Effect of Nerve Growth Factor and Fibroblast Growth Factor on PC12 Cells: Inhibition by Orthovanadate

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Abstract. Sodium orthovanadate, an inhibitor of protein tyrosine phosphatases, causes increased levels of tyrosine phosphorylation and blocks, at noncytotoxic concentrations, the differentiative response of rat pheochromocytoma (PC12) cells to β-nerve growth factor (βNGF) and basic fibroblast growth factor (bFGF) in a reversible manner. It also prevents growth factor-induced neurite proliferation in primed cells and causes the retraction of previously formed neurites, even in the presence of βNGF or bFGF. It is equally effective in blocking neurite proliferation by 8-Br-cAMP. Zinc chloride and ammonium molybdate, two other inhibitors of tyrosine phosphatases, also cause parallel decreases in neurite proliferation. Orthovanadate generally reduces the transcription of immediate early response genes (TIS 8 and c-*fos*) and secondary response genes (ornithine decarboxylase (ODC), acetylcholinesterase (AChE) and SCG 10) induced by βNGF, bFGF, EGF, and PMA, albeit in a variable fashion. There was no observed effect on the kinetics of expression as judged by TIS 8 induction by βNGF and protein kinase C (PKC) downregulation did not change the levels of inhibition by orthovanadate seen in control cells. Orthovanadate does not affect the production of diacylglycerol induced by βNGF or bFGF. These observations are consistent with the view that growth factor stimulation of differentiation in PC12 cells involves at least one other PKC independent pathway, and that cAMP and PMA (and their active analogs) activate tyrosine kinases (albeit probably secondarily), which are at least partially responsive for their actions. Although the exact site(s) of action of orthovanadate that lead to the inhibition of growth factor-induced neurite proliferation are unknown, the results presented suggest that it prolongs tyrosine phosphorylations by nonreceptor tyrosine kinases that act downstream from the receptor kinases.

β-NERVE growth factor (βNGF),[1] basic fibroblast growth factor (bFGF) and many of their homologs act as potent neurotrophic factors on a variety of neuronal cells in vitro (Levi and Alema, 1991; Wagner, 1991). At least in the case of the NGF-like agents (neurotrophins), these effects extend to many of the same cells in vivo. One cultured cell line, responsive to both factors, was established from a rat pheochromocytoma by Greene and Tischler (1976). Designated PC12, these cells adopt a differentiated phenotype resembling sympathetic neurons when treated by βNGF or bFGF (acidic or basic) that is reversed to a chromaffin-cell phenotype when they are withdrawn. Among other responses, they induce neurite proliferation, neurite regeneration, changes in ion fluxes, electrical excitability and cholinergic activities (Greene and Tischler, 1982). PC12 cells also possess receptors for and are stimulated by other external ligands such as EGF, interleukin-6 and carbachol (a muscarinic agonist) but these do not necessarily lead to neuronal differentiation (Greene and Tischler, 1982; Satoh et al., 1988).

The intracellular signals responsible for the cellular responses to βNGF and bFGF in any target cell are incompletely understood. In PC12 cells, both factors clearly stimulate receptor bound tyrosine kinases leading to autophosphorylation and the induction of a broad variety of intracellular protein phosphorylations (on both tyrosine and serine/threonine residues) that results in the stimulation of phospholipase C_γ (Vetter et al., 1991), protein kinase C (PKC) (Cremins et al., 1986; Contreras and Guroff, 1987; Heasley and Johnson, 1989; Altin and Bradshaw, 1989; Pessin et al., 1991), and phosphatidyl inositol-3-kinase (Raffioni and Bradshaw, 1992). In addition, obligatory roles for c-*src* and c-*ras* that are apparently independent of these pathways have also been identified (Alema et al., 1985; Bar-Sagi and Feramisco, 1985; Kremer et al., 1991). In fact, as judged by immediate-early response gene induction (Kujubu et al., 1987; Damon et al., 1990; Sigmund et al., 1990; Altin et al., 1991), both PKC-dependent and independent pathways are operative for both factors.

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1. *Abbreviations used in this paper*: AChE, acetylcholinesterase; βNGF, β-nerve growth factor; DG, diacylglycerol; ODC, ornithine decarboxylase; PI, phosphoinositide; PKC, protein kinase C; PTPase, protein tyrosine phosphatase; TIS, tetradecanoyl phorbol acetate-induced sequences.

Although the stimulation of membrane bound receptor tyrosine kinases in a ligand dependent fashion appears essential to initiate these neurotrophic responses, the resulting protein tyrosine phosphorylations are relatively transitory and appear to function as short-lived intermediates in the signal cascade(s). In this respect, the neurotrophic factor responses may resemble the regulation of the cell cycle in marine invertebrates and yeast which is controlled by the phosphorylation/dephosphorylation of $p34^{CDC\ 28/CDC\ 2}$ kinase (Nurse, 1990; Matsushime et al., 1991).

To further probe the role of tyrosine phosphorylations in the βNGF/bFGF action on PC12 cells, we have examined the effect of orthovanadate on various growth factor induced responses. This compound is an inhibitor of protein tyrosine phosphatases (PTPases) that, in concert with the kinases, control the level of tyrosine phosphorylation. As an example of this function, membrane PTPase activity increases as the rate of cell proliferation decreases, reaching a maximum when cells become contact inhibited (Brautigan and Pinault, 1991; Pallen and Tong, 1991). By analogy with phosphodiesterase inhibitors, which prolong cAMP-dependent signals, PTPase inhibition might also be expected to enhance βNGF/bFGF responses. However, orthovanadate has proven to be a strong inhibitor of neurite proliferation and regeneration and markedly affects, in a quantitative, but not temporal fashion, the expression of several immediate early and secondary response genes. It was without effect, however, on the production of diacylglycerol (DG). These findings support the view that tyrosine phosphorylation signals are probably generally (and necessarily) transitory and that multiple signalling pathways exist in response to both growth factors.

Materials and Methods

Materials

βNGF and EGF were prepared by the method of Mobley et al. (1976) and Savage and Cohen (1972), respectively. bFGF used in all experiments was an analog with all half-cystine residues replaced by serines (Fox et al., 1988) (kindly provided by Dr. Michael Fox, Amgen, Inc., Thousand Oaks, CA). PMA, 8-Br-cAMP, and sodium orthovanadate were from Sigma Chemical Co. (St. Louis, Mo.). Stock solutions of sodium orthovanadate (5 mM) was prepared according to Brown and Gordon (1984).

Cell Culture

PC12 cells were obtained from Dr. D. Schubert (Salk Institute, San Diego, CA). All cells were maintained as monolayer cultures in 150 cm^2 tissue culture flasks (Bellco Glass, Inc., Vineland, NJ) in DME containing 10% horse serum, 5% FCS, and 1% Fungi-bact solution (complete medium) in a 5% CO_2 humidified atmosphere. Cells were subcultured once a week by shaking the flask and replating in a 1:4 to 1:6 ratio. The medium was changed every 3 d.

Neurite Outgrowth and Neurite Regeneration

PC12 cells used for induction of morphological differentiation were subcultured in collagen-coated 35 mm 6-well cluster dishes at a density of ~0.1 × 10^6 cells/well. Cells were first grown in complete medium for 6 h, rinsed once with DME, and then cultured in DME containing 1% horse serum and 1% Fungi-bact solution. βNGF (100 ng/ml), bFGF (10 ng/ml) or 8-Br-cAMP (1 mM) were added to the medium in the presence or absence of various concentrations of inhibitors. After 24 or 48 h of treatment, 2 to 3 random microscopic fields per well were photographed using a Nikon Diaphot microscope. The negatives were examined under a microfiche reader. Responsive cells were defined as those bearing neurites at least 2 cell diam in length and with clearly defined growth cones. At least 200 cells were scored per negative. For neurite regeneration experiments, cells were primed in complete media containing 50 ng/ml βNGF for 7 d. Neurites were sheared from cell bodies by vigorously shaking the flask followed by a brief centrifugation and resuspension. Cells were then replated as described above for neurite outgrowth. The proportion of responsive cells was examined one day after the readdition of βNGF with or without orthovanadate.

Measurement of PTPase Activity

PC12 cells were cultured in 60 mm collagen-coated dishes and pretreated with or without PTPase inhibitors (sodium orthovanadate, zinc chloride, or ammonium molybdate) for 24 h. Cells were rinsed with PBS and solubilized in 0.15 ml lysis buffer containing 50 mM Hepes (pH 7.4), 10% glycerol, 4 mM EDTA, 1 mM dithiothreitol, 1% NP-40, 0.5 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The cell lysate was centrifuged at 14,000 *g* for 15 min and the supernatant was used for PTPase assay. The synthetic peptide raytide (Oncogene Science, Uniondale, NY), phosphorylated on tyrosine residues, was used as the substrate in the PTPase assay. Phosphorylation was performed in 0.4 ml containing 40 μg of raytide, 50 mM Hepes (pH 7.4), 10 μg of A-431 cell membrane particulates, 1 μg/ml EGF, 0.25% NP-40, 10 mM $MnCl_2$, 0.5 mM orthovanadate, 25 μM ATP, and 150 μCi of [γ-^{32}P]ATP for 1 h at 30°C. The reaction mixture was spotted on P-81 phosphocellulose paper and washed 3 times for 5 min with 0.5% phosphoric acid. Phosphorylated raytide was eluted from the P-81 paper by 10 ml 0.5 M ammonium bicarbonate and lyophilized. Phosphatase assay was performed essentially as described by Streuli et al. (1990) except 1 μg of cellular protein was used for each assay and enzyme samples were incubated with substrate at 25°C for 10 min.

Immunoblot Analysis

PC12 cells were grown in 100 mm collagen-coated dishes and pretreated with or without 20 μM orthovanadate for 24 h. After stimulation with growth factors for 5 min, cells were rinsed with PBS and solubilized in 100 μl 2 × SDS sample buffer (Laemmli, 1970) with the addition of 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM orthovanadate. Samples were boiled for 5 min and 100 μg of proteins was separated by an 10% SDS/PAGE, transferred to an Immunobilon-P membrane (Millipore Corp., Bedford, MA), and probed with antiphosphotyrosine antiserum (Zymed Labs, South San Francisco, CA) and ^{125}I-protein A. The membrane was subjected to autoradiography at −80°C for 6 h.

Measurement of Ornithine Decarboxylase Activity

PC12 cells were grown in 60 mm collagen-coated dishes in complete media for 2 d. 1 d before the experiment, media were changed to DME containing 1% horse serum with or without 10 μM sodium orthovanadate. On the day of the experiment, growth factors were added directly to the media for 5 h. Cells were then rinsed with PBS and harvested. Preparation of cell extracts and estimation of ornithine decarboxylase (ODC) activity were essentially the same as described by Djurhuus (1981). Protein concentration was determined by the method of Bradford (1976) using BSA as a standard. ODC activities were expressed as nmol putrescine/mg protein/h.

Measurement of Acetylcholinesterase Activity

PC12 cells were grown in 100 mm collagen-coated dishes under similar conditions as for neurite outgrowth experiment. Cells were treated with growth factors with or without 10 μM orthovanadate for 3 d. Cell extracts were prepared and acetylcholinesterase (AChE) activities were estimated as described by Rieger et al. (1980). Enzyme activities were expressed as nmol thiocholine/mg protein/min.

Measurement of Diacylglycerol

PC12 cells were grown in 100 mm collagen-coated dishes in complete media for 2 d. Cells were rinsed once with DME and changed to DME in the presence or absence of 10 μM orthovanadate and continuously grown overnight. After stimulation with growth factors for 5 min, cells were extracted as described by Bligh and Dyer (1959) except that 1 M NaCl was used as the upper phase to prevent acid-catalyzed isomerization of sn-diacylglycerols (DG). Two samples of the final $CHCl_3$ phase were dried under a stream of N_2 for DG and phospholipid measurements. Sn-1,2-DG was estimated essentially as described by Preiss et al. (1986), as modified by Wright et al. (1988). In all assays, the conversion of a known quantity of sn-DG

was included as a control. Total phospholipid was estimated according to the method of Ames and Dubin (1960). Data were expressed as nmol of DG/100 nmol of total phospholipids (mole percent).

Northern Blot Analyses

PC12 cells were grown in 150 mm collagen coated dishes in complete media for 2 d. 1 d before the experiment, cells were fed with fresh media in the presence or absence of 20 μM orthovanadate. On the day of the experiment, growth factors were added directly to the media for the indicated time. Cells were at a density ~60–70% confluency. To determine SCG 10 expression, cells were harvested 24 h after the addition of growth factor and orthovanadate. Because the induction of SCG 10 by growth factors was inhibited at high cell density, the cell density was maintained at ~30% confluency. Total cellular RNA (10 μg/lane), isolated as described by Chomczynski and Sacchi (1987), was fractionated on 0.9 or 1.0% agarose gels containing 2.2 M formaldehyde and transferred to a nitrocellulose membrane (Sambrook et al., 1989). DNA probes used were: SCG 10, a 1.9-kb *Eco* RI fragment from the clone SCG 10-3 (Stein et al., 1988*a*); v-*fos*, a 1.0-kb *Pst* I fragment isolated from *p-fos.1* (Curran et al., 1982); CHOb, a 0.74-kb Bam HI fragment (Lim et al., 1987); and TIS 8, a full-length cDNA (Lim et al., 1987). (SCG 10-3 was kindly provided by Dr. D. J. Anderson, California Institute of Technology, Pasadena, CA; the v-*fos*, TIS 8, and pCHOb probes were a generous gift from Dr. Harvey Herschman, University of California, Los Angeles, CA). Radioactive probes were prepared using a multiprime DNA labeling system by Amersham Corp. (Arlington Heights, IL). RNA blots were prehybridized and hybridized as described by Lim et al. (1987) and were exposed to Kodak X-Omat AR films at −80°C with intensifying screens for 24 to 48 h. TIS 8 blots were cohybridized with the pCHOb probe; other blots were first hybridized with the SCG 10-3 or c-*fos* probes, washed to remove these probes, and then hybridized with the pCHOb probe to normalize RNA loading differences. Densitometric scanning of the corresponding autoradiograms was used to semiquantitate the expression of these genes after normalization.

Data Analysis

Values were reported as mean and SEM. The unpaired *t* test was used for comparisons between two treated groups and the paired *t* test was used for comparisons between treated groups (expressed as fold of control) and control (set as 1.0). Significance level was set at $\alpha = 0.05$.

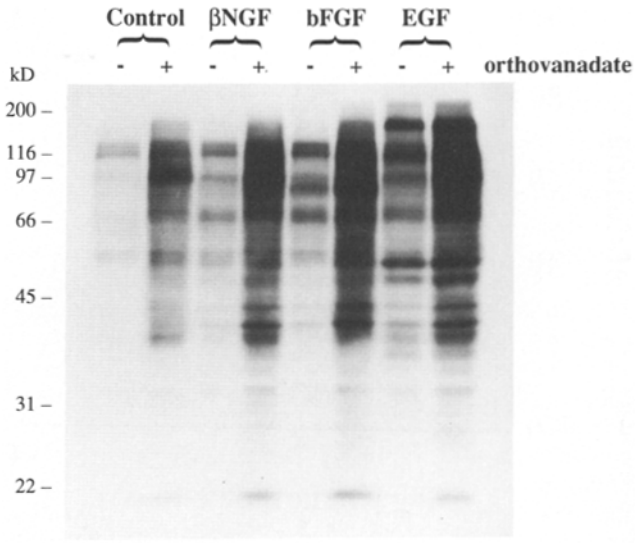

Figure 1. Effect if orthovanadate on increased growth factor-stimulated tyrosine phosphorylation in PC12 cells. PC12 cells pretreated with or without 20 μM orthovanadate for 24 h were stimulated with βNGF (100 ng/ml), bFGF (10 ng/ml), or EGF (3 ng/ml) for 5 min. 100 μg of total cellular proteins were separated by 10% SDS PAGE and immunoblotted with antiphosphotyrosine antibody and ^{125}I-protein A. The migration of molecular weight standards is shown on the left.

Results

Increase of Growth Factor-stimulated Protein Tyrosine Phosphorylation by Orthovanadate in PC12 Cells

The effect of orthovanadate on growth factor-induced protein tyrosine phosphorylation in PC12 cells is shown in Fig. 1. Consistent with its potent protein tyrosine phosphatase inhibitor activity, the levels of tyrosine phosphorylation detected by immunoblot analysis after 5 min are markedly enhanced in all orthovanadate treated samples (including unstimulated cells). Within the limits of this analysis, it appears that the pattern of phosphorylation is not altered with respect to any factor although a few phosphorylated proteins are only detectable in the orthovanadate-pretreated cells. However, the pattern of tyrosine phosphorylation for each growth factor is distinct with each factor showing at least some unique bands.

Inhibition by Orthovanadate of Neurite Outgrowth Induced by βNGF, bFGF, and 8-Br-cAMP

The induction of neurites by βNGF and bFGF serves as a convenient marker for the differentiative action of these growth factors on PC12 cells. Although a relatively long term response, it is clearly connected to a variety of intracellular changes including the rapid, ligand-dependent phosphorylation of tyrosine residues in a variety of proteins, modifications that are among the earliest observed effects. Thus, orthovanadate, a potent inhibitor of PTPases, might be expected to affect positively growth factor induced processes such as neurite proliferation. However, as shown in Figs. 2 *a* and 3, incubation of PC12 cells with NGF or bFGF for 2 days in the presence of 1 μM orthovanadate results in inhibition of neurite outgrowth; at 21.5 μM, the inhibition is essentially complete. The EC_{50} for the inhibition of neurites induced by bFGF was 5.67 $\pm$ 0.07 μM ($n = 5$) while that for βNGF was 3.44 $\pm$ 0.05 μM ($n = 5$). Importantly, the concentration of orthovanadate that gave complete inhibition (21.5 μM) was not toxic for PC12 cells (Fig. 3, *A1–A3*); at higher concentrations, some cytotoxicity was observed.

Orthovanadate also affected the neurite formation produced by 8-Br-cAMP. Although analogs of cAMP are not as potent in their ability to stimulate differentiation in PC12 cells as are the neurotrophic growth factors and may employ an entirely separate mechanism, the response to 1 mM 8-Br-cAMP (Fig. 3 *D1*) after 2 d of treatment is quite pronounced. As seen in Fig. 3, *D2* and *D3*, orthovanadate also inhibits the ability of this cAMP analog to elicit neurites; the magnitude of inhibition is comparable to that seen with the growth factors (Fig. 2 *a*, and 3, *B2, B3, C2* and *C3*).

The inhibitory effect of orthovanadate was also observed on preformed neurites. As shown in Fig. 4, treatment of PC12 cells for 2 d with βNGF (100 ng/ml), followed by removal of the medium (as well as the ligand) and replacement with fresh medium containing βNGF and 0, 4.6, and 10 μM orthovanadate results in the loss of the preformed neurites (formed after 2 d exposure of βNGF) in the samples containing the inhibitor. Thus, the processes required to maintain neurites are also affected in the same manner by or-

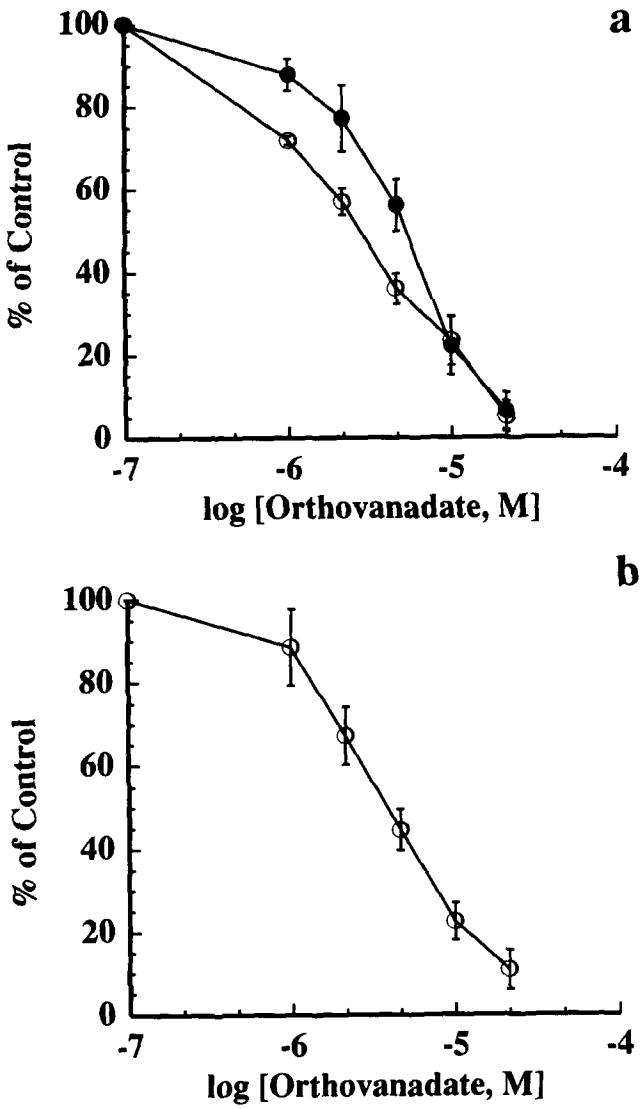

Figure 2. Dose-dependent inhibition of orthovanadate on βNGF or bFGF induced neurite outgrowth (*a*) and neurite regeneration (*b*). (*a*) PC12 cells were cultured for 2 d in DME containing 1% horse serum, βNGF (100 ng/ml) or bFGF (10 ng/ml) in the presence of various concentrations of orthovanadate. The % of responsive cells in PC12 cells without orthovanadate treatment (control): 68 ± 6% (n = 5) for βNGF and 80 ± 5% (n = 5) for bFGF. The proportion of responsive cells in the presence of orthovanadate was normalized in each experiment and expressed as percent of the control. -○-, NGF; -●-, bFGF. (*b*) PC12 cells were primed with 50 ng/ml βNGF for 7 d and replated. Neurite bearing cells were scored 1 d after the readdition of βNGF (100 ng/ml) and addition of various concentrations of orthovanadate. In the absence of orthovanadate, 82 ± 4% (n = 4) of the cells was responsive. 77 ± 5% (n = 4) of the βNGF responsive cells were actionomycin D (1 nM) resistant. In both panels, values are averages of triplicate determinations from four to five separate experiments. Vertical bars represent SEM.

thovanadate as those necessary to initiate the response. Similar results were observed for bFGF (data not shown).

The inhibitory effect of orthovanadate was also seen in cells that had been primed by earlier exposure to βNGF (Figs. 2 *b*, and 5). As previously documented (Greene and Tischler, 1982), when PC12 cells are treated with βNGF for 1 wk, they respond to new additions of βNGF and elaborate neurites immediately; maximal neurite generation is observed within 24 h. This effect of βNGF is not blocked by actinomycin D (1 nM) (Fig. 5 *C*). However, as with naive cells, orthovanadate, at 21.5 μM, effectively blocks βNGF induction of neurites in these cells indicating that the inhibition by orthovanadate does not depend on new protein synthesis.

Although orthovanadate is a good inhibitor of neurotrophic factor induced neurite proliferation in PC12 cells, its effects are clearly reversible. As shown in Fig. 6, PC12 cells previously treated with either βNGF or bFGF in the presence of orthovanadate (10 μM) can be induced to produce neurites by washing (to remove the inhibitor) followed by the addition of more growth factor. The treated cells appeared as responsive as naive ones.

Inhibition of Neurite Proliferation Correlates with the Inhibition of PTPase Activities

In addition to its PTPase inhibitor activity, orthovanadate has been reported to affect other enzymatic activities in vivo. Orthovanadate at the concentration used in this study does not affect (Na^+,K^+)-ATPase activity in A431 cells (Cassel et al., 1984), rat myocardial cells (Werdan et al., 1980), intact red blood cells (Cantley, 1978) or adipocytes (Dubyak and Kleinzeller, 1980). In addition, in the intact red cell, 50 μM vanadate has no effect on the (Ca^{++},Mg^{++})-ATPase activity (Szasz et al., 1981) and 0.5 mM vanadate is required to stimulate Ca^{++} pump (Varecka and Carafoli, 1982).

To further test the relation of phosphatase inhibitors and neurite outgrowth, other substances known to inhibit protein tyrosine phosphatases were tested for their effect on neurite outgrowth. As with orthovanadate, a good correlation with zinc chloride and ammonium molybdate, two other known PTPase inhibitors, was observed (Fig. 7). However, they were less potent than orthovanadate. This may be due to multiple forms of PTPase that have different sensitivities to the various inhibitors (Tonks et al., 1988). After a 24-h treatment with 20 μM orthovanadate, nearly 80% of βNGF-induced neurite outgrowth and PTPase activity was inhibited. In the presence of the highest nontoxic concentration of zinc (250 μM) or molybdate (600 μM), only 40 and 50–60%, respectively, of βNGF-induced neurite outgrowth and PTPase activity were inhibited.

Effects of Orthovanadate on Acetylcholinesterase and Ornithine Decarboxylase Activity

In addition to morphological changes, βNGF and bFGF also induce cholinergic responsiveness in PC12 cells by regulating acetylcholinesterase (AChE) and cholineacetyltransferase activities (Greene and Rein, 1977; Schubert et al., 1977; Lucas et al., 1979, 1980; Rieger et al., 1980; Greene and Rukenstein, 1981). Thus, the induction of AChE activity by neurotrophic factors is a biochemical marker for neuronal differentiation in PC12 cells. As shown in Fig. 8 *a*, after exposure to βNGF and bFGF for 3 d, the activity of AChE per milligram of protein increased by 2.01 ± 0.22 (n = 5) and 2.23 ± 0.17 (n = 5)-fold, respectively. When 10 μM orthovanadate was coincubated, the response to βNGF and bFGF was inhibited by 79.88 ± 5.69 and 61.80 ± 14.61%, respectively, reducing the levels in treated cells to essentially those of control values.

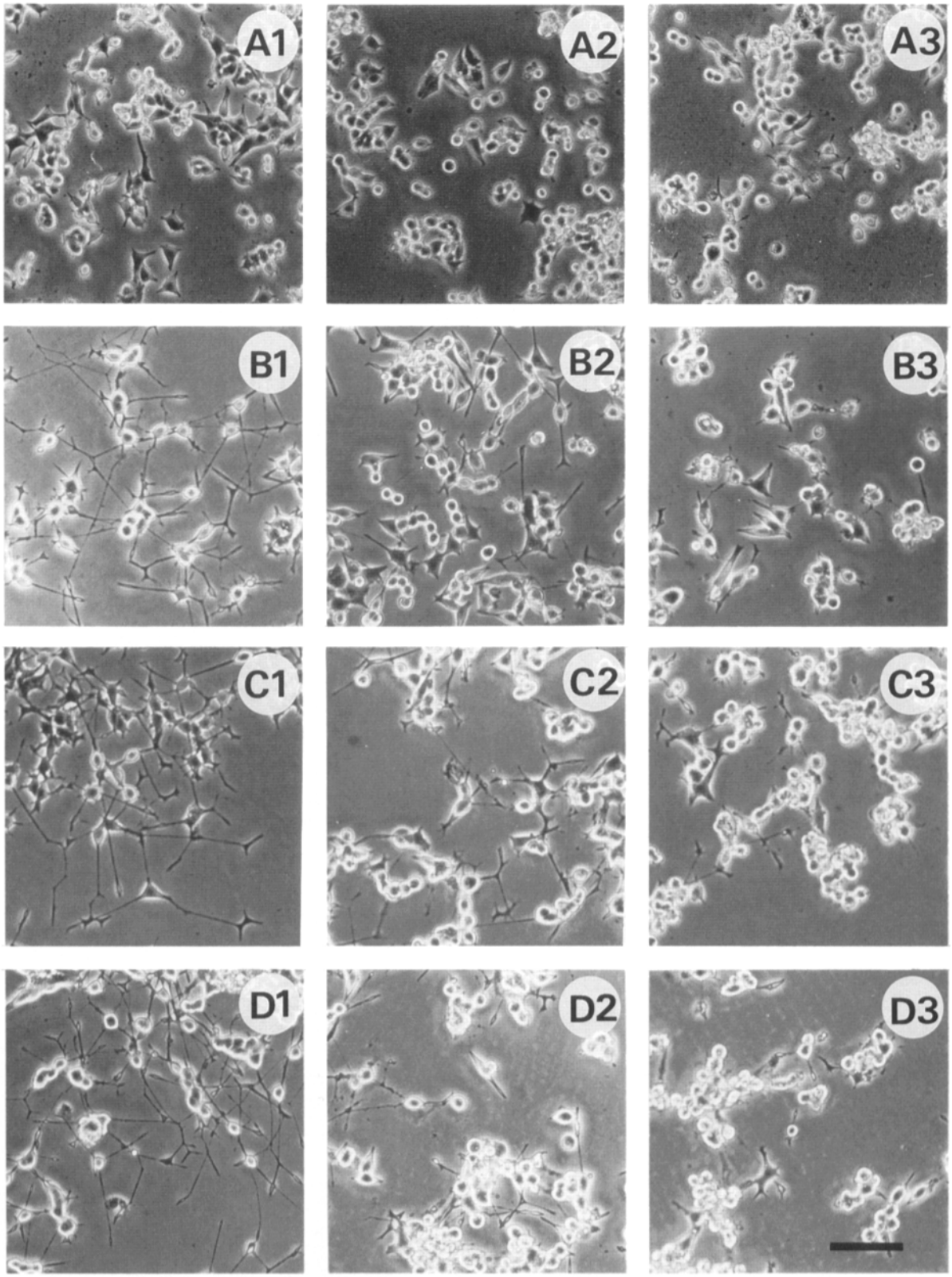

Figure 3. The inhibition of neurite proliferation induced by βNGF, bFGF, or 8-Br-cAMP by orthovanadate. Cells were treated with no factor (*A1–A3*), βNGF (100 ng/ml) (*B1–B3*), bFGF (10 ng/ml)(*C1–C3*), or 8-Br-cAMP (1 mM)(*D1–D3*) and 0 (*A1, B1, C1, D1*), 10 μM (*A2, B2, C2, D2*), or 21.5 μM (*A3, B3, C3, D3*) orthovanadate. Other details are given in Materials and Methods. Bar, 100 μm.

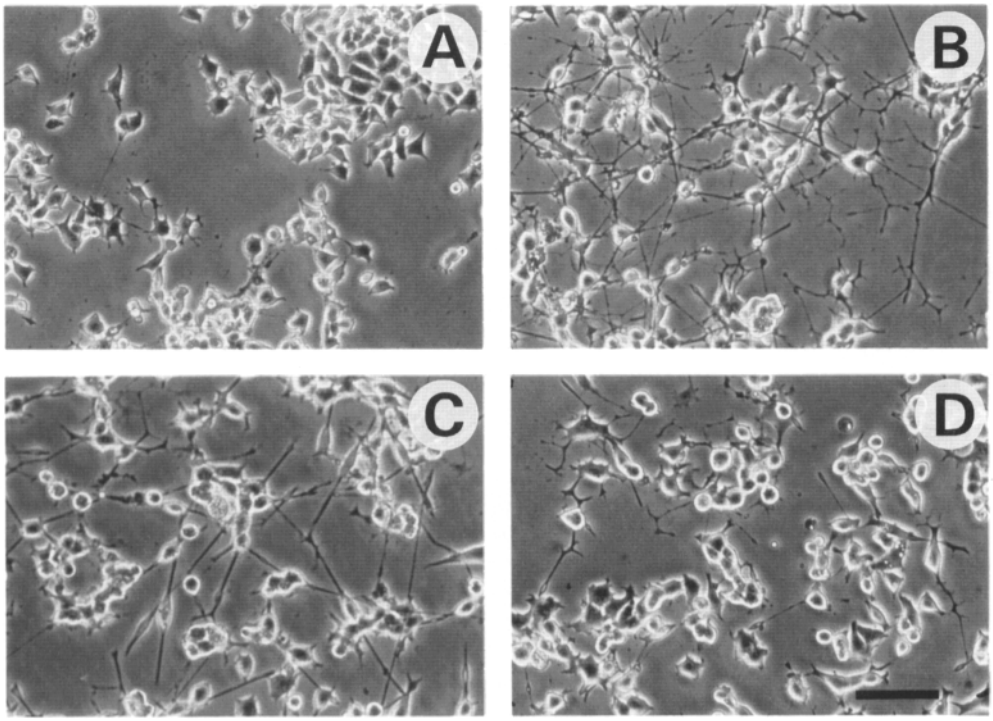

Figure 4. Effect of orthovanadate on PC12 cells pretreated with βNGF. Cells were cultured in the presence of βNGF (100 ng/ml) for 2 d and the medium replaced with fresh medium containing 0 (*B*), 4.6 μM (*C*) and 10 μM (*D*) orthovanadate with βNGF (100 ng/ml). Control cells in culture for 4 d with no addition of NGF or orthovanadate (*A*). Bar, 100 μm.

Increase in ODC induced by βNGF, bFGF, and EGF represents a short-lived, transcription-dependent response in PC12 cells. However, the induction of ODC activity does not appear to be required for neurite proliferation by βNGF (Feinstein et al., 1985). Inhibition of either the induction or the enzymatic activity of ODC does not alter other responses to βNGF (Greene and McGuire, 1978). ODC activity, which was greatly increased after a 5-h incubation with βNGF or bFGF, was only partially blocked by 10 μM orthovanadate pretreatment (Fig. 8 *b*). Increasing the orthovanadate concentration to 20 μM did not produce further inhibition (data not shown). In contrast, EGF, which also induces an increase in ODC, albeit more modest, was essentially completely abolished by the orthovanadate.

Effect of Orthovanadate on Diacylglycerol Production

In PC12 cells, βNGF and bFGF stimulate the hydrolysis of

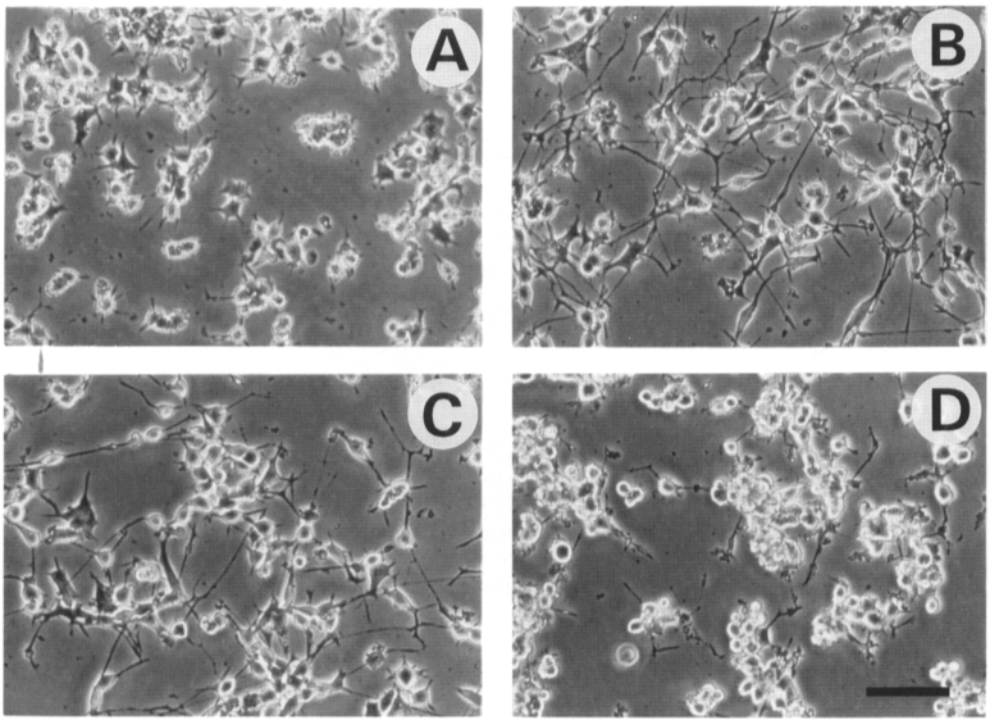

Figure 5. The inhibition of βNGF-induced neurite proliferation by orthovanadate in PC12 cells primed by previous exposure to βNGF. Cell culture conditions were the same as described in Fig. 2. (*A*) Control cells primed with βNGF (50 ng/ml) for 7 d and placed in DME containing 1% horse serum for 1 d; (*B*) primed cells (treated as in *A*, after 1 d readdition of βNGF; (*C*) primed cells (as in *B*) with the addition of actinomycin D (1 nM); (*D*) primed cells (as in *B*) with the addition of orthovanadate (21.5 μM). Bar, 100 μm.

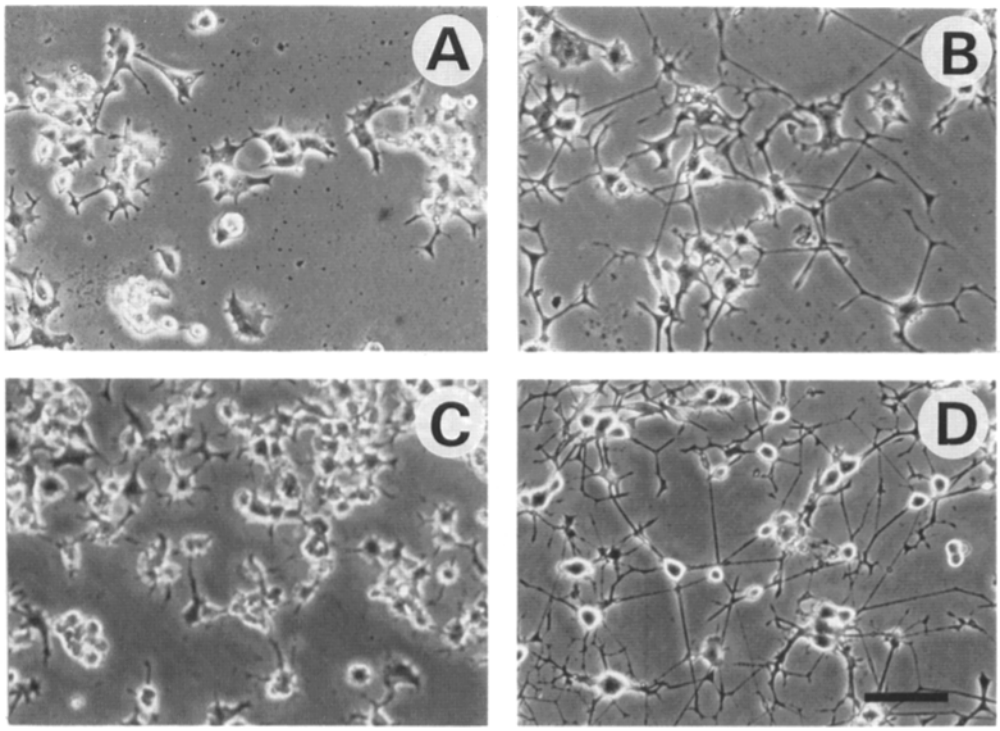

Figure 6. Reversal of the inhibition of neurite outgrowth by orthovanadate. Cells were cultured for 2 d in DME containing 1% horse serum, 10 μM orthovanadate and βNGF (*A* and *B*) or bFGF (*C* and *D*). In the control group, (*A* and *C*), cells were washed free of orthovanadate and growth factors and cultured without further additives for 2 d. In the test group (*B* and *D*), cells were treated in the same fashion except that βNGF (*B*) and bFGF (*D*) were added after washing. Bar, 100 μm.

phospholipids to produce DG (Contreras and Guroff, 1987; Altin and Bradshaw, 1990). A portion of the DG arises from phosphoinositide (PI), resulting in the production of inositol-1,4,5-trisphosphate, with the remainder, particularly at longer times after growth factor induction, being derived predominantly from phosphatidylcholine (Pessin et al., 1991). The formation of DG from PI occurs by the activation of phospholipase C_γ, an enzyme known to be phosphorylated on tyrosine (Nishibe et al., 1990; Goldschmidt-Clermont et al., 1991). To test the effect of orthovanadate on this process, PC12 cells were treated with 10 μM inhibitor and DG production in response to a 5-min stimulation by βNGF and bFGF, was measured. Although the level of DG was elevated ~1.3- to 1.4-fold under these conditions, there was no effect by orthovanadate relative to controls (data not shown). Similarly, EGF caused about the same increase and was not affected by the phosphatase inhibitor either. Thus, the rapid onset of phospholipase activation, which is itself a relatively transient event (Pessin et al., 1990), is not affected by prolonging tyrosine phosphorylation.

Effect of Orthovanadate on the Induction of Immediate Early Response Genes

Several immediate early response genes, that are characterized by a rapid, transient and cycloheximide-superinducible expression, can be induced by EGF, PMA, βNGF, bFGF, or K^+-induced depolarization in PC12 cells (Herschman, 1991). The products of some of these genes, such as TIS 8 and c-*fos*, have been identified as nuclear proteins which may act as transcriptional activators to mediate growth factor responses (Herschman, 1991). However, the signaling pathways involved in their induction are not fully delineated and may involve more than one pathway (Altin et al., 1991). As shown in Fig. 9, basal levels of TIS 8 and c-*fos* mRNA are very low, as has been previously reported (Kujubu et al., 1987), and orthovanadate treatment (20 μM for 18 h) did not alter these levels. pCHOb mRNA expression, which has been shown not to be regulated by growth factors (Altin et al., 1991), was used to normalize mRNA loading and orthovanadate had no effect on its expression, as compared with the intensity of ethidium bromide stained 28S and 18S ribosomal RNA bands, suggesting that orthovanadate does not act as a nonspecific transcription inhibitor. In contrast to the basal condition, the induction of TIS 8, a putative transcription factor (Herschman, 1991), by βNGF, bFGF, or EGF (after 30 min) was reduced by 62.0 $\pm$ 7.3 (n = 8) %, 42.5 $\pm$ 9.8 (n = 7) %, and 53.6 $\pm$ 6.9 (n = 7) %, respectively, in cells pretreated with 20 μM orthovanadate (Fig. 9 *a*).

Orthovanadate had an even greater effect on the induction of c-*fos*, also identified as a transcription factor (Herschman, 1991) (Fig. 9 *b*). The response induced by βNGF was virtually eliminated and that by bFGF, nearly so. The amount of c-*fos* induced by EGF was also greatly reduced by orthovanadate, although this factor did not induce as much transcript, in the absence of orthovanadate, at this time point. Interestingly, orthovanadate also dramatically affected the transcription induced by the phorbol ester, PMA, of both TIS 8 and c-*fos*. TIS 8 was reduced by 54.2 $\pm$ 7.2 (n = 7) for the controls (which was equivalent to that of the growth factors (Fig. 9 *a*) and c-*fos* was completely blocked. However, the induction of c-*fos* by PMA, in the absence of orthovanadate was much less than that observed for βNGF and bFGF stimulation at 30 min, but was similar to that observed for EGF. These observations suggest that either a part of the PMA induction mechanism depends on protein tyrosine phosphorylation or that orthovanadate affects activities other than PTPases.

The time course of TIS 8 response to βNGF was also examined in cells pretreated with or without orthovanadate (Fig. 10). In the absence of the inhibitor, the maximal re-

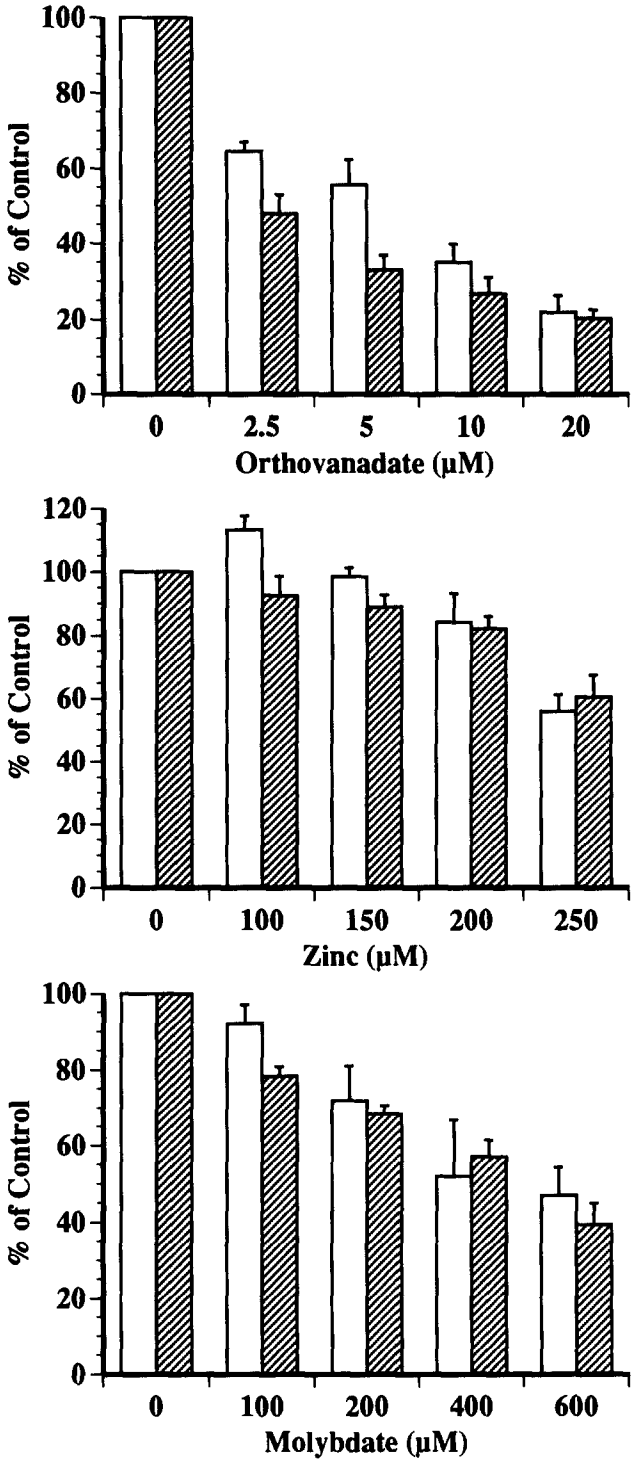

Figure 7. Effect of orthovanadate, zinc, and molybdate on PTPase activities and βNGF-induced neurite proliferation. PC12 cells were pretreated with or without various concentrations of orthovanadate, zinc, or molybdate and in the presence or absence of βNGF (100 ng/ml) for 24 h. In the control PC12 cells, 61.9 ± 3.1% cells bear neurites after 100 ng/ml βNGF stimulation for 24 h. Neurite outgrowth (*open columns*) induced by βNGF in the presence of PTPase inhibitors was expressed as percent of control. PTPase activities (*hatched columns*) were measured as described in the section of Materials and Methods and the activity in the presence of inhibitor was expressed as percent of that in the untreated control. Vertical bars represent the SEM. (n = 6).

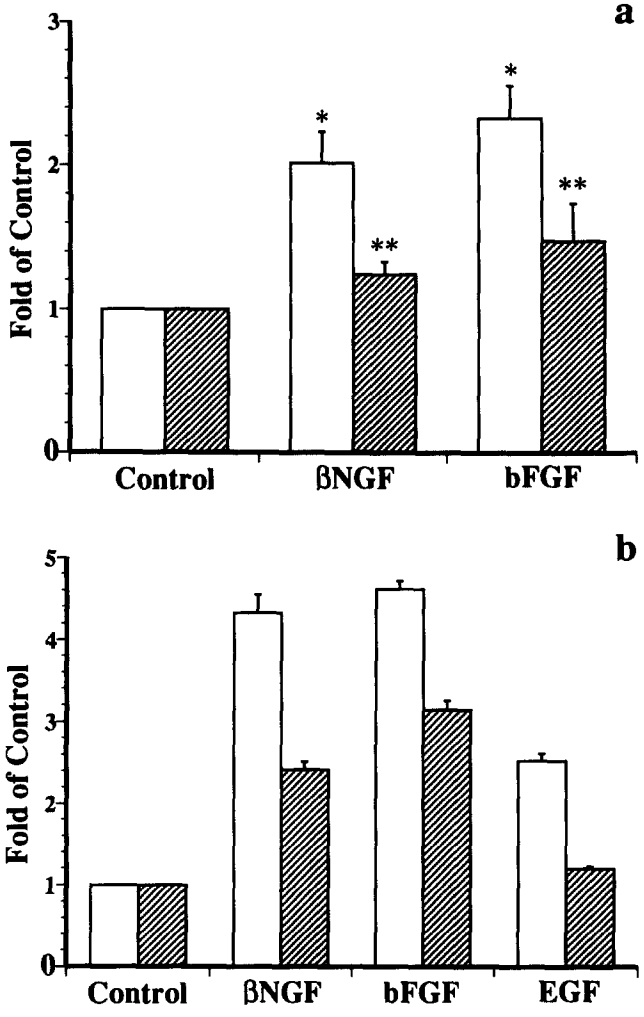

Figure 8. The effect of orthovanadate on the stimulation of acetylcholinesterase (AChE) (*a*) and ornithine decarboxylase (ODC) (*b*) by growth factors in PC12 cells. (*a*) Cells were cultured in DME containing 1% horse serum for 3 d in the presence of βNGF (100 ng/ml) or bFGF (10 ng/ml) and in the presence (*hatched*) or absence (*open*) of 10 μM orthovanadate. The basal AChE activities in the absence of βNGF or bFGF were: 4.36 ± 0.60 nmol/mg protein/min (n = 5) in normal PC12 cells and 4.88 ± 0.70 nmol/mg protein/min (n = 4) in PC12 cells incubated with 10 μM orthovanadate. Values are averages of triplicate determinations from four to five separate experiments. Vertical bars represent the SEM. (*)$p < 0.05$ compared with the control level; (**)$p < 0.05$ compared with the βNGF or bFGF treated group in the absence of orthovanadate. (*b*) Cells were pretreated with 10 μM orthovanadate for 18 h in DME containing 1% horse serum. ODC activities were measured after 5 h stimulation with βNGF (100 ng/ml), bFGF (10 ng/ml) or EGF (10 ng/ml). The basal ODC activities (expressed as nmol putrescine formed/mg protein/h) were 0.61 ± 0.05 for normal cells and 0.73 ± 0.02 for cells treated with orthovanadate. Vertical bars represent standard deviations.

sponse of TIS 8 transcription to NGF induction was reached at 1 h and the response was essentially zero in 3 h. In the presence of orthovanadate, the diminished TIS 8 response followed a similar time course (the loading of the 2- and 3-h time points was decreased, as judged by the CHOb marker, making it difficult to evaluate whether significant transcripts

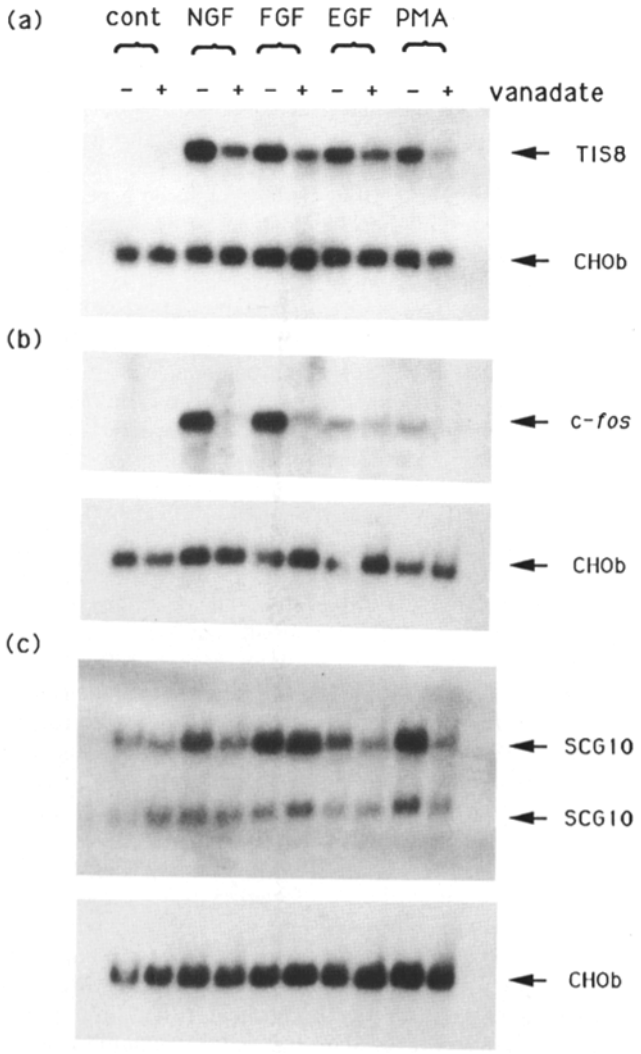

Figure 9. Northern blot analyses of the effect of orthovanadate on the expression of TIS 8 (*a*), c-*fos* (*b*) and SCG 10 (*c*). For TIS 8 and c-*fos* expression, PC12 cells were grown in complete medium pretreated with or without 20 μM orthovanadate for 18 h. Cells were stimulated with βNGF (100 ng/ml), bFGF (10 ng/ml), PMA (50 nM) or EGF (3 ng/ml) for 30 min. Controls (*cont*) were cells without growth factor stimulation. For SCG 10 expression, PC12 cells were cultured in complete medium containing growth factor and in the presence or absence of 20 μM orthovanadate for 24 h. Total RNA was extracted from the cells and fractionated on 0.9% (*TIS 8* or c-*fos*) or 1.0% (*SCG 10*) formaldehyde agarose gels (10 μg per lane). The TIS 8 blot (*a*) was cohybridized with TIS 8 and pCHOb probes. The c-*fos* and SCG 10 blots (*b* and *c*) were stripped after hybridization with the first probe and rehybridized with the pCHOb probe, resulting in two different exposures.

were still detectable at 120 min). Thus, the decrease in transcription is not the result of an altered kinetic profile.

The TIS 8 response to NGF or bFGF stimulation was not significantly changed in PC12 cells in which PKC had been down regulated (1 μM PMA for 24 h) as previously reported (Damon et al., 1990; Sigmund et al., 1990; Altin et al., 1991) (Fig. 11). Furthermore, orthovanadate inhibited TIS 8 induction by βNGF in PKC-downregulated cells to the same extent as in normal PC12 cells, suggesting that the effect of orthovanadate on NGF-induced TIS 8 expression was largely

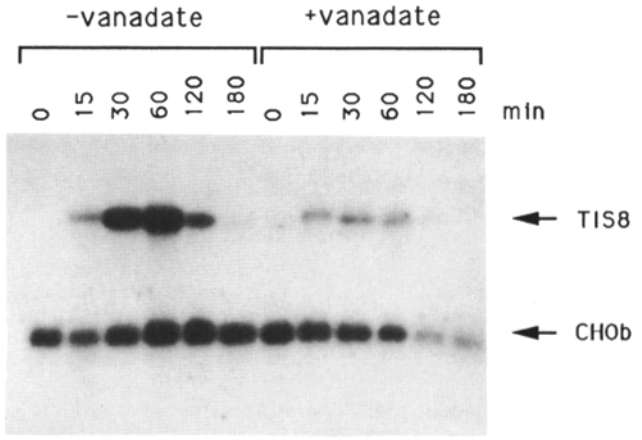

Figure 10. Time course for the induction of TIS 8 transcripts by βNGF in the presence or absence of orthovanadate. PC12 cells were grown in complete medium, pretreated with or without 20 μM orthovanadate for 18 h and stimulated with βNGF (100 ng/ml) for the indicated time. Other conditions as in Fig. 9.

independent of PKC-mediated mechanisms. As also shown in Fig. 11, PMA induced down-regulation of PKC has little or no effect on EGF induction of TIS 8; the decreased response produced by orthovanadate is similarly unaffected.

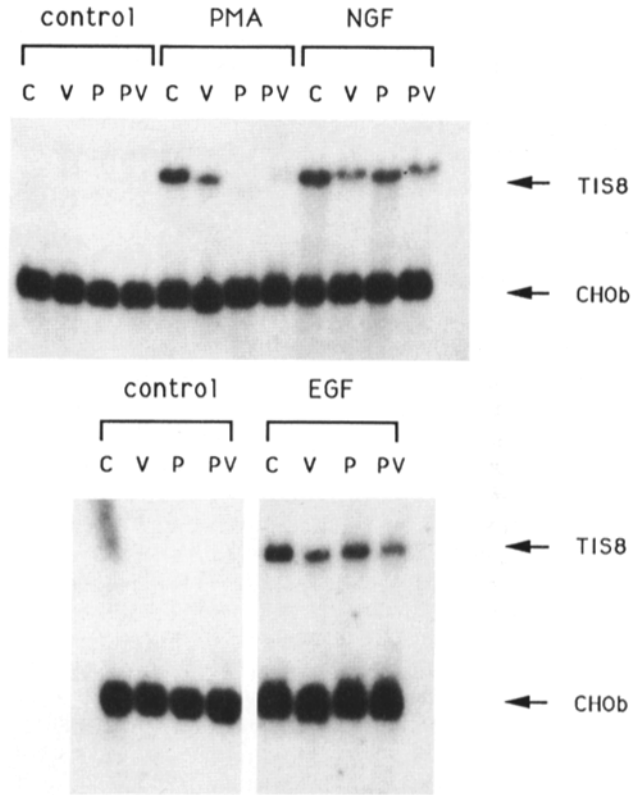

Figure 11. Effect of orthovanadate on the induction of TIS 8 in PKC-down-regulated PC12 cells. PC12 cells were grown in complete medium (*C*) and pretreated with 20 μM orthovanadate (*V*), or 1 μM PMA (*P*), or 1 μM PMA and 20 μM orthovanadate (*PV*) for 24 h. Cells were then stimulated with PMA (50 nM), βNGF (100 ng/ml), or EGF (10 ng/ml) for 30 min under the same conditions as described in Fig. 9.

Effect of Orthovanadate on the Induction of SCG 10

Growth factors also regulate the expression of several secondary response genes (also called middle-response genes or late-response genes) such as GAP-43 (Federoff et al., 1988), transin (Machida et al., 1989, 1991), neurofilament protein (Lindenbaum et al., 1988) and SCG 10 (Stein et al., 1988*a*; *b*). The induction of this class of genes is transcription and protein synthesis dependent and their expression follows the same time course as the induction of neurite outgrowth. Thus, their gene products are considered to be associated with the neuronal phenotype. However, as with the immediate early response genes, the intracellular mechanisms responsible for their induction are mostly unknown. SCG 10, a neural-specific gene that encodes a membrane-bound protein, accumulates in the growth cones of developing neurons (Stein et al., 1988*b*) and its induction in PC12 cells is stimulated by βNGF, bFGF, EGF, and PMA (Fig. 9 *c*). (There are two transcripts at 1.1 and 2.2 kb detected that are somewhat variable in their response; the smaller one was generally stimulated to a lesser degree and was less affected by orthovanadate). However, treatment for one day increased SCG 10 mRNA levels in PC12 cells only at low cell density (<30% confluency); at higher cell density, the induction of SCG 10 by βNGF or bFGF was inhibited, although the response of neurite outgrowth to these factors (βNGF and bFGF) was not impaired.

Addition of orthovanadate (20 μM for 24 h) did not significantly affect the basal expression of SCG 10 (larger transcript). However, orthovanadate at the same concentration did reduce the induction of SCG 10 expression by βNGF, EGF or PMA, but not by bFGF (Fig. 9 *c*). Although the induction of SCG 10 may be required for morphological differentiation in PC12 cells, clearly its expression alone does not produce the differentiated state.

Discussion

The induction of biological responses by many growth factors and hormones appears to involve the initial stimulation of a receptor tyrosine kinase which leads to its autophosphorylation and to the phosphorylation of a number of protein substrates (Cantley et al., 1991; Friesel et al., 1989; Hempstead et al., 1991; Kaplan et al., 1991*a*; *b*; Klein et al., 1991). These modifications, in turn, activate a number of intracellular processes, including an array of additional kinases, that ultimately lead to modulations in gene expression and characterize the phenotypic response to the stimulus. It is not entirely clear whether, in any of these cases, signals other than those emanating from the receptor kinase are induced. In the case of βNGF, which interacts with two membrane-bound receptors in PC12 cells (as well as other responsive cells) (Bothwell, 1991), this is a distinct possibility because one of the receptors, designated p75, does not contain a tyrosine kinase (Johnson et al., 1986; Radeke et al., 1987). However, it is presently unknown (and still controversial) what the role of this entity is, if any, in βNGF signal transduction. Nonetheless, substantial evidence supports the view that receptor-mediated tyrosine phosphorylation is the principal initiator of most growth factor actions. It follows that compounds known to affect these activities would be expected to also affect individual steps in the signalling pathway and probably the overall response.

Sodium orthovanadate, which inhibits PTPases and thereby the cleavage of the phosphotyrosine residues produced by the activation of the receptor tyrosine kinases, is potentially such a compound (Nechay et al., 1986). By analogy to the phosphodiesterases that control the levels of cAMP produced by adenylate cyclase-linked receptors (Robison et al., 1971), orthovanadate might be expected to affect, in a generally positive manner, growth factor responses and indeed, orthovanadate stimulates DNA synthesis in quiescent human fibroblasts (Carpenter, 1981; Smith, 1983), old human lymphocytes (Marini et al., 1987), subpopulations of thymus cells (Ramanadham and Kern, 1983), bone cells (Lau et al., 1988), and it potentiates the estrogen stimulation of proliferation of a mouse leydig cell line (Sato et al., 1987). Orthovanadate also will replace interleukin-3 as a growth factor in a mouse cell line (Tojo et al., 1987). In addition, it inhibits terminal differentiation of murine Friend erythroleukemia cells (English et al., 1983) and it mimics the effect of aFGF inhibiting some differentiative processes in BC_3H1 cells. However, it does not at concentrations of 10 μM for 2 d, induce neurotrophic responses in PC12 cells despite the fact that it greatly increases intracellular protein tyrosine phosphorylations (Maher, 1989).

As described in this report, orthovanadate is actually inhibitory of a number of processes induced by three growth factors, βNGF, bFGF, and EGF, all of which bind to and activate tyrosine kinase containing receptors in PC12 cells. The growth factor induced expression of several genes ranging from putative transcriptional regulators (TIS 8 and c-*fos*) to proteins involved in specialized functions (AChE, ODC and SCG 10) were variously affected by the inhibitors. In some cases the stimulation was essentially obliterated (c-*fos* and AChE) while in others it was only partially affected (TIS 8, SCG 10, and ODC). There was some variation also observed between factors, e.g., orthovanadate had little effect on the bFGF induction of SCG 10, whereas both the βNGF and EGF effects were substantially lowered. In contrast, the EGF stimulation of ODC was completely blocked while those induced by βNGF and bFGF were only partially inhibited. These differences are not likely to be due to kinetic alterations in the response as judged by the induction of TIS 8 by βNGF (Fig. 10). These observations underscore previous findings (Altin et al., 1991) that the tyrosine phosphorylations arising from individual growth factor receptors must be sufficiently unique to account for the variations seen in the gene responses.

It is important to note that the products of these genes are not necessarily required for neurite outgrowth. AChE induction by βNGF and bFGF is transcription-dependent (Greene and Rukenstein, 1981; Rydel and Greene, 1987) and results in a more than twofold increase in the activity with the appearance of the 16S form. However, AChE activity is not induced by EGF or dibutyryl cAMP, even though the latter can induce neurite proliferation (see below). Similarly, ODC transcription is rapidly increased by βNGF and bFGF; however, increased ODC activity does not appear to be essential for neurite proliferation. At the same time, SCG 10, a protein associated with neurite proliferation, is induced by both EGF and PMA, neither of which can induce neurites. Clearly neither the induction of these genes nor the inhibition of their expression by orthovanadate is selectively associated with the morphological responses (or the inhibition thereof).

It was also observed that the effects of PMA, a phorbol derivative that is considered to function through the activation of PKC, were tempered by orthovanadate. The induction of SCG 10 was substantially reduced and the two putative transcriptional activators, TIS 8 and *c-fos*, were even more drastically lowered. However, the effect of orthovanadate on the induction of TIS 8 expression by βNGF or EGF in cells that had been PKC down-regulated (by previous exposure to PMA) was not significantly different than in untreated cells arguing that the effect of orthovanadate is not directly on PKC. It has been reported that in addition to PKC-dependent serine/threonine-phosphorylation, PMA stimulates protein tyrosine phosphorylation of a 42-kD protein in chicken embryo fibroblasts (Vila and Weber, 1988) and in Swiss 3T3 cells (Kazlauskas and Cooper, 1988). More recently, a MAP kinase was identified in PC12 cells. The MAP kinase kinases are activated by NGF and are dependent on serine/threonine phosphorylation for activity. They can promote phosphorylation of serine/threonine and tyrosine residues on MAP kinases (Gomez and Cohen, 1991). In addition, membrane PTPase activity may be regulated by PKC (Brautigan and Pinalult, 1991). These observations are consistent with the view that PMA effects are, at least in part, also induced through tyrosine phosphorylations probably involving soluble kinases as opposed to membrane-bound enzymes. These findings also support previous reports (Damon et al., 1990; Sigmund et al., 1990; Altin et al., 1991) that induction of immediate early response genes by βNGF and bFGF requires a signal transduction pathway other than PKC. This alternative pathway also is likely to involve tyrosine kinases and therefore may be susceptible to orthovanadate effects.

The effect of orthovanadate on the inhibition of neurite proliferation was reversible and independent of the state of differentiation, i.e., inhibited cells would produce neurites when orthovanadate was removed (and the cells stimulated by the appropriate growth factor) and preformed neurites were retracted when orthovanadate was added even in the presence of growth factors. Orthovanadate also inhibited the production of neurites from primed cells (previously exposed to βNGF), a transcriptional-independent event. These findings clearly support the conclusion that orthovanadate interferes with the signal transduction cascade as its effect is equivalent to removing the growth factor itself. It is, of course, well established that the differentiative actions of βNGF and bFGF on PC12 cells are entirely reversible and that the maintenance of neurites requires the continued presence of the factors (Greene and Tischler, 1982). However, it should be noted that the results presented here do not definitively establish that the effect of orthovanadate on differentiation is via the inhibition of protein tyrosine phosphatases although the parallel effects of zinc and molybdate and the excellent correlation between inhibitions of neurite proliferation and phosphatase activity support this view.

If the effects of orthovanadate on differentiation arise from phosphatase inhibition, it is possible that multiple enzymes are involved. However, several of the observations suggest that the effect is more likely related to the action of downstream kinases (nonreceptor tyrosine kinases) than those associated with the receptors themselves. In general, ligand-induced tyrosine phosphorylation is transient and is rapidly superseded by serine/threonine phosphorylations in PC12 cells (H. Hondermarck, S. Raffioni, Y. Y. Wu and R. A. Bradshaw, unpublished observations). The lack of effect of orthovanadate on DG production suggests that it does not result in the significant inhibition of phospholipase C_γ that initiates the cleavage phosphoinositide. This enzyme is known to be phosphorylated by the βNGF receptor in PC12 cells, an event that presumably leads to its activation (Goldschmidt-Clermont et al., 1991; Kim et al., 1991; Vetter et al., 1991; Rhee, 1991; Obermeier et al., 1993). However, most DG induced by βNGF and bFGF in PC12 cells arises from the cleavage of phosphatidylcholine (Pessin et al., 1991) and the pathway and locations are not yet defined. It may arise from the stimulation of PKC by the DG formed from PI or it may involve enzymes directly stimulated by the receptor kinase (as with phospholipase C_γ). Thus, the dependence on tyrosine phosphorylation may be direct or indirect; in either case, it is not apparently affected by orthovanadate supporting the conclusion that the inhibitions observed are not due to the perpetration of receptor-mediated phosphorylations.

Further support for this view is provided by the observations that the neurite induction by 8-Br-cAMP is inhibited by orthovanadate and the production of DG is not. The induction of neurite outgrowth by βNGF or bFGF is not mediated by a cAMP-dependent pathway (Frazier et al., 1973; Chijiwa et al., 1990; Damon et al., 1990; Glowacka and Wagner, 1990; Ginty et al., 1991) nor is there any direct connection of cAMP with any tyrosine kinase containing receptor. Rather cAMP (or appropriate analogs) stimulates intracellular kinases that likely in turn induce one or more tyrosine kinases (probably in part overlapping those induced by βNGF and bFGF) that leads to the observed, albeit modest, neurite proliferation. Of course, it is also possible that the effect of the orthovanadate on the cAMP response is entirely unrelated to phosphatase inhibition. Because cAMP (and its analogs) do not induce as robust a response as βNGF and bFGF, the kinase activations are probably not entirely overlapping. However, they may involve the same orthovanadate sensitive step(s).

The conclusion that orthovanadate may regulate the tyrosine phosphorylation of proteins stimulated by βNGF or bFGF, downstream from their receptors, is also consistent with both our phosphotyrosine immunoblot data and other observations. It has been reported that exposure of PC12 cells to βNGF or bFGF rapidly stimulated threonine and tyrosine phosphorylation of MAP kinases followed by an increase in their activities (Anderson et al., 1990; Tsao et al., 1990; Boulton et al., 1991; Miyasaka et al., 1991; Schanen-King et al., 1991; Seger et al., 1991; Tsao and Greene, 1991). The MAP kinase activities can be completely inactivated by treatment with either phosphatase 2A, a protein phosphatase specific for phosphoserine and phosphothreonine, or CD45, a PTPase, and the inactivation is preventable by okadaic acid or orthovanadate, respectively (Anderson et al., 1990; Gomez and Cohen, 1991). Moreover, it was reported by Tsao and Greene (1991) that orthovanadate treatment (0.5 to 1.0 mM) resulted in the activation MAP kinase activity in vivo. NGF down-regulated MAP kinase activity can be restimulated by orthovanadate and okadaic acid. Tyrosine and threonine phosphorylation of MAP kinase have been implicated in the regulation of cell growth and differentiation in PC12 cells (Boulton et al., 1991). In addition, $pp60^{src}$ has also been implicated in the regulation of cell differentiation in PC12 cells (Alema, 1985; Rausch et al., 1989; Kremer et al., 1991) and v-*src* constructs will rescue NGF nonresponsive PC12 cell mutants (Eveleth et al., 1989). It has

been suggested in a recent report by Zheng et al. (1992) that overexpression of a protein tyrosine phosphatase activates pp60^{src} and leads to transformation of rat embryo fibroblast. In PC12 cells, it is possible that orthovanadate treatment increases the phosphorylation of the carboxy-terminal negative regulatory (Tyr-527) site of pp60^{src} and decreases the pp60^{src} activities (Cooper et al., 1986; Kmiecik and Shalloway 1987; Piwnica-Worms et al., 1987; Cartwright et al., 1987), which is required for the actions of βNGF and bFGF (Kremer et al., 1991). Finally, regulation of the cell cycle specific proteins and kinases by growth factors may be partly responsible for the stimulation of cell growth and differentiation (Matsushime et al., 1991). Orthovanadate treatment might have affected the activity of a specific tyrosine phosphatase, such as CDC25, which directly activates p34^{CDC2} (Strausfeld et al., 1991; Dunphy and Kumagai, 1991; Gautier et al., 1991).

Although our observations do not specifically identify the step(s) that is sensitive to PTPase inhibition, they strongly support the view that they affect the substrates of non-receptor kinases and that these enzymes are essential in growth factor induced responses. They further suggest that agents such as PMA and 8-Br-cAMP may also be able to activate these entities, at least to some extent, and that these stimulations are essential for their various responses as well.

We thank Drs. Dave Anderson, Cal Tech, and Harvey Herschman, University of California at Los Angeles, for generously providing probes and Dr. Michael Fox, AMGEN (Thousand Oaks, CA), for the FGF derivative used in this work. We gratefully acknowledge the assistance of Mrs. Gale Trudeau in the preparation of this manuscript.

This work was supported by research grants from the U.S. Public Health Services (AG-09735-10) and the American Cancer Society (BE-41L).

Received for publication 20 March 1992 and in revised form 11 January 1993.

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