NGF-Induction of the Metalloproteinase Transin/Stromelysin in PC12 Cells: Involvement of Multiple Protein Kinases

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Abstract. In previous work, we found that nerve growth factor (NGF) induced expression of the mRNA transcript encoding the metalloproteinase transin/stromelysin in PC12 cells. Transin was found, moreover, to be a "late" gene product whose expression correlated with neurite extension. In this study, various aspects of the NGF intracellular signaling pathway in PC12 cells are investigated. We show that the protein kinase inhibitor staurosporine, but not various other kinase inhibitors, specifically blocked the NGF induction of transin. Preliminary characterization of this staurosporine-sensitive kinase suggest that it does not correspond to a tyrosine kinase, nor various serine kinases, and that it is involved both at the transcriptional and posttranscriptional levels of transin gene regulation. In contrast to these effects of staurosporine, various activators of protein kinases C and A augmented the NGF induction of transin. Similar effects of these kinase inhibitors and activators were also observed with the expression of various immediate-early genes that have been proposed to mediate the transcriptional effects of NGF, including c-*fos* and c-*jun*. These data suggest, therefore, that the NGF induction of transin mRNA expression involves multiple protein kinases acting at a number of postreceptor regulatory steps in the NGF signaling pathway.

Nerve growth factor (NGF)[1] is a polypeptide growth factor that promotes the survival and differentiation of various neuronal populations during development (see Levi-Montalcini, 1987). One experimental model for studying the effects of NGF is the PC12 rat pheochromocytoma cell line (Greene and Tischler, 1976). In the absence of NGF, PC12 cells resemble normal adrenal chromaffin cells by various morphological and biochemical criteria (Greene and Tischler, 1976; Greene et al., 1985). After addition of NGF to the culture medium, PC12 cells undergo a transformation over a period of hours to days into neuronal cells, elaborating neurites and expressing various "late" gene products associated with the neuronal phenotype (Greene, 1984), including the neurofilament protein NF-M (Lindenbaum et al., 1988), GAP-43 (Federoff et al., 1988), and SCG-10 (Stein et al., 1988). A major issue, however, has concerned the nature of the intracellular signal responsible for the NGF-regulation of these various late genes.

Various protein kinases have recently been implicated as part of the intracellular signaling mechanism for NGF. The high-affinity NGF receptor has been shown, for example, to contain two ligand-binding proteins, one of which corresponds to the *trk* tyrosine kinase protooncogene (Chao et al., 1986; Klein et al., 1991; Hempstead et al., 1991; Kaplan et al., 1991). In addition, various serine protein kinases have also been found to influence the effects of NGF in PC12 cells (Mann et al., 1989; Brady et al., 1990; Costello et al., 1990; Damon et al., 1990; Glowacka and Wagner, 1990), and may act at various post-receptor steps in the NGF signaling pathway. The most compelling evidence involves cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC) (Cremins et al., 1986; Mann et al., 1989; Glowacka and Wagner, 1990; Kalman et al., 1990). NGF has been shown to activate both of these kinases within a few minutes (Heasley and Johnson, 1989). Various kinase inhibitors, and in particular inhibitors with some specificity for PKC, block completely the NGF induction of neurites and various neuronal gene products (Hama et al., 1986; Hall et al., 1988; Koizumi et al., 1988; Hashimoto and Hagino, 1989; Lazarovici et al., 1989). Studies using a variant subclone of PC12 cell line lacking PKA activities (i.e., the A126-1B2 subclone) show that these cells express some, but not all, of the effects of NGF (Brady et al., 1990).

Other early events which have been suggested to be part of the NGF signaling pathway include the transient expression of various "immediate-early" (IE) gene products, including Fos, Jun, and NGFI-A (Greenberg et al., 1985; Kruijer et al., 1985; Milbrandt, 1986; Kujubu et al., 1987; Bartel et al., 1989; Wu et al., 1989; Sheng and Greenberg, 1990). mRNA transcripts encoded by these genes have been found to increase within 10–15 min of NGF addition, and then to decrease to basal levels within hours. The proteins encoded by these IE genes are also expressed transiently

1. *Abbreviations used in this paper*: 2-AP, 2-aminopurine; CAT, chloramphenical acetyltransferase; cDNA, complementary DNA; dbcAMP, N^6,2'-*O*-dibutyryladenosine 3',5'-cyclic monophosphate; DOG, dioctanylglycerol; FGF, fibroblast growth factor; IE genes, immediate-early genes; NF-M, middle molecular weight protein of the neurofilament triplet; NGF, nerve growth factor; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; 6-TG, 6-thioguanine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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(Curran and Morgan, 1985) and are believed to recognize specific regulatory elements in the genome and act as transcriptional activators (Rauscher et al., 1988; Mitchell and Tijan, 1989; Morgan and Curran, 1989; Gizang-Ginsberg and Ziff, 1990; Sheng and Greenberg, 1990).

The nature of the coupling between IE and late gene expression is, however, unclear. Depolarization of PC12 cells with high K^+ or the addition of various growth factors other than NGF, for example, induce expression of *c-fos*, *c-jun*, and NGFI-A mRNA transcripts in PC12 cells (Bartel et al., 1989; Wu et al., 1989), but do not induce neuronal differentiation (Greene et al., 1985; Machida et al., 1989). It has been suggested, therefore, that combinations of specific IE gene products may act in concert as tertiary messengers in NGF signaling, coupling early events (e.g. kinase activation) into long-term responses involving differential transcription of late genes (Greene et al., 1985; Levi et al., 1988; Sheng and Greenberg, 1990). Alternatively, induction of these IE genes may not be involved in neuronal differentiation at all, but may direct other events associated with these growth factors, such as changes in cellular metabolism or mitogenesis (Greene et al., 1985; Boonstra et al., 1987).

Transin (or "stromelysin-1" or "matrix metalloproteinase-3" [Matrisian, 1990]) is a metalloproteinase under transcriptional control by various growth factors in a variety of cell types (Matrisian et al., 1986; Machida et al., 1988; Kerr et al., 1988, 1990). In rat fibroblasts, transin is induced by epidermal growth factor (EGF) (Matrisian et al., 1986), and this EGF induction requires activation of PKC (McDonnell et al., 1990). In PC12 cells, in contrast, transin is induced by NGF, but not EGF (Machida et al., 1989). In the absence of NGF, transin mRNA levels are undetectable in PC12 cells, but increase over 100-fold in 24 h after addition of NGF (Machida et al., 1989), making transin one of the most NGF-responsive gene products described (e.g., Leonard et al., 1987; Federoff et al., 1988; Stein et al., 1988; see Milbrandt, 1986, 1987). Nuclear run-on assays showed, moreover, that this increase in transin mRNA levels is at least partly due to de novo transcription (Machida et al., 1989).

Various aspects of transin's regulation by NGF are characteristic of the late gene products (Greene et al., 1985; Machida et al., 1989). The time course of transin mRNA expression, for example, coincides closely with that of the mRNA encoding neurofilament protein, NF-M (Lindenbaum et al., 1988; Machida et al., 1989). In addition, the NGF induction of transin mRNA expression requires protein synthesis, presumably due to a requirement for induction of various transcriptional activator proteins.

There are two reasons to believe a priori that the regulation of transin by NGF in PC12 cells may involve specific IE gene products. First, the promoter region of the transin gene contains DNA consensus sequences corresponding to binding sites for various IE genes induced in PC12 cells by NGF (Matrisian et al., 1985; Bartel et al., 1989). These sequences include an NGFI-A recognition sequence (also known as zif 268, egr-1, Krox 24 and TIS 8; i.e., GCGG/TGGGCG) at position −408 to the transcription start site which is recognized by the NGF-IA gene product (Christy and Nathans, 1989), and an AP1 consensus sequence (i.e., TGAGTCA) at position −68. AP1 sequences are believed to bind the transcriptional activating proteins, Fos and Jun, and members of their gene families (Kouzarides and Ziff, 1988; Halazonetis et al., 1988; Rauscher et al., 1988). The NGFI-A and AP1 consensus sequences are found, moreover, in a 750-bp region of the transin promoter region, which confers NGF responsiveness in transient transfection experiments (Machida et al., 1989). A second reason to believe that various IE genes, and members of the Fos and Jun families in particular, are involved in transin regulation comes from the studies of Kerr et al. (1988). In these experiments, the EGF induction of the transin gene in fibroblasts was inhibited by the expression of anti-sense *c-fos* transcripts or by cotransfection with synthetic copies of the AP1 sequence, presumably titrating out the effects of AP1 binding proteins.

In this paper, we examine various aspects of the intracellular signaling pathway by which NGF induces various late gene products, as well as neurite extension, in PC12 cells. We show that the NGF-induction of transin gene expression requires the activation of a potentially novel staurosporine-sensitive protein kinase, which acts both at the transcriptional and posttranscriptional levels. Activation of various other serine protein kinases, on the other hand, do not seem to be necessary for transin expression, but do serve to potentiate the effects of NGF. These studies suggest, therefore, that the NGF induction of transin gene expression involves multiple protein kinases that act at various steps in the signaling pathway and that may mediate different NGF-associated events.

Materials and Methods

Protein Kinase Inhibitors and Activators

Staurosporine was purchased from Kamiya Biomedical Co. (Thousand Oaks, CA). H-7, H-8, and HA-1004 were obtained from Seikagaku America, Inc. (St. Petersburg, FL). All other protein kinase inhibitors and activators were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Stock cultures of PC12 cells were grown in "basal medium," which consisted of Leibowitz L15 culture medium (Gibco Laboratories, Grand Island, NY) supplemented with 6 mg/ml D-(+)-glucose and a vitamin mixture (Mains and Patterson, 1973) (Sigma Chemical Co.), as well as the following Gibco products: FBS (5%), horse serum (5%), glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml). For all experiments, $\sim 5 \times 10^5$ cells were plated onto 10 cm Primaria dishes (Falcon Labware, Oxnard, CA). The next day, the culture medium was removed and replaced with "N2-supplemented" medium (Bottenstein and Sato, 1979), consisting of basal medium without serum, but supplemented with the following Sigma products: insulin (5 μg/ml), transferrin (100 μg/ml), sodium selenite (30 nM), putrescein (100 μM), and progesterone (20 nM). The next morning, NGF (50 ng/ml) and the various PKC activators or inhibitors were added directly to this N2-supplemented medium.

Morphometric Analysis

Random microscopic fields were photographed using a Zeiss IM35 inverted microscope. The negatives were then examined, and the proportion of PC12 cells expressing neurites determined (neurites were operationally defined as cell processes at least two cell diameters in length). Typically, three to five cultures were examined per condition, and ten photographs taken per culture. Statistical comparisons were made using the Newman-Keuls multi-comparison test, and statistically significant differences were defined as $P < 0.05$.

RNA Blot Analysis

Cells were harvested and total cellular RNA was isolated, as described previously (Machida et al., 1988, 1989). RNA preparations (10–20 μg/lane) were then subjected to electrophoresis in 1.2% agarose gels containing

2.2 M formaldehyde and blotted onto Nytran membranes (Schleicher & Schuell, Keene, NH). The positions of the ribosomal RNA bands were then visualized by acridine orange staining as viewed on an ultraviolet light transilluminator. Prehybridization and hybridization were performed as described previously (Machida et al., 1988, 1989), using approximately 10^8 cpm/μg of ^{32}P-labeled nick-translated pTR1 plasmid containing a 1.6-kb transin cDNA insert. After hybridization for 16 h at 42°C, the membranes were washed briefly in 2× SSC buffer (Maniatis et al., 1982) containing 0.1% SDS (SDS) at room temperature followed by a last wash with 0.2× SSC buffer containing 0.1% SDS at 60°C. These membranes were then exposed to X-OMAT X-ray film (Eastman Kodak, Rochester, NY) with intensifying screens for 1-2 d at −80°C. For stripping, the RNA blots were treated for 1 h at 70°C with a solution consisting of 50% formamide, 50 mM EDTA and 100 mM Tris buffer (pH 7.4). In some cases, semiquantitative analysis of mRNA levels was performed, using a scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA). In these experiments, autoradiographic densities of the cyclophilin mRNA band in each lane served as an internal control for RNA loading artifacts (Danielson et al., 1988; Machida et al., 1989).

Nuclear Run-On Assays

Preparation of nuclei and transcriptional run-on assays were carried out as described previously (Machida et al., 1988, 1989). Briefly, nuclei containing similar amounts of radioactivity (2×10^7 cpm/ml) were hybridized for 2 d at 42°C with either transin, NF-M, cyclophilin or pBR322 DNA (10 μg) immobilized on Nytran membranes. After hybridization, the membranes were washed two times in 2× SSC buffer at room temperature and then in 0.2× SSC buffer (with 0.1% SDS) at 60°C. The hybridized membranes were then exposed to X-OMAT x-ray film (Eastman Kodak) with intensifying screens for 9 d at −70°C, and then the films were scanned as described above. Negative controls in these studies included blots with immobilized pBR322 DNA and showed no detectable hybridization.

Transient Transfection Assays

The method of Gorman et al. (1982), as detailed in Machida et al. (1989), was used. Briefly, PC12 cells were first plated at a density of 2×10^5 cells per 10 cm plate. The next day, fresh medium containing a calcium phosphate precipitate (Gorman et al., 1982) with 7 μg of p750TRCAT (Kerr et al., 1988) was added to the cultures. After a 4-h incubation, the medium was removed and the cells were treated with 15% glycerol in HBSS (Gibco Laboratories) for 2 min. The cells were then washed twice and cultured overnight in "N2-supplemented" medium. The next day, NGF and/or the various PKC inhibitors or activators were added to the culture medium, and the cultures were then incubated an additional 24 h. At that time, the cells were harvested and the extracts assayed for chloramphenicol acetyltransferase (CAT) activity using the method of Gorman et al. (1982), as modified by Seasholtz et al. (1988). After autoradiography, radioactive spots corresponding to acetylated and nonacetylated forms of chloramphenicol were cut out and counted in a Beckman scintillation counter (Beckman Instruments, Fullerton, CA).

Results

Effects of Kinase Inhibitors on the NGF Induction of Transin and NF-M mRNA Expression

To determine whether the NGF induction of transin mRNA expression required kinase activation, various protein kinase inhibitors were screened for their ability to inhibit this induction. In these experiments, PC12 cells were cultured for 24 h in NGF-containing medium supplemented with these various inhibitors, and then the cultures were harvested and RNA blotting studies performed. Fig. 1 *A* is an autoradiograph from two such experiments and shows that the nucleotide analogues 2-aminopurine (2-AP) and 6-thioguanine (6-TG) partially blocked the NGF induction of transin (compare lanes *J* and *K*, respectively, with lane *I*), but that 5 nM staurosporine completely blocked this induction (compare lane *C* with lane *B*). In contrast, various other kinase inhibitors had little or no effect on the NGF induction of transin, including the PKC inhibitors sphingosine and H-7 and the cyclic nucleotide–dependent kinase inhibitors H-8 and HA-1004. It should be noted that none of these inhibitors induced transin mRNA expression in the absence of NGF. It should also be noted that these concentrations of H-8 and HA-1004 were able to inhibit cyclic nucleotide–dependent protein kinase activities in broken cell preparations of PC12 cells, as well as in live cultures (data not shown). These data

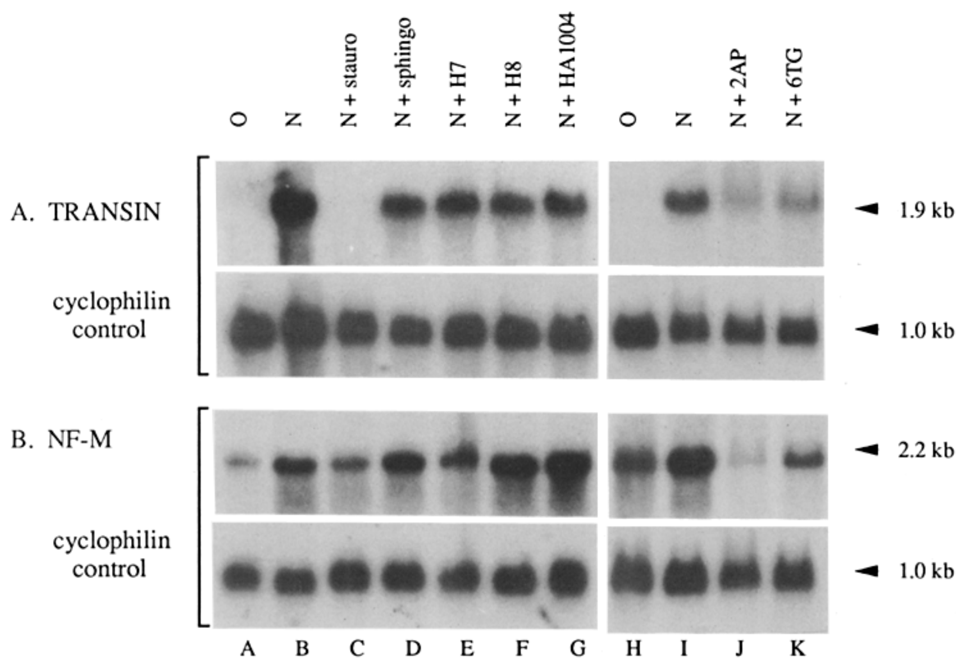
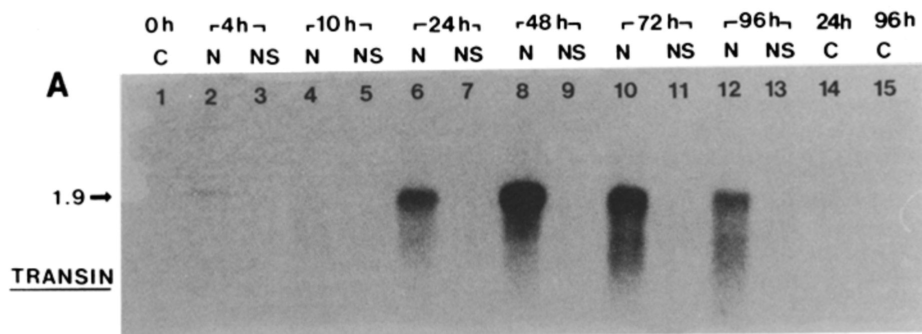
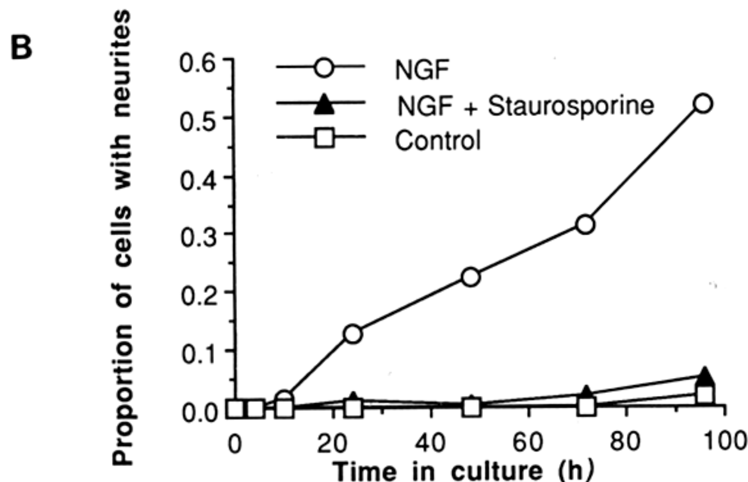


Figure 1. RNA blot showing the effects of various protein kinase inhibitors on the NGF induction of transin mRNA levels (*A*) and NF-M mRNA levels (*B*). PC12 cells were cultured for 24 h in the absence or presence of NGF (50 ng/ml) or NGF plus various protein kinase inhibitors (staurosporine, 5 nM; sphingosine, 5 μM; H-7, 6 μM; H-8, 2 μM; HA-1004, 4 μM). At the end of this period, the cultures were harvested and RNA blot analysis was performed. In all cases, blots probed for transin or NF-M were stripped (Machida et al., 1989) and reprobed for cyclophilin to control for RNA loading artifacts. Note that staurosporine completely blocked the NGF induction of transin mRNA and NF-M mRNA expression above basal levels. Also note that 2AP and 6-TG partially inhibited the NGF-induction of both transin and NF-M mRNA levels.





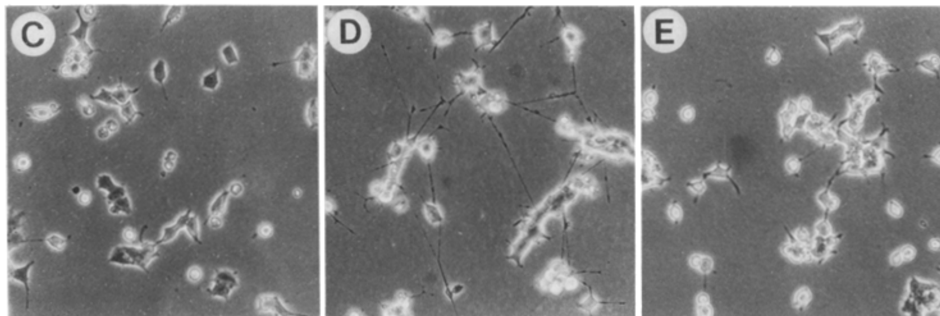


Figure 2. Effects of staurosporine on the NGF-induced time course of transin mRNA expression (*A*) and neurite outgrowth (*B–E*). PC12 cells were cultured for various periods of time in the presence of NGF alone (lanes *N*) or NGF plus staurosporine (10 nM; lanes *NS*), and then harvested for RNA analysis. Note that staurosporine completely blocked the NGF induction of both transin mRNA expression and neurites.

suggest that activation of neither the cyclic nucleotide-dependent protein kinases nor PKC are necessary for the NGF induction of transin gene expression.

Additional evidence that activation of cAMP-dependent protein kinase (PKA) is not involved in the NGF induction of transin mRNA levels come from studies using the A126-1B2 subclone of PC12, which lacks detectable PKA II activities (VanBuskirk et al., 1985). In these studies, A126-1B2 cells were found to display normal levels of NGF-induced transin mRNA expression (data not shown).

To determine whether the effects of these kinase inhibitors were specific to transin, or whether other NGF late genes would be similarly affected, duplicate RNA blots to those described above were probed for expression of the mRNA encoding the neurofilament protein "NF-M"–another NGF-inducible late gene product in PC12 cells (Lindenbaum et al., 1988). Fig. 1 *B* shows that 2-AP was most effective at inhibiting the NGF induction of NF-M, reducing mRNA levels below basal levels of expression (compare lane *J* with lanes *H* and *I*). This inhibitory effect of 2AP was also seen with basal levels of NF-M expression (data not shown). In addition, 6TG and staurosporine were also found to partially inhibit the NGF-induction of NF-M (i.e., compare lane *K* with *I* and lane *C* with *B*), although the extent of inhibition varied somewhat between experiments. In contrast, neither sphingosine, H7, H8, nor HA-1004 were found to affect the NGF induction of NF-M mRNA levels. These experiments were performed three times with qualitatively similar results.

Effects of Staurosporine on Neurite Extension

Since staurosporine was most effective at blocking the NGF induction of transin, further characterization of its effects on PC12 cells were carried out. Previously, Hashimoto and

Hagino (1989) had shown that staurosporine had differential effects on neurite formation in a subclone of PC12 cells (i.e., PC12h) cultured in serum-containing medium, with lower concentrations of staurosporine (3–10 nM) blocking the NGF induction of neurites, and higher concentrations (e.g., 100 nM) inducing neurites in the absence of NGF (see also Tishler et al., 1990, 1991). To determine whether staurosporine had similar effects on neurite formation under our serum-free culture conditions, and to correlate these effects with transin mRNA expression, cultures were treated with either NGF alone or NGF plus staurosporine. At various periods of time, the cultures were photographed and then harvested for RNA blot analysis. Fig. 2 *A* is an RNA blot showing the time course of transin expression following addition of either NGF (lanes *N*) or NGF plus low concentrations of staurosporine (lanes "NS"), and demonstrates that staurosporine blocked completely the NGF-induction of the transin transcript at all time points. Fig. 2 *B* represents the morphometric analysis of neurite extension in these same cultures, and shows that staurosporine also inhibited the NGF induction of neurites. Whereas over half of the PC12 cells in NGF-treated cultures expressed neurites at 96 h, only 3% of the cells expressed neurites in the presence of NGF plus 10 nM staurosporine. *C–E* are photomicrographs from representative cultures at 4 d and show that the NGF/staurosporine-treated cultures (*E*) had similar cell densities to those in NGF-supplemented medium (*D*), as well as control cultures (*C*). These photomicrographs also show that the staurosporine-treated cultures appeared healthy, and did not display any of the characteristic features of cytotoxicity, such as vacuoles or cell detachment. In contrast, similar experiments using the relatively higher concentrations of staurosporine reported to elicit neurite extension (i.e., 100 nM; Hashimoto and Hagino, 1989) proved to be cytotoxic under our culture conditions (data not shown).

Effects of PKC Downregulation on the NGF Induction of Transin mRNA Expression

Staurosporine has been reported to be a potent inhibitor of various protein kinases in rat cells, with somewhat higher specificity for PKC (Tamaoki et al., 1988). To determine, therefore, whether activation of PKC was necessary for the NGF induction of transin mRNA expression, a second strategy was used to deplete PC12 cells to PKC. In these experiments, cultures were pretreated with various concentrations of the phorbol ester 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), which has been shown to downregulate PKC in a variety of cell types (Fournier and Murray, 1987; Inagaki et al., 1986; Cooper et al., 1989), including PC12 cells (Sigmund et al., 1990; Vyas et al., 1990). Previous studies by Matthies et al. (1987) had found, for example, that treatment of PC12 cells with 1.0 μM TPA resulted in the loss of >95% of PKC activity, but did not affect significantly the levels of other kinases. In our experiments, PC12 cell cultures were pretreated for 24 h with various concentrations of TPA, and then NGF was added to this medium for an additional 24-h period. At that time, the cultures were harvested for RNA blot analysis. Fig. 3 shows that there were no significant differences in the NGF-induced levels of transin mRNA after pretreatment with various concentrations of TPA, including 1.0 μM (i.e., compare lane *F* with lane *B*). Similarly, PKC downregulated cells displayed no significant decrease in the NGF induction of NF-M mRNA expression or neurite extension (data not shown). These data indicate, therefore, that activation of PKC is probably not a necessary step in the NGF-induction of various late gene products, nor in the elaboration of neurites (see also Damon et al., 1990).

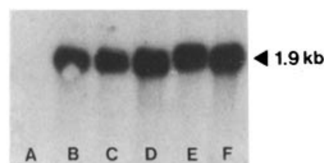


Figure 3. RNA blot showing that PKC downregulated PC12 cells did not display decreased NGF-induced levels of transin mRNA. PC12 cells were cultured for 24 h in the absence (lanes *A* and *B*) or presence of various concentrations of TPA (lane *C*, 10 nM; lane *D*, 100 nM; lane *E*, 500 nM; lane *F*, 1000 nM). At the end of this period, NGF was added to the culture medium corresponding to lanes *B–F* for an additional 24 h, and then the cells were harvested for RNA blot analysis. Note that even the highest concentrations of TPA did not significantly reduce the NGF-induction of transin mRNA expression (compare lane *F* with lane *B*).

Effects of Kinase Activators on the NGF Induction of Transin and NF-M mRNA Expression

Although activation of PKC or the cyclic nucleotide–dependent protein kinases do not seem to be necessary for NGF action, they may still serve to augment the inductive effects of NGF on late gene expression in PC12 cells, as had been previously shown with neurite extension (Hall et al., 1988). To test this possibility, various protein kinase activators were screened for their ability to synergize with NGF in inducing transin and NF-M mRNA expression. In these experiments, PC12 cells were cultured for 24 h in NGF-containing medium supplemented with various kinase activators and then the cultures were harvested and RNA blotting studies performed. Fig. 4 *A* shows that low concentrations of TPA, which activate PKC in PC12 cells (Heasley and Johnson, 1989), augmented the NGF-induction of transin mRNA levels (compare lanes *C* with *B*). Similarly, addition of the PKA activators dibutyryl cAMP (dbcAMP) and forskolin also served to augment the NGF-induction of transin (compare lanes *D* and *E*, respectively, with lane *B*). In contrast, vanadate, which is believed to potentiate the actions of various tyrosine kinases by inhibiting phosphotyrosyl phosphatases (Swarup et al., 1982; Yonemoto et al., 1987), had no significant effect in this regard (compare lane *F* with lane *B*). It should be noted that none of these agents induced detectable levels of transin mRNA expression in the absence of NGF either alone (lanes *K–M*) or in various combinations (lanes *G–J*). This experiment has been performed three times with similar results.

When the effects of these kinase activators on transin mRNA induction were compared with those of NF-M, some differences were noted. Fig. 4 *B* shows that TPA and vanadate each augmented the NGF-induction of NF-M (compare lanes *C* and *F* with lane *B*), but that neither of the PKA activators – dbcAMP or forskolin – had a significant effect in this regard (compare lanes *D* and *E* with lane *B*). In the absence of NGF, vanadate also increased the basal levels of NF-M expression somewhat (compare lanes *H* and *K* or lanes *M*

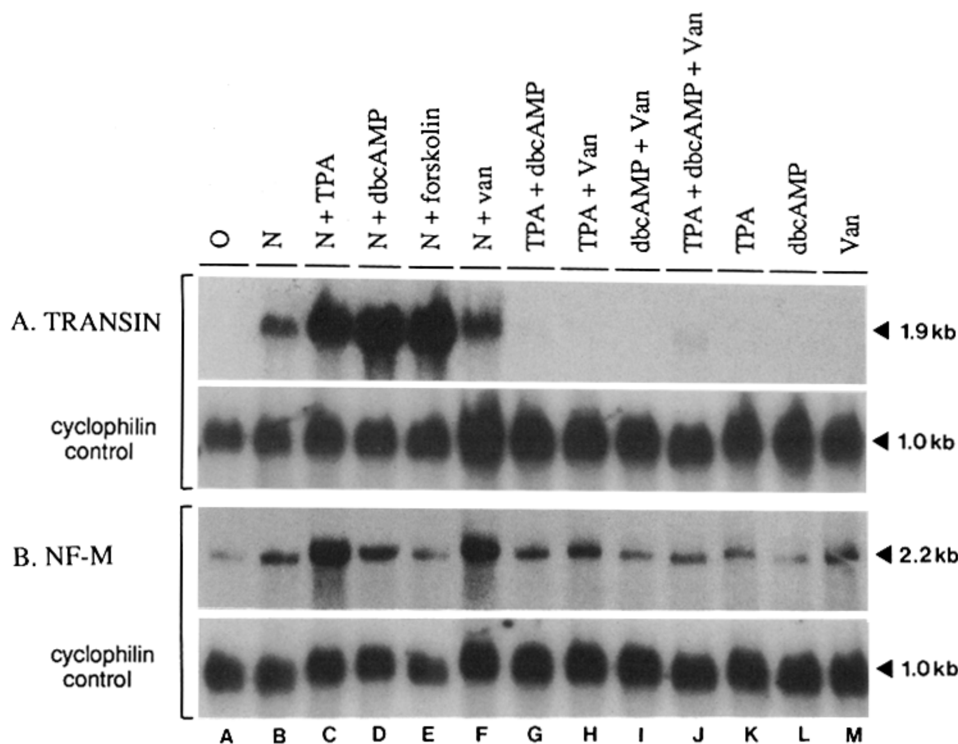


Figure 4. Effects of various protein kinase activators on the NGF induction of transin mRNA levels (*A*) and NF-M mRNA levels (*B*). PC12 cells were cultured for 24 h in the absence (lanes *A* and *H*) or presence of NGF (50 ng/ml) (lanes *B* and *I*) or NGF plus various protein kinase activators, including TPA (lane *C*; 10 nM), dibutyryl cAMP (1.0 mM; lane *D*), forskolin (10 μM; lane *E*), vanadate (10 μm; lane *F*). Other cultures were treated with these kinase activators alone (lanes *K–M*) or in combination (lanes *G–J*). Note that the PKC activator TPA augmented the NGF induction of both transin and NF-M mRNA expression; and that the PKA activators dbcAMP and forskolin augmented the NGF induction of transin, but not NF-M, mRNA levels. Also note that vanadate (10 μM; lane *F*) augmented the NGF-induction of NF-M, but not transin, mRNA levels.

and *A*). This experiment was performed three times with similar results.

Transient Transfection Studies

In previous work, we found that the recombinant plasmid p750TRCAT conferred NGF responsiveness in transient transfection assays (Machida et al., 1989). This plasmid contains 750-bp of the promoter region immediately adjacent to the transcriptional start site of the transin gene fused to the reporter gene chloramphenicol acetyltransferase (CAT) (Kerr et al., 1988). To determine whether this region of the transin promoter mediated the effects of these kinase activators and inhibitors, PC12 cells were transfected with the p750TRCAT plasmid, cultured for 24 h in the presence of NGF plus or minus various kinase activators or inhibitors, and then assayed for CAT activity. Fig. 5 is a representative experiment of four such treatments, and shows that the PKC activators TPA and dioctanoylglycerol (DOG) augmented the NGF induction of CAT activities in transfected PC12 cells (i.e., compare lanes *NT* and *ND* with lane *N*). Similar augmentation of CAT-induced by NGF was observed with the PKA activators dibutyryl cAMP and forskolin (data not shown). Fig. 5 also shows that staurosporine inhibited the NGF induction of CAT activity by ∼25% (i.e., compare lane *NS* with lane *N*), and that this inhibition was statistically significant ($P < 0.05$). This inability of staurosporine to block completely the inductive effects of NGF in these transient transfection assays may indicate either that some of staurosporine's effects are mediated by a regulatory element(s) not present in the 750-bp transin promoter region, or that staurosporine acts posttranscriptionally.

Effects of Kinase Inhibitors on Immediate–Early Gene Expression

The transient transfection studies described above suggest that the transin promoter contains DNA sequences which are responsive to these kinases. This 750-bp region is known to contain DNA binding sites for transcription factors encoded by the IE genes, NGFI-A and the AP1 proteins, Fos and Jun. If these IE gene products are also part of the NGF signaling pathway, then the NGF induction of these various IE gene products should be similarly affected by the same set of protein kinase inhibitors and activators. To test this prediction, PC12 cells were cultured for 30 min in the presence of NGF plus various kinase inhibitors, and then the cultures were harvested for RNA analysis. Fig. 6 represents a quantitative analysis of the autoradiographic densities from three experiments in which these RNA blots were probed for c-*fos* (*black bars*), c-*jun* (*striped bars*), and NGFI-A transcripts (*stippled bars*). To facilitate comparisons, autoradiographic density values were normalized to cyclophilin mRNA levels as an internal control, and then were calculated as a percentage of cultures treated with NGF alone. These experiments show that staurosporine or 2-AP inhibited significantly the NGF induction of the c-*fos* and c-*jun* transcripts, but did not affect induced levels of the NGFI-A transcript. In contrast, neither the PKC inhibitor sphingosine, nor the cyclic nucleotide–dependent protein kinase inhibitors H-7, H-8, and HA-1004 significantly affected the NGF induction of any of these IE genes.

To determine whether activation of PKC would augment the NGF-induction of these IE genes, experiments were performed using the PKC activator, TPA. In these studies, PC12

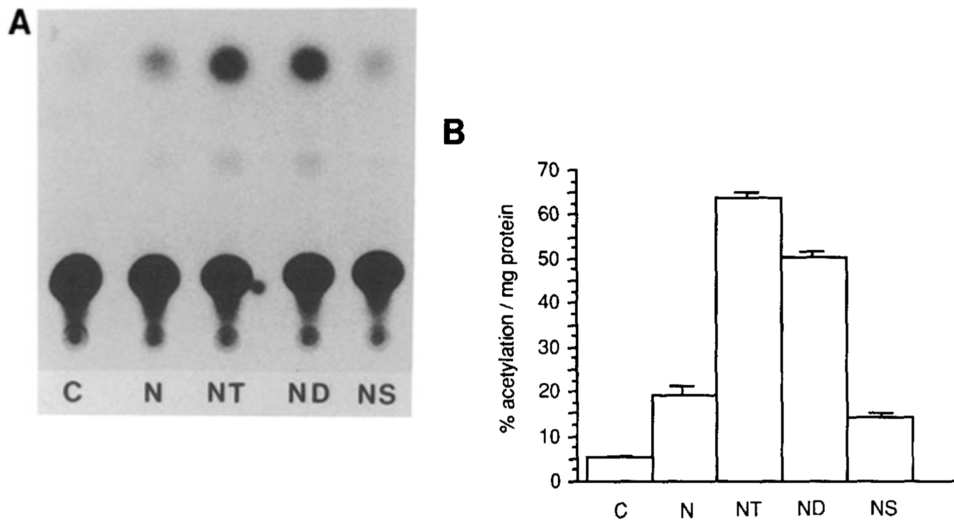


Figure 5. Transient transfection assay using p750TRCAT. PC12 cells were transfected and then cultured for 24 h in the presence of NGF (lanes *N*), NGF plus TPA (lanes *NT*), NGF plus dioctanylglycerol (lanes *ND*), or NGF plus staurosporine (lanes *NS*). *A* is a representative experiment; *B* represents the results of four separate experiments and shows the mean values plus the standard errors of the mean. Note that *NT*, *ND*, and *NT* cultures all expressed CAT-specific activities which differed to a statistically significant degree (i.e., $P < 0.05$) from those of *N* cultures, as determined using a Newman-Keuls multicomparison test.

cells were cultured for various periods of time in the presence of NGF (lanes *N*), NGF plus TPA (lanes *NT*), TPA alone (lanes *T*), or without any additives (lanes *C*), and then harvested for RNA. Fig. 7 shows that TPA augmented the NGF-induction of transcripts for all three of these IE genes (e.g., compare lane *6* with lane *3*), ranging from about threefold (i.e., over NGF alone) for c-*fos* and NGFI-A (*A* and *C*, respectively) to about fivefold for the c-*jun* transcripts (*B*). In the absence of NGF, TPA also induced these transcripts, albeit at lower levels of expression (compare lane *8* with lane *9*), as had been reported earlier (Kujubu et al., 1987). Similar results showing augmentation of the NGF-induction of IE gene expression were also seen when the PKC activator, DOG, was substituted for TPA (data not shown).

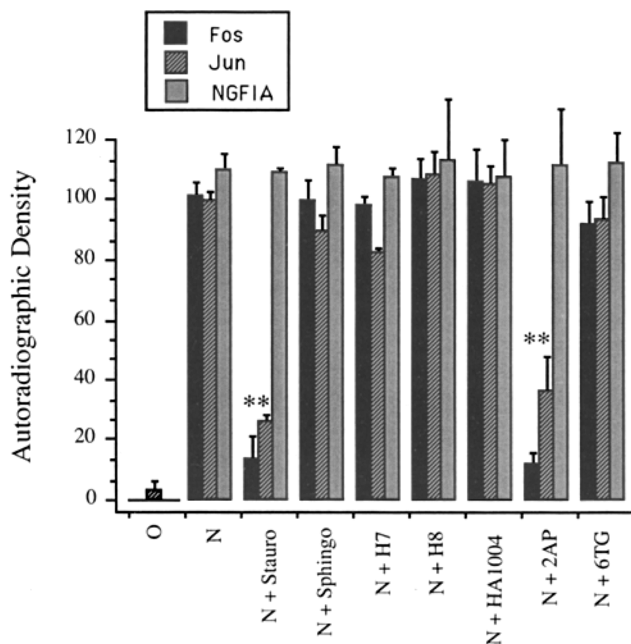


Figure 6. Effects of various protein kinase inhibitors on the NGF induction of c-*fos* mRNA levels (*black bars*), c-*jun* mRNA levels (*striped bars*), or NGFI-A mRNA levels (*stippled bars*). PC12 cells were cultured for 30 min in the presence of NGF alone (lane *N*) or NGF plus one of the protein kinase inhibitors described in the legend to Fig. 1. Densitometric analysis of autoradiographs from three experiments were scanned, and then the raw data were adjusted for differences in cyclophilin mRNA densities. Cultures treated with NGF alone were set at 100 (arbitrary units), and statistical comparisons were performed using the Newman-Keuls multicomparison test. Error bars refer to standard errors of the mean; asterisks mark conditions in which there was a statistically significant difference (i.e., $P < 0.05$) from cultures treated with NGF alone.

Effects of Staurosporine Addition after IE Gene Expression

It has been suggested that kinase activation is likely to be upstream of IE gene expression in the NGF signaling pathway (Levi et al., 1988). If so, then the addition of staurosporine after the transient expression of the IE genes has occurred would have little effect on transin mRNA expression. To test this prediction, staurosporine was added either 30 minutes following NGF-addition (i.e., at the peak of both mRNA and protein expression of the Fos and Jun gene products [Curran and Morgan, 1985; Greenberg et al., 1985; see also Fig. 7]) or 4 h after NGF addition (i.e., after Fos and Jun mRNA and protein levels have returned to basal levels of expression). At the end of 24 h, the cultures were harvested and RNA blotting studies performed. Fig. 8 *A* shows that addition of staurosporine either 30 min (lane *C*) or 4 h after NGF addition (lane *D*) completely blocked the NGF induction of transin mRNA expression. In contrast, NF-M mRNA levels did not seem to be affected significantly by the delayed addition of staurosporine at 4 h (Fig. 8 *B*, lane *D*). This experiment was performed three times with similar results. These data indicate, therefore, that at least some of the inhibitory effects of staurosporine on transin mRNA expression occur after the Fos and Jun proteins are no longer present.

Nuclear Run-On Experiments

To test directly whether staurosporine inhibited de novo transin mRNA expression, nuclear run-on assays were then performed. In these experiments, PC12 cultures were treated for 4 h with NGF in the presence or absence of staurosporine, and then the cells were harvested and nuclei isolated. Fig.

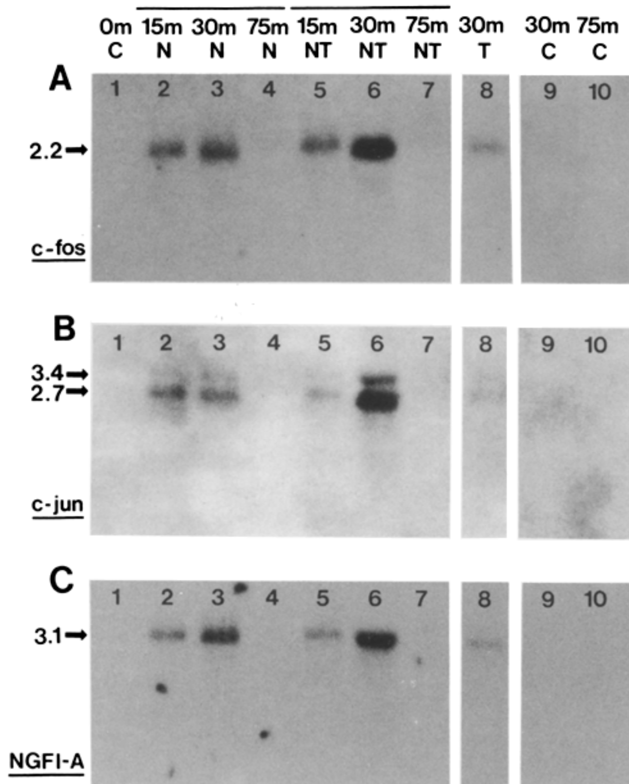


Figure 7. Effects of the PKC activator TPA on the NGF induction of c-*fos* (*A*), c-*jun* (*B*) or NGFI-A levels (*C*). PC12 cells were cultured for various periods of time in the absence (lanes marked *C*) or presence of NGF (lanes marked *N*) or NGF plus 10 nM TPA (lanes marked *NT*), and then were harvested for RNA blot analysis. Note that TPA augmented the NGF-induced expression of all three IE transcripts, and with a similar time course.

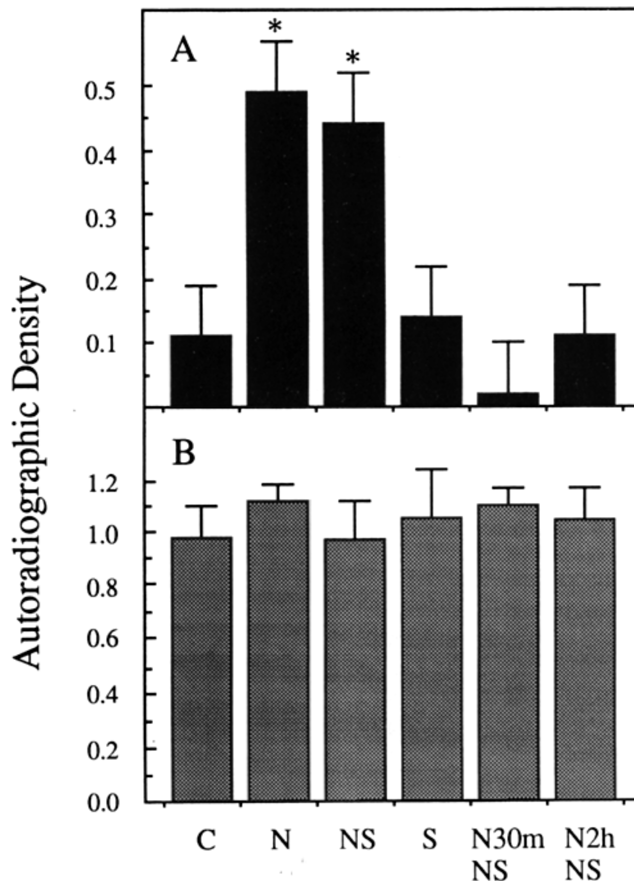


Figure 9. Nuclear run-on assays showing the effects of staurosporine on de novo transin mRNA expression (*A*) and cyclophilin mRNA expression (*B*) as a control. PC12 cells were treated for a period of 4 h with fresh medium (*C*), NGF (50 ng/ml; *N*), NGF plus staurosporine (5 nM; *NS*), staurosporine (*S*), NGF alone for 30 min followed by addition of staurosporine (*N30m/NS*), or NGF alone for 2 h followed by addition of staurosporine (*N2h/NS*). At the end of this 4-h treatment, the cells were harvested, nuclei isolated, nuclear run-on assays performed, and autoradiographs prepared, as described in Materials and Methods. Error bars refer to standard errors of the mean of four densitometric scans. Asterisks refer to conditions in which there was a statistically significant difference ($P < 0.05$) from cultures treated with fresh medium alone (i.e., *C*), as determined by *t* test analysis.

9 *A* shows that NGF increased significantly the rate of transin mRNA expression, and that the presence of staurosporine did not significantly inhibit this NGF induction (compare conditions *N* and *NS*). Control cultures, in which cells were treated with staurosporine alone (*S*), were not significantly different from those of untreated cultures (*C*). Negative controls in these experiments included analysis of the rates of cyclophilin mRNA expression, and showed that neither NGF nor staurosporine, either alone or in combination, affected this rate of transcription (Fig. 9 *B*). To determine whether the delayed addition of staurosporine affected the rate of NGF-induced transin mRNA expression, some cultures were given staurosporine at various times after the addition of NGF. Fig. 9 *A* shows that addition of staurosporine at either 30 min (*N30m/NS*) or 2 h (*N2h/NS*) blocked the NGF-induced increased rate of transin mRNA expression. And again, controls for the rate of cyclophilin mRNA production showed no significant differences (Fig. 9 *B*). These experiments were performed twice with qualitatively similar results.

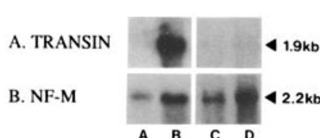


Figure 8. Effects of staurosporine-addition after IE gene expression has occurred on NGF-induced transin and NF-M mRNA expression. PC12 cell cultures were cultured in the absence (lane *A*) or presence of NGF (lanes *B–D*), and then staurosporine (5 nM) was added to the culture medium at either 30 min (lane *C*) or 4 h (lane *D*). At the end of 24 h, the cultures were harvested for RNA blot analysis. Note that addition of staurosporine at 4 h blocked the NGF-induction of transin mRNA levels, but had little effect on NGF-induced NF-M mRNA levels.

Discussion

In this paper, we examine the intracellular signaling mechanism whereby NGF induces transin gene expression in PC12 cells, and in particular, the possible roles of various protein kinases and IE gene products. Our results suggest that activation of a staurosporine-sensitive protein kinase (or kinases) is necessary for this induction, and that this kinase may act

at a number of postreceptor steps in the NGF signaling pathway, including both transcriptional and posttranscriptional steps. These data are consistent with the notion that the NGF signaling pathway involves multiple protein kinases which regulate the various effects of NGF.

The NGF Induction of Transin Involves a Staurosporine-sensitive Serine Kinase

There are several reasons to believe that activation of a novel, staurosporine-sensitive protein kinase is a necessary step in the NGF signaling pathway inducing transin gene expression, and probably NF-M expression as well. We found, for example, that low concentrations of staurosporine completely blocked the NGF-induction of both transin and NF-M, but did not affect expression of a non-NGF-regulated gene product–cyclophilin. Although staurosporine has a broad specificity at higher concentrations, it should be noted that the concentrations used in these studies (i.e., 5 nM) is below the K_i for inhibition of many protein kinases, including the cyclic nucleotide-dependent protein kinases, myosin light chain kinase (Tamaoki et al., 1986) and the platelet-derived growth factor receptor tyrosine kinase (Secrist et al., 1990). In contrast to these effects of staurosporine, various other kinase inhibitors had no significant effect on the NGF induction of transin, including inhibitors of the cyclic nucleotide-dependent protein kinases (e.g., H8, HA1004). Further evidence that activation of PKA, in particular, was not involved in transin induction came from studies using the A126-1B2 subclone of PC12 (VanBuskirk et al., 1985) in which NGF was found to induce normal levels of transin mRNA expression in these PKA-deficient cells.

The notion that PKC is not the target of staurosporine's inhibitory effects is based on two observations. First, the PKC inhibitors sphingosine and H-7 were found to have no significant effect on the NGF induction of transin mRNA expression at concentrations reported to block neurite extension in PC12 cells (Hall et al., 1988). Second, downregulation of PKC by pretreatment with high concentrations of TPA also failed to inhibit the NGF induction of transin mRNA levels. The lack of a direct role for PKC activation in transin gene regulation is supported by observations that PKC-downregulated PC12 cells show unaffected NGF induction of various early genes such as ornithine decarboxylase (Reinhold and Neet, 1986), late gene products such as N-CAM, GAP-43, and SGC10 (Doherty et al., 1988; Costello et al., 1990; Sigmund et al., 1990), and neurite outgrowth (Reinhold and Neet, 1986; Sigmund et al., 1990). On the other hand, the induction of other NGF-induced late genes in PC12 cells does seem to be affected by PKC downregulation, including tyrosine hydroxylase (Vyas et al., 1990). It should be noted, however, that treatment with 1.0 μM TPA for 24 h does not completely downregulate PKC, but leaves $\sim$5% of the original activity (Matthies et al., 1987). If activation of PKC was a necessary step in the NGF induction of transin and NF-M gene expression, however, it seems likely that such a dramatic loss of PKC activity would produce an observable decrease in accumulated mRNA levels. On the other hand, TPA may not downregulate all isoforms of PKC (e.g., Cooper et al., 1989). It is possible, for example, that a non-downregulated isoform of PKC, which may comprise <5% of the PKC activity in PC12 cells, may mediate entirely the NGF induction of transin.

In any case, the notion that PKC is not involved in the NGF induction of transin mRNA expression in PC12 cells is in stark contrast to the finding that PKC-downregulation completely blocks the EGF induction of transin in fibroblasts (McDonnell et al., 1990). Indeed, this significant difference in the effects of PKC downregulation on transin mRNA expression indicates that the signaling pathways for these two growth factors may be quite distinct, at least at the level of protein kinase involvement. The findings, however, that staurosporine inhibits the NGF induction of c-*fos*, c-*jun*, and transin expression in PC12 cells, and that c-*fos* and c-*jun* induction are likely to be directly involved in the EGF-induction of transin in fibroblasts (Kerr et al., 1988) suggest that Fos and Jun may be common elements in the NGF- and EGF-signaling pathways.

The conclusion that PKC activation is not involved in the NGF induction of transin, of course, leaves open the question of how staurosporine acts. It seems likely, however, that staurosporine acts via inhibition of one or another serine kinase given reports of its relatively high affinity for this class of kinase (Tamaoki et al., 1986). This staurosporine-sensitive kinase(s) is probably not, however, one of the cyclic nucleotide-dependent protein kinases, since none of the isoquinoline sulfonamide derivatives (e.g., H-8, etc.) had any observable effect on the NGF induction of transin. It also seems unlikely that the staurosporine-sensitive kinase(s) includes PK-N–an NGF-responsive protein kinase (Rowland et al., 1987). This conclusion is based on the report that 6-TG is a more effective inhibitor of PK-N than 2-AP (Volonte et al., 1989), yet we found the opposite order of effectiveness in blocking transin mRNA expression (see Fig. 1 *A*). On the other hand, other NGF-responsive serine kinases (e.g., Blenis and Erickson, 1986; Matsuda et al., 1986; Brady et al., 1990; Landreth et al., 1990; Miyasaka et al., 1990), as well as the *trk* protooncogene tyrosine kinase, cannot yet be ruled out as possible targets of staurosporine's effects.

The Staurosporine-sensitive Protein Kinase Acts Both Transcriptionally and Posttranscriptionally

These studies also suggest that staurosporine is likely to act at multiple steps in the NGF signaling pathway for transin gene expression, and at least one of those steps is posttranscriptional. That is, when staurosporine was added simultaneously with NGF, it blocked the accumulation of transin mRNA as measured by RNA blot analysis, but had no significant effect on the rate of transin mRNA production as measured by nuclear run-on assays. This would suggest that staurosporine acts either to increase the rate of transin mRNA degradation or to block transin mRNA elongation or processing. Additional evidence that the rate of transin mRNA transcription is not affected by the simultaneous addition of staurosporine with NGF can be gleaned from the transient transfection studies. In these experiments, staurosporine was observed to have only a small effect on the NGF-induction of CAT activities in p750TRCAT-transfected cells, yet produced a dramatic decrease in steady-state transin mRNA levels, presumably due to the lack of effect of staurosporine on the stability of CAT transcripts. Alternatively, the p750TRCAT plasmid may simply not have contained DNA sequences responsive to staurosporine.

In contrast to its effects with simultaneous addition, staurosporine had qualitatively different effects on transin

gene expression when it was added after NGF. In these experiments, the addition of staurosporine either 30 min or 2 h after NGF blocked transin mRNA expression, and this block occurred at the transcriptional level. In nuclear run-on assays, for example, the rate of transin mRNA expression was no different from that of non–NGF-treated cultures. These data indicate, therefore, that staurosporine has different effects on the NGF induction of transin gene expression, and that these different effects follow different time courses. It is not known, however, whether all of these effects of staurosporine are due to inhibition of a single protein kinase or whether multiple kinases are involved.

These studies provide conflicting evidence, however, as to whether Fos and Jun expression are intermediates in the NGF signaling pathway. On the one hand, the inhibition of transin mRNA expression by staurosporine correlated with its effect on c-*fos* and c-*jun* mRNA expression. Similarly, the various PKC and PKA activators augmented the NGF induction of c-*fos* and c-*jun* expression as it did with transin mRNA expression (see below). On the other hand, Fos and Jun protein levels have been shown to return to basal levels within 2 h after addition of NGF (Curran and Morgan, 1985), yet addition of staurosporine at this delayed time point still blocked the de novo transcription of transin mRNA. These experiments would indicate, therefore, that the posttranscriptional effects of staurosporine on the NGF induction of transin mRNA expression are likely not to involve Fos or Jun. The possibility remains that some other, as yet unidentified IE gene product is involved. If so, this IE gene product would most likely be stably expressed and would require continual kinase activation.

Activation of PKC or PKA Augment the NGF Induction of Transin

Although activation of PKC is probably not a necessary step for the NGF induction of transin mRNA expression, PKC activation does potentiate the effects of NGF. This conclusion is based on the observations that low concentrations of TPA, which activate PKC in PC12 cells (Heasley and Johnson, 1989), synergized with NGF to induce higher levels of transin mRNA expression. In addition, both TPA and DOG act synergistically with NGF to induce CAT activities in transient transfection studies with pTR750CAT. Similarly, various activators of PKA, such as dbcAMP and forskolin, were also found to augment the NGF induction of transin, and augmented the NGF induction of CAT activity in transient transfection assays. The absence of a cAMP-responsive element in this 750-bp region of the transin promoter suggests, however, that this effect may be indirect. In any case, these data suggest that activation of any one of several kinases may serve to upmodulate the expression of transin. These findings are consistent, moreover, with the observations that various PKC and PKA activators augment the NGF induction of various other late gene products, as well as neurites, in PC12 cells (Guroff et al., 1981; Mann et al., 1989; Costello et al., 1990; Damon et al., 1990; Glowacka and Wagner, 1990). It should be noted, however, that none of these PKC and PKA activators, alone or in various combinations, induced transin mRNA expression or neurites in the absence of NGF. This would suggest that activation of these kinases does not substitute for this staurosporine-sensitive kinase in the NGF induction of transin.

The NGF Induction of Transin and NF-M Gene Expression May Involve Different Sets of Kinases

Interestingly, the NGF induction of transin and NF-M transcripts seemed to respond differently to different sets of pharmacological agents. Although the NGF-induction of both transin and NF-M were blocked by staurosporine and the purine analogues, for example, 2-AP was found to have a much more dramatic effect on NF-M than transin expression. This differential effect of 2-AP on the expression of different gene products is consistent with earlier reports, showing selective inhibition of various IE gene products, including c-*fos* and c-*myc* (Zinn et al., 1988).

Studies using dbcAMP and forskolin showed that PKA activation upmodulated the NGF induction of transin mRNA levels, but had no significant effect on NGF-induced NF-M mRNA levels. Vanadate, on the other hand, augmented the NGF-induction of NF-M, but had no significant effect on transin, suggesting that activation of some tyrosine kinase may be involved in NF-M expression, but not that of transin. Since the *trk* tyrosine kinase of the high affinity NGF receptor is likely to be responsible for the NGF induction of both transin and NF-M, it seems likely that effect of vanadate on NF-M specifically involves some other tyrosine kinase-mediated event in the NGF signaling pathway.

Expression of Transin May Be Functionally Linked to Neurite Extension

These and other studies also provide evidence that transin expression and neurite extension are correlated with one another. In earlier work, we showed that subclones of PC12 that elaborated neurites in response to the fibroblast growth factors (FGF) were also shown to express transin mRNA transcripts in response to these same growth factors (Machida et al., 1989). Activators of PKC and PKA, moreover, augmented the NGF induction of both transin mRNA expression and neurite outgrowth in PC12 cells (see also End et al., 1982; Glowacka and Wagner, 1990). These studies are consistent with earlier observations that various PKA and PKC activators augmented the NGF-induced expression of various neuronal traits, such as GAP-43 (Costello et al., 1990), Thy-1 (Doherty et al., 1988), and N-CAM (Mann et al., 1989). Thus, the effects of various protein kinase activators and inhibitors on transin mRNA expression and neurite outgrowth correlate with one another, suggesting that the pathways involved in their regulation may be functionally linked.

If transin is involved in neurite extension, it seems most likely that it would serve to degrade extracellular matrix barriers at the growth cone, as has been described for other metalloproteinases (see Pittman, 1985; Pittman et al., 1989). This possibility is consistent with the categorization of transin as one member of a large metalloproteinase family involved in extracellular matrix remodeling (Matrisian, 1990). Recently, we have shown that the transin protein is also synthesized and released into the culture medium by NGF-treated PC12 cells (Ciment, M., unpublished observations).

The observation that different sets of protein kinases have differential effects on the NGF-induced expression of transin and NF-M may reflect, moreover, a general cellular strategy for being able to modulate independently the levels of different gene products to fit conditions in the cellular microen-

vironment. One might imagine, for example, that neuronal cells extending neurites into an environment in which axonal growth is impeded by extracellular matrix barriers might require higher levels of secreted proteases, such as transin, and lower levels of axonal structural elements, such as NF-M. It is of interest to note, therefore, that laminin has been reported to stimulate neurite extension via a pathway involving PKC (Bixby, 1989). More direct evidence for a role of transin in axonal outgrowth, however, awaits additional studies.

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