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Nerve growth factor induces protein-tyrosine phosphorylation

(phosphotyrosine/PC12 cells)

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ABSTRACT **When the sympathetic nerve-like cell line PC12 is exposed to nerve growth factor (NGF), there is a rapid and transient phosphorylation of tyrosine residues in cellular proteins, as demonstrated by immunoblotting of cell extracts with high-affinity polyclonal antibodies specific for phosphotyrosine residues. Epidermal growth factor (EGF), which does not cause the morphological differentiation of PC12 cells that is produced by NGF, also induces protein-tyrosine phosphorylation. The methyltransferase inhibitor, 5′-methylthioadenosine, which is known to block the NGF-mediated morphological differentiation of PC12 cells, also inhibits the induction of protein-tyrosine phosphorylation by NGF. 5′-Methylthioadenosine has no effect, however, on EGF-stimulated phosphorylation of tyrosine residues in cellular proteins. In addition, low temperature markedly slows the rate of protein-tyrosine phosphorylation stimulated by NGF, but it has no effect on the time course of protein-tyrosine phosphorylation induced by EGF. These data suggest that NGF and EGF induce protein-tyrosine phosphorylation in PC12 cells by different mechanisms.**

The phosphorylation of tyrosine residues in proteins is thought to mediate the mitogenic response of cultured cells to several growth factors. The plasma membrane receptors for insulin (1), platelet-derived growth factor (2), and epidermal growth factor (EGF) (3) all have intrinsic protein-tyrosine kinase activities (EC 2.7.1.112) as does the viral transforming protein $pp60^{v\text{-}src}$ (4). These data suggest a similar mode of action between some mitogenic growth factors and certain viruses that immortalize cells. Nerve growth factor (NGF) is a protein that promotes the survival and differentiation of several types of neurons (5), but it has little mitogenic activity (6). NGF, the mitogenic growth factors, and the transforming viruses do, however, induce a common set of metabolic changes in responsive cells. These include an elevation of ATP levels, more rapid nutrient and ion transport, and changes in the rates of macromolecular synthesis (7–11). To ask if NGF also shares with mitogenic growth factors and transforming viruses the ability to stimulate tyrosine-specific protein phosphorylation, the effect of NGF on protein-tyrosine phosphorylation in the clonal rat sympathetic nerve-like cell line PC12 was examined. In the presence of NGF, PC12 cells extend neurites (12), increase their ability to synthesize neurotransmitters (13), and become more electrically excitable (14). In addition, NGF induces the phosphorylation of serine and threonine residues in a subset of cytoplasmic and nuclear proteins in PC12 cells (15–18). The data presented here show that NGF also stimulates the phosphorylation of tyrosine residues in a limited number of proteins in PC12 cells.

MATERIALS AND METHODS

β-NGF and basic fibroblast growth factor (FGF) were obtained from D. Schubert (Salk Institute). NGF was used at 50 ng/ml, and basic FGF was used at 15 ng/ml. EGF was obtained from G. Gill (University of California, San Diego) and was used at 12 ng/ml. Phorbol 12-myristate 13-acetate (PMA), insulin, and dibutyryl-cAMP were obtained from Sigma. PMA was used at 125 ng/ml, insulin was used at 1 μM, and dibutyryl-cAMP was used at 1 mM. 5′-Methylthioadenosine (MeSAdo) was obtained from Sigma and was used at 3 mM. PC12 cells were obtained from D. Schubert and were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 5% horse serum. To examine the effect of growth factors on protein-tyrosine phosphorylation, cells were grown on polylysine-coated dishes in serum-containing medium; the culture medium was aspirated and was replaced by the chemically defined N2 medium (19). Only exponentially dividing cultures were used at a density of 5×10^5 cells per 35-mm dish. The different agents were added to the N2 medium, and after the indicated periods of time, the cells were rinsed rapidly with phosphate-buffered saline (P_i/NaCl) and were solubilized on the dish in 100 μl of $NaDodSO_4$ sample buffer (20) containing 0.1 mM sodium orthovanadate, 10 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 0.2 trypsin inhibitor units of aprotinin. The cells were then scraped into microcentrifuge tubes, sonicated briefly, and boiled for 3 min.

Equal amounts of cellular proteins were separated by $NaDodSO_4$/PAGE on 7.5% separating gels. The proteins were transferred to nitrocellulose (21), and the transfers were blocked with 3% bovine serum albumin in P_i/NaCl, incubated with anti-phosphotyrosine antibodies (2 μg/ml) for 4 hr, rinsed with P_i/NaCl, and incubated for 60 min with ^{125}I-labeled protein A (ICN) at 0.5 μCi/ml (1 Ci = 37 GBq). The nitrocellulose sheets were washed, dried, and autoradiographed on Kodak blue brand film with an intensifying screen for 1–4 days at −70°C. The molecular mass standards were myosin (200 kDa), vinculin (120 kDa), α-actinin (100 kDa), bovine serum albumin (68 kDa), and actin (43 kDa).

To prepare the high-affinity anti-phosphotyrosine antibodies, the insoluble material obtained after sonication of bacteria expressing the v-*abl*-encoded transforming protein in its tyrosine phosphorylated form was used to immunize rabbits (22). The immune serum was purified on an affinity column prepared by linking *O*-phospho-L-tyrosine (Sigma) to Affi-Gel 15 (Bio-Rad). The antibodies specifically bound to the phosphotyrosine groups of the column were eluted with 40 mM phenylphosphate in P_i/NaCl. After dialysis against P_i/NaCl and the addition of 0.3% bovine serum albumin, the antibodies were frozen.

RESULTS

Induction of Protein-Tyrosine Phosphorylation by NGF. To assay for the ability of NGF to stimulate the phosphorylation of tyrosine residues in proteins, anti-phosphotyrosine anti-

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Abbreviations: NGF, nerve growth factor; EGF, epidermal growth factor; FGF, basic fibroblast growth factor; PMA, phorbol 12-myristate 13-acetate; MeSAdo, 5′-methylthioadenosine.

bodies were used to detect proteins in immunoblotting experiments. These antibodies react specifically with proteins containing phosphotyrosine when used for immunoprecipitation (22, 23), immunoblotting (23–25), and immunofluorescence microscopy (26). They recognize known tyrosine-phosphorylated proteins such as talin (23), vinculin (23), and $pp60^{v\text{-}src}$ in Rous sarcoma virus-transformed cells. The reaction of the antibodies with cellular proteins is not inhibited by tyrosine sulfate (26). When exponentially dividing PC12 cells were exposed to NGF at 50 ng/ml for various times, lysed, run on 7.5% $NaDodSO_4$/polyacrylamide gels, transferred to nitrocellulose, and probed with anti-phosphotyrosine antibodies, a rapid increase in the extent of protein-tyrosine phosphorylation was observed. Unstimulated cells (Fig. 1, 0 time) have two major tyrosine-phosphorylated proteins of 180 and 150 kDa. The extent of protein-tyrosine phosphorylation is significantly higher after exposure to NGF for 2.5–5 min and then steadily declines. The level of total protein-tyrosine phosphorylation is increased 7-fold over control cells as determined by scanning the autoradiograms with a densitometer. After 6 hr in the continual presence of NGF, the levels of phosphotyrosine-containing proteins are indistinguishable from controls (data not shown). The concentration for half-maximal stimulation of protein-tyrosine phosphorylation by NGF is 5 ng/ml, which, under the conditions employed here, is similar to that for neurite extention. The major proteins whose tyrosine residues are phosphorylated in response to NGF have molecular masses of 150, 120, 60, and 55 kDa. The pattern was the same at NGF concentrations of 5 and 50 ng/ml. Transfers of proteins from NGF-treated cells that were probed with the anti-phosphotyrosine antibody in the presence of 10 mM phosphotyrosine exhibited no labeled bands (data not shown). These data show that NGF rapidly and reversibly stimulates the phosphorylation of tyrosine residues in a set of PC12 proteins.

Protein-Tyrosine Phosphorylation in PC12 Cells in Response to Other Growth Factors and Dibutyryl-cAMP. As outlined above, PC12 cells respond to a variety of mitogenic growth factors with an increased metabolic rate but not with an enhanced rate of cell division. To test if EGF or insulin alter protein phosphotyrosine levels, cells were exposed to these growth factors for increasing lengths of time. Fig. 2*A* shows that EGF rapidly induces an increase in the phosphotyrosine content of proteins of 180, 150, 120, 60, and 55 kDa. The band at 180 kDa may correspond to the EGF receptor. This represents an 8-fold increase in total protein phosphotyrosine levels over untreated cells. The overall protein phosphotyrosine levels and the time course of the EGF response are very similar to those caused by NGF. When proteins from NGF- and EGF-treated cells are coelectrophoresed and probed with the anti-phosphotyrosine antibodies, the tyrosine-phosphorylated proteins from each appear to have

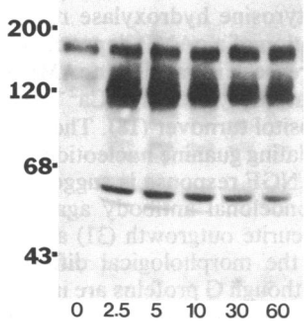

FIG. 1. Immunoblot, with antibodies specific for phosphotyrosine, of extracts of PC12 cells that had been exposed to NGF (50 ng/ml) for various times. The exposure time to NGF is given in minutes. Molecular mass markers (in kDa) are at left.

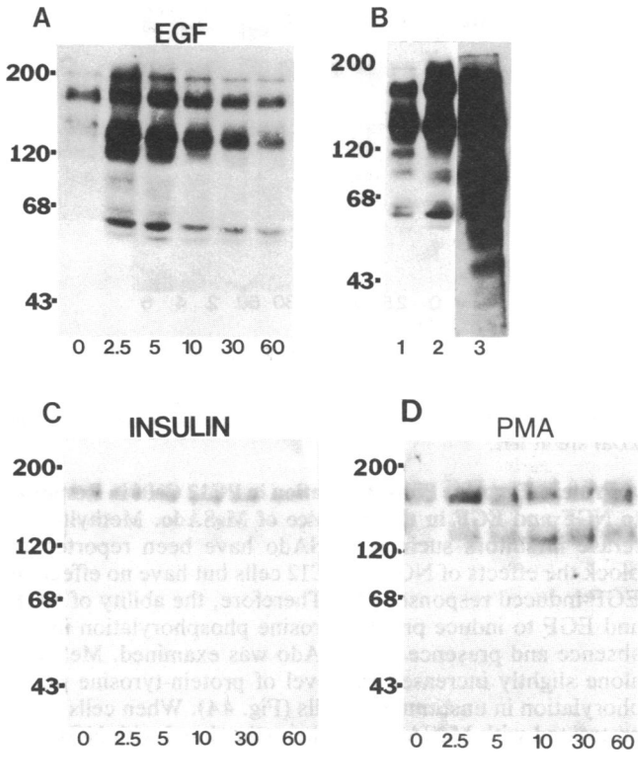

FIG. 2. Immunoblots, with antibodies specific for phosphotyrosine, of extracts of PC12 cells that had been exposed to EGF at 12 ng/ml (*A*), 1 μM insulin (*C*), or PMA at 125 ng/ml (*D*) for various times. The exposure time is given in minutes. (*B*) A comparison of the phosphotyrosine-containing proteins from EGF-treated (lane 1; 12 ng/ml; 2.5 min) and NGF-treated (lane 2; 50 ng/ml; 2.5 min) cells. Also shown is an equal amount of protein from an extract of Rous sarcoma virus-transformed mouse 3T3 cells (lane 3). Molecular mass markers (in kDa) are at left.

similar molecular masses (Fig. 2*B*, lanes 1 and 2). However, the band at 180 kDa generally appears much stronger in the EGF-treated cells as compared with NGF-treated cells. Although treatment of PC12 cells with either of these agents greatly increases the level of phosphotyrosine in proteins as compared with unstimulated cells, the overall level of protein-tyrosine phosphorylation is still well below that found in cells transformed by Rous sarcoma virus (Fig. 2*B*, lane 3). Phorbol esters are mitogenic for some cells and can stimulate protein-tyrosine phosphorylation in responsive cells (27). PMA is, however, a weaker inducer of protein-tyrosine phosphorylation in PC12 cells (Fig. 2*D*); it causes only a 3-fold increase in total protein phosphotyrosine levels. In contrast to EGF, insulin is totally ineffective at stimulating protein-tyrosine phosphorylation although it does have a variety of metabolic effects on these cells (Fig. 2*C*).

Unlike EGF, PMA, and insulin, which do not stimulate the morphological differentiation of PC12 cells, dibutyryl-cAMP is effective in causing the elaboration of neurites (28, 29). When cells were exposed to 1 mM dibutyryl-cAMP, there was a stimulation of tyrosine phosphorylation in proteins with molecular masses of 70 and 150 kDa, but both the time course and magnitude (2-fold above control cells) of the response differ from that of NGF-treated cells (Fig. 3). In addition, a phosphorylated protein of 70 kDa is not observed in NGF-treated cells. The increase in protein-tyrosine phosphorylation is maximal at 60 min and returns to baseline by 2 hr. Exposure of PC12 cells to FGF or membrane depolarization does not give any reproducible increase in protein-tyrosine phosphorylation although both treatments induce the morphological differentiation of the cells (data not shown).

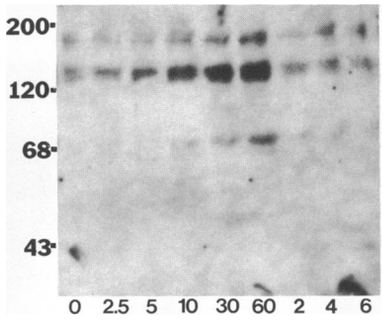

FIG. 3. Immunoblot, with antibodies specific for phosphotyrosine, of extracts of PC12 cells that were treated with 1 mM dibutyryl-cAMP for various times. The exposure time to dibutyryl-cAMP is given in minutes (0–60) and hours (2–6). Molecular mass markers (in kDa) are at left.

Protein-Tyrosine Phosphorylation in PC12 Cells in Response to NGF and EGF in the Presence of MeSAdo. Methyltransferase inhibitors such as MeSAdo have been reported to block the effects of NGF on PC12 cells but have no effect on EGF-induced responses (30). Therefore, the ability of NGF and EGF to induce protein-tyrosine phosphorylation in the absence and presence of MeSAdo was examined. MeSAdo alone slightly increased the level of protein-tyrosine phosphorylation in unstimulated cells (Fig. 4*A*). When cells were pretreated with MeSAdo and then stimulated with NGF, no increase in protein-tyrosine phosphorylation was seen (Fig. 4*A*). The absence of NGF-induced protein-tyrosine phosphorylation in the presence of MeSAdo was not due to a slower rate of phosphorylation since a 1-hr exposure to NGF in the presence of MeSAdo gave the same result (Fig. 4*A*). In contrast, preincubation with MeSAdo increased the induction of protein-tyrosine phosphorylation by EGF (Fig. 4*B*).

Protein-Tyrosine Phosphorylation in PC12 Cells in Response to NGF and EGF at 7°C. To further compare the NGF- and EGF-stimulated phosphorylation of tyrosine residues in proteins, the time courses of protein-tyrosine phosphorylation induced by NGF and EGF at 7°C were examined. EGF still caused a rapid increase in the level of phosphotyrosine in proteins (Fig. 4*D*), whereas NGF did not (Fig. 4*C*). At 7°C, NGF-stimulated phosphorylation of tyrosine on proteins was not apparent until 60 min after addition of the agent.

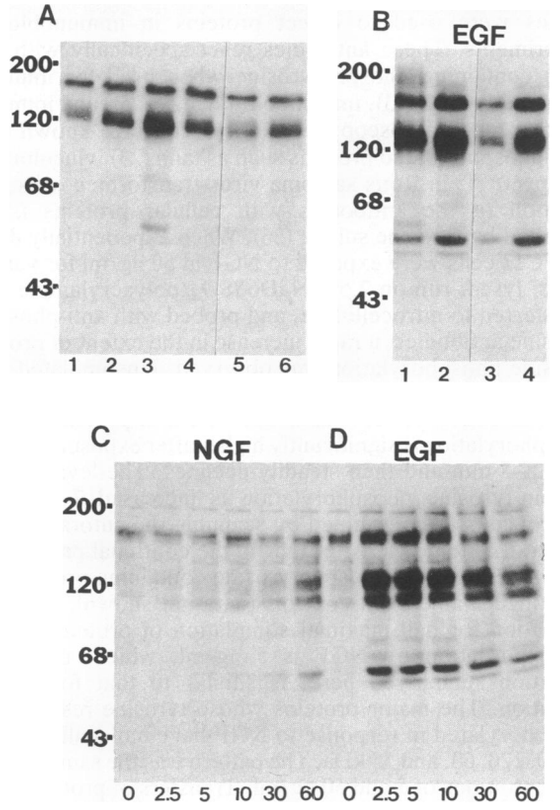

FIG. 4. (*A*) Immunoblot, with antibodies specific for phosphotyrosine, of extracts of PC12 cells that were untreated (lanes 1 and 2) or treated with NGF at 50 ng/ml (lanes 3–6) for 2.5 min (lanes 3 and 4) or 60 min (lanes 5 and 6) in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 3 mM MeSAdo. (*B*) Immunoblot, with antibodies specific for phosphotyrosine, of extracts of PC12 cells that were treated with EGF at 12 ng/ml for 2.5 min (lanes 1 and 2) or 10 min (lanes 3 and 4) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 3 mM MeSAdo. (*C* and *D*) Immunoblots, with antibodies specific for phosphotyrosine, of extracts of PC12 cells that were treated with NGF at 50 ng/ml (*C*) or EGF at 12 ng/ml (*D*) for various times at 7°C. The exposure time is given in minutes. Molecular mass markers (in kDa) are at left.

DISCUSSION

The above data show that NGF stimulates the tyrosine phosphorylation of a limited number of proteins in the sympathetic nerve cell line PC12. The phosphorylation event is rapid; it reaches maximal levels between 2.5 and 5 min, followed by a slow decline to baseline levels (Fig. 1). EGF also stimulates extensive protein-tyrosine phosphorylation, whereas insulin and phorbol esters have little or no effect (Fig. 2). Of the reagents or conditions in addition to NGF that stimulate the morphological differentiation of PC12 cells (neurite outgrowth), only dibutyryl-cAMP causes protein-tyrosine phosphorylation, albeit with a slower time course than NGF (Fig. 3).

There is an alteration in the phosphorylation of several PC12 proteins after the addition of NGF, as well as EGF, insulin, and dibutyryl-cAMP to PC12 cells (15, 16). The time course of this response to NGF is slower than protein-tyrosine phosphorylation, with a maximal response occurring at 15 min, and high levels of phosphorylation are maintained as long as NGF is present. The identified proteins that are phosphorylated include tyrosine hydroxylase, ribosomal protein S6, histones H1 and H3, and the nonhistone chromosomal and cytoplasmic high mobility group 17 protein. The molecular masses of these proteins are different from those found to be phosphorylated on tyrosine residues. The best studied of these proteins is tyrosine hydroxylase, which is phosphorylated on serine residues (17, 18). The data presented here show that NGF can also induce a protein-tyrosine kinase, suggesting that NGF causes the activation of several classes of protein kinases that phosphorylate distinct subsets of proteins in the responsive cell.

How are these kinases activated by NGF? Serine phosphorylation of tyrosine hydroxylase results from the independent stimulation of a cAMP-dependent protein kinase by means of an increase in intracellular cAMP and the activation of a protein kinase C by means of Ca^{2+} mobilization and/or phosphatidylinositol turnover (18). The involvement of protein kinase regulating guanine nucleotide-binding proteins (G proteins) in the NGF response is suggested by the observations that a monoclonal antibody against ras p21 inhibits NGF-induced neurite outgrowth (31) and that activated ras protein causes the morphological differentiation of PC12 cells (32, 33). Although G proteins are involved in phosphatidylinositol turnover, the induction of protein-tyrosine phosphorylation is, however, likely to be independent of phosphatidylinositol turnover since elevated external potassium stimulates phosphatidylinositol turnover (11) but not protein-tyrosine phosphorylation. It is also unlikely that cAMP-

dependent protein kinases are causal for the NGF-induced tyrosine phosphorylation of proteins because, although dibutyryl-cAMP stimulates protein-tyrosine phosphorylation, the time course is much slower than with NGF and the molecular masses of some of the phosphorylated proteins are different.

Since the rapid tyrosine phosphorylation of proteins by growth factors has only been reported for molecules whose receptors are themselves protein-tyrosine kinases (34), the most straightforward explanation of these results is that the NGF receptor itself is a protein-tyrosine kinase. However, a NGF receptor has been sequenced, and it has no sequence similarity with protein-tyrosine kinases (35, 36). Since there are two forms of the NGF receptor, distinguished on the basis of their relative molecular ligand affinities (37), it is possible that the unidentified high-affinity receptor is a protein-tyrosine kinase. Consistent with this possibility is the finding that NGF does not induce protein-tyrosine phosphorylation in the JS-1 schwannoma cell line (data not shown), which expresses only the low-affinity NGF receptor (38).

The experiment with the methyltransferase inhibitor MeSAdo, as well as the low-temperature studies, suggest that EGF and NGF induce protein-tyrosine phosphorylation in the PC12 cells by different mechanisms. These data also indicate that the induction of protein-tyrosine phosphorylation by NGF is not due to contamination of the NGF preparation with EGF. Since the EGF receptor is known to be a protein-tyrosine kinase (3), these data suggest that either the high-affinity NGF receptor is a different type of protein-tyrosine kinase than the the EGF receptor or that the NGF receptor is not itself a protein-tyrosine kinase but activates one. In the latter case, the NGF–receptor complex might have to bind to the protein-tyrosine kinase in order to activate it, and this could be the significance of the inhibition of phosphorylation at low temperature. Since the protein-tyrosine phosphorylation patterns induced by NGF and EGF are so similar, the protein-tyrosine kinase activated by NGF could be the EGF receptor. However, EGF induces tyrosine phosphorylation of a 180-kDa protein, which may be its receptor, whereas NGF does not. The correlation between the ability of MeSAdo to inhibit the morphological changes caused by NGF and to inhibit the protein-tyrosine phosphorylation induced by NGF suggests that the specific NGF-induced protein tyrosine phosphorylations may play a role in the morphological changes, whereas the protein-tyrosine phosphorylations induced by EGF do not. In this connection, MeSAdo has also been found to block the transformation of chicken embryo fibroblasts by Rous sarcoma virus, another inducer of protein-tyrosine phosphorylation (39).

In conclusion, the finding that NGF induces rapid phosphorylation of tyrosine residues in proteins in PC12 cells should help to elucidate the mechanism whereby NGF alters the morphology and physiology of these cells.

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