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Molecules and Cells

Podophyllotoxin Induces CREB Phosphorylation and CRE-Driven Gene Expression via PKA but Not MAPKs

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CRE-driven luciferase reporter is commonly used in drug screening systems involving G protein-coupled receptors (GPCRs). In a screen campaign designed to search for melanocortin-4 receptor (MC4R) agonists, podophyllotoxin, a microtubules disruptor, was found to induce cAMP-responsive element (CRE)-driven reporter expression. MC4R was not involved because podophyllotoxin induced CREB activation and CRE-driven transcription in cells not expressing MC4R. Previous studies indicated that intracellular calcium, PKA, and MAPKs are involved in CREB phosphorylation and activation. Our studies revealed that podophyllotoxin did not affect intracellular calcium level and the phosphorylation state of p38. Podophyllotoxin induced JNK and ERK activation, but blockade of JNK and ERK activation with specific inhibitors had no effect on podophyllotoxin-induced CREB activation and CRE-regulated gene expression. Further experiments revealed that H89, a specific inhibitor of PKA, significantly inhibited podophyllotoxin-induced CREB activation. Podophyllotoxin itself did not alter intracellular cAMP level. Taken together, podophyllotoxin induces CREB activation and CRE-driven gene expression via PKA activation by a cAMP-independent mechanism.

INTRODUCTION

The melanocortin-4 receptor (MC4R) plays pivotal roles in the control of body-weight homeostasis, sexual behavior and autonomic functions (Jia, 2005; Loos et al., 2008). This has evoked enormous interests in identifying MC4R modulators for the treatment of multiple disorders. To search for new MC4R agonists, we employed a cAMP-responsive element (CRE)-driven luciferase reporter system because, after stimulation by an agonist, MC4R couples to Gαs protein and activates adenylyl cyclase, leading to increased intracellular levels of cAMP (Lee et al., 2001; Mountjoy et al., 1992).

We screened a library containing 48,000 pure compounds and approximately 4,000 herbal extract fractions. Unexpectedly, we found that podophyllotoxin and its derivatives could induce luciferase expression in cells transfected with MC4R and the CRE-driven reporter system. Our further analysis, however, indicated that MC4R was not involved in the induction of luciferase expression by podophyllotoxin and its derivatives, as podophyllotoxin could activate CRE binding protein (CREB) and CRE-driven transcription in cells not expressing MC4R.

Podophyllotoxin is a natural product isolated from *Podophyllum peltatum* and *Podophyllum emodi*. It binds to the colchicine site of tubulin and potently inhibits microtubule assembly (Damayanthi and Lown, 1998; Sackett, 1993). Podophyllotoxin exerts profound effects on important cellular processes such as cell cycle arrest and apoptosis (Damayanthi and Lown, 1998; Tseng et al., 2002). At the molecular level, podophyllotoxin could induce *c-jun* N-terminal kinase (JNK) phosphorylation (Shtil et al., 1999; Tseng et al., 2002). Its derivative, etoposide, is a potent inhibitor of topoisomerase II and an inducer of DNA damages (Hande, 1998). Etoposide can also activate extracellular signal-regulated kinase (ERK), JNK and p38 in selected tumor cell lines (Boldt et al., 2002). GP7, a new spin-labelled derivative of podophyllotoxin, activates the caspase signalling pathway by releasing cytochrome-C (Qi et al., 2004; 2007). GL133, another podophyllotoxin-derived compound, decreases ERK phosphorylation and subsequently inhibits cyclin D1 transcription (Lin et al., 2001). However, there has been no previous report on the involvement of podophyllotoxin in CRE-driven gene expression.

In this study, we report that podophyllotoxin could induce CREB phosphorylation and drive CRE-mediated gene expression. Interestingly, other microtubule-depolymerizing agents, such as colchicine, vincristine and vinorelbine, also induce CREB phosphorylation while microtubule stabilizing agent such as taxol exerts no such effect. Calcium mobilization assay and further studies with various kinase inhibitors have revealed that intracellular calcium and mitogen activated protein kinases (MAPKs) are not involved in podophyllotoxin-induced CREB activation. By contrast, inhibition of protein kinas A (PKA) almost totally abolishes podophyllotoxin-induced CREB phosphorylation and CRE-driven gene expression. Podophyllotoxin does not induce intracellular cAMP elevation. The exact mechanisms whereby podophyllotoxin acts through PKA warrant further investigation.

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MATERIALS AND METHODS

Reagents

The human MC4R expression vector was obtained from the UMR cDNA Resource Center (USA) and the *pCRE-Luc* reporter plasmid, which contains 4 copies of *CRE* in front of the luciferase gene, was purchased from Stratagene (USA). G418, α-MSH, forskolin, podophyllotoxin, taxol, etopside, colchicines and γ-lumicolchicine (Sigma, USA), nocodazole, vincristin, vinorelbine, H89, SB202190, SB203580, U0126, PD98059 and SP600125 (CalBiochem, USA), and fluo-4 AM (Invitrogen, USA) were used. Luciferase substrates were purchased from Promega (USA). Mouse anti-phospho-ERK1/2 antibody (Santa Cruz Biotechnology, USA), antibodies against ERK1/2, phospho-CREB, CREB, JNK, p38 and GAPDH (Cell Signaling, USA) and antibodies against phospho-JNK and phospho-p38 (Beyotime, China) were used for immunoblotting or immunofluorescent studies.

Cell culture and transfection

CHO or HEK293 cells were maintained in F12 nutritional medium or Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 mg/L penicillin, and 100 mg/L streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Cells were co-transfected with plasmids encoding MC4R and the *pCRE-Luc* reporter by electroporation. To generate stable cell lines, transfected cells were seeded into 10-cm dishes and 1 mg/ml G418 was added to the culture medium 24 h later. The selection medium was changed every 3 days until colonies were formed. A single colony was isolated, expanded, and tested with calcium mobilization assay to confirm the expression and proper function of the transfected genes.

Luciferase assay

Cells transfected with the *pCRE-Luc* reporter were seeded at a density of 5×10^4 cells per well onto a 96-well plate and incubated overnight. After the removal of medium, 50 μl of fresh medium containing compounds at various concentrations were added with 0.5% DMSO as a negative control. After 5 h incubation, 50 μl of Dual-Glo™ luciferase substrate were added. The plate was gently shaken for 10 min to ensure that the cells were completely lysed. Eighty μl of the cellular lysate were transferred to a white opaque 96-well plate and the luminescence signal was measured with an Envision multi-label plate reader (PerkinElmer, USA).

Calcium assay

Calcium assay was performed as previously described (Zhu et al., 2008). Briefly, cells transfected with MC4R and $G\alpha_{16}$ were seeded onto 96-well plates at a density of 5×10^4 cells per well and incubated overnight. After the cells were incubated with 2 μM fluo-4 AM in Hanks' balanced salt solution (HBSS) (pH 7.4) at 37°C for 50 min, excess dye was removed and the cells were rinsed with HBSS. In the antagonist detection mode, 50 μl HBSS containing known antagonist (SHU9119) or podophyllotoxin were added. After a 10-min incubation at room temperature, 25 μl α-MSH were dispensed into the well with a FlexStation II microplate reader (Molecular Devices, USA) and intracellular calcium change was recorded at an excitation wavelength of 485 nm and an emission wavelength of 525 nm. In the agonist detection mode, 50 μl HBSS were added to the dye-loaded cells, and 25 μl of known agonist (α-MSH) or podophyllotoxin were added and calcium change was measured by FlexStation II.

Western blotting studies

Cells were seeded onto 6 or 12-well plate for 24 h and serum-starved overnight. After their exposure to various drugs for appropriate periods of time, the cells were lysed, sonicated, and boiled at 95 to 100°C for 5 min in the sample buffer (62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% bromophenyl blue, pH 6.8). Aliquots of proteins were resolved by SDS-PAGE on 10% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were first blocked with 5% milk in TBST (20 mM Tris, 0.137 M NaCl, and 0.05% v/v Tween, pH 7.6) for 1 h followed by incubation with antibodies against phospho-ERK1/2, phospho-CREB (serine 133), phospho-JNK, phospho-p38, or ERK1/2, and CREB, JNK, and p38 at 4°C overnight. GAPDH was used as a loading control. The membranes were rinsed thrice in TBST and incubated with appropriate secondary antibodies conjugated to HRP for 1 h. The signal was developed with the ECL plus reagents (GE Healthcare) and images were captured and analyzed with the ChemiDoc™ Imaging Station (Bio-Rad).

cAMP measurement

Cells were detached with 0.04%-EDTA in PBS and adjusted to a density of 6×10^5 cells/ml using the stimulation buffer (1 × HBSS containing 5 mM HEPES, 0.1% bovine serum albumin, and 0.5 mM IBMX, pH 7.4). Aliquots of cells (3,000 cells/6 μl) were added to the components diluted by the stimulation buffer containing 1% DMSO (vehicle), 20 μM forskolin (positive control), or compounds of interest and incubated at room temperature for 30 min. The reaction was terminated and the cAMP production was detected using LANCE cAMP kits (Perkin Elmer) according to manufacturer's instructions.

Immunofluorescent microscopy

Cells were seeded onto 96-well plates at a density of 3.5×10^4 cells/well and incubated overnight. After serum starvation for 2 h, the cells were stimulated with 1% DMSO (vehicle) or compounds of interest for 30 min. After fixation with 4% formaldehyde in PBS and permeabilization with 0.3% Triton X-100, cells were incubated overnight with antibodies against CREB and phospho-CREB (serine 133) at 4°C. On the next day, the cells were washed and incubated with appropriate secondary antibodies conjugated to Alexa Fluor 555 for 1 h at room temperature. Finally, cell nuclei were stained with Hoechst 33342 for 10 min at room temperature. Fluorescent images were captured with an Olympus IX51 inverted fluorescent microscope. The fluorescent intensity was measured with an ArrayScan 4.0 HCS Reader (Cellomics), which can automatically identify and outline each cell. Experiments were run in triplicates, and > 1,000 cells from each well were analyzed.

Reverse transcription-PCR (RT-PCR)

Cells were seeded onto 6-well plate and grown overnight to reach ~80% confluency. Cells were treated with various microtubule-binding agents for 6 h and total RNA was extracted with Trizol reagents (Invitrogen). RT-PCR was carried out with the following primers: NR4A2 (sense) 5′-AACGCCAGTCTCCCGCCTCT-3′, NR4A2 (anti-sense) 5′-GGTTGGACCTGTATGCTAATCG-3′, GAPDH (sense) 5′-CTCAGACACCATGGGGAAGGTGA-3′ and GAPDH (anti-sense) 5′-ATGATCTTGAGGCTGTTGTCATA-3′.

Data analysis

All data were statistically analyzed with GraphPad Prism software (GraphPad, USA). Non-linear regression analyses were performed to generate dose-response curves and EC_{50} or IC_{50}. Two-tailed Student's *t*-test was applied to analyze differences.

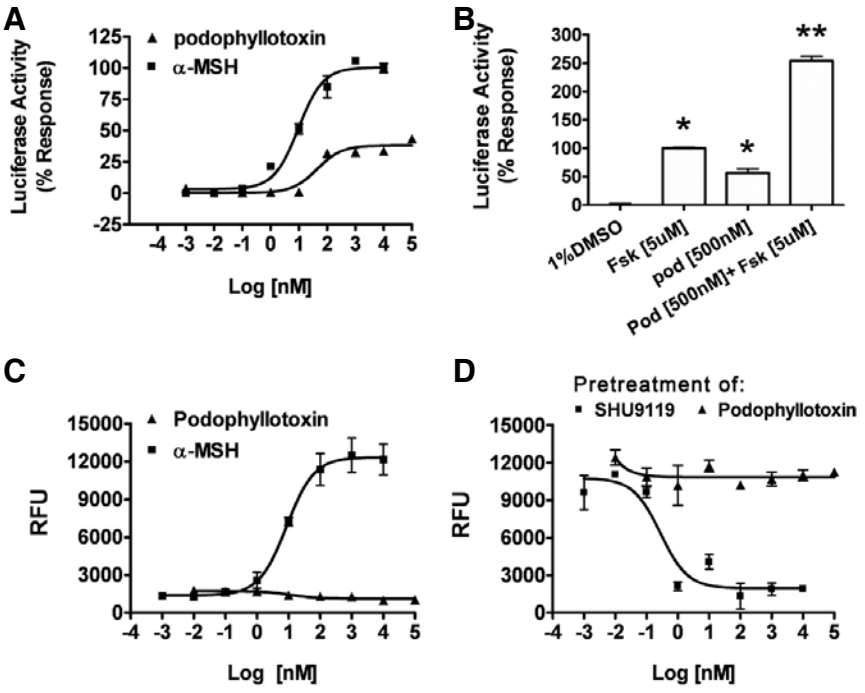

Fig. 1. Podophyllotoxin-induced CRE-driven luciferase expression is independent of MC4R. (A) Podophyllotoxin and α-MSH induced dose-dependent luciferase expression in CHO/MC4R/CRE-*Luc* cells. (B) In HEK293 cells transiently transfected with pCRE-*Luc* plasmid, podophyllotoxin (Pod) also induced significant luciferase expression. Forskolin (Fsk) was used as a positive control. Combination treatment of podophyllotoxin and forskolin revealed a synergistic effect. Data are presented as mean ± S.E.M. (n = 3). * $P < 0.0001$ versus DMSO control, ** $P < 0.0001$ versus forskolin alone. (C) α-MSH induced dose-dependent intracellular calcium elevation in HEK293 cells transiently transfected with MC4R and $G_{\alpha16}$. Podophyllotoxin at concentrations up to 100 μM did not cause any change in intracellular calcium. (D) HEK293 cells expressing MC4R and $G_{\alpha16}$ were pretreated with various concentrations of SHU9119 and podophyllotoxin, and then stimulated with 100 nM α-MSH. SHU9119 inhibited α-MSH induced calcium elevation in a dose-dependent manner. In contrast, podophyllotoxin had no effect on α-MSH induced calcium response. All dose-response curves are representative of three independent experiments, each carried out in triplicate.

RESULTS

Podophyllotoxin-induced CRE-driven luciferase expression is independent of MC4R

To search for small molecule agonists of MC4R, we developed a CHO cell line (CHO/MC4R/CRE-Luc) stably transfected with MC4R and pCRE-*Luc* reporter gene. We screened a library containing 48,000 pure compounds and approximately 4,000 fractions isolated from traditional Chinese medicinal herbs. Interestingly, podophyllotoxin and its derivatives were found to induce luciferase expression in this cell line. Further study indicated podophyllotoxin induced luciferase expression in a dose dependent manner, with an EC_{50} value of 44 nM. The maximum luciferase expression stimulated by podophyllotoxin was approximately 35% of the maximum response evoked by the MC4R ligand, α-MSH (Fig. 1A). These results raised a question whether podophyllotoxin, a known tubulin binding natural compound, induced CRE-driven luciferase expression via an interaction with MC4R. Subsequently, we carried out reporter assays in cells that did not express MC4R. We found that podophyllotoxin induced luciferase expression in HEK293 cells transiently transfected with pCRE-*Luc* plasmids alone (Fig. 1B). Without the expression of MC4R, 500 nM podophyllotoxin was still able to induce CRE-driven reporter expression and the response was approximately half of that induced by 5 μM forskolin, which served as a positive control. Interestingly, podophyllotoxin and forskolin seemed to be synergistic in luciferase gene induction since the combination treatment significantly enhanced reporter expression (2.5 fold of forskolin treatment, Fig. 1B).

To further determine the relationship between podophyllotoxin and MC4R, we carried out calcium assays in HEK293 expressing MC4R and $G\alpha_{16}$, a G protein commonly used to enhance the coupling of GPCRs to the phospholipase C and calcium pathways (Berridge, 1993; Zhu et al., 2008). As shown in Fig. 1C, α-MSH induced a dose-dependent elevation of intracellular calcium with an EC_{50} of 8.7 nM, which is in agreement with the EC_{50} of the luciferase assays (9.7 nM). In contrast, podophyllotoxin did not induce intracellular calcium changes at any concentrations tested. In the antagonist testing mode, we found SHU9119, a MC4R antagonist, dose-dependently inhibited α-MSH (100 nM)-induced calcium response while podophyllotoxin had no such effect (Fig. 1D). These results indicated that podophyllotoxin is not a ligand for MC4R and the induction of CRE-driven luciferase expression by podophyllotoxin is independent of MC4R.

Podophyllotoxin and other microtubule depolymerizing agents induce CREB phosphorylation and downstream gene transcription

The initiation of CRE-driven gene expression requires an orderly binding of transcription factors that form a transcription complex at the CRE site, and CREB phosphorylation at serine 133 is a key event required for recruiting different transcription effectors (Shaywitz and Greenberg, 1999). To answer the question whether podophyllotoxin induces CREB phosphorylation at serine 133, immunofluorescent staining was carried out in HEK293 cells with antibodies against total CREB and the phosphorylated form of CREB (serine 133). As shown in Figs. 2A and 2B, the phosphorylation level of CREB was very low at the resting state. Treatment of these cells with 50 μM forskolin or 500 nM podophyllotoxin for 30 min induced a significant increase in the level of CREB phosphorylation. In contrast, α-MSH had no such effect since the cells were not expressing MC4R.

Podophyllotoxin is known to bind to and depolymerize microtubules (Andreu and Timasheff, 1982; Sackett, 1993). We were curious whether this CREB phosphorylation effect is specific to podophyllotoxin or common to all microtubule binding compounds. We tested a few other microtubule binding compounds including taxol, colchicine, nocodazole, vincristine, and vinorel-

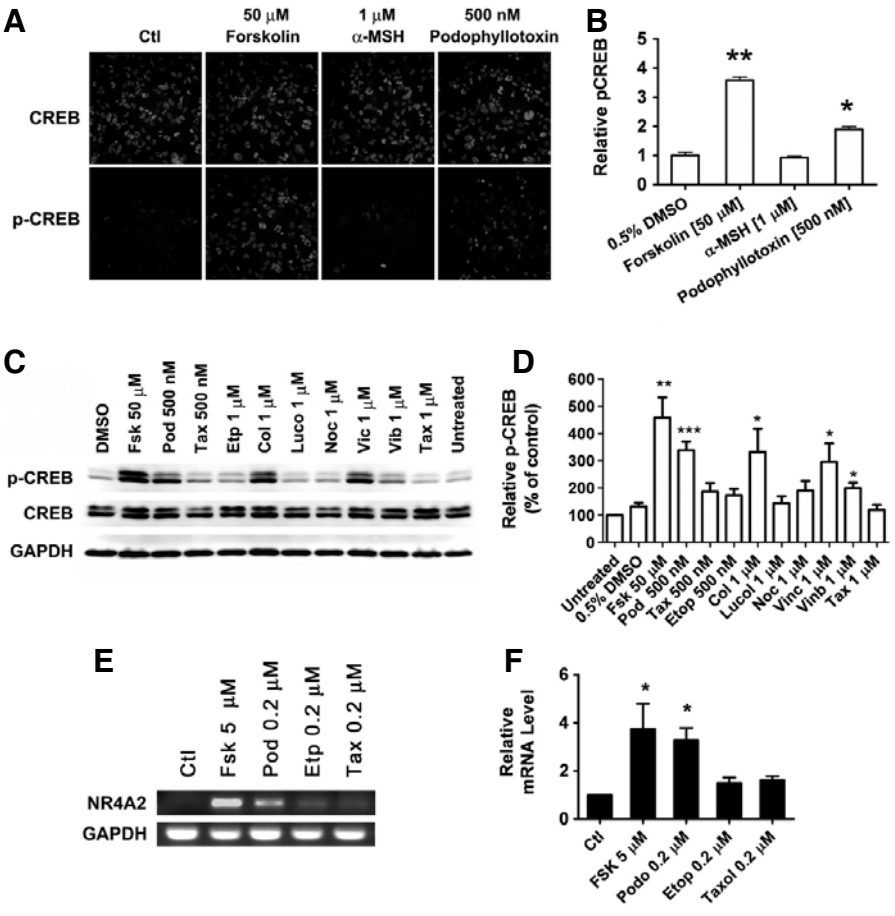

Fig. 2. Podophyllotoxin and other microtubule-depolymerizing agents induce CREB phosphorylation and downstream gene transcription. (A) Representative immunofluorescent images of phospho-CREB (Ser133) and total-CREB in HEK293 cells treated with forskolin, α-MSH or podophyllotoxin for 30 min. (B) Statistical analysis of the immunofluorescent staining. Podophyllotoxin and forskolin significantly induced CREB phosphorylation at Ser133 in HEK293 cells, α-MSH had no such effect. Data are representative of three independent experiments, each run in triplicate, and approximately 1000 cells from each well were analyzed. ** $P < 0.005$; * $P < 0.05$ versus DMSO control. (C) Western blotting analysis of CREB phosphorylation at Ser133 in HEK293 cells stimulated with various microtubule binding compounds for 60 min. (D) Statistical analysis of the immunoblots. Forskolin (Fsk), podophyllotoxin (Pod), colchicine (Col), vincristine (Vic), and vinorelbine (Vib) significantly induced CREB phosphorylation. But taxol (Tax), etoposide (Etp), γ-lumicolchicine (Lucol) and nocodazole (Noc) had no significant effect. Data are presented as mean ± S.E.M. ($n = 4$). *** $P < 0.001$; ** $P < 0.005$; * $P < 0.05$ versus 0.5% DMSO control. (E) RT-PCR analysis of the mRNA levels of NR4A2 and GAPDH after treatment of microtubule binding compounds. (F) Statistical analysis of the RT-PCR results. Data are presented as mean ± S.E.M. ($n = 3$). * $P < 0.05$ versus 0.5% DMSO control.

bine. γ-Lumicolchicine and etoposide were included as controls. γ-Lumicolchicine is a structural isomer of colchicine and does not bind tubulin or interfere with microtubule formation. Etoposide is a podophyllotoxin derivative. It arrests cell growth by inhibiting DNA topoisomerase II (Hande, 1998; Wilstermann et al., 2007), but does not inhibit tubulin polymerization. Interestingly, all microtubule-depolymerizing agents, except nocodazole, induced significant CREB phosphorylation (Figs. 2C and 2D) compared to DMSO after 1 h treatment. By constrast, taxol, a microtubule stabilizer, had no such effect. Two control compounds, etoposide and γ-lumicolchicine, which are structurally related to podophyllotoxin and colchicines but do not bind to tubulin, did not induce CREB phosphorylation either.

To further verify the function of activated CREB, we examined the expression level of endogenous genes regulated by CREB. NR4A2 is a member of the steroid/thyroid hormone family of nuclear receptors that respond to the activation of T cells (Mages et al., 1994). The gene encoding NR4A2 contains a CREB binding element (Conkright et al., 2003). We examined the expression level of NR4A2 by RT-PCR after treatment with various microtubule binding agents (Figs. 2E and 2F). Our results indicated that, similar to the positive control (forskolin), podophyllotoxin induced a significant elevation of the mRNA level of NR4A2. But etoposide and taxol had no such effect. These results were consistent with the CREB phosphorylation profile. Taken together, the above results indicate that CREB phosphorylation at serine 133 might be a common phenomenon after treatment with microtubule-depolymerizing agents, including podophyllotoxin. Additionally, the activation of CREB eventually leads to the transcription of endogenous CRE-containing genes.

p38 and JNK/SAPK are not involved in podophyllotoxin-induced CREB activation and CRE- driven gene expression

MAPKs are reported to phosphorylate CREB at serine 133 (Johannessen et al., 2004; Shaywitz and Greenberg, 1999). Generally, each of the MAPKs subfamilies activates a unique, although overlapping, spectrum of cellular targets under different conditions. Thus, it is necessary to determine whether MAPKs contribute to podophyllotoxin-induced CREB phosphorylation and which subtype might be involved. Specially, p38 and JNK are activated by lipopolysaccharide, inflammatory cytokines, heat shock, radiation and other environmental stresses (Chang and Karin, 2001; Johnson and Lapadat, 2002). Microtubule catastrophe is inarguably an intracellular stress for all cells. We were interested to know whether p38 and JNK are involved in podophyllotoxin-induced CREB activation.

We first applied SB202190, a p38 inhibitor, in the luciferase assay. As indicated in Fig. 3A, SB202190 had no effect on podophyllotoxin (500 nM)-induced luciferase expression at concentrations up to 10 μM, nor did it affect the luciferase activity stimulated by forskolin, which is known to act on the PKA pathway. Then we tested whether podophyllotoxin induced p38 activation. P38 phosphorylation is a prerequisite for its activa-

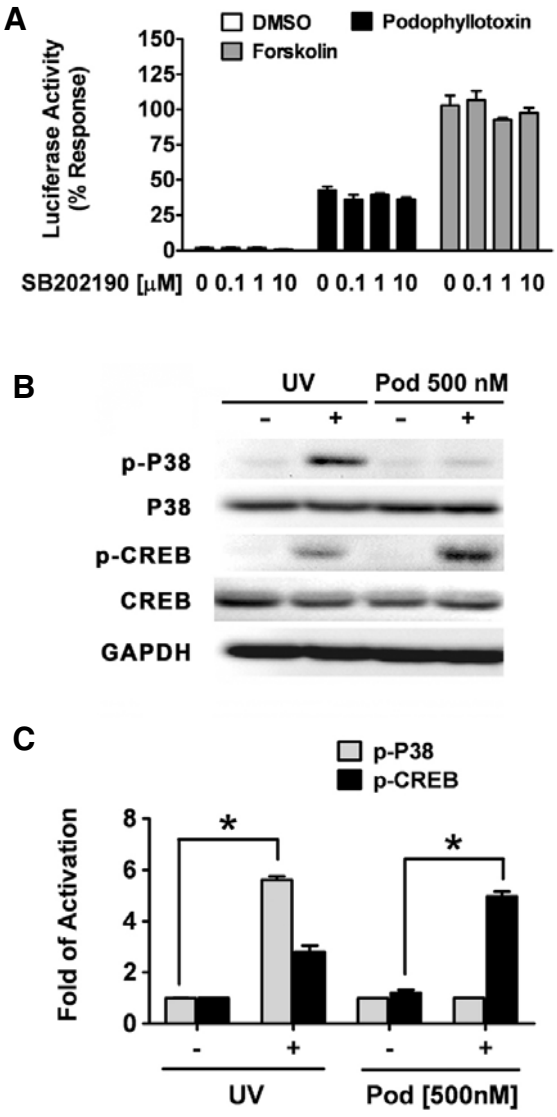

Fig. 3. P38 is not involved in podophyllotoxin-induced CREB activation and CRE-driven gene expression. (A) HEK293 cells transiently transfected with pCRE-Luc plasmids were pretreated with SB202190 for 30 min; thereafter, 500 nM podophyllotoxin was added. After 5 h, the luciferase activity was measured. Forskolin (10 μM) was used as positive control of luciferase activity. SB202190 had no effect on podophyllotoxin-induced CRE-driven luciferase expression. Data are presented as mean ± S.E.M. (n = 3). (B) Representative Western blotting results of p38 phosphorylation. Podophyllotoxin stimulation for 1 h failed to induce p38 phosphorylation but stimulated CREB phosphorylation in HEK293 cells. UV exposure for 15 min was used as a positive control of p38 phosphorylation. (C) Statistical analysis of the immunoblots. Data are presented as mean ± S.E.M. (n = 3), * P < 0.005.

tion (Raingeaud et al., 1995). Western blotting analysis revealed that treatment of podophyllotoxin (500 nM) for 1 h evoked significant CREB phosphorylation, but failed to induce p38 phosphorylation in HEK293 cells (Figs. 3B and 3C). As a control, 15 min UV irradiation induced significant p38 and CREB phosphorylation. These results indicated that podophyllotoxin did not induce p38 phosphorylation and activation. Thus, p38 is not likely to be involved in podophyllotoxin-induced CREB activation.

JNK is another MAPK that is preferentially activated by stress signals (Chang and Karin, 2001; Deak et al., 1998). Western blotting studies revealed that JNK phosphorylation pattern induced by microtubule binding compounds and their analogs was very similar to the CREB phosphorylation pattern, i.e, all microtubule destabilizing agents, including podophyllotoxin, colchicines, nocodazole, vincristine and vinorelbine, induced JNK phosphorylation. However, taxol, etoposide and lumicolchicine did not have any effect (Figs. 4A and 4B).

Bu et al. (2008) reported that attenuation of JNK activity reduces CREB phosphorylation. To test whether podophyllotoxin-induced JNK activation is upstream of CREB phosphorylation, we applied SP600125, a JNK kinase MKK4 inhibitor, with forskolin as a positive control for CREB phosphorylation and negative control for JNK activation. We found that pre-treating HEK293 cells with 5 μM SP600125 for 30 min completely blocked podophyllotoxin-stimulated JNK phosphorylation (Figs. 4C and 4D). However, CREB phosphorylation was not changed even when the SP600125 concentration was raised to 25 μM (Figs. 4C and 4E). Therefore, JNK activation does not function upstream of podophyllotoxin-induced CREB activation.

Podophyllotoxin-induced ERK1/2 activation is not required for CREB activation and CRE-driven reporter expression

ERK is another major subfamily of MAPKs and is reported to play a role in CREB activation (Deak et al., 1998). Previous studies also indicate that podophyllotoxin and other microtubule binding agents are able to induce ERK activation (Samarakoon et al., 2009; Schmid-Alliana et al., 1998; Stone and Chambers, 2000). Therefore, we tested whether ERK1/2 played a role in podophyllotoxin-induced CREB activation. Two MEK inhibitors, U0126 and PD98059, were applied. Because MC4R was proved not to interact with podophyllotoxin, we still used the CHO/MC4R/CRE-*Luc* stable cell line for luciferase assay. Cells were pre-incubated with various concentrations of U0126 and PD98059 for 15-30 min. Without removing the inhibitors, cells were stimulated with 500 nM podophyllotoxin and luciferase activity was measured after 5 h. As shown in Fig. 5A, treatment with U0126 or PD98059 failed to suppress podophyllotoxin-induced luciferase expression. α-MSH (500 nM) was used as a positive control to stimulate luciferase expression.

Immunoblotting analysis revealed that podophyllotoxin and colchicines could induce ERK1/2 phosphorylation, but taxol had no such effect. These results are consistent with previous reports (Qiu et al., 2006; Samarakoon et al., 2009; Shtil et al., 1999). U0126 at 10 μM not only significantly blocked podophyllotoxin and colchicine-induced ERK1/2 phosphorylation, but also completely suppressed the basal phosphorylation level of ERK1/2 (Figs. 5B and 5D). However, U0126 failed to inhibit podophyllotoxin and colchicine-induced phosphorylation of CREB at serine 133 (Figs. 5B and 5E). This result was confirmed by PD98059, a selective inhibitor of MEK1, which suppressed podophyllotoxin-induced ERK1/2 phosphorylation in a dose-dependent manner (Figs. 5C and 5G), but had no effect on CREB phosphorylation at serine 133 (Figs. 5C and 5F). These results demonstrate that podophyllotoxin and other microtubule destabilizing agents can induce ERK1/2 phosphorylation. But ERK phosphorylation does not contribute to CREB phosphorylation and CRE-driven gene expression stimulated by podophyllotoxin.

PKA is critically involved in podophyllotoxin-induced CREB activation and CRE- driven gene expression

PKA is a typical CREB kinase. When cAMP binds to the two

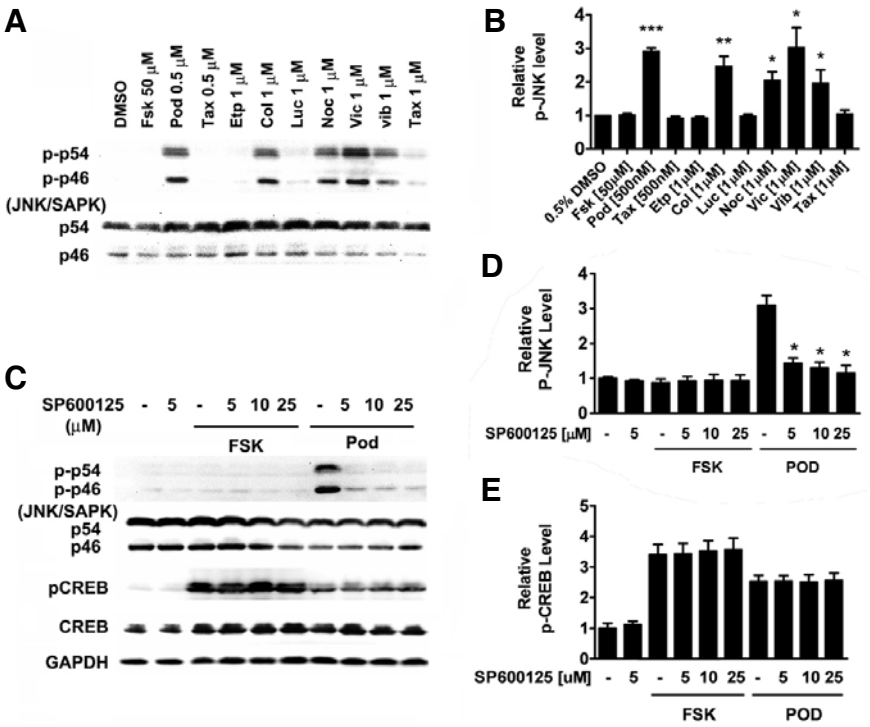

Fig. 4. JNK/SAPK activation is not required for podophyllotoxin-induced CREB phosphorylation. (A) Representative Western blot of JNK/SAPK phosphorylation in HEK293 cells stimulated with various microtubule binding agents for 60 min. (B) Statistical analysis of the immunoblots from (A). Podophyllotoxin (Pod), colchicine (Col), nocodazole (Noc), vincristine (Vic), and vinorelbine (Vib) significantly induced JNK phosphorylation. Forskolin (Fsk), taxol (Tax), etoposide (Etop), and γ-lumicolchicine (Lucol) had no significant effect. Data are represented as mean ± S.E.M. ($n = 3$). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus DMSO control. (C) Western blotting result of SP600125's effect on JNK and CREB phosphorylation. (D, E) Statistical analysis of the immunoblots from (C). SP600125 significantly blocked podophyllotoxin-stimulated JNK phosphorylation (D), but had no effect on CREB phosphorylation (E). Data are represented as mean ± S.E.M. ($n = 3$). * $P < 0.05$ versus SP600125 negative control.

regulatory subunits of PKA, the catalytic subunits of PKA are released and translocated to the nucleus. CREB family members are among the best characterized nuclear substrates of PKA (Gullingsrud et al., 2006; Shaywitz and Greenberg, 1999). To determine whether PKA is involved in the signaling pathway of CREB phosphorylation induced by podophyllotoxin, H89, a selective inhibitor of PKA, was applied in luciferase assay and Western blotting analysis. *In vitro*, the IC_{50} of H89 for PKA is approximately 50 nM. But *in vivo* inhibition of substrate phosphorylation by PKA requires 10 to 30 μM H89 (Chijiwa et al., 1990). In CHO/MC4R/CRE-Luc cells, H89 at 16.7 μM almost completely inhibited podophyllotoxin or forskolin-induced lu-ciferase expression (Fig. 6A). Western blotting also confirmed that H89 dose-dependently suppressed podophyllotoxin or forskolin-induced phosphorylation of CREB at serine 133 (Figs. 6B and 6C). H89 almost completely inhibited podophyllotoxin-induced CREB phosphorylation at 10-20 μM. The efficient inhibition of CREB phosphorylation by H89 suggests that PKA is the main upstream effector modulated by podophyllotoxin. However, cAMP assay indicated that podophyllotoxin was not able to induce intracellular cAMP elevation as forskolin did in HEK 293 cells (Fig. 6D) and CHO/MC4R/CRE-Luc cells (data not shown). The exact mechanism whereby podophyllotoxin induces CREB activation via PKA needs further elucidation.

DISCUSSION

Reporter gene assays are commonly used in high-throughput drug screening. However, it has certain disadvantages. The expression of the reporter gene is usually controlled by the activity of a certain signaling pathway and modification of many signaling molecules along this pathway could lead to changes in the expression of the reporter gene, sometimes generating many off-target hits. In our case, we applied a CRE-driven luciferase reporter system to screen for MC4R agonists, and found unexpectedly that podophyllotoxin induced luciferase expression independently of MC4R.

Podophyllotoxin is a natural product isolated from *Podophyllum peltatum* and *Podophyllum emodi* and has long been known to possess medicinal properties. Since 1940, *Podophyllum* resin has been used topically for various skin lesions such as warts and condylomas (Beutner and von Krogh, 1990; Kaplan, 1942). Today, podophyllotoxin continues to be used worldwide as a first-line treatment for condyloma acuminate and as a component of some cathartic herbal preparations (Beutner and von Krogh, 1990; Gordaliza et al., 1994; 2004).

The most well-known molecular target for podophyllotoxin is tubulin. Podophyllotoxin binds to the colchicine site of tubulin and potently inhibits microtubule assembly (Damayanthi and Lown, 1998; Sackett, 1993). Podophyllotoxin exerts profound effects on such important cellular processes as cell cycle control and apoptosis (Damayanthi and Lown, 1998; Huang et al., 1999; Tseng et al., 2002). However, previous attempts to use podophyllotoxin in the treatment of cancer were mostly unsuccessful due to the complicated side effects such as nausea, vomiting, and damage of normal tissues. Therefore, podophyllotoxin has been used as a lead compound for drug design to obtain more potent and less toxic anticancer agents.

Podophyllotoxin and its derivatives are reported to activate various intracellular signaling pathways. For example, podophyllotoxin could induce JNK phosphorylation in human breast cancer cell line MCF-7 and human promyelocytic leukemia cell line HL-60 (Shtil et al., 1999; Tseng et al., 2002). Its derivative, etoposide, also activates ERK, JNK and p38 in selected tumour cell lines even though topoisomerase II is the main molecular target of etoposide (Boldt et al., 2002). GL133, a new derivative of podophyllotoxin, was reported to inhibit cyclin D1 gene transcription via the ERK pathway (Lin et al., 2001).

To our knowledge, so far, there has been no report on the involvement of podophyllotoxin in CREB activation and CRE-driven gene expression. More interestingly, we found that other microtubule-depolymerizing agents, such as colchicine, vincris-

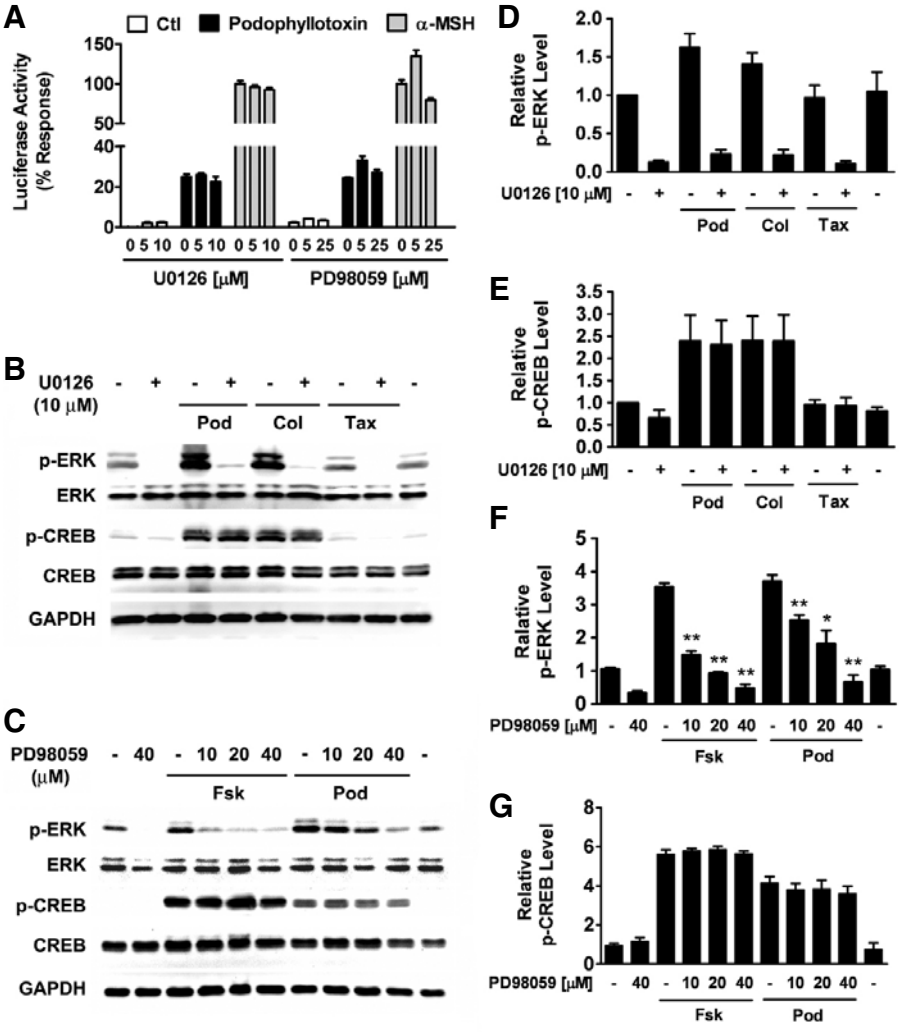

Fig. 5. Podophyllotoxin-induced ERK1/2 activation does not contribute to CREB activation and CRE-driven reporter expression. (A) In CHO/MC4R/CRE-Luc cells, pretreatment of U0126 and PD98059 failed to suppress podophyllotoxin-induced luciferase expression. α-MSH (500 nM) was used as a positive control of luciferase activity. (B) Representative Western blotting result of U0126's effect on podophyllotoxin and colchicine-induced ERK and CREB phosphorylation. (C) Representative Western blotting result of PD98059's effect on podophyllotoxin and forskolin-induced ERK and CREB phosphorylation. (D, E) Statistical analysis of the immunoblots from (B). Podophyllotoxin (Pod) and colchicine (Col), but not taxol (Tax), significantly induced ERK and CREB phosphorylation. U0126 at 10 μM almost totally inhibited ERK phosphorylation, but it had no effect on CREB phosphorylation. (F, G) Statistical analysis of the immunoblots from (C). PD98059 dose-dependently inhibited podophyllotoxin (Pod) and Forskolin (Fsk) stimulated ERK phosphorylation, but did not block CREB phosphorylation. Data are represented as mean ± S.E.M. ($n = 3$). * $P < 0.02$, ** $P < 0.001$ versus PD98059 negative controls.

tine, and vinorelbine, were also able to induce CREB phosphorylation like podophyllotoxin. We were interested in finding out which pathways might be upstream of CREB activation induced by podophyllotoxin.

Previous studies with specific protein kinase inhibitors indicate that various protein kinases, including PKA, PKB/Akt (Du and Montminy, 1998; Liang et al., 2008), PKC (Choi et al., 2007; Cramer et al., 2008) , PKG (Gudi et al., 1997; Lu et al., 1999; Pilz and Casteel, 2003), GSK (Tyson et al., 2002), CaMKs (Francis et al., 2008; Sato et al., 2006; Tai et al., 2008), ERK (Choi et al., 2007; Deak et al., 1998), p38 (Kitamura et al., 2002; Swarthout et al., 2002), and PI3K (Moeenrezakhanlou et al., 2007) can phosphorylate and activate CREB in various cell types upon stimulation. Therefore, using a similar approach, we investigated which pathway might be involved in podophyllotoxin-induced CREB activation.

The calcium dependent kinases (CaMKs) were first ruled out because podophyllotoxin did not induce changes in the intracellular calcium content. P38 and JNK are the major stress-related kinases. Podophyllotoxin-induced microtubule destabilization is no doubt a stress for all cells; therefore, we investigated whether p38 and JNK are involved in podophyllotoxin-induced CREB activation. Western blotting assays indicated that p38 was not activated by podophyllotoxin treatment (Figs. 3B and 3C). Reporter assay also revealed that p38 inhibitor, SB202190, had no effect on podophyllotoxin-induced luciferase expression (Fig. 3A). Thus, p38 does not likely play a role here. In contrast, JNK was indeed activated by podophyllotoxin and other tubulin polymerization inhibitors (Figs. 4A and 4B). Blocking JNK phosphorylation with SP600125, however, failed to inhibit podophyllotoxin-induced CREB phosphorylation, indicating that JNK is not an upstream effector of CREB phosphoryaltion (Figs. 4C, 4D, and 4E).

Then we turned to investigate whether ERK is mediating podophyllotoxin-induced CREB activation. We found that both podophyllotoxin and colchicine induced ERK and CREB phosphorylation. The microtubule stabilizing agent taxol had no such effect (Fig. 5B). But studies with MEK inhibitors U0126 and PD98059 indicated that blocking ERK phosphorylation had no effect on podophyllotoxin or colchicine induced CREB phosphorylation and reporter expression (Fig. 5).

The application of PKA inhibitor H89 indicated that PKA is critically involved in podophyllotoxin-mediated CREB phosphorylation and CRE-driven gene expression (Figs. 6A, 6B and 6C). The classical model of PKA activation requires the elevation of intracellular cAMP level. PKA exists as a holoenzyme comprised of two regulatory subunits (PKAr) and two catalytic subunits (PKAc). Two molecules of cAMP bind to each regula-

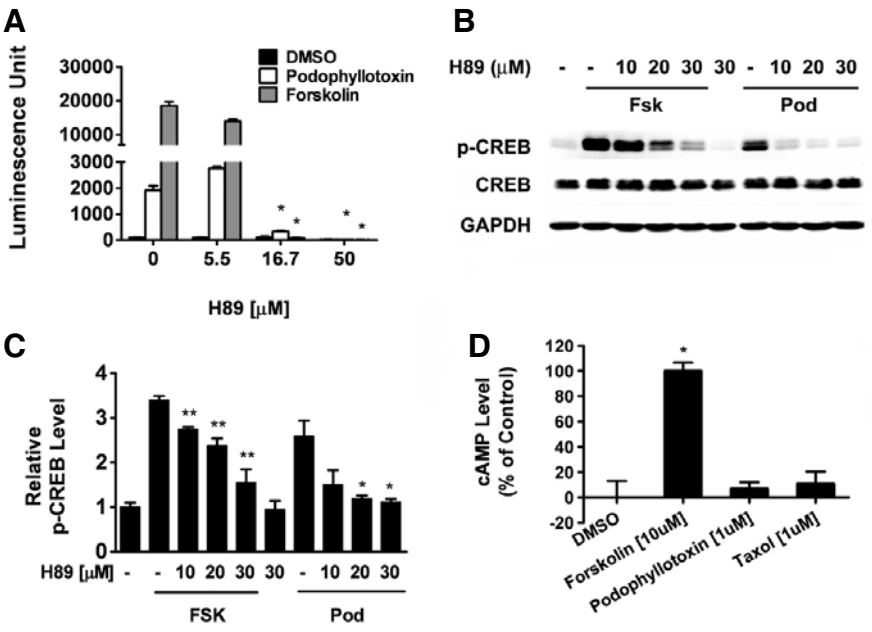

Fig. 6. PKA is critically involved in podophyllotoxin-induced luciferase expression and CREB phosphorylation. (A) H89, PKA inhibitor, suppressed podophyllotoxin-induced CRE-driven luciferase expression. In CHO/MC4R/CRE-Luc cells, H89 at 16.7 μM concentration almost totally inhibited podophyllotoxin (500 nM) or forskolin (50 μM)-induced luciferase expression. Data are represented as mean ± S.E.M. (n = 3). * P < 0.001 versus H89 negative control. (B) Representative Western blot result of H89's effect on forskolin (Fsk) and podophyllotoxin (Pod)-induced CREB phosphorylation. (C) Statistical analysis of immunoblots from (B). H89 inhibited podophyllotoxin-induced phosphorylation of CREB in a dose-dependent manner. Data are represented as mean ± S.E.M. (n = 3). * P < 0.05, ** P < 0.01 versus H89 negative controls.

tory subunit, allowing the release of the active catalytic subunits (Gullingsrud et al., 2006; Shabb, 2001). To our surprise, the cAMP assay revealed that podophyllotoxin had no effect on intracellular cAMP level. These findings suggest that podophyllotoxin may activate PKA in a cAMP-independent manner, which subsequently leads to CREB activation and CRE-driven gene expression.

Cyclic-AMP-independent activation of PKA has also been reported recently. For instance, a multi-protein complex of $G\alpha_{13}$/AKAP110/PKAr/PKAc was identified (Niu et al., 2001). The active $G\alpha_{13}$ can induce the release of PKAc from this complex, resulting in a cAMP-independent PKA activation. PKAc plays important roles in hypertonicity-dependent transcription factor activation. These activities can be enhanced by the expression of PKAc but not by cAMP elevation (Ferraris et al., 2002). The phosphorylation of the p65/RelA subunit of NF-κB by PKA is also independent of cAMP. Both NF-κB and PKAc are inactivated by binding to IκB, forming an NF-κB/IκB/PKAc complex. Upon stimulation, IκB is phosphorylated and targeted for degradation, PKAc is released in an active form and phosphorylates p65 (Zhong et al., 1997). Moreover, second messenger lipids, such as phosphatidylserine, sphingosine and dimethylsphingosine, have been found to modulate the activity of PKC (Igarashi, 1997; Merrill and Stevens, 1989).

Apart from the catalytic subunit of PKA, other proteins were found to interact with PKAr, the regulatory subunit of PKA. One particular interesting case is the identification of a high molecular weight complex containing α/β-tubulin and PKAr I from mouse brain extracts (Kurosu et al., 2009). This interaction between tubulin and PKAr is believed to be important for the subcellular localization of PKA. But the influence on PKAc activity by such tubulin-PKAr interaction is not very clear. It remains to be elucidated whether podophyllotoxin-induced PKA activation is mediated by a lipid-based second messenger or by disrupting microtuble homeostasis, thus affecting PKA localization and activity via protein-protein interaction.

In summary, our study revealed for the first time that podophyllotoxin and other microtubule-depolymerizing agents can induce CREB activation and CRE-driven gene expression. CaMKs, p38, ERK and JNK do not contribute to podophyllotoxin-induced CREB activation. Podophyllotoxin activates PKA in a cAMP-independent manner and subsequently activates CREB and CRE-driven gene expression.

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