Involvement of cAMP in nerve growth factor-triggered p35/Cdk5 activation and differentiation in PC12 cells

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Chen MC, Lin H, Hsu FN, Huang PH, Lee GS, Wang PS. Involvement of cAMP in nerve growth factor-triggered p35/Cdk5 activation and differentiation in PC12 cells. Am J Physiol Cell Physiol 299: C516–C527, 2010. First published May 12, 2010; doi:10.1152/ajpcell.00534.2009.—The signaling mechanisms underlying cell differentiation have been extensively studied with the use of rat PC12 cells as a model system. Nerve growth factor (NGF) is a trophic factor inducing PC12 cell differentiation through the activation of the p35/cyclin-dependent kinase 5 (Cdk5) complex. It has been reported that adenylyl cyclase activation and cAMP production may be involved in NGF-dependent actions. Our previous results indicated that cAMP activates the p35/Cdk5 complex in reproductive cells. Therefore, the role of cAMP in NGF-triggered p35/Cdk5 activation and PC12 differentiation was interesting to explore. Our results indicate that roscovitine, a molecular inhibitor of Cdk5, blocks cAMP-triggered PC12 differentiation, which was evaluated by neurite initiation, a decrease in proliferation, and cell cycle G1 arrest. The following data show that cAMP treatment increased NGF-induced cAMP activity through p35 upregulation. cAMP downstream components, protein kinase A (PKA) and phosphorylated cAMP response element binding protein (CREB), are involved in this regulation. The immunocytochemical results indicate that PKA inhibition disrupted cAMP-triggered p35/Cdk5 localization in PC12 cells. In addition, adenylyl cyclase inhibition was found to diminish NGF-induced intracellular cAMP production, CREB phosphorylation, and p35 expression. The cAMP antagonist and the PKA inhibitors reduced NGF-induced p35 expression. Finally, NGF-triggered PC12 differentiation was partially decreased by adenylyl cyclase or PKA inhibitors. In conclusion, these results demonstrate that cAMP may play a role in NGF-p35/Cdk5-dependent PC12 differentiation.

adenosine 3',5'-cyclic monophosphate; nerve growth factor; cyclin-dependent kinase 5

THE PC12 CELL LINE WAS CLONED from rat adrenal pheochromocytoma and is characterized based on its capacity to cease proliferation and promote differentiation when exposed to nerve growth factor (NGF) (8, 11, 18). In recent decades, the PC12 cell line became a useful model system for investigating the genes and proteins involved in the effects of NGF on cell differentiation (31, 42). NGF signals through two different types of receptors to promote differentiation: TrkA, a receptor tyrosine kinase, and p75NGFR, a low-affinity neurotrophin receptor (4). The finding that Src and Ras enhance NGF-induced differentiation led to the identification of Ras and Src signaling pathways that are a part of the total differentiation response to NGF (6). NGF was also reported to upregulate cyclin D1 expression and promote neurite outgrowth through Ras/ERK pathway (25). In addition to NGF, cAMP is also thought to be an important stimulator of PC12 differentiation (36). A recent report suggests that NGF and cAMP have a differential and synergistic effect on PC12 differentiation through transcriptional regulation (27). Furthermore, several lines of evidence indicate that NGF-triggered responses are correlated to cAMP-related signals (9, 13, 32, 39). NGF induces cAMP formation via a p75NGFR/G protein-mediated mechanism (13). Also, cAMP response element binding protein (CREB) appears to mediate NGF-induced Bcl-2 expression and promotes sympathetic neuron survival (32). A recent study revealed that NGF-like neurotrophins elevate cAMP and overcome inhibition by myelin-associated glycoprotein (MAG) (9). Stessin et al. (39) identified soluble adenylyl cyclase as a mediator of NGF signaling and revealed the existence of distinct pathways leading to cAMP-dependent signal transduction.

Cyclin-dependent kinase 5 (Cdk5) plays important roles in the development of the central nervous system and neurodegenerative diseases (7). Association with its regulatory partner, p35, is required for Cdk5 activation, which maintains the physiological functions of Cdk5 (7, 44). The expression of the p35/Cdk5 complex in extraneuronal cells has been linked to various biological functions (19, 26, 37, 41). Our previous results (14, 20, 22, 23) indicated that Cdk5 regulates the fate of neuronal cells as well as tumor cells. In addition, we also identified that cAMP modulates Cdk5 kinase activity in mouse Leydig cells (21). Alternatively, NGF induces p35 expression through ERK/Egr-1 activation and causes Cdk5 activity-dependent differentiation of PC12 cells (12). In light of these findings, the involvement of cAMP in NGF-induced p35 expression and PC12 differentiation becomes interesting to investigate. Our present data indicate that NGF treatment stimulates intracellular cAMP production and causes p35 expression and Cdk5 activation, which induces growth arrest and neurite initiation of PC12 cells.

MATERIALS AND METHODS

Cell culture. The PC12 cell line (ATCC no. CRL-1721) was purchased from the Culture Collection and Research Center, Taiwan. PC12 cells were cultured in Ham’s F-12K medium (Sigma, St. Louis, MO) supplemented with 5% fetal bovine serum (Invitrogen, Carlsbad, CA), 10% horse serum (Invitrogen), 1 mM sodium pyruvate (Sigma), and penicillin-streptomycin (Sigma) at 37°C in a humidified atmosphere with 5% CO2.

Measurement of neurite outgrowth. Cells were seeded on 24-well culture plates and treated for 48 h with CAMP or NGF with or without specific inhibitors. Neurite outgrowth was measured with an optical
microscope, and five random fields were observed in each culture dish. A neurite is defined as an extension longer than the diameter of two cell bodies (3, 38, 40). The mean number of neurites per 100 cells for each well was calculated in 3 independent experiments. The values are expressed as means ± SE.

Western blotting. Cell lysates were extracted with lysis buffer as previously described (20, 21), and the proteins were analyzed by Western blotting (20–40 μg/lane). Antibodies included anti-Cdk5 antibody (DC-17, Upstate, Lake Placid, NY), anti-p35 antibody (sc-820, Santa Cruz Biotechnology, Santa Cruz, CA), anti-actin antibody (Chemicon, Temecula, CA), and peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch Laboratory, West Grove, PA). Enhanced chemiluminescence (ECL) detection reagent (PerkinElmer, Shelton, CT) was used to detect the immunoreactive proteins.

In vitro Cdk5 kinase assay. Cdk5 protein was immunoprecipitated from PC12 cell lysate with a specific antibody (DC-17, Upstate). The kinase assay was performed by washing immunoprecipitates three times with kinase reaction buffer [in mM: 50 HEPES (pH 7.0), 10 MgCl₂, and 1 DTT]. ExactaCruz matrix beads (sc-45039, Santa Cruz Biotechnology) bound to target proteins were incubated in kinase reaction buffer containing 10 μg of substrate (histone H1, 14-155, Upstate) and 20 μCi of [γ-32P]ATP (NEG502Z001MC, PerkinElmer) in a final volume of 40 ml at 30°C for 30 min.

Measurement of cAMP. PC12 cells were primed for 30 min with 1 mM 3-isobutyl-1-methylxanthine (IBMX, phosphodiesterase inhibitor) to prevent the degradation of cAMP before other treatments. PC12 cells were then scraped and lysed with lysis buffer (no. 250006, Cell Biolabs, San Diego, CA). The intracellular content of cAMP in cell lysates was measured with a cAMP ELISA kit (Cell Biolabs).

Reverse transcription-polymerase chain reaction. Total RNA was isolated from PC12 cells with the Spin Column RNA Miniprep Kit according to the manufacturer’s instructions (Genermark). Reverse transcription-polymerase chain reaction (RT-PCR) was performed.
with the One-Step RT-PCR Kit (Genemark). Primer sets were designed and synthesized according to database sequences (p35: 5'-taggatccgccatcaccctgcgagcgagggac-3' and 5'-tagtcgactcaccgatccagccccag-3'; GAPDH: 5'-tgatgacatcaagaaggtggtgaag-3' and 5'-tccttggaggccatgtgggccat-3'; β-actin: 5'-tgccgacagatagatgcagaa-3' and 5'-gcggatccacacggagtact-3'). The RT-PCR reactions were performed with 10 pmol of each primer and the following program: 30 min at 50°C and 2 min at 94°C, followed by 18 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, ending with a final extension at 72°C for 7 min. The amplified PCR products were separated by electrophoresis on a 0.8% agarose gel and visualized by ethidium bromide staining. A 1-kb DNA ladder (Genemark) was used as a size marker.

**MTT assay.** A modified colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to quantify the proliferation of PC12 cells (14). Yellow MTT compound (M5655, Sigma) is converted by living cells to blue formazan, which is soluble in dimethyl sulfoxide (DMSO, D8418, Sigma). The intensity of blue staining in the culture medium proportionally represented the number of living cells and was measured by an optical density reader (Anthos-2001) at 570 nm (20).

**Analysis of cell cycle distribution.** Propidium iodide staining was used to measure the DNA content of PC12 cells as previously described (14). DNA content was quantified by flow cytometry (FACScalibur, BD). The percentage of cells in the G1 phase of the cell cycle was determined with Cell Quest software (BD) (14).

**Immunocytochemistry.** After two phosphate-buffered saline (PBS) washes, PC12 cells cultured on coverslips were fixed for 10 min in 4% paraformaldehyde and 2% sucrose in PBS at room temperature (RT). After fixation, the cells were permeabilized in PBS buffer containing 0.3% Triton X-100 and 3% bovine serum albumin (USB, Cleveland, OH) for 2 min at RT. A PBS wash and a subsequent block step in 3% BSA-PBS were performed for an additional 15 min at RT. Antibodies used for immunostaining included anti-Cdk5 (DC-17, Upstate), anti-p35 (SC-820, Santa Cruz Biotechnology), and anti-StAR (FL-285, Santa Cruz Biotechnology). Images were scanned and recorded with Leica confocal microscopy (TCS NT).

**Reagents.** 8-Bromoadenosine 3',5'-cyclic monophosphate (8-BrcAMP, B7880), 8-bromoadenosine-3',5'-cyclic monophosphorothioate, Rp-isomer (Rp-8-BrcAMPS, B2432), 2',5'-dideoxycytidine (ddAdo, D7408), H-89 (B1427), IBMX (I7018), KG-501 (70845), KT-5720 (K3761), roscovitine (RV, R7772), and SQ-22536 (SQ, S153) were all purchased from Sigma. Recombinant NGF (no. 450-1) was purchased from PeproTech (Rocky Hill, NJ).

**Data analysis.** All values are denoted as means ± SE. In all cases, the Student’s t-test was used. A difference between two means was considered statistically significant with \( P < 0.05 \).

**RESULTS**

Cdk5 inhibition blocks cAMP-induced differentiation of PC12 cells. RV is a Cdk5 molecular inhibitor and prevents NGF-triggered differentiation (12). On the basis of analysis of differentiation indicators such as neurite initiation (defined as extensions longer than 2 cell body diameters), proliferation, and the cell cycle G1 distribution of PC12 cells, our results confirmed the RV inhibitory effects on NGF-triggered PC12 differentiation (Fig. 1A). In addition to the inhibition of NGF-triggered responses, RV blocked the morphological changes induced by 48-h treatment with 8-BrcAMP (a permeable analog of cAMP) in PC12 cells (Fig. 1B, top left). The quantitative results indicate that RV treatment significantly prevented Fig. 2. 8-BrcAMP induces p35 protein expression and cyclin-dependent kinase 5 (Cdk5) activity in PC12 cells. PC12 cells were treated with 8-BrcAMP (0.05 or 0.1 mM) or NGF (100 ng/ml) for 48 h. Cdk5 and p35 proteins in cell lysates were analyzed by Western blotting with specific antibodies. Expression of β-actin served as the internal control. Cdk5 activity was detected via an in vitro kinase assay after immunoprecipitation from lysates, and histone H1 was used as the specific substrate. Quantitative results of 8-BrcAMP effects on p35 protein expression and Cdk5 activity are shown at bottom. **P < 0.01 vs. 8-BrcAMP = 0 group.

**Fig. 2.** 8-BrcAMP induces p35 protein expression and cyclin-dependent kinase 5 (Cdk5) activity in PC12 cells. PC12 cells were treated with 8-BrcAMP (0.05 or 0.1 mM) or NGF (100 ng/ml) for 48 h. Cdk5 and p35 proteins in cell lysates were analyzed by Western blotting with specific antibodies. Expression of β-actin served as the internal control. Cdk5 activity was detected via an in vitro kinase assay after immunoprecipitation from lysates, and histone H1 was used as the specific substrate. Quantitative results of 8-BrcAMP effects on p35 protein expression and Cdk5 activity are shown at bottom. **P < 0.01 vs. 8-BrcAMP = 0 group.
Fig. 3. 8-Br-cAMP-induced cAMP response element binding protein (CREB) phosphorylation, p35 protein expression, and changes in the differentiation indicators are attenuated by protein kinase A (PKA) inhibition. A: PC12 cells were treated with 8-Br-cAMP (0.1 mM) and PKA inhibitors [H-89 (10 μM) or KT-5720 (1 μM)] for 48 h. Protein levels of phospho (p)-CREB, CREB, and p35 were detected by Western blotting with specific antibodies. Expression of β-actin served as the internal control. Quantitative results of protein levels are shown at bottom. **P < 0.01 vs. 8-Br-cAMP alone group.

B: PC12 cells were treated with 8-Br-cAMP (0.1 mM) with or without KT-5720 (1 μM) for 48 h. C: PC12 cells were treated with 8-Br-cAMP (0.1 mM) with or without H-89 (10 μM) for 48 h. Treatment groups are listed at top left. Neurite initiation was measured as described in MATERIALS AND METHODS (n = 3). Cell proliferation was evaluated by MTT assay as described in MATERIALS AND METHODS (n = 4). The control value was adjusted to 100%. PC12 cells were stained by propidium iodide, and the G1 phase distribution in cell cycle was analyzed by flow cytometry as described in MATERIALS AND METHODS (n = 4). *P < 0.05, **P < 0.01 vs. control group (a); +P < 0.05, ++P < 0.01 vs. 8-Br-cAMP group (b).
8-BrcAMP-triggered neurite initiation (Fig. 1B, top right). Alternatively, a decrease in cell proliferation and cell cycle arrest are phenomena of cell differentiation progress (24, 35). Therefore, MTT assay and flow cytometric analysis were performed, and the results showed that 8-BrcAMP treatment decreased PC12 cell proliferation (Fig. 1B, bottom left), while cell death was unaffected (data not shown). In addition, 8-BrcAMP-triggered G1 phase arrest of PC12 cells was also observed (Fig. 1B, bottom right). In regard to the influence of Cdk5 inhibition, RV treatment significantly prevented 8-BrcAMP-induced morphology changes, neurite initiation, decrease in cell proliferation, and cell cycle arrest. These findings suggest that Cdk5 activation may be involved in 8-BrcAMP-induced changes of PC12 cells. Subsequently, the effects of Cdk5 on the regulation of Cdk5 activity were explored. p35 is the major regulator of Cdk5 and is associated with PC12 differentiation (12); therefore, the involvement of 8-BrcAMP in p35-dependent Cdk5 activation was investigated. Our results indicate that a 48-h treatment with 0.1 mM 8-BrcAMP significantly increased p35 protein expression and Cdk5 activity in PC12 cells (Fig. 2, top). NGF treatment (100 ng/ml) was used as a positive control for the induction of p35/Cdk5 activation in PC12 cells. The quantitative results of cAMP-dependent regulation on p35 expression and Cdk5 activation are also shown (n = 3; Fig. 2, bottom). These results indicate that cAMP may regulate p35/Cdk5 activation and also suggest the possibility that p35/Cdk5 activation involves cAMP-triggered PC12 differentiation.

**PKA inhibition partially blocks 8-BrcAMP-induced PC12 differentiation.** Protein kinase A (PKA) is a common downstream component of cAMP signaling in neurons (43). Here, we used two molecular inhibitors (H-89 and KT-5720) to inhibit 8-BrcAMP-dependent activation of PKA. The serine-133 phosphorylation of CREB (PKA downstream target) and p35 protein expression were then monitored. The data indicate that both PKA inhibitors suppressed 8-BrcAMP-induced CREB phosphorylation and p35 protein expression were then monitored. The data indicate that both PKA inhibitors suppressed 8-BrcAMP-induced CREB phosphorylation and p35 protein expression in PC12 cells (Fig. 3A, top). The quantitative results of the effects of PKA inhibitors are shown in Fig. 3A, bottom (n = 3). In addition to CREB phosphorylation and p35 expression, the effects of PKA inhibitors on 8-BrcAMP-triggered differentia-
tion indicators are also reported in Fig. 3, B (for KT-5720) and C (for H-89). We discovered that PKA inhibition was able to reverse the differentiation-related actions (including neurite initiation, cell proliferation, and cell cycle distribution) induced by 8-BrcAMP. Alternatively, immunocytochemical analysis was utilized to investigate the subcellular localization of the p35/Cdk5 protein in PC12 cells. Corresponding to the results of the Western blot, the immunocytochemical images indicate that p35 protein levels in the 8-BrcAMP group were apparently higher than the levels in the control group; however, Cdk5 levels in both groups were comparable (Fig. 4, left and center). Although few processes of PC12 cells were observed in the control group, p35 protein was absent in these regions, which suggests that the original processes in the control group are p35/Cdk5 independent (arrows in control group, Fig. 4). After 8-BrcAMP treatment, the morphology of neurite initiation was shown, and the colocalization of p35 and Cdk5 proteins was observed in the periphery of PC12 cells, especially in the termini of the neurites (arrows in 8-BrcAMP group, Fig. 4). These data suggest that cAMP may modulate the function of p35/Cdk5 in the periphery (or termini) of PC12 cells and cause neurite initiation partially through PKA activation.

**CREB blockade downregulates cAMP-induced p35 expression.**

As shown by the results in Fig. 3, cAMP may stimulate p35 expression through CREB activation; therefore, the importance of CREB activation in cAMP-dependent p35 expression was subsequently examined. A blocker of CREB activation, KG-501, was recently reported (1, 28) and was utilized in our experiments. KG-501 treatment for 48 h in culture medium of PC12 cells was found to dose-dependently inhibit both p35 mRNA and protein expression induced by 8-BrcAMP (Fig. 5, top). The quantitative results of KG-501 effects on p35 mRNA and protein expression are shown in Fig. 5, bottom (n = 3).

**Adenylyl cyclase inhibition blocks NGF-stimulated cAMP production in PC12 cells.** NGF induces cAMP elevation in neuronal cells (9, 39); therefore, NGF itself is a possible upstream regulator that controls cAMP production and the...
subsequent differentiation of PC12 cells. We found that 48-h treatment with NGF elevated the accumulation of intracellular cAMP, while the addition of IBMX (a phosphodiesterase inhibitor; 1 mM) to NGF treatment inhibited the drastic digestion of cAMP by phosphodiesterase (Fig. 6). In addition, the NGF-stimulated increase in cAMP was significantly blocked by adenyl cyclase inhibitors [SQ (10 μM) and ddAdo (50 μM)] (Fig. 6). These data suggest that adenyl cyclase is involved in NGF-increased cAMP production in PC12 cells.

Inhibition of cAMP pathway interferes with actions of NGF in PC12 cells. Adenylyl cyclase inhibitors (ddAdo and SQ), cAMP antagonist [Rp-8-BrcAMPS (21)], and PKA inhibitors (H-89 and KT-5720) were used to investigate the involvement of adenyl cyclase, cAMP, and PKA in NGF-induced responses in PC12 cells. When adenyl cyclase was inhibited, we found that NGF-induced CREB phosphorylation and p35 mRNA/protein expression were significantly decreased (Fig. 7, A–C). Furthermore, cAMP antagonism also significantly di-

Fig. 7. NGF-triggered CREB phosphorylation and p35 expression are prevented by adenylyl cyclase inhibitors, a cAMP antagonist, and PKA inhibitors. A–C: PC12 cells were treated with NGF (100 ng/ml) with or without (C) adenyl cyclase (AC) inhibitors [SQ (10 μM) or ddAdo (50 μM)] for 48 h. D and E: PC12 cells were treated with NGF (100 ng/ml) with or without 8-bromoadenosine-3’,5’-cyclic monophosphorothioate, Rp-isomer (Rp-8-BrcAMPS, 0.05 or 0.1 μM) for 48 h. F: PC12 cells were treated with NGF (100 ng/ml) with or without PKA inhibitors [H-89 (10 μM) or KT-5720 (KT, 1 μM)] for 48 h. RNA was extracted from cell lysates, and semiquantitative RT-PCR was performed as described in MATERIALS AND METHODS. Protein was extracted from cell lysates, and Western blotting was performed. Expression levels of β-actin and GAPDH served as internal controls. The quantitative results of the indicated experiments are shown. *P < 0.05, **P < 0.01 vs. NGF alone group.
minished NGF-induced p35 mRNA/protein expression levels (Fig. 7, D and E). Finally, when PKA was inhibited, NGF-induced p35 protein expression was reduced (Fig. 7F). The quantitative results of CREB phosphorylation, p35 expression, and Cdk5 activation are shown in their respective panels (n = 3) in Fig. 7. In addition to the above findings, the effects of adenylyl cyclase inhibition on PC12 differentiation were further identified. The differentiation indicators of PC12 cells described in Fig. 1 were observed after NGF treatment for 48 h. Inhibition of adenylyl cyclase (by SQ, 10 μM) was shown to inhibit NGF-triggered morphology changes (Fig. 8A, top left) and effectively prevent NGF-induced neurite initiation (Fig. 8A, top right). The results also show that 48-h treatment with NGF reduced PC12 proliferation, and SQ reversed the reduction without cell death (Fig. 8A, bottom left). G1 phase distribution of PC12 cells increased after 48-h treatment with NGF, and the distribution was reversed by cotreatments with SQ (Fig. 8A, bottom right). In addition to SQ inhibition, PKA inhibition (by KT-5720 and H-89) was also tested, and the PKA inhibitors prevented the actions of NGF (Fig. 8, B and C). These results suggest that the one of the numerous pathways triggered by NGF that promote PC12 differentiation may involve cAMP production, PKA activation, CREB phosphorylation, p35 expression, and Cdk5 activation. A schematic representation of our findings is provided in Fig. 9.

**DISCUSSION**

The PC12 cell line is a popular tool for investigating the mechanisms underlying neuronal differentiation. Upon treatment with NGF, PC12 cells differentiate into sympathetic-like neurons (11, 18, 35). p35/Cdk5 activation was previously believed to be a main cause of NGF-induced PC12 differentiation, in which NGF mediates p35/Cdk5 activation through the ERK pathway (12). Some reports suggest that cAMP is also involved in NGF-induced responses of PC12 cells (9, 13, 39). However, cAMP was found to both induce the transactivation of the NGF receptor as well as modulate ERK1/2 activation.
Fig. 8. Inhibition of adenylyl cyclase and PKA block NGF-induced PC12 differentiation. A: PC12 cells were treated with NGF (100 ng/ml) with or without adenylyl cyclase inhibitor (SQ, 10 μM) for 48 h. Treatment groups in A: a, control group; b, NGF group; c, NGF+SQ group; d, SQ group. Cell morphology was recorded with an optical microscope (×40). B: PC12 cells were treated with NGF (100 ng/ml) with or without KT-5720 (1 μM) for 48 h. Treatment groups are listed at top left. C: PC12 cells were treated with NGF (100 ng/ml) with or without H-89 (10 μM) for 48 h. Treatment groups are listed at top left. Neurite initiation was measured as described in MATERIALS AND METHODS (n = 3). Cell proliferation was evaluated by MTT assay as described in MATERIALS AND METHODS (n = 4). The control value was adjusted to 100%. PC12 cells were stained by propidium iodide, and the G1 phase distribution in the cell cycle was analyzed by flow cytometry (n = 4). **P < 0.01 vs. control group (a); +P < 0.05, ++P < 0.01 vs. NGF alone group (b).
protein and Cdk5 activity induced by cAMP and NGF are (10, 27), our data showed that the increasing levels of p35 neurites initiated by cAMP or NGF are reportedly different increase in proliferation, and G1 phase arrest (Fig. 1 NGF-induced morphology changes, neurite initiation, a de-
crease in NGF were monitored. Our data indicate that RV prevented lation exists among NGF, cAMP, and p35/Cdk5 in promoting the differentiated PC12 cells. In addition, the literature indicates that NGF-triggered MEK/ERK activation may also contribute to p35 expression and Cdk5 activation. In addition to p35, there are likely some alternative regulators to Cdk5 activity in NGF-induced PC12 differentiation, which are not PKA/p35 dependent.

and neurite outgrowth (30). Therefore, a complicated correlation exists among NGF, cAMP, and p35/Cdk5 in promoting the differentiation of PC12 cells; however, the link between cAMP and p35/Cdk5 in neuronal differentiation remains unclear. Our previous findings indicate that cAMP regulates p35/Cdk5 activity in mouse Leydig cells. Here we determined that cAMP also acts as a mediator in the NGF pathway and promotes p35/Cdk5-dependent differentiation of PC12 cells.

RV inhibits NGF-dependent differentiation through Cdk5 inhibition (12). To understand whether our evaluation of PC12 differentiation is appropriate, the effects of RV on the actions of NGF were monitored. Our data indicate that RV prevented NGF-induced morphology changes, neurite initiation, a decrease in proliferation, and G1 phase arrest (Fig. 1A). In regard to cAMP, our data show that RV blocked cAMP-induced PC12 differentiation (Fig. 1B). Although RV partially rescues NGF/cAMP-differentiated PC12 cells from cell death induced by withdrawal of trophic factors (15), our data demonstrate the involvement of p35/Cdk5 in cAMP-dependent PC12 differentiation. Several lines of evidence indicate that cAMP may cause apoptosis of various tumor cell lines (2, 5, 17); however, we did not detect any cell death by Trypan blue staining and sub-G1 phase distribution (data not shown). Although the neurites initiated by cAMP or NGF are reportedly different (10, 27), our data showed that the increasing levels of p35 protein and Cdk5 activity induced by cAMP and NGF are comparable (Figs. 1, 2, and 8). These findings imply that even though different signaling pathways play a role in differentiation, NGF and cAMP do share partial downstream p35/Cdk5 signaling components. A recent report indicates the synergistic effect of NGF and dibutyryl cAMP (DBcAMP) on neurite outgrowth; it also indicates that >100 genes appeared to be similarly regulated by both NGF and DBcAMP. These findings suggest that these genes may contribute to the induction of neuronal differentiation by NGF through the downstream activation of the cAMP pathway (27). In addition, it has been reported that the GC box-binding protein Sp1 contributes to the regulation of p35 expression in neurons (34), and Sp1 was also found to be modulated by cAMP-dependent protein kinase (PKA) (33). Together with our findings, these findings imply that cAMP may be an upstream regulator of p35 expression in PC12 cells. Alternatively, cAMP regulates a PKA-independent signaling pathway that mediates neuritogenesis through Egr1 in PC12 cells (31). Therefore, the detailed mechanisms of cAMP-dependent p35/Cdk5 regulation in cell differentiation are still unclear and are worthy of further investigation.

To unravel the above enigma, the involvement of PKA and CREB in cAMP-p35/Cdk5 regulation was investigated by utilizing their molecular inhibitors. In addition to the common PKA inhibitors (H-89 and KT-5720) utilized, KG-501 is a new and original blocker of CREB activation that blocks cAMP induction of CREB-dependent target gene transcription through interference of the binding between the kinase-inducible domain (KID) domain of CREB and the KIX domain of the CREB-binding protein (1). Our results suggest that cAMP may regulate p35 expression, at least through PKA and CREB activation (Fig. 3). Supporting our findings, although the authors do not mention this point, Ravni and colleagues (31) showed that PKA inhibitor (H-89) significantly decreases forskolin (adenylyl cyclase activator)-induced total length of neurites, and there is a tendency that the number of neurites induced by forskolin per cell is affected by H-89. Alternatively, several lines of evidence indicate that p35/Cdk5 colocalization may be important for neuronal differentiation (12, 45). Our previous results (14, 20, 23) also show that the localization of the p35/Cdk5 protein complex can be an indicator of cell fate in both neuronal cells and tumor cells. Therefore, it is worthwhile to understand whether the p35/Cdk5 localization in PC12 cells changed after cAMP treatment. The immunocytochemical results indicate that cAMP increased p35 protein levels in PC12 cells and p35/Cdk5 colocalization in the periphery (arrows in 8-BrcAMP group, Fig. 4). In the termini of neurites, 8-BrcAMP-induced colocalization of p35/Cdk5 may promote neuritogenesis. Moreover, inhibition of PKA both prevented the 8-BrcAMP-induced increase of the p35 protein level and eliminated p35/Cdk5 colocalization (8-BrcAMP+KT group, Fig. 4). Interestingly, although inhibition of PKA decreased p35 expression in the termini, a few processes still existed (arrows in 8-BrcAMP+KT group, Fig. 4), which supports the previous findings indicating that the cAMP-dependent, PKA-independent pathway mediates neuritogenesis in PC12 cells (31). In addition to PKA inhibition, a CREB activation blocker (KG-501) significantly prevented 8-BrcAMP-induced p35 expression (Fig. 5). These results suggest that PKA and CREB activation were involved in cAMP-induced p35 expression and PC12 differentiation.
After understanding that cAMP acts via p35/Cdk5 to promote PC12 differentiation, the correlation of NGF and cAMP production in PC12 cells becomes the next question that needs to be further examined. Several lines of evidence indicate that NGF induces cAMP formation in neuronal cells including PC12 cells (9, 13, 39). Here we provided evidence indicating that NGF stimulates intracellular cAMP production through activating adenylyl cyclase (Fig. 6). However, we observed that NGF-induced stimulation of cAMP production was not obviously detected in the absence of IBMX treatment (data not shown). This observation may explain why the relationship between cAMP and NGF was previously questionable. Subsequently, the involvement of adenylyl cyclase activity, downstream cAMP, and PKA activation on NGF-induced p35 expression was confirmed (Fig. 7). The blockade of NGF-induced morphological changes by inhibition of adenylyl cyclase or PKA was also identified (Fig. 8). Therefore, we suggest that cAMP plays a role in NGF-induced p35/Cdk5 activation and PC12 differentiation. Alternatively, it has been demonstrated that p35 and Cdk5 expression in human neuroblastoma cells is regulated by activating Erk/Egr-1 and PKA/CREB, respectively (16). However, our present data provide evidence showing that PKA/CREB is important for cAMP-regulated p35 expression, while Cdk5 protein expression only slightly increased after cAMP treatment (Fig. 2). Correspondingly, our previous results in Leydig cells (21) also support the idea that Cdk5 is upregulated by cAMP. Importantly, H-89 did not fully reverse NGF-induced actions in Fig. 7F and Fig. 8C, which suggests that there are still additional PKA-independent regulations under NGF stimulation. ERK-related inhibitors were used to determine whether ERK-dependent steps are involved in NGF-induced Cdk5 activation. The results revealed that ERK inhibition partially blocked NGF-induced p35 expression and subsequent Cdk5 activation (Supplemental Fig. S1), which corresponds to the study of Harada et al. (12).1 Our unpublished data also indicated that some kinases might be involved in NGF-induced Cdk5 activation through direct phosphorylation. In addition, another Cdk5 activator, p39, is also a possible regulator of NGF-induced Cdk5 activation (44). On the other hand, PKA phosphorylation of Src is essential for cAMP and NGF signaling in PC12 cells, and PKA was identified as an important downstream target of NGF (29). Here we used molecular inhibitors to identify that cAMP plays a role in activating p35/Cdk5, and this regulation is one pathway that NGF acts through to promote the differentiation of PC12 cells. These observations imply that differentiation is a process that occurs through the simultaneous activation of multiple parallel signaling events rather than a single master pathway (31). This study sheds light on the involvement of cAMP in NGF-p35/Cdk5-related development of the central nervous system and provides a new target for future research in neurobiology.

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1 Supplemental Material for this article is available online at the Journal website.


