatcaatcactattcatgtgtgagcttggcc-3’    |
| CREB-RNAi F      | 5’-gctgaatcaatcactatggagatgtgctcct-3’        |
| CREB-RNAi R      | 5’-gctgaatcaatcactatggagatgtgctcct-3’        |

Overexpression

| PKAC1-OE F       | 5’-tactcagagtctgggcaaaactgtgctgccacc-3’      |
| PKAC1-OE R       | 5’-tactcagagtctgggcaaaactgtgctgccacc-3’      |
| CREB-OE F        | 5’-tactcagagtctgggcaaaactgtgctgccacc-3’      |
| CREB-OE R        | 5’-tactcagagtctgggcaaaactgtgctgccacc-3’      |

Prokaryotic expression

| PKAC1-exp F  | 5’-tactcagagtctgggcaaaactgtgctgccacc-3’      |
| PKAC1-exp R  | 5’-tactcagagtctgggcaaaactgtgctgccacc-3’      |

Western Blotting—The total protein from the cells or larvae was extracted using 1 × phosphate-buffered saline (1 × PBS; 140 mM NaCl and 10 mM sodium phosphate, pH 7.4) supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF). The protein was collected by centrifuging the lysate at 10,000 g at 4 °C for 10 min, and its concentration was measured according to the method of Bradford (74). A total of 20 µg of the total proteins from each sample was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred onto a nitrocellulose membrane. The membrane was incubated with blocking buffer (2% skim milk in Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl)) for 1 h at room temperature and then incubated for 2–4 h with the primary antibodies diluted 1:100 or 1:1000 in blocking buffer. The membrane was washed three times for 10 min each with TBST and then washed three times for 5 min each with TBS. The target bands were visualized using NBT/BCIP (10 ml of TBS, 45 µl of 5% p-nitro-blue tetrazolium chloride, and 35 µl of 5% 5-bromo-4-chloro-3-indolyl phosphate) by incubating in the dark for 10 min. The densities of the bands on the Western blots were acquired by Quantity One software, and the statistical analyses were based on three independent biological experiments. The H. armigera β-actin, as a quantitative control, was detected by Western blotting using a rabbit polyclonal antiserum against H. armigera β-actin that was prepared in our laboratory.

dsRNA Synthesis and Gene Knockdown—The dsRNAs were produced using the MEGAscriptTM RNAi kit (Ambion, Austin, TX). The PCR templates of the genes for the dsRNA synthesis were provided according to the manufacturer’s instructions (the PCR primers are shown in Table 1). The reaction was performed at 37 °C for 4 h, and DNase I was then added to remove the DNA templates. The dsRNA concentrations were measured by spectrophotometry at 260 nm. dsGFP was transcribed from the green fluorescent protein DNA template and used as a nonspecific control. For gene knockdown in cells, the HaEpi cells were cultured in 6-well plates to 80% confluence and then transfected with the dsRNAs using the RNAfectin transfection reagent (TIANGEN) for 6–12 h at 27 °C. The final concentration of the dsRNA was 1 µg/ml. The cells were replenished with fresh medium following the transfection. Finally, the proteins or mRNAs were used in the subsequent experiments. For gene knockdown in larvae, H. armigera larvae (30 larvae per group) were injected with dsPKAC1 and dsGFP (control; 1 µg/larva) at 6 h after the appearance of the sixth instar, and three independent experiments were performed. The transcription levels of the 20E-responsive genes were detected by qRT-PCR.

Overexpression of Proteins in the HaEpi Cell Line—The open reading frame (ORF) of the genes was amplified from H. armigera with specific primers (Table 1) and then inserted into the pIEx-4-RFP-His vector (the red fluorescent protein was subcloned into pIEx-4-His vector, Invitrogen) through double enzyme digestion. The HaEpi cells were cultured in 6-well plates under normal growth conditions until they reached 80% confluence. Then the reconstructed plasmids were transfected into the cells using DNAfectin and then cultured at 27 °C for 12 h. After being cultured with complete culture medium for 24 h, the cells were used in the subsequent experiments. The pIEx-4-RFP-His vector was transfected as a negative control. The overexpressed proteins from recombined pIEx-4-RFP-His or pIEx-4-His vector were detected by Western blotting using the mouse monoclonal antibody against RFP tag or His tag (Zhongshan, Beijing, China), respectively.

20E-responsive RFP Reporter Plasmid—The DNA fragment between −1875 and −795 bp of the 5′-flanking region before the initiation codon ATG (+1) of H. armigera HR3 (20) was inserted into the pIEx-4-RFP-His plasmid (the structure of pIEx-4-RFP-His plasmid is hr5-IE1-RFP-His) using the SapI and PsiI restriction enzymes (Thermo Fisher Scientific, Lithuania), and this fragment replaced the hr5 enhancer region to produce the pHR3-P-IE1-RFP-His reporter plasmid. The HaEpi cells were transfected with the pH3-P-IE1-RFP-His plasmid and then treated with 20E (0.1, 0.2, 0.5, 1, 2, and 5 µM) for 12 h. DMSO-treated cells served as a solvent control. The
total proteins from the overexpressing cells were used for the Western blotting analyses. The pIE1-RFP-His plasmid was generated by deleting the hr5 enhancer region of the pIEx-4-RFP-His plasmid using the SapI and PsiI restriction enzymes and linking the two termini with T4 DNA ligase (Thermo Fisher Scientific) after treatment with Pfu DNA polymerase (TIANGEN). The pIE1-RFP-His plasmid was overexpressed as a control in the HaEpi cells as described above.

Site-directed Mutagenesis of the CREB Phosphorylation Sites—Using the corresponding primers (Table 1), the wild-type or serine 143 to alanine (S143A) mutant (obtained by site-directed mutagenesis of CREB in vitro) CREB ORF was amplified from H. armigera and named CREB-RFP-His and CREB-S143A-RFP-His, respectively. The HaEpi cells were transfected with CREB-RFP-His and CREB-S143A-RFP-His, respectively. Subsequently, the cells were incubated with 2 μM 20E or DMSO (solvent control) for 30 min. CREB-RFP-His and CREB-S143A-RFP-His were purified using a Ni²⁺-NTA affinity column from the overexpressing cells and then used for Western blotting using the phospho-(Ser/Thr) PKA substrate antibody (Cell Signaling Technology).

Co-immunoprecipitation—The cells were transfected with the CREB-RFP-His plasmid and then incubated with 2 μM 20E or DMSO (solvent control) for 30 min. The proteins were extracted from the cells using radioimmunoprecipitation assay buffer containing 0.1 M Tris-HCl buffer, pH 8, 150 mM NaCl, and 1% Nonidet P-40. The supernatant was harvested by centrifugation at 12,000 g for 10 min (4 °C). The supernatant was added to Protein A resin to remove the nonspecific binding proteins, and the supernatant was harvested by centrifugation. The RFP antibody (1 μl) and 1 × PBS (400 μl) were incubated with the Protein A resin for 30 min at room temperature to form the resin-antibody complex, and the resin was then washed three times with 500 μl of 1 × PBS. The cell lysate was added to the resin-antibody complex and incubated for 2–4 h at 4 °C with gentle shaking. The resin was harvested by centrifugation and washed three times with 1 × PBS. Finally, the resin was treated with SDS-PAGE loading buffer and boiled for 10 min. After centrifugation at 12,000 × g for 10 min at 4 °C, the protein samples were loaded onto an SDS-polyacrylamide gel for Western blotting using anti-RFP (Zhongshan) and anti-PKAC1 antibodies against CREB-RFP-His and PKAC1, respectively. RFP-His was overexpressed from the pIEx-4-RFP-His plasmid in the HaEpi cells as a negative control as described above.

Electrophoretic Mobility Shift Assay (EMSA)—CREB-RFP-His was overexpressed by transfecting the HaEpi cells with the pEx-CREB-RFP-His plasmid, and the cells were harvested by centrifugation. The resin was then washed by centrifugation and resuspended in 5 × binding buffer. The resin was added to Protein A resin to remove the nonspecific binding proteins, and the supernatant was harvested by centrifugation. The RFP antibody (1 μl) was added to Protein A resin to remove the nonspecific binding proteins, and the supernatant was harvested by centrifugation. The RFP antibody (1 μl) and 1 × PBS (400 μl) were incubated with the Protein A resin for 30 min at room temperature to form the resin-antibody complex, and the resin was then washed three times with 500 μl of 1 × PBS. The cell lysate was added to the resin-antibody complex and incubated for 2–4 h at 4 °C with gentle shaking. The resin was harvested by centrifugation and washed three times with 1 × PBS. Finally, the resin was treated with SDS-PAGE loading buffer and boiled for 10 min. After centrifugation at 12,000 × g for 10 min at 4 °C, the protein samples were loaded onto an SDS-polyacrylamide gel for Western blotting using the phospho-(Ser/Thr) PKA substrate antibody (Cell Signaling Technology).

PKAC1 Knockdown Repressed the Pupation Rate and 20E-responsive Gene Expression—To identify the function of PKAC1 in larval development, RNA interference was performed in larvae. After the dsPKAC1 injection, 42.2% of the larvae formed larval-pupal chimeras and failed to pupate,
whereas 6.67% of the dsGFP injection control group failed to pupate (Fig. 2, A and B). The expression levels of the 20E-responsive genes, including EcRB1, USP1, and HHR3, were decreased in the larval midgut after PKAC1 knockdown, and the interference efficiency of PKAC1 knockdown was 88% (Fig. 2C). In HaEpi cells, the 20E-induced expression levels of EcRB1, USP1, and HHR3 were also decreased after PKAC1 knockdown, and the interference efficiency of PKAC1 knockdown was 53% (Fig. 2D). The PKAC1 levels in larval midgut and HaEpi cells were decreased as shown by Western blotting analyses (Fig. 2, E and F). Therefore, PKAC1 is involved in the 20E-triggered metamorphosis and 20E-responsive gene transcription.

20E Induced PKAC1 Phosphorylation and Promoted Its Nuclear Translocation via ErGPCR2—To study the mechanism of PKAC1 in 20E-responsive gene transcription, the responses of PKAC1 to 20E induction were investigated. Western blotting showed that there are two PKAC1 bands. The density of the upper band was increased in HaEpi cells within 15 min of 20E treatment compared with the DMSO control (Fig. 3 upper band was increased in HaEpi cells within 15 min of 20E). These results illustrate that the upper band from the HaEpi cell extracts is phosphorylated PKAC1, and the PKAC1 maintains a phosphorylated form in the fat body (Fig. 3B). Threonine 197 (Thr-197) is a key phosphorylation site for PKAC1 (48), and Thr-200 in H. armigera PKAC1 is conserved as mammalian Thr-197 by homology analysis. To identify the 20E-induced phosphorylation site, a site-specific point mutation was investigated in vitro. PKAC1 and its mutant, PKAC1-T200A (where Thr-200 was mutated to alanine), were overexpressed from the pIEx-4-His vector in the HaEpi cells. As shown by Western blotting, 20E induced the phosphorylation of PKAC1-His but not PKAC1-T200A-His (Fig. 3C). Western blotting demonstrated the molecular weight of PKAC1-His after plEx-PKAC1-His overexpression in HaEpi cells (Fig. 3D). These results suggest that 20E induced PKAC1 phosphorylation at Thr-290.

To identify the 20E-induced pathway that is upstream of PKAC1 phosphorylation, the GPCR inhibitor suramin (49) and receptor tyrosine kinase inhibitor SU6668 (50) were added to the HaEpi cells. Compared with the 20E-treated cells, suramin, but not SU6668, significantly inhibited 20E-induced PKAC1 phosphorylation. The knockdown of ErGPCR1 blocked the larval-pupal transition and 20E-responsive gene expression (1 μg of dsPKAC1/larva was injected into sixth instar at 6 h). B, statistical analysis of the phenotypes in A. The abnormal pupae indicate the larval-pupal chimera. C, expression levels of the 20E-responsive genes in larval midgut after dsPKAC1 knockdown with dsGFP as a negative control. D, expression levels of the 20E-responsive genes after dsPKAC1 knockdown in HaEpi cells with dsGFP as negative control. β-Actin was used as a quantitative control. The values are expressed as the means ± S.D. (n = 3). * denotes significant differences as determined by Student’s t test, *p < 0.05.
illustrated that PKAC1-RFP-His was primarily localized in the cytoplasm in the DMSO-treated controls. However, PKAC1-RFP-His could be detected in the nucleus when the HaEpi cells were treated with 20E for 0.5 h. In the RFP-overexpressing control cells, the RFP was distributed throughout the cell in the absence of hormonal induction (Fig. 4A). The ratios of RFP distributed in the cytoplasm and nucleus were analyzed by the relative density acquired by Quantity One software (Fig. 4B). The RFP-His and PKAC1-RFP-His molecular weights were detected by Western blotting using protein ladder (Fig. 4C). As shown by Western blotting, PKAC1-RFP-His was localized in the cytosol in the DMSO controls but translocated into the nucleus in the 20E-treated cells. The protein also had a higher molecular mass, indicating phosphorylation. Compared with the PKAC1-RFP-His protein in the cytoplasm, the RFP-His protein did not exhibit a shift in its subcellular localization in the 20E-treated cells (Fig. 4D). These results suggest that PKAC1 translocates to the nucleus upon 20E stimulation.

20E Regulated CREB Phosphorylation via PKAC1—The PKA-mediated phosphorylation of CREB was investigated to determine the mechanism of PKAC1 action in the nucleus following 20E induction. Using a sequence homology alignment analysis, a conserved PKA serine phosphorylation site (RRPSY) (51) was identified at amino acid 143 (Ser-143) in H. armigera CREB that was in the phosphorylated kinase-inducible activation domain of CREB (Fig. 5A). CREB-RFP-His was overexpressed in HaEpi cells and detected by Western blotting using a protein ladder (Fig. 5B). To confirm the 20E-induced phosphorylation of CREB at Ser-143, CREB-RFP-His and the mutant CREB-S143A-RFP-His were overexpressed from the pIEx-4-RFP-His plasmid. The protein input showed that CREB-RFP-His and CREB-S143A-RFP-His were equally expressed in both the experimental and control groups. However, Western blotting using anti-phospho-(Ser/Thr) PKA substrate antibody showed that 20E induced PKA-mediated CREB-RFP-His phosphorylation but did not induce PKA-mediated CREB-S143A-RFP-His mutant phosphorylation (Fig. 5C). These results suggest that 20E regulates PKA-mediated CREB phosphorylation at Ser-143.

To identify the pathway by which 20E regulates CREB phosphorylation, various inhibitors and RNAi were used to block the 20E-triggered nongenomic signaling in the CREB-RFP-His-overexpressing HaEpi cells. Western blotting showed that CREB-RFP-His was equally expressed in all samples in the input proteins, and 20E induced CREB phosphorylation (Fig. 5D, lanes 1 and 2). The GPCR inhibitor suramin and the PKA inhibitor H89 repressed 20E-induced CREB phosphorylation (Fig. 5D, lanes 3 and 4), but the PKC inhibitor chelerythrine...
chloride (CC) did not affect this phosphorylation (Fig. 5D, lane 5). ErGPRC2 and PKAΔ1 knockdown suppressed 20E-induced CREB phosphorylation compared with the dsGFP transfection control, but ErGPRC1 knockdown had no effect (Fig. 5D, lanes 6–9). These results indicate that PKA-mediated CREB phosphorylation is regulated by 20E via ErGPRC2 and PKA signaling.

To determine whether CREB phosphorylation was mediated by PKAΔ1 following 20E treatment, the interaction between the CREB and PKAΔ1 proteins was examined by overexpressing CREB-RFP-His in the HaEpi cells. The expression of CREB-RFP-His and endogenous PKAΔ1 was uniform in the input from the HaEpi cells. However, in the co-immunoprecipitation with an antibody against RFP that bound to CREB-RFP-His, the co-precipitated PKAΔ1 was only detected in the 20E-induced cells. pEx-4-RFP-His was overexpressed to exclude the possibility of protein interactions with the RFP-His tag (Fig. 5E). These results suggest that the PKAΔ1 directly phosphorylates CREB following 20E treatment.

The Phosphorylated CREB Bound to CRE to Enhance Gene Transcription following 20E Treatment—CREB regulates gene expression by binding to CRE at the promoter region of its target genes (52). In a previous study, the 5′-flanking region of *H. armigera* HR3 (~1878 to ~795 bp before the initiation codon ATG), containing an EcRE box (5′-ggtgccgtaaatgctag-3′, ~1405 to ~1391 bp before the initiation codon ATG), was cloned into the pEx-4-RFP-His vector (hr5-IE1-RFP-His) by replacing the original hr5 enhancer and IE1 promoter in the plasmid to produce a pEx-HR3pro-RFP plasmid (CRE-EcRE-RFP-His). This plasmid was used to detect the expression of EcRB1-USP1-dependent gene transcription following 20E treatment (20). Using sequence homology analysis, a highly conserved CRE sequence (5′-tgacgtca-3′) (53) was found at ~1690 to ~1683 bp upstream of the 5′-flanking region before the initiation codon ATG of *H. armigera* HR3. To investigate the function of CREB in 20E-induced gene transcription, an EMSA was performed using the nucleoproteins from the CREB-RFP-His-overexpressing cells. The pEx-4-RFP-His plasmid was separately overexpressed to confirm that CREB mediated the binding and not the RFP tag (Fig. 6A, lanes 1 and 2). Compared with the DMSO-treated, CREB-RFP-His-overexpressing nucleoproteins, the 5′-Dig-labeled CRE probe (5′-ttttcgtctaatgcaatgctag-3′) was shifted from the 20E-induced nucleoprotein (Fig. 6A, lanes 3 and 4). The unlabeled CRE probe (5′-ttttcgtctaatgcaatgctag-3′) was used as a competitive probe and decreased this shifted band (Fig. 6A, lane 5). A mutated, unlabeled CRE-M probe (5′-ttttcgtctaatgcaatgctag-3′) did not decrease this shifted band (Fig. 6A, lane 6). The anti-RFP antibody, which recognized CREB-RFP-His, produced a supershifted band (Fig. 6A, lane 7). Compared with dsGFP, PKAΔ1 knockdown repressed CREB binding to the CRE element (Fig. 6A, lanes 8 and 9). The expression of the CREB-RFP-His and RFP-His proteins involved in the EMSA were validated by Western blotting (Fig. 6B and C). To confirm that the PKA-mediated phosphorylation of CREB at Ser-143 contributed to CREB binding to CRE, the PKA phosphorylation site mutant of CREB (CREB-S143A-RFP-His) was overexpressed using the pEx-4-RFP-His plasmid. CREB-RFP-His bound to CRE following 20E treatment, but CREB-S143A-RFP-His did not. The expression of the CREB-RFP-His and CREB-S143A-RFP-His proteins involved in the EMSA was validated by Western blotting (Fig. 6D). The results indicate that 20E triggers PKA to phosphorylate CREB via PKAΔ1, enabling CREB to bind to CRE.

To study the function of CRE in 20E-triggered gene expression, the DNA fragment from ~1878 to ~795 bp upstream
before the initiation codon ATG that contained both the CRE and EcRE sequences was inserted into the promoter region of the pIE1-RFP-His (hr5-IE1-RFP-His) vector by replacing the original hr5 enhancer with a 20E-inducible activation domain (pKID). The resulting plasmid retained the IE1 sequence for the basic expression of the RFP protein in the absence of 20E induction (Fig. 7A, B, and C). The pIE1-RFP-His (hr5-IE1-RFP-His) plasmid without the CRE-EcRE insert was used as a control (Fig. 7D). The RFP-His protein was expressed in the absence of 20E, and the expression levels of RFP-His from the pHR3-P-IE1-RFP-His (CRE-EcRE-IE1-RFP-His) plasmid increased with increasing 20E concentrations in the transfected HaEpi cells. However, the RFP-His expression did not increase in the presence of 20E in the cells transfected with the pIE1-RFP-His (IE1-RFP) plasmid (Fig. 7E). Therefore, the 20E-induced RFP-His expression is dependent on the CRE and EcRE sequences in the 5’-flanking region of the H. armigera HR3 DNA fragment.

To address the contribution of CRE to the 20E-induced RFP-His expression from the pHR3-P-IE1-RFP-His plasmid, the
CRE sequence was deleted from the pH3-P-IE1-RFP-His (CRE-EcRE-IE1-RFP-His) plasmid to produce a CRE-free pH3-mCRE-P-IE1-RFP-His plasmid (EcRE-IE1-RFP-His; Fig. 7F). Compared with the 20E-induced RFP-His expression from the pH3-P-IE1-RFP-His (with CRE-EcRE-IE1-RFP-His) plasmid, the 20E-induced RFP-His expression from the pH3-mCRE-P-IE1-RFP-His (with EcRE-IE1-RFP-His) plasmid was significantly reduced (Fig. 7G). The results suggest that CRE enhances the 20E-responsive gene transcription based on the EcRE sequence.

20E Regulated the 20E-responsive Gene Expression via the Nongenomic Pathway—To demonstrate that the RFP expression from the pH3-P-IE1-RFP-His plasmid is regulated by 20E-triggered nongenomic signaling, the inhibitors and dsRNAs that can block 20E-triggered nongenomic signaling, including the GPCRs inhibitor suramin, the PKA inhibitor H89 (55), the PLC inhibitor U73122 (56), the PKC inhibitor CC (54), and two ErGPCR dsRNAs (dsErGPCR1 and dsErGPCR2), were used in the next set of experiments. Compared with the positive control, the 20E-treated group, all the inhibitors repressed the RFP-His expression in the HaEpi cells; compared with the negative control, the dsGFP-transfected group, ErGPRC1 and ErGPRC2 knockdowns repressed the 20E-induced RFP-His expression (Fig. 8A). PKAC1 or CREB knockdown also decreased RFP-His expression from the pH3-P-IE1-RFP-His plasmid in the 20E-treated HaEpi cells compared with the dsGFP-transfected negative control group (Fig. 8B). Furthermore, the expression levels of the endogenous 20E-responsive genes, including EcR-B1, USP1, and HHR3, decreased after CREB knockdown in the HaEpi cells (Fig. 8C). These results indicate that 20E acts via the ErGPRC1, ErGPRC2, PLC, PKC, and PKA/CREB nongenomic signaling pathway to regulate gene transcription depending on the EcRE and CRE located in the promoter region of HHR3.

20E Increased cAMP via ErGPCR2 to Promote PKA-mediated CREB Phosphorylation and Enhance 20E-induced Gene Transcription—The dissociation of PKAC from PKAR is dependent on the binding of cAMP to PKAR (30, 31), and the activated PKAC phosphorylates CREB in the nucleus (32). Therefore, the 20E-induced intracellular cAMP levels were detected in the HaEpi cells. Compared with the DMSO-treated cells, the concentration of intracellular cAMP in the 20E-treated HaEpi cells was increased at 15 min (Fig. 9A). ErGPCR2 knockdown in HaEpi cells by transfecting dsErGPCR2 significantly reduced the 20E-induced intracellular cAMP concentrations (Fig. 9B). These results suggest that 20E increases the intracellular concentration of cAMP via ErGPCR2.

To confirm the function of the increased cAMP levels in 20E-induced CREB phosphorylation, dbcAMP (a mimic molecule of cAMP) was used to mimic the increased intracellular cAMP induced by 20E. dbcAMP treatment in CREB-RFP-His-overexpressing cells enhanced the PKA-mediated phosphorylation of CREB compared with the H2O control (Fig. 9C). In correlation, the results of the qRT-PCR analyses showed that the addition of 0.1 or 0.5 mM dbcAMP alone did not induce HHR3 expression in HaEpi cells, but the simultaneous addition of 20E significantly increased the 20E-induced expression of HHR3 (Fig. 9D). dbcAMP enhanced the 20E-induced RFP-His expression from the pH3-P-IE1-RFP-His-report plasmid in the HaEpi cells (Fig. 9E), but the expression of RFP-His from the pH3-P-mCRE-RFP-His mutated report plasmid was not enhanced (Fig. 9F). These results suggest that dbcAMP alone could not initiate the transcription of HHR3, but it could enhance the 20E-induced transcription of HHR3.

To analyze the possible signaling pathways to activate CRE by 20E-induced CREB, the vector pCRE-IE1-RFP-His that only contains the CRE from the upstream region of HHR3 was con-
structed by deleting the EcRE in pH3-P-IE1-RFP-His (Fig. 10A). The kinases inhibitors involved in cAMP and Ca\(^{2+}\) signaling, including the cAMP production inhibitor bupivacaine hydrochloride (57), the PKA inhibitor H89 (54), the exchange protein directly activated by cAMP (EPAC) inhibitor ESI-09 (58), the PKC inhibitor CC (55), and the CaMKII and CaMKIV inhibitor KN-93 (59, 60), were used, respectively, to analyze the relative signaling pathways that may take part in 20E-induced gene expression. The results of Western blotting showed that 20E enhanced the RFP-His expression from the pCRE-IE1-RFP-His plasmid in HaEpi cells, and bupivacaine hydrochloride, H89, ESI-09, and KN-93 repressed RFP-His expression, but CC did not (Fig. 10B). These results suggest that 20E may activate CRE via cAMP, PKA, CaMKII, and CaMKIV signaling pathways, and the PKC signaling pathway is not involved in 20E-induced CRE activation.

**Discussion**

In addition to the well known nuclear receptor-mediated genomic pathway, GPCR-Ca\(^{2+}\)-PKC and GPCR-cAMP-PKA pathways are involved in animal steroid hormone signaling. It is known that 20E regulates USP phosphorylation via the ErG-PC1/ErGPCR2-Ca\(^{2+}\)-PKC nongenomic pathway and initiates gene transcription through the EcR-USP genomic pathway, which is dependent on the EcRE in the promoter region of HHR3 (24, 25). However, the output of activating the cAMP/ PKA pathway in animal steroid hormone signaling and the relationship between the nongenomic PKC and PKA pathways are unclear. The present study shows that steroid hormone 20E signals through the GPCR-cAMP-PKA pathway to enhance 20E-induced gene transcription based on the GPCR-Ca\(^{2+}\)-PKC-activated EcR-USP pathway.

**20E Induces PKAC1 Phosphorylation and Nuclear Translocation via ErGPCR2**—The mammalian steroid hormone estrogen binds to a GPCR (GPR30) and causes adenylyl cyclase activation to produce cAMP, thereby activating PKA, which in turn phosphorylates CREB (61). Previous studies have demonstrated that 20E triggered an increase in the intracellular cAMP concentrations within 30 s in the anterior silk glands of the silkworm (28). We observed that 20E induced PKAC1 phosphorylation and nuclear translocation, which was accompanied by a 20E-induced increase of cAMP, suggesting that there is a conserved mechanism of cAMP-induced PKAC phosphorylation and nuclear translocation in insects. 20E induces PKAC1

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**FIGURE 7. 20E enhanced gene expression via CRE.** A, the 5'-flanking region before the initiation codon ATG of the *H. armigera* HHR3 sequence. B, the structure of pEx-4-RFP-His. C, the structure of pH3-P-IE1-RFP-His. D, the structure of pIE1-RFP-His. E, Western blotting analysis of the RFP-His expression from the pH3-P-IE1-RFP-His plasmid in HaEpi cells following incubation with 20E (0.1, 0.2, 0.5, 1, 2, and 5 μM) for 12 h; the pIE1-RFP-His plasmid was used as a control. F, the structure of pH3-mCRE-P-IE1-RFP-His. G, the expression of RFP-His from the pH3-mCRE-P-IE1-RFP-His and pH3-P-IE1-RFP-His plasmids in the presence of 2 μM 20E for 12 h. The total proteins were used for the Western blotting analyses with an antibody against RFP. β-Actin was used as a loading control using the antiserum against *H. armigera* β-actin. A 7.5% SDS-polyacrylamide gel was used. * and ** denote *p* < 0.05 and *p* < 0.01, respectively. Error bars represent S.D.
phosphorylation via ErGPCR2. ErGPCR2 is a cell membrane receptor, and the PKA pathway is a typical cell membrane pathway; therefore, the steroid hormone 20E transmits signals via a cell membrane receptor-mediated nongenomic pathway prior to initiating gene transcription through the EcR-USP genomic pathway.

In mammalian cells, the phosphorylation of Thr-197 in the activation loop of PKAC is necessary for the maturation and optimal biological activity of PKA (62, 63). The 3-phosphoinositide-dependent protein kinase is involved in PKAC phosphorylation at Thr-197 (48, 64). In the present study, PKAC1 expression was elevated, and it was phosphorylated during molting and metamorphosis. In HaEpi cells, 20E also induced PKAC1 phosphorylation at Thr-200 at 15 min via ErGPCR2. Because Thr-200 is the conserved amino acid for mammalian Thr-197 as shown by homology analysis, 20E likely induces PKAC1 phosphorylation by 3-phosphoinositide-dependent protein kinase. This requires confirmation.

**PKAC1 Phosphorylates CREB to Enhance EcR-USP-initiated Gene Transcription following 20E Treatment**—When PKAC enters the nucleus, it regulates a number of cellular processes, including motility, metabolism, neurotransmitter release, and transcription, by the reversible phosphorylation of key substrates (65), such as the transcription factor CREB (66). CREB is an important basic leucine zipper transcription factor (67) that contains a leucine zipper at the C terminus for DNA binding and an N-terminal transcriptional activation domain. The N terminus of the protein has two domains that are necessary for its function as a transcriptional activator: the kinase-inducible domain and glutamine-rich flanking sequences. The kinase-inducible domain comprises a plurality of conserved serine phosphorylation sites (RRPSY), which can be phosphorylated by PKA, MAPK, CaMKs, and PKC, and has different functions (68, 69). In the present study, we demonstrated that PKAC1 directly phosphorylates CREB at the conserved serine 143 (RRPSY) in the kinase-inducible domain via the 20E-triggered ErGPCR2 and PKAC1 nongenomic signaling pathway within 30 min. Moreover, a consensus CRE nucleotide sequence (5’/H11032-tgacgtca-3’/H11032) was found upstream of the EcRE sequence in the 5’-flanking region of the H. armigera HR3 gene.

The activated CREB protein binds to the CRE sequence in the promoter region of genes that contain the consensus nucleotide sequence 5’-tgacgtca-3’; this was found upstream of the EcRE sequence in the 5’-flanking region of the H. armigera HR3 gene. The activated CREB protein binds to the CRE sequence in the promoter region of genes that contain the consensus nucleotide sequence 5’-tgacgtca-3’, thereby increasing or decreasing the transcription of the downstream genes (33, 34). The genes whose transcription is selectively regulated by CREB following 20E treatment include the yolk protein precursor genes with CREB as a potent transcriptional repressor (37) and Fascin1 in D. melanogaster mushroom body remodeling with CREB as an up-regulated transcriptional coactivator (70). We found 20E induced CREB binding to CRE via the 20E-triggered non-
genomic signaling pathway to enhance 20E-induced gene transcription. These data indicate that 20E triggered the ErGPCR2, PKAC1, and CREB signaling pathway to enhance 20E-responsive gene transcription.

The Relationship between the 20E-induced PKC and PKA Signaling Pathways—The two main pathways initiated by GPCRs are the cAMP-triggered PKA pathway and the Ca\(^{2+}\)-triggered PKC pathway (71). The steroid hormones may activate PKC and PKA pathways. For example, estrogen increases calcium and activates PI3K/PI3K and PLC/PKC signaling (72). Diethylstilbestrol induces the expression and activation of melanin-related enzymes by activating cAMP/PKA signaling (73). 20E also activates the PKC pathway in *B. mori* (22, 23) and *H. armigera* (18, 24) and the PKA pathway in *D. melanogaster* (25). The PKC pathway regulates USP phosphorylation, formation of the EcR-USP transcription complex (25, 26), and acetylation of USP1 (2) for 20E pathway gene expression. However, the function of the 20E-induced PKC pathway and its relationship with the 20E-induced PKA pathway are unclear.

20E induces the expression of 20E-responsive genes, which depends on the formation of the EcRBP1-USP1 transcription complex (47). The formation of the EcRBP1-USP1 transcription complex is regulated by 20E-regulated phosphorylation of USP1 via activating the ErGPCR1, Ca\(^{2+}\), PKC signaling pathway (25). In the present study, 20E increased cAMP and induced PKA-mediated CREB phosphorylation, suggesting that 20E triggered the cAMP/PKA/CREB pathway. However, the cAMP analog dbcAMP alone could not initiate HHR3 expression in HaEpi cells, but dbcAMP significantly enhanced the 20E-induced HHR3 expression. Furthermore, dbcAMP increased 20E-induced RFP expression from the pHR3-P-IE1-RFP-His plasmid that contains CRE. However, dbcAMP could not increase the 20E-induced RFP expression from the CRE-deleted pHR3-P-mCRE-IE1-RFP-His plasmid. These data sug-
ggest that the 20E-triggered cAMP/PKA/CREB pathway can only enhance gene transcription when the EcR-USP pathway is activated by the 20E-induced PKC pathway. In additional, KN-93 and ESI-09 can repress the reporter protein FLP expression from the EcRE-deleted pCRE-IE1-RFP-His plasmid that contains CRE only, suggesting the involvement of CaMKII/ CaMKIV and EPAC signaling pathways in regulating CREB signaling under 20E induction. This needs further study.

Conclusions—20E increases intracellular CaM, PKAC1 phosphorylation, and nuclear translocation via ErGPRC2. The phosphorylated PKAC1 directly phosphorylates CREB in the nucleus, which then binds to CRE to enhance 20E-induced gene transcription based on the PKC-activated EcR-USP pathway (Fig. 11).

Author Contributions—Y.-P. J. designed, performed, and analyzed the experiments in this manuscript and drafted the preliminary version of this manuscript. D. W., D.-J. D., and X.-L. H. assisted in the laboratory experiments described in this manuscript. J.-X. W. participated in the design and coordination of the study. X.-F. Z. conceived the study and helped to draft the final version of this manuscript. All authors reviewed the results and approved the final version of the manuscript.

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FIGURE 10. The possible signaling pathway involved in 20E-induced CREB-dependent gene expression. A, the structure of pCRE-IE1-RFP-His. B, the signaling pathways involved in CREB signaling were analyzed by Western blotting. The HaEpi cells were transfected with pCRE-IE1-RFP-His plasmid for 24 h and then treated with bupivacaine hydrochloride (24 h) and then treated with bupivacaine hydrochloride (BH, 3 μM), H89 (10 μM), ESI-09 (4 μM), CC (5 μM), and KN-93 (1 μM) for 0.5 h followed by incubation with 20E (2 μM) for 6 h. The total proteins were used for the Western blotting analyses using an antibody against RFP. β-Actin was used as a loading control. *p < 0.05, **p < 0.01, and n.s. denote p < 0.05, p < 0.01, and no significant difference, respectively. Error bars represent S.D.

FIGURE 11. A model for the role of the 20E-induced CAMP/PKA/CREB signaling in 20E-regulated gene expression. 1, 20E mediates PKAC1 phosphorylation via ErGPRC2. 2, 20E triggers an increase in the intracellular CAMP concentrations, which promotes the dissociation of PKAC1 and PKAR. 3, then the liberated phosphorylated PKAC1 (P-PKAC1) enters the nucleus to bind to and phosphorylate CREB. 4, the PKA-phosphorylated CREB (P-CREB) binds to CRE to enhance EcR1-USP1-initiated gene transcription, which is regulated by the 20E-induced GPCR, Ca^{2+}, PKC-, and P-USP1 signal pathway (24, 25). P-USP1, phosphorylated USP1.
Steroid Hormone Activates CREB Pathway

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