The Cyclic Adenosine Monophosphate-dependent Protein Kinase (PKA) Is Required for the Sustained Activation of Mitogen-activated Kinases and Gene Expression by Nerve Growth Factor

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Induction of neuronal differentiation of the rat pheochromocytoma cell line, PC12 cells, by nerve growth factor (NGF) requires activation of the mitogen-activated protein (MAP) kinase or extracellular signal-regulated kinase (ERK). cAMP-dependent protein kinase (protein kinase A (PKA)) also can induce differentiation of these cells. Like NGF, the ability of PKA to differentiate PC12 cells is associated with a sustained activation of ERKs. Here we show that maximal sustained activation of ERK1 by NGF requires PKA. Inhibitors of PKA partially blocked activation of ERK1 by NGF but had no effect on activation of ERK1 by EGF. Inhibition of PKA also reduced the ability of NGF and cAMP, but not EGF, to activate the transcription factor Elk-1, reduced the induction of both immediate early and late genes after NGF treatment, and blocked the nuclear translocation of ERK1 induced by NGF. We propose that PKA is an important contributor to the activation of ERK1 by NGF and is required for maximal induction of gene expression by NGF.

Nerve growth factor (NGF) promotes the differentiation of sympathetic and sensory neurons that is characterized by morphological features of neuronal differentiation (neurite formation) and changes in gene expression including the late gene transin (1–3). This differentiation has been examined extensively in the rat pheochromocytoma cell line, PC12 cells, a well-studied model of growth factor actions (4, 5). In PC12 cells, neuronal differentiation by NGF requires activation of the mitogen-activated protein (MAP) kinases (also called extracellular signal-regulated protein kinases, or ERKs) (6). The mechanisms by which NGF activates ERKs have been the subject of many studies. Upon NGF binding, activation of the NGF receptor, TrkA, triggers the assembly of a multimeric protein complex that includes the small monomeric G protein Ras (7). Ras activation triggers a cascade of phosphorylations on protein kinases that lie upstream of the ERKs (8). Epidermal growth factor (EGF) also induces ERK activation via Ras. Unlike that of NGF, the EGF activation of Ras and ERK triggers a mitogenic program within PC12 cells. The ability of NGF to trigger neuronal differentiation instead of proliferation is thought to depend, in part, on its ability to activate ERKs for long, sustained periods. Sustained activation of ERKs may be required for the translocation of ERKs into the nucleus where they induce a distinct set of gene expression (9). In contrast, ERK activation after EGF stimulation is transient. This is a consequence of the rapid termination of signals to ERK via a short feedback loop involving an ERK-dependent phosphorylation of the Ras activator SOS (10). This loop uncouples Ras-dependent activation of ERKs from upstream activators (11). The activity of this feedback loop is reflected in the transient activation of Ras by EGF. Interestingly, although NGF induces a sustained activation of ERKs, Ras activation after NGF treatment of PC12 cells is terminated rapidly (7). Since NGF activation of ERK is sustained despite the rapid inactivation of Ras, NGF may utilize Ras-independent pathways that are not inactivated rapidly to allow the sustained activation of ERKs. Here we test this hypothesis by examining the requirement of PKA, cAMP-dependent protein kinase, for NGF activation of ERK1.

We have recently identified a novel Ras-independent pathway by which cAMP induces sustained activation of ERKs in PC12 cells (12). This pathway involves the Ras-related small G protein Rap1 as well as the cAMP-dependent protein kinase, PKA. In this study, we examine the possibility that PKA also participates in NGF signaling to the MAP kinase cascade by providing NGF a Ras-independent pathway to ERKs. We show that inhibition of PKA can inhibit signaling of NGF to ERK, to the transcription factor Elk-1, and to a specific marker gene of differentiation and can block the nuclear translocation of ERK1 induced by NGF. In addition, we demonstrate that NGF as well as PKA can activate the small G protein Rap1 and that this activation is blocked by the PKA inhibitor PKI. Therefore, we propose that PKA participates in NGF signaling to ERK, in part via the activation of Rap1, and that this pathway contributes to the sustained activation of ERKs that characterizes NGF signaling.

EXPERIMENTAL PROCEDURES

Materials—PC12-GR5 cells were kindly provided by Rae Nishi (Oregon Health Sciences University, Portland, Oregon). A126-1B2 cells, PKA-deficient PC12 cells, and stromelysin-1 (transin) cDNA were provided by Gary Ciment (Oregon Health Sciences University). Plasmids encoding Elk-1/Gal-4, 5xGal4-E1b/luciferase, protein kinase inhibitor (cPKI), and loss-of-function mutant of PKI, cPKImut, were gifts of Rich...
and Maurer (Oregon Health Sciences University). Agarose-conjugated ERK1 (c-16) used in immunoprecipitations was purchased from Santa Cruz Biotechnology Inc. NGF was from Boehringer Mannheim. EGF was from Sigma. Forskolin, H89, and 8-CPT-cAMP were purchased from DiBulloch.

**Cell Culture—**PC12 cells and A126-IB2 cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% horse serum and 5% phenylmethylsulfonyl fluoride was used to identify transfectants. PC12 cells were maintained with 10% horse serum and 5% fetal calf serum on 100-mm plates to 50–60% confluence at 37 °C in 5% CO2 before harvesting. For immune complex assays and Northern blotting, cells were deprived of serum and maintained in Dulbecco’s modified Eagle’s medium for 16 h at 37 °C in 5% CO2 before treatment with various reagents. 10 μM H89 was added to the calcium phosphate transfection kit (Life Technologies, Inc.) 15 min before transfection with NGF (50 ng/ml) or forskolin (10 μM). Lipid-modified PKI peptide (sPKI) was added at 5 μM, 10 min before treatment with NGF.

**Transient Transfections and Luciferase Assay—**60–80% confluent PC12 cells were co-transfected with the indicated cDNAs using a calcium phosphate transfection kit (Life Technologies, Inc.) according to the manufacturer’s instructions. The vector pcDNA3 (Invitrogen Corp.) was added to each set of transfections to ensure that each plate received the same amount of DNA. Four h after transfection, cells were glycerol-shocked and allowed to recover in serum-containing media overnight. Cells were then starved overnight in supplemented serum-free media (N2) that contained Dulbecco’s modified Eagle’s medium with 5 mg/ml insulin, 100 μg/ml transferrin, 30 μM sodium selenite, 100 μM putrescine, 10 ng/ml heparin, 0.5 μM ascorbic acid, 100 ng/ml apotransferrin, 30 μM sodium fluoride, 10 μg/ml basic fibroblast growth factor from CalBiochem. NGF was from Sigma. Forskolin, H89, and 8-CPT-cAMP were purchased from DiBulloch.

**Northern Blotting using this cRNA probe were done as described previously (13).** MKP-2 riboprobe synthesis and analysis by Northern blotting was done as previously described (13). RNase Protection was done as described previously (13). MKP-2 riboprobe synthesis and Northern blotting using this cRNA probe were done as described previously (13). All filters were scanned and quantitated using a Molecular Dynamics PhosphorImager 445SI. The GTP fraction was calculated as follows: (GTP counts/3)/(GTP counts/3 + (GDP counts/2)). The addition of a preclearing step using activated charcoal. Nucleotide samples were spotted on a polyethyleneimine-cellulose chromatography plate along with GTP and GDP standards (Sigma), resolved in 1 M KH2PO4 (pH 3.4) at room temperature, and analyzed using a Molecular Dynamics PhosphorImager 445SI. The GTP fraction was calculated as follows: (GTP counts/3)/(GTP counts/3 + (GDP counts/2)).

**Results**

**Activation of ERK1 by cAMP and NGF Is Reduced by the Inhibitor of PKA, H89—**Both NGF and agents that activate PKA, including forskolin and the cAMP analog 8-CPT-cAMP, induce sustained activation of ERK1 in PC12 cells (Fig. 1). ERK1 activation by forskolin peaked by 20 min and remained elevated for at least one h. 8-CPT-cAMP induced a similar inhibition of ERK1 (Fig. 1A). The activation of ERK1 by forskolin was completely blocked by an inhibitor of PKA, H89 (17) (Fig. 1A), suggesting that the actions of cAMP on ERK1 require PKA. NGF activation of ERK1 was inhibited at multiple time points (20, 40, and 60 min of stimulation) in the presence of the PKA inhibitor H89 (Fig. 1, B and C). In contrast, EGF activation of ERKs was not blocked by H89 at 5 or 20 min (and only minimally blocked at 10 min) in the data presented in Fig. 1D, suggesting that H89 was preferentially acting on kinases downstream of NGF at this concentration.

**NGF Activation of ERK1 Is Reduced by the Protein Kinase Inhibitor PKI—**PKA can be inhibited by the protein kinase inhibitor PKI (PKA, a physiological inhibitor of PKA (18, 19). To test whether this specific inhibitor of PKA could alter NGF activation of ERK1, we treated wild type PC12 cells with a peptide corresponding to PKI sequences from amino acids 5 to 22 that had been shown to be a specific inhibitor of PKA (18, 19). This peptide was modified by the addition of a stearyl group at the amino terminus (sPKI) to allow penetration into the cell (20). In vivo, sPKI inhibited NGF stimulation of ERK1 minimally at early time points but showed significant inhibitory effects at later time points (20 and 40 min) compared with NGF-treated cells not receiving peptide (Fig. 2A). The addition of sPKI in vitro completely inhibited 8-CPT-cAMP-stimulated PKA activity (Fig. 2A, right panel), demonstrating that the addition of the stearyl group did not interfere with the ability of PKI to inhibit PKA catalytic activity. Unrelated peptides that contained the stearyl modification did not alter the ability of NGF to activate ERK1 at the time points examined, suggesting-
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A. Misra-Press, H. Yao, and P. J. S. Stork, unpublished observations.

MBP by ERK1 after stimulation of PC12 cells by NGF in the absence of H89 (top panel) or the presence of H89 (bottom panel). B. Autoradiograms showing the phosphorylation of MBP by ERK1 after stimulation of PC12 cells by EGF. Top panel, the time course of EGF actions on ERK1 in the absence of H89. Bottom panel, the time course of EGF actions on ERK1 in the presence of H89. The position of the phosphorylated MBP is indicated.
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Fig. 2. ERK1 activation by NGF is blunted in A126-1B2 cells and inhibited by PKI peptide (sPKI) in PC12 cells. A, inhibition of NGF-stimulated ERK activation by PKI peptide. PC12 cells were pretreated with or without sPKI peptide (20 μM) for 20 min and then treated with NGF (50 ng/ml) or forskolin (Forsk., 10 μM) for the time indicated. Cells were lysed at the indicated times, and ERK1 kinase assays were performed as described in Fig. 1A. Right panel, the inhibition of PKA in vitro by purified PKI protein and sPKI peptide is shown to control for the function of sPKI. H89 was utilized as a positive control for PKA inhibition. B, ERK1 activation in PKA-deficient cells. A126-1B2 cells were treated with NGF in the same way as with PC12 cells. Equal amounts of protein was assayed for ERK1 activity. The time course of ERK1 activation by NGF in these cells was compared with wild type (wt) PC12 cells. Note that NGF-induced ERK1 activity at later time points was reduced in A126-1B2 cells. The inability of 8-CPT-cAMP to activate ERKs in these cells is shown as a control.

Maximal Induction of the Late Gene Transin by NGF Requires PKA—The expression of the metalloprotease transin (stromelysin) is stimulated by NGF but not EGF and has been used as a marker for neuronal differentiation of PC12 cells (1, 30, 31). Its induction by NGF is dependent on Ras, the MAP kinase kinase Raf, and ERKs (13, 32). It has been shown that this induction requires multiple transcription factors, including those of the Ets family (33, 34). NGF induces low levels of transin mRNA in PC12 cells within 4 h (30), and by 24 h of stimulation, expression levels could be easily detected by Northern blot (Fig. 3C). 8-CPT-cAMP could not stimulate the expression of transin in the absence of additional agents (30) (Fig. 3C). However, 8-CPT-cAMP dramatically enhanced the ability of NGF to stimulate transin (data not shown), suggesting that NGF and PKA are synergistic in their induction of transin gene expression. The synergistic action of NGF and 8-CPT-cAMP on transin expression reflects the action of these agents on neurite outgrowth as well (35–37). The participation of PKA signaling in NGF induction of transin mRNA was examined using H89. Preincubation with H89 blocked the induction of transin mRNA by NGF, suggesting that PKA activation was required for NGF induction of this gene (Fig. 3C). Neither treatment with 8-CPT-cAMP or H89 alone stimulated transin mRNA to detectable levels (Fig. 3C). These data demonstrate that maximal induction of specific immediate early and late genes by NGF may require PKA.

NGF Does Not Stimulate Detectable PKA Kinase Activity in PC12 Cells—Since we observed the involvement of PKA in NGF signaling, we determined whether NGF could stimulate total cellular PKA activity directly. Based on the kinetics shown in Fig. 2, we treated cells with NGF over a time course extending from 5 to 40 min and examined total PKA activity retained within lysates prepared from those cells. We could not detect increases in PKA activity after NGF treatment at any of the time points examined (Fig. 4A). Similar results were seen after EGF treatment (Fig. 4B). In contrast, 8-CPT-cAMP induced a 5-fold increase in PKA activity at the time points examined (Fig. 4B). The activity within all lysates could be stimulated in vitro by cAMP except cells pretreated with H89, demonstrating the presence of PKA and the action of H89 on PKA in this experiment (Fig. 4B). These results are consistent with previous reports showing that adenyl cyclase and PKA activities are not significantly increased after NGF stimulation of these cells (38, 39). Taken together, these data raise the possibility that a fraction of the total cellular pool of PKA is activated by NGF. Alternatively, basal activity of PKA may play a permissive role in NGF regulation of ERKs.

NGF Activation of the B-Raf Activator Rap1 Requires PKA—The PKA activation of ERK in PC12 cells requires Rap1, a small GTP-binding protein in the Ras superfamily. Rap1 is a selective activator of one of the isoforms of MAP kinase kinase kinase Raf, and the expression of B-Raf is required for Rap1 to activate ERKs (12). We show data that suggest that NGF utilizes PKA to activate ERK. It is possible that PKA participates in the NGF stimulation of ERK via its actions on Rap1. To test this hypothesis, we examined the GTP loading of transfected histidine-tagged Rap1 after transfection in PC12 cells. Both 8-CPT-cAMP and NGF increased GTP loading of Rap1 in this assay (Fig. 5), raising the possibility that Rap1 participates in NGF signaling. Activation of Rap1 by both NGF and 8-CPT-cAMP was reduced to basal levels by the co-transfection of cPKI, suggesting that PKA contributes to the activation of Rap1 by both NGF and 8-CPT-cAMP. Since Rap1 mediates the ability of PKA to activate ERKs in PC12 cells (12), the activation of Rap1 by PKA may contribute to NGF activation of ERKs in these cells.4

PKI Can Block the Induction of Neurites Induced by 8-CPT-cAMP but Not NGF—We have previously shown that Rap1 activation is not required for the elaboration of neurites seen after NGF treatment of PC12 cells (12). However, it is possible that PKA may have other actions in NGF-treated cells that are required for the differentiation phenotype. To determine whether neuronal differentiation by NGF was dependent on

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H89 Blocks Nuclear Translocation of ERK1 by NGF—Nuclear translocation of ERKs in PC12 cells has also been associated with their sustained activation and can be detected by immunofluorescence after NGF but not EGF treatment of these cells (6). Nuclear localization of ERK1 was seen 90 min after NGF treatment (Fig. 7C). In untreated cells, ERK1 was mainly in cytoplasm (Fig. 7A). The nuclear staining seen after NGF treatment was blocked by pretreatment with H89 (Fig. 7E), suggesting that PKA was required for this action of NGF. Parallel samples prepared and incubated with normal rabbit serum showed no nonspecific staining (Fig. 7, B, D, and F).

DISCUSSION

The ability of NGF to stimulate differentiation of PC12 cells has been associated with a sustained activation of ERKs. In contrast, EGF activation of ERKs was rapidly terminated (9). The signaling pathways that are selectively activated by NGF to permit sustained activation of ERKs have not been fully elucidated. PKA also stimulates neuronal differentiation of PC12 cells and induces a sustained activation of ERKs (41). In this study we examined the possibility that PKA contributes to the sustained activation of ERKs seen after NGF treatment. We showed that maximal activation of ERKs by NGF was diminished in PC12 cells in three independent assays using two inhibitors of PKA, PKI and H89, and in a clonal isolate of PC12 cells that is deficient in cAMP signaling.

PKA has been previously proposed to be required for a subset of NGF actions in PC12 cells (42). Inhibiting PKA blocks selected actions of NGF at transcriptional (43, 44) as well as post-transcriptional levels (45, 46). NGF can augment cAMP production in some neuronal cells (47) and has been reported to stimulate the production of cAMP in PC12 membranes (48, 49). However, neither the direct demonstration of an increase in adenyl cyclase nor in PKA activity by NGF in PC12 cells has been established (38, 39). We were also not able to detect PKA activation by NGF in PC12 cells (at 5, 10, 15, 20, and 40 min), raising the possibility that only a fraction of the total cellular PKA might be activated by the NGF signaling complex. This may be achieved by the activation of a distinct subcellular pool of PKA, either directly or indirectly, through the regulation of specific adenylyl cyclases or phosphodiesterases (50). Alternatively, PKA might be recruited to specific subcellular compartments in response to NGF to form a signal complex with activators of downstream effectors of NGF, such as Rap1.

Subcellular localization of PKA with specific signaling complexes may be achieved via specific targeting proteins. For example, PKA may be anchored to multienzyme complexes via a diverse family of PKA-anchoring proteins (20, 51, 52). In addition, the adaptor molecule Grb2 has been implicated in targeting PKA to growth factor receptors (53). Other kinases are also targeted to components of the NGF receptor signaling complex (54, 55). One of these kinases, protein kinase N, has been reported to activate PKA directly (56, 57). Therefore, it is possible that PKA may participate in a signaling complex through a direct interaction with these proteins that associate with the NGF receptor/tyrosine kinase cascade.
In PC12 cells, NGF induces a sustained activation of ERKs that is associated with neuronal differentiation (9). The inhibition of PKA by H89 or PKI in these cells or through the use of PC12-derived PKA-deficient cells blocked the sustained activation of ERKs by NGF significantly more than it blocked the rapid, initial portion of ERK activation. However, neither method of PKA inhibition interfered with the ability of NGF to induce differentiation (Fig. 6) (13, 43, 60, 61). Therefore, PKA does not appear to be required for NGF induction of neurites in PC12 cells. Furthermore, although sustained activation of ERK is sufficient for triggering differentiation, it is not necessary (58, 59). Other signals than ERK activation might also be required for triggering this process, as suggested by others (62).

The ability of PKA to augment NGF signaling may be most important in the regulation of gene expression during differentiation. Previous reports have suggested that the induction of selected genes and proteins by NGF requires PKA (30, 39, 42, 44–46). Much of the transcriptional actions of PKA in neuronal cells are mediated by specific sites present in the promoter of many cAMP-responsive genes, called cAMP-responsive elements (63). Transcriptional activation by cAMP can occur via phosphorylation of a PKA-responsive site within the transactivation domain of the cAMP-responsive element binding protein (64). Another transcription factor, Elk-1, can also be activated by PKA in neuronal cells via PKA activation of the MAP kinase cascade (12). Elk-1 is a member of the Ets family of transcription factors and is activated by ERKs by direct phosphorylation (23, 24, 65). Stimulation of ERK and Elk-1 is required for full activation of the serum response element within the c-fos promoter (66–68) and other immediate early genes that are activated by NGF (69, 70). We show here that maximal activation of Elk-1 by NGF but not EGF requires PKA. Therefore, PKA may contribute to the specificity of NGF transcriptional effects via its activation of Elk-1.

PKA modulates NGF induction of representative early and late genes as well. We show here that one immediate early gene, MKP-2, also requires PKA for its maximal induction by NGF. The induction of late genes by NGF may also be regulated by PKA. One example is transin, whose expression is a...
Fig. 6. Inhibition of PKA does not interfere with neurite outgrowth. A, PC12 cells were transfected with RSV-β-gal and cPKI and treated with 8-CPT-cAMP or NGF for 2 days as indicated. Cells were then stained with X-gal as described under “Experimental Procedures.” β-Galactosidase was used as a marker for transfected cells; therefore, blue cells represent transfected cells. Cells with neurites were counted from both blue and white cells. PKI and β-galactosidase-transfected cells were treated with 8-CPT-cAMP (left panel) or NGF (right panel). The percentage of neurite-bearing cells is included in the text.

Fig. 7. Inhibition of PKA blocks nuclear translocalization of ERK1 by NGF. PC12 cells were left untreated (A and B) or treated with NGF (50 ng/ml) for 90 min (C–F). Cells were treated with NGF alone (C and D) or after a 15-min pretreatment with H89 (10 μM) (E and F). After fixation, cells were incubated with ERK1 antiserum (A, C, and E) or normal rabbit serum (B, D, and F) and visualized by fluorescent microscopy.

marker for neuronal differentiation (1, 30, 71). This may be mediated in part by NGF activation of ERKs, since the activation of the ERK substrate, Ets, has been implicated in transin activation (13, 34, 72).

One mechanism by which PKA might influence NGF signals to the nucleus is via regulation of nuclear translocation of ERKs. We have previously demonstrated that forskolin, an activator of adenylyl cyclase, could promote the nuclear translocation of ERKs in EGF-treated PC12 cells (13). Here, we show data that suggest that PKA may be required for the translocation of ERKs by cells treated with NGF alone. Since nuclear translocation of ERKs correlates with their sustained activation, the involvement of PKA in NGF signaling may be a consequence of its ability to augment the sustained activation of ERKs by NGF. It is also possible that PKA may have additional protein targets that help regulate the subcellular localization of ERKs in a manner that is independent of its action on ERK activity. In either case, the ability of PKA to increase the nuclear concentration of ERK proteins after NGF treatment may account, in part, for the PKA-dependent activation of Elk-1 by NGF as well as NGF induction of the genes encoding transin and MKP-2.

Although Ras was required for the actions of both NGF and EGF on ERKs in PC12 cells, the mechanisms that distinguish NGF and EGF signaling to ERKs are not known. In particular, it is not known how NGF can activate ERK for extended periods. The activation of Ras by both EGF and NGF is rapidly terminated, suggesting that sustained activation of ERKs by NGF involves pathways that are downstream or independent of Ras. We propose that the activation of PKA by NGF allows NGF to activate Ras-independent pathways that are not rapidly terminated. One potential Ras-independent pathway that is activated by PKA involves the small G protein Rap1. Rap1 has been recently shown to be required for the sustained activation of ERKs by PKA in PC12 cells (12). We show here that Rap1 is activated after NGF treatment and that this activation requires PKA. The ability of NGF to activate Rap1 distinguishes it from EGF (12). Rap1 activation stimulates ERKs in PC12 cells via its direct activation of the MAP kinase kinase kinase B-Raf, the only known effector of Rap1 (Fig. 6) (12). We suggest that the activation of Rap1 by NGF may account for the high level of B-Raf activity seen after NGF treatment of PC12 cells (73, 74). We propose that NGF activation of ERK may be enhanced by its activation of Rap1 via the action of PKA.
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