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# Nerve growth factor stimulates the hydrolysis of glycosylphosphatidylinositol in PC-12 cells: A mechanism of protein kinase C regulation

(diacylglycerol/second messenger/protooncogene/signal transduction)

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**ABSTRACT** **Treatment of PC-12 pheochromocytoma cells with nerve growth factor (NGF) results in the differentiation of these cells into a sympathetic neuron-like phenotype. Although the initial intracellular signals elicited by NGF remain unknown, some of the cellular effects of NGF are similar to those of other growth factors, such as insulin. We have investigated the involvement of a newly identified inositol-containing glycolipid in signal transduction for the actions of NGF. NGF stimulates the rapid generation of a species of diacylglycerol that is labeled with [$^3$H]myristate but not with [$^3$H]arachidonate. NGF stimulates [$^3$H]myristate- or [$^{32}$P]phosphate-labeled phosphatidic acid production over the same time course. Although NGF alone has no effect on the turnover of inositol phospholipids, it does stimulate the hydrolysis of glycosylphosphatidylinositol. The NGF-dependent cleavage of this lipid is accompanied by an increase in the accumulation of its polar head group, an inositol phosphate glycan, which is generated within 30–60 sec of NGF treatment. In an unresponsive PC-12 mutant cell line, neither the diacylglycerol nor inositol phosphate glycan response is detected. A possible role for the NGF-stimulated diacylglycerol is suggested by the inhibition of NGF-dependent c-*fos* induction by staurosporin, a potent inhibitor of protein kinase C. These results suggest that, like insulin, some of the cellular effects of NGF may be mediated by the phospholipase C-catalyzed hydrolysis of glycosylphosphatidylinositol.**

Despite the extensive characterization of the trophic and differentiative properties of nerve growth factor (NGF; refs. 1 and 2), the molecular mechanisms of signal transduction remain poorly understood. Early reports suggested a role for cAMP as a second messenger (3); however, cAMP appears to be neither necessary nor sufficient for many NGF-stimulated activities (4, 5). Similarly, phosphatidylinositol turnover has been implicated in NGF action (6, 7); however, the intracellular mobilization of calcium that is expected with inositol trisphosphate production is not observed in response to NGF (8). On the other hand, recent studies have shown that the diacylglycerol/protein kinase C pathway may play some role, since phorbol esters, the tumor-promoting activators of protein kinase C, share certain biological activities of NGF in PC-12 cells (9–12), and protein kinase C inhibitors can block certain actions of NGF (13, 14).

The cellular effects of NGF are reminiscent of the pleiotropic actions of insulin. Recent studies on the mechanism of signal transduction for insulin have led to the proposal that some of the actions of the hormone might be mediated by the hydrolysis of a glycosylphosphatidylinositol, resulting in the generation of a structurally distinct diacylglycerol and an inositol phosphate glycan (15). Because of similarities in some of the biological actions of insulin and NGF, we explored the possible effects of NGF on the metabolism of glycosylphosphatidylinositol in PC-12 cells.

## EXPERIMENTAL PROCEDURES

**Materials.** All reagents were from Sigma except for tissue culture supplies (GIBCO), 2.5S NGF (Collaborative Research), Bio-Gel P-2 (Bio-Rad), [2,6-$^3$H]inositol (American Radiolabeled Chemicals, Saint Louis, MO), [$^3$H]glucosamine, [9,10-$^3$H]myristic acid, and [5,6,8,9,11,12,14,15-$^3$H]-arachidonic acid (New England Nuclear). The analytical SAX HPLC column was from Whatman. Silica gel G plates were from Analtech. DNA polymerase I and all restriction enzymes were from Boehringer Mannheim. Staurosporin was from Kamiya Biochemical (Thousand Oaks, CA).

**Cell Culture.** PC-12 cells (gift of L. Greene, Columbia University, New York) were adapted to grow on plastic 100-mm culture dishes or 3.5 × 1 cm six-well dishes in Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) fetal bovine serum and 5% (vol/vol) horse serum. Cells were incubated with 3 $\mu$Ci (1 Ci = 37 GBq) of [$^3$H]inositol, [$^3$H]glucosamine, or tritiated fatty acid per ml for 24 hr in 70% inositol-free RPMI 1640 medium containing 25% DMEM, 3.7% fetal bovine serum, and 1.3% horse serum.

**Extraction and Analysis of Lipids.** After preincubation with the appropriate isotopes, cells were washed three times with 1 ml of Hanks' buffer (pH 7.4) and equilibrated in 1 ml of the same buffer for 30 min prior to the addition of 50 ng of NGF per ml. At the designated time intervals, reactions were terminated by the addition of 2 ml of chloroform/methanol/6 M HCl, 200:100:1 (vol/vol), followed by 1 ml of 50 mM formic acid. After centrifugation at 500 × *g* for 5 min, the upper aqueous phase was saved for further analysis of the water-soluble products. The lower organic phase was evaporated under nitrogen and resuspended in chloroform/methanol, 2:1 (vol/vol). Diacylglycerol and phosphatidic acid (16) and glycosylphosphatidylinositol (17) were analyzed by TLC.

**Analysis of Inositol Glycan Production.** The aqueous phase from the first extraction was evaporated to remove methanol and passed through a $C_{18}$ Sep-Pak column to remove any residual lipids. After lyophilization, the sample was redissolved in water and further chromatographed on an analytical Partisil 10 SAX HPLC column. The column was subjected to a 5-min isocratic elution in 20 mM triethylamine formate (pH 4.5) followed by a linear 25-min gradient of 20 mM–1 M triethylamine formate (pH 4.5) at 1 ml/min.

**RNA Analysis.** Total RNA was isolated from PC-12 cells by guanidinium isothiocyanate treatment followed by centrifu-

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Abbreviation: NGF, nerve growth factor.

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gation in a cushion of CsCl. Equal amounts of RNA were subjected to electrophoresis in 1% agarose gels containing 2.2 M formaldehyde/20 mM Mops buffer/1 mM EDTA. After transfer to nitrocellulose paper, hybridization to a v-*fos* probe was carried out in 50% formamide containing 5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7), 10% dextran sulfate, and 100 μg of salmon sperm DNA per ml at 42°C. Washes were carried out with 0.5× standard saline citrate (SSC) at 50°C.

## RESULTS

**NGF Stimulates Production of Myristate-Labeled Diacylglycerol.** The production of diacylglycerol in response to NGF was evaluated in PC-12 cells. Cells were labeled with [$^3$H]myristic acid or [$^3$H]arachidonic acid and exposed to NGF for various time intervals (Fig. 1). In [$^3$H]myristate-labeled cells (Fig. 1 *Upper*), NGF caused a rapid increase in labeled diacylglycerol, which peaked by about 2–3 min, declined to basal levels by 5 min, and thereafter slowly increased. This biphasic pattern was consistently observed in several experiments, although occurrence of the peak of radioactivity varied from 0.5 to 3 min. In contrast to the rapid increase in [$^3$H]myristate-labeled diacylglycerol, NGF did not cause any significant increase in the [$^3$H]arachidonate-labeled species (Fig. 1 *Lower*).

After its formation, diacylglycerol is rapidly phosphorylated to produce phosphatidic acid. The time course of phosphatidic acid production was evaluated in cells labeled with [$^3$H]myristate for 24 hr or [$^{32}$P]orthophosphate for 3 hr (Fig. 2). Cells were washed and exposed to NGF, and phosphatidic acid production was monitored at different time intervals. Both [$^3$H]myristate- and [$^{32}$P]orthophosphate-labeled phosphatidic acid production rapidly increased in response to NGF over a time course similar to that observed for diacylglycerol. NGF had no detectable effect on the production of [$^3$H]arachidonate-labeled phosphatidic acid. These labeling experiments do not indicate the mass of the diacylglycerol or phosphatidic acid that is generated in response to NGF.

**NGF Stimulates the Hydrolysis of Glycosylphosphatidylinositol.** Since inositol phospholipids derived from mammalian cells are known to largely contain 1-stearoyl-2-arachidonoylglycerol as the hydrophobic domain, it is likely that the NGF-stimulated species of diacylglycerol arises from alternative sources. A glycosylated form of phosphatidylinositol has been identified recently that appears to contain almost exclusively saturated fatty acids in the glycerolipid domain—predominantly myristate or palmitate (16–19).

After a 24-hr preincubation with [$^3$H]glucosamine (1 μCi/ml), PC-12 cells were washed and exposed to 50 ng of NGF per ml at different time intervals (Fig. 3). After phase separations, glycosylphosphatidylinositol was quantitated by TLC (Fig. 3 *Upper*). NGF caused nearly a 70% depletion in cpm of glycosylphosphatidylinositol within 2 min, followed by a slower increase over the next 10–20 min. This pattern of

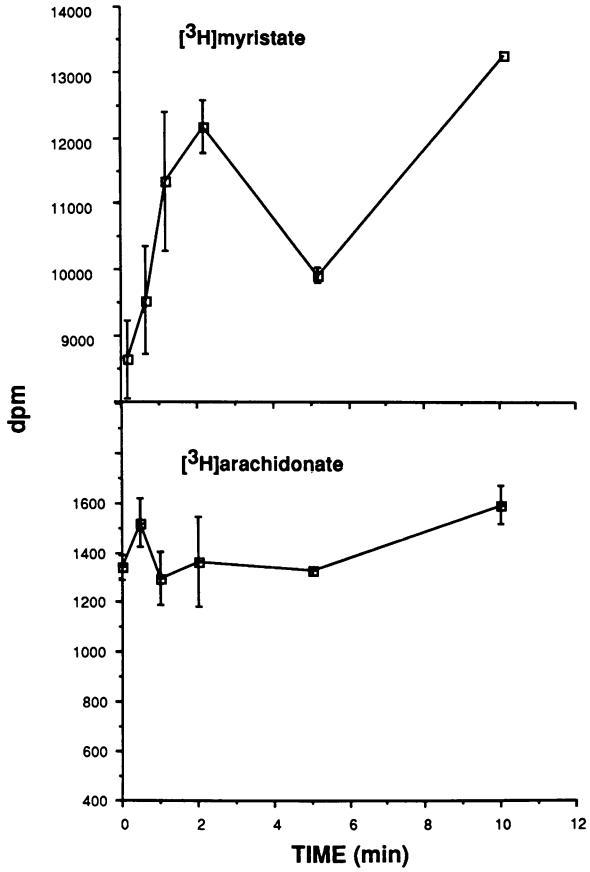


FIG. 1. NGF stimulates [$^3$H]myristate-labeled diacylglycerol in PC-12 cells. PC-12 cells cultured in six-well plates were incubated for 24 hr with 3 μCi of [$^3$H]myristic acid (*Upper*) or [$^3$H]arachidonic acid (*Lower*) per ml. Cells were washed and exposed to 50 ng of NGF per ml for the designated intervals. After the incubations, cells were extracted, and labeled diacylglycerol was quantitated by thin-layer chromatography as described. Data are expressed as the means of triplicate determinations ± SD and were repeated in several experiments.

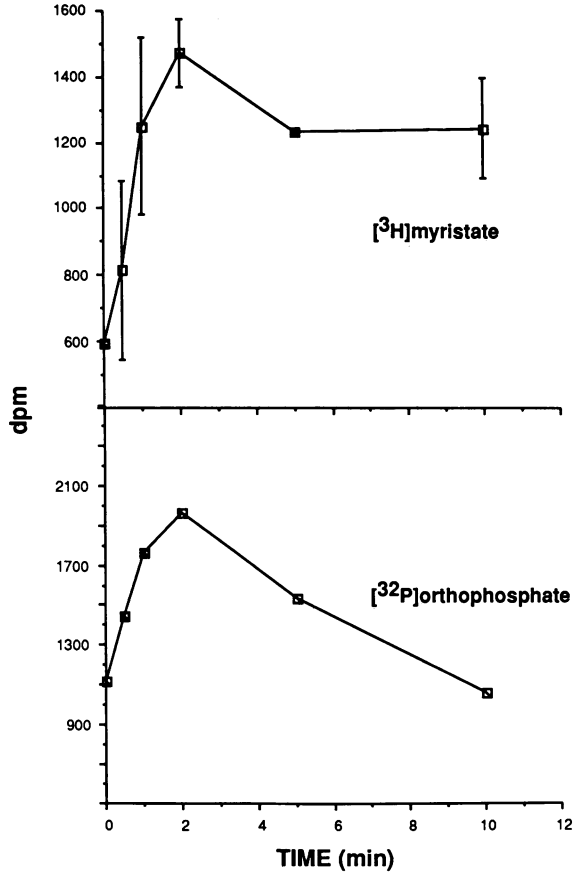


FIG. 2. NGF stimulates phosphatidic acid production in PC-12 cells. PC-12 cells cultured in six-well plates were incubated with 3 μCi of [$^3$H]myristic acid per ml for 24 hr (*Upper*) or with 10 μCi of [$^{32}$P]orthophosphate per ml for 3 hr (*Lower*). Cells were washed and exposed to 50 ng of NGF per ml for the designated intervals. After the incubation, cells were extracted, and labeled phosphatidic acid was quantitated by thin-layer chromatography as detailed. Results are the means of triplicate determinations, which were repeated in several experiments.

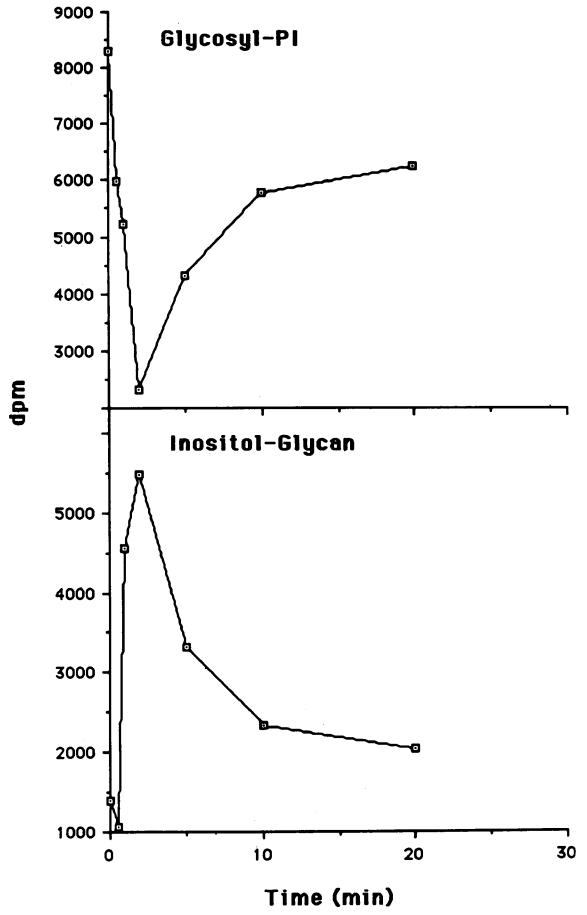


FIG. 3. NGF stimulates the hydrolysis of glycosylphosphatidylinositol in PC-12 cells. Plates (100 mm) of PC-12 cells were incubated for 24 hr with 3 $\mu$Ci of [$^3$H]glucosamine per ml. Cells were washed and exposed to 50 ng of NGF per ml for the designated intervals. After the incubations, cells were extracted and phase separated as described above. (*Upper*) Labeled glycosylphosphatidylinositol was quantitated by thin-layer chromatography. (*Lower*) Labeled inositol glycan in the aqueous phase was quantitated by SAX HPLC. Results are the means of triplicate determinations that were repeated in several experiments.

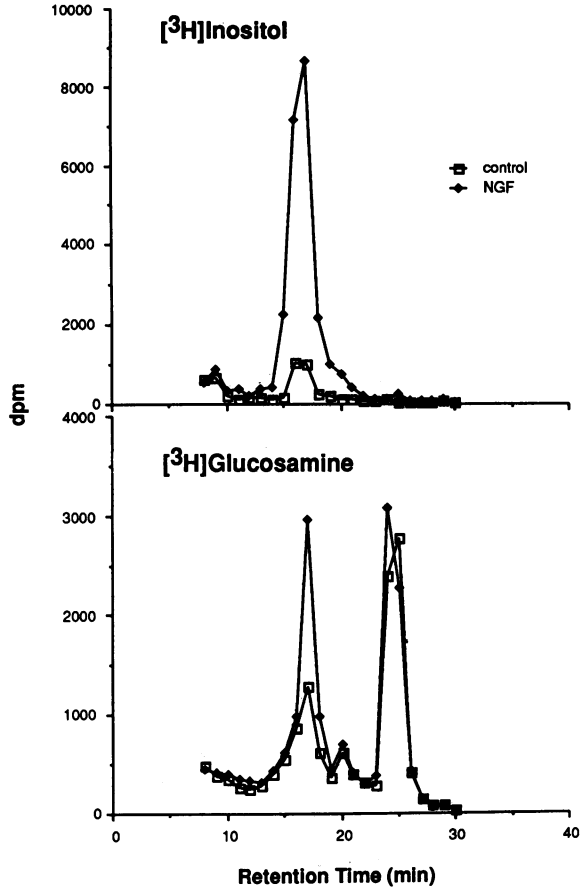


FIG. 4. NGF stimulates inositol glycan production in PC-12 cells. Plates (100 mm) of PC-12 cells were incubated with [$^3$H]inositol (*Upper*) or [$^3$H]glucosamine (*Lower*) as described in Fig. 3. Cells were washed and exposed to 50 ng of NGF per ml or buffer alone for 2 min. The incubations were stopped by extraction of cells with chloroform/methanol/HCl. Aqueous phases were chromatographed in a SAX HPLC column as described. One-minute fractions were collected and assayed for radioactivity.

apparent resynthesis of glycosylphosphatidylinositol in NGF-treated PC-12 cells is similar to that observed in $BC_3H1$ cells (16, 17), H-4 hepatoma cells (18), or T lymphocytes (19) after exposure to insulin.

The phospholipase C-catalyzed cleavage of glycosylphosphatidylinositol should also result in the production of the polar head group of the lipid, inositol phosphate glycan. The upper, aqueous phases from the above experiment were purified and chromatographed on a SAX HPLC column as described in *Experimental Procedures* (Fig. 3 *Lower*). NGF caused a 3- to 4-fold increase in the accumulation of the [$^3$H]glucosamine-labeled inositol glycan within 1–2 min. A similar pattern was observed in [$^3$H]inositol-labeled cells (not shown). Fig. 4 illustrates the SAX HPLC profile for the aqueous products. NGF treatment increased the incorporation of [$^3$H]inositol (Fig. 4 *Upper*) and [$^3$H]glucosamine (Fig. 4 *Lower*) into a peak that was eluted at 17 min. The elution of this peak is identical to the retention time for the inositol glycan produced in $BC_3H1$ cells in response to insulin (16).

NGF rapidly stimulated the accumulation of the inositol glycan (Fig. 3 *Lower*). The effect was maximal at 1–2 min and declined thereafter. This biphasic effect of NGF was consistently observed in several experiments, although the peak of radioactivity varied from 0.5 to 3 min, as described above for diacylglycerol production. Although NGF rapidly stimulated the production of the inositol glycan, there was no effect of the hormone on the generation of other inositol phosphates throughout a 20-min incubation. In contrast, exposure of these cells to carbachol for 2 min led to a 3-fold increase in the accumulation of inositol 1,4,5-trisphosphate (not shown). These data are consistent with the lack of effect of NGF on the mobilization of intracellular calcium in these cells (8), indicating no effect on the hydrolysis of phosphatidylinositol 4,5-bisphosphate.

**NGF Fails to Stimulate Diacylglycerol or Inositol Glycan Production in Receptor-Negative Mutant Cells.** To evaluate the specificity of NGF action in this cell type, a PC-12 genetic variant that lacks the NGF receptor was examined. NR18 cells were selected after ethylmethanesulfonate mutagenesis of PC-12 cells and are unresponsive to NGF treatment (20). The production of both [$^3$H]myristate-labeled diacylglycerol and inositol glycan was assayed after exposure to NGF (Table 1). In control PC-12 cells, NGF caused a 3-fold increase in the intracellular production of the inositol glycan and almost a 2-fold increase in the generation of [$^3$H]-myristate-labeled diacylglycerol. In contrast, there was an insignificant effect of the hormone on inositol glycan or diacylglycerol production in the receptor-negative cell line.

**Staurosporin Blocks Induction of c-*fos* Gene Expression by NGF.** One of the earliest responses to NGF treatment in PC-12 cells is the transient induction of the c-*fos* protooncogene (12, 21–23). We tested whether this early response can

Table 1. NGF-stimulated generation of inositol glycan and diacylglycerol (DAG) in normal and receptor-negative cells

| Cell type | NGF | [$^3$H]Myristate-labeled DAG, dpm | [$^3$H]Inositol glycan, dpm |
|---|---|---|---|
| PC-12 | − | 3467 ± 191 | 688 ± 52 |
| PC-12 | + | 6724 ± 407 | 1858 ± 77 |
| NR18 | − | 3945 ± 157 | 800 ± 70 |
| NR18 | + | 3554 ± 284 | 907 ± 91 |

PC-12 or NR18 cells were labeled in 100-mm dishes with 1 μCi of [$^3$H]myristate or [$^3$H]inositol per ml for 24 hr in DMEM. Cells were washed and exposed to NGF (50 ng/ml) or buffer alone for 2 min. Cells were extracted with chloroform/methanol/HCl, and diacylglycerol and inositol glycan were quantitated as described.

be attenuated by staurosporin, a potent inhibitor of protein kinase C activity (24). PC-12 cells were treated with a variety of agents including phosphatidic acid, phospholipase C, and NGF for 30 min. Total RNA was prepared and tested for the steady-state levels of c-*fos* mRNA by blot-hybridization (Northern) analysis (Fig. 5*A*). Exposure of cells to 50 ng of NGF per ml led to an enormous accumulation of c-*fos* mRNA. Treatment with phosphatidic acid (50 μg/ml) caused a modest increase in c-*fos* induction (Fig. 5*A*). Interestingly, treatment of cells with NGF in the presence of 2.5 nM staurosporin resulted in a level of c-fos RNA that was ≈1/10th that in cells treated with NGF alone.

The dose dependence of the effect of staurosporin upon the level of NGF-stimulated c-*fos* induction was evaluated (Fig. 5*B*). PC-12 cells were simultaneously treated with 50 ng of NGF per ml and increasing concentrations of staurosporin. A decrease in c-fos mRNA occurred with an $IC_{50}$ of 2 nM staurosporin, similar to the concentration range previously reported for the inhibition of protein kinase C (24). Equal amounts of RNA were verified by hybridization of the same blot with a probe for the NGF receptor mRNA (data not shown).

## DISCUSSION

One of the major obstacles in elucidating the mechanisms of signal transduction for NGF has been the wide variety of cellular changes induced by the hormone. Although the precise molecular events in NGF action remain uncertain, numerous reports have indicated a role for protein phosphorylation (25–34). The involvement of both cAMP-dependent kinase (27–29) and calcium/phospholipid-dependent kinases (10–14, 29) has been suggested; however, considerable uncertainty remains concerning the biochemical mechanisms that link the NGF receptor to the regulation of these kinases. Although an early report suggested that NGF stimulates cAMP production (3), stimulation of adenylate cyclase activity has not been observed (35). Similarly, NGF stimulation of phosphatidylinositol turnover occurs only in the presence of other agonists (36, 37) and may merely reflect nonspecific increases in phospholipid synthesis. There is no evidence to support a direct activation of the phosphatidylinositol polyphosphate-specific phospholipase C by NGF, since inositol trisphosphate is not generated in response to the hormone, and there is no indication of increased intracellular calcium mobilization (8).

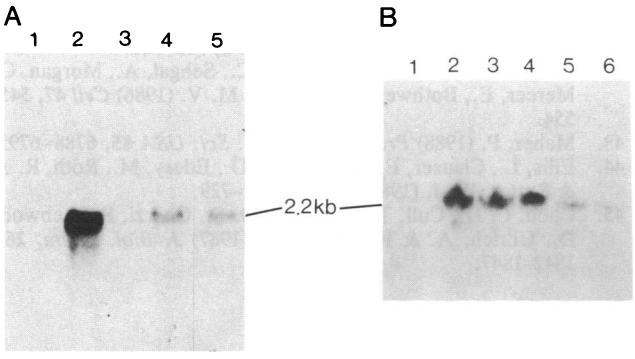


FIG. 5. Staurosporin blocks NGF-induced c-fos mRNA production in PC-12 cells. (*A*) PC-12 cells were treated with the following for 30 min at 37°C: no treatment (lane 1), NGF at 50 ng/ml (lane 2), phospholipase C at 1 μg/ml (lane 3), phosphatidic acid at 50 μg/ml (lane 4), and NGF at 50 ng/ml plus staurosporin at 2.5 nM (lane 5). Equal amounts of total cellular RNA were extracted and electrophoretically separated on 2.2 M formaldehyde/1% agarose gels, transferred to nitrocellulose, and analyzed with a nick-translated v-*fos* probe. (*B*) c-*fos* induction was measured as described above in the presence of NGF at 50 ng/ml plus increasing concentrations of staurosporin: no treatment (lane 1), NGF alone (lane 2), NGF plus 0.3 nM staurosporin (lane 3), NGF plus 1 nM staurosporin (lane 4), NGF plus 3 nM staurosporin (lane 5), and NGF plus 10 nM staurosporin (lane 6). kb, Kilobases.

Since phosphatidylinositol polyphosphate hydrolysis appears not to be involved in kinase C activation by NGF, it seemed likely that diacylglycerol is formed from another pathway. The results presented in this report indicate that diacylglycerol is produced in response to NGF in PC-12 cells. Although it is not possible to determine the mass or identify the source of diacylglycerol, the observations reported here suggest that the hydrolysis of glycosylphosphatidylinositol may be involved. The turnover of this lipid is observed over a time course similar to that for the production of the myristate-labeled diacylglycerol and phosphatidic acid, as well as the inositol phosphate glycan. The specificity of this response is supported by the inability of NGF to stimulate the production of diacylglycerol or inositol glycan in a receptor-negative mutant PC-12 cell line. These experiments suggest that like insulin, the NGF-stimulated species of diacylglycerol may result at least in part from the hydrolysis of glycosylphosphatidylinositol.

The observations that the inositol-glycan may mediate some of the effects of insulin on lipid and carbohydrate metabolism (15) suggest that this molecule may also account for some of the anabolic effects of NGF (1, 2). Furthermore, the hydrolysis of glycosylphosphatidylinositol may represent a mechanism whereby protein kinase C may be directly regulated by NGF without phosphatidylinositol polyphosphate hydrolysis or calcium mobilization, similar to what has been proposed for insulin (15). This possibility has been tested by examining a short-term response to NGF—induction of c-*fos* gene expression. The kinase C inhibitor staurosporin caused the dose-dependent inhibition of c-*fos* induction by NGF, consistent with the finding that the c-*fos* gene regulation may be influenced by protein kinase C (38). Similarly, NGF-stimulated neurite outgrowth was blocked by the kinase C inhibitors K252A (34) and sphingosine (14). Although these results do not directly prove that NGF action involves the stimulation of kinase C, they suggest that active protein kinase C is necessary for the NGF-dependent induction of c-*fos*.

The apparent similarities in the structure, function, and origin of NGF and insulin have been noted (39). Indeed, NGF and insulin elicit a number of similar cellular responses, including maintenance of neuronal viability in culture and differentiation of neuroblastoma cells (40), induction of neurite formation in PC-12 cells and cultured embryonic sensory neurons (41), regulation of amino acid and glucose uptake (2), and c-*fos* induction (12) in PC-12 cells. Clear differences in the actions of insulin and NGF also exist. The phosphorylation pattern induced by NGF in PC-12 cells was qualitatively dissimilar to that produced by insulin, although some overlap was observed (27). In addition, whereas NGF has been reported to increase cAMP levels in PC-12 cells, insulin generally produces the opposite effect in adipose and

liver cells (15). Thus, the actions of insulin and NGF may depend upon both common and divergent biochemical pathways.

Significant differences exist in the basic structures of the NGF and insulin receptors. The NGF receptor is a cysteine-rich, negatively charged glycoprotein with a single transmembrane segment (42). There was no evidence for a tyrosine kinase domain in the cytoplasmic region of the receptor, although tyrosine phosphorylation in PC-12 cells has been detected in response to NGF (43). The predicted structure of the NGF receptor is unlike the fundamental structure of receptors known to couple to G proteins (seven membrane spanning domains) and also unlike many other growth factor receptors and protooncogene products that contain a tyrosine kinase activity. In the case of insulin, tyrosine kinase activity appears to be necessary for receptor function (44, 45). The differences in the basic structures of the NGF and insulin receptors raise important questions concerning the precise biochemical events distal to receptor binding that converge to elicit similar biological responses.

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