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Nerve Growth Factor Stimulation of p42/p44 Mitogen-Activated Protein Kinase in PC12 Cells: Role of $G_{i/o}$, G Protein-Coupled Receptor Kinase 2, β-Arrestin I, and Endocytic Processing

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ABSTRACT

In this study, we have shown that nerve growth factor (NGF)-dependent activation of the p42/p44 mitogen-activated protein kinase (p42/p44 MAPK) pathway in PC12 cells can be partially blocked by pertussis toxin (which inactivates the G proteins $G_{i/o}$). This suggests that the Trk A receptor may use a G protein-coupled receptor pathway to signal to p42/p44 MAPK. This was supported by data showing that the NGF-dependent activation of p42/p44 MAPK is potentiated in cells transfected with G protein-coupled receptor kinase 2 (GRK2) or β-arrestin I. Moreover, GRK2 is constitutively bound with the Trk A receptor, whereas NGF stimulates the pertussis toxin-sensitive binding of β-arrestin I to the TrkA receptor-GRK2 complex. Both GRK2 and β-arrestin I are involved in clathrin-mediated endocytic signaling to p42/p44 MAPK. Indeed, inhibitors of clathrin-mediated endocytosis (e.g., monodansylcadaverine, concanavalin A, and hyperosmolar sucrose) reduced the NGF-dependent activation of p42/p44 MAPK. Finally, we have found that the G protein-coupled receptor-dependent component regulating p42/p44 MAPK is required for NGF-induced differentiation of PC12 cells. Thus, NGF-dependent inhibition of DNA synthesis was partially blocked by PD098059 (inhibitor of MAPK kinase-1 activation) and pertussis toxin. Our findings are the first to show that the Trk A receptor uses a classic G protein-coupled receptor-signaling pathway to promote differentiation of PC12 cells.

Nerve growth factor (NGF) promotes the survival and differentiation of sensory and sympathetic neurons. NGF also induces growth arrest of PC12 cells, which then accumulate in the G_1 phase of the cell cycle and subsequently undergo differentiation. NGF binds to a specific high-affinity tyrosine kinase receptor, Trk A. Binding of NGF to Trk A induces autophosphorylation of the receptor on specific tyrosine residues. The subsequently phosphorylated sites on the receptor act as acceptors for the recruitment and assembly of signaling complexes, such as Grb-2, phospholipase C γ, and PI3K to elicit intracellular responses. For instance, the binding of the SH2 containing protein Shc, Grb-2, and mSos site to phospho-Tyr-490 on the Trk A receptor elicits Ras-dependent activation of the p42/p44 MAPK pathway.

The novel neuronal substrate FRS2 also uses the same docking site on the Trk A receptor as the SH2-containing protein Shc and has been implicated in the stimulation of the Ras-dependent p42/p44 MAPK pathway by forming a complex with the tyrosine phosphatase SHP-2, and associated adaptor proteins Grb-2, mSos, and Crk. This is achieved via its association with C3G and leads to the sustained activation of the small G protein Rap1. Rap1 then complexes to and activates B-raf, resulting in subsequent downstream stimulation of the p42/p44 MAPK pathway (York et al., 1998). Because NGF activates Ras and c-Raf transiently and B-Raf in a sustained manner, the prevalent view is that transient activation of p42/p44 MAPK leads to cell proliferation, whereas a more prolonged activation of this kinase pathway by NGF promotes cell differentiation (Marshall, 1995; Tombes et al., 1998).

Recent studies have shown that the insulin-like growth factor-1 (IGF-1) can use the G proteins, $G_{i/o}$ to stimulate activation of p42/p44 MAPK in fibroblasts (Luttrell et al. 1995). This was established using pertussis toxin (which inactivates $G_{i/o}$) and the C-terminal domain of β-adrenergic

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ABBREVIATIONS: NGF, nerve growth factor; MAPK, mitogen-activated protein kinase; IGF, insulin-like growth factor; GRK, G protein-coupled receptor kinase; Grb-2, growth factor receptor binding protein; PI3K, phosphoinositide 3-kinase; LPA, lysophosphatidic acid; PDGF, platelet-derived growth factor; G_i, inhibitory G protein; Gab1, growth factor receptor binding protein associated binder; HRP, horseradish peroxidase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; NP-40, Nonidet P-40; EGF, epidermal growth factor; GPCR, G protein-coupled receptor; mSos, son of sevenless; c-Src, cellular Src tyrosine kinase; MDC, monodansylcadaverine.

kinase (which sequesters $G_{\beta\gamma}$ subunits). Both reduced the IGF-1-dependent activation of p42/p44 MAPK. These agents also reduced fibroblast growth factor-dependent activation of p42/p44 MAPK in fibroblasts and promoted differentiation (Fedorov et al. 1998). Recent studies have also implicated the involvement of G protein-coupled receptor kinase 2 (GRK2) and β-arrestin I and II in regulating IGF-1- and β-adrenergic receptor-stimulated p42/p44 MAPK activation via a process that involves clathrin-mediated endocytosis of receptor-signal complexes (Daaka et al. 1998; Ahn et al. 1999; Lin et al. 1999). GRK2 is activated in an agonist- and G protein-dependent manner. β-arrestin I/II are clathrin adaptor proteins that promote dynamin II-mediated internalization of receptor signal complexes containing c-Raf-MAPK kinase-1 for subsequent activation of p42/p44 MAPK.

Certain G protein-coupled receptor agonists have also been shown to stimulate the tyrosine phosphorylation/*trans*-activation of growth factor receptors. The subsequently phosphorylated sites on the receptor act as acceptors for the recruitment and assembly of signaling complexes such as Grb-2, phospholipase C γ, and PI3K to elicit mitogenic responses. For instance, LPA has been shown to *trans*-activate the EGF receptor and $p185^{neu}$ to stimulate p42/p44 MAPK activation in Cos-7 cells (Daub et al., 1996), whereas angiotensin II can induce platelet-derived growth factor (PDGF) receptor *trans*-activation in vascular smooth muscle (Linsemen et al. 1995).

We have shown that the PDGF can also use G_i-dependent and -independent routes to promote stimulation of c-Src and p42/p44 MAPK in cultured airway smooth muscle cells (Conway et al. 1999; Rakhit et al. 2000). Furthermore, c-Src inhibitors abolished the PDGF-dependent activation of p42/p44 MAPK in these cells. We have suggested that G_i might recruit c-Src near the PDGF receptor tyrosine kinase for activation. Furthermore, PDGF stimulates a G_i-mediated tyrosine phosphorylation of the Grb-2 associated binding protein, Gab1. This promotes the binding of tyrosine-phosphorylated PI3K1a to Gab1 and is required for dynamin II mediated endocytic stimulation of the p42/p44 MAPK pathway (Rakhit et al. 2000). Moreover, Gab1 binds to and activates PI3K (Kaplan and Millar, 1997; Korhonen et al., 1999) in response to NGF in neuronal cells. This raises the possibility that the Trk A receptor may also use classic GPCR-dependent signaling to regulate activation of the p42/p44 MAPK pathway. However, to date there is no direct evidence to support this proposed model.

Therefore, in this article, we have investigated whether the NGF-dependent activation of p42/p44 MAPK involves a classic GPCR signaling pathway. We have also evaluated whether this pathway has an important role in regulating the differentiation of PC12 cells.

Experimental Procedures

Materials. All biochemicals, including NGF, were from Sigma Chemical Co. (Dorset, UK). [^{3}H]Thymidine, [γ-^{32}P]ATP (3000 Ci/mol), MAPK Biotrak assay kits, and enhanced chemiluminescence reagents were from Amersham Pharmacia Biotech (Bucks, UK). Cell culture supplies were from Life Technologies (Paisley, UK). Anti-phospho-p42/p44 MAPK antibodies were from New England Biolabs (Beverly, MA). Anti-p42/p44 MAPK and HRP-linked anti-phosphotyrosine antibodies were from Transduction Laboratories (Lexington, KY). Anti-Trk A phospho-Tyr-490 and Trk A antibodies were from New England Biolabs (Beverly, MA). Anti-FLAG antibody was from Stratagene (La Jolla, CA). Anti-β-arrestin I antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Reporter HRP-anti-mouse/rabbit antibodies were from the Scottish Antibody Production Unit (Carluke, Scotland). pRK5-GRK2 and pcDNA3-βarr1 FLAG cDNA plasmid constructs and anti-GRK2 antibodies were kind gifts from Professor R. Lefkowitz (Duke University, Durham, NC).

Cell Culture. PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS). Cells were placed in DMEM supplemented with 0.1% (v/v) fetal calf serum (FCS) for 24 h before experimentation. In some cases, pertussis toxin (0.1 μg/ml) was added to the DMEM supplemented with 0.1% (v/v) FCS.

Transfection. PC12 cells were transiently transfected with β-arrestin I or GRK2 plasmid constructs. Cells at 90% confluence were placed in DMEM containing 2% (v/v) FCS and transfected with 2 to 4 μg of plasmid construct after complex formation with LipofectAMINE 2000, according to the Manufacturer's instructions. The cDNA containing media was then removed after 24 h at 37°C, and the cells incubated for a further 24 h in DMEM supplemented with 0.1% (v/v) FCS before addition of agonists.

p42/p44 MAPK Assays. The phosphorylation and activation of p42/p44 MAPK was detected by Western blotting using an anti-phospho-p42/p44 MAPK antibody. p42/p44 MAPK activity was also measured in PC12 cell lysates using a specific p42/p44 MAPK peptide substrate ($EGFR^{661–680}$ peptide synthesized to contain one phosphorylation site) as we described previously (Conway et al. 1999).

Blotting. Immunoblotting was performed as we described previously (Conway et al., 1999; Rakhit et al. 1999). Briefly, nitrocellulose membranes were blocked for 2 h at 4°C in 10 mM phosphate-buffered saline and 0.1% (v/v) Tween-20 containing 5% (w/v) nonfat dried milk and 0.001% (w/v) thimerosal. The nitrocellulose sheets were then incubated overnight at 4°C in blocking solution containing antibodies. The sheets were then washed with phosphate-buffered saline and 0.1% (v/v) Tween-20 before incubation with HRP-linked anti-rabbit mouse antibodies in blocking solution for 2 h at room temperature. In the case of HRP-linked anti-phosphotyrosine antibodies, no secondary antibody was used. After washing the blots as above, immunoreactive proteins were visualized using the enhanced chemiluminescence detection kit and were quantified using densitometry.

Immunoprecipitation of Trk A. The medium was removed and cells lysed in ice-cold immunoprecipitation buffer (1 ml) containing 20 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% (v/v) Nonidet P-40 (NP-40), 10% (v/v) glycerol, 1 mg/ml bovine serum albumin, 0.5 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, antipain, pepstatin, and aprotinin; pH 8) for 10 min at 4°C. The material was harvested, centrifuged at 22,000*g* for 5 min at 4°C and 200 μl of cell lysate supernatant (equalized for protein, 0.5 to 1 mg/ml) taken for immunoprecipitation with antibodies (5 μg of anti-Trk A-490 phosphotyrosine or Trk A antibodies and 30 μl of 1 part immunoprecipitation buffer and 1 part protein A Sepharose CL4B, pre-equilibrated with lysis buffer. After agitation for 2 h at 4°C, the immune complex was collected by centrifugation at 22,000*g* for 15 s at 4°C. Immunoprecipitates were washed twice with buffer A [containing 10 mM HEPES, pH 7, 100 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 0.5% (v/v) NP-40] and once in buffer A without NP-40. Samples were taken for Western blotting with HRP-linked anti-phosphotyrosine or anti-GRK2 or β-arrestin I antibodies

DNA Synthesis. [^{3}H]Thymidine incorporation studies were performed as described by Rakhit et al. (1999).

cAMP Assays. Intracellular cAMP was measured using a cAMP enzyme-linked immunosorbent assay kit as described by the manufacturer (Amersham Pharmacia Biotech).

Results

The NGF-Dependent Activation of p42/p44 MAPK. The treatment of PC12 cells with NGF stimulated p42/p44 MAPK activation in a dose-dependent manner (Fig. 1A). p42/p44 MAPK was activated ~5- to 6-fold above basal using a maximal concentration of 50 ng/ml NGF, measured using a specific p42/p44 MAPK peptide ($EGFR^{661–680}$ peptide substrate assay) (Fig. 1B). Using 5 ng/ml NGF, the activation of p42/p44 MAPK was sustained for at least 30 min (Fig. 1C). The inhibitor of MAP kinase-1 activation, PD098059 markedly reduced the NGF-dependent activation of p42/p44 MAPK (Fig. 1D).

NGF Uses a Classic GPCR-Mediated Pathway to Stimulate the p42/p44 MAPK Pathway. The treatment of PC12 cells with the bacterial toxin, pertussis toxin (which inactivates the G proteins $G_{i/o}$) at 0.1 μg/ml for 24 h reduced the NGF-dependent activation of p42/p44 MAPK by ~80% at a low concentration of NGF (5 ng/ml) (Fig. 1A). In contrast, only p42 MAPK activation was reduced by pertussis toxin at a high concentration of NGF (50 ng/ml) (Fig. 1A).

Pertussis toxin also seems to have a more profound effect during the early phase activation of p42/ MAPK (0–5 min) at low concentrations of NGF (5 ng/ml). The effect of pertussis toxin diminished after 10-min stimulation, whereas after 30 min, no effect was observed (Fig. 1C). Pertussis toxin had no effect on the expression level of p42 MAPK (Fig. 1, A and C).

The specificity and action of pertussis toxin in inactivating $G_{i/o}$-mediated signaling was further characterized using lysophosphatidate, which binds to a G_i-coupled receptor, and EGF, which does not use these G proteins. Figure 1E shows that the LPA-dependent stimulation of p42/p44 MAPK was markedly reduced by pretreating cells with pertussis toxin, whereas the response to EGF was unaffected. Under the conditions used in these experiments, pertussis toxin partially inactivated G_i. This was evidenced from experiments showing that the LPA-induced reduction in basal cAMP levels was partially blocked by the toxin (Fig. 2).

In some cell types, G_i can tonically inhibit adenylyl cyclase. Under these conditions, pertussis toxin could potentially increase cAMP formation by disinhibiting adenylyl cyclase. This is an important consideration because cAMP has been

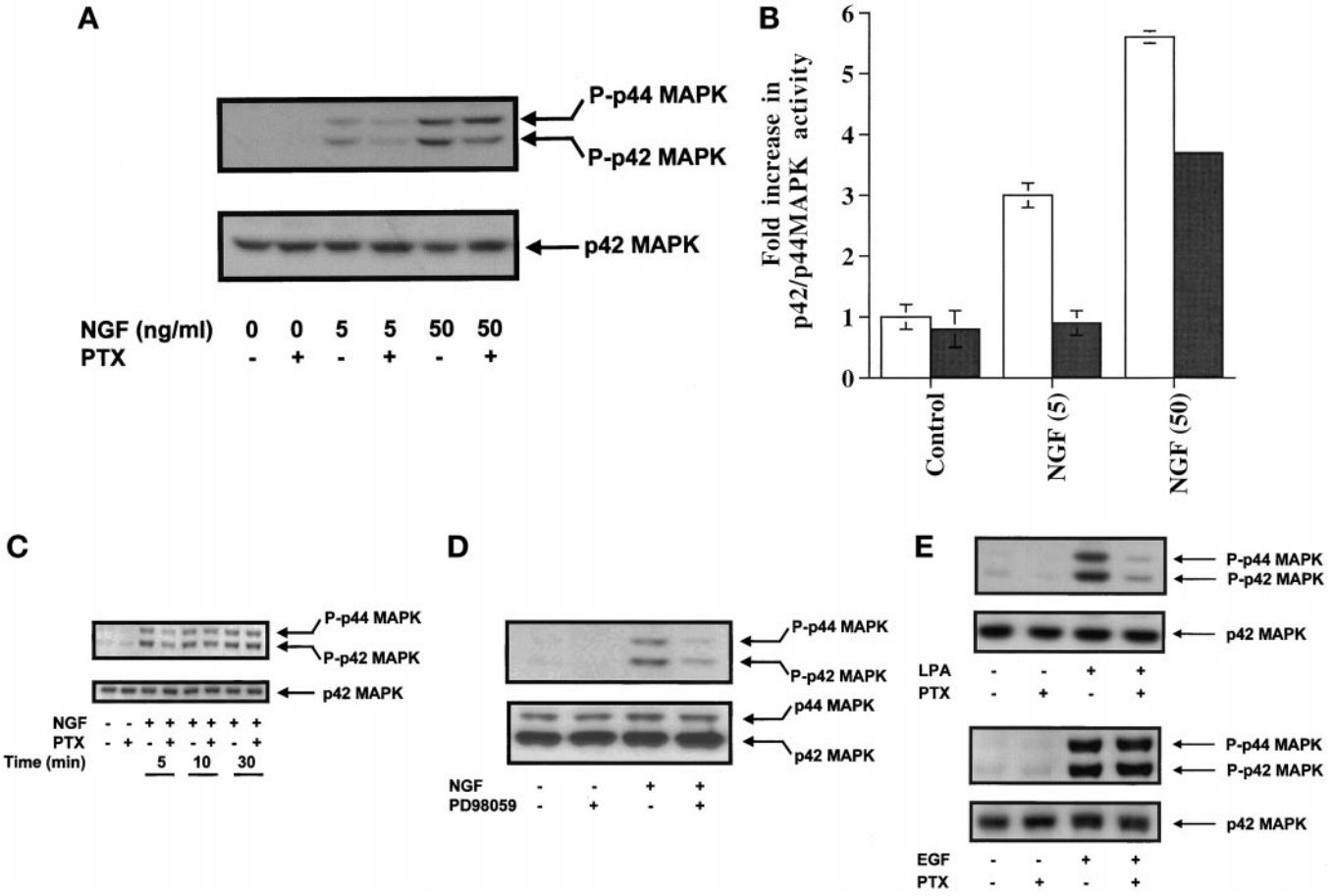

Fig. 1. The effect of pertussis toxin on the NGF-dependent activation of p42/p44 MAPK in PC12 cells. PC12 cells were pretreated with and without pertussis toxin (0.1 μg/ml, 24 h) or PD090859 (50 μM, 15 min) before stimulation with NGF (5–50 ng/ml), LPA (5 μM), or EGF (100 nM) for the indicated times. Cell lysates were then prepared for Western blotting with antibodies that cross-react the phosphorylated/activated forms of p42/p44 MAPK. The results show the effect of (A) pertussis toxin on the concentration-dependent activation of p42/p44 MAPK by NGF (t = 5-min stimulation); (B) pertussis toxin on p42/p44 MAPK activation measured using a specific p42/p44 MAPK peptide ($EGFR^{661–680}$ peptide substrate assay) (control, open bar; pertussis toxin, shaded bar). Results are expressed as -fold increases in p42/p44 MAPK activity above basal in control cells (means ± S.E.M., for n = 3 experiments). C, pertussis toxin on the time course of p42/p44 MAPK activation. D, PD098059 on the NGF (5 ng/ml, 5 min)-dependent activation of p42/p44 MAPK. E, pertussis toxin on LPA and EGF (t = 5 min) stimulated p42/p44 MAPK activation. Western blots were stripped and reprobed with antibodies that cross-react with either total p42 MAPK alone or p42/p44 MAPK to ensure equal protein loading. A, C, D and E are representative results of an experiment performed three times.

shown (via PKA) to block p42/p44 MAPK activation in some cell types, which could potentially explain the effect of pertussis toxin on NGF signaling in PC12 cells. Furthermore, the chronic effect of cAMP/PKA could theoretically induce a major shift in the metabolic set of the cell that leads to alterations in Trk A receptor signaling. However, we found that this is not the case in these cells. This was based upon several lines of evidence. First, the chronic treatment of cells with pertussis toxin had no effect on basal cAMP formation in PC12 cells (Fig. 2), thereby ruling out the possibility that pertussis toxin acts via this route. Furthermore, stimulation of PC12 cells with NGF actually reduced cAMP levels in a pertussis toxin-sensitive manner, suggesting that the Trk A receptor may also use G_i to inhibit adenylyl cyclase (Fig. 2). Second, the acute treatment of cells with forskolin (activator of adenylyl cyclase) increased rather than decreased p42/p44 MAPK activation (Fig. 3, top). The increase in p42/p44 MAPK activation with forskolin was additive with NGF (Fig. 3, top). This contrasts with pertussis toxin, which was inhibitory. Third, chronic treatment of cells with forskolin to theoretically induce a major shift in the metabolic set of the cell had no effect on the NGF-dependent activation of p42/p44 MAPK (Fig. 3, bottom).

Therefore, taken together, these results provide strong evidence that pertussis toxin abrogates NGF-dependent activation of p42/p44 MAPK by modulating $G_{i/o}$ rather than by altering cAMP/PKA signaling

We also investigated whether NGF induces the release of epinephrine, which could in turn function as an autocrine by binding to receptors linked to $G_{i/o}$. This possibility was ruled out based upon data showing that the receptor antagonists, phentolamine (nonspecific α-adrenoceptor antagonist), and yohimbine (α_2-adrenoceptor antagonist) have no effect on the activation of p42/p44 MAPK by NGF (Fig. 4).

Tyrosine Phosphorylation of the Trk A Receptor. We next investigated whether the Trk A receptor uses $G_{i/o}$ to promote phosphorylation of tyrosine sites on the receptor. This would enable the recruitment of signal complexes to the receptor, thereby initiating stimulation of the p42/p44 MAPK pathway. To evaluate this, we assessed the effect of pertussis toxin on NGF-stimulated phosphorylation of Tyr-490 in the Trk A receptor. Tyr-490 is the relevant phosphorylation site because it functions as an adaptor site for Grb2/mSos complex as well as FRS2, both of which are required for activation of the p42/p44 MAPK pathway. The pretreatment of PC12 cells with pertussis toxin had no effect on NGF-stimulated Tyr-490 phosphorylation, which was detected in anti-Trk A phospho-Tyr-490 immunoprecipitates with HRP-linked anti-phosphotyrosine antibodies (Fig. 5). The tyrosine-phosphorylated protein (molecular mass, 140 kDa) was also immunostained with specific anti-Trk A antibody (equal

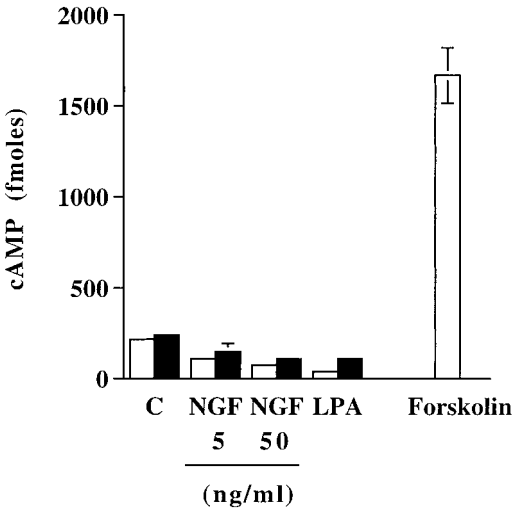

Fig. 2. Pertussis toxin has no effect on basal cAMP and reversed NGF- and LPA-mediated reduction in cAMP. PC12 cells were pretreated with (shaded bar) and without (open bar) pertussis toxin (0.1 μg/ml, 24 h) before stimulation with NGF (5–50 ng/ml) or LPA (5 μM). The histogram shows that pertussis toxin has no effect on basal cAMP and partially blocks the NGF- and LPA-induced reduction in cAMP. Forskolin (50 μM, 10 min) was used as a positive control for the stimulation of cAMP formation. These are representative results of an experiment performed three times.

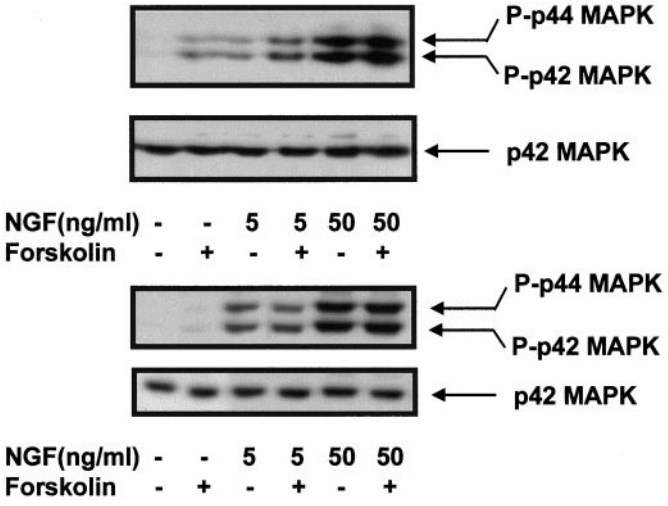

Fig. 3. The effect of forskolin on the NGF-dependent activation of p42/p44 MAPK in PC12 cells. PC12 cells were pretreated with and without forskolin (50 μM, 15 min or 24 h) before stimulation with NGF (5–50 ng/ml) for 5 min. The Western blots show the acute (top) and chronic (bottom) effect of forskolin on the NGF-dependent activation of p42/p44 MAPK. Western blots were stripped and reprobed with antibodies that cross-react with total p42 MAPK to ensure equal protein loading. These are representative results of an experiment performed three times.

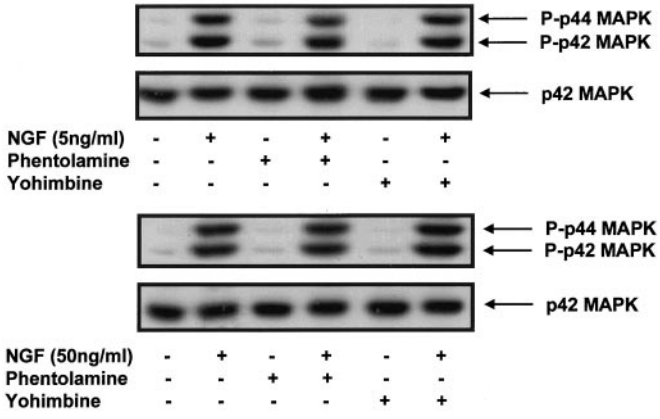

Fig. 4. Epinephrine does not contribute to the activation of p42/p44 MAPK by NGF. PC12 cells were pretreated with phentolamine (10 μM, 15 min) or yohimbine (10 μM, 15 min) before stimulation with NGF (5 and 50 ng/ml, 5 min). The results show that phentolamine and yohimbine have no effect on the NGF-dependent activation of p42/p44 MAPK. Western blots were stripped and reprobed with antibodies that cross-react with total p42 MAPK to ensure equal protein loading. These are representative results of an experiment performed three times.

amounts of the protein were immunopreciptated from each sample) (data not shown).

Taken together, these data are consistent with a model in which the Trk A receptor uses $G_{i/o}$ as coupling protein(s) to elicit activation of the p42/p44 MAPK pathway, rather than regulation of Trk A tyrosine phosphorylation.

Role of PI3K. Certain G protein-coupled receptor agonists use a PI3K-dependent pathway to stimulate p42/p44 MAPK in mammalian cells. Moreover, several growth factor receptors that regulate p42/p44 MAPK activation via a $G_{i/o}$-dependent mechanism also seem to require PI3K (Rakhit et al., 2000). Consistent with these findings, we show here that the NGF (5 ng/ml)-dependent stimulation of p42/p44 MAPK has a mandatory requirement for PI3K, because the stimulation of p42/p44 MAPK was reduced by ~70% by pretreating PC12 cells with the PI3K inhibitor wortmannin (Fig. 6).

The Effect of GRK2 and β-Arrestin I on the NGF-Dependent Activation of p42/p44 MAPK. We next investigated whether transfecting cells with GRK2 and β-arrestin I can increase the NGF-dependent activation of p42/p44 MAPK. These proteins play a key role in regulating p42/p44 MAPK activation in response to G protein-coupled receptors (Daaka et al. 1998; Ahn et al. 1999; Lin et al. 1999).

Fig. 7A, top, shows the expression of recombinant GRK2 (molecular mass, 80 kDa) and FLAG-β-arrestin I (molecular mass, 55 kDa) in PC12 cells transfected with respective plasmid constructs and detected with anti-FLAG and GRK2 antibodies. FLAG-β-arrestin I was not detected in vector-transfected cells. Figure 7A, bottom, shows that the NGF-dependent activation of p42/p44 MAPK was potentiated ~3- to 5-fold in cells overexpressing either GRK2 or β-arrestin I. This stimulation was not further enhanced when cells were cotransfected with both proteins compared with either alone.

These results clearly show that GRK2 and β-arrestin I participate in Trk A signaling. However, to establish a role for the endogenous forms, it was also necessary to show that these proteins are expressed in PC12 cells and that they form a complex with the Trk A receptor. Figure 7b shows that GRK2 and β-arrestin I are expressed in PC 12 cells and can be immunostained in cells lysates that have been Western blotted with anti-GRK2 and β-arrestin I antibodies, respectively. Furthermore, both endogenous and recombinant GRK2 are constitutive bound in a complex with the Trk A

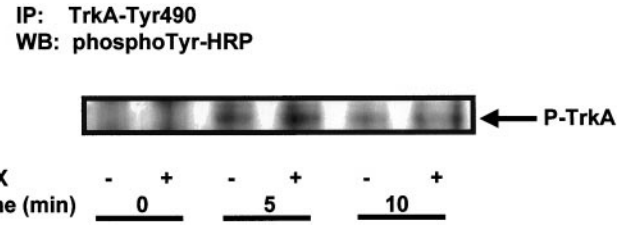

Fig. 5. Pertussis toxin has no effect on NGF-dependent Trk A tyrosine phosphorylation (Tyr-490) in PC12 cells. PC12 cells were pretreated with and without pertussis toxin (0.1 μg/ml, 24 h) before stimulation with NGF (50 ng/ml) for 0, 5, and 10 min. Trk A (Mr = 140kDa) was immunoprecipitated with anti-Trk A phospho-Tyr-490 antibodies and samples subjected to Western blotting with HRP-linked anti-phosphotyrosine antibodies. The results show that pertussis toxin had no effect on the NGF-dependent phosphorylation Tyr-490 in the Trk A receptor. This is a representative result of an experiment performed three times.

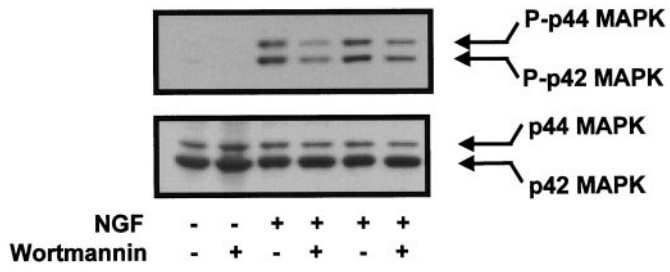

Fig. 6. The effect of wortmannin on the NGF-dependent activation of p42/p44 MAPK in PC12 cells. PC12 cells were pretreated with and without wortmannin (50 nM, 15 min) before stimulation with NGF (5 ng/ml) for 5 min. The Western blot shows the effect of wortmannin on the NGF-dependent activation of p42/p44 MAPK. The blot was stripped and reprobed with antibodies that cross-react with total p42/p44 MAPK to ensure equal protein loading. This is a representative result of an experiment performed three times.

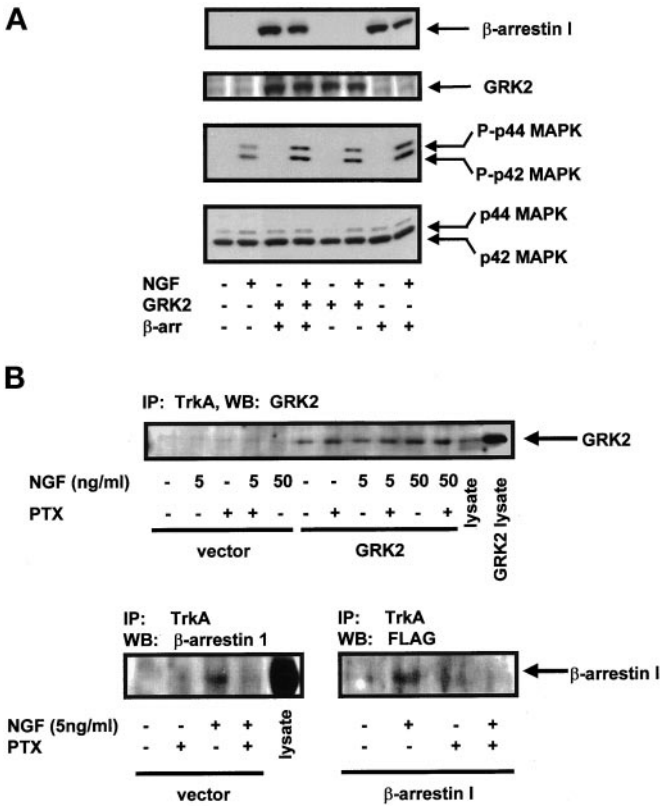

Fig. 7. The effect of GRK2 and β-arrestin I on the NGF-dependent activation of p42/p44 MAPK. PC12 cells were transiently transfected with plasmid constructs containing no insert (vector alone), pRK5-GRK2, and pcDNA3-βarr1 FLAG cDNA. PC12 cells were pretreated with and without pertussis toxin (0.1 μg/ml, 24 h) before stimulation with NGF (5 and 50 ng/ml) for 5 min. A, top, Western blot showing the expression of recombinant GRK2 and FLAG-β-arrestin I in pRK5-GRK2 and pcDNA3-βarr1 FLAG transfected versus vector-transfected cells. A, bottom, Western blot showing the effect of GRK2 and β-arrestin I on the NGF-(5 ng/ml) dependent activation of p42/p44 MAPK. The blots were restripped and probed with antibodies that cross-react with total p42/p44 MAPK to ensure equal protein loading. B, top, Western blot showing that endogenous and recombinant GRK2 are bound in a complex with the Trk A receptor. GRK2 was detected in anti-Trk A receptor immunoprecipitates by Western blotting with anti-GRK2 antibodies. Endogenous and recombinant GRK2 in cell lysates are also shown as positive controls for the antibody. B, bottom, Western blot showing that NGF promotes the binding of β-arrestin I to the GRK2-Trk A receptor complex in a pertussis toxin-sensitive manner. Endogenous β-arrestin I was detected in anti-Trk A receptor immunoprecipitates by Western blotting with anti-β-arrestin I antibodies, and recombinant β-arrestin I was detected with anti-FLAG antibodies. Endogenous β-arrestin I in cell lysates is shown as a positive control for the antibody. These are representative results of an experiment performed three times.

receptor in these cells (Fig. 7B, top). Low but detectable levels of endogenous GRK2 were found associated with the Trk A receptor; this was increased in GRK2-transfected cells. GRK2 was detected in anti-Trk A receptor immunoprecipitates by Western blotting with anti-GRK2 antibodies. In Fig. 7B, bottom, we show that NGF stimulates the binding of endogenous and recombinant β-arrestin I to the Trk A receptor-GRK2 complex, which is prevented by pretreating cells with pertussis toxin. Endogenous β-arrestin I was detected in anti-Trk A receptor immunoprecipitates by Western blotting with anti-β-arrestin I antibodies, whereas recombinant β-arrestin I was detected with anti-FLAG antibodies. In all cases, the immunoprecipitation of Trk A was not altered by the various treatments (data not shown).

The Effects of Inhibitors of Clathrin Mediated Receptor Endocytosis. Recent studies have confirmed that certain G protein-coupled receptor agonists use β-arrestin I as a clathrin adaptor to mediate endocytosis of receptor signal complexes (which include c-Raf-MAPK kinase-1) to trigger activation of p42/p44 MAPK. PC12 cells were therefore preincubated with the inhibitors of clathrin-mediated endocytosis, such as concanavalin A (which prevents receptor clustering), hyperosmolar sucrose (which blocks clathrin association), and monodansylcadaverine (MDC) to establish their effect on NGF-stimulated p42/p44 MAPK activation. Figure 8a shows that concanavalin A and hyperosmolar sucrose markedly reduced the NGF-dependent activation of p42/p44 MAPK (~80–90% inhibition). Figure 8B shows the concentration-dependent inhibition of the NGF-dependent stimulation of p42/p44 MAPK by MDC.

The Effect of NGF on DNA Synthesis. We next investigated whether the G protein-coupled receptor-dependent component regulating p42/p44 MAPK is required for NGF-induced differentiation of PC12 cells. The data is shown in Fig. 9. First, we found that NGF induces a marked reduction in [^{3}H]thymidine incorporation into DNA. This was dose-dependent with maximal inhibition at 10 to 50 ng/ml NGF (Fig. 9A). Pretreatment of cells with PD098059 or pertussis

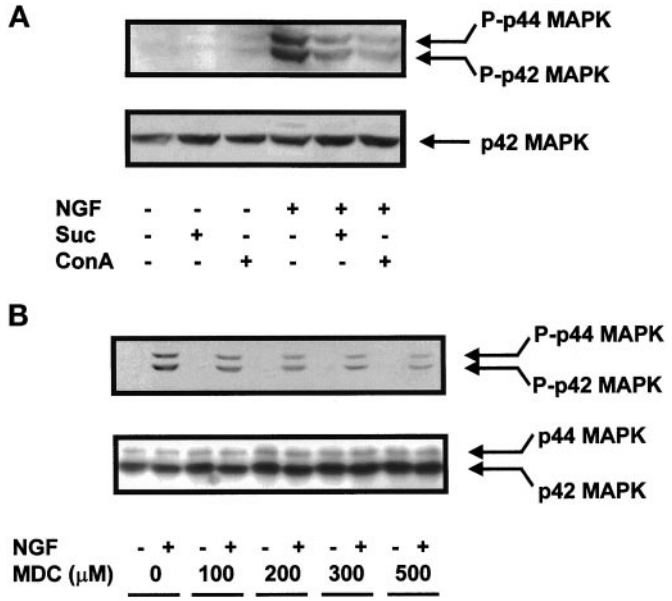

Fig. 8. The effect of inhibitors of clathrin-mediated endocytosis on the NGF-dependent activation of p42/p44 MAPK in PC12 cells. PC12 cells were pretreated with and without concanavalin A (0.25 mg/ml), sucrose (0.4 M) or monodansylcadervine (100–500 μM) for 15 min before stimulation with NGF (5 ng/ml) for 5 min. Western blots showing (A) the effect of concanavalin A and sucrose on the NGF-dependent activation of p42/p44 MAPK. B, the concentration-dependent effect of MDC on the activation of p42/p44 MAPK by NGF. The blots were stripped and reprobed with antibodies that cross-react with either total p42 MAPK or both p42/p44 MAPK to ensure equal protein loading. These are representative results of an experiment performed three times.

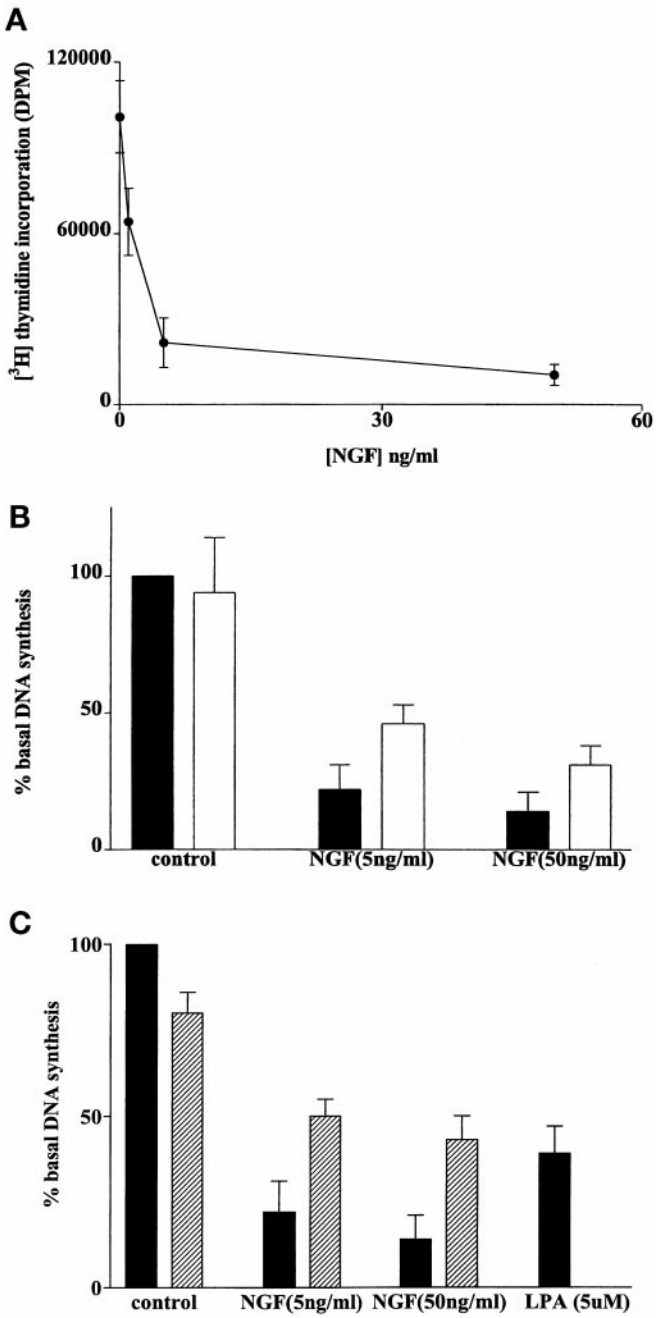

Fig. 9. NGF-induced inhibition of DNA synthesis. PC12 cells were pretreated with and without pertussis toxin (0.1 μg/ml, 24 h) or PD090859 (50 μM) for 15 min before stimulation with NGF (1, 5 and 50 ng/ml) or LPA (5 μM) for 24 h. A, the graph shows the concentration-dependent inhibition of DNA synthesis by NGF. Results are expressed in dpm of [^{3}H]thymidine incorporation (means ± S.D. for $n = 3$ experiments). Histograms showing the effect of (B) pertussis toxin (control, shaded bar; pertussis toxin, open bar) and (C) PD098059 (control, shaded bar; PD098059, hatched bar] on the NGF-induced inhibition of [^{3}H]thymidine incorporation into DNA synthesis. Results are expressed percentage of basal DNA synthesis (means ± S.D. for $n = 3$–5 experiments). $P < 0.05$ for NGF/pertussis toxin and NGF/PD090859 versus NGF alone.

toxin at concentrations that reduced the NGF-dependent stimulation of p42/p44 MAPK activation blocked the NGF-induced inhibition of DNA synthesis (Fig. 9, B and C). In both cases, DNA synthesis was not completely restored to the basal level (~50–60% of basal level). Significantly, inhibition of DNA synthesis was also observed in response to LPA, thereby confirming that G protein-coupled receptor agonists can indeed induce growth arrest of PC12 cells (Fig. 9C).

Discussion

We have reported that the pretreatment of PC12 cells with pertussis toxin (which inactivates the G proteins $G_{i/o}$) reduces the NGF-dependent activation of p42/p44 MAPK. Because pertussis toxin was without effect on the NGF-stimulated phosphorylation of Tyr-490 in the Trk A receptor, we suggest that the Trk A receptor can use $G_{i/o}$ as coupling proteins to regulate p42/p44 MAPK activation. The mechanism by which the Trk A receptor interacts with $G_{i/o}$ is not known. However, other studies have reported that growth factor receptors can interact with G proteins (Rothenberg and Kahn, 1988; Luttrell et al., 1990). Recently, IGF-1 has been shown to activate G_i to release $\beta\gamma$ subunits (Hallak et al. 2000), which in turn initiates activation of the p42/p44 MAPK pathway (Luttrell et al. 1995).

Additional evidence showing that the Trk A receptor uses classic G protein-coupled receptor signaling pathways was obtained using cells transiently transfected with GRK2 or β-arrestin I. Both recombinant proteins potentiated the NGF-dependent stimulation of p42/p44 MAPK. Thus, endogenous GRK2 and β-arrestin I might also behave in a similar manner. This was supported by the finding that both endogenous and recombinant GRK2 are constitutively bound in a complex with the Trk A receptor. Moreover, NGF stimulates a pertussis toxin-sensitive association of β-arrestin I with the Trk A receptor-GRK2 complex. This interaction is similar with the role of GRK2 in regulating G protein-coupled receptor signaling processes, where it is activated by $G_{\beta\gamma}$ and catalyzes the phosphorylation of ligand bound G protein-coupled receptors, thus creating a binding site for β-arrestin I/II. This leads to an uncoupling of the receptor from its G protein, followed by rapid receptor internalization and stimulation of p42/p44 MAPK.

A number of GPCR agonists and growth factor receptors that can use $G_{i/o}$ to stimulate p42/p44 MAPK do so via a PI3K-dependent pathway in mammalian cells. Consistent with this, we found that the NGF-dependent activation of p42/p44 MAPK was blocked by ~70% by pretreating cells with the PI3K inhibitor, wortmannin. Other growth factors (e.g., PDGF) stimulate a G_i-mediated tyrosine phosphorylation of Gab1 required for binding of PI3K1a and subsequent dynamin II-mediated stimulation of the p42/p44 MAPK pathway (Rakhit et al. 2000). Moreover, NGF also seems to stimulate the binding of PI3K to Gab1 (Kaplan and Millar, 1997; Korhonen et al., 1999). This may be important in regulating NGF-dependent endocytic signal processes in the cell. Indeed, we show here that the NGF-dependent activation of p42/p44 MAPK can be blocked by ~80% with endocytosis inhibitors.

An interesting finding in the current study was that at low concentrations of NGF (5 ng/ml), pertussis toxin substantially reduced the activation of p42/p44 MAPK, whereas at a high concentration of NGF (50 ng/ml), only p42 MAPK activation was affected. Therefore, whereas NGF seems to elicit a more robust activation of p42/p44 MAPK at high receptor occupancy, the requirement of p44 MAPK activation for $G_{i/o}$ seems to be surmounted. Importantly, these findings suggest that the two MAPK isoforms may be differentially regulated at high Trk A occupancy. The ability of growth factors to surmount the requirement of $G_{i/o}$ has been reported before [e.g., insulin (Luttrell et al. 1995)]. These authors found that insulin at low insulin receptor density stimulated p42/p44 MAPK activation in a pertussis toxin-sensitive manner in fibroblasts, whereas at high insulin receptor density, insulin stimulated a more robust activation of p42/p44 MAPK that was insensitive to pertussis toxin.

Finally, we have found that the GPCR-dependent component regulating p42/p44 MAPK in response to NGF is required for differentiation of PC12 cells. This was based on experiments showing that PD098059 (inhibitor of MAPK kinase-1 activation) and pertussis toxin partially blocked NGF-induced inhibition of DNA synthesis. Indeed, other agonists that use G protein-coupled receptor signaling pathways to regulate p42/p44 MAPK, such as LPA, also induce differentiation of PC12 cells. The fact that PD098059 and pertussis toxin did not fully restore DNA synthesis suggests that NGF may also use additional pathways to promote growth arrest.

In conclusion, our findings show for the first time that the Trk A receptor can use $G_{i/o}$ to promote efficient activation of the p42/p44 MAPK pathway required for cell differentiation. This seems to involve GRK2-β-arrestin-I and endocytosis of Trk A receptor signal complexes. These findings are novel and significant as they break the conventional paradigm for growth factor receptor signaling and strengthen an emerging model that such growth factor receptors can indeed, use classic GPCR signaling pathway to stimulate p42/p44 MAPK.

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