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Cyclic AMP Induces Transactivation of the Receptors for Epidermal Growth Factor and Nerve Growth Factor, Thereby Modulating Activation of MAP Kinase, Akt, and Neurite Outgrowth in PC12 Cells*

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In PC12 cells, a well studied model for neuronal differentiation, an elevation in the intracellular cAMP level increases cell survival, stimulates neurite outgrowth, and causes activation of extracellular signal-regulated protein kinase 1 and 2 (ERK1/2). Here we show that an increase in the intracellular cAMP concentration induces tyrosine phosphorylation of two receptor tyrosine kinases, *i.e.* the epidermal growth factor (EGF) receptor and the high affinity receptor for nerve growth factor (NGF), also termed Trk_A. cAMP-induced tyrosine phosphorylation of the EGF receptor is rapid and correlates with ERK1/2 activation. It occurs also in Panc-1, but not in human mesangial cells. cAMP-induced tyrosine phosphorylation of the NGF receptor is slower and correlates with Akt activation. Inhibition of EGF receptor tyrosine phosphorylation, but not of the NGF receptor, reduces cAMP-induced neurite outgrowth. Expression of dominant-negative Akt does not abolish cAMP-induced survival in serum-free media, but increases cAMP-induced ERK1/2 activation and neurite outgrowth. Together, our results demonstrate that cAMP induces dual signaling in PC12 cells: transactivation of the EGF receptor triggering the ERK1/2 pathway and neurite outgrowth; and transactivation of the NGF receptor promoting Akt activation and thereby modulating ERK1/2 activation and neurite outgrowth.

Neuronal development, differentiation, survival, and repair are subject to regulation by many different external signals under physiological and pathological conditions. For instance, the high affinity receptor for nerve growth factor (NGFR),[1] a receptor tyrosine kinase (RTK) also termed Trk_A, is an important mediator of development, differentiation and survival of neurons (1, 2).

The rat pheochromocytoma cell line PC12 is the best studied model of neuronal differentiation and survival. In these cells, nerve growth factor (NGF) causes survival upon serum-withdrawal and promotes neurite outgrowth. Activation of the epidermal growth factor receptor (EGFR), another RTK, can induce both proliferation and differentiation (3, 4); the latter response is strongly increased in EGFR-overexpressing cells (5). Activation of the extracellular signal-regulated kinases 1/2 (ERK1/2) pathway appears to play an important role in growth factor-mediated PC12 cell differentiation (5, 6). The mechanism of ERK1/2 activation by RTKs is well established and involves receptor autophosphorylation, recruitment of adaptor proteins such as Shc and Grb2 to the receptor, and activation of guanine nucleotide exchange factors acting on and thereby activating the small GTPase Ras. Active Ras recruits Raf kinases to the membrane, which leads to their activation and subsequent triggering of the ERK pathway (7).

Studies on the pro-survival effect of NGF in PC12 cells show that activation of phosphatidylinositol 3-kinase (PI3K) is critical for its protective effect (8). Upon activation, PI3K phosphorylates membrane phosphoinositides at the D-3 position. These 3′-phosphorylated phospholipids act as second messengers that mediate the diverse cellular functions of PI3K. One of the major targets of these lipid second messengers is the serine/threonine kinase Akt/protein kinase B (9). The amino terminus of Akt contains a pleckstrin homology domain that is thought to directly bind the phospholipid products of PI3K activation. This binding recruits Akt to the plasma membrane and induces a conformational change that allows the phosphorylation of Akt by the phosphoinositide-dependent kinases I and II at the residues Thr-308 and Ser-473, respectively (10), which results in the full activation of its kinase activity. The critical importance of Akt in NGF-induced survival has been demonstrated (11, 12).

Receptors acting through an elevation of the intracellular cAMP level ($[cAMP]_i$) are important mediators of neuronal differentiation and survival. Cyclic AMP can induce biological responses such as neuronal survival or differentiation on its own or it can potentiate the effects of RTKs (13–15). In PC12 cells, elevation of $[cAMP]_i$ induces morphological changes similar to NGF and survival in serum-free media (16, 17).

ERK1/2 activity is regulated by the cAMP-signaling pathway: whereas cAMP inhibits ERK1/2 in non-neuronal cells (18,

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[1] The abbreviations used are: NGFR, nerve growth factor receptor; NGF, nerve growth factor; $[cAMP]_i$, intracellular cAMP concentration; CPT-cAMP, 8-(4-chlorphenylthio)-cAMP; DMEM, Dulbecco's modified Eagle medium; EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated protein kinases 1 and 2; EGFR, EGF receptor; HB-EGF, heparin-binding EGF-like growth factor; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; RTK, receptor tyrosine kinase.

19), it activates ERK1/2 in neurons and PC12 cells (20–25). Activation of ERK1/2 by the cAMP signaling pathway is important for several cellular functions. For example, activation of ERK1/2 by cAMP is critical for long-term potentiation (26, 27). cAMP-induced ERK1/2 activation in PC12 cells has been proposed to be mediated by a Ras-dependent pathway (21, 28) or a Ras-independent pathway, in which cAMP causes Rap1 activation, which then activates B-Raf (24). The latter model is supported by the findings that Rap1 activates B-Raf *in vitro* (29), and elevation of $[cAMP]_i$ level stimulates Rap1 by direct activation of guanine nucleotide exchange factors acting on Rap1 and enhancing its GTP loading (30–32). However, several studies failed to show an essential role of Rap1 in cAMP-stimulated ERK1/2 activation in PC12 cells (33–37). A further possibility is that cAMP-induced ERK1/2 activation involves activation of Src kinases (37).

An increase in $[cAMP]_i$ may induce cellular survival by several distinct mechanisms such as phosphorylation of Bad (38) or glycogen synthase kinase-3 (39, 40). In addition, cAMP has been shown to activate Akt when this enzyme is overexpressed in 293 cells (41, 42). However, in sympathetic ganglion neurons as well as in PC12 cells, cAMP-induced survival appears to be Akt-independent (17, 43).

It has previously been shown that G protein-coupled receptors can utilize RTKs to modulate ERK1/2 activity (44, 45). Moreover, a recent study reports that activation of the adenosine A_{2A} receptor, a typical G_s-coupled receptor, leads to tyrosine phosphorylation of NGFR and thereby causes activation of Akt (46). In the present study, we investigated in PC12 cells the possible involvement of the EGFR and NGFR in cAMP-induced modulation of ERK1/2 and Akt cascades, neurite outgrowth, and survival upon serum withdrawal. Our results show that cAMP induces tyrosine phosphorylation of the EGFR, which mediates activation of the ERK pathway and neurite outgrowth; and activation of NGFR that mediates cAMP-induced Akt activation. cAMP-induced activation of Akt is not essential for its strong pro-survival effect, but modulates activation of ERK1/2 and neurite outgrowth.

EXPERIMENTAL PROCEDURES

Reagents—Forskolin, human recombinant EGF, Hoechst 33342, monoclonal anti-actin, affinity-purified horseradish peroxidase-conjugated anti-mouse, anti-rabbit, anti-goat, and anti-sheep IgG were obtained from Sigma. Anti-Shc, Grb2, Gab1, Ras, and anti-EGFR used for immunoblotting were from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-phosphotyrosine and anti-Shc were obtained from Transduction Laboratories (Lexington, KY). Enhanced chemiluminescence reagents, protein G-Sepharose, and x-ray films were obtained from Amersham Biosciences. LipofectAMINE 2000, Dulbecco's modified Eagle medium (DMEM), OptiMEM, fetal calf serum, and horse serum were from Invitrogen. NGF was obtained from Promega (Madison, WI). Neutralizing anti-NGF, AG1478, PD165393, K252a, 8-Br-cAMP, and 8-(4-chlorphenylthio)-cAMP (CPT-cAMP) were obtained from Calbiochem (San Diego, CA). The antibodies recognizing dually phosphorylated activated ERK1/2, pY-490-NGFR, pY-674/675-NGFR, as well as anti pS-473-Akt were from Cell Signaling (Beverly, MA). Goat polyclonal anti-EGFR used for immunoprecipitation, monoclonal anti-NGFR, anti-Akt, monoclonal anti-NGFR, and anti-ERK2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pY-1086-EGFR antibody was from BIOSOURCE (Camarillo, CA). Neutralizing anti-heparin-binding EGF-like growth factor (HB-EGF) was from R & D Systems (Minneapolis, MN).

Cell Culture—Parental PC12 cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). EGFR-overexpressing PC12 cells were kindly provided by Dr. P. Cohen (University of Dundee, UK). All PC12 cell lines were grown in DMEM containing 10% horse serum and 5% fetal calf serum. Panc-1 cells were cultured in DMEM containing 10% fetal calf serum. Human mesangial cells were isolated and cultured as described previously (47). Cells were serum-starved overnight prior to their exposure to stimuli in serum-free DMEM.

DNA Constructs—The cDNA encoding dominant-negative Akt (K179M-Akt) in pCMV6 expression vector was kindly provided by Dr. T. Franke (Columbia University, New York, NY). The cDNA containing dominant-negative Ras (N17Ras) in pUSE was from Upstate Biotechnology.

Transfection—PC12 cells were transiently transfected using LipofectAMINE 2000 according to the instructions of the manufacturer. Similar to a recent study (36), the efficiency of the transfection as monitored by transfecting green fluorescent protein (GFP, Clontech, Palo Alto) exceeded 50%. Expression of the constructs was verified by immunoblotting.

Detection of Neurite Outgrowth—Cells grown in 24-well dishes were exposed to forskolin, CPT-cAMP, EGF, NGF, or vehicle for 24 h in serum-containing DMEM. Cells were visualized by phase-contrast microscopy, and representative cells were photographed with a CCD camera. Images were prepared using Adobe Photoshop 6.0 software.

Cell Death Assay/Detection of Apoptosis—Cells grown in 24-well dishes were switched to serum-free DMEM, and forskolin, CPT-cAMP, or growth factors were added to the media. After 24 h, cell death was quantified by measuring lactate dehydrogenase (LDH) released from injured cells into the media by using the Cytox 96 Cytotoxicity Assay kit (Promega). LDH values were expressed as the percent of the full kill reference, *i.e.* LDH activity after a freeze/thaw cycle. Apoptotic cells were assessed by Hoechst 33342 staining. Normal nuclei show faint delicate chromatin staining, nuclei at the early stage of apoptosis display increased condensation and brightness, and nuclei at the late stage of apoptosis exhibit chromatin condensation and nuclear fragmentation.

Immunoprecipitation of the EGFR—For the experiments, 80% confluent serum-starved cells were used. About 3×10^6 cells grown in culture flasks were incubated with indicated agents at 37 °C. At specified times, the incubation was stopped by the addition of lysis buffer (50 mM Hepes, pH 7.0, 100 mM NaCl, 0.2 mM $MgSO_4$, 0.5 mM Na_3VO_4, 0.4 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin). The EGFR was immunoprecipitated by the addition of anti-EGFR antibody. After an incubation for 2 h at 4 °C with gentle agitation, protein G-Sepharose was added and the incubation was continued for 2 h. Immunoprecipitates were washed three times in lysis buffer, resuspended in 2× SDS sample buffer, boiled for 4 min, and separated on SDS-polyacrylamide gels under reducing conditions.

Immunoblotting—Gel-resolved proteins were electrotransferred to polyvinylidene difluoride sheets, and immunoblotting was performed as recently described (48, 49). Antigen-antibody complexes were visualized using horseradish peroxidase-conjugated antibodies and the enhanced chemiluminescence system. X-ray films were scanned and processed by Adobe Photoshop 6.0 software.

Reproducibility of Results—Results are representative of at least three experiments on different occasions giving similar results.

RESULTS

To investigate whether the EGFR participates in cAMP-induced signaling, we examined the effects of forskolin, a direct activator of adenylyl cyclase, and of membrane-permeable cAMP analogs (8-Br-cAMP, CPT-cAMP) on tyrosine phosphorylation of the EGFR by anti-phosphotyrosine immunoblotting of EGFR immunoprecipitates. As illustrated in Fig. 1, *A* and *B*, forskolin and 8-Br-cAMP caused rapid and transient tyrosine phosphorylation of the EGFR with a maximum after 3–5 min. A similar result was obtained in EGFR-overexpressing PC12 cells, when cellular lysates were analyzed by immunoblotting with an antibody that recognizes specifically the tyrosine phosphorylated EGFR (Fig. 1*C*).

The effect of cAMP on tyrosine phosphorylation of the NGFR was analyzed by immunoblotting of cell lysates with antibodies recognizing specifically phosphorylated forms of the NGFR. Forskolin induced tyrosine phosphorylation of the NGFR as revealed by immunoblotting of cellular lysates with an antibody recognizing specifically pY-674/675-NGFR in the activation loop (Fig. 1*D*). A similar result was obtained when immunoblotting was performed with an antibody recognizing pY-490-NGFR, the phosphorylation of which is crucial for NGF-induced ERK1/2 activation, differentiation, and activation of PI3K (50, 51). Compared with its effect on EGFR tyrosine phosphorylation, the kinetics of forskolin-induced tyrosine phosphorylation of NGFR was less rapid and more sustained

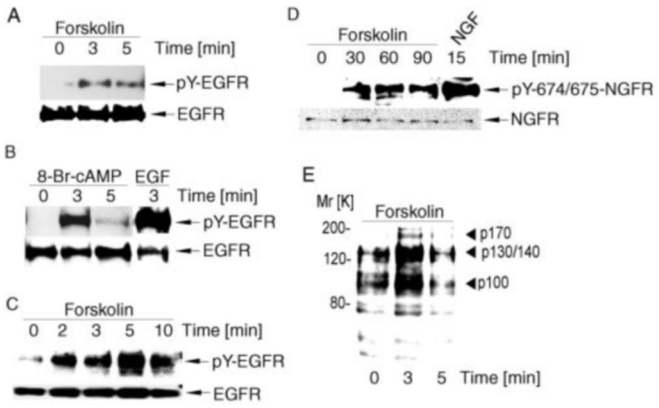

FIG. 1. **Forskolin and 8-Br-cAMP induce tyrosine phosphorylation of the EGFR and NGFR.** Parental (*A*, *B*, and *E*) or EGFR- (*C*) or NGFR-overexpressing PC12 cells (*D*) were exposed to forskolin (20 μM), 8-Br-cAMP (200 μM), EGF (10 ng/ml), or NGF (10 ng/ml) for the indicated time periods at 37 °C in serum-free DMEM. The incubation was terminated by replacement of the medium with lysis buffer. *A* and *B*, cell lysates were immunoprecipitated with anti-EGFR antibody followed by analysis of the immunoprecipitates by anti-phosphotyrosine immunoblotting. Antigen-antibody complexes were visualized by horseradish peroxidase-conjugated antibodies and the enhanced chemiluminescence system. To determine loading, the blot was stripped of the antibody and reprobed with anti-EGFR. *C*, cell lysates from EGFR-overexpressing cells were analyzed by immunoblotting with an antibody recognizing pY-1086-EGFR. The blot was stripped and reprobed with anti-EGFR. *D*, cell lysates from NGFR-overexpressing cells were analyzed by immunoblotting with an antibody recognizing pY-674/675-NGFR. The blot was stripped and reprobed with anti-NGFR. *E*, cell lysates from parental cells were analyzed by immunoblotting with anti-phosphotyrosine immunoblotting.

(maximum after 60 min). Thus, our data indicate that cAMP induces tyrosine phosphorylation of the receptors for EGF and NGF in PC12 cells.

Analysis of total cellular lysates from PC12 cells stimulated with forskolin for 3–5 min by anti-phosphotyrosine immunoblotting shows that forskolin induced rapid increase in tyrosine phosphorylation of several protein bands (Fig. 1*E*). Major forskolin-responsive bands migrated at 170, 130/140, and 100 kDa. pp170 comigrated with the EGFR. Longer periods of stimulation of the cells with forskolin did not result in detectable increase in protein tyrosine phosphorylation. These data indicate that forskolin induces rapid tyrosine phosphorylation of several proteins in addition to the EGFR and NGFR.

To assess whether cAMP-mediated EGFR tyrosine phosphorylation is a general phenomenon or whether it is confined to PC12 cells, we studied the effect of forskolin on EGFR tyrosine phosphorylation in Panc-1 cells, a pancreatic carcinoma cell line with moderate EGFR expression. As illustrated in Fig. 2, incubation of Panc-1 cells with forskolin also caused tyrosine phosphorylation of the EGFR. In human mesangial cells, however, we did not detect forskolin-induced EGFR tyrosine phosphorylation (data not shown). Thus, cAMP-induced EGFR tyrosine phosphorylation appears to occur in some, but not all EGFR-expressing cell types.

RTK activation involves complex formation of the EGFR with the adaptor proteins Grb2, Shc, and Gab1, and tyrosine phosphorylation of SH2-domain-containing substrates such as Shc (7). Immunoprecipitation of Shc and analysis of the immunoprecipitates with anti-phosphotyrosine showed that forskolin caused tyrosine phosphorylation of Shc (Fig. 3*A*). The specific EGFR tyrosine kinase inhibitor AG1478 abolished forskolin-induced tyrosine phosphorylation of Shc. To investigate whether cAMP-induced tyrosine phosphorylation of the EGFR is accompanied by recruitment of adaptor proteins to the EGFR, the cells were stimulated with forskolin and EGFR

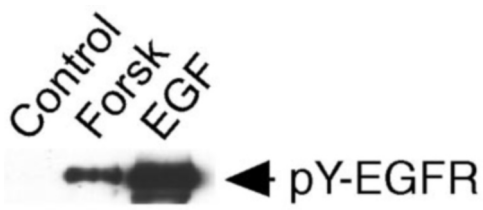

FIG. 2. **Forskolin induces tyrosine phosphorylation of the EGFR in Panc-1 cells.** Serum-starved Panc-1 cells were exposed to forskolin (20 μM) or EGF (10 ng/ml) for 3 min at 37 °C in serum-free DMEM. The incubation was terminated by replacement of the medium with lysis buffer. Cell lysates were immunoprecipitated with anti-EGFR antibody followed by analysis of the immunoprecipitates by anti-phosphotyrosine immunoblotting. Antigen-antibody complexes were visualized by horseradish peroxidase-conjugated antibodies and the enhanced chemiluminescence system.

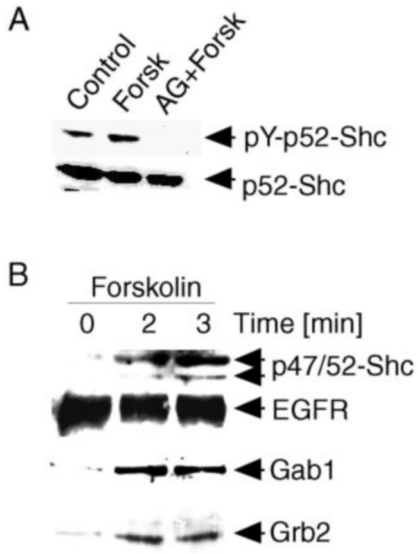

FIG. 3. **Forskolin induces tyrosine phosphorylation of Shc and complex formation of the EGFR with Shc, Grb2, and Gab1.** PC12 cells were exposed to forskolin (20 μM) in the presence or absence of AG1478 (250 nM) for 3 min (*A*) or the indicated time periods (*B*) at 37 °C in serum-free DMEM. The incubation was terminated by replacement of the medium with lysis buffer. *A*, cell lysates were immunoprecipitated with anti-Shc followed by analysis of the immunoprecipitates by anti-phosphotyrosine immunoblotting. *B*, cell lysates were immunoprecipitated with anti-EGFR followed by analysis of the immunoprecipitates by anti-Shc, anti-Grb2, and anti-Gab1 immunoblotting. Antigen-antibody complexes were visualized by horseradish peroxidase-conjugated antibodies and the enhanced chemiluminescence system. The blots were stripped of the antibodies and reprobed with anti-Shc (*A*) and anti-EGFR (*B*), respectively.

immunoprecipitates were analyzed by anti-phosphotyrosine, anti-Shc, anti-Grb2, and anti-Gab1. As shown in Fig. 3*B*, forskolin increased the amount of Shc, Grb2, and Gab1 coprecipitating with the EGFR, indicating that activation of adenylyl cyclase induces complex formation of the EGFR with Grb2, Shc, and Gab1. In contrast, our attempts to detect adaptor proteins in NGFR immunoprecipitates were unsuccessful because the immunoprecipitation of the NGFR was insufficient.

To investigate the possible involvement of the EGFR or NGFR in cAMP-induced ERK1/2 activation as well as neurite outgrowth, we tested the effects of forskolin in parental and EGFR overexpressing PC12 cells. ERK1/2 activity was detected by immunoblotting of cellular lysates with an antibody recognizing the dually phosphorylated active form of ERK1/2. Forskolin and CPT-cAMP caused robust neurite outgrowth in EGFR-overexpressing cells (Fig. 4*B*), whereas their effect on morphology of parental PC12 cells was minor (Fig. 4*A*). Forskolin- and CPT-cAMP-induced ERK1/2 activation were more

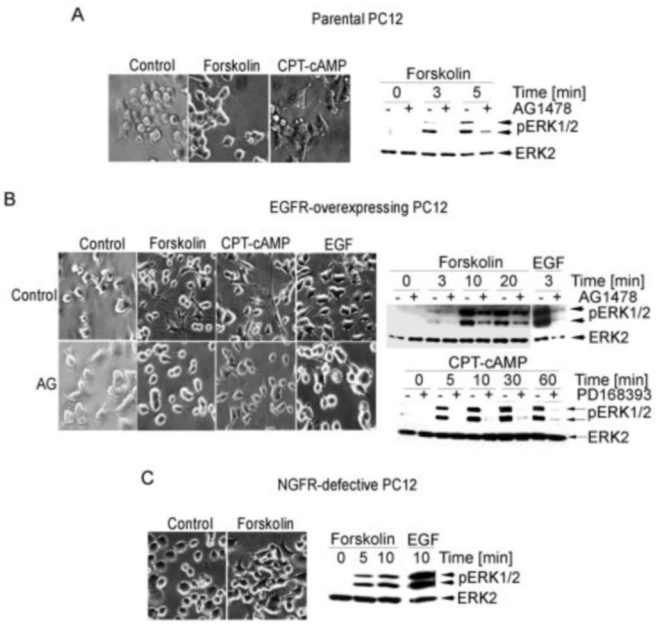

FIG. 4. **cAMP-stimulated ERK1/2 activation and neurite outgrowth are inhibited by AG1478 and enhanced by EGFR overexpression.** ERK1/2 activation: Parental (*A*), EGFR-overexpressing (*B*), or NGFR-defective (*C*) PC12 cells were exposed to forskolin (20 μM), 8-(4-chlorphenylthio)-cAMP (*CPT-cAMP*, 200 μM), or EGF (100 ng/ml) in the presence or absence of AG1478 (250 nM) (*AG*) or PD165393 (500 nM) for the indicated time periods at 37 °C in serum-free DMEM. The incubation was terminated by replacement of the medium with lysis buffer. Lysates were analyzed by anti-phospho-ERK1/2 immunoblotting. Equal loading of the lanes was controlled by reprobing of the blots with anti-ERK2. Neurite outgrowth: PC12 cells were treated with forskolin, CPT-cAMP, or EGF in the presence or absence of AG1478 for 48 h. Phase-contrast images of representative cells were taken with a CCD camera.

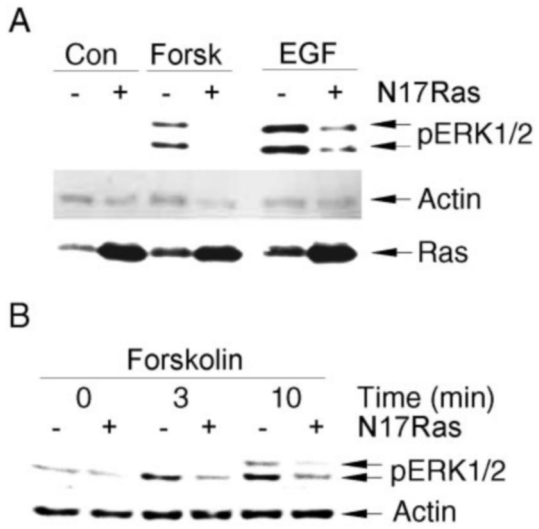

FIG. 5. **Expression of dominant-negative Ras (N17Ras) abolishes forskolin-induced ERK1/2 activation.** Parental (*A*) or EGFR-overexpressing (*B*) PC12 cells grown in 24-well dishes were transfected with N17Ras (+) or empty vector (−). On the next day, cells were switched to serum-free DMEM, and the cells were incubated with forskolin (20 μM) or EGF (10 ng/ml) for 3 min (*A*) or the indicated time (*B*) at 37 °C in serum-free DMEM. The incubation was terminated by replacement of the medium with lysis buffer. Lysates were analyzed by anti-phospho-ERK1/2 immunoblotting. The loading of the lanes was controlled by anti-actin immunoblotting. Overexpression of N17Ras was confirmed by anti-pan-Ras immunoblotting; *Con*, control

pronounced and sustained in cells overexpressing the EGFR (Fig. 4, *A* and *B*), and its kinetics correlated well with EGFR tyrosine phosphorylation (Fig. 1, *A–C*). EGFR tyrosine kinase inhibition by AG1478 strongly reduced ERK1/2 activation as well as neurite outgrowth in response to forskolin or membrane-permeable cAMP analogs (Fig. 4, *A* and *B*), whereas this manipulation had no effect on NGF-induced responses (data not shown). Similarly, PD165393, another EGFR tyrosine kinase inhibitor, abolished forskolin and CPT-cAMP-induced ERK1/2 phosphorylation (Fig. 4*B*). This indicates that activation of the EGFR is involved in cAMP-induced ERK1/2 activation and neurite outgrowth. However, the inhibitory effect of AG1478 was less pronounced on cAMP-driven responses compared with its effect on the EGF response (Fig. 4*B*), indicating that the effects of cAMP are not entirely dependent on a functional EGFR and that additional mechanisms may participate in cAMP-induced ERK1/2 activation and neurite outgrowth. To investigate whether the NGFR participates in cAMP-induced ERK1/2 activation and neurite outgrowth, we studied the effect of forskolin on these responses in a NGFR-defective PC12 cell line. As illustrated in Fig. 4*C*, the ability of forskolin to induce ERK1/2 activation and neurite outgrowth was not impaired in NGFR-defective cells as compared with parental cells, indicating that the NGFR is not essential for cAMP-induced ERK1/2 activation and neurite outgrowth. In support of this assumption, cAMP-induced ERK1/2 activation and neurite outgrowth were not inhibited by the NGFR tyrosine kinase inhibitor K252a (data not shown).

Previous studies have provided evidence that $G_{i/q}$-coupled receptors can induce EGFR transactivation by proteolytic cleavage of EGF-like transmembrane precursor pro-HB-EGF by metalloproteinase activity (52, 53). Moreover, a recent study shows that the proforms of NGF and of brain-derived neurotrophic factor are secreted and cleaved extracellularly by the proteases and can thereby activate neurotrophin receptors (54). To investigate whether cAMP-induced tyrosine phosphorylation of the RTKs involves ligand-dependent mechanisms, we studied the effect of neutralizing anti-HB-EGF antibody on forskolin-induced EGFR tyrosine phosphorylation and ERK1/2 activation. Moreover, we tested the effect of anti-NGF antibody on forskolin-induced phosphorylation of the NGFR and Akt. All neutralizing antibodies had virtually no effect on forskolin responses, suggesting that cAMP-induced EGFR and NGFR activation are HB-EGF- and NGF-independent.

In PC12 cells, the mechanism of ERK1/2 activation by a rise in $[cAMP]_i$ has been claimed to be Ras-dependent (21, 28) or Ras-independent, but Rap1-dependent (24). We examined this controversial issue in parental and EGFR-overexpressing PC12 cells using transient overexpression of dominant-negative Ras (N17Ras) and detection of forskolin-induced ERK1/2 phosphorylation. Expression of N17Ras was verified by anti-Ras immunoblotting (Fig. 5*A*) and inhibited forskolin-induced ERK1/2 phosphorylation in parental (Fig. 5*A*) as well as in EGFR-overexpressing cells (Fig. 5*B*). Expression of N17Ras inhibited forskolin-induced ERK1/2 phosphorylation to a similar extent as the EGF response. These data indicate the involvement of Ras in cAMP-induced ERK1/2 activation.

Another response elicited by activated EGFR and NGFR is stimulation of Akt, which mediates the pro-survival effects of RTK, including that of NGF in neuronal cells by activation of Akt (12). Because cAMP induces tyrosine phosphorylation of both the EGFR and NGFR, we investigated whether cAMP-induced tyrosine phosphorylation of these RTKs is coupled to activation of Akt. Akt activation was detected by immunoblotting of cellular lysates with an antibody that specifically recognizes pS-473-Akt. Forskolin or CPT-cAMP increased Akt phosphorylation (Fig. 6, *A* and *B*). The kinetics of forskolin/CPT-cAMP-induced phosphorylation of Akt was considerably slower than that for Akt activation by EGF and NGF and

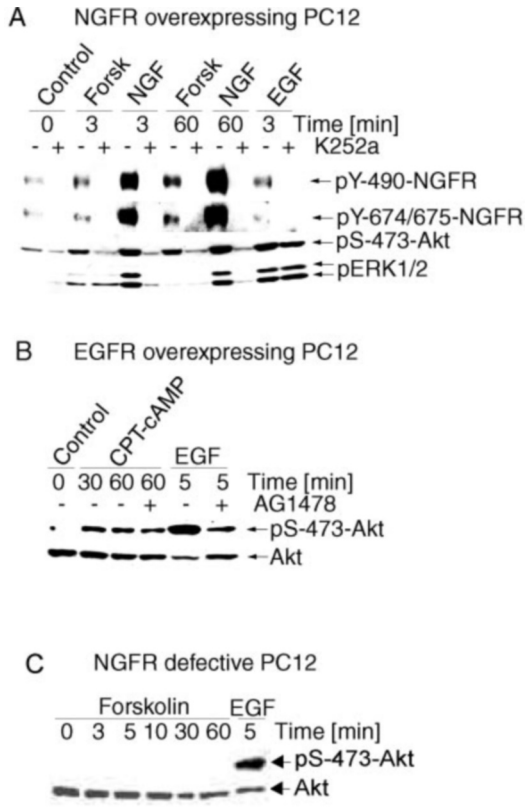

FIG. 6. **Forskolin/CPT-cAMP induces activation of Akt by a NGFR-dependent mechanism.** NGFR-overexpressing (*A*), EGFR-overexpressing (*B*), or NGFR-defective (*C*) cells were incubated with forskolin (*Fork*, 20 μM), NGF (100 ng/ml), or EGF (100 ng/ml) in the presence or absence of K252a (100 nM) or AG1478 (250 nM) at 37 °C in serum-free DMEM for the indicated time. Lysates were then immunoblotted with the indicated antibodies. In *B* and *C*, the blots were stripped and reprobed with anti-Akt.

followed the kinetics of cAMP-induced phosphorylation of the NGFR on tyrosine 490 and tyrosines 674/675 (Fig. 6*A*). It did not correlate with cAMP-induced ERK1/2 activation (Fig. 6*A*) and EGFR tyrosine phosphorylation (Fig. 1, *A–C*). The NGFR tyrosine kinase inhibitor K252a blocked forskolin-induced Akt phosphorylation without influencing ERK1/2 phosphorylation, whereas AG1478 inhibited forskolin-induced ERK1/2 phosphorylation without influencing phosphorylation of Akt in response to forskolin (Fig. 6, *A* and *B*). In NGFR-defective cells, forskolin had no effect on Akt phosphorylation, whereas EGF caused rapid and strong activation of Akt (Fig. 6*C*). Thus, cAMP-induced activation of Akt depends critically on NGFR activation and is EGFR-independent, although activation of the EGFR by EGF causes Akt activation. These data suggest that the response patterns of EGFR and NGFR stimulated by cAMP only partially overlap with those induced by their cognate ligands, *i.e.* the cAMP-activated EGFR is coupled to ERK1/2 activation but not to activation of Akt, whereas the opposite holds for NGFR.

To investigate whether the restricted response pattern of cAMP-activated EGFR and NGFR are caused by differential efficacies in their coupling to the ERK1/2 and Akt cascade, we examined the dose-response curves for EGF and NGF to elicit activation of ERK1/2 and Akt. As can be inferred from Fig. 7, the dose-response curves of the growth factors to induce ERK1/2 and Akt phosphorylation were almost identical. Thus, the partial responses of cAMP-activated EGFR and NGFR may not be caused by differential efficacies in their coupling to the ERK1/2 and Akt cascades.

An increased level of $[cAMP]_i$ as well as EGF and NGF are

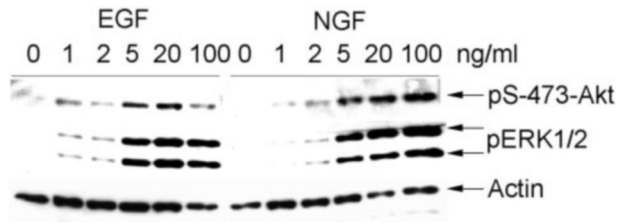

FIG. 7. **Concentration-dependent phosphorylation of ERK1/2 and Akt by EGF and NGF.** PC12 cells were exposed to the indicated concentration of EGF or NGF for 10 min at 37 °C in serum-free DMEM. The incubation was terminated by replacement of the medium with lysis buffer. Lysates were analyzed by anti-phospho-ERK1/2 immunoblotting. The loading of the lanes was controlled by anti-actin immunoblotting.

important pro-survival factors in PC12 cells and post-mitotic neurons (12, 14–17, 55, 56). Activation of Akt plays a key role in NGF-induced survival upon serum withdrawal (12). Here, we investigated whether cAMP-induced NGFR-dependent Akt activation contributes to the pro-survival effect of an elevated $[cAMP]_i$ as revealed by LDH release into the media and the number of Hoechst-positive cells. To this end, we investigated the effect of forskolin and dominant-negative Akt (K179M-Akt) on cell death of parental and NGFR-defective PC12 cells in serum-free media. As shown in Fig. 8, forskolin increased the survival of both parental and NGFR-defective cells. The trophic effect of forskolin appeared to be less pronounced in NGFR-defective cells. Expression of K179M-Akt did not reduce forskolin-induced pro-survival effect in both parental as well as NGFR-defective PC12 cells, whereas the effects of EGF and NGF were strongly reduced. Thus, our data indicate that the pro-survival effect of cAMP is independent of NGFR-dependent activation of Akt. AG1478 did not reduce forskolin-induced pro-survival effect (data not shown), indicating that the EGFR does not participate in cAMP-induced trophic effect as well.

Examining the effect of Akt on forskolin/CPT-cAMP-induced ERK1/2 activation and neurite outgrowth in PC12 cells, we found that expression of K179M-Akt significantly increased these responses (Fig. 9, *A* and *B*). This result suggests that Akt imposes an inhibitory effect on cAMP-induced ERK1/2 activation and neurite outgrowth. Thus, cAMP-induced activation of Akt may modulate ERK1/2 activation rather than cellular survival.

DISCUSSION

G_s-coupled receptors and its messenger cAMP are important modulators of growth, differentiation, and survival in the nervous system. In the present study, we show for the first time that cAMP can induce tyrosine phosphorylation of the receptors for NGF and EGF, and that these events may importantly modulate cellular homeostasis. A number of studies have shown that various stimuli such as G protein-coupled receptors (44), integrin (57), cytokine receptors (58), or membrane depolarization (59) can induce tyrosine phosphorylation of the EGFR. Moreover, it has been shown that ERK1/2 activation in response to G protein-coupled receptors, including the G_s-coupled β_2-adrenergic receptor in COS cells, depends on tyrosine phosphorylation of the EGFR (44, 60). cAMP-induced EGFR activation observed in the present study occurs in the absence of G-coupled receptor agonists. Thus, the mechanisms of EGFR tyrosine phosphorylation by elevated $[cAMP]_i$ in PC12 cells and through stimulation of the β_2-adrenergic receptor in COS cells are clearly different. Recent studies report the tyrosine phosphorylation of the NGFR by A_{2A} adenosine and pituitary adenylate cyclase-activating polypeptide receptors (46, 61). Here, we provide experimental evidence for a novel type of cAMP activity that is transactivation of two distinct RTKs in PC12

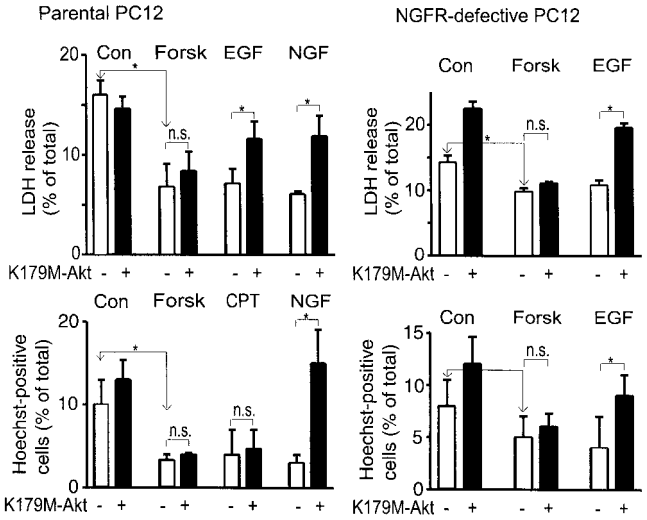

FIG. 8. **Effect of K179M-Akt expression on cAMP-induced cell death in serum-free media.** Parental or NGFR-defective PC12 cells grown in 24-well dishes were transfected with K179M-Akt (+) or empty vector (−). On the next day, cells were switched to serum-free DMEM, and forskolin, CPT-cAMP (*CPT*), EGF, or NGF were added. After 24 h, 100 μl aliquots of the supernatant were analyzed for LDH activity as described under "Experimental Procedures." Apoptotic cells were assessed by Hoechst 33342 (200 nM) staining. The values shown are means ± S.E. of the mean of 4 experiments. Statistical significance was calculated by ANOVA (*, $p < 0.05$); *Con*, control; *n.s.*, not significant.

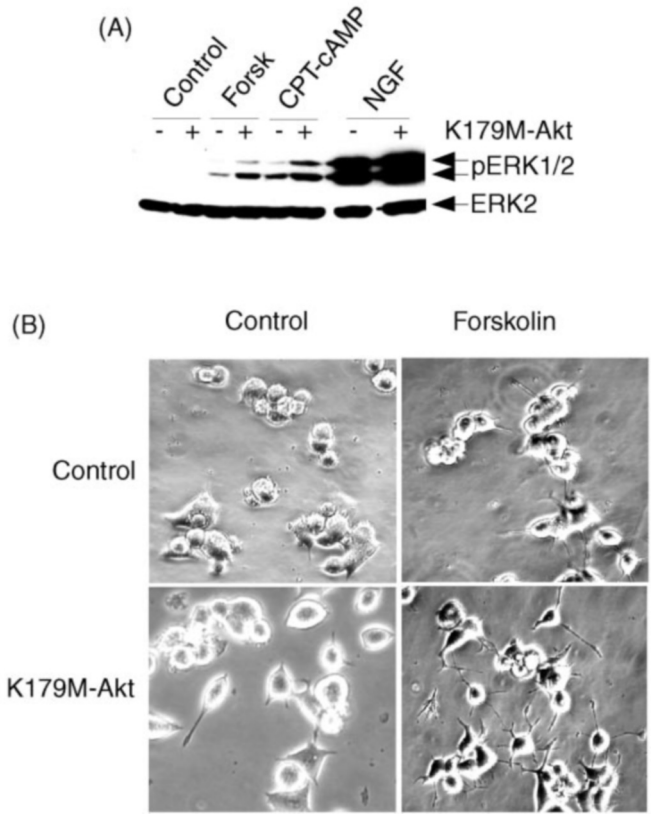

FIG. 9. **Expression of K179M-Akt enhances forskolin/CPT-cAMP-induced ERK1/2 activation and neurite outgrowth.** PC12 cells were transfected with K179M-Akt (+) or empty vector (−). On the next day the cells were incubated with forskolin (*Forsk*, 20 μM), CPT-cAMP (200 μM), or NGF (100 ng/ml) for 3 min, followed by immunoblotting with anti-phospho-ERK1/2 (*A*). Equal loading of the lanes was controlled by anti-ERK2 immunoblotting. *B*, cells were incubated with forskolin for 24 h. Representative cells were then photographed with a CCD camera.

cells. The time course of cAMP-induced tyrosine phosphorylation of the NGFR is relatively slow compared with $G_{i/q}$-coupled receptor-induced RTK tyrosine phosphorylation, but resembles NGFR tyrosine phosphorylation upon stimulation of the A_{2A} or pituitary adenylate cyclase-activating polypeptide receptor (46, 61). A recent study has shown that elevation of $[cAMP]_i$ by forskolin can induce tyrosine phosphorylation of the receptor for brain-derived neurotrophic factor, termed Trk_B (62). Thus, cAMP appears to be an important modulator of neurotrophin receptor signaling.

The present study also shows that forskolin-induced tyrosine phosphorylation of the EGFR is accompanied by rapid tyrosine phosphorylation of the adaptor protein Shc and recruitment of Shc, Grb2, and Gab1 to the EGFR. These events are well known to occur upon EGF-induced activation of the EGFR and thus support the assumption that cAMP induces activation of the EGFR in PC12 cells. Furthermore, these signaling intermediates might be critical for cAMP-mediated signal transmission, and the recruitment of these proteins to the EGFR could be involved in cAMP-induced activation of ERK1/2 and other responses such as differentiation, induction of neurite outgrowth, and survival in PC12 cells (63).

The mechanisms of cAMP-induced tyrosine phosphorylation of the EGFR and the NGFR remains to be established. One possibility is that cAMP-induced tyrosine phosphorylation of the RTKs involves ligand-dependent mechanisms. Previous studies have provided evidence that $G_{i/q}$-induced receptors can induce EGFR transactivation by proteolytic cleavage of EGF-like transmembrane precursor HB-EGF by metalloproteinase activity (52, 53). Moreover, a recent study shows that the proforms of NGF and of brain derived neurotrophic factor are secreted and cleaved extracellularly by proteases and can thereby activate neurotrophin receptors (54). However, we did not observe any effect of neutralizing anti-HB-EGF antibody on forskolin-induced EGFR tyrosine phosphorylation or ERK1/2 activation. Likewise, immunoneutralizing anti-NGF antibody did not ablate forskolin-induced tyrosine phosphorylation of the NGFR and Akt. Thus, cAMP-induced EGFR and NGFR activation appears to be HB-EGF and NGF-independent. Similar to our observations, tyrosine phosphorylation of the NGFR in response to pituitary adenylyl cyclase activating peptide, which is coupled to activation of adenylyl cyclase, is not inhibited by anti-NGF antibody (61).

cAMP-induced ERK1/2 activation in PC12 cells has been reported to occur through a Ras-dependent pathway (21) or a Ras-independent, Rap1-dependent pathway (24). The stimulatory effect of cAMP on ERK1/2 appears to depend on expression of the 95-kDa splice variant of B-Raf (24, 29, 64–67). Activated protein kinase A (PKA) has been shown to phosphorylate the Ras-related small GTPase Rap1 (68), and elevation of $[cAMP]_i$ activates Rap1 (30). Recent studies have shown that cAMP activates guanine nucleotide exchange factors acting directly on and thereby activating Rap1 independently of PKA activation (31, 32, 69). However, activation of cAMP-responsive Rap1-guanine nucleotide exchange factors is not sufficient to account for cAMP-induced ERK1/2 activation, because cAMP-induced ERK1/2 activation in PC12 cells is critically dependent on PKA (24). Several lines of evidence indicate that activation of Rap1 alone is insufficient to account for cAMP-induced ERK1/2 activation in PC12 cells: a number of studies failed to demonstrate B-Raf or ERK1/2 activation by Rap1 or that Rap1 inhibition ablates cAMP-induced ERK1/2 activation (33, 35–37). Furthermore, it has been reported that the ability of cAMP to activate Rap1 does not correlate with its capacity to activate B-Raf (34). Thus, it is unlikely that Rap1 activation alone can account for cAMPs ability to activate B-Raf and consecutively ERK1/2 in PC12 cells. A recent study has provided evidence for the involvement of Src kinases in cAMP-induced ERK1/2 acti-

vation in PC12 cells (37). In the present study, we show that cAMP induces tyrosine phosphorylation of the EGFR and that cAMP-induced activation of ERK1/2 is EGFR-dependent. Furthermore, we confirmed that cAMP-induced ERK1/2 activation is Ras-dependent in PC12 cells (21, 28). The involvement of the EGFR in cAMP-induced ERK1/2 activation provides a rationale for the Ras dependence of cAMP-induced ERK1/2 activation.

Several different mechanisms appear to mediate ERK1/2 activation in response to adenylyl cyclase-coupled receptors. For instance, the mechanism of cAMP-induced ERK1/2 activation is cell-specific. The β_2-adrenergic receptor-stimulated activation of ERK1/2 in COS cells requires assembly of a large signaling complex and is EGFR-, Src-, and Ras-dependent (60, 70). However, β_2-adrenergic receptor-stimulated ERK1/2 activation in COS cells is not mimicked by membrane-permeable cAMP analogs (71) and apparently involves switching in coupling of the receptor from G_s to G_i (72). In S49 mouse lymphoma cells, the β-adrenergic receptor appears to stimulate ERK1/2 through activation of Rap1, but not Ras (64).

The data of the present study provide evidence that activation of the EGFR mediates at least in part cAMP-induced neurite outgrowth in PC12 cells. Because expression of dominant-negative mutants of MEK or ERK1/2 inhibit cAMP-induced neurite outgrowth in PC12 cells (24), one mechanism by which the activated EGFR in conjunction with cAMP could increase neurite outgrowth is activation of ERK1/2.

Our finding that cAMP-induced Akt activation correlates with tyrosine phosphorylation of the NGFR and is not altered by EGFR tyrosine kinase inhibition suggests that the NGFR, but not the EGFR is involved in cAMP-induced activation of Akt. Thus, it seems that cAMP does not cause full activation of the EGFR as observed in response to EGF stimulation. In contrast to the cAMP-activated EGFR, the cAMP-activated NGFR is coupled to Akt activation, but not to activation of the ERK1/2 cascade, whereas NGF elicits both responses. Thus, cAMP does not cause full activation of NGFR as well. The fractional responses of cAMP-activated EGFR and NGFR are unlikely to be caused by different efficacies in their coupling to the ERK1/2 and Akt cascades, because the ligand-activated EGFR and NGFR activate ERK1/2 and Akt with similar potency. At present, we have no explanation for our finding that cAMP-activated RTKs apparently elicit only partial biological responses compared with their stimulation by the cognate ligands. It has recently been reported that internalization is required for the NGFR to elicit ERK1/2 activation and differentiation, whereas survival appears to be normal when endocytosis is impaired by the expression of thermosensitive dynamin (73). Moreover, NGF covalently cross-linked to beads to prevent internalization increased phosphorylation of Akt, but not of ERK1/2 in cultured rat sympathetic neurons (74). Thus, it is possible that signaling specificity is generated by routing the receptors to different subcellular compartments.

The membrane-permeable cAMP analog CPT-cAMP has positive effects on the survival of superior cervical ganglion neurons (55). Similarly, an increase in $[cAMP]_i$ increases cell survival upon serum withdrawal in PC12 cells by an Akt-independent pathway that may involve PKA-dependent activation of atypical protein kinase C (17). The present study confirms that cAMP-mediated survival is mediated by an Akt-independent pathway, although cAMP causes Akt activation in PC12 cells. Alternative mechanisms by which cAMP foster cellular survival may include PKA-dependent phosphorylation and inactivation of glycogen synthase kinase-3 (39, 40), as well as PKA-induced phosphorylation of Bad (38, 75). Whether these mechanisms play a role in cAMP-induced survival in PC12 cells remains to be established.

Whereas cAMP-induced Akt activation appears to be of minor importance in mediating survival response, the present study suggests that one role of cAMP-induced NGFR-dependent activation of Akt is suppression of ERK1/2 activation and neurite outgrowth. This is concluded from our finding that dominant-negative Akt strongly increases cAMP-induced ERK1/2 activation and neurite outgrowth. In agreement with an inhibitory role of Akt in PC12 cell differentiation, expression of dominant-negative Akt has recently been shown to enhance NGF-induced differentiation (76). Because Akt has been reported to inhibit activation of c-Raf-1 and B-Raf (77, 78), Akt may inhibit cAMP-induced ERK1/2 activation and neurite outgrowth at the level of B-Raf, the major Raf isoform expressed in PC12 cells.

In summary, the present study demonstrates that activation of the cAMP pathway involves stimulation of two receptor tyrosine kinases, *i.e.* the EGFR triggering activation of the ERK pathway and cellular differentiation, and the NGFR mediating activation of Akt. Thus, our results shed new light on the mechanisms by which elevated $[cAMP]_i$ can modulate ERK and Akt signaling pathways and thereby regulate neuronal differentiation and survival.

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