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Role of protein phosphatase 2A in PTTH-stimulated prothoracic glands of the silkworm, *Bombyx mori*

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ABSTRACT

In the present study, the roles of a major serine/threonine protein phosphatase 2A (PP2A) in prothoracicotropic hormone (PTTH)-stimulated prothoracic glands (PGs) of *Bombyx mori* were evaluated. Immunoblotting analysis showed that *Bombyx* PGs contained a structural A subunit (A), a regulatory B subunit (B), and a catalytic C subunit (C), with each subunit undergoing development-specific changes. The protein levels of each subunit were not affected by PTTH treatment. However, the highly conserved tyrosine dephosphorylation of PP2A C subunit (PP2Ac), which appears to be related to activity, was increased by PTTH treatment in a time-dependent manner. We further demonstrated that phospholipase C (PLC), Ca^{2+}, and reactive oxygen species (ROS) are upstream signaling for the PTTH-stimulated dephosphorylation of PP2Ac. The determination of PP2A enzymatic activity showed that PP2A enzymatic activity was stimulated by PTTH treatment both *in vitro* and *in vivo*. Okadaic acid (OA), a specific PP2A inhibitor, prevented the PTTH-stimulated dephosphorylation of PP2Ac and reduced both basal and PTTH-stimulated PP2A enzymatic activity. The determination of ecdysteroid secretion showed that treatment with OA did not affect basal ecdysteroid secretion but did significantly inhibit PTTH-stimulated ecdysteroid secretion, indicating that PTTH-stimulated PP2A activity is involved in ecdysteroidogenesis. Treatment with OA stimulated the basal phosphorylation of the extracellular signal-regulated kinase (ERK) and 4E-binding protein (4E-BP) without affecting PTTH-stimulated ERK and 4E-BP phosphorylation. From these results, we hypothesize that PTTH-regulated PP2A signaling is a necessary component for the stimulation of ecdysteroidogenesis, potentially by mediating the link between ERK and TOR signaling pathways.

1. Introduction

Ecdysteroids, synthesized and secreted by the prothoracic glands (PGs), play critical roles in regulating insect growth, molting, and metamorphosis (Agui et al., 1979; Thummel, 2001; Smith and Rybczynski, 2012; De Loof et al., 2015). The ecdysteroid biosynthetic activity of PGs is mainly stimulated by prothoracicotropic hormone (PTTH), a neuropeptide produced by brain neurosecretory cells (Ishizaki and Suzuki, 1994; Marchal et al., 2010; Smith and Rybczynski, 2012; De Loof et al., 2015). PTTH activates ecdysteroidogenesis in PGs through a complex signaling network which contains various protein phosphorylations (Smith and Rybczynski, 2012). In eukaryotic cells, it has been well documented that protein phosphorylation is an integral component of signal transduction pathways and regulated by the fine interplay of protein kinases and phosphatases (Pawson and Scott, 2005). The key roles played by protein kinases and protein phosphorylations in regulating PTTH-stimulated ecdysteroidogenesis in PGs are well documented. Upon binding to the PTTH receptor torso, a receptor tyrosine kinase, PTTH initiates a signaling transduction network (Rewitz et al., 2009b, 2013; Marchal et al., 2010; Smith and Rybczynski, 2012; De Loof et al., 2015). Previous studies indicated that numerous protein kinases, including protein kinase A (PKA), protein kinase C (PKC), tyrosine kinase, and p70 S6 kinase are involved in PTTH-stimulated ecdysteroidogenesis in PGs (Smith et al., 1984, 1985, 2003; Song and Gilbert, 1997; Rybczynski and Gilbert, 2006; Smith and Rybczynski, 2012). In addition, extracellular signal-regulated kinase (ERK) phosphorylation, phosphatidylinositol 3-kinase (PI3K)/adenosine 5′-monophosphate-activated protein kinase (AMPK)/target of rapamycin (TOR) signaling, and reactive oxygen species (ROS) were found to be involved in PTTH's stimulation of ecdysteroidogenesis (Rybczynski et al., 2001; Lin and Gu 2007; Gu et al., 2010, 2011, 2012, 2013; Hsieh et al., 2013; Hsieh et al., 2014). More recently, our study further demonstrated that PTTH stimulates the phosphorylation of histone H3 at serine 10 in *Bombyx mori* PGs (Gu and Hsieh, 2015), thus

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leading to the increased immediate early gene expression of *Bombyx HR38* (Gu et al., 2016).

In contrast to the widely studied roles of phosphorylation events, the dephosphorylation processes involved in ecdysteroidogenesis of PGs are not so extensively studied. Although the previous study demonstrated that in *Manduca sexta* PGs, protein phosphatases (PPs), which were sensitive to okadaic acid (OA) or calyculin A, were required for PTTH-stimulated ecdysteroidogenesis (Song and Gilbert, 1996), there was no attempt to characterize the involved PPs. In the tobacco budworm larvae infected with parasitoid wasps, associated bracoviruses induced overexpression of tyrosine phosphatase in fat body and PGs, resulting in the inactivation of PGs (Falabella et al., 2006).

Reversible protein phosphorylation, mediated by kinases and phosphatases, is a common mechanism utilized to regulate basic processes in eukaryotes (Pawson and Scott, 2005). Protein phosphatase 2A (PP2A) is a multimeric serine/threonine phosphatase that has been demonstrated to be highly conserved during the evolution of eukaryotes (Millward et al., 1999; Janssens and Goris, 2001; Kiely and Kiely, 2015; Sangodkar et al., 2016). PP2A is a well-studied protein phosphatase that has been shown to regulate a wide array of biological processes including signal transduction, cell differentiation and development, protein translation, and apoptosis (Janssens and Goris, 2001; Janssens et al., 2005). PP2A is a trimeric holoenzyme, composed of a core dimer plus a third subunit. The holoenzyme consists of a 36-kDa catalytic C subunit (PP2Ac), a structural A subunit (PP2AA), and a third variable regulatory B subunit (PP2AB). The B subunit is variable, ranging from 54 to 130 kDa, and serves to confer distinct properties on the enzyme for substrate specificity (Janssens and Goris, 2001; Janssens et al., 2005; Kiely and Kiely, 2015; Sangodkar et al., 2016). It has been demonstrated that PP2A activity is modulated by either non-covalent interaction with regulatory subunits, heat stable inhibitors or lipids, or covalent post-translational modifications such as phosphorylation and methylation (Sents et al., 2013; Seshacharyulu et al., 2013). Phosphorylation of the PP2Ac on tyrosine 307 has been reported to be responsible for PP2A inactivation (Chen et al., 1992; Guo and Damuni, 1993; Longin et al., 2007).

To begin to explore a potential role for PP2A in insect ecdysteroidogenesis, the present study examined the changes in protein expression patterns of PP2A A, B, and C subunits in PGs during the last larval instar and studied the effects of PTTH. The mRNA expression levels of the PP2A catalytic β subunit and regulatory subunit were also examined. We found that PTTH treatment did not affect the protein levels of each subunit, but stimulated the highly conserved tyrosine dephosphorylation of PP2Ac. We further demonstrated the relationship between the PTTH-stimulated dephosphorylation of PP2Ac and other known PTTH-regulated signaling pathways. Using a specific PP2A inhibitor OA, the functional significance of PP2A activity in PTTH-stimulated ecdysteroidogenesis was assessed.

2. Materials and methods

2.1. Experimental animals

Larvae of an F1 racial hybrid, Guofu × Nongfong of *B. mori* were reared on fresh mulberry leaves at 25 °C under a 12-L: 12-D photoperiod. A developmentally synchronous population of larvae was obtained by collecting the newly ecdysed last instar larvae shortly after lights-on, and this was designated as day 0. Larvae used in the present study wandered on day 7, and underwent pupal ecdysis around day 11.

2.2. Reagents

A23187, thapsigargin, diphenylene iodonium (DPI), N-acetylcysteine (NAC), and OA were supplied by Sigma-Aldrich (St. Louis, MO, USA). All other reagents used were of analytical grade. Grace's insect cell culture medium was purchased from Invitrogen (Carlsbad, CA, USA). A mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) inhibitor (U0126), a specific inhibitor of phospholipase C (PLC) (U73122), a PI3K inhibitor (LY294002), and a cell-permeable AMPK activator (5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside, AICAR) were purchased from Calbiochem (San Diego, CA, USA). Recombinant *B. mori* PTTH (PTTH) was produced by infection of *Spodoptera frugiperda*-SF21 cells with the vWTPTTHM baculovirus as described previously (O'Reilly et al., 1995). The same PTTH as that previously reported (O'Reilly et al., 1995; Gu et al., 2011, 2012, 2013) was used in the present study. Extracellular fluid from cells infected with vWTPTTHM was used as the PTTH source, and it was diluted 500 times with medium. Each incubation (50 µl) contained about 0.15 ng PTTH. In addition, extracellular fluid from cells infected with wild-type *Autographa californica* nuclear polyhedrosis virus (WT AcMNPV) was diluted 500 times with medium and used as control medium.

Anti-PP2Ac subunit antibody (610556) was purchased from Transduction Laboratories™ (Franklin Lakes, NJ, USA). Anti-PP2AA (#2039), PP2AB subunit (#2290), anti-phospho-ERK (#9101), anti-phospho-4E-BP1 (Thr37/46) (#9459), and anti-α-tubulin (#2144) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-PP2Ac antibody (sc-271903) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A horseradish peroxidase (HRP)-linked goat anti-rabbit and anti-mouse second antibodies were purchased from PerkinElmer Life Sciences (Boston, MA, USA).

2.3. In vitro incubation of PGs and in vivo injection of PTTH

PGs from day-6 last instar larvae or other stages were dissected in lepidopteran saline (Hsieh et al., 2013, 2014). Considering that PGs between days 6 and 8 showed the highest responsiveness in PTTH-stimulated ERK phosphorylation and ecdysteroid secretion (Gu et al., 1996; Lin and Gu, 2007), we used PGs from day-6 last instar larvae for most experiments. Following dissection, the medium was replaced with fresh medium (with or without any inhibitors), and a 30-min preincubation period was initiated. After preincubation, PGs were rapidly transferred to fresh medium (with or without experimental materials, such as an inhibitor or PTTH) and then incubated for 1 h with gentle shaking. To study the *in vivo* effect of PTTH on PP2A enzymatic activity, day-6 last instar larvae were injected with 10 µl saline containing 0.3 µl of the original PTTH solution. Larvae injected with 10 µl saline containing diluted extracellular fluid from cells infected with WT AcMNPV were used as the controls.

2.4. Western blot analysis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as previously described (Lin and Gu, 2007; Gu et al., 2011, 2012, 2013). Briefly, the treated or control PGs were individually homogenized in lysis buffer (10 mM Tris and 0.1% Triton x100) at 4 °C, then boiled in an equal volume of SDS sample buffer for 4 min, followed by centrifugation at 15,800 *g* for 3 min to remove any particulate matter. Aliquots of the supernatants were loaded onto SDS gels. Following electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes using an Owl (Portsmouth, NH, USA) Bandit™ Tank Electroblotting System and then washed with Tris-buffered saline (TBS) for 5 min at room temperature. Blots were blocked at room temperature for 1 h in TBS containing 0.1% Tween 20 (TBST) and 5% (w/v) nonfat powdered dry milk, followed by washing three times for 5 min each with TBST. Blots were incubated overnight at 4 °C with the primary antibody in TBST with 5% bovine serum albumin (BSA). Blots were then washed three times in TBST for 10 min each and further incubated with the HRP-linked second antibody in TBST with 1% BSA. Following three additional washes, immunoreactivity was visualized by chemiluminescence using Western Lightning Chemiluminescence Reagent *Plus* from PerkinElmer Life Sciences. Films exposed to the chemiluminescent reaction

were scanned and quantified using an AlphaImager Imaging System and AlphaEaseFC software (Alpha Innotech, San Leandro, CA, USA).

2.5. PP2A phosphatase activity assay

PP2A activity was measured with the fluorescent-based RediPlate™ 96 EnzChek® Serine/Threonine Phosphatase Assay kit (Life Technologies) following the manufacturer's protocol. PP2A activity can be distinguished from PP1, PP2B, and PP2C by adding different metal ions, such as $NiCl_2$, $MnCl_2$, and Ca^{2+} to the assay buffer, which can differentially enable phosphatase activities (Cohen, 1991). In the present study, as suggested by the manufacturer's protocol, $NiCl_2$ was added as an additional reaction buffer component to specifically detect PP2A activity. Gland lysates (2 PGs for each datum point) were prepared by using low detergent buffer (1% Nonidet P-40, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and complete protease inhibitor cocktail). A total of 50 µl lysates were incubated with 1X PP2A phosphatase reaction buffer for 30 min at 37 °C. Fluorescence intensity was measured using excitation at 355 nm and emission at 485 nm.

2.6. Enzyme immunoassay (EIA) for ecdysteroid measurements

Ecdysteroids released into the medium were extracted with methanol and measured using 20-hydroxyecdysone EIA kit (Cayman Chemical/Sanbio, Uden, the Netherlands) as previously described (Marchal et al., 2012; Koyama et al., 2014).

2.7. RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was extracted from a pool of 4–6 *Bombyx* PGs for each time point. Total RNA from PGs was extracted using the TRI Reagent (Molecular Research Center, OH, USA) according to the manufacturer's protocol. The quantity of extracted RNA was assessed with a UV1101 photometer (Biotech, Cambridge, UK) and/or by electrophoresis on 1% (w/v) agarose gels. First-strand complementary DNA (cDNA) was synthesized using an iScript cDNA synthesis kit (Bio-Rad, CA, USA).

For the qRT-PCR analysis, total RNA was extracted from PGs (Young et al., 2012). The PCR was carried out in a 20 µl reaction volume containing 10 µl of SYBR1 Green Realtime PCR Master Mix (Bio-Rad), 2 µl of a first-strand cDNA template, and 8 µl of the primers. The iQ5 Real-Time PCR Detection System (Bio-Rad) was used according to the manufacturer's instructions. The PCR primers were designed according to parameters (no primer dimers and a product length of no more than 200 bp) outlined in the manual of the SYBR1 Green Realtime PCR Master Mix. The annealing temperature for all reactions was 59.5 °C. Transcript levels were normalized to the *Bombyx ribosomal protein 49* (*rp49*) mRNA levels. C_T values were set against a calibration curve. The $\Delta\Delta C_T$ method was used to calculate relative abundances. *Bombyx mori rp49* was chosen as a reference gene. The qRT-PCR was performed using the following primers: PP2A catalytic subunit β isoform forward, 5′ CCAACTACTGCTATAGATGT-3′ and reverse, 5′CAGGAATGAATACTTG AGTG-3′; PP2A regulatory subunit forward, 5′-GTCAAACTCAGCAAT TCC-3′ and reverse, 5′-TACAGGTTTCGTTTCCAT-3′; and *rp49* forward, 5′-CAGGCGGTTCAAGGGTCAATAC-3′ and reverse, 5′-TGCTGGGCTCT TTCCACGA-3′.

3. Results

3.1. Expression of PP2A subunits

PP2A exists *in vivo* as a heterotrimer composed of A, B, and C subunits (Janssens and Goris, 2001). Our previous study demonstrated that silkworm eggs contain each subunit which undergoes differential

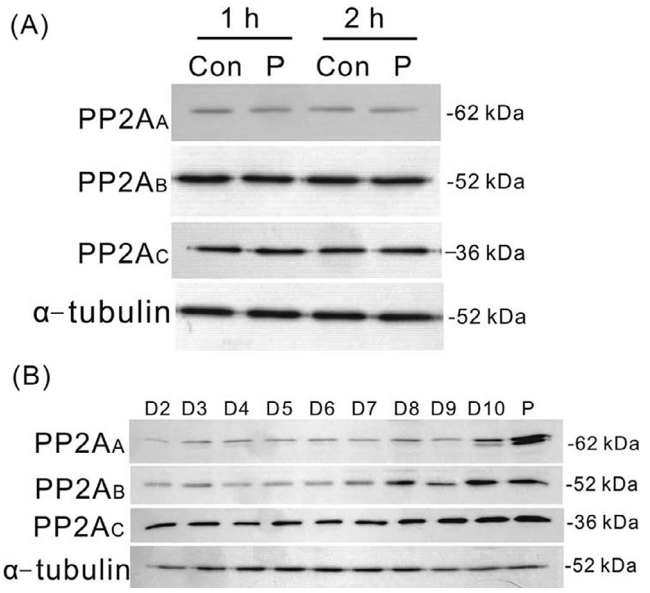

Fig. 1. Effects of PTTH on protein levels of PP2A A, B, and C subunits (A) and their changes during the last larval instar and pupation stage. (A) Effects of PTTH. PGs were treated with PTTH (P) or incubated with control medium (Con) for the indicated time points. (B) Changes in the protein levels of PP2A-A, -B, and -C subunits during the last larval instar and pupation stage. P, pupation. Lysates of PGs during different days (from day 2 (D2) to pupation day) were individually prepared and subjected to immunoblotting analysis with anti-PP2AA, anti-PP2AB, anti-PP2AC, and anti-α-tubulin antibodies. Results shown are from representative experiments of 3–5 independent experiments. The MW markers are shown on the right side of the gel.

regulation during the embryonic diapause process (Gu et al., 2017). However, it is not clear whether the A, B, and C subunits exist in the PGs of silkworm larvae. In the present study, the presence of each subunit of PP2A in *B. mori* PGs was confirmed by using commercial antibodies against mammalian PP2A subunits and Western blot analysis (Gu et al., 2017). As shown in Fig. 1A, the antibodies for PP2A structural A and regulatory B subunit are highly specific, detecting a single protein band of the expected molecular weights (A, 62-kD and B, 52-kD). The PP2AC subunit antibody specifically detects a single 36-kD protein band consistent with the molecular weight of the PP2AC subunit. Treatment with PTTH for 1 and 2 h *in vitro* did not affect the protein levels of each subunit.

We further examined changes in the protein levels of each PP2A subunit during development. As shown in Fig. 1B, PP2A A, B, and C subunit protein levels were found to be developmentally regulated in PGs during the last larval instar, with the highest protein levels being detected during the later stages of the last larval instar. The presence of two bands for the A subunit of PP2A during the later stages of the last larval instar may have been due to different isoforms, as previously reported in mammalian systems (Janssens and Goris, 2001; Seshacharyulu et al., 2013). These results clearly demonstrated that PGs contained the PP2A A, B, and C subunits and that PTTH treatment during the short incubation periods (1 or 2 h) did not affect the protein levels of each subunit.

A further experiment was conducted to examine the effect of PTTH on the mRNA expression levels of the PP2A catalytic β subunit and regulatory subunit. Upon treatment with PTTH for either 1 h or 2 h *in vitro*, mRNA expression levels of catalytic β subunit and regulatory subunit were not greatly changed (Fig. 2), indicating that PTTH did not exert its action on their transcript levels.

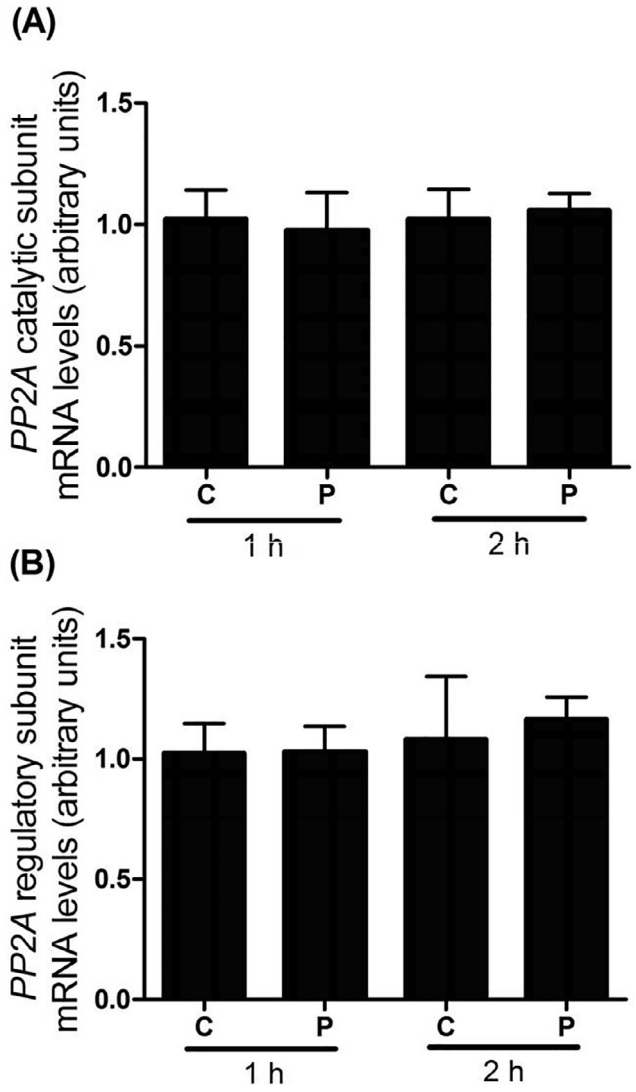

Fig. 2. Changes in the mRNA expression levels of PP2A catalytic subunit (β isoform) (A) and regulatory subunit (B) in PGs upon treatment with PTTH *in vitro*. Glands from day-6 last instar larvae were treated with PTTH (P) or incubated with control medium (C) for the indicated time periods. Gland extracts were then prepared and mRNA expression levels were determined by a qRT-PCR. Each bar represents the mean + SD (N = 4 biological replicates).

3.2. Effect of PTTH on the tyrosine dephosphorylation of PP2Ac and its developmental changes

Although PTTH treatment did not affect the protein levels of each subunit, it is unclear whether or not PTTH increases the protein dephosphorylation of the PP2Ac subunit. Previous study demonstrated that the PP2Ac subunit can be tyrosine phosphorylated and that this highly conserved phosphorylation of the PP2Ac subunit appears to be related to catalytic activity (Chen et al., 1992; Guo and Damuni, 1993). To examine whether PTTH treatment affects the tyrosine phosphorylation of PP2Ac, PGs from day-6 last instar larvae were treated with PTTH for different periods and then the tyrosine phosphorylation of PP2Ac was examined by Western blot analysis. As shown in Fig. 3A, PTTH treatment increased dephosphorylation of PP2Ac in a time-dependent manner with the highest stimulation being detected after 1 h treatment. However, a decrease in PTTH stimulatory effect was detected after 2 or 4 h treatment.

To examine whether PTTH-stimulated dephosphorylation of PP2Ac undergoes changes in different developmental stages, PGs from day-1, −7, and −10 last instar larvae were isolated and then challenged with PTTH. As shown in Fig. 3B, PTTH slightly stimulated dephosphorylation of PP2Ac in PGs from day-1 last instar larvae. However, PTTH dramatically increased dephosphorylation of PP2Ac in PGs from day-7 last instar larvae. For day-10 last instar larvae (1 day before pupation), a decreased response in dephosphorylation of PP2Ac upon PTTH stimulation was detected, indicating that refractoriness of gland cells to the PTTH may occur at this stage.

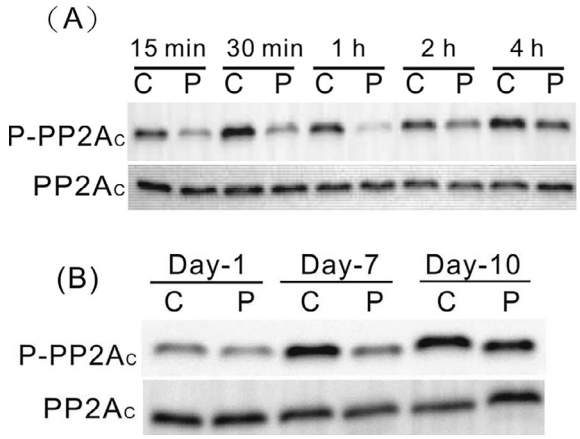

Fig. 3. Time- and stage-dependent effects of tyrosine dephosphorylation of PP2Ac by PTTH. (A) Time-dependent effects. (B) Stage-dependent effects. PGs from day-6 (for A) or from day-1, -7, and -10 last instar larvae (for B) were preincubated in control medium for 30 min, then transferred to medium with PTTH (P), or control medium (C), and incubated for the indicated time points (for A) or for 1 h (for B). Gland lysates were individually prepared and subjected to an immunoblot analysis with anti-phosphorylated PP2Ac (P-PP2Ac) and anti-PP2Ac (PP2Ac) antibodies. Results shown are representative of 3–5 independent experiments.

3.3. Signaling pathway involved in the PTTH-stimulated dephosphorylation of PP2Ac and effect of ecdysone

Previous studies demonstrated that MAPK/ERK and AMPK/TOR are two signaling pathways regulated by PTTH in both *M. sexta* and *B. mori* and that PLC and Ca^{2+} are upstream signaling of these two signaling pathways (Rybczynski et al., 2001; Lin and Gu, 2007; Gu et al., 2011, 2012, 2013). We further investigated the correlation between the PTTH-stimulated dephosphorylation of the PP2Ac subunit and the above PTTH signaling pathways. As shown in Fig. 4, PTTH-stimulated dephosphorylation of PP2Ac was prevented in Ca^{2+}-free saline and blocked by U73122, a potent and specific inhibitor of PLC. In addition, a weak increase in tyrosine dephosphorylation of PP2Ac was also detected when PGs were treated with agents (either A23187 or thapsigargin) that directly elevated the intracellular Ca^{2+} concentration, thereby indicating the involvement of Ca^{2+} and PLC.

Fig. 5 showed that pretreatment with U0126 (an inhibitor of MEK) and LY294002 (a specific PI3K inhibitor), failed to block the PTTH-stimulated dephosphorylation of PP2Ac, indicating that PTTH-stimulated ERK and PI3K signaling pathways were not related to tyrosine dephosphorylation. A chemical activator of AMPK (AICAR) also did not block the PTTH-stimulated dephosphorylation of PP2Ac, showing that AMPK is not involved.

We further investigated the effect of modulation of the redox state on PTTH-stimulated tyrosine dephosphorylation of PP2Ac. In the presence of either an antioxidant (NAC) or DPI, PTTH-stimulated dephosphorylation of PP2Ac was blocked, indicating the involvement of ROS (Fig. 6).

These results showed that PLC, Ca^{2+}, and ROS are upstream signaling pathways for the PTTH-stimulated dephosphorylation of PP2Ac, which is not related to MAPK/ERK and AMPK/TOR.

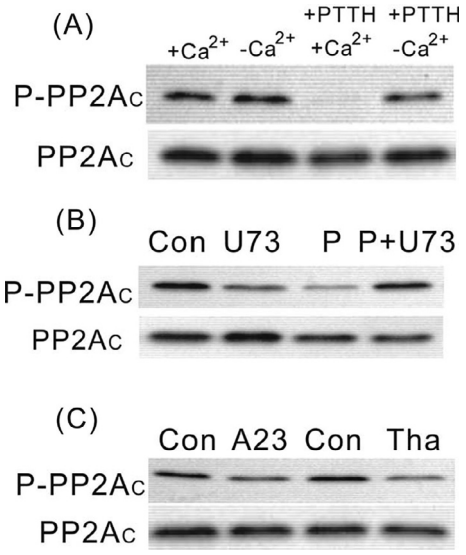

Fig. 4. Effect of external Ca^{2+} (A) and U73122 (B) on the PTTH-stimulated dephosphorylation of PP2Ac and effects of A23187 and thapsigargin (C). (A) Effect of external Ca^{2+}. PGs were preincubated in Ca^{2+}-free saline (with 5 mM EGTA) for 30 min, then transferred to either control saline (+Ca^{2+}), saline with the PTTH (+PTTH + Ca^{2+}), Ca^{2+}-free saline (-Ca^{2+}), or Ca^{2+}-free saline with PTTH (+PTTH-Ca^{2+}), and incubated for 60 min. (B) Effect of U73122. PGs were pretreated with either 50 μM U73122 or control Grace's medium for 30 min, then transferred to Grace's medium containing the same dose of inhibitor with or without PTTH, and incubated for 60 min. Con, PGs incubated in control medium; P, PGs incubated in medium containing PTTH only; U73, PGs incubated in medium containing U73122 only; P + U73, PGs incubated in medium containing both PTTH and U731226. (C) Effect of A23187 and thapsigargin. PGs were pretreated with control Grace's medium for 30 min, then, transferred to control Grace's medium (Con), medium containing 50 μM A23187 (A23), or 10 μM thapsigargin (Tha), and incubated for 30 min. Gland lysates were individually prepared and subjected to an immunoblot analysis with anti-phosphorylated PP2Ac (P-PP2Ac) and anti-PP2Ac (PP2Ac) antibodies. Results shown are representative of 3–5 independent experiments.

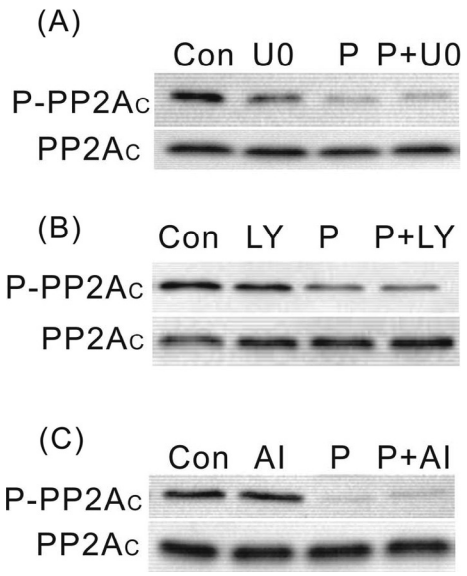

Fig. 5. Effects of U0126 (A), LY294002 (B), and AICAR (C) on the PTTH-stimulated dephosphorylation of PP2Ac. PGs were pretreated with either 10 μM U0126, 50 μM LY294002, 1 mM AICAR, or control medium for 30 min, then transferred to medium containing the same dose of each inhibitor with or without PTTH, and incubated for 60 min. Con, PGs incubated in control medium; P, PGs incubated in medium containing PTTH only; U0, PGs incubated in medium containing U0126 only; P + U0, PGs incubated in medium containing both PTTH and U0126; LY, PGs incubated in medium containing LY294002 only; P + LY, PGs incubated in medium containing both PTTH and LY294002; AI, PGs incubated in medium containing AICAR only; P + AI, PGs incubated in medium containing both PTTH and AICAR. Gland lysates were individually prepared and subjected to an immunoblot analysis with anti-phosphorylated PP2Ac (P-PP2Ac) and anti-PP2Ac (PP2Ac) antibodies. Results shown are representative of 3–5 independent experiments.

In *Bombyx* larvae, PGs predominantly secrete ecdysone (Kiriishi et al., 1990). Upon secretion into the hemolymph, ecdysone is converted to 20-hydroxyecdysone in peripheral tissues (Smith and Rybczynski, 2012). To rule out the possibility that dephosphorylation of PP2Ac is indirectly induced by ecdysone as a result of PTTH-stimulated ecdysone biosynthetic activity, PGs were treated *in vitro* with ecdysone at different concentrations (0, 10, 100, and 1000 ng/ml) for 1 h, and dephosphorylation of PP2Ac was examined. As shown in Fig. 7, dephosphorylation of PP2Ac did not change due to treatment with ecdysone. This result confirms that the PTTH-stimulated dephosphorylation of PP2Ac is direct.

3.4. Stimulatory effect of PTTH on PP2A enzymatic activity

The tyrosine phosphorylation of PP2Ac reduces its catalytic activity (Chen et al., 1992; Guo and Damuni, 1993). The present finding that PTTH stimulated dephosphorylation of PP2Ac implies the possibility that PTTH may activate PP2A activity. To confirm that treatment with PTTH may affect PP2A enzymatic activity, we directly determined PP2A enzymatic activity in extracts of PGs and examined the effect of PTTH by using the RediPlate™ 96 EnzChek® Serine/Threonine Phosphatase Assay kit, as shown in a previous study (Gu et al., 2017). When PGs were treated with PTTH for 30 min *in vitro*, PP2A enzymatic activity significantly increased compared to that of control PGs, indicating that PTTH has a stimulatory effect on PP2A enzymatic activity (Fig. 8A). In subsequent experiments, we examined the *in vivo* activation of PP2A enzymatic activity by PTTH. Day-6 last instar larvae were injected with PTTH. Thirty minutes later, PGs were quickly dissected

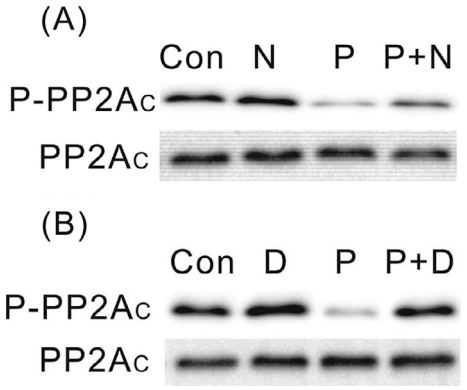

Fig. 6. Effects of NAC (A) and DPI (B) on the PTTH-stimulated dephosphorylation of PP2Ac. PGs were pretreated with either NAC (4 mM), DPI (5 μM), or vehicle alone for 30 min, then transferred to medium containing the same dose of each inhibitor with or without PTTH, and incubated for 60 min. Con, PGs incubated in control medium; N, PGs incubated in medium containing NAC; P, PGs incubated in medium containing PTTH; P + N, PGs incubated in medium containing both PTTH and NAC; D, PGs incubated in medium containing DPI; P + D, PGs incubated in medium containing both PTTH and DPI. Gland lysates were individually prepared and subjected to an immunoblot analysis with anti-phosphorylated PP2Ac (P-PP2Ac) and anti-PP2Ac (PP2Ac) antibodies. Results shown are representative of 3–5 independent experiments.

out and PP2A activity was examined and compared to control larvae. Result (Fig. 8B) shows that a PTTH injection significantly increased PP2A activity compared to controls. The result that the elevated PP2A activity by a challenge with PTTH *in vivo* was quite similar to the *in vitro* experiment, verifying the *in vitro* stimulation of PP2A activity by PTTH.

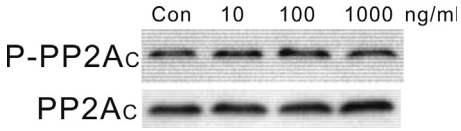

Fig. 7. Effects of ecdysone on dephosphorylation of PP2Ac. PGs were pretreated with control medium for 30 min, then transferred to control medium (Con), or medium containing 10, 100, or 1000 ng/ml of ecdysone, and incubated for 1 h. Gland lysates were individually prepared and subjected to an immunoblot analysis with anti-phosphorylated PP2Ac (P-PP2Ac) and anti-PP2Ac (PP2Ac) antibodies. Results shown are representative of 3–5 independent experiments.

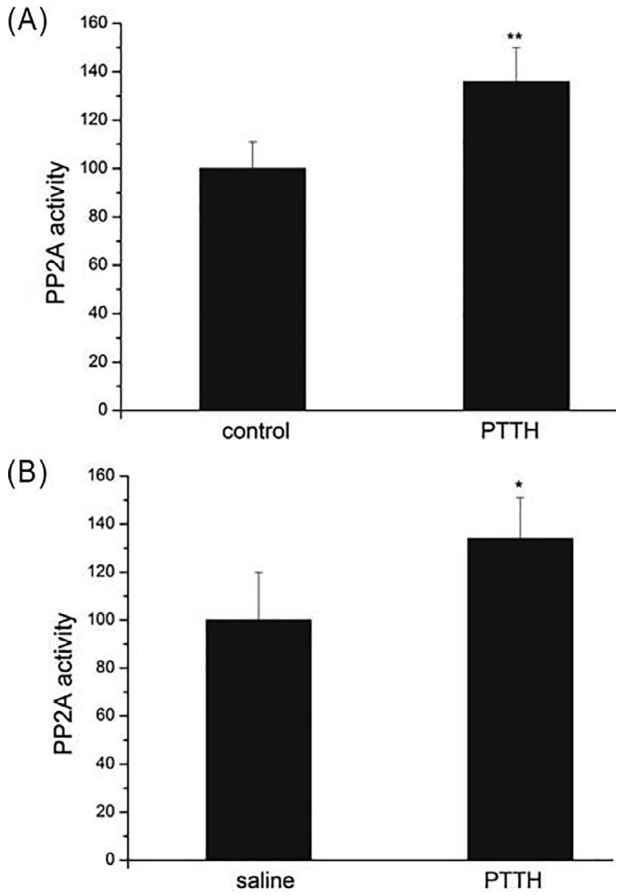

Fig. 8. Changes in PP2A enzymatic activity in PGs upon treatment with PTTH both *in vitro* (A) and *in vivo* (B). (A) Effect of *in vitro* PTTH treatment. PGs from day-6 last instar larvae were treated with PTTH (PTTH) or incubated with control medium (control) for 30 min. (B) Effect of *in vivo* PTTH injection. Day-6 last instar larvae were injected with saline containing PTTH (PTTH) or with saline containing diluted extracellular fluid from cells infected with WT AcMNPV (saline). Thirty minutes later, PGs were quickly dissected out and PP2A activity was examined and compared to that of control larvae. PP2A enzymatic activity was determined by the RediPlate™ 96 EnzChek® Serine/Threonine Phosphatase Assay kit and normalized to controls. Each bar represents the mean + SEM of three independent assays from multiple larvae. Asterisks indicate a significant difference compared to the control (by Student's *t*-test, $^{*}p < 0.05$; $^{**}p < 0.01$).

3.5. Effect of OA on PTTH-regulated PP2A signaling and ecdysteroid secretion

To examine whether PTTH-stimulated PP2A enzymatic activity is linked to ecdysteroid secretion, a specific PP2A inhibitor, OA, was used. PGs from day-6 last instar larvae were pretreated with OA, then treated with PTTH. As shown in Fig. 9A, pretreatment with OA prevented the stimulation of dephosphorylation of PP2Ac by PTTH. In addition,

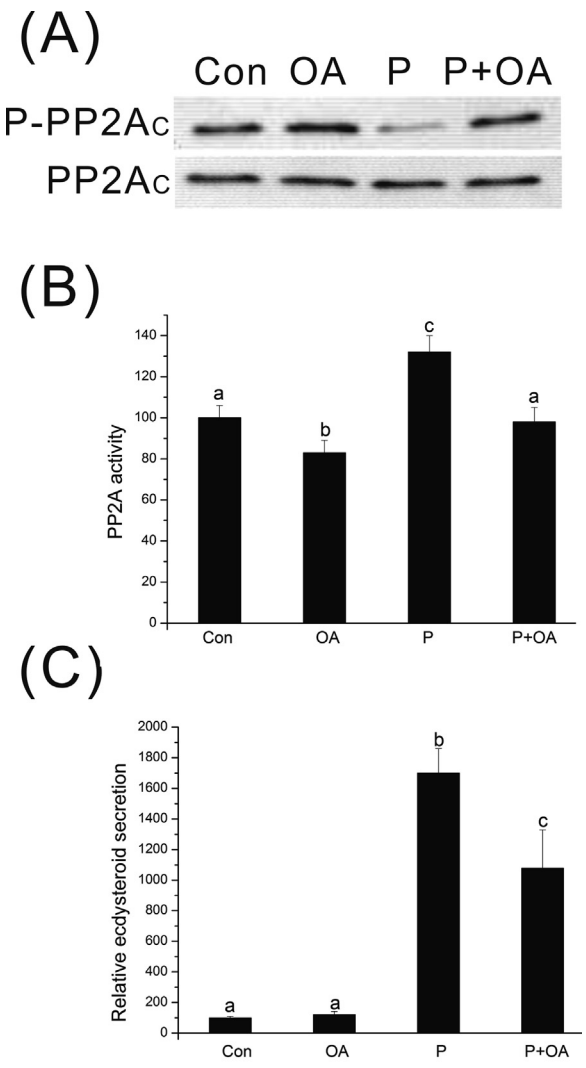

Fig. 9. Effects of OA on the PTTH-stimulated dephosphorylation of PP2Ac (A), PTTH-stimulated PP2A enzymatic activity (B) and ecdysteroid secretion (C). PGs from day-6 last instar larvae were pretreated with 100 nM OA or vehicle alone for 30 min, then transferred to medium containing the same dose of each inhibitor with or without PTTH. Con, PGs incubated in control medium; P, PGs incubated in medium containing PTTH only; OA, PGs incubated in medium containing OA only; P + OA, PGs incubated in medium containing both PTTH and OA. (A) Effects of OA on PTTH-stimulated tyrosine dephosphorylation of PP2Ac. Gland lysates were individually prepared and subjected to an immunoblot analysis with anti-phosphorylated PP2Ac (P-PP2Ac) and anti-PP2Ac (PP2Ac) antibodies. Results shown are representative of 3–5 independent experiments. (B) Effects of OA on PP2A enzymatic activity. Each bar represents the mean + SEM of three independent assays. (C) Effects of OA on ecdysteroid secretion. Incubation was maintained for 30 min for PP2A enzyme activity, tyrosine dephosphorylation of PP2Ac, and ecdysteroid secretion for 60 min. Ecdysteroids released into the medium during the 1 h incubation were quantified and normalized to the controls. Each bar for ecdysteroid determination represents the mean + SEM of four independent assays. Different letters above the bars indicate a significant difference (ANOVA followed by a Tukey's multiple comparisons test, $p < 0.05$).

PTTH-stimulated PP2A enzymatic activity was also prevented by pretreatment with OA (Fig. 9B). These results clearly indicated that the stimulation of dephosphorylation of PP2Ac by PTTH might be, at least in part, responsible for PTTH-stimulated PP2A activity. Moreover, when ecdysteroid secretion was examined in PGs treated with both OA and PTTH, it was found that OA treatment partly inhibited PTTH-stimulated ecdysteroid secretion (Fig. 9C), thus clearly confirming the

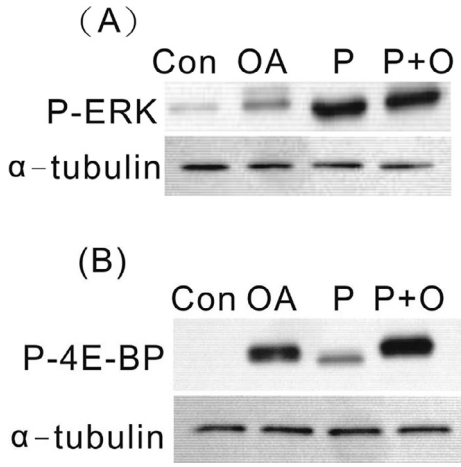

Fig. 10. Effects of OA on the PTTH-stimulated phosphorylation of ERK (A) and 4E-BP (B). PGs from day-6 last instar larvae were pretreated with 100 nM OA or vehicle alone for 30 min, then transferred to medium containing the same dose of OA with or without PTTH, and incubated for 60 min. Con, PGs incubated in the control medium; P, PGs incubated in medium containing PTTH only; OA, PGs incubated in medium containing OA only; P + OA, PGs incubated in medium containing both PTTH and OA. Gland lysates were individually prepared and subjected to an immunoblot analysis with anti-phospho-ERK (P-ERK), anti-phospho-4E-BP1 (Thr37/46) (P-4E-BP), and anti-α-tubulin (α-tubulin) antibodies. Results shown are representative of 3–5 independent experiments.

involvement of PP2A activity in PTTH-stimulated ecdysteroidogenesis. In addition, treatment with OA inhibited basal PP2A enzymatic activity, but not basal ecdysteroid secretion.

3.6. Effect of OA on basal and PTTH-stimulated ERK and 4E-BP phosphorylation

To study the effects of OA on ERK and TOR signaling pathway, PGs were pre-incubated with OA, then challenged with or without PTTH. Fig. 10 showed that treatment with OA increased basal ERK and 4E-BP phosphorylation without affecting PTTH-stimulated ERK and 4E-BP phosphorylation.

4. Discussion

The present study provided the first evidence that PP2A is involved in PTTH-stimulated ecdysteroidogenesis in *B. mori* PGs. Our results showed that the protein levels of PP2A A, B, and C subunits are developmentally regulated in *B. mori* PGs during the last larval instar, with the relatively higher levels being detected during later stages. Our study further showed that although treatment with PTTH during the short incubation period did not affect the protein levels of each subunit, it did greatly stimulate the tyrosine dephosphorylation of PP2Ac, thus leading to increased PP2A enzymatic activity. Ecdysone did not affect the tyrosine dephosphorylation of PP2Ac, indicating the direct activation of PTTH on dephosphorylation of PP2Ac. Moreover, OA, a specific PP2A inhibitor, not only blocked the stimulation of dephosphorylation of PP2Ac by PTTH, inhibited PTTH-stimulated PP2A enzymatic activity, but partially attenuated PTTH-stimulated ecdysteroid secretion. These results clearly indicate that PP2A is an important signaling involved in PTTH-stimulated ecdysteroidogenesis in *B. mori* PGs (Fig. 11). Although OA- and calyculin A-sensitive PPs were previously reported in *M. sexta* PGs (Song and Gilbert, 1996), to our knowledge, this is the first study to clearly demonstrate that PP2A plays a partial role in regulating PTTH-stimulated ecdysteroidogenesis in an insect endocrine system.

PP2A is an essential serine/threonine phosphatase found in all eukaryotic organisms (Janssens and Goris, 2001; Janssens et al., 2005;

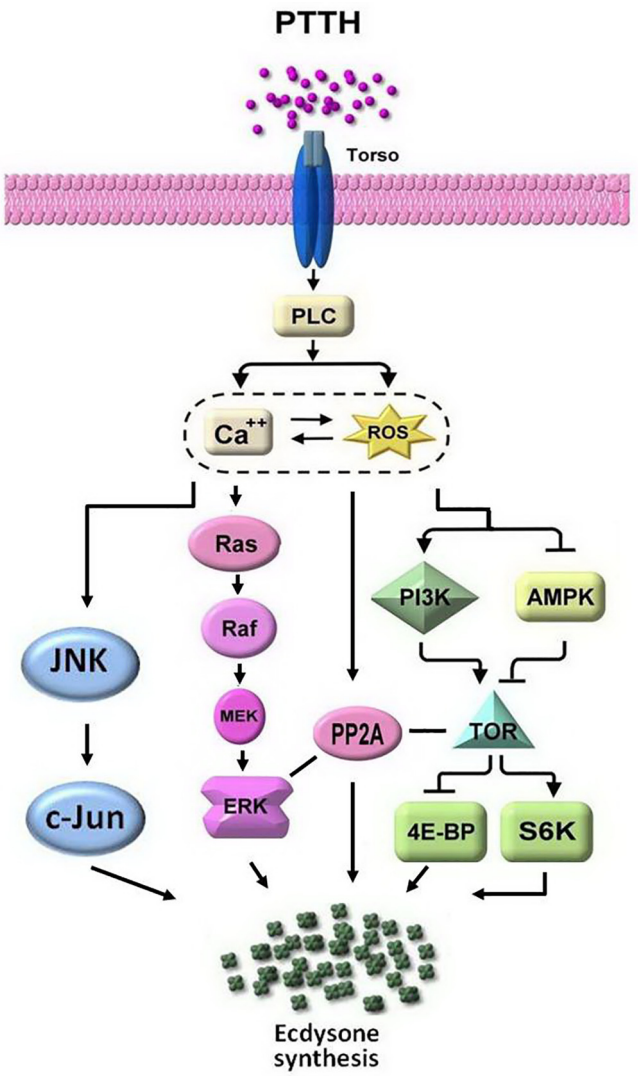

Fig. 11. Our current understanding on the signaling network involved in PTTH-stimulated ecdysteroidogenesis in PGs. See text for details.

Weber et al. 2015). It was documented that the tyrosine phosphorylation of PP2Ac is responsible for more than 90% of the phosphatase activity of PP2A and that this phosphatase is inactive when tyrosine 307 is phosphorylated (Chen et al., 1992; Guo and Damuni, 1993; Janssens and Goris, 2001; Seshacharyulu et al., 2013). The stimulation of tyrosine phosphorylation of PP2Ac and inhibition of PP2A enzymatic activity by OA was also previously reported (Chen et al., 1992; Cristóbal et al., 2014). In the present study, we showed that PTTH treatment stimulated dephosphorylation of PP2Ac and increased PP2A enzymatic activity, and that pretreatment with OA prevented the stimulation of PP2Ac dephosphorylation by PTTH and reduced PTTH-stimulated PP2A enzymatic activity. Moreover, we have demonstrated that PLC, Ca^{2+}, and ROS are upstream signaling for the PTTH-stimulated dephosphorylation of PP2Ac. In addition, in both *Manduca* and *Bombyx*, it has been demonstrated that Ras/ERK MAPK signaling cascade is involved in PTTH-stimulated ecdysteroidogenesis (Rybczynski et al., 2001; Lin and Gu, 2007). Our recent studies show that PI3K/AMPK/TOR signaling is another distinct signaling pathway involved in PTTH-stimulated ecdysteroidogenesis in *B. mori* PGs (Gu et al., 2011, 2012, 2013). In the present study, we found that pretreatment with either U0126 (an inhibitor of MEK), AICAR (an activator of AMPK), or LY294002 (an inhibitor of PI3K) did not prevent the PTTH-stimulated dephosphorylation of PP2Ac. These results clearly showed that ERK and

PI3K/AMPK are not upstream signaling pathways for PTTH-stimulated dephosphorylation of PP2Ac. In *Manduca*, it was reported that treatment with either OA or calyculin A stimulated the basal phosphorylation level of ribosomal protein S6, but inhibited PTTH-stimulated ecdysteroidogenesis without affecting the PTTH-stimulated phosphorylation of ribosomal protein S6 (Song and Gilbert, 1996). Similar results were also obtained in the present study. We found that treatment with OA greatly increased the basal phosphorylation levels of ERK and 4E-BP without affecting PTTH-stimulated ERK and 4E-BP phosphorylation. However, PTTH-stimulated ecdysteroidogenesis was significantly inhibited. These results indicate that PTTH-regulated ERK and AMPK/TOR signaling pathways are necessary but not sufficient for the stimulation of ecdysteroidogenesis. In addition, considering the stimulation of basal phosphorylation levels of both ERK and 4E-BP by OA, we hypothesize that PTTH-regulated PP2A signaling may provide possible link between ERK and AMPK/TOR, two distinct signaling pathways downstream of torso activation (Fig. 11).

PP2A is a ubiquitously expressed protein phosphatase that makes up ~ 1% of all cellular proteins and plays an important role in the regulation of cell growth, metabolism, and a diverse set of cellular proteins, including metabolic enzymes, ion channels, hormone receptors, and various kinase cascades (Janssens and Goris, 2001; Weber et al., 2015). Although the present study clearly demonstrated the partial contribution of PP2A to PTTH-stimulated ecdysteroidogenesis in *B. mori* PGs, the exact PP2A target is not clear at the present time. In mammalian systems, it has been well demonstrated that protein phosphorylation/dephosphorylation is a critical regulatory system of signal transduction that controls many aspects of cellular functions, and that PP2A can modulate the activities of several kinases, particularly phosphorylase kinase, MAPK/ERK, PKA, protein kinase B (PKB), PKC, and p70 S6 kinase (Janssens and Goris, 2001). PP2A has both positive and negative effects on these signaling pathways, depending on the cell type (Lechward et al., 2001; Seshacharyulu et al., 2013). In steroidogenic tissues of mammals, it has been documented that phosphorylation/dephosphorylation-dependent signaling pathways are required for the stimulation of steroid biosynthesis through the activation of protein kinases and PP. Inhibition of PP1 and PP2A activities appears to block cyclic AMP-induced steroid production primarily through preventing the increased expression of the steroidogenic acute regulatory protein (Burns et al., 2000; Jones et al., 2000; Paz et al., 2016). It was reported that the intracellular transport of cholesterol to important cellular processing sites is defective upon treatment with OA, thus resulting in inhibition of steroidogenesis (Azhar et al., 1994). In *Manduca* PGs, a phosphoproteomic study showed that PTTH not only stimulates the phosphorylations of several key pathway kinases, but influences the phosphorylation levels of many proteins involved in endosomal trafficking, constituents of the cytoskeleton, and regulators of transcription and translation, as well as Spook (a possible rate limiting enzyme in the ecdysone synthetic pathway) (Rewitz et al., 2009a). We hypothesize that precisely regulated PP2A may play roles in keeping the balance of phosphorylation and dephosphorylation of several key components involved in PTTH-stimulated ecdysteroidogenesis and that pretreatment with PP2A inhibitor OA may interfere with this cycle of events by inhibiting the latter process. Further studies are needed to clarify the downstream targets of PTTH-regulated PP2A signaling. In addition, PTTH-stimulated tyrosine dephosphorylation of PP2Ac appears to be development-specific, with the highest stimulation being detected on day 7 of the last larval instar (Fig. 3B). This result is consistent with changes in PTTH-stimulated ERK phosphorylation demonstrated in the previous study (Lin and Gu, 2007).

In conclusion, we provide evidence that PP2A is partially involved in PTTH-stimulated ecdysteroidogenesis in *B. mori* PGs. Treatment with PTTH increased tyrosine dephosphorylation of PP2Ac, leading to increased PP2A enzymatic activity. Pretreatment with OA not only blocked the stimulation of dephosphorylation of PP2Ac by PTTH and inhibited PTTH-stimulated PP2A enzymatic activity, but also significantly attenuated PTTH-stimulated ecdysteroid secretion, indicating that PP2A is an another important signaling component involved in PTTH-stimulated ecdysteroidogenesis.

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