The steroid hormone 20-hydroxyecdysone induces phosphorylation and aggregation of stromal interacting molecule 1 for store-operated calcium entry

Cai-Hua Chen, Yu-Qin Di, Qin-Yong Shen, Jin-Xing Wang, and Xiao-Fan Zhao

From the 4Shandong Provincial Key Laboratory of Animal Cells and Developmental Biology, School of Life Sciences, Shandong University, Qingdao 266237, China and the 5Department of Entomology, College of Plant Protection, Northwest A & F University, Yangling 712100, China

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Oligomerization of stromal interacting molecule 1 (STIM1) promotes store-operated calcium entry (SOCE); however, the mechanism of STIM1 aggregation is unclear. Here, using the lepidopteran insect and agricultural pest cotton bollworm (Helicoverpa armigera) as a model and immunoblotting, RT-qPCR, RNA interference (RNAi), and ChiP assays, we found that the steroid hormone 20-hydroxyecdyscöne (20E) up-regulates STIM1 expression via G protein–coupled receptors (GPCRs) and the 20E nuclear receptor (EcRB1). We also identified an ecdysone-response element (EcRE) in the 5′-upstream region of the STIM1 gene and also noted that STIM1 is located in the larval midgut during metamorphosis. STIM1 knockdown in larvae delayed puation time, prevented midgut remodeling, and decreased 20E-induced gene transcription. STIM1 knockdown in a H. armigera epidermal cell line, HaEpi, repressed 20E-induced calcium ion influx and apoptosis. Moreover, 20E-induced STIM1 clustering to puncta and translocation toward the cell membrane. Inhibitors of GPCRs, phospholipase C (PLC), and inositol trisphosphate receptor (IP3R) repressed 20E-induced STIM1 phosphorylation, and we found that two GPCRs are involved in 20E-induced STIM1 phosphorylation. 20E-induced STIM1 phosphorylation on Ser-485 through protein kinase C (PKC), and we observed that Ser-485 phosphorylation is critical for STIM1 clustering, interaction with calcium release-activated calcium channel modulator 1 (Orai1), calcium ion influx, and 20E-induced apoptosis. These results suggest that 20E up-regulates STIM1 phosphorylation for aggregation via GPCRs, followed by interaction with Orai1 to induce SOCE, thereby promoting apoptosis in the midgut during insect metamorphosis.

Cytosolic calcium, a key secondary signaling messenger in almost every cell, regulates a wide range of biological processes (1), such exocytosis, cell proliferation, and apoptosis (2). However, the concentration of cytosolic Ca2+ is well-controlled at a low concentration (~nanomolar) compared with that in the extracellular environment (~millimolar) by storing Ca2+ in the intracellular calcium store of endoplasmic reticulum (ER),2 or excluding Ca2+ outside cells, because it binds water less tightly and precipitates phosphate (3). The cytosolic Ca2+ concentration can be increased for signaling by the depletion of the intracellular Ca2+ store that triggers Ca2+ influx into cells from the outside environment. This kind of Ca2+ entrance is called store-operated calcium entry (SOCE) (4), and the channel is called the intracellular store-operated Ca2+ channel (SOC) or Ca2+ release-activated channel (CRAC) (5).

Stromal interacting molecule (STIM1), as an intracellular calcium sensor locates on the ER, is essential for SOCE (6, 7). The N terminus of STIM1 is inside the ER lumen and C terminus lies outside the ER (8). When ligands bind to cell membrane receptors, such as G protein–coupled receptors (GPCRs), phospholipase C (PLC), and inositol trisphosphate receptor (IP3R) repressed 20E-induced STIM1 phosphorylation, and we found that two GPCRs are involved in 20E-induced STIM1 phosphorylation. 20E-induced STIM1 phosphorylation on Ser-485 through protein kinase C (PKC), and we observed that Ser-485 phosphorylation is critical for STIM1 clustering, interaction with calcium release-activated calcium channel modulator 1 (Orai1), calcium ion influx, and 20E-induced apoptosis. These results suggest that 20E up-regulates STIM1 phosphorylation for aggregation via GPCRs, followed by interaction with Orai1 to induce SOCE, thereby promoting apoptosis in the midgut during insect metamorphosis.

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1To whom correspondence should be addressed. E-mail: xfzhao@sdu.edu.cn

2The abbreviations used are: ER, endoplasmic reticulum; SOCE, store-operated Ca2+ entry; STIM1, stromal interacting molecule; GPCR, G protein–coupled receptors; PLC, phospholipase C; IP3, inositol 1,4,5-trisphosphate; PM, plasma membrane; 20E, 20-hydroxyecdyscöne; CaMKII, Ca2+/calmodulin–dependent protein kinase II; PCD, programmed cell death; qRT-PCR, quantitative real-time reverse transcription PCR; Tg, thapsigargin; SERCA, sarcoplasmic/endoplasmic reticulum Ca2+–ATPase; WGA, wheat germ agglutinin; DPBS, Dulbecco’s phosphate-buffered saline; co-IP, co-immunoprecipitation; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; Xec, xestospongin; RNAi, RNA interference; FBS, fetal bovine serum; AP, alkaline phosphatase; λPPase, λ-phosphatase; DAPI, 4′,6-diamidino-2-phenylindole; H&E, hematoxylin and eosin; GFP, green fluorescent protein; RFP, red fluorescent protein.

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Oligomerization of STIM1 promotes SOCE in humans; however, the upstream regulator of STIM1 aggregation and the outcome are unclear.

Phosphorylation as a post-translational modification plays an important role in protein functions. Various phosphorylation sites of STIM1 have been detected in mammals (18), and the outcomes of different STIM1 phosphorylations are different in various cellular processes. For example, estrogen inhibits oligomerization and Ser-575 phosphorylation of STIM1 to reduce SOCE in human bronchial epithelial cells (19). Phosphorylation of STIM1 on Ser-486 and Ser-668 during mitosis in HEK293 cells represses SOCE (20). The phosphorylation of STIM1 on Ser-575, Ser-608, and Ser-621 is required for SOCE in HEK293 cells (21). However, the phosphorylation and mechanism of STIM1 in insects are unclear.

Steroid hormones, such as mammal estrogen and insect molting hormone 20-hydroxyecdysone (20E), are small fat-soluble molecules. They can diffuse into cells through the PM and bind with estrogen receptors in mammals (22), and the 20E nuclear receptor (EcRB1) in insects (23), to regulate gene expression for animal development. In recent years, studies have shown that estrogen induces rapid cellular responses, such as rapid calcium increase, via GPCRs (24). 20E also induces Ca\(^{2+}\) increase via GPCRs in Bombyx mori (25) and Helicoverpa armigera (26). Such a rapid signaling pathway acting before gene transcription is called a non-genomic pathway. In H. armigera, ecdysone-responsive GPCRs, ErGPCR-1 (26) and ErGPCR-2 (27) on the cell membrane, have been identified to participate in the 20E-non-genomic pathway by inducing rapid calcium entry, and protein phosphorylation in transcription complexes for gene transcription during insect development (28, 29). Dependent on the increased intracellular Ca\(^{2+}\) levels, 20E causes a switch from autophagy to apoptosis in the midgut of H. armigera during metamorphosis (30). Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) is activated by Ca\(^{2+}\) to regulate transcription factor ultraspircal (USP1) acetylation for gene expression (31). 20E, via GPCRs, induces Ca\(^{2+}\) influx through the Orai1 channel in H. armigera (32); however, currently available data are insufficient to determine the mechanism of steroid hormone-induced Ca\(^{2+}\) entry, without understanding the role of the key initiator STIM1 in the process. The 20E is also produced by various plants, such as Cyanotis vaga, to disrupt development of insect pests (33), however, the mechanism is unclear.

To address these questions, the present study focused on STIM1 using the agricultural pest, cotton bollworm (Lepidoptera H. armigera), as a model. We revealed that 20E, via GPCRs and EcRB1, up-regulated STIM1 expression. 20E, via GPCR, PLC, IP\(_3\), and PKC signaling, induced the phosphorylation of STIM1 at Ser-485, which was essential for aggregation of STIM1 and calcium influx leading to apoptosis. STIM1 is essential for the degradation of the larval midgut and 20E-regulated gene expression. Our study proved that 20E induces SOCE via a GPCR-mediated nongenomic pathway to induce insect metamorphosis.

Results

**STIM1 was up-regulated and exists in a phosphorylated form during metamorphosis**

We prepared rabbit-derived polyclonal antibodies against the STIM1 and examined their specificity by Western blotting. We found that the antiserum had the ability to recognize a 65-kDa molecular weight (Mr) protein specifically, which was consistent with the predicted Mr of STIM1. However, the pre-serum of rabbit did not recognize any protein (Fig. 1A), which suggests that the antibodies had specificity to STIM1 of H. armigera. The expression profiles of STIM1 in certain tissues from fifth instar larvae to the pupal stage were detected using Western blotting to verify the involvement of STIM1 in larval development and metamorphosis. STIM1 was detected in the epidermis, midgut, and fat body, with a marked increase in its level at the metamorphic stage from sixth instar larvae to the late pupae (Fig. 1B). The data were quantified based on three independent experiments (Fig. 1C). The Mr of STIM1 was decreased by treatment with APPase, suggesting that STIM1 was phosphorylated posttranslationally (Fig. 1D). These data suggested that STIM1 plays an important role in insect metamorphosis.

**20E up-regulates STIM1 expression through a membrane receptor and a nuclear receptor**

The increased expression level of STIM1 suggested that 20E might up-regulate STIM1 expression, because the 20E titer increases during metamorphosis in lepidopteran insects (34). To verify the up-regulation of STIM1 by 20E, we examined the level of STIM1 in the midgut using Western blotting after 20E injection into larvae. The results showed that the level of STIM1 was increased by 20E injection in a time- and concentration-dependent manner (Fig. 2, A and B). Quantitative analysis confirmed that the expression of STIM1 was increased by 20E induction significantly (Fig. 2, C and D). The results demonstrated that 20E increased the expression level of STIM1 in the midgut.

GPCRs, such as ErGPCR-1 (26) and ErGPCR-2 (27), transmit 20E signals in the cell membrane to promote formation of the EcRB1/USP1 complex for gene transcription (29, 35). Therefore, we knocked down the expression of ErGPCR-1, ErGPCR-2, EcRB1, and USP1, respectively, in HaEpi cells and detected STIM1 expression to address the signaling axis of 20E up-regulated STIM1 expression. The qRT-PCR results showed that the 20E-promoted expression of STIM1 was repressed after knockdown of ErGPCR-1, ErGPCR-2, EcRB1, and USP1 (Fig. 3, A–D). The interference efficiencies were detected after transfection of dsRNA alone (Fig. 3E). The results suggested that 20E, via the ErGPCR-1 and ErGPCR-2 cell membrane GPCRs and EcRB1/USP1 nuclear receptor transcription complex signaling axis, up-regulates STIM1 expression.

To confirm the above hypothesis, the Ec-R binding element (EcRE) was analyzed and an EcRE was predicted in the 5’-upstream genomic DNA sequence of STIM1 in the genome of H. armigera.
$H.\ armigera$ using the JASPAR website (http://jaspar.genereg.net)\(^3\) (64). We identified an EcRE in the promoter region of STIM1, 5'-GCGGTTAATGCATTA-3', which is very similar to the previously identified EcRE, 5'-GGGGTCAATGAACTGA-3', in the promoter region of HR3 (28) with a few base differences (Fig. 3F). ChIP experimental analysis proved that EcRB1 could bind to the EcRE in the promoter region of STIM1 (Fig. 3G). These results showed that the different base had no effect on the binding of EcRB1 and EcRE. These data indicated that 20E directly promotes STIM1 transcription by EcRB1 binding to the EcRE.

**STIM1 is located in the larval midgut during metamorphosis**

To identify the function of STIM1 in the larval midgut, we first examined the localization of STIM1 in the midgut. STIM1 was subjected to immunohistochemistry using polyclonal antibodies. We found that little STIM1 distributed in the midgut of sixth instar 72-h larvae. Anti-rabbit IgG (AP) was used as the secondary antibody. \(\beta\)-Actin was used as a protein quantity control. 5F, fifth instar feeding larvae; 5M, fifth instar molting larvae; 6th-6 h to 6th-120 h represent sixth instar larvae at the corresponding hours. P-0 d to P-8 d indicate 0- to 8-day-old pupae. F, feeding; M, molting; MM, metamorphic molting; P, pupae. The molecular weight markers were indicated on the left. G, the immunoreactive protein bands were subjected to densitometry analyses using Quantity One software based on three independent biological experiments. The bars indicate the mean ± S.D. of three independent experiments. D, examination of STIM1 phosphorylation. The λ-phosphatase (λ-PP)-treated proteins extracted from epidermis, midgut, and fat body of sixth 72-h larvae. The gel concentrations were 7.5% for all experiments. Molecular weight markers are shown on the left.

**Figure 1. Western blot analysis of the expression and phosphorylation of STIM1 in different tissues during development.** A, examination of the specificity of the polyclonal antibodies against $H.\ armigera$ STIM1 by Western blotting. M, maker; lane 1, pre-serum; lane 2, antiserum. The proteins were from the midgut of sixth instar 72-h larvae. Anti-rabbit IgG (AP) was used as the secondary antibody. B, expression profiles of STIM1 in the epidermis, midgut, and fat body were detected using anti-STIM1 antibodies. \(\beta\)-Actin was used as the protein quantity control. 5F, fifth instar feeding larvae; 5M, fifth instar molting larvae; 6th-6 h to 6th-120 h represent sixth instar larvae at the corresponding hours. P-0 d to P-8 d indicate 0- to 8-day-old pupae. F, feeding; M, molting; MM, metamorphic molting; P, pupae. The molecular weight markers were indicated on the left. C, the immunoreactive protein bands were subjected to densitometry analyses using Quantity One software based on three independent biological experiments. The bars indicate the mean ± S.D. of three independent experiments. D, examination of STIM1 phosphorylation. The λ-phosphatase (λ-PP)-treated proteins extracted from epidermis, midgut, and fat body of sixth 72-h larvae. The gel concentrations were 7.5% for all experiments. Molecular weight markers are shown on the left.

**Figure 2. Western blotting shows the up-regulated level of STIM1 by 20E in the midgut.** A, 20E was injected into larvae at the sixth instar 6-h larvae (6th-6 h larvae) at different concentrations for 24 h. An equal amount of diluted DMSO was injected as the control. \(\beta\)-Actin was detected as the protein quantity control. B, injection of 500 ng of 20E per sixth 6-h larva for different times. DMSO was used as the solvent control and \(\beta\)-actin as the protein quality control. All gel concentrations were 7.5%. The molecular weight markers near the bands are indicated. C and D, the immunoreactive protein bands in A and B were subjected to densitometry analyses using Quantity One software. The bars indicate the mean ± S.D. Significant differences were calculated using Student’s t test (*, \(p < 0.05\)) based on three biological replicates.

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expressed in the larval midgut, and might be related to midgut apoptosis during metamorphosis.

**STIM1 knockdown suppressed metamorphosis, midgut programmed cell death (PCD), and 20E-induced gene expression**

**STIM1** was knocked down by injecting *dsSTIM1* into fifth and sixth instar larvae to explore the function of STIM1 during insect metamorphosis. Compared with that in the *dsGFP* injection control group, pupation was delayed by about 30 h in the *dsSTIM1* injection group (Fig. 5A and B). The efficiency of RNAi of *STIM1* was confirmed by Western blotting (Fig. 5C). In *H. amigera*, the midgut turns red at 72 h of the sixth instar larvae when it occurs apoptosis during metamorphosis (36, 37). However, the midgut did not turn red after injection of *dsSTIM1* for 96 h and the larval midgut did not separate from imaginal midgut, compared with the larvae injected with the control *dsGFP* injection (Fig. 5D and E). qRT-PCR analysis showed that the expression of 20E-response genes, *HR3*, *EcRB1*, *USP1*, and *BurZ7*, and apoptosis-related genes, *Caspase-3* and *Caspase-6*, were repressed in the midgut after interference by *dsSTIM1* in the larval midgut (Fig. 5F). Similar results were observed in HaEpi cells after knockdown of *STIM1* (Fig. 5G). These results demonstrated that STIM1 participates in the larval midgut PCD and metamorphosis.

To address the mechanism of STIM1’s participation in 20E-promoted gene expression and metamorphosis, the intracellular Ca²⁺ levels were examined after *STIM1* was knocked down in HaEpi cells. 20E promoted the release of intracellular-stored Ca²⁺ ions and thus caused Ca²⁺ influx (Fig. 6A). However, the release of intracellular Ca²⁺ was unaffected, but the influx of extracellular Ca²⁺ was repressed (Fig. 6B) when *STIM1* was knocked down. Besides, 20E-induced apoptosis was also detected using a TUNEL assay after knockdown of *STIM1*.5/6 (Fig. 6C). The statistical analysis of the ratio of apoptotic cells showed similar results (Fig. 6D). The efficacy of *STIM1* knockdown by RNAi was confirmed using qRT-PCR (Fig. 6E). These data suggested that STIM1 participates in 20E-
promoted SOCE, which is necessary for 20E pathway gene expression and apoptosis.

20E-induced STIM1 aggregation

To demonstrate the mechanism of STIM1’s function in 20E-promoted SOCE, we examined the subcellular localization of STIM1 under regulation by 20E using overexpression of STIM1-GFP in HaEpi cells. Thapsigargin (Tg), as an agonist of SOC, triggers stored calcium release, but inhibits the calcium pump sarcoplasmic/endoplasmic reticulum Ca$_{2+}$-ATPase (SERCA) on the ER will suppress calcium back into the ER from the cytosol \(^{38}\). Therefore, we used Tg as a positive control of calcium-store depletion. By staining the cell membrane with wheat germ agglutinin (WGA), we found that 20E promoted STIM1-GFP to form oligomers and move toward the PM, where they were located below the PM. The aggregation of STIM1-GFP disappeared after the addition of calcium into DPBS followed by 20E treatment that refilled the intracellular calcium store. Tg promoted similar aggregation and subcellular translocation of STIM1-GFP to 20E; however, the addition of calcium did not eliminate the Tg-induced aggregation of STIM1-GFP because Tg blocked the refill of the calcium store (Fig. 7A). The percentage of STIM1-aggregated cells were calculated and suggested the same results (Fig. 7B). The overexpressed STIM1-GFP and GFP tag proteins were detected by Western blotting (Fig. 7C). These data suggested that 20E induces STIM1 aggregation and cell membrane translocation upon depletion of stored calcium.

20E-induced STIM1 phosphorylation via GPCRs

Considering STIM1 was phosphorylated in larvae, the pathway by which 20E might stimulate STIM1 phosphorylation was analyzed using various inhibitors and knockdown of GPCRs. There was no effect on the molecular sizes of the overexpressed GFP-tag control (Fig. 8A, a). However, overexpressed STIM1-GFP appeared on the upper band under 20E regulation in Ca$_{2+}$-free conditions. Moreover, the amount of the upper band was reduced by the addition of calcium and eliminated by aPPase treatment (Fig. 8A, b), suggesting that 20E-induced STIM1 phosphorylation under conditions of calcium shortage. The GPCR and ryanodine receptor inhibitor Suramin (39), the PLC inhibitor U73122 (40), the IP$_3$R inhibitor xestospongin C (XeC) (41), and the PKC inhibitor chelerythrine chloride (CC) (42) all repressed 20E-induced STIM1 phosphorylation (Fig. 8B). Moreover, knockdown of ErGPCR-1 and ErGPCR-2 using RNAi inhibited 20E-induced phosphorylation of STIM1 (Fig. 8C). The efficacies of knockdown of ErGPCR-1 and ErGPCR-2 were confirmed using qRT-PCR (Fig. 8, D and E). These results suggested that 20E promotes STIM1 phosphorylation through the GPCRs, PLC, IP$_3$R, and PKC signaling axis.

In addition, anti-pSer, anti-pThr, and anti-pTyr antibodies were used to detect the type of STIM1 phosphorylation in the epidermis and midgut. The anti-pSer antibody detected a band from the purified STIM1. However, the anti-pThr and anti-pTyr antibodies did not detect a band (Fig. 8F), which suggested that STIM1 phosphorylation occurred on serine residues.

Phosphorylation of STIM1 was required for STIM1 aggregation and calcium influx

The phosphorylation site of STIM1 was identified to confirm the outcome of phosphorylation of STIM1. STIM1 phosphorylation was serine phosphorylation and was regulated by PKC (as confirmed by the inhibitor experiments and antibodies detection); therefore, we predicted the PKC-phosphorylation

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**Figure 4.** STIM1 is located in the larval midgut during metamorphosis. H&E staining showing the tissue histology of the midgut at different developmental stages. Immunohistochemistry was used to identify the location of STIM1. IM, imaginal midgut; LM, larval midgut. The green fluorescence indicates STIM1 detected using an anti-STIM1 antibody. The blue fluorescence indicates the nuclei stained with DAPI. Rabbit pre-serum was used as a control for the antibody. Yellow bar = 50 μm.
Three PKC-type serine phosphorylation sites, Ser-360, Ser-395, and Ser-485, were predicted using NetPhos. We constructed three mutant plasmids in which the possible phosphorylated serines were replaced by alanine (S360A, S395A, and S485A) and overexpressed the mutant proteins in HaEpi cells. The phosphorylation of STIM-GFP WT and mutants were detected by Western blotting under 20E or DMSO treatments. Under 20E induction, the upper band was detected in the WT, Ser-360 mutant, and Ser-395 mutant, but not in the S485A mutant, compared with that in the DMSO control (Fig. 9A), which suggested that 20E-induced STIM1 phosphorylation at Ser-485. To explore the outcomes of STIM1 phosphorylation, STIM1-GFP and the STIM1-S485A-GFP mutant were overexpressed in HaEpi cells and various cellular processes were examined. The results showed that the STIM1-S485A-GFP mutant could not aggregate under 20E induction in HaEpi cells compared with that in STIM1-GFP overexpressing cells (Fig. 9B and C). In addition, DMSO, as a solvent control, did not induce Ca\(^{2+}\) release or influx in GFP and STIM1-GFP overexpressing cells. In contrast, 20E induced normal Ca\(^{2+}\)/H\(^{11000}\) release in GFP, STIM1-GFP, and STIM1-S485A-GFP mutant overexpressing cells; however, 20E triggered much more Ca\(^{2+}\)/H\(^{11000}\) influx in STIM1-GFP overexpressing cells than that in GFP or STIM1-S485A-GFP mutant overexpressing cells (Fig. 9D), which suggested that Ser-485 was critical for the function of STIM1 in calcium entry. Co-immunoprecipitation (co-IP) using antibodies against GFP detected STIM1-GFP and Orai1-RFP together under 20E induction, but did not detect STIM1-S485A-GFP and Orai1-RFP together under 20E induction (Fig. 9E), which suggested that the interaction between STIM1 and Orai1 relies on phosphorylation of Ser-485 in STIM1.
High levels of 20E induce an intracellular calcium increase for apoptosis (43). To determine the effect of STIM1 phosphorylation on 20E-induced apoptosis, we overexpressed the STIM1-GFP and mutation type STIM1-S485A-GFP. Apoptosis by the TUNEL assay shows that overexpression of STIM1-GFP increased apoptosis under 5 μM 20E induction for 48 h. However, overexpression of the mutant STIM1-S485A-GFP did not increase apoptosis under same conditions (Fig. 10, A and B). These results suggested that Ser-485 phosphorylation of STIM1 is required for 20E-induced apoptosis.

Discussion

20E can induce Ca\(^{2+}\) entry; however, the mechanism is unclear. In the present study, we demonstrated that 20E, via cell membrane receptor GPCRs and the ecdysone nuclear receptor EcRBI, induced STIM1 expression for Ca\(^{2+}\) influx. 20E-induced STIM1 phosphorylation at Ser-485 through the GPCRs, PLC, IP\(_3\), R, and PKC signaling axis to promote STIM1 aggregation, interaction with Orai1 for Ca\(^{2+}\) entry, and finally, apoptosis. Therefore, 20E via STIM1 induces SOCE for apoptosis in the midgut during metamorphosis.

20E up-regulates STIM1 expression through GPCRs and nuclear receptor EcRBI

STIM1 is a Ca\(^{2+}\) sensor in cells that initiates Ca\(^{2+}\) entry when the cellular Ca\(^{2+}\) store is depleted (44). STIM1 is expressed in a range of human cells and tumor cells to suppress growth (18). The expression of STIM1 can be positively or negatively regulated by androgens (45), nuclear factor-kappa B (NF-κB) (46), and glucocorticoid inducible kinase 1 (SGK1) (47). In the present study, we found that steroid hormone 20E up-regulates STIM1 expression through GPCRs and the 20E nuclear receptor, EcRBI. This is the first report that GPCRs participate in the expression of STIM1, which was induced by steroid hormone.

20E has a high titer during insect metamorphosis (34) and regulates gene transcription through genomic (23) and nongenomic pathways (25). Studies have shown that 20E via the nongenomic pathway directs the genomic pathway for gene expression (29, 48). ErGPCR-1 and ErGPCR-2 transmit the 20E signal by the nongenomic pathway and finally regulate gene expression via the EcRBI/USP1-mediated genomic pathway.
for apoptosis in the midgut during metamorphosis in H. armigera (26, 27). In the present study, we demonstrated that membrane receptors ErGPRC-1 and ErGPRC-2 are critical for the expression of STIM1, which was up-regulated by 20E in the larval midgut. The results further verified that 20E regulates gene expression through the nongenomic pathway.

We found the nuclear receptor EcRB1 is also necessary for 20E-induced STIM1 expression. To date, most genes in the 20E pathway have been found to be up-regulated by 20E via the EcRB1/USP1 transcription complex binding to EcRE, for example, HR3 (49). The EcRE1 in the MHR3 promoter region of Manduca sexta (50), 5'-GGGGTCAATGAAACC-3', is highly conserved with the EcRE in the HHR3 promoter region of H. armigera, 5'-GGGGTCAATGAAACCG-3' (29). In the present study, we identified an EcRE in the promoter region of STIM1, 5'-GCGGTTAATGCACTTA-3', which is very similar to the EcRE in the HHR3 promoter. All these EcREs have a conservative core sequence 5'-7AATG10-3'. The EcRE of STIM1 also has the core site, so it may be a canonical binding site for EcR. In addition, an EcRE was predicted in the promoter region of STIM1 from B. mori, Drosophila melanogaster, Plutella xylostella, and Apis florea. The sequences of the EcRE only have one base difference between H. armigera and B. mori (Table 1). These data indicated that the EcRE in the promoter region of STIM1 is widespread in insects.

### 20E promotes STIM1 phosphorylation and aggregation for SOCE via the nongenomic pathway

20E regulates insect metamorphosis not only by transcriptional mechanisms, but also by posttranslational mechanisms, such as protein phosphorylation. Protein phosphorylation of calponin (51), CDK10 (28), USP1 (29), and EcRB1 (35) is involved in the 20E signaling transduction pathway. Our results revealed that via GPCR, PLC, and IP₃R signaling, 20E induces intracellular Ca²⁺ release from the ER. GPCR-triggered intracellular Ca²⁺ release binds to and activates PKC in humans (3). Our results showed 20E increases Ca²⁺ and promotes PKC phosphorylation of STIM1 on Ser-485 via GPCRs.

It is known that Ca²⁺ depletion inside the ER lumen induces STIM1 aggregation (52). The function of STIM1 phosphorylation is different in various cellular processes, based on the phosphorylation sites (19). In the present study, we demonstrated that 20E induces phosphorylation of STIM1 at Ser-485, which is critical for STIM1 aggregation and the subsequent interaction with the Orai1 channel to trigger SOCE. The phosphorylation of STIM1 at Ser-485 requires Ca²⁺ and promotes PKC phosphorylation (Fig. 8B), which explains the gapped mechanism between Ca²⁺ depletion inside the ER lumen and STIM1 aggregation in the cytosol.

### 20E-induced SOCE for midgut apoptosis via STIM1

The increased intracellular Ca²⁺ induced by steroids has multiple functions. In hepatocytes, the intracellular Ca²⁺ level is increased by estrogen to induce ERK activation for growth (53). In Drosophila, the expression of STIM1 in embryonic and larval tissues regulate growth and patterning of imaginal discs (54). In B. mori, 20E mediates PCD through the Ca²⁺–PKC–caspase-3 pathway (25). In H. armigera, 20E triggers extracellular Ca²⁺ influx into cells through the nongenomic pathway to...
promote autophagy and the switch from autophagy to apoptosis (30). The increased Ca\(^{2+}\) triggers 20E-response gene transcription and apoptosis by mediating the formation of EcRB1/USP1 transcriptional complexes and binding to EcRE (29). Ca\(^{2+}\) induction of apoptosis depends on the Ca\(^{2+}\) concentration (43). In the present study, we confirmed that 20E induces Ca\(^{2+}\) influx via SOCE for apoptosis in the midgut during metamorphosis in *H. armigera*. We also observed an increase in STIM1 levels in the epidermis and fat body. Orai1 was also observed to increase in the epidermis and fat body in *H. armigera* (32). However, the epidermis and fat body do not undergo apoptosis during metamorphosis. Thus, the mechanism needs to be further clarified in a future study.

**Conclusion**

20E-induced Ca\(^{2+}\) release and STIM1 phosphorylation at Ser-485 through the GPCRs, PLC, IP\(_3\)R, and PKC signaling axis, which caused STIM1 aggregation. Aggregated STIM1 moves toward the ER-PM junction and interacts with Orai1 on the PM for Ca\(^{2+}\) entry. EcRB1 binds to EcRE to up-regulate the transcription of STIM1 and other 20E pathway genes in a positive feedback manner. Thus, 20E induces apoptosis in the midgut during metamorphosis via SOCE (Fig. 11).

**Materials and methods**

**Insects and HaEpi cells**

Cotton bollworms, *H. armigera*, were cultured in our laboratory at 27 ± 1 °C with a photoperiod of 14 h light/10 h dark. The larvae were reared on an previously described artificial diet (55). The HaEpi cell line was derived from the epidermis of *H. armigera* in our laboratory (56) and has been used for research of hormone regulation, RNAi, and overexpression of proteins (29, 35). The HaEpi cells at 50 passages without mycoplasma contamination were cultured in Grace’s medium with 10% fetal bovine serum (FBS) (Biological Industries, Beit-Haemek, Israel) at 27 °C for experiments.
The antibodies for experiments

A fragment of STIM1 (encoding amino acids 1–208) was expressed in Escherichia coli Rosetta, to be used as antigen. Rabbit polyclonal antibodies recognizing STIM1 were prepared in our laboratory according to a previously reported method (57). Rabbit polyclonal antibodies against β-actin and other
**20E promotes SOCE via GPCRs**

![Diagram](image)

### Table 1

| Species          | Sequence (5'→3') |
|------------------|------------------|
| *H. armigera*    | 5′-gcggTcAtGcAtTA-3′ |
| *B. mori*        | 5′-gcggTcAtGcAtTA-3′ |
| *D. melanogaster*| 5′-gatTcAtGcAtTA-3′ |
| *P. xylostella*  | 5′-aagTcAtGcAtTA-3′ |
| *A. florea*      | 5′-aatTcAtGcAtTA-3′ |

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**Protein extraction and Western blotting**

The total protein of tissues was extracted using extraction buffer (40 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.5). After grinding the tissue completely, the homogenate was centrifuged at 10,000×g at 4 °C for 10 min. The supernatant was collected, and Bradford’s method was used to determine the protein concentration. (Yeasen, Shanghai, China). After the protein concentrations were detected, an appropriate amount of loading buffer was added to the lysate and then the samples were boiled for 10 min. For each sample, 50 μg of protein was subjected to SDS-PAGE. The proteins were electrophoretically transferred onto nitrocellulose membranes. Protein Ladder (Thermo Fisher Scientific, Vilnius, Lithuania) markers were used to identify the molecular weights of target proteins. The membranes were incubated at room temperature in blocking buffer (5% fat-free powdered milk in Tris-buffered saline (TBS, 10 mM Tris-HCl, pH 7.5)) for 1 h. Primary antibodies were diluted with blocking buffer and incubated with the membranes overnight at 4 °C (anti-STIM1, 1:100; β-actin, 1:5000; anti-GFP, -RFP, and -His, 1:5000). The membrane was washed three times with 1×TBST (0.1% Tween 20 in 1×TBS) for 10 min each time, and the corresponding secondary antibody conjugated to alkaline phosphatase (AP) (anti-rabbit or anti-mouse IgG (ZSGB-BIO, Beijing, China)) was diluted 1:5000 with the same blocking buffer. The protein signal was observed in 10 ml of 0.1% 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sangon, Shanghai, China) in the dark for 20 min.

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**λ-Phosphatase (λPPase) treatment**

Proteins were extracted from tissues or cells using RIPA buffer. Forty microliters of protein (2 mg/ml) was incubated with 0.5 μl of λ- phosphatase (APP), 5 μl of 10X buffer, and 5 μl of MnCl₂ (10 mM) at 30 °C for 30 min, according to the manufacturer’s specifications (New England Biolabs, Beijing, China). The proteins were subjected to SDS-PAGE using 7.5% low concentration gels for Western blot analysis. Protein phosphorylation was examined via variations in molecular mass. β-Actin was detected as the protein quality control. Controls were performed by the same method without APPase.

**Hormonal regulation in larvae**

20E was dissolved in DMSO at a storage concentration of 20 mM (9.6 mg/ml). 20E was diluted to 10, 20, 40, and 100 ng/μl using sterile 1× PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) and injected 5 μl into the sixth 6-h larval hemocoel for different mRNA targets (1, 3, 6, 12, and 24 h). The proteins were then extracted from the larval midguts. Western blotting was then used to analyze STIM1 expression. An equal amount of DMSO was used as the solvent control.

### dsRNA synthesis

RNAi primers with the T7 promoter (Table 2) were designed for PCR. About 500 bp of the target gene cDNA was used as the template to synthesize dsRNA. Both strands of the dsRNA were synthesized in one reaction according to the method described in the MEGAscript RNAi kit (Thermo Fisher Scientific, Waltham, MA). The purity of dsRNA is critical for the efficacy of RNAi in larvae. We used the phenol/chloroform method to purify the dsRNA according to a previous study (35).

Long dsRNAs suppress the expression of a target gene in worms specifically (59), which can be broken down into several smaller fragments of the dsRNAs in vivo for different mRNA targets (60). Long dsRNAs also work well in lepidopteran insects (61).
**RNAi in HaEpi cells**

HaEpi cells were cultured in Grace’s medium with 10% FBS (Biological Industries, Beit-Haemek, Israel) at 27 °C to about 80% confluence in 6-well plates, and then 2 μg of dsRNA was transfected into the cells using the QuickShuttle-enhanced transfection reagent (Biodragon Immunotech, Beijing, China) in 2 ml of Grace’s medium with 10% FBS for 24 h. 20E (2 μg transfection reagent) was injected twice into fifth instar 12-h larvae (1 μg), and the experiments were repeated for 3 times. Injection of dsGFP was used as the control. The detailed method was described previously (35).

**RNAi in larvae**

The dsRNA was diluted to the appropriate concentration with nuclease-free PBS. Sterile dsRNA was injected twice into the larval hemocoel after the larvae were immobilized on ice. The first injection was into fifth instar 12-h larvae (1 μg), and the second was into sixth instar 6-h larvae (2 μg). Each group contained 30 larvae and the experiments were repeated three times. Injection of dsGFP was used as the control. The detailed method was described previously (35).

**qRT-PCR**

The corresponding primers were designed (Table 2) for qRT-PCR using first strand cDNA as a template. The program was 95 °C 15 min; 95 °C 15 s, 60 °C 60 s, 78 °C reading plate 2 s; 40 cycles; 65 to 95 °C analysis dissolution curve, interval at 0.5 °C. After obtaining the cycle threshold \( C_t \) value, the relative mRNA expression levels were calculated using the formula 

\[
2^{-\Delta\Delta C_t} = 2^{-\Delta C_t} \text{experimental group} - 2^{-\Delta C_t} \text{control group}
\]

where \( \Delta C_t \) experimental group and \( \Delta C_t \) control group indicates the difference between the \( C_t \) of the gene and the average \( C_t \) of the ACTB (β-actin) in the experimental group. The \( \Delta C_t \) control group indicates the difference between the \( C_t \) value of the gene and the average \( C_t \) of ACTB in the control group.

**ChIP assay**

The EcRB-binding element (EcRE) in the 5′-upstream genomic DNA sequence of STIM1 in the genome of H. armigera was analyzed using the JASPAR website.3 HaEpi cells were transfected with pIEx-4-EcRB1-RFP-His plasmid for 72 h and then treated with 20E or DMSO for 3 h. After incubating at 37 °C for 10 min in 1% formaldehyde, 0.125 M glycine was then added at 25 °C for 10 min to terminate the cross-linking. The cells were washed twice with 1 × PBS and then suspended in SDS lysis buffer. The DNA was broken into fragments of 200–

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**Table 2**

**Oligonucleotide sequences of PCR primers**

| Primer name       | Sequence (5′-3′)               |
|-------------------|--------------------------------|
| **qRT-PCR**       |                                |
| STIM1 RT F        | ggtgacacctccccctgtactg         |
| STIM1 RT R        | ttctccactctccccctcccc          |
| EcRB1 RT F        | tggacccgtatgtagga              |
| EcRB1 RT R        | atttgcccgtacgctgcct            |
| USP1 RT F         | gttcttggccgactttttggc          |
| USP1 RT R         | ccggtatcgtgcctactcctctctct    |
| HR3 RT F          | tcacgacctcaacacggcacccccta     |
| HR3 RT R          | gactttgctgtcctccctccctcgc     |
| BrZ7 RT F         | gttcatcgctctactcgccgat        |
| BrZ7 RT R         | cctatctccctttgaccatgactctct   |
| Caspase-3 RT F    | gggacagggtgagagggaa            |
| Caspase-3 RT R    | ggaggcctatgtagtgcctggt        |
| Caspase-6 RT F    | gtctgtatcgcctctactggtccagcttgctg         |
| Caspase-6 RT R    | ccggaattctccgcttacctctctct    |
| RPL27-RT F        | aagagttctcactcctctctcctctct   |
| RPL27-RT R        | gctcttgctgcctgactactctctctc   |
| β-Actin RT F      | cctggtatcgtgcctctactggtccagcttgctg         |
| β-Actin RT R      | cctggtgagagtgagagggaa          |
20E promotes SOCE via GPCRs

1000 bp by sonication (250 W for 9 s, interval of 9 s, 10 times). After centrifugation, 30 μl of Protein A was added to the supernatants followed by centrifugation to clean the nonspecifically recognized proteins. The sample was divided into three equal parts. One part was used as the input control, one was incubated without antibodies as a control, and the last part was treated with anti-RFP antibodies to precipitate EcRB1-RFP and the EcRB1-RFP–bound ecdysone response element (EcRE) fragments in the *H. armigera STIM1* promoter. Nonspecific IgG from mouse was used as a control. The EcRE fragments were purified using a ChIP Assay Kit (Beyotime) according to the manufacturer’s instructions. The enrichment of EcRE fragments in different experiments was analyzed using qRT-PCR with ChIP assay primers (primer EcRE) (Table 2). Results were presented as percentage of enrichment of EcRE fragments in samples relative to the input (EcRE fragments in the sample before RFP antibody immunoprecipitation); % input = (sample precipitated by RFP antibody – sample without antibody)/input × %.

**Hematoxylin and eosin (H&E) staining of tissues and immunohistochemistry**

The larval midguts (24 h, sixth at 48 h, and sixth at 96 h) were isolated, fixed overnight with 4% paraformaldehyde at 4 °C, and then dehydrated in a gradient ethanol series. The tissues were embedded in paraffin, sliced into 7-μm sections using a paraffin slicing machine, and adhered to gelatin-coated glass slides. The slides were dried at 42 °C overnight and subsequently dewaxed and dehydrated in a gradient ethanol series. Hematoxylin and eosin were used to stain nuclei and cytoplasm following the method described in our previous work (63). For immunostaining, the tissue slices were blocked using 2% bovine serum albumin (BSA) for 1 h and then incubated overnight with serum (pre-serum or anti-STIM1 serum: 1:50) at 4 °C. The cells were then treated with 20E (2 μM in DPBS) for 30 min. The cells were washed three times. Positive signals were observed using an Olympus BX51 fluorescence microscope (Shinjuku-ku, Tokyo, Japan).

**Calcium ion detection in HaEpi cells**

HaEpi cells were incubated in culture dishes with fresh Grace’s medium with 10% FBS for 24 h and reached 80% confluence. AM ester Calcium Crimson™ dye (Invitrogen) was added to the medium to final concentration 3 μM for 30 min at 27 °C. The cells were washed with Ca²⁺-free 1× DPBS (2.7 mM KCl, 1.5 mM KH₂PO₄, 138 mM NaCl, and 8 mM Na₂HPO₄) three times and kept in 500 μl of DPBS. The fluorescence of cells before any treatment (every 6 s for 60 s as background) was detected using a Zeiss LSM 700 laser confocal microscope (Carl Zeiss, Oberkochen, Germany). Then, 500 μl of DPBS with 4 μM 20E or DMSO was added dropwise onto the plate over 60 s. Subsequently, 1 ml of DPBS with Ca²⁺ (1 mM) and 20E (2 μM) were added at 240 s. Each image’s fluorescence intensity was collected for 420 s using Image pro-plus software (Media Cybernetics, Rockville, MD).

**Overexpression of proteins**

The plEx-4-GFP/RFP-His vector that was fused with green (GFP) or red fluorescent protein (RFP) was used for the experiments in the insect cell line. The open reading frames (ORFs) of the target genes (*STIM1, Orai1*, and *EcRB1*) were amplified using primers (Table 2) and inserted into the vector. Five micrograms of recombinant plasmids were transfected into HaEpi cells using the QuickShuttle-enhanced transfection reagent (Biodragon Immunotech). All recombinant plasmids in the experiments contained a His tag. Cell fluorescence was observed using a Carl Zeiss LSM 700 laser scan confocal microscope (Thornwood, NY).

**Identification of the phosphorylation type of STIM1**

The phosphorylation sites were predicted using NetPhos. The epidermis and midgut were isolated from the sixth 72-h larvae. Total proteins were lysed using RIPA buffer and ground completely. STIM1 was isolated using immunoprecipitation with anti-STIM1 antibodies and detected by SDS-PAGE. The three anti-phosphorylation antibodies, a mouse monoclonal antibody (mAb) recognizing phosphoserine (anti-pSer) (Abcam, Cambridge, United Kingdom), rabbit polyclonal antibodies (pAbs) recognizing phosphothreonine (anti-pThr) (Immunechem, Burnaby, Canada), and a mouse mAb recognizing phosphotyrosine (anti-pTyr) (Biodragon, Beijing, China), which were diluted 1:100 with 2% BSA and used to detect the phosphorylation type of STIM1. Anti-mouse (AP) secondary antibodies (ZSGB-BIO, Beijing, China) were used to detect anti-pSer and anti-pTyr. Anti-rabbit (AP) secondary antibody (ZSGB-BIO, Beijing, China) was used to detect anti-pThr.

**Co-IP**

Plasmid constructs plEx-4-STIM1-GFP-His or STIM1 mutant plEx-4-STIM1-S485A-GFP-His and plEx-4-Orai1-RFP-His were co-transfected into HaEpi cells for 72 h, respectively. The cells were washed three times with Ca²⁺-free DPBS. The cells were then treated with 20E (2 μM in DPBS) for 30 min. The cells were washed on ice for 30 min using RIPA buffer. The supernatant was collected through centrifugation at 12,000 × g for 10 min at 4 °C. The supernatant was added to protein A resin to eliminate nonspecific binding and harvested by centrifugation (1,000 × g for 2 min). The lysate was incubated with anti-GFP antibodies (1:1,000) overnight at 4 °C and then with Protein A resin for 1 h. The precipitate was washed with RIPA buffer three times. Finally, the resin was treated with SDS-PAGE loading buffer and boiled for 10 min. Western blotting was used to analyze the proteins with their correspondent antibodies.

**TUNEL assay in HaEpi cells**

The apoptosis was detected in HaEpi cells using the TUNEL Apoptosis kit (RIBOBIO, Guangzhou, China). The HaEpi cells were fixed within 4% paraformaldehyde for 15 min after treatment with 20E or an equal amount of DMSO for appropriate times. Then, the cells were incubated with 0.5% Triton X-100 for 10 min at room temperature. After washing three times with PBS, 100-μl reaction mixtures covered the cells and were incu-
bated for 2 h at 37 °C. The reaction was terminated by 200 μl of 2× SSC (300 mM NaCl, 30 mM Na3 citrate+2H2O, pH 7.0) The nuclei were stained with 1 μg/ml of 4',6-diamidino-2-phenylindole (DAPI) for 10 min, and the resulting signals were observed using a Olympus FV3000 laser confocal microscope.

**Statistical methods**

Student’s t test was used for the statistical analysis. The p value was calculated through paired and two tailed analysis (*, p < 0.05; **, p < 0.01; ***, p < 0.001). The value is indicated as the mean ± S.D., n ≥ 3. The density of the immunoreactive protein bands of the Western blots was quantified using Quantity One software (Bio-Rad). Three biological replicates and three technical replicates were performed for all experiments.

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**Note added in proof**—The wrong images were used in the dsGFP treated with 20E in Fig. 6C in the version of this article that was published as a Paper in Press on August 14, 2019. This error has now been corrected and does not affect the results or conclusions of this work.

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