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Activation of cAMP-response element-binding protein is positively regulated by PKA and calcium-sensitive calcineurin and negatively by PKC in insect

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ABSTRACT

The cAMP response element binding protein, CREB, is a G protein-coupled receptor (GPCR) signal-activated transcription factor implicated in the control of many biological processes. In the current study, we constructed a cAMP response element (CRE)-driven luciferase assay system for GPCR characterization in insect cells. Our results indicated that Gs-coupled *Bombyx* adipokinetic hormone receptor (AKHR) and corazonin receptor could effectively initiate CRE-driven luciferase transcription, but forskolin, a reagent widely used to activate adenylyl cyclase in mammalian systems, failed to induce luciferase activity in insect cells co-transfected with a CRE-driven reporter construct upon agonist treatment. Further investigation revealed that the specific protein kinase C (PKC) inhibitors exhibited stimulatory effects on CRE-driven reporter transcription, and blockage of Ca^{2+} signals and inhibition of Ca^{2+}-dependent calcineurin resulted in a significant decrease in the luciferase activity. Taken together, these results suggest that PKC likely acts as a negative regulator to modulate CREB activation; in contrast, Ca^{2+} signals and Ca^{2+}-dependent calcineurin, in addition to PKA, essentially contribute to the positive regulation of CREB activity. This study presents evidence to elucidate the underlying molecular mechanism by which CREB activation is regulated in insects.

1. Introduction

The cyclic AMP-response element binding protein (CREB) functions as a stimulus-induced transcriptional activator in organisms ranging from *Aplysia* to *Drosophila* to humans (Frank and Greenberg, 1994) and plays a critical role in a variety of cellular processes, including proliferation, differentiation, and adaptive responses (Shaywitz and Greenberg, 1999). CREB family members are also believed to be important for learning and memory (Yin et al., 1995) and for the hormonal regulation of gluconeogenesis (Hanson and Reshef, 1997).

CREB belongs to a family of transcription factors that includes CREB (Gonzalez et al., 1989; Hoeffler et al., 1988), cAMP-response element modulator (CREM) (Foulkes et al., 1991), and activating transcription factor 1 (ATF1) (Hai et al., 1989). The primary structure of CREB consists of two highly conserved regions, a basic region/leucine zipper (bZIP) dimerization domain, and a 60-residue kinase-inducible domain (KID) (Brindle et al., 1993; de Groot et al., 1993). Within KID, a critical residue, serine 133 (Ser-133), has been identified to be specifically phosphorylated by cAMP-dependent protein kinase A (PKA). Phosphorylation at Ser-133 facilitates the binding of CREB to CREB-binding protein (CBP), which is followed by the promotion of transcription via the recruitment of RNA polymerase II complexes to CRE-containing promoters (Gonzalez and Montminy, 1989; Gonzalez et al., 1989; Montminy and Bilezikjian, 1987; Seamon et al., 1981). In addition to PKA, calcium/calmodulin-dependent kinases (CaMKs) (Sheng et al., 1990; Sun et al., 1994), mitogen- and stress-activated kinase MSK1 (Wiggin et al., 2002), protein kinase C (PKC) (Xie and Rothstein, 1995), the CREB kinase RSK-2 (Ginty et al., 1994), and the stress-activated kinase P38 (Tan et al., 1996) have also been shown to phosphorylate CREB. A recent study revealed that, in flies, phosphorylation of dCREB2 at Ser-231 and casein kinase (CK)-targeted serine residues prevent DNA binding and that dCREB2 activation requires a dephosphorylation step at both Ser-231 and the CK sites, followed

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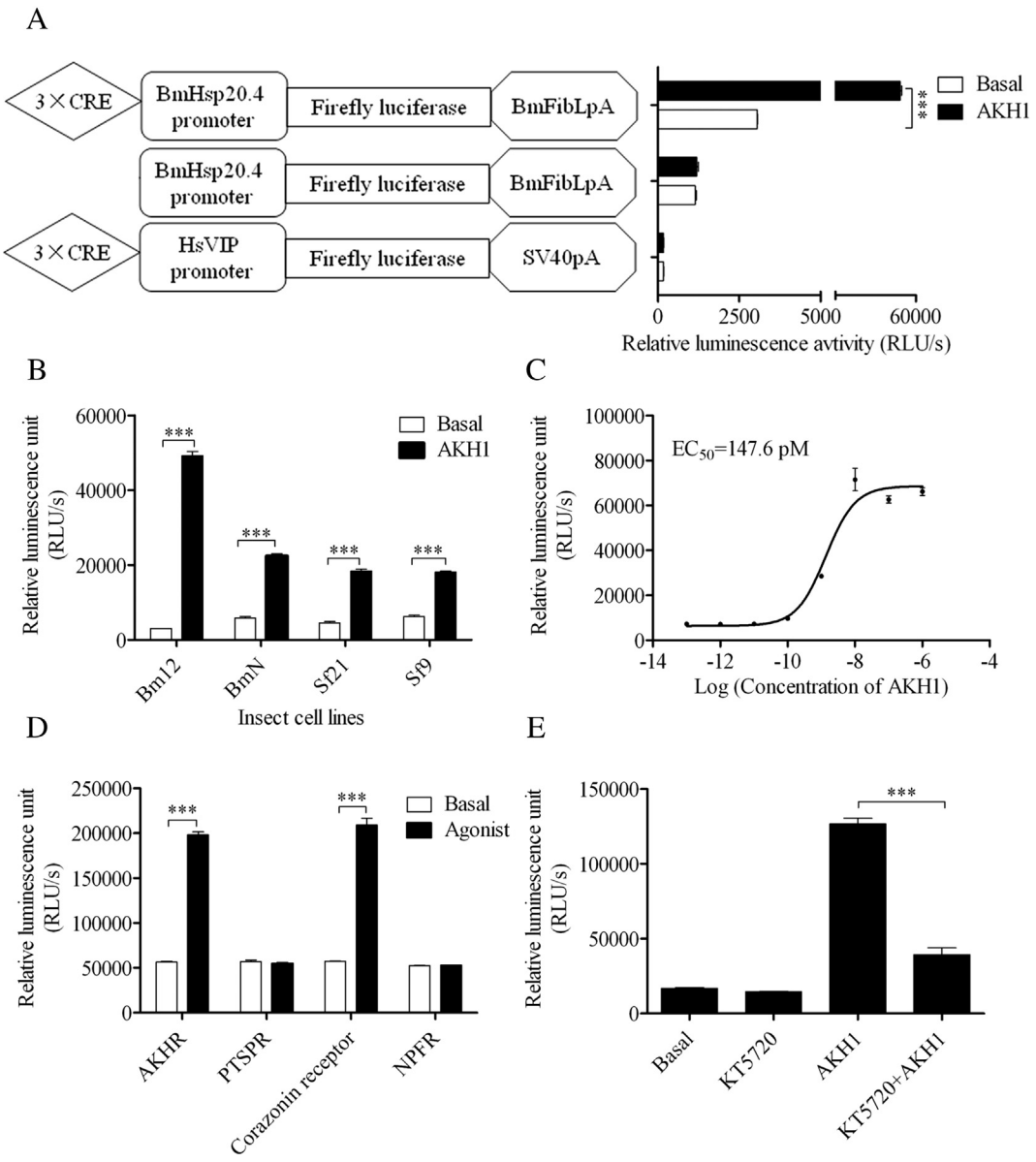

Fig. 1. CRE-driven luciferase expression induced by the Gs-coupled receptor. (A) The structures of the reporter plasmids with or without CRE are shown. Abbreviations: 3 × CRE, three tandem CRE consensuses; BmHsp20.4, *Bombyx mori* heat shock protein 20.4; BmFibLp(A) *Bombyx mori* fibroin protein light chain polyadenylation signal; HsVIP, *Homo sapiens* vasointestinal peptide; SV40p(A), SV40 polyadenylation signal. These constructs were cloned into the pBluescript KS (+) vector. The luciferase activity in Bm12 cells transiently co-transfected with pBmFlag-AKHR and pBmCRE-Luc was determined in response to treatment with 100 nM AKH1 (A), different doses of AKH1 (C), and 1 nM AKH1 with or without 1 h pre-treatment of PKA inhibitor (KT5720, 2 μM) (E). The construct without CRE was used as a control. (B) CRE-driven luciferase expression induced by 1 nM AKH1 in Bm12, BmN, Sf21, and Sf9 cell lines. (D) AKH1 (1 nM), DmSP (1 μM), corazonin (100 pM), and NPF1 (1 μM) induced CRE-driven luciferase activity in Bm12 cells transiently transfected with the pBmFlag vector containing AKHR (adipokinetic hormone receptor), PTSPR (prothoracicostatic peptide receptor), corazonin receptor, and NPFR (neuropeptide F receptor), respectively.

by at least one specific phosphorylation step at Ser-231 (Horiuchi et al., 2004).

In this study, we aimed to construct a CRE-driven luciferase system for assaying GPCR-mediated cAMP in an insect cell system. To our surprise, although *Bombyx* AKHR could initiate CRE-driven luciferase transcription, forskolin had no stimulatory effect in this system. Thus, we further investigated the possible involvement of signaling pathways other than cAMP/PKA in CREB phosphorylation and found that Ca^{2+} influx and calcineurin, a calcium/calmodulin-dependent phosphatase (also known as PP2B), are required for the activation of CREB-induced gene transcription. This result suggests that a novel regulatory mechanism, distinctive from that in mammals, for the regulation of CREB-involved gene transcription might exist in insects.

2. Material and methods

2.1. Material

Bombyx mori adipokinetic hormone 1 (AKH1), corazonin, Neuropeptide F1 (NPF1), and *Drosophila melanogaster* sex peptide (DmSP) were synthesized by Gel Company, Shanghai, China. Forskolin, KT5720, Thapsigargin, SKF-96365, 8-CPT and FK506 were purchased from Sigma–Aldrich (St. Louis, USA). U0126, phorbol 12-myristate 13-acetate (PMA), and BAPTA-AM were obtained from TOCRIS, UK. Chelerythrine chloride (CC) was purchased from Merck, Germany. The reagents for PCR were obtained from Invitrogen, USA. Restriction enzymes and DNA ligase were purchased

from TAKARA (Dalian, China). EGTA and cyclosporine A (CsA) were purchased from Beyotime, China.

2.2. Plasmid construction and molecular cloning

The immediate-early gene promoter (IE1) and homologous region 3 (Hr3) of *Bombyx mori* nucleopolyhedrovirus (BmNPV) and promoter of BmAcitnA3 were used to replace the corresponding sites of pFlag-CMV-3 (Chen et al., 2004). The resulted vector was designated as pBmIE1-Flag (with the Flag tag upstream of the multiple clone site, also labeled as pBmFlag) (Fig. S1). BmAKHR (accession number: NM_001043584), BmPTSPR (*Bombyx* prothoracicostatic peptide receptor, accession number: NM_001114874), *Bombyx* corazonin receptor (accession number: AB330442), and NPF receptor (NPFR, BNGR-A4, accession number: AB330424) were inserted into the multiple clone site to generate pBmFlag-AKHR, pBmFlag-PTSPR, pBmFlag-corazonin receptor, and pBmFlag-NPFR, respectively. pBmCRE-Luc was generated using the promoter of BmHsp20.4 (*B. mori* heat shock protein 20.4, accession number: EU350577) and the BmFibL (*B. mori* fibroin light chain, accession number: NM_001044023) polyadenylation signal (Xie et al., 2009), which were amplified from silkworm (P50) genomic DNA by PCR; these sequences were used to replace pVIP (promoter of vasointestinal peptide) and the SV40 polyadenylation signal of pCRE-Luc (Li et al., 2010), respectively (Fig. 1A and Fig. S2). All constructs were sequenced to verify the correct sequence and orientation.

2.3. Cell culture and transfection

The Bm12 cell line and Sf9 cell line were kindly provided by Dr. Chuanxi Zhang (Zhejiang University) and Dr. Sheng Li (Chinese academy of Sciences), and the BmN and Sf21 cell lines were provided by Dr. Zhifang Zhang. The insect cell lines were cultured in TC100 insect medium (Applichem, Darmstadt, Germany) with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, China) and incubated at 28 °C. The HEK293 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Transfection was performed using X-tremeGENE HP (Roche, Mannheim, Germany) following the manufacturer's instructions.

2.4. Luciferase activity assay

Transfected Bm12 or HEK293 cells were seeded in 96-well plates overnight and treated with specific concentrations of stimuli in TC100 basic medium (without FBS) for 4 h at 28 °C. Luciferase activity was detected using a firefly luciferase kit (Promega, Madison, WI). For the cAMP concentration, Bm12 cells over-expressing AKHR were stimulated with AKH1 (1 μM) and forskolin (10 μM) for 10 min; the cell lysis and ELISA assays were performed following the instructions of the Cyclic AMP Assay kit (RD, USA).

2.5. Intracellular calcium measurement

Calcium mobilization was monitored using a TECAN infinite F200PRO (Germany). The Bm12 cells transfected with pBmFlag-AKHR were suspended using a pipette, washed twice with HBM buffer (pH 6.2) (Kukkonen et al., 1996) containing 5 mM $CaCl_2$ and 0.01% bovine serum albumin, and resuspended in HBM. The cells were then loaded with 3 μM fura-2 acetoxymethyl ester derivative (Fura-2/AM) (Dojindo, Japan) for 30 min at 28 °C. The cells were washed once in HBM, resuspended in HBM, incubated at 28 °C for 15 min, washed twice in HBM, and then resuspended in HBM at a concentration of 1×10^6 cells/ml. The cells were separated into 96-well black plates (Corning, USA) at 1×10^5 cells/well; these cells were stimulated with the indicated concentration of ligand. Calcium flux was indicated by the ratio (340/380) of OD_{510} excited at 340 and 380 nm, respectively. Cells transfected with the empty vector were stimulated in parallel as a negative control.

2.6. Western blot analysis

For examining the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), transfected cells were plated in a 24-well plate and incubated overnight. After 1 h pre-treatment with or without 1 μM U0126 (TOCRIS, UK), the cells were incubated for 10 min with the indicated concentration of AKH1. The cells were then lysed with RAPI buffer (Beyotime, China) containing of one tablet of complete protease inhibitor (Roche Applied Science) per 100 ml at 4 °C on a rocker for 30 min and then scraped. The proteins were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to a PVDF membrane (Millipore, Billerica, MA), and immunoblotted using rabbit monoclonal antiphospho-ERK1/2 antibodies (1:1000) (Cell Signaling technology, Danvers, MA). The membranes were next probed with horseradish peroxidase (HRP)-labeled secondary antibodies, and chemiluminescent detection was performed using horseradish peroxidase-substrate (Cell Signaling technology, Danvers, MA). For the phospho-PKC substrate assay, the cells were treated as indicated and harvested as above. Immunoblotting was performed using rabbit polyclonal antibody (1:3000) against phospho-PKC substrate (Cell Signaling technology, Danvers, MA). The membrane processing was the same as for the ERK assay. The blots were stripped and reprobed using a polyclonal anti-beta-Actin (1:5000) (Huabio, China) as a control for protein loading.

2.7. Data analysis

The data were analyzed using the software Prism (GraphPad Software, San Diego, CA), and all data are expressed as the mean ± SEM (Standard error of mean). The results were analyzed using a one-way analysis of variance (ANOVA), followed by a statistical significance determination using Student's *t*-test in which a probability value (*P* value) less than 0.05 was considered significant and less than 0.01 was considered very significant ($^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$). The EC_{50} values were obtained using nonlinear curve fitting. All pictures and data shown are representative of at least three independent experiments.

3. Results

3.1. Gs-coupled AKHR can induce CRE-driven reporter transcription in insect cells

The CRE-driven reporter-gene assay has been widely used to pharmacologically characterize GPCRs and ligands in mammalian cell systems. To establish a CRE-driven reporter-gene system to evaluate GPCR signaling in insect cells, we constructed an assay system consisting of the firefly luciferase coding region under the control of a minimal BmHsp20.4 promoter containing three CREs. Bm12 cells were transiently co-transfected with the reporter construct and BmAKHR and incubated for 48 h, followed by stimulation with AKH1 for 4 h. As shown in Fig. 1A, agonist stimulation induced a significant increase in the luciferase signal in the insect cells transfected with pBmCRE-Luc, whereas the control cells transfected with a construct without 3 × CREs or with the HsVIP promoter failed to trigger luciferase transcription in response to AKH1 treatment (Fig. 1A and B). AKH1 exhibited stimulatory effects on CRE-driven luciferase transcription in a dose-dependent

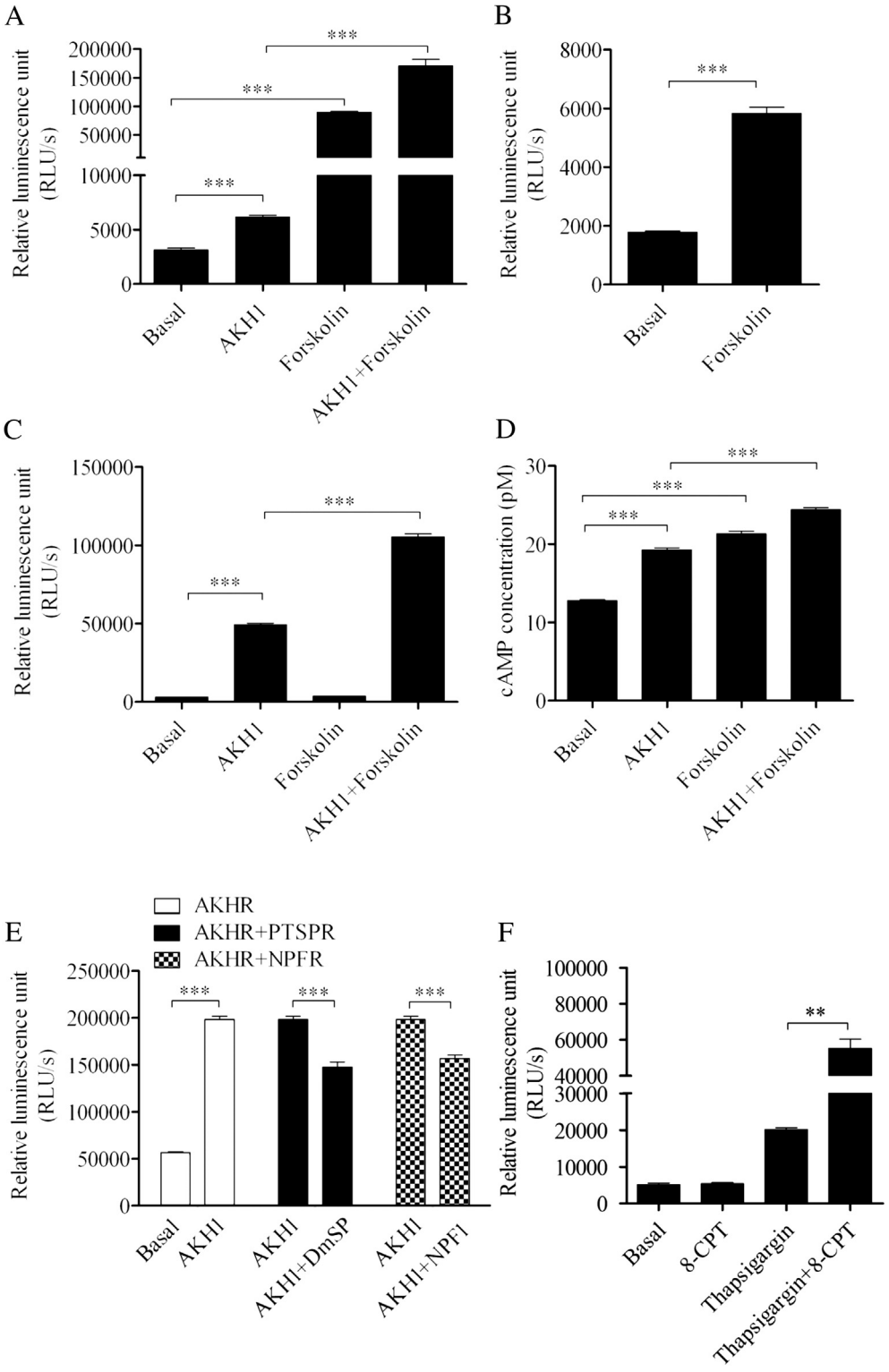

Fig. 2. Effects of forskolin on CRE-driven luciferase expression in HEK293 and Bm12 cells. The luciferase activities in HEK293 cells (A) and Bm12 cells (C) transiently co-transfected with AKHR and CRE-Luc (pCRE-Luc and pBmCRE-Luc, respectively) were determined in response to AKH (100 nM), forskolin (10 μM), or both. (B) Forskolin (10 μM) induced luciferase activity in HEK293 cells transiently transfected with the pBmCRE-Luc. (D) The cAMP concentration stimulated by AKH1 (1 μM), forskolin (10 μM), or both in Bm12 cells transiently transfected with pBmFlag-AKHR was assessed using a commercially available ELISA kit (RD, USA). (E) Effects of DmSP (1 μM) and NPF1 (1 μM) on CRE-driven luciferase expression induced by AKH1 (1 nM) in Bm12 cells transiently co-transfected with pBmFlag-AKHR and pBmFlag-PTSPR or pBmFlag-NPFR, respectively. (F) Effects of cAMP mimic (8-CPT, 30 μM) on CRE-driven luciferase expression in Bm12 cells transfected with pBmCRE-Luc. The concentrations of Thapsigargin was 100 nM.

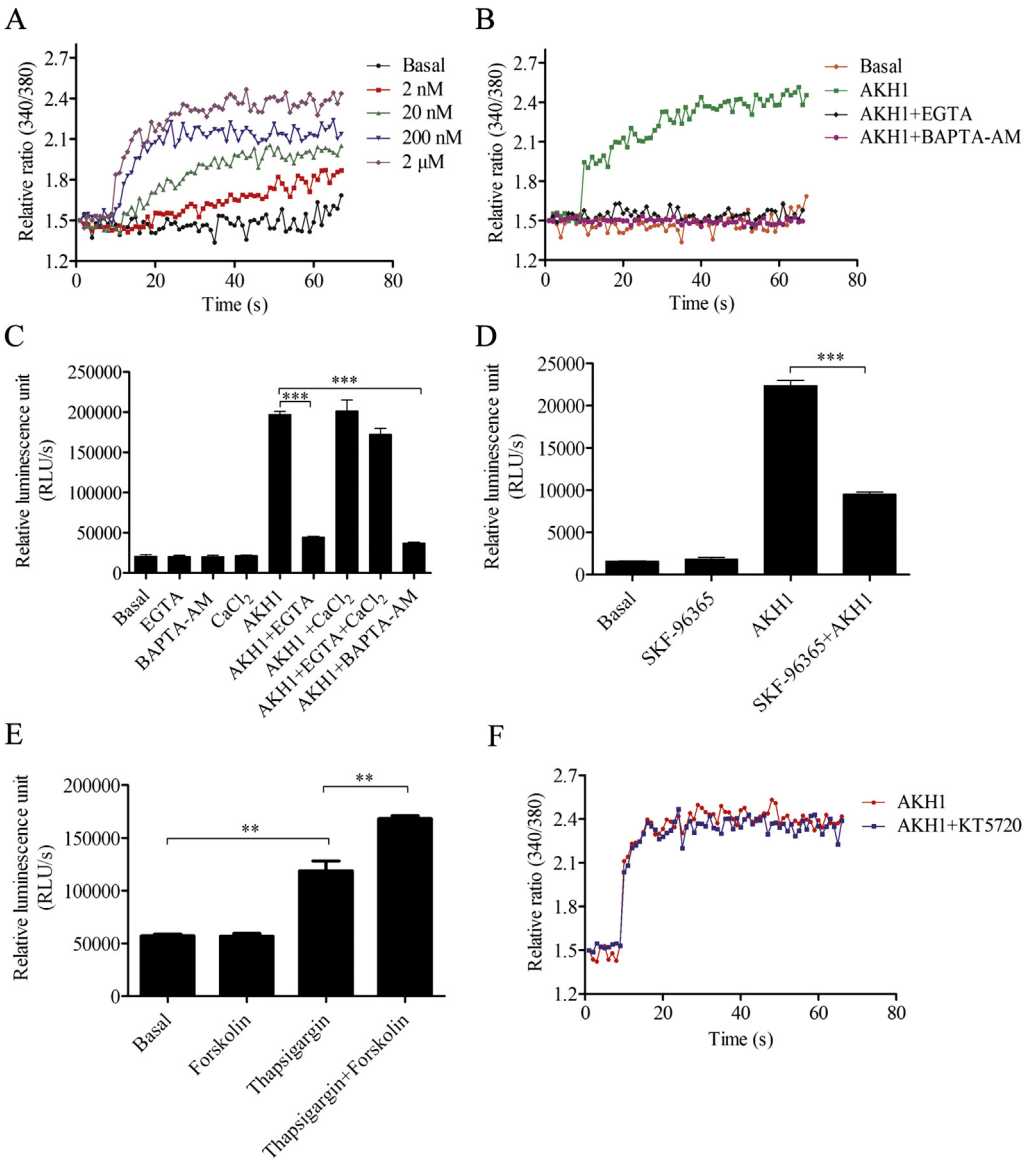

Fig. 3. Calcium mobilization and its effects on CRE-driven luciferase expression. Calcium mobilization in Bm12 cells transiently transfected with pBmFlag-AKHR was detected in response to the indicated concentration of AKH1 (A) and AKH1 (2 μM) with or without calcium chelators (EGTA [5 mM] and BAPTA-AM [40 μM]) (B) and PKA specific inhibitor (KT5720 [2 μM]) (F). A 30-min pre-treatment of BAPTA-AM was necessary, whereas EGTA was added at the beginning of detection. Effects of EGTA (5 mM), $CaCl_2$ (5 mM), BAPTA-AM (40 μM) (C), SKF96365 (50 μM) (D) on the luciferase activity induced by AKH1 (1 nM) in Bm12 cells transiently co-transfected with pBmFlag-AKHR and pBmCRE-Luc. A 1-h pre-treatment was necessary for SKF-96365. (E) Luciferase activity induced by thapsigargin (100 nM), forskolin (10 μM), or both in Bm12 cells transiently transfected with the pBmCRE-Luc.

manner, with EC_{50} values of 147.6 pM in Bm12 cells (Fig. 1C). Our data revealed that, in addition to AKHR, Gs- and Gq-dually coupled *Bombyx* corazonin receptor also could stimulate CRE-driven gene transcription. In contrast, Gi-coupled BmPTSPR and NPFR were unable to induce luciferase activity upon agonist treatment (Fig. 1D). The stimulation of CRE-driven luciferase transcription was inhibited by the pretreatment of 2 μM of the PKA-specific inhibitor KT5720 (Fig. 1E). Collectively, these data suggest that Gs-coupled receptors induce the activation of CREB via the cAMP/PKA signaling pathway in insect cells.

3.2. Forskolin is not sufficient to induce CRE-driven gene transcription in insect cells

Forskolin, a diterpene isolated from the roots of the Indian plant *Coleus forskohlii*, activates adenylate cyclase and is commonly used to raise the intracellular levels of cAMP for the evaluation of Gi-coupled GPCR activity in mammalian systems. In HEK293 cells transiently expressing AKHR, both forskolin and AKH1 showed stimulatory effects on CRE-driven reporter transcription (Fig. 2A and B). However, unexpectedly, AKH1, but not forskolin, could induce a significant increase in CRE-driven luciferase activity in insect Bm12 cells (Fig. 2C). To confirm whether forskolin can activate adenylate cyclase and trigger intracellular cAMP production in insect cells, cAMP was directly assayed by ELISA. As shown in Fig. 2D, both AKH1 (1 μM) and forskolin (10 μM) effectively triggered intracellular cAMP accumulation in Bm12 cells. Moreover, in the Bm12 cells co-expressing AKHR and Gi-coupled PTSPR or NPFR, treatment with *Bombyx* PTSP or *Bombyx* NPF peptides was found to induce a concentration-dependent inhibition of AKH1-stimulated luciferase activity (Fig. 2E). In addition, treatment of 8-CPT, a potent cAMP mimic, led to no increase in luciferase activity, but

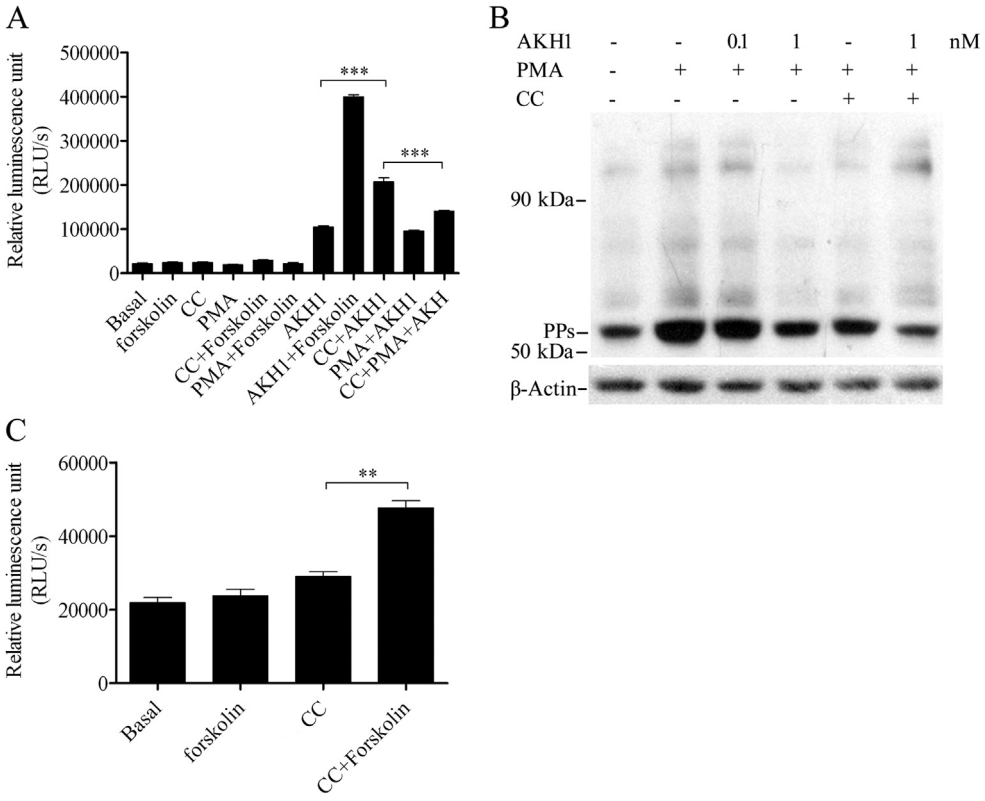

Fig. 4. Roles of PKC on CRE-driven luciferase expression. (A) The effects of PKC inhibitor (chelerythrine chloride [CC], 5 μM) and agonist (phorbol 12-myristate 13-acetate [PMA], 1 μM) on the luciferase activity induced by AKH1 (1 nM) and forskolin (10 μM) in Bm12 cells transiently co-transfected with pBmFlag-AKHR and pBmCRE-Luc. Pre-treatment with CC 20 min prior to incubation with PMA, AKH1, and forskolin was necessary. (B) PPs (phosphor-PKC substrate) induced by AKH1 (indicated concentration), PMA (1 μM), and CC (5 μM) in Bm12 cells transiently transfected with pBmFlag-AKHR was detected using a commercially available antibody (Cell Signaling technology, Danvers, MA). The concentration of AKH1 is labeled at the top, and the protein molecular weight marker is listed on the left. β-actin was probed as a control for protein loading. Pre-treatment with CC was performed for 20 min prior to a 15 min-incubation with AKH1 and PMA. (C) Luciferase activity induced by forskolin (10 μM) after an overnight pre-treatment with CC (5 μM).

when in the presence of thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA), 8-CPT stimulated a significant increase in CRE-driven luciferase transcription (Fig. 2F). Taken together, our results indicate that the cAMP-dependent PKA signaling pathway is insufficient to activate CREB in insect cells.

3.3. Calcium signaling is required for GPCR-mediated CRE-driven luciferase transcription

AKHR has been demonstrated to trigger a significant Ca^{2+} mobilization in insect cells in response to AKH (Gade and Auerswald, 2003). Accordingly, we next evaluated the role of intracellular Ca^{2+} mobilization in the regulation of CREB activation. In Bm12 cells transiently expressing AKHR, AKH1 treatment induced a rapid increase of intracellular Ca^{2+} in a dose-dependent manner, and the induction of Ca^{2+} mobilization was completely blocked by the extracellular calcium chelator EGTA and intracellular calcium chelator BAPTA-AM (Fig. 3A and B). Indeed, both EGTA and BAPTA-AM exhibited strong inhibitory effects on AKHR-mediated CRE-driven luciferase activity, and this inhibition could be attenuated by the addition of $CaCl_2$ (Fig. 3C). Furthermore, our data demonstrated that pretreatment with SKF-96365, an inhibitor of voltage-gated calcium channels, resulted in a significant suppression of AKH-induced luciferase activity (Fig. 3D). In addition, a transient endoplasmic reticulum Ca^{2+} release induced by thapsigargin led to an increase in CRE-driven luciferase transcription, and together with forskolin, thapsigargin exhibited a synergistic effect on luciferase activity (Fig. 3E). To assess the role of PKA in Ca^{2+} mobilization, our data indicated that treatment of KT5720, an inhibitor of PKA, resulted in no change in calcium flux induced by AKH1 (Fig. 3F). Thus, it is most likely that, in addition to PKA, a Ca^{2+} signal, from both intracellular and extracellular Ca^{2+} mobilization, is essentially required for the activation of CREB in insect cells.

3.4. PKC appears to constitutively and negatively regulate CREB activation

It is well established that GPCRs can activate Ca^{2+}-dependent protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) via the PLC/Ca^{2+} signaling pathway. Previous data has demonstrated that Ca^{2+} has been shown to act as a signal for CREB activation via a PKC-dependent pathway in vertebrates (Guo and Feng, 2012; Muthusamy and Leiden, 1998; West et al., 2001). Therefore, we investigated whether PKC is involved in CREB activation in insect cells. As shown in Fig. 4A, pretreatment with CC, a potent PKC inhibitor, resulted in a significant enhancement of AKHR-induced CRE-driven luciferase transcription. In contrast, PMA, an activator of PKC, had no stimulatory effect on the CRE-driven reporter signal but did counteract the CC-caused enhancement of the luciferase signal in Bm12 cells expressing AKHR (Fig. 4A). A western blot analysis demonstrated that PMA exhibited a stimulatory effect on PKC activity, whereas AKH1, with or without CC, appeared to inhibit the PKC-induced phosphorylation of its substrates via the AKHR signaling pathway (Fig. 4B). Furthermore, the overnight treatment of cells with the PKC inhibitor CC stimulated CRE-driven luciferase transcription in the

presence of forskolin (Fig. 4C). These results suggested the possible role of PKC in the negative regulation of CREB activation.

3.5. Calcium-dependent calcineurin is required for CREB activation

Mitogen-activated protein kinase (MAPK) has been well documented to be coupled to CREB phosphorylation (Roberson et al., 1999; Sgambato et al., 1998; Xing et al., 1996). We, therefore, investigated the role of the ERK1/2 signaling pathway in AKHR-induced CREB activation. AKHR-induced ERK1/2 phosphorylation was significantly inhibited by MEK inhibitor U0126 treatment for 1 h, but the CRE-driven luciferase signal was unchanged by a 5-hour incubation of the cells with U0126 (Fig. 5A and B). These results suggested that the ERK1/2 pathway is not involved in the AKHR-mediated activation of CREB. Moreover, to further assess the role of calcineurin (PP2B) in the regulation of CREB activation, two inhibitors selective for PP2B, FK506 and cyclosporine A (CsA), were used in this study. As shown in Fig. 5C, the application of either inhibitor resulted in complete inhibition of AKH1 (1 nM)- or thapsigargin (100 nM)-mediated CRE-driven luciferase transcription. These results strongly suggest that calcium-dependent calcineurin is likely involved in AKHR-induced CREB activation.

4. Discussion

Intracellular cAMP accumulation results in CREB activation and CRE-related gene transcription through cAMP-dependent PKA upon activation of Gs-coupled GPCRs. The CRE-driven reporter assay has been widely used in academia and industry to detect GPCR signaling pathways and to pharmacologically characterize ligands and receptors in mammalian cell systems. Forskolin is a potent stimulator of adenylyl cyclase activity and is widely used as a tool to stimulate intracellular cAMP formation for determining Gi-coupled receptor activation. In the present study, we constructed a CRE-driven luciferase system using the BmHsp20.4 promoter and BmFibL polyadenylation signal to assay for GPCR-mediated cAMP production in insect cells. Our current data clearly showed that neuropeptide AKH could induce a significant increase in luciferase activity in both Bm12 and sf21 cells co-transfected with the AKHR and CRE-driven luciferase construct. Conversely, forskolin treatment led to no significant change in luciferase activity, even though intracellular cAMP formation was detectable in response to forskolin stimulation by a cAMP ELISA kit. Previous study indicated that the treatment of lepidopteran Bm5 cells with forskolin also resulted in the accumulation of cAMP. However, CRE-linked reporter constructs are not activated by forskolin in lepidopteran or dipteran cell lines (Douris et al., 2006; Poels et al., 2004). A previous in vivo study also demonstrated that the forskolin-dependent activation of PKA is not an important pathway for CREB phosphorylation in Schwann cells (Rahmatullah et al., 1998). Taken together, these results suggest that other signaling pathways, including Ca^{2+}/PKC and ERK1/2, may also be critically involved in CREB phosphorylation and activation.

Accumulating evidence has demonstrated that AKHR triggers a significant increase in both intracellular cAMP and Ca^{2+} in response to AKH (Gade and Auerswald, 2003). To evaluate the role of the Ca^{2+} signaling pathway in the regulation of CREB activation, we next investigated whether intracellular and extracellular Ca^{2+} are involved in the stimulation of CRE-driven luciferase transcription by AKH. AKH treatment elicited a rapid increase in intracellular Ca^{2+} and CRE-driven luciferase activity in a dose-dependent manner through AKHR. This effect was blocked by the calcium chelator EGTA, and the addition of $CaCl_2$ could rescue EGTA-inhibited luciferase activity. In addition, SKF-96365, an inhibitor of voltage-gated calcium channels, exhibited an inhibitory effect on AKH-stimulated luciferase activity. Additionally, thapsigargin, a tight-binding inhibitor of SERCA, was observed to be able to elicit a rapid increase in intracellular Ca^{2+} and induced a significant

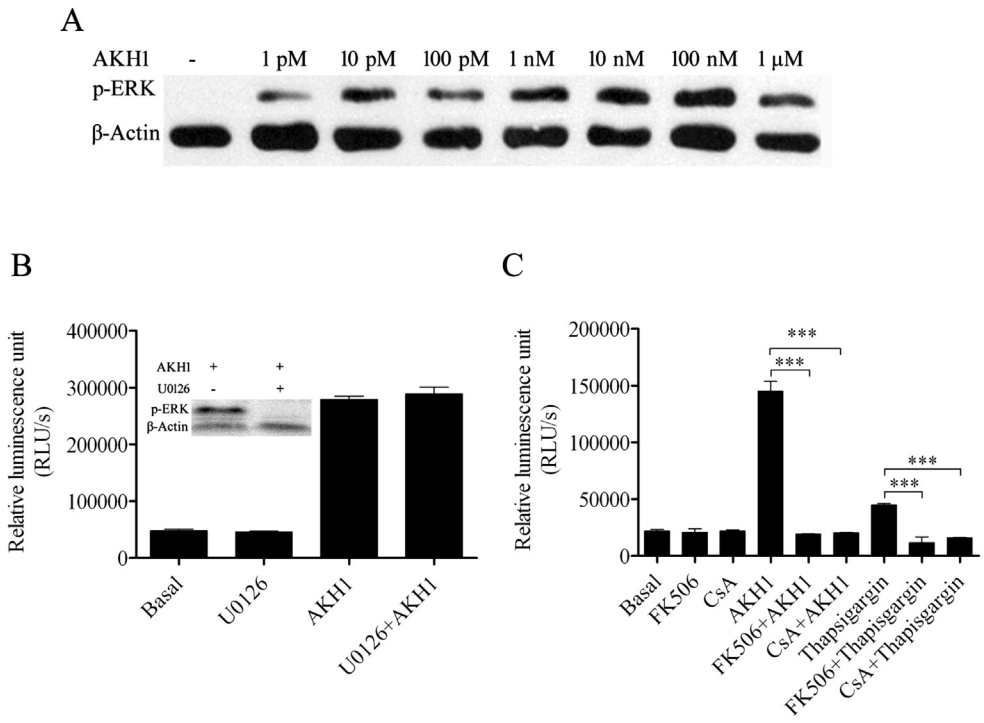

Fig. 5. Involvement of PP2B, but not ERK, in the activation of CREB. The phosphorylation of ERK in response to different doses of AKH1 (A) and ERK inhibitor (U0126, 1 μM) (inset of B) was detected by western blotting in Bm12 cells transiently transfected with pBmFlag-AKHR. Effect of ERK inhibitor (U0126, 1 μM) (B) and PP2B inhibitors (FK506, 10 μM and cyclosporine A [CsA], 10 μM) (C) on CRE-driven luciferase expression stimulated by AKH1 (1 nM) or thapsigargin (100 nM) in Bm12 cells transiently co-transfected with pBmFlag-AKHR and pBmCRE-Luc. A 1-h pre-treatment of U0126 (1 μM) and a 30-min pre-treatment of FK506 (or CsA) was performed.

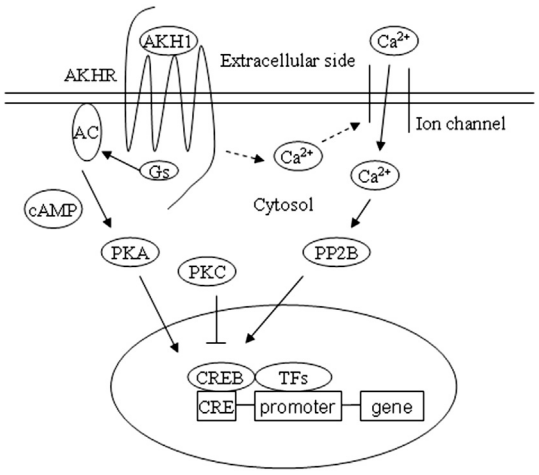

Fig. 6. Schematic diagram of the regulation of CREB activation in an insect system. PKC is shown to constitutively phosphorylate specific serine residues of CREB to generate an inactive state. The agonist-mediated activation of a Gs-coupled receptor signals to CREB via AC/cAMP/PKA and Ca^{2+}/PP2B signaling cascades. CREB then binds to CREs in target genes to initiate transcription via the recruitment of transcription factors with RNA polymerase II complexes. Gs, G protein of stimulation; AC, adenylate cyclase; PKA, protein kinase A; PKC, protein kinase C; PP2B, protein phosphatase 2B; CREB, cAMP-response element-binding protein; TFs, transcription factors.

increase in luciferase activity. Previous data revealed that AKH-bound AKHR caused an influx of Ca^{2+} via channels into the fat body of *Locusta migratoria* (Van Marrewijk et al., 1991; Vroemen et al., 1995), and the inhibitors of Ca^{2+} channels verapamil and nifedipine inhibited the adipokinetic effect of the endogenous AKH in *Manduca sexta* in vivo (Arrese et al., 1999). The influx of extracellular Ca^{2+} has been found to be essential for lipid release from the fat body of locusts (Lum and Chino, 1990; Zhiwei et al., 1990) and moths (Arrese et al., 1999) and also to play a critical role in hyperprolinemic signaling in vitro and in vivo (Auerswald and Gade, 2001a, b). In *Drosophila* Schneider 2 (S2) cells, CRE-driven reporter gene expression is poorly regulated by intracellular cAMP changes, and a Ca^{2+}-induced elevation of CRE-dependent luciferase activity was detected after stimulation with thapsigargin (Poels et al., 2004). Collectively, these data suggest that, distinct from vertebrates, CREB activation in insects is both cAMP- and Ca^{2+}-dependent.

It is well known that, in addition to PKA, GPCRs can activate Ca^{2+}-dependent PKC, Ca^{2+}/CaMKII, and ERK1/2. Treatment with high concentrations of autocamtide-2 related inhibitory peptide (AIP) was found to exert no suppressive effect on thapsigargin-mediated CRE-dependent reporter expression, suggesting that CaMKII is likely not involved in CREB activation (Poels et al., 2004). Although ERK1/2 has been confirmed to activate CREB in PC12 (Grewal et al., 2000) and CHO cells (Klinger et al., 2002), in the current study, pretreatment with U0126, a selective inhibitor of MEK1/2, resulted in a significant inhibition of AKH-mediated ERK1/2 phosphorylation but no change in CRE-linked luciferase activity. To our surprise, CC, a potent PKC inhibitor, showed a stimulatory effect on AKH-triggered luciferase activity via the inhibition of PKC activity. Moreover, the most commonly used phorbol ester PMA could activate PKC, but exhibited an inhibitory effect on the CC-mediated enhancement of luciferase activity. Our data are consistent with the observation that treatment with Ro-31-8220, an inhibitor of PKC and ribosomal S6 kinases, failed to result in a decrease in thapsigargin-induced luciferase expression (Poels et al., 2004). Recent data have revealed that serine residues 108, 111, 114, 117, and 121 of *Drosophila* dCREB2 are targets for casein kinases (CKs) and that the constitutive phosphorylation of these CK sites is likely to occur in vivo (Horiuchi et al., 2004). Thus, it is possible that the insect CREB activation is negatively regulated by constitutive phosphorylation and that PKC may be responsible for the constitutive phosphorylation of CREB.

The dCREB2 protein from *Drosophila* extracts has been shown to be unable to bind to CRE sites, unless treated with phosphatase (Horiuchi et al., 2004). In the present study, pretreatment with the PP2B inhibitors FK506 and CsA resulted in the complete suppression of AKHR and thapsigargin-induced CRE-dependent luciferase expression. In combination with the recent observation that the phosphorylated CREB was identified as an inactive form for binding to DNA in *Drosophila* (Horiuchi et al., 2004), our results provide evidence to suggest that PKC is more likely to constitutively phosphorylate CREB for negative regulation and that PP2B-mediated dephosphorylation in addition to the PKA-induced phosphorylation of Ser-231 positively regulates CREB activation in insects (Fig. 6). However, the residues thought to be the targets for PKC and PP2B have not been identified. It will be of great interest to elucidate the exact mechanism by which the transcriptional responses to cAMP and Ca^{2+} in insects are finely regulated.

Our current data have led us to conclude that, in insects, inactive CREB exists in a state in which certain serine and threonine residues are phosphorylated by PKC. Upon Gs-coupled receptor ligand binding, the intracellular cAMP level is elevated, followed by activation of PKA, leading to CREB phosphorylation at Ser-231; in addition, a significant Ca^{2+} mobilization, most likely from both intracellular and extracellular Ca^{2+} stores, is triggered, resulting in the activation of Ca^{2+}-dependent PP2B, followed by CREB dephosphorylation at certain serine residues. Active CREB then binds to a conserved CRE, TGACGTCA, on DNA and initiates the transcription of target genes by recruiting the co-activator CBP, together with RNA polymerase II complexes (Fig. 6). Our study provides convincing evidence for the different insect and vertebrate mechanisms to regulate CREB activation.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2013.08.011.

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