

# Nerve Growth Factor and Fibroblast Growth Factor Regulate Neurite Outgrowth and Gene Expression in PC12 Cells via Both Protein Kinase C- and cAMP-independent Mechanisms

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**Abstract.** Nerve growth factor (NGF), acidic fibroblast growth factor (aFGF), and basic fibroblast growth factor (bFGF) promote the survival and differentiation of a variety of peripheral and central neurons. The signal transduction mechanisms that mediate the actions of these factors in neuronal cells are not well understood. We examined the effect of a deficiency in protein kinase C (PKC) and/or cAMP second messenger systems on the actions of NGF, aFGF, and bFGF in the pheochromocytoma (PC12) cell line. Activation of PKC was not required for NGF, aFGF, and bFGF to maximally induce ornithine decarboxylase (ODC), transcription of the early response genes, d2 and d5, or neurite outgrowth. In a PC12 cell mutant that is deficient in cAMP responsiveness (A126-1B2), all three growth factors maximally induced the transcription of d5 and neurite outgrowth, but aFGF and bFGF did not induce significant increases in ODC. NGF and

aFGF maximally induced the transcription of d2 in A126-1B2 cells, but bFGF-induced d2 transcription was attenuated. NGF, aFGF, and bFGF maximally induced neurite outgrowth and d5 transcription in A126 cells that were made deficient in PKC. The d2 transcriptional response was substantially reduced in cells deficient in both PKC and cAMP responsiveness. These observations lead us to conclude that (a) cAMP- and PKC-dependent events are, at least in part, causally linked to NGF, aFGF, and bFGF induction of both ODC and transcription of d2 and may control functionally redundant pathways; (b) NGF, aFGF, and bFGF can elicit neurite outgrowth and increase transcription of d2 and d5 in PC12 cells via mechanisms that are independent of both PKC and cAMP; (c) NGF, aFGF, and bFGF can induce ODC in the absence of PKC; and (d) aFGF and bFGF require cAMP responsiveness to induce ODC in PC12 cells.

**N**ERVE growth factor (NGF),<sup>1</sup> acidic fibroblast growth factor (aFGF), and basic fibroblast growth factor (bFGF) promote survival and differentiation of specific classes of neuronal cells (5, 13, 21, 27, 32, 38, 40, 41, 43, 45, 46). NGF and the fibroblast growth factors (FGFs) are found in the peripheral and central nervous system in developing and adult organisms (14, 26, 28). The ability of each of these factors to act upon neuronal cells and their presence throughout the nervous system suggest that they may play a critical role in the development and/or maintenance of the nervous system.

To study the biochemical mechanisms underlying the actions of NGF and FGFs on neuronal cells, a stably transformed pheochromocytoma cell line (PC12) is frequently used. In this model system, the addition of NGF or the FGFs

induces PC12 cells to undergo biochemical and morphological changes characteristic of sympathetic neuronal differentiation (16, 39). The specific transduction mechanisms that mediate many NGF- and FGF-induced changes in PC12 cells are not known. It has been reported that protein kinase C (PKC) and cAMP second messenger systems are activated in response to NGF (1, 6, 8, 20, 22). NGF has also been reported to activate a tyrosine kinase in PC12 cells (29), and bFGF has been observed to increase tyrosine phosphorylations in 3T3 cells (7, 12, 23). However, no causal relationships have been established between the activated second messenger systems and the many observed growth factor actions.

In this study, we evaluated the role of PKC and cAMP second messenger systems in mediating NGF, aFGF, and bFGF induction of several cellular events. First, we studied three responses that are initiated within 15 min of exposure to NGF or the FGFs: the induction of ornithine decarboxylase (ODC) and the genes d2 and d5. The enzyme ODC, which catalyzes the conversion of ornithine to putrescine, is the

1. *Abbreviations used in this paper:* aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; dbcAMP, dibutyryl adenosine 3':5'-cyclic monophosphate; FGF, fibroblast growth factor; NGF, nerve growth factor; ODC, ornithine decarboxylase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

rate-limiting step in polyamine synthesis. We measured increases in enzyme activity that have been attributed to transcriptional induction of the ODC gene (11). The genes d2, a putative transcriptional activator (31), and d5, whose product has not been characterized, are also rapidly induced by NGF and the FGFs (3). The kinetics of these early responses suggest that these genes may be primary targets of second messenger systems that are regulated by the growth factors. NGF and FGF induction of neurite outgrowth was also examined. This morphological change, which requires at least 24 h of growth factor exposure, is the probable convergence of many cellular processes involved in differentiation.

The use of cells that had deficiencies in particular second messenger systems permitted us to directly evaluate whether PKC- and cAMP-dependent transduction mechanisms play a causal role in mediating specific growth factor actions. Furthermore, we used a novel approach of combining a pharmacologically induced deficiency in PKC with a genetic deficiency in cAMP responsiveness to evaluate the potential interactions between these two second messenger systems. Our observations suggest that NGF and the FGFs induce neuronal differentiation and elevations in d2 and d5 transcription in PC12 cells via second messenger systems that are not cAMP and PKC dependent. However, either PKC or cAMP responsiveness is required for NGF and the FGFs to maximally activate d2 transcription. Our data also indicate that NGF, but not aFGF and bFGF, can induce ODC via cAMP- and PKC-independent mechanisms. We suggest that NGF, aFGF, and bFGF act via at least three second messenger systems including those that involve cAMP and PKC.

## Materials and Methods

### Materials

NGF was prepared as described by Mobley et al. (31a). aFGF and bFGF were purchased from F.G.F. Co. (LaJolla, CA) or purified from bovine brain as described by Lobb et al. (27a). Phorbol 12-myristate 13-acetate (PMA) and dibutyryladenine 3':5'-cyclic monophosphate (dbcAMP) were from Sigma Chemical Co. (St. Louis, MO), and [<sup>3</sup>H]ornithine and [ $\alpha$ -<sup>32</sup>P]cytidine triphosphate were from New England Nuclear (Boston, MA).

### ODC Assay

Cells were incubated in DME containing 1% horse serum and indicated additives for 5 h. Cells were then washed and harvested into sucrose Hepes buffer (0.32 M sucrose, 10 mM Hepes; 4°C). The pellet was resuspended and lysed by shearing through a 21-gauge needle in a solution containing 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.1 mM EDTA, 5 mM DTT, and 40  $\mu$ M pyridoxal phosphate at 4°C. The cell lysate was centrifuged at 8,800 g for 15 min (4°C), and the supernatant fraction was assayed for ODC as described by Djurhuus (9). The amount of ornithine converted to putrescine was determined at 0, 20, 40, 60, and 80 min by spotting an aliquot of assay mixture onto P81 cation exchange paper (Whatman Inc., Clifton, NJ) and washing in 0.10 M NH<sub>4</sub>OH (pH 11). Activities (nanomoles putrescine per milligram per hour) were calculated from the slopes of linear regressions correlating putrescine production with time.

### Nuclear Run-on Analysis

Cells were incubated with indicated additives for 20 min, the time required for maximal induction of the transcription of d2 and d5 (3). Nuclei were isolated and frozen in glycerol, and nuclear run-on assays were performed as described by Greenberg et al. (15) and modified by Cho et al. (3). Frozen nuclei were thawed and incubated with ribonucleotide triphosphates, one being radiolabeled ( $\alpha$ -<sup>32</sup>P]cytidine triphosphate). Transcription was allowed to proceed for 30 min and then RNA transcripts were isolated. Transcripts were hybridized to d2, d5, and alpha-tubulin DNA (5  $\mu$ g DNA

per slot). Transcription of alpha-tubulin DNA was assessed as a normalization control since the transcription of this DNA is not affected under our experimental conditions. Nuclear run-on data was quantitated as described by Suissa (39).

### Neurite Outgrowth Assay

Cells (2–4  $\times$  10<sup>4</sup> cells/ml), grown in DME supplemented with 10% FCS (Flow Laboratories, Inc., McLean, VA) and 5% horse serum (Hazleton Biologics, Inc., Lenexa, KS) at 37°C in 10% CO<sub>2</sub>, were plated in 24-well culture dishes in the presence of additives as indicated in figure legends and scored for neurite outgrowth 72 h later. A neurite was identified as a process whose length was 1.5 times the cell body length and had a clearly definable growth cone. 200 cells were counted per well, and the percentage of cells with neurites was calculated.

### Statistics

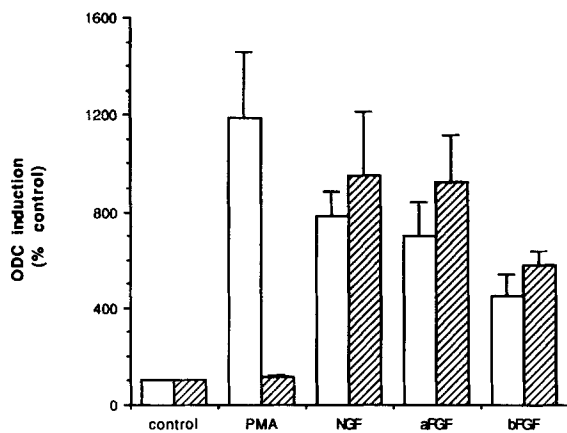
Data were compared using unpaired *t* tests. Differences were considered significant if *P*  $\leq$  0.05.

## Results

Activation of PKC and cAMP-dependent protein kinase in PC12 cells produces many, but not all, of the changes induced by NGF and the FGFs. This suggests that PKC or cAMP-dependent protein kinase may at least in part mediate several of these growth factor actions. It is clear that activation of PKC alone is not adequate to elicit neurite outgrowth (10; data not shown), indicating that other signal transduction systems must be used by NGF and FGF to elicit this response. End et al. (10) observed that the induction of ODC by NGF was greater in the presence of activators of PKC. Likewise, Guroff et al. (17) observed that the induction of ODC by NGF was greater in the presence of cAMP analogues. This observation indicates that NGF can induce ODC via a PKC- and/or cAMP-independent mechanism.

### Is PKC Required for NGF, aFGF, and bFGF Actions in PC12 Cells?

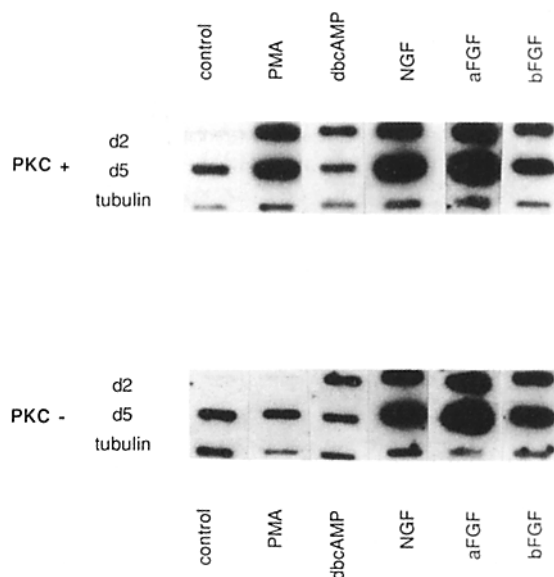
Although PKC activation is not sufficient for FGF or NGF induction of neurite outgrowth (data not shown) and although PKC activation may not be sufficient to account for FGF- or NGF-induced increases in ODC, we do not know whether PKC activation is necessary for these and other NGF and FGF actions. If this second messenger system was required, measured responses should be reduced or eliminated in cells deficient in PKC. We examined the effect of a 24-h PMA pretreatment on the ability of NGF and the FGFs to elicit specific PC12 cell responses. This pretreatment has been used in PC12 cells to downregulate PKC activity to <5% of control levels (30). First, we determined whether PKC activation was required for NGF, aFGF, and bFGF to induce the enzyme ODC. Increases in activity of this enzyme have been attributed to rapid increases in transcription of the ODC gene (11), suggesting that this response may be an early event in growth factor signal transduction and that the ODC gene may be a primary target for regulation by known second messenger systems. In cells that had not been downregulated, PMA, NGF, aFGF, and bFGF stimulated ODC  $\sim$ 10-fold (Fig. 1, *open bars*). In PKC-deficient cells, PMA no longer induced ODC, indicating that PKC was indeed downregulated; however, NGF, aFGF, and bFGF stimulated ODC to levels that were not significantly different from those observed in cells that were not pretreated with PMA (Fig. 1,



**Figure 1.** NGF, aFGF, and bFGF induce ODC in PC12 cells that are deficient in PKC. Open bars represent the ODC inductions, expressed as percents of controls, measured in PC12 cells that had not been preexposed to PMA. Hatched bars represent ODC inductions measured in PC12 cells that had been pretreated for 24 h with 1  $\mu$ M PMA. Data represent the means  $\pm$  SEM of at least three experiments.

*hatched bars*, and Table I). These data indicate that PKC activation was not required for NGF, aFGF, and bFGF induction of ODC.

We also determined whether PKC activation was required for NGF, aFGF, and bFGF to increase transcription of the genes d2 and d5. Like ODC, these genes are also rapidly induced by these growth factors and could also represent primary targets for regulation of PKC and/or cAMP. Furthermore, d2 is a putative transcriptional regulator and as such may represent a subsequent mediator of NGF and FGF signal transduction. The transcription of the gene d2 was increased by PMA, dbcAMP, NGF, aFGF, and bFGF approximately fivefold in PC12 cells that had not been downregulated (Fig. 2, *top*). In PKC-deficient cells, the response to PMA was eliminated, but the responses to all other agents were unaffected (Fig. 2, *bottom*, and Table I). PMA, NGF, aFGF, and bFGF also increased transcription of the gene d5 approximately twofold in cells that had not been downregulated; downregulation eliminated the induction by PMA but



**Figure 2.** NGF, aFGF, and bFGF increase transcription of d2 and d5 in PC12 cells that are deficient in PKC. PKC+ data were obtained from cells that had not been preexposed to PMA. PKC- data were obtained from PC12 cells that had been exposed to 1  $\mu$ M PMA for 24 h before indicated additions. Data are representative of at least two experiments. Additions were as follows: 100 nM PMA, 1 mM dbcAMP, 100 ng/ml NGF, 100 ng/ml aFGF, and 10 ng/ml bFGF.

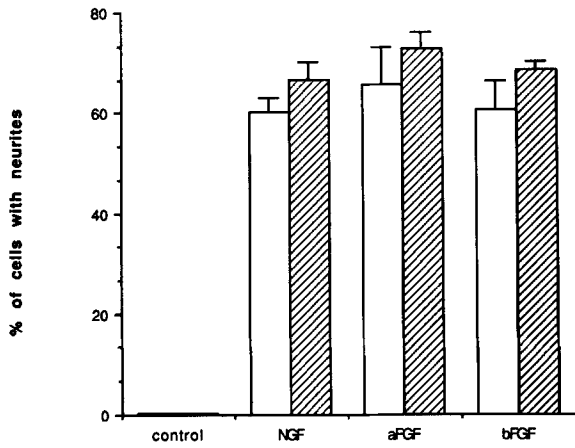
did not reduce or eliminate NGF, aFGF, and bFGF responses (Fig. 2 and Table I). As has been reported previously (3), dbcAMP did not elevate transcription of d5 in PC12 cells that had not been downregulated, and this lack of response was also observed in PKC-deficient cells (Fig. 2). Thus, our data indicate that PKC is not required for NGF, aFGF, and bFGF to increase transcription of d2 and d5.

Finally, we determined if PKC was required for the induction of neurite outgrowth by NGF, aFGF, and bFGF. This response is most closely associated with the sympathetic neuronal phenotype and is thus most characteristic of differentiation. NGF, aFGF, and bFGF induced comparable neurite outgrowth from PC12 cells that had not been pretreated

**Table I. Summary of Growth Factor-induced Responses in PC12 Cells Deficient in PKC and cAMP Second Messenger Systems**

Growth factor	Cell type	Neurite outgrowth	ODC	d2	d5
NGF	PC12	60.2 (100)	7.74 (100)	4.17 (100)	1.78 (100)
	PC12/PKC-	66.4 (110)	7.22 (93)	4.85 (116)	4.03 (226)
	A126	95.0 (158)	3.40 (44)	3.92 (94)	2.34 (131)
	A126/PKC-	90.26 (150)	3.00 (39)	2.66 (64)	3.65 (205)
aFGF	PC12	65.4 (100)	7.51 (100)	5.33 (100)	2.64 (100)
	PC12/PKC-	72.6 (111)	8.32 (111)	7.29 (137)	2.43 (95)
	A126	59.9 (91)	2.06 (28)	4.78 (90)	1.91 (72)
	A126/PKC-	61.2 (103)	1.95 (25)	1.87 (45)	2.94 (111)
bFGF	PC12	60.5 (100)	4.74 (100)	4.33 (100)	1.98 (100)
	PC12/PKC-	68.6 (113)	6.36 (134)	5.27 (121)	4.62 (233)
	A126	65.3 (108)	1.22 (26)	2.18 (50)	2.05 (104)
	A126/PKC-	56.8 (87)	1.36 (29)	1.54 (36)	2.14 (108)

Responses are expressed as stimulated versus control cells and are also expressed as percent of PC12 cell responses in parentheses. For neurite outgrowth, the numbers not in parentheses indicate the absolute percent of cells with neurites. PKC- refers to cells that had been pretreated with 1  $\mu$ M PMA to downregulate PKC.

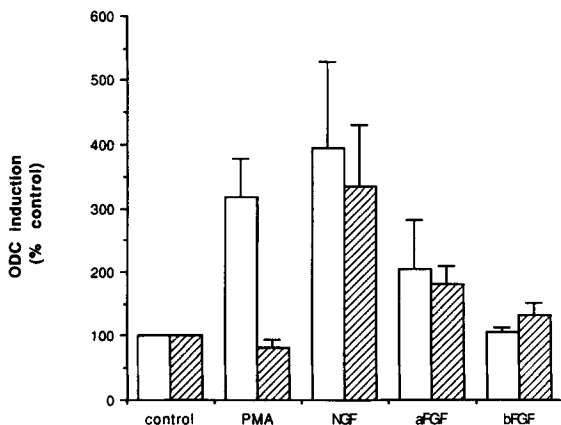


**Figure 3.** NGF, aFGF, and bFGF induce neurite outgrowth from PC12 cells that are deficient in PKC. Open bars represent neurite outgrowth from PC12 cells that had not been preexposed to PMA. Hatched bars indicate neurite outgrowth from PC12 cells that had been exposed to 1  $\mu$ M PMA for 24 h before growth factor addition and at 24-h intervals for the duration of the neurite outgrowth assay. Data represent the means  $\pm$  SEM of at least three experiments.

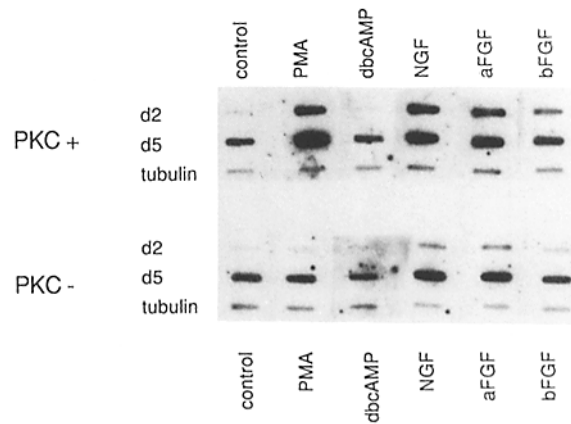
with PMA. The ability of these growth factors to induce neurite outgrowth was not compromised in cells that were rendered PKC deficient (Fig. 3 and Table I). We verified that PKC was still downregulated at the time of our neurite outgrowth assay by showing that PMA did not induce ODC (data not shown). Thus, our data strongly suggest that although PKC may be activated in response to NGF and FGF, acute activation of PKC is not essential for the rapid induction of gene transcription (ODC, d2, and d5) or the induction of neurite outgrowth.

#### Is the cAMP Second Messenger System Required for NGF, aFGF, and bFGF Actions in PC12 Cells?

A126-1B2 cells are a mutant line derived from PC12 cells that are deficient in cAMP-dependent protein kinase II activ-



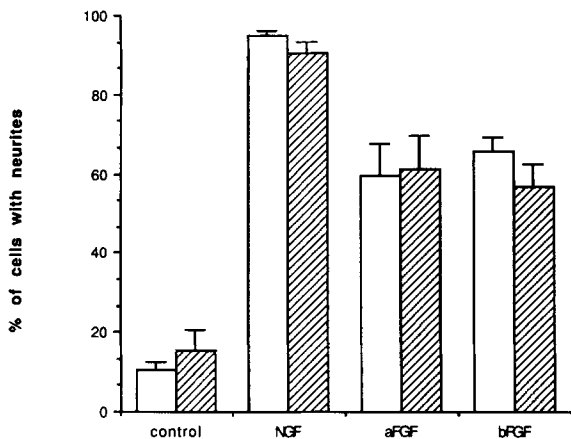
**Figure 4.** NGF but not aFGF and bFGF induce ODC in cells deficient in cAMP responsiveness. Open bars represent ODC inductions, expressed as percents of controls, in A126-1B2 cells. Hatched bars represent ODC inductions in A126-1B2 cells that had been downregulated with 1  $\mu$ M PMA for 24 h before growth factor addition. Data represent the means  $\pm$  SEM of at least three experiments.



**Figure 5.** NGF, aFGF, and bFGF increase transcription of d2 and d5 in PC12 cells that are deficient in PKC and cAMP responsiveness. PKC+ data were obtained from A126-1B2 cells that had not been preexposed to PMA. PKC- data were obtained from A126-1B2 cells that had been exposed to 1  $\mu$ M PMA for 24 h before the indicated additions. Data are representative of three experiments. Additions were as follows: 100 nM PMA, 1 mM dbcAMP, 100 ng/ml NGF, 100 ng/ml aFGF, and 10 ng/ml bFGF.

ity. Agents that act as cAMP agonists or increase cAMP levels do not induce ODC, but increases in ODC activity are observed in response to NGF (44). To assess the requirement for cAMP-dependent processes in mediating the effects of NGF, aFGF, and bFGF, we studied regulation of gene expression and neurite outgrowth by these growth factors in A126-1B2 cells.

As expected, PMA, but not dbcAMP, induced ODC in A126-1B2 cells (Fig. 4; data not shown). As has been reported previously (44), NGF induced ODC, but the inductions observed in the A126-1B2 cells are somewhat reduced (to 44% of control) but not eliminated (Fig. 4, open bars, and Table I). The observed inductions of ODC by aFGF and bFGF in A126-1B2 cells were not statistically significant ( $P > 0.05$ ) (Fig. 4, open bars, and Table I). These data indicate



**Figure 6.** NGF, aFGF, and bFGF induce neurite outgrowth from PC12 cells that are deficient in PKC and cAMP responsiveness. Open bars indicate neurite outgrowth from A126-1B2 cells. Hatched bars indicate neurite outgrowth from A126-1B2 cells that were pre-treated with 1  $\mu$ M PMA for 24 h before growth factor addition. Data represent the means  $\pm$  SEM of at least three experiments.

that NGF, in part, and aFGF and bFGF require cAMP responsiveness to induce ODC.

In A126-1B2 cells, dbcAMP did not induce the transcription of d2 or d5 (Fig. 5, *top*), which is consistent with the resistance of these cells to cAMP analogues. NGF, PMA, and aFGF increased the transcription of d2 and d5 to levels that were not measurably different from those in wild-type PC12 cells. bFGF also increased d2 transcription in A126-1B2 cells, but the increases were less than those observed in PC12 cells (Fig. 5, *top*, and Table I). This reduced responsiveness of bFGF in A126-1B2 cells was reproduced in three out of three experiments. Furthermore, in a single experiment, bFGF induced d2 as well as NGF and aFGF in PC12 cells but not in A126-1B2 cells. NGF, aFGF, and bFGF also maximally induced neurite outgrowth from A126 cells (Fig. 6, *open bars*, and Table I). These findings indicate that (a) cAMP responsiveness is required for aFGF and bFGF to induce ODC; (b) cAMP responsiveness is required for NGF to maximally induce ODC; (c) bFGF, but not aFGF and NGF, require cAMP responsiveness to maximally induce d2; and (d) cAMP responsiveness is not required for NGF or the FGFs to increase d5 transcription and to induce neurite outgrowth.

#### Can NGF, aFGF, and bFGF Act in PC12 Cells Independently of Both PKC and cAMP Second Messenger Systems?

We studied A126-1B2 cells that had been downregulated with PMA to determine the effect of compromising both the cAMP and the PKC signal transduction systems on NGF and FGF actions. Downregulation of PKC by a 24-h pretreatment of A126 cells with PMA yields cells that are deficient in both of these second messenger systems. If NGF, aFGF, and bFGF elicit responses in the downregulated A126 cells, it must be via a second messenger system that is both PKC and cAMP independent.

PMA induction of ODC in A126-1B2 cells was abolished by PMA pretreatment. However, pretreatment did not significantly affect the ability of NGF to induce this enzyme (Fig. 4, *hatched bars*, and Table I). Thus, NGF can induce ODC via a pathway that is independent of both PKC and cAMP responsiveness.

PMA pretreatment of A126 cells abolished PMA stimulation of d2 and d5 transcription; thus, neither gene was induced by treatment of the cells with either PMA or dbcAMP (Fig. 5, *bottom*), demonstrating that these cells were functionally deficient in PKC and cAMP second messenger systems. The ability of NGF, aFGF, and bFGF to increase transcription of d2 was substantially reduced, but not eliminated, in cells deficient in both PKC and cAMP responsiveness (Fig. 5, *bottom*, and Table I). The removal of each second messenger system individually did not compromise d2 transcriptional induction in response to aFGF or NGF (Fig. 2, *bottom*, Fig. 5, *top*, and Table I), but the removal of both systems did. These observations suggest that either the PKC or cAMP second messenger system, but not both, is required for NGF and aFGF to maximally elevate transcription of the putative transcriptional activator, d2. The response to bFGF in cells deficient in both second messenger systems was slightly less than that observed in cells deficient in cAMP responsiveness alone (Fig. 5 and Table I). The residual d2 induction in downregulated A126-1B2 cells suggests that NGF,

aFGF, and bFGF can elicit a response via a mechanism that is both PKC and cAMP independent. The growth factor-induced increases in d5 transcription were unaffected by downregulation of A126 cells, indicating that neither cAMP responsiveness nor PKC are required to maximally elicit this response, again suggesting that NGF, aFGF, and bFGF can act via a non-PKC-dependent and non-cAMP-dependent mechanism.

NGF, aFGF, and bFGF maximally induced neurite outgrowth in A126 cells that were deficient in PKC (Fig. 6, *hatched bars*, and Table I), suggesting that neither PKC nor a cAMP-dependent process is essential for growth factor-induced neurite outgrowth.

#### Discussion

Our goal in the present study was to determine if there is a causal link between cAMP and/or PKC second messenger systems and the actions of three neuronal growth factors. To do this, we examined the effects of NGF and the FGFs on PC12 cells that were deficient in either one or both of these second messenger systems. Results of these studies indicate that neither PKC nor cAMP are required for NGF- and FGF-induced changes in morphology and transcriptional induction of the gene d5. However, increases in the transcription of d2, a gene that encodes a putative transcriptional regulator, were significantly compromised by the absence of both PKC and cAMP responsiveness. Also, aFGF and bFGF did not significantly induce ODC in A126-1B2 cells that are deficient in cAMP responsiveness. Thus, although PKC- and/or cAMP-dependent processes partially mediate some NGF, aFGF, and bFGF actions, alternate second messenger systems must mediate other key responses, such as neurite outgrowth. These results suggest a model of NGF and FGF action that is schematically depicted in Fig. 7.

In spite of the fact that cAMP and/or PKC activation have been implicated in some of the actions of NGF and FGF (6,

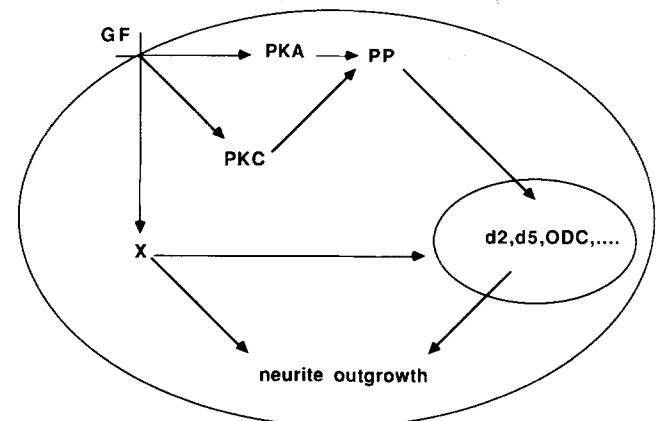


Figure 7. Proposed model of NGF and FGF signal transduction in PC12 cells. Occupation of growth factor (GF; i.e. NGF, aFGF, bFGF) receptors transduces signals via three second messenger pathways: PKC dependent, PKA dependent, and X (an unidentified signal transduction pathway). PKC and/or PKA phosphorylate a protein or proteins (PP) that permit growth factor-dependent regulation of gene expression. Activation of X results in regulation of gene expression and neurite outgrowth. This activation, in the absence of PKC and PKA pathways, produces maximal induction of d5 and neurite outgrowth but only partial induction of d2 and ODC.

8, 20), our findings clearly indicate the lack of a causal role for both of these second messenger systems in mediating neurite outgrowth and the transcriptional induction of d5. The role of cAMP-dependent processes and PKC activation in mediating FGF actions in PC12 cells has not previously been studied. Observations of Richter-Landsberg and Jastorff (34) and Rydel and Greene (36) support our evidence indicating that the cAMP second messenger system is not required for NGF induction of neurite outgrowth. Observations similar to ours regarding the role of PKC in mediating NGF induction of neurite formation have been reported by Reinhold and Neet (33), who demonstrated NGF-induced neurite outgrowth in PMA downregulated PC12 cells grown in suspension. In contrast to these observations, sphingosine inhibition of NGF-induced neurite outgrowth in PC12 cells led to the suggestion of a requirement for PKC in eliciting this response (19); however, the documented lack of specificity of sphingosine as an inhibitor of PKC makes these results difficult to interpret. For example, sphingosine inhibition of thyrotropin-releasing hormone binding to pituitary cells has been shown to be independent of PKC (47). Thus, sphingosine may act to inhibit neurite outgrowth from PC12 cells by a mechanism that is not dependent upon PKC. Alternatively, sphingosine may be more effective than phorbol ester downregulation in eliminating PKC. This latter alternative would suggest that there may be residual PKC activity in PMA downregulated cells. If this is so, the PKC levels must be below the limits of sensitivity of the PKC assay (30). Further, these residual levels of PKC must be below that required to elicit increases in ODC activity and gene transcription since a stimulator of PKC no longer elicited these responses in PMA downregulated cells (Figs. 2, 3, 5, and 6). Since neurite outgrowth and d5 induction proceed in the absence of both PKC and cAMP responsiveness, NGF, aFGF, and bFGF must use an alternate signal transduction mechanism or mechanisms to elicit these responses.

Elimination of either the PKC- or cAMP-dependent system alone had no effect on the d2 response to NGF and aFGF. However, the combined absence of both signal transduction systems reduced NGF and aFGF stimulation of d2 transcription. That the d2 response was not abolished when only one signal transduction system was compromised suggests a control mechanism that is complex. Consistent with our observations is the presence of putative cAMP- and PKC-dependent regulatory elements in d2-like genes (2, 4). The existence of more than one regulatory mechanism would allow for cooperativity that could account for partial reductions in responses. Further, the residual response that we observed in cells deficient in both of these systems suggests the presence of at least one additional regulatory region in this gene. Lim et al. (26a) found a 40% reduction in FGF-induced increases in d2 (which they refer to as TIS 8) expression after PMA downregulation of PKC in 3T3 cells. The difference in the effect of downregulation on FGF-induced d2 transcription in 3T3 versus PC12 cells might be attributable to divergent functions of the FGFs in the two different cell types.

The differential sensitivity of the neurite outgrowth, d5, ODC, and d2 responses to deficiencies in second messenger systems suggests that these responses are differentially regulated. We observed that a lack of PKC and cAMP responsiveness did not affect growth factor-induced neurite outgrowth but did reduce the induction of d2. This suggests that acute

elevations in d2 transcription are not essential for growth factor-induced differentiation. An alternative explanation for this difference is that maximal increases in d2 transcription are not required for differentiation and that the residual level of d2 transcription is sufficient.

Our data and those of others indicate that NGF and FGF elicit many of the same responses from PC12 cells (36, 37, 42, 45). Furthermore, our data indicate that in nearly all cases NGF and FGF responses are comparably affected by deficiencies in PKC and cAMP second messenger systems. This supports the idea that NGF, aFGF, and bFGF use the same group of second messenger systems to transduce their signals. However, NGF but not the FGFs induced ODC in cells that were deficient in cAMP responsiveness, and ODC levels measured in the presence of FGF and NGF are greater than those measured in the presence of either factor alone (data not shown), suggesting that FGF and NGF use at least one distinct second messenger system to induce ODC. This subtle difference between NGF and FGF action may help elucidate the nature of the differential effects of these two growth factors on neurite formation and survival in chromaffin cells (5). Koizumi et al. (24) have shown that K-252a, an in vitro protein kinase inhibitor, inhibits many actions of NGF on PC12 cells but not those of FGF, again suggesting that NGF and the FGFs use at least one distinct signal transduction system.

We have provided convincing evidence that cAMP and/or PKC second messenger systems are not required for NGF- or FGF-induced differentiation (neurite outgrowth) in PC12 cells. This observation in conjunction with our findings of a cAMP- and PKC-independent increase in d2 transcription indicates the involvement of other second messenger systems in mediating growth factor actions. Among known second messenger systems, tyrosine kinase activation has been implicated in mediating both NGF (29) and FGF (7, 12, 23, 25, 35) actions, but a causal link between tyrosine kinase activation and differentiation has not been established. Other potential mediators of growth factor actions include cyclic guanosine monophosphate *ras*-like G proteins, Ca<sup>2+</sup>, pH, and phospholipids (1, 18). The lack of an "all-or-none" role for one second messenger system emphasizes the fact that multiple second messenger systems operate singularly or concomitantly to mediate a variety of growth factor actions.

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