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# G-protein αq participates in the steroid hormone 20-hydroxyecdysone nongenomic signal transduction

Jing Ren, Xiang-Ru Li, Peng-Cheng Liu, Mei-Juan Cai, Wen Liu, Jin-Xing Wang, Xiao-Fan Zhao *

*Shandong Provincial Key Laboratory of Animal Cells and Developmental Biology, School of Life Science, Shandong University, Jinan 250100, China*

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ABSTRACT

The nuclear receptor-mediated genomic pathways of the animal steroid hormones are well known. However, the cell membrane receptor-mediated nongenomic pathways of the animal steroid hormones are little understood. In this study, we report the participation of a G-protein alpha q (Gαq)[1] subunit in the 20E nongenomic pathway in the cell membrane and regulating gene expression during molting and metamorphosis in a lepidopteran insect, *Helicoverpa armigera*. 20E-induced phosphorylation of Gαq was detected using two-dimensional electrophoresis techniques. Knockdown of *Gαq* by injecting double-stranded RNA suppressed the development of larvae, delayed metamorphosis, and inhibited 20E-induced gene expression. Gαq was distributed throughout the cell, and migrated toward the plasma membrane upon 20E induction. Gαq was necessary in the 20E-induced intracellular $Ca^{2+}$ release and extracellular $Ca^{2+}$ influx. The protein kinase C (PKC) inhibitor could repress 20E-induced phosphorylation of cyclin-dependent kinase 10 (CDK10) and transcription factor ultraspiracle (USP1). PKC inhibitor could repress the Gαq phosphorylation and membrane trafficking. These results suggest that Gαq participates in 20E signaling in the cell membrane at the pre-genomic stage by modulating the increase of the intracellular $Ca^{2+}$ and phosphorylation of CDK10 and USP1 in 20E transcription complex to regulate gene transcription.



## 1. Introduction

The animal steroid hormones are lipid-soluble molecules, they act via genomic mechanism by diffusing freely into the target cell membranes and binding with the nuclear receptors to regulate gene transcription [1]. Steroid hormones also initiate signaling via nongenomic mechanism by membrane-associated receptors [2]. The nuclear receptor-mediated genomic pathways of the animal steroid hormones are well known. However, the cell membrane receptor-mediated nongenomic pathways of the animal steroid hormones are little understood. Recent studies report that some steroid hormones have membrane receptors, such as estrogen receptor G-protein-coupled receptor 30 (GPR30) in mammals [3] and brassinolide kinase receptor in plants [4]. Insect steroid hormone 20-hydroxyecdysone (20E) regulates molting and metamorphosis [5]. It is unknown whether 20E has a plasma membrane receptor [6].

The classical theory of 20E action is known via a genomic pathway, that is, 20E binds to its nuclear receptor ecdysteroid receptor (EcR) and forms a heterodimeric transcription complex with ultraspiracle (USP) [7], followed by modulation of gene transcription, including transcription factors *Br*, *HR3*, *E74*, and *E75* [8]. However, 20E might have nongenomic pathways [9], and has been found to mediate programmed cell death (PCD) through $Ca^{2+}$ signals via a nongenomic pathway in cell membranes [10]. The 20E-$Ca^{2+}$-PKC-caspase-3-like protease pathway induces PCD in silkworm silk glands [11]. 20E regulates small GTPase Rab 4b quickly moving toward the cell membrane [12] and calponin fast subcellular translocation and phosphorylation in

*Abbreviations:* 20E, 20-hydroxyecdysone; Gαq, G-protein alpha q; GPCR, G-protein-coupled receptor; EcRB1, ecdysone receptor; USP, ultraspiracle; *HR3*, hormone receptor 3; *Br*, broad isoform 7; PKC, protein kinase C; CDK10, cyclin-dependent kinase 10; CC, PKC inhibitor chelerythrine chloride; λPP, lambda protein phosphatase; PMA/TPA, phorbol-12-myristate-13-acetate/12-O-tetradecanoyl-phorbol 13-acetate; DMSO, dimethyl sulfoxide; GFP, green fluorescence protein; WGA, wheat germ agglutinin; RNAi, RNA interference.

* Corresponding author. Tel.: +86 531 88364620; fax: +86 531 88364620.
*E-mail address:* xfzhao@sdu.edu.cn (X.-F. Zhao).

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*Helicoverpa armigera* [13]. In *Drosophila melanogaster*, a G-protein-coupled receptor (GPCR) DmDopEcR with structural homology to vertebrate β-adrenergic-like receptors can be activated rapidly by ecdysteroids [14]. Previous research indicated that 20E via GPCR regulates non-canonical steroid action to $Ca^{2+}$/calmodulin-responsive cAMP signaling in the *Drosophila* adult brain [15]. GPCR might be involved in the 20E pathway before genomic response [10]. USP is the heterodimeric protein of EcR, which is PKC-phosphorylated by 20E regulation in *D. melanogaster* [16]. In *H. armigera*, an ErGPCR-Gαq-PLCG1-$Ca^{2+}$-PKC, no genomic pathway is reported recently [17,18]. These data suggest that a nongenomic pathway exists in 20E signaling, but its mechanism is not fully understood.

Heterotrimeric guanine nucleotide-binding proteins (G-proteins) play roles in various GPCRs pathways [19]. G-proteins comprise Gα subunits and Gβ/γ subunits. Gα subunits are grouped into four main classes according to the primary sequence and produced second messengers: Gαs stimulates cAMP production; Gαi inhibits cAMP production; Gα12 activates RhoA GTPases, and Gαq activates phospholipase C-beta (PLC-β) [20]. PLC-β hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 promotes intracellular $Ca^{2+}$ release [21]. The intracellular $Ca^{2+}$ release may induce the extracellular $Ca^{2+}$ influx [22]. $Ca^{2+}$ and DAG activate PKC by binding to the C1 and C2 domains of PKC, respectively [23]. PKC can regulate protein phosphorylation in various signaling pathways [24] to control gene expression [25].

*H. armigera* belongs to lepidopteran noctuidae, which is a severe agricultural pest. It has six instars and the generation period is about one month [26]. It is a medium-sized insect and can be reared in the laboratory with artificial diet. The larval molting time can be easily counted by each ecdysis when a white head capsule of the newly hatched larva appears. An epidermal cell line of *H. armigera* (HaEpi) was established in our previous work [27]. The RNA interference was not only successful in the larvae either by feeding the dsRNA expressing *Escherichia coli* or injecting the dsRNA, but also practicable in the HaEpi cells [28,29]. We used this insect model to investigate the function of Gαq in the nongenomic pathway of 20E. Our data demonstrate that Gαq plays important roles in 20E nongenomic pathway.

## 2. Materials and methods

### 2.1. Insects

The cotton bollworms *H. armigera* were fed with an artificial diet on a light/dark schedule of 14:10 h. The cotton bollworms were obtained from the Henan Agricultural University. The larvae were fed with an artificial diet according to the previously described methods [30].

### 2.2. Two-dimensional gel electrophoresis (2-DE)

The *Helicoverpa* epidermal cell line HaEpi was cultured according to the method [27]. After treatment by 1 μM 20E for 30 min, the cells were collected and the protein was used for first-dimensional isoelectric focusing on nonlinear immobiline IPG Drystrip (pH 3–10) (GE Healthcare, San Francisco, CA, USA). After electrophoresis by sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), the gels were stained with the Pro-Q Diamond Phosphoprotein Stain following the manufacturer's instructions (Molecular Probes, USA) and the images were captured using a Typhoon Trio$^+$ System (GE Healthcare, USA). The same gels were stained with colloidal Coomassie Brilliant Blue G-250 to detect total protein spots. The putative phosphoprotein spots were manually picked from the gel and used for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis with an ABI 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, USA). The MASCOT search engine (version 1.9, Matrix Science, http://www.matrixscience.com/search_form_select.html) was used to search the tandem mass spectra against the NCBInr Metazoa Database. High confidence identifications had statistically significant search scores (greater than 95% confidence, equivalent to the MASCOT expected value, $P < 0.05$).

### 2.3. Cloning and phylogenetic analysis of Gαq

The full sequence of *Gαq* cDNA was cloned from the library of *H. armigera* larvae. Two special primers, *Gαq*EXPF and *Gαq*EXPR (Table S1), were used to amplify the complete open reading frame (ORF) of *Gαq*. Protein translation and prediction was achieved by ExPASy software (http://www.au.expasy.org/). Domain prediction was performed using SMART software (http://www.smart.embl-heidelberg.de/). Sequence alignment and phylogenetic tree analyses were conducted using the MEGA 4.0 software (http://mega.software.informer.com/4.0/) and GENDOC computer program (http://www.psc.edu/biomed/genedoc/).

### 2.4. Recombinant expression of Gαq and preparation of antiserum

A 1059 bp ORF encoding *Gαq* from *H. armigera* was inserted into the expression vector pET-30a(+), and the protein was expressed in *Escherichia coli* BL21 (DE3) host cells in the form of an inclusion body. Buffer A (50 mM Tris–HCl, pH 8.0, 5 mM EDTA) and buffer B (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 2 M urea) were used to wash the inclusion bodies for two times, respectively. The washed inclusion bodies were dissolved in buffer C (0.1 M Tris–HCl, pH 8.0, 8 M urea). The protein was then refolded in dialysis buffer (0.1 M Tris–HCl, pH 8.0, 5 mM EDTA, 5 mM cysteine) for 1 h at 37 °C, and the target recombinant protein was purified to homogeneity using a $Ni^{2+}$-NTA affinity column. The rabbit polyclonal antiserum was prepared as previously described method [31].

### 2.5. Western blot

The total protein of various tissues was extracted using TBS (10 mM Tris–HCl, pH 7.5; 150 mM NaCl with 1 mM phenylmethanesulfonyl fluoride) from three larvae to eliminate the individual variation. The protein concentration was measured according to the Bradford method [32]. Equal amount of protein (20 μg) for each sample was loaded on SDS-PAGE. Protein was electrophoretically transferred onto a nitrocellulose membrane. The membrane was incubated with blocking buffer (2% skim milk powder dissolved in TBS) for 1 h at room temperature. Anti-*Helicoverpa* Gαq was diluted to 1:100 in blocking buffer in TBS. Then the secondary antibody of alkaline phosphatase conjugated goat anti-rabbit IgG was diluted 1:1000 in the blocking buffer. The protein signal was visualized by 45 μL of nitroblue tetrazolium (75 μg/μL) and 35 μL of 5-bromo-4-chloro-3-indolyl phosphate (50 μg/μL) (Sigma) in 10 mL of TBS in the dark at room temperature. The gel concentration was 12.5% unless otherwise stated.

### 2.6. Hormonal treatment

20E (Sigma, St. Louis, MO, USA) was first dissolved to 20 mM in dimethyl sulfoxide (DMSO), and diluted in PBS (140 mM NaCl, 10 mM sodium phosphate, pH 7.4) for further use. The sixth instar 6 h larvae were chosen to be injected with the hormone (500 ng/larva) in the hemocoel, because the titer of 20E was low at this stage according to *Manduca sexta* [7]. The controls were