Calcineurin-mediated Dephosphorylation of Acetyl-coA Carboxylase is Required for Pheromone Biosynthesis Activating Neuropeptide (PBAN)-induced Sex Pheromone Biosynthesis in Helicoverpa armigera*

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Chemical signaling plays a critical role in the behavior and physiology of many animals. Female insects, as many other animals, release sex pheromones to attract males for mating. The evolutionary and ecological success of insects therefore hinges on their ability to precisely mediate (including initiation and termination) pheromone biosynthesis. Pheromone biosynthesis activating neuropeptide (PBAN) acts directly on pheromone glands to regulate sex pheromone production using Ca2+ and cyclic-AMP as secondary messengers in the majority of species. However, the molecular mechanism downstream of the secondary messengers has not yet been elucidated in heliothine species. The present study shows that calcineurin, protein kinase A (PKA) and acetyl-coA carboxylase (ACC) are key components involved in PBAN-induced sex pheromone biosynthesis in Helicoverpa armigera using PBAN-dependent phosphoproteomics in combination with transcriptomics. RNAi-mediated knockdown and inhibitor assay demonstrated that calcineurin A is required for PBAN-induced ACC activation and sex pheromone production. Calcineurin-dependent phosphoproteomics and in vitro calcineurin phosphorylation assay further revealed that calcineurin regulated ACC activity by dephosphorylating ser84 and ser92. In addition, PKA-dependent phosphoproteomics and activity analysis revealed that PKA reduces the activity of AMP-activated protein kinase (AMPK), a negative regulator of ACC by phosphorylating the conserved ser92. Taken together, our findings indicate that calcineurin acts as the downstream signal of PBAN/G-protein receptor/Ca2+ to activate ACC through dephosphorylation while inactivating AMPK via PKA to reduce ACC phosphorylation, thus facilitating calcineurin activation of ACC. Molecular & Cellular Proteomics 16: 10.1074/mcp.RA117.000065, 2138–2152, 2017.

Insects are the most successful and largest group of organisms in the world. One of the most important reasons for their success is their mating and reproductive capabilities. Insect mating, especially in lepidopteran moths, is enabled through the production of sex pheromones for communication. Volatile sex-pheromones are commonly synthesized and emitted by females at specific times of the day and are perceived by males in search of mates to guide their orientation to receptive females. The evolutionary and ecological success of insects therefore hinges on their ability to precisely mediate initiation and termination of pheromone biosynthesis (1). Based on the findings in cockroaches (2), the neuroendocrine regulation of sex pheromone biosynthesis in moths was initially thought to be controlled by the corpora allata and its secretory product, juvenile hormone. However, subsequent studies found that a neuroendocrine factor other than juvenile hormone regulated sex pheromone biosynthesis (3). Subsequently a novel neuropeptide termed “pheromone biosynthesis activating neuropeptide” (PBAN)1 was identified and shown to regulate the

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1 The abbreviations used are: PBAN, Pheromone Biosynthesis Activating Neuropeptide; AC, adenylyl cyclase; ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP activated protein kinase; CaM, calcium calmodulin; cAMP, cyclic AMP; CC, corpora cardiaca; CT, Cross Threshold; CysA, cyscloporine A; DTT, dithiothreitol; FAS, fatty acid synthetase; GO, Gene Ontology; GPCR, G-protein coupled receptor; ITRAQ, Isobaric tags for relative and absolute quantification; KO, KEGG Ortholog database; PK, protein kinase A; SAMS, the synthetic peptide substrate with the amino acid sequence HMRSAMSGLHLVKRF used to assay AMPK; SDS, sodium dodecyl sulfate; SOG, suboesophageal ganglion; Sp-cAMPS, adenosine 3',5'-cyclic monophosphate sodium salt monohydrate TiO2.
diel periodicity of sex pheromone biosynthesis in *Helicoverpa zea* (4–5). PBAN is produced in the subesophageal ganglion and acts directly on the pheromone glands (PGs) of adult female moths to stimulate sex pheromone biosynthesis. Because the first identification of PBAN in *H. zea* (5), PBAN signal transduction has been extensively studied (1, 6–14). In all moth species studied to date, extracellular calcium ions are essential for PBAN-induced sex pheromone biosynthesis. The absence of extracellular calcium or the presence of calcium-calmodulin inhibitors or calcium blockers was found to abolish pheromone-tropic activity in incubated PGs from *H. armigera, H. zea, H. virescens, Argyrotaenia velutinana, Ostrinia nubilalis,* and *Bombyx mori* (1, 6–11). Calcium ionophores can mimic the pheromone-tropic activity of PBAN in *H. armigera, B. mori, H. virescens, Spodoptera litura, A. velutinana,* and *O. nubilalis* (1, 6, 7, 9, 12–14). The implication that the PBAN-receptor is a G-protein coupled receptor was determined pharmacologically and subsequent identification and characterization confirmed that the PBAN-receptor belongs to the G-Protein coupled receptor family (14, 15). Studies clearly indicated that PBAN triggers extracellular calcium influx after binding to its G protein-coupled receptor, calcium ionophores stimulated levels of intracellular cyclic adenosine-3, 5-monophosphate (cAMP) (12). In addition to using calcium as a secondary messenger, cAMP has been also implicated in several species indicating that the PBAN-triggered signal transduction cascade is species-dependent. For example, cAMP was found to be necessary in PBAN signaling in *heliothine* species (1). Intracellular cAMP levels increased dramatically following even a short PBAN stimulation time (1 min) in *H. armigera, H. zea,* and *H. virescens* (6, 7, 11, 16–17). The increase in cAMP levels triggered by PBAN is calcium-dependent in *H. armigera* and occurs downstream of calcium influx because calcium-free incubation medium prevented a PBAN-triggered increase in intracellular cAMP levels and ionophores induce significant elevations of cAMP (12). These findings confirmed that PBAN acts via calcium ions and cAMP to stimulate downstream events and therefore to regulate pheromone biosynthesis in *heliothine* species.

In contrast, direct PBAN stimulation or incubation with 3-isobutyl-1-methylxanthine (IBMX), a competitive nonselective phosphodiesterase inhibitor, followed by PBAN stimulation failed to increase intracellular cAMP levels in *Bombyx mori* (18). Similarly, incubation with forskolin, an adenylyl cyclase activator, significantly increased intracellular cAMP levels, but had no effect on sex pheromone production in *B. mori* (18). Similar results were also observed with *S. litura* and *O. nubilalis* (11, 19). These findings clearly demonstrate that in these species, cAMP is not involved in the PBAN signaling pathway. The differences in the intracellular secondary messenger cascades for PBAN signaling between *heliothine* species and *B. mori* may be because of species differences, resulting in activating different enzymatic steps of sex pheromone biosynthesis.

Lepidopteran sex pheromones are produced in the PGs in response to PBAN stimulation by *de novo* biosynthesis of fatty acid from acetyl-CoA (involving acetyl-CoA carboxylase and fatty acid synthase) followed by desaturation, chain-shortening, fatty acyl reduction and oxidation or acetylation (20). Sex pheromone biosynthetic enzymes directly regulated by PBAN have been studied using labeled precursors and intermediates and evidences indicate that the PBAN-regulated enzymes vary between species. For example, in *B. mori,* *Thaumetopoea pityocampa,* *S. littoralis,* and *Manduca sexta,* PBAN has been shown to regulate the fatty-acyl reductase step (21–24). Subsequent studies have also confirmed that PBAN accelerates the rapid lipolysis of cytoplasmic triacylglycerols in *B. mori* (25). In contrast, in *H. zea,* *S. exigua,* *Mamestra brassicae* and *A. velutinana,* PBAN has been shown to stimulate sex pheromone biosynthesis before the fatty acid synthesis step (6, 26–29). In *H. armigera,* the use of labeled precursors and inhibitor analysis confirmed that PBAN directly regulates ACC activity (30). Interestingly, PBAN controls two enzymes in *H. virescens*: one at the initial step and the other at the terminal step of the pheromone biosynthesis process (31). From the current literature, it appears that the rate-limiting step regulated by PBAN in *heliothine* species is the fatty acid biosynthesis and fatty acid reduction or both. In addition, the differing enzymes regulated by PBAN appear to be linked to the presence or absence of adenylyl cyclase activity, because those species in which the initial steps of fatty acid biosynthesis can be modified by PBAN are dependent on cAMP production whereas those species that rely on PBAN activation of the terminal steps are not (18). Although the rate-limiting enzymes regulated by PBAN have been identified in some species, including *H. armigera* (30), detailed molecular mechanisms leading to enzyme activation by PBAN have yet to be elucidated. In this work, quantitative phospho-proteomics, transcriptomics, and RNA interference were used to investigate the molecular mechanism underlying PBAN-regulated sex pheromone biosynthesis in *H. armigera.*

**EXPERIMENTAL PROCEDURES**

**Insects—** *H. armigera* larvae were reared on an artificial diet at 26 °C under a 16 h light/8 h dark cycle. Newly-emerged adults in the first photophase were referred to as 1 day old adults.

**Chemicals—** *H. armigera* PBAN was obtained from Bachem (AG, Bubendorf, Switzerland). The sex pheromone component, Z11-hexadecenal (Z11-16Ald), was purchased from Sigma (St. Louis, MO) and used to make a calibration curve for quantitative pheromone measurements for gas chromatography/mass spectrometry (GC/MS). 5-(tetradecyloxy)-2-furoic acid (Tofu, an ACC inhibitor), 5-aminoimidazole-4-carboxamide1-β-D-ribofuranoside (AICAR, an AMPK activator), adenosine 3’, 5’-cyclic monophosphate sodium salt mono hydrate (Sp-cAMPS, a PKA activator), cyclosporine A (CysA, a calcineurin inhibitor), and n-hexane were obtained from Sigma (St. Louis, MO).

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Titanium dioxide; Tofu, 5-(tetradecyloxy)-2-furoic acid; UA, uric acid.
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Sample Preparation and RNA Isolation—To fully cover all genes involved in sex pheromone biosynthesis, PGs at 3 different developmental time points (48 h before emergence, 0 h (new emergence) and 48 h (48 h after emergence)) were collected. Each sample included at least 500 PGs for extracting enough total RNA. Total RNA was extracted from the obtained samples.

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The purity of the obtained RNA was determined using Nanodrop spectrophotometer (IMPLEN, CA) and agarose gel electrophoresis. RNA concentrations were measured using a Qubit® RNA Assay Kit in a Qubit®2.0 Fluorometer (Life Technologies, CA). RNA integrity was assessed using an RNA Nano 6000 Assay Kit with the Agilent Bioanalyzer (Agilent Technologies, CA).

cDNA Synthesis, Sequencing and Data Analysis—Samples for transcriptomic analysis were prepared using a NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB) according to the manufacturer’s instructions. Briefly, mRNA was purified from total RNA extracted using Oligo(dT) magnetic beads followed by mRNA fragmentation in the presence of divalent cations at an elevated temperature in a NEBNext First Strand Synthesis Reaction Buffer (5×). Fragmented mRNA was then used as the template for first strand cDNA synthesis using a random hexamer primer and M-MuLV Reverse Transcriptase (RNaseH−) followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. Second strand cDNA products were amplified after end-repair and ligation with NEBNext adaptors containing hairpin loop structures. To preferentially select cDNA fragments of 150–200 bp in size, library fragments were further purified using an AMPure XP system (Beckman Coulter, Beverly, MA). Then, 3 μl of USER Enzyme (NEB) was added to the size-selected, adapter-ligated cDNA. The mixture was incubated at 37 °C for 15 min, then incubated for 5 min at 95 °C before PCR. PCR was carried out using Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. The cDNA library was finally generated after purification of the PCR products (using an AMPure XP system). Subsequent assessment of library quality was performed using an Agilent Bioanalyzer 2100.

Clustering of index-coded samples was performed on a cBot Cluster Generation System by TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer’s instructions. After cluster generation, library preparations were sequenced on an Illumina HiSeq 2500 platform and paired-end reads were obtained.

Clean data were obtained from raw data generated by filtering reads containing adapter, reads containing poly-N sequences and low-quality reads. The Q20, Q30, GC-content and sequence duplication levels of the clean data were calculated. All downstream analyses were performed on high quality, clean data. Transcriptome assembly was accomplished using Trinity software (version: trinityrnaseq_r20131110) with min_kmer_cov set to 2 by default (32). All other parameters in Trinity were set to their default values. The transcriptomic data set has been deposited in GenBank with BioSample accession (SAMN05768739). Corresponding protein sequence database was obtained by blastx search using the NCBI non-redundant protein sequences (Nr), NCBI non-redundant nucleotide sequences (nt), Protein family (Pfam), Clusters of Orthologous Groups of proteins (KOG/COG), a manually annotated and reviewed Gene Ontology (GO), and Gene Ontology (GO). GO analyses (http://www.geneontology.org) were conducted according to a previously reported method (33). Metabolic pathways were analyzed according to the KEGG pathway database (http://www.genome.jp/kegg/pathway.html).

Protein Sample Preparation and Isobaric Tags for Relative and Absolute Quantification (iTRAQ) Labeling—Newly emerged females were first decapitated, and then maintained for 24 h to assure PBAN depletion. PGs were collected from the decapitated females and incubated at room temperature in Grace’s Insect Medium. After 1 h of incubation, medium was replaced by 2 ml fresh Grace’s Insect Medium containing 10 pmol PBAN. Samples were incubated for different time points (0 min (referred to as C0), 10 min (referred to as T10) and 30 min (referred to as T30)). Three biological replicates were prepared for T10 and T30 respectively, whereas a duplicate was prepared for C0. Each replicate included at least 1000 PGs to obtain enough PG protein for phosphoproteomic analysis. PBAN-treated PG samples were harvested and completely homogenized with SDBT buffer (4% Sodium dodecyl sulfate (SDS), 1 mM dithiothreitol (DTT), 150 mM Tris-HCl pH 8.0), heated at 100 °C for 5 min and then cooled to room temperature. Supernatants were transferred to new tubes, and protein concentrations were determined using the Bicinchoninic Acid (BCA) assay (Bio-Rad, Berkeley, CA).

Protein digestion was performed according to the previously described FASP procedure described previously (34). The resulting peptide mixture was labeled with 8-plex iTRAQ reagent following the manufacturer’s instructions (Applied Biosystems, Foster City, CA). Briefly, 200 μg of PG proteins were incorporated into 30 μl SDBT buffer for each sample. Ultrafiltration (Microcon units, 30 kDa) was performed to remove detergent, DTT and other low molecular weight components using uric acid (UA) buffer (8 M Urea, 150 mM Tris-HCl pH 8.0). Then, 100 μl of UA buffer (containing 0.05M iodoacetamide) was added to block reduced cysteine residues. Samples were incubated for 20 min in the dark. Filters were washed with 100 μl UA buffer three times, followed by two washes with 100 μl DS buffer (50 mM triethylammonium bicarbonate at pH 8.5). Finally, 2 μg trypsin (Promega, Madison, WI) dissolved in 40 μl DS buffer was added to each filtrate, and protein suspensions were digested overnight at 37 °C. The resulting peptides were collected as a filter via centrifugation. Peptide content was estimated via absorbance at 280 nm. An extinction coefficient of 1.1 was assumed for a 0.1% (g/l) peptide solution; this coefficient was calculated based on the frequencies of tryptophan and tyrosine in the sample.

iTRAQ labeling was carried out according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). Each iTRAQ reagent was dissolved in 70 μl of ethanol and added to different peptide mixtures. C0 samples were labeled with reagents 115, 116, and 117. T30 samples were labeled with reagents 118, 119, and 121. After labeling, samples were multiplexed and vacuum dried.

Enrichment of Phosphorylated Peptides by the Titanium dioxide (TiO2) Beads—The mixture of labeled peptides was concentrated in a vacuum concentrator and resuspended in 500 μl loading buffer (2% glutamic acid/65% acetonitrile (ACN)/2% trifluoroacetic acid (TFA)). TiO2 beads were then added and the samples were agitated for 40 min. The peptide mixture was then centrifuged for 1 min at 5000 g, generating the first set of beads. The second set of beads was obtained by mixing the supernatant from the first centrifugation with another volume of TiO2 beads, which were collected as before. Both sets of beads were washed and washed three times with 50 μl of washing buffer I (30% ACN/3% TFA), and then washed three times with 50 μl of washing buffer II (80% ACN/0.3% TFA) to remove any non-adsorbed material. Phosphopeptides were finally eluted with 50 μl of elution buffer (40% ACN/15% NH4OH (35), followed by lyophilization and MS analysis.

NanoLC-MS/MS—A phosphopeptide solution (5 μl) obtained from above step was mixed with 15 μl of 0.1% (v/v) TFA, and then a 10 μl aliquot of each fraction was injected into a Q Exactive MS (Thermo Finnigan, California, CA) equipped with Easy nLC (Proxeon Biosys-
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Terms, now Thermo Fisher Scientific) for nanoLC-MS/MS analysis. The peptide mixture was loaded onto a C18-reversed phase column (15 cm long, 75 μm inner diameter, RP-C18 3 μm, packed in-house) in buffer A (0.1% formic acid in water). Separation was performed using a linear gradient of buffer B (80% ACN and 0.1% formic acid) over 240 min at a flow rate of 250 nL/min, the gradient and flow rate were controlled using IntelliFlow technology. Peptides were eluted from the column using a gradient of 0–60% buffer B from 0 min to 200 min, 60% to 100% buffer B from 200 min to 216 min, and 100% buffer B from 216 min to 240 min.

MS spectra were acquired using a data-dependent top10 method to dynamically choose the most abundant precursor ions from survey scan (300–1800 m/z) for high energy collisional Collision (pAGC). The dynamic exclusion duration was set to 40.0 s. Survey scans were acquired at a resolution of 70,000 at an m/z of 200. The resolution for HCD spectra was set to 17,500, at an m/z of 200. The normalized collision energy was 27 eV, and the under-fill ratio was defined as 0.1%. The underfill ratio specifies the minimum percentage of the target value likely to be reached at the maximum fill time. The instrument was run with peptide recognition mode enabled.

Data Analysis—MS/MS spectra were searched against the amino acid sequence database (16, 250 sequences) translated from the transcriptomic unigenes database obtained from H. armigera PGs (ncbi Accession number: BioSample: SAMN05767839) using Mascot 2.2 engine embedded in Proteome Discoverer 1.4. Parameters for protein identification were set as follows: peptide mass tolerance = 20 ppm; enzyme = trypsin; MS/MS tolerance = 0.1 Da; missed cleavage = 2; fixed modification: carbamidomethyl (C), iTRAQ8plex (K) and iTRAQ8plex (N-term); and variable modification: oxidation (M).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (36) partner repository with the data set identifier PXD005645 (Username: reviewer11457@ebi.ac.uk, Password: FM9IaRe6), PXD006034 (Username: reviewer50400@ebi.ac.uk, Password: FM9IaRe6), PXD006380 (Username: reviewer50400@ebi.ac.uk, Password: FM9IaRe6), and PXD006380 (Username: reviewer50400@ebi.ac.uk, Password: FM9IaRe6). Synthesized dsRNA was then eluted in diethyl pyrocarbonate-treated nuclease-free water, and resulting dsRNA concentrations were measured using a BioPhotometer (Eppendorf, Hauppauge, NY). The dsRNA of enhanced green fluorescent protein (EGFP), a classical RNAi control, was used as a negative control.

For differentially expressed phosphoproteins, protein-protein interaction networks were constructed using http://string-db.org/. GO functional enrichment and KEGG pathway analyses were performed using CytoScape software version 2.6.2 (http://www.cytoscape.org/).

Data Repository—The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (36) partner repository with the data set identifier PXD005645 (Username: reviewer11457@ebi.ac.uk, Password: FM9IaRe6), PXD006034 (Username: reviewer96628@ebi.ac.uk, Password: ISVII3ME) and PXD006380 (Username: reviewer50400@ebi.ac.uk, Password: 9WEa6HW2).

Immunoprecipitation and Western Blot—To explore the interaction between calcineurin A and ACC, the nucleotide sequences for calcineurin A (his-tagged) and ACC (flag-tagged) were synthesized by Sangon Biotech Corporation (Shanghai, China). The proteins were expressed using the TntTM T7 Insect Cell Extract Protein Expression System (Promega, Madison, WI) according to the manufacturer’s recommendation. Expressed proteins were then subjected to immunoprecipitation.

Before incubation with protein A/G beads, expressed proteins were incubated with anti-His or anti-Flag antibodies for 2 h at 4 °C. Protein A/G beads were washed at least three times with 1 ml wash buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 1% Triton X-100, 1 mM EDTA). Co-IP complexes were eluted with 2X protein sample buffer and boiled for 5 min. To detect interactions between ACC and Calcineurin A, Western blots were performed with anti-His or anti-flag antibodies according to previously described methods (37).

Quantitative Real-Time PCR—First-strand cDNA was synthesized from the total RNA (1 μg) extracted from each sample using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China). This first-strand cDNA was used as the template for real-time PCR. The primers used for real-time PCR analysis are listed in supplemental Table S1. The H. armigera ribosomal protein gene 18S was used as an internal standard for normalization (38). Real-time PCR was carried out using SYBR Green Supermix (TaKaRa) on an Applied Biosystems 7500 Fast Real-Time PCR system (ABI, Carlsbad, CA) according to the manufacturer’s instructions. The thermal cycler conditions used for real-time PCR were: 95 °C for 4 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 20 s. The specificity of the SYBR green PCR signal was confirmed using agarose gel electrophoresis and melting curve analysis. mRNA expression was quantified using the comparative Cross Threshold (CT, the PCR cycle number that crosses the signal threshold) method (39). The CT of the 18S gene was subtracted from the CT of the target gene to obtain ΔCT. Normalized fold changes in target gene mRNA expression were expressed as 2−ΔΔCT, in which ΔΔCT is equal to ΔCT_untreated sample − ΔCT_control.
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As for PBAN-induced Z11-16 Ald production, data were indicated using ng/gland. As for RNAi-treated samples, the data were indicated with relative percentage of control.

Inhibitor analysis—The obtained PG samples as described above (see Protein Sample Preparation) was incubated with the fresh Grace’s Insect Medium (containing corresponding inhibitors and 10 pmol PBAN). PGs were collected after 60 min of incubation with PBAN and extracted in n-hexane. Control females were incubated without inhibitors.

Analysis of the Calcineurin-dependent Phosphoproteome by Mass Spectrometry—The obtained PG samples as described above was incubated with the fresh Grace’s Insect Medium containing 5 μM CysA and 10 pmol PBAN. PG samples were collected after 10 min and 30 min of PBAN incubation, and samples were subjected to quantitative phosphoproteomics analysis according to method described above. Control females were incubated without calcineurin inhibitor.

PG samples incubated without inhibitor were labeled with reagents 113, 114, and 115. PG samples incubated with calcineurin inhibitor were labeled with reagents 116, 117, and 118.

ACC Activity Analysis—ACC activity was assayed using an ACC Activity Assay Kit (GenMed, Shanghai, China) according to previous methods (42). ACC catalyzes the ATP-dependent carboxylation of acetyl-CoA, forming malonyl-CoA, ADP and orthophosphate. The rate of ADP production was assayed by coupling the ACC reaction with pyruvate kinase and lactate dehydrogenase. ACC activity was determined by measuring NADH oxidation at 340 nm (μmol NADH/min/mg of PG protein) according to previous methods (42).

Effect of Calcineurin on ACC Activity—The obtained PG samples as described above was incubated with 2 ml fresh Grace’s Insect Medium containing 5 μM CysA and 10 pmol PBAN. PGs were collected after various incubation times in PBAN (0, 10, 30, 60, and 90 min) and subjected to ACC activity analysis. Control females were incubated with PBAN only.

Effect of Calcineurin on ACC Activity Sites—Synthetic peptides (LRLPPNS(p)GTQLPMSQGTVIHSQR and LLRPNSGTQPSMS(p)-QGTVIHSQR), possible targets of calcineurin according to phosphoproteomic analysis, were incubated with calcineurin protein in reaction buffer (0.5 mg/ml acetylated BSA, 50 mM Tris-HCl (pH 7.4), 1 mM NiCl2, 10 μg/ml calmodulin) for 15 min at 30 °C. Treated peptides were then subjected to LC/MS/MS to determine changes in phosphorylation status. The peptide that was not treated with calcineurin was used as the control.

Identification of the PKA-dependent Phosphoproteome—The obtained PG samples as described above was incubated with the fresh Grace’s Insect Medium containing 0.5 mmol Sp-cAMPS. Samples were incubated for different periods of time (0 min, 10 min and 30 min). Three biological replicates were prepared for each incubation time condition membrane of newly emerged females that had been decapitated. Treated females were maintained for 48 h under normal conditions, and then dissected and incubated in Grace’s Insect Medium. After 1 h of incubation, the medium was replaced with the fresh Grace’s Insect Medium (containing 10 pmol PBAN). PGs were collected after different incubation times with PBAN (0, 10, 30, 60, 90 min) and then subjected to AMPK activity analysis.

The Effect of AMPK on ACC Activity and Sex Steroid Metabolism—The obtained PG samples as described above were incubated with the fresh Grace’s Insect Medium containing 5 mmol of 5-aminomidozale-4-carboxamide-1-beta-D-ribofuranoside (AICAR), an AMPK activator. PGs were collected after different incubation times with AICAR (0, 10, 30, 60, and 90 min) and subjected to ACC activity analysis.

Newly emerged females were decapitated and maintained for 24 h as described above. PGs were then dissected and incubated with Grace’s Insect Medium. After 1 h of incubation, the medium was replaced with fresh Grace’s Insect Medium containing 5 mmol AICAR and 10 pmol PBAN. PGs were collected 60 min after PBAN treatment, extracted in n-hexane and the main component was quantified as described above.

Experimental Design and Statistical Rationale—As for PBAN-regulated phosphoproteomics, three biological replicates were prepared for T10 and T30 respectively, a duplicate were prepared for C0. As for calcineurin-regulated phosphoproteomics, three biological replicates were prepared for controls and treatments respectively. As for PKA-regulated phosphoproteomics, three biological replicates were prepared for 2 treatments respectively, a duplicate were prepared for control. Every replicate included at least 1000 PGs to obtain enough PG protein for phosphoproteomic analysis.

The phosphopeptide ratios were normalized by dividing the median ratio of all peptides identified. Significantly changed phosphopeptides were identified using a Student’s t-tests (a two-sided p < 0.05) and fold changes (>1.2 or <0.8333).

Quantitative real-time PCR and GC/MS experiments were performed in triplicates. The results are expressed as means ± standard deviation (M ± S.D.). The real-time PCR and GC/MS results were compared using Student’s t-tests or one-way ANOVA variance analysis (Tukey’s test).

RESULTS

Transcriptomic Sequencing and Assembly—To identify the major components in the PBAN signaling pathway and, because no genomic data are available in H. armigera, PG transcriptomes at different time points of PG development were generated. Transcriptomic sequencing of H. armigera PGs yielded 126,362,406 raw sequencing reads (>12.6 Gb). A total of 114,067,170 clean sequencing reads were generated after noise filtration. Trinity assembly generated 40,381 unigenes with a mean length of 901 bp, a N50 length of 1851 bp and a N90 length of 304 bp (Table I). This transcriptomic data
set is the largest set reported for insect PGs to date (44). Of the 40,381 unigenes obtained, 10,335 unigenes (accounting for 25.59% of the transcriptome assembly) were longer than 1000 bp (Table I and supplemental Table S2).

The 40,381 obtained unigenes were searched against five databases (Nr, Nt, Swiss-Port, COG and GO). All 40,381 unigenes were annotated in at least one of these referred databases (supplemental Table S3). Of 40,381 unigenes, 13,650 unigenes (33.8%) could be assigned a GO classification. Each unigene could be assigned to one or more of the functional groups of three biological processes (supplemental Fig. S1). Of the 13,650 annotated unigenes, genes related to cellular process were most abundant (60.39%), followed by genes related to binding and metabolic processes (supplemental Fig. S1).

Candidate Genes in *H. armigera* PGs with Putative Functions in Sex Pheromone Biosynthesis—Homologous searches identified a total of 123 transcripts belonging to 18 unigenes, including ACC, fatty acid synthase, desaturase, fatty acyl reductase, alcohol oxidase, aldehyde reductase, acyl-coA oxidase, aldehyde dehydrogenase, acetyltransferase, calcineurin, PBAN receptor, protein kinase A, protein kinase C, etc. (supplemental Table S4). The obtained transcriptomic data therefore provided a solid foundation for further investigations of the PBAN signaling pathway using phosphoproteomics.

**Characterization of the PBAN-dependent Phosphoproteome**—PBAN-mediated phosphorylation events were identified by using iTRAQ-based quantitative proteomics to analyze phosphopeptides (supplemental Fig. S2). In total, 2234 phosphopeptides representing 787 different phosphoproteins were identified with scores higher than 1.3 (confidence level of 95%) and False discovery rates (FDRs) lower than 0.01 (supplemental Table S5). In addition, supplemental material shows the tandem mass spectra for each peptide reported.

Using quantitative comparison of the phosphopeptides in different samples (T10 versus C0, T30 versus C0 and T30 versus T10) via a Student’s t-tests (a two-sided *p* < 0.05,) and fold changes (>1.2 or <0.833), a total of 139 phosphopeptides (47 upregulated and 92 downregulated) were found to be differentially expressed between the T10 and C0 samples. The expression levels of 225 phosphopeptides (68 upregulated and 157 downregulated) were altered significantly in T30 compared with C0 whereas the expression levels of 86 phosphopeptides (24 upregulated and 62 downregulated) were different in T30 compared with T10 (Fig. 1A and supplemental Table S6). GO analysis of differentially expressed phosphoproteins confirmed that most of the differentially expressed phosphoproteins were related to cellular components, catalytic activity and binding for molecular function, and cellular and metabolic processes (supplemental Fig. S3).

An integrated analysis of all differentially expressed phosphoproteins regulated by PBAN was performed using Cytoescape (v2.8.3). The protein-protein interaction network is shown in Fig. 1B. Apparently, increase in calcineurin appears to be related to the cAMP signaling pathway and associated with fatty acid biosynthesis via the interaction with ACC.

**Calcineurin and ACC Interaction Analysis**—The protein-protein interaction network generated based on PBAN-dependent phosphoproteomics revealed that calcineurin can interact with ACC. To confirm the interaction between calcineurin and ACC, His-tagged calcineurin was coexpressed with Flag-tagged ACC in the Insect Cells Extract Protein Expression System. Immunoprecipitation analysis confirmed that calcineurin was coprecipitated with ACC (Fig. 1C), thus, demonstrating that calcineurin interacts with ACC directly.

**Effect of Calcineurin on Sex Pheromone Production**—Based on phosphoproteomics, calcineurin is involved in PBAN-regulated signaling (Fig. 2A and 2B). Quantitative PCR (qPCR) analysis of calcineurin expression pattern revealed that the transcript level of calcineurin A, the catalytic subunit of calcineurin, began to increase at ~48 h and continued to increase until 48 h (Fig. 2C), a key stage of sex pheromone biosynthesis. The calcineurin inhibitor, CysA and RNAi-mediated knockdown of calcineurin A both significantly reduced sex pheromone production (Fig. 2D–2F). These findings clearly demonstrate that calcineurin acts as a signal downstream of PBAN/receptor/calcium to influence sex pheromone biosynthesis in *H. armigera*.

**Effect of ACC on Sex Pheromone Production**—Following PBAN stimulation, 4 ACC peptides were found to be phosphorylated (supplemental Fig. S4.) and 1 dephosphorylated (Fig. 3A and Fig. 3a’) indicating that ACC is involved in the PBAN signal transduction cascade. ACC transcript expression in female PGs is significantly upregulated at ~48h females and is continually expressed at a high level in female PGs at 48 h (Fig. 3B). This ACC expression pattern is like that of calcineurin A. Inhibitor analysis and RNAi-mediated knockdown of ACC significantly decreased sex pheromone productions (Fig. 3C–3E).

**Effect of Calcineurin on ACC Activity**—The dependence of the pheromontropic response of PGs on PBAN is shown in Fig. 4A. Following PBAN stimulation, sex pheromone production rapidly accumulates and reaches saturation at 60 min. Similarly, ACC activity began to increase at 10 min and
reached its highest level at 60 min following PBAN stimulation (Fig. 4B). RNAi-mediated knockdown of calcineurin A as well as CysA treatment counteracted the increase in PBAN-induced ACC activity (Fig. 4C and 4D). These results demonstrate that PBAN induces ACC activity via calcineurin.

Identification of the Calcineurin-dependent Phosphoproteome—Calcineurin-dependent dephosphorylation events that occur in response to PBAN stimulation were identified by analyzing PBAN-stimulated alterations in phosphopeptides from PGs incubated with and without a calcineurin inhibitor CysA (supplemental Fig. S5).

A total of 2,875 phosphopeptides were detected in 6 samples (2 treatments with 3 replicates) (supplemental Table S7) after 10 min of incubation with PBAN, whereas 91 phosphopeptides were found to be differentially expressed following incubation with calcineurin inhibitor compared with the control. The phosphorylation levels of 38 phosphopeptides were significantly decreased following treatment of calcineurin inhibitor (Fig. 5A and supplemental Table S8). Similarly, after 30 min incubation with PBAN, a total of 2,001 phosphopeptides were detected in 6 samples (2 treatments with 3 replicates) (supplemental Table S9) whereas 59 phosphopeptides (including 29 upregulated and 30 downregulated) were found to be differentially expressed following treatment with calcineurin inhibitor compared with the control. These differentially expressed phosphopeptides detected after 10 min and 30 min incubation with PBAN represent the signaling events that are influenced by calcineurin.

GO analysis of differentially expressed phosphoproteins confirmed that most of these phosphoproteins are related to cell and organelle for cellular component, to binding and catalytic activity for molecular function and to metabolic process and single-organism process for biological process (supplemental Fig. S6). Protein-protein interaction of calcineurin-regulated signaling network identified several signaling and metabolic pathways that respond to calcineurin, including fatty acid biosynthesis and the cAMP signaling pathway (Fig. 5B). This analysis indicates that these processes are regulated by calcineurin, and agree with the processes regulated by PBAN. These results suggest that calcineurin acts via ACC to regulate sex pheromone synthesis.

Characterization of Calcineurin-dependent Phosphorylated ACC Peptides—PBAN stimulation significantly altered the phosphorylation level of 5 ACC peptides. Four peptides exhibited increased phosphorylation levels (supplemental Fig. S4) whereas one peptide (LLRPPNsGTLOQPSMsQGTVIHSQR) exhibited a decreased level of phosphorylation (dephosphorylation). Because calcineurin significantly increased ACC activity (Fig. 4C) and ACC activity is dependent on dephosphorylation, the effect of calcineurin on ACC phosphorylation was investigated by analyzing the calcineurin-dependent phosphoproteome. Results confirmed that only a single peptide, LLRPPNsGTLOQPSMsQGTVIHSQR, exhibited an increased phosphorylation level following calcineurin inhibitor treatment (Fig. 5C). This peptide was also found to be dephosphorylated following PBAN stimulation (Fig. 3). The phosphorylation sites regulated by calcineurin and PBAN in the LLRPPNsGTLOQPSMsQGTVIHSQR (small s represents phosphorylation serine site) peptide fragment are ser7 and ser15 (ser84 and ser92 in the ACC sequence). The effects of calcineurin on two synthetic ACC phosphopeptides with different phosphor-serine residue (LLRPPNsGTLOQPSMSQGTVIHSQR and LLRPnPNSGTLOQPSMsQGTVIHSQR) in vitro were further investigated by LC/MS/MS. The results further confirmed that calcineurin can dephosphorylate the ser7 and ser15 sites (Fig.

Fig. 1. Characterization of the PBAN-dependent phosphoproteome. A, Differentially expressed phosphopeptides after PBAN treatment. B, PBAN-mediated signal network. Protein interaction networks of the differentially expressed proteins induced by PBAN were constructed by using string website (https://string-db.org/) with high confidence (>0.7). Arrow represents interaction between calcineurin A and ACC. C, Interaction of ACC with calcineurin.
Supplemental Table S11. Table listing differentially expressed phosphopeptides following 10 min or 30 min incubation with PKA activator compared with control samples (0 min of incubation). Statistically significant differences are denoted by different capital letters (ANOVA and Tukey’s test, $p < 0.01$).

A total of 2,447 phosphopeptides were detected in 8 samples (supplemental Table S11). 196 phosphopeptides (137 upregulated and 62 downregulated peptides) were found to be differentially expressed following 10 min of incubation with a PKA activator compared with a sample incubated for 0 min. A total of 104 phosphopeptides (75 upregulated and 29 downregulated peptides) differed significantly after 30 min of incubation with PKA activator compared with control samples (0 min of incubation). When compared with samples incubated for 10 min with a PKA activator, the samples incubated for 30 min had 268 differentially expressed phosphopeptides (122 upregulated and 146 downregulated peptides) (Fig. 6C).

GO analyses revealed binding in molecular function and metabolic processes and signaling for biological processes were most significantly enriched after 10 min or 30 min treatment with PKA activator (supplemental Fig. S9). These results indicate signaling events impacted by PKA (Fig. 6D and supplemental Table S12). A series of biological process in PG tissues were found to be affected by the PKA activator, including...
protein translation, regulation of transcription, protein phosphorylation and dephosphorylation etc. (Fig. 6D). 5’ adenosine monophosphate-activated protein kinase (AMPK) was also altered in the presence of a PKA activator (supplemental Table S12 and supplemental Fig. S10), suggesting that AMPK serves as a downstream signal of PKA and takes part in PBAN-regulated sex pheromone biosynthesis.

Effect of PKA on AMPK Activity—AMPK activity decreased following PBAN stimulation, similar to the result observed for PKA activator stimulation (Fig. 7A and 7B). RNAi-mediated knockdown of PKA C1 significantly weakens the decrease of PBAN-induced AMPK activity (Fig. 7C). These results indicate that PBAN-induced decrease of AMPK activity contributes to the PKA signal.

PKA activator also increased ACC activity and augmentation of AMPK activity using an AMPK activator decreased ACC activity (Fig. 7D and 7E). It is well known that AMPK acts upstream of ACC kinase by phosphorylating ser79 in M. musculus at ACCs. This ser79 site is a homolog of ser92 in the Helicoverpa ACC, which has been confirmed to be dephosphorylated by calcineurin (LLRPPNsGTLQSpsQGTVIHSQR). Treatment with the AMPK activator (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (ACIAR)) further confirmed that AMPK acts as a negative regulator of ACC to influence sex pheromone biosynthesis (Fig. 7F). These findings clearly demonstrate that AMPK, the downstream signal of PBAN/receptor/PKA, inhibits ACC activity via phosphorylation and thereby influences sex pheromone biosynthesis in H. armigera.

Fig. 3. ACC positively regulates sex pheromone biosynthesis in H. armigera PGs. A, ACC MS spectrum identified in the PBAN-regulated phosphoproteome. (a’) Differentially expressed phosphopeptides identified in the PBAN-regulated phosphoproteome. B, Developmental expression profile of ACC. C, Effect of ACC inhibitor on sex pheromone biosynthesis. Bars indicate the mean ± S.D. of three biological replicates for independent experimental animals (n = 12). Statistically significant differences are denoted by different capital letters (ANOVA and Tukey’s test, p < 0.01). D, Effects of ACC dsRNA on ACC transcript level. Significance of comparisons are marked with *** (p < 0.001) as determined by Student’s t test. E, Effect of ACC knockdown on sex pheromone biosynthesis. Bars indicate the mean ± S.D. of three biological replicates for independent experimental animals (n = 12). Statistically significant differences are denoted by different capital letters (ANOVA and Tukey’s test, p < 0.001).
Calcineurin positively regulates ACC activity by dephosphorylating ser84 and ser92. A, Differentially expressed phosphopeptides identified in the calcineurin-regulated phosphoproteome. B, Calcineurin-regulated signaling network. Protein interaction networks of the differentially expressed proteins regulated by calcineurin were constructed by using string website (https://string-db.org/) with high confidence (r>0.7). Arrow represents interaction between calcineurin A and marker proteins in two pathways (fatty acid synthesis pathway (ACC) and cAMP/PKA pathway (PKA regulatory subunit II)). C, Effect of calcineurin inhibitor CysA on ACC activity. Ck represents control females that were incubated with PBAN only. ACC activity was shown as \( \mu \text{mol NADH/min/mg of PG protein} \). Bars indicate the mean ± S.D. of three biological replicates for independent experimental animals (n = 30). Statistically significant differences are denoted with ** (p < 0.01) or *** (p < 0.001) as determined by Student’s t test.

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FIG. 7. PKA regulates sex pheromone biosynthesis by inhibiting AMPK activity in *H. armigera* PGs. A, Effect of PBAN stimulation on AMPK activity. Bars indicate the mean ± S.D. of three biological replicates for independent experimental animals (*n* ≥ 30). Statistically significant differences are denoted by different capital letters (ANOVA and Tukey’s test, *p* < 0.001). B, Effect of PKA activator on AMPK activity. Bars indicate the mean ± S.D. of three biological replicates for independent experimental animals (*n* ≥ 12). Statistically significant differences are denoted by different capital letters (ANOVA and Tukey’s test, *p* < 0.01). C, Effect of AMPK activator on ACC activity. Bars indicate the mean ± S.D. of three biological replicates for independent experimental animals (*n* ≥ 30). Statistically significant differences are denoted by different capital letters (ANOVA and Tukey’s test, *p* < 0.01). D, Effect of AMPK activator on sex pheromone production. Bars indicate the mean ± S.D. of three biological replicates for independent experimental animals (*n* ≥ 20). Statistically significant differences are denoted by different capital letters (ANOVA and Tukey’s test, *p* < 0.01).

FIG. 6. Identification of PKA-regulated phosphopeptides in *H. armigera* PGs. A, Effect of PKA C1 dsRNA on PKA C1 transcript levels. Bars indicate the mean ± S.D. of three biological replicates. Statistically significant differences are denoted with *** (*p* < 0.001) as determined by Student’s *t*-test. B, Effect of PKA C1 dsRNA on sex pheromone biosynthesis. Bars indicate the mean ± S.D. of three biological replicates for independent experimental animals (*n* ≥ 12). Statistically significant differences are denoted by different capital letters (ANOVA and Tukey’s test, *p* < 0.001). C, Differentially expressed phosphopeptides identified in the PKA-regulated phosphoproteome. D, PKA-mediated signal network. Protein interaction networks of the differentially expressed proteins induced by PKA were constructed by using string website (https://string-db.org/) with high confidence (>0.7). Arrow represents interaction between AMPK and ACC.
DISCUSSION

In most moths, sex pheromone production and emission for mating are precisely regulated by PBAN (5). PBAN signal transduction has been extensively studied in several insect models. In *B. mori*, PBAN signal transduction was elucidated in impressive detail: on binding to its G-protein coupled receptor (45), PBAN activates calcium influx and subsequently activates Ca\(^{2+}\)/calmodulin-dependent protein kinase II. This kinase then phosphorylates lipid storage droplet protein-1 (Lsd-1), a homolog of perilipin in mammals (25). The phosphorylation of Lsd-1 facilitates and promotes the release of the lipase activator complex, which activates TAG lipase and leads to the generation of bombykol precursors (25). In *helothine* species, however, the detailed molecular mechanisms of PBAN signal transduction have not been fully elucidated because of insufficient genomic information. Most importantly, the molecular mechanisms underlying PBAN signal transduction in *helothine* species is different from that in *B. mori* because of differences in intracellular secondary messenger cascades and in the enzymatic steps regulated by PBAN (6, 16–18). In our endeavor to explore PBAN signal transduction in *helothine* species, transcriptomic sequencing of PGs was performed with *H. armigera*, as *H. armigera* is an extensively studied model for PBAN action. Transcriptomic analysis yielded 40,381 unigenes, the largest transcriptomic data set for the insect PGs examined to date (44). Most importantly, an extensive list of candidate genes potentially involved in sex pheromone biosynthesis was generated and this list facilitated further characterization of the PBAN signal transduction in *H. armigera*.

Because the PBAN receptor is a G-protein coupled receptor and PBAN uses cAMP and calcium ions as secondary messengers, it was necessary to characterize the phosphoproteins that undergo phosphorylation/dephosphorylation resulting from secondary messenger signals initiated by PBAN interaction with its receptor. PBAN-dependent phosphoproteomics identified a series of phosphopeptides that undergo phosphorylation/dephosphorylation in response to PBAN stimulation, including cAMP-dependent protein kinase RII and ACC. KEGG analysis demonstrated that the cAMP signaling pathway and fatty acid biosynthesis are involved in PBAN-dependent phosphorylation/dephosphorylation cascades. These results are consistent with previous findings, which found that PBAN uses cAMP as secondary messenger for signal transduction and that ACC is the rate-limiting step that is directly regulated by PBAN in *H. armigera* (30). In addition, the calcium signaling pathway was found to be involved in PBAN-dependent phosphorylation/dephosphorylation cascades. Calcineurin, a Ca\(^{2+}\)/calmodulin-dependent protein phosphatase, is a highly conserved ubiquitous phosphatase. Calcineurin consists of two subunits (catalytic A and regulatory B) (46), and the activity of this phosphatase is strictly controlled by Ca\(^{2+}\) and calmodulin. Our results demonstrated that calcineurin acts as a downstream marker of calcium/calmodulin signaling and PBAN-dependent phosphoproteomics also demonstrated its involvement in PBAN signaling transduction. These results provide evidence that calcineurin participates in PBAN-regulated sex pheromone biosynthesis. Further functional analysis revealed that RNAi-mediated knockdown and inhibitor analysis of calcineurin A significantly decreased sex pheromone production in present study. These results indicated that calcineurin acts as a downstream signal of PBAN/Ca\(^{2+}\) signaling to regulate sex pheromone biosynthesis in *H. armigera*.

Because calcineurin is required for PBAN-regulated sex pheromone biosynthesis, the question arises how does it regulate sex pheromone biosynthesis in *H. armigera*? Because calcineurin is a protein phosphatase and acts via dephosphorylation, calcineurin-dependent dephosphorylation events were thus identified by analyzing phosphopeptides in calcineurin-inhibited PGs. Many phosphopeptides were found to be more abundant in calcineurin-inhibited PGs, these peptides are likely substrates for calcineurin regulation. KEGG analysis demonstrated that the fatty acid biosynthesis pathway is involved in calcineurin-regulated biological process. This finding is consistent with the PBAN-dependent phosphoproteomic results that the fatty acid biosynthesis pathway is activated following PBAN stimulation. Taken together, these results demonstrate that calcineurin regulation of fatty acid biosynthesis is involved in PBAN-regulated sex pheromone biosynthesis. GO and protein-protein interaction analysis further assured that calcineurin regulates the fatty acid biosynthesis pathway via acetyl-coA carboxylase.

Lepidopteran sex pheromones are biosynthesized de novo in PGs cells from acetyl-CoA via the fatty acid biosynthesis pathway (20). Therefore, it is not surprising that the target for PBAN regulation is ACC, a rate-limiting enzyme that plays a critical role in the regulation of fatty acid metabolism. Our result confirmed that ACC activity increases following PBAN stimulation (Fig. 4B). This increase in ACC activity during the sex pheromone biosynthesis process is consistent with sex pheromone production (Fig. 4A). PBAN-induced ACC activity can be counteracted with calcineurin inhibitor, indicating that calcineurin acts as an upstream regulator, influencing sex pheromone biosynthesis by regulating ACC activity. In animals, the mechanism that regulates ACC activity is widely accepted and relatively conserved: ACC is rapidly mediated by reversible phosphorylation (47). AMPK phosphorylates and inactivates ACC, whereas other phosphatases dephosphorylate and activate ACC. Eight phosphorylation sites have been identified in the ACC from rats to date, including serines 23, 25, 29, 77, 79, 95, 1200 and 1215 (48). Critical phosphorylation sites are rapidly phosphorylated during certain physiological processes, suppressing ACC activity and influencing fatty acid synthesis. Ser79 and ser1200 are the most important phosphorylation sites for inhibition, and are substrates for AMPK (49). AMPK phosphorylation of ser1200 and ser1215 does not have any additional effect on ACC activity.
cAMP-dependent protein kinase (PKA) phosphorylates ser77 and ser1200; however, ser77 does not appear to be phosphorylated in hepatocytes exposed to glucagon (51). It is well-accepted that ser79 is the dominant inhibitory site for ACC activity (50). In the present study, only one peptide (LLRPPNsGTLQPSMsQGTVIHSQR) was found to be significantly dephosphorylated following PBAN stimulation. The corresponding phosphorylation sites for this peptide in H. armigera PG ACC sequence are ser84 and ser92 (LLRPPNs(84)GTLQPSMs(92)QGTVIHSQR). Our sequence analysis demonstrated that ser92 in H. armigera PGs appears to be equivalent to ser79 in M. musculus. However, no equivalent to ser84 in H. armigera PGs has been found in the M. musculus ACC sequence. This result indicates that PBAN-regulated dephosphorylation of ser92 is probably required for ACC activation. In the present study, calcineurin was confirmed to act as a downstream signal of PBAN/calcium/calmodulin signaling for the regulation of sex pheromone biosynthesis. In addition, PBAN- and calcineurin-dependent phosphoproteomics demonstrated that ACC is a probable target for calcineurin. Further inhibitor analysis also confirmed that calcineurin inhibitors CysA abolish PBAN-induced ACC activity. Protein-protein interaction studies and in vitro calcineurin treatments provided further evidence that calcineurin is an upstream phosphatase of ACC, directly dephosphorylating ser84 and ser92 in ACC to activate this protein.

If calcineurin acts as a downstream signal of PBAN/calcium/calmodulin signaling to regulate sex pheromone biosynthesis via ACC activation, what role does the cAMP/PKA signal play in PBAN-regulated sex pheromone biosynthesis in H. armigera? PKA-dependent phosphoproteomics was employed to investigate downstream events caused by PKA. A series of phosphoproteins were found to be mediated by PKA, one of which is beta subunit of AMPK. AMPK is a heterodimeric kinase that consists of three subunits: catalytic subunit α, regulatory subunits β and γ (52). In mammals, AMPK is activated by its upstream kinase via phosphorylation of the α catalytic subunit at thr172 residue (53). AMPK activity can also be regulated by phosphorylation of the regulatory β subunit. Several phosphorylated residues were identified in the β subunit of rat AMPKs (ser24/ser 25, ser 108, ser182, ser96, ser 101, thr80, thr158 ser174, and ser177). Phosphorylation of s24, s25 and s182 does not affect AMPK activity but results in nuclear redistribution of the subunit. Phosphorylation of s108 was found to increase AMPK activity (54). However, no additional studies have been performed to determine the roles of other phosphorylated sites (including ser96, ser101, thr80, thr158 ser174, and ser177). From our present study, it is unclear whether the identified phosphorylated sites play important roles in AMPK activity, however the effect of the PKA signal on AMPK activity was measured. As expected, subsequent experiments confirmed that PBAN stimulation and PKA activator inhibit AMPK activity.

In M. musculus AMPK phosphorylates and inactivates ACC at ser79 (47–51). Our present study revealed that the ser92 site in H. armigera PGs is in fact homologous to ser79 in mouse a conserved domain in mouse and H. armigera. Additionally, activation of AMPK decreased ACC activity and subsequently reduced sex pheromone production. These results thus demonstrate that the PBAN/G-protein/cAMP/PKA signaling pathway inhibits AMPK activity, influencing ACC activity and concomitant sex pheromone production. Based on our results, we propose a model for PBAN regulated sex pheromone biosynthesis in H. armigera in which PBAN acts via its secondary messengers (calcium ions and cAMP) to regulate sex pheromone biosynthesis. Calcineurin, once activated by the PBAN/receptor/calcium signal pathway, activates ACC.
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via dephosphorylation and thereby stimulates sex pheromone biosynthesis. In parallel, the cAMP/PKA signal pathway decreases AMPK activity, leading to enhanced dephosphorylation of ACC thereby increasing ACC activity, and thus stimulating sex pheromone biosynthesis. For the first time, we provide a clear overview of how PBAN secondary messengers (calcium ions and cAMP) synchronously regulate sex pheromone biosynthesis (Fig. 8).

Despite these unequivocal results, more studies are required to elucidate details in the PBAN signal transduction pathway. For example, does PKA regulate AMPK activity indirectly or directly? How does the phosphorylation of ser84 in ACC affect its activity and is this phosphorylation site a target site for AMPK?

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DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomcentral.proteomexchange.org) via the PRIDE partner repository (http://www.ebi.ac.uk/pride/archive/) with the data set identifier PXD005645, PXD006034 and PXD006380.

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