Molecular Biology of the Cell
Vol. 3, 323–333, March 1992

Testing the In Vivo Role of Protein Kinase C and c-Fos in Neurite Outgrowth by Microinjection of Antibodies into PC12 Cells

Joseph G. Altin,*† Richard Wetts,‡§ Karl T. Riabowol,‖ and Ralph A. Bradshaw*

*Department of Biological Chemistry, College of Medicine and ‡Department of Physiology and Biophysics, and the Developmental Biology Center, University of California, Irvine, California 92717; and ‖Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Submitted to Cell Regulation August 28, 1991; Accepted December 2, 1991

To define the molecular bases of growth factor-induced signal transduction pathways, antibodies known to block the activity of either protein kinase C (PKC) or the *fos* protein were introduced into PC12 cells by microinjection. The antibody against PKC significantly inhibited neurite outgrowth when scored 24 h after microinjection and exposure to nerve growth factor (NGF). Microinjection of antibodies to *fos* significantly increased the percentage of neurite-bearing cells after exposure to either NGF or basic fibroblast growth factor (bFGF) but inhibited the stimulation of DNA synthesis by serum, suggesting that in PC12 cells, *fos* is involved in cellular proliferation. Thus, activation of PKC is involved in the induction of neurite outgrowth by NGF, but expression of the *fos* protein, which is induced by both NGF and bFGF, is not necessary and inhibits neurite outgrowth.

INTRODUCTION

Despite considerable knowledge about several polypeptide growth factors and their receptors, the molecular signaling mechanism(s) involved in differentiation and proliferation remains to be clearly delineated. The rat pheochromocytoma cell line PC12 has been used widely to study neuronal differentiation (Greene and Tischler, 1982) and is a useful system in which to distinguish those intracellular signals that may be specific for inducing differentiation on the one hand from those that induce proliferation on the other. PC12 cells proliferate as round chromaffin-like cells when grown in standard culture conditions, but on addition of nerve growth factor (NGF) or basic fibroblast growth factor (bFGF), the cells stop dividing, extend numerous processes, and display characteristics of fully differentiated sympathetic neurons (Green and Tischler, 1982; Togari *et al.*, 1985; Rydel and Green, 1987; Schubert *et al.*, 1987). Neuronal differentiation of PC12 cells can be induced by the *ras* or *src* transforming proteins (Alema *et al.*, 1985; Bar-Sagi and Feramisco, 1985; Satoh *et al.*, 1987; Eveleth *et al.*, 1989; Rausch *et al.*, 1989). Microinjection of antibodies that block the function of either the *ras* or the *src* proteins inhibits the induction of neurite outgrowth by NGF or by bFGF in both fused (Hagag *et al.*, 1986) and native PC12 cells (Altin *et al.*, 1991b), suggesting that these proteins are essential components of signal transduction pathways leading to differentiation in response to NGF and bFGF in PC12 cells.

In certain cell types, some actions of the *ras* protein may involve protein kinase C (PKC) (Nishizuka, 1986; Lacal *et al.*, 1987; Huang *et al.*, 1988; Lacal, 1990). Consistent with this view, both NGF and bFGF increase the production of 1,2-diacylglycerol, a physiological activator of protein PKC, in PC12 cells (Altin and Bradshaw, 1990) and NGF increases PKC activity (Cremins *et al.*, 1986; Hama *et al.*, 1986; Heasley and Johnson, 1989). Evidence suggesting that activation of PKC is necessary for induction of neurite outgrowth by NGF comes from the findings that sphingosine, an inhibitor of PKC, inhibits neurite outgrowth in PC12 cells (Hall *et al.*, 1988). In addition, tetradecanoyl phorbol acetate (TPA, an activator of PKC) induces neurite outgrowth in chick sensory ganglia explants (Hsu *et al.*, 1989) and potentiates

† Present address: Division of Cell Biology, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. 2601, Australia.
§ Present address: Division of Biology, Beckmann Institute, 139-74 California Institute of Technology, Pasadena, CA 91125.

the action of NGF in PC12 cells (Burstein *et al.*, 1982). However, some reports indicate that treatment of PC12 cells with TPA alone does not induce neurite outgrowth (Burstein *et al.*, 1982; but see Glowacka and Wagner, 1990) and that down-regulation of PKC by prolonged treatment with TPA does not block neurite outgrowth by NGF (Reinhold and Neet, 1989). These latter observations suggest that activation of PKC may be neither necessary nor sufficient for induction of this response.

Another potential signal for differentiation of PC12 cells is the oncogene c-*fos*, which is rapidly and transiently induced in these cells by both NGF and bFGF (Curran and Morgan, 1985; Milbrandt, 1986; Sheng *et al.*, 1988) and belongs to a class of genes referred to as immediate early genes. These genes are induced rapidly in many cell types by a number of mitogenic and differentiative stimuli in the presence of inhibitors of protein synthesis (for a recent review, see Sheng and Greenberg, 1990). The c-*fos* gene encodes a highly phosphorylated protein of 380 amino acids that localizes in the nucleus where it interacts with members of the *jun* family of proteins. The *fos* and *jun* protein families make up a major part of the activator protein-1 (AP-1) transcriptional complex that plays a role in regulating the transcription of genes possessing AP-1 promoters (Chiu *et al.*, 1988; Sheng and Greenberg, 1990). Increased AP-1 DNA-binding activity has been found in nuclear extracts of *ras*-infected PC12 cells, suggesting a possible involvement of *fos* and *jun* in the action of *ras* (Sassone-Corsi *et al.*, 1989; Wu *et al.*, 1989). That *fos* is an effector downstream of *ras* is supported also by studies in other cell types, NIH 3T3 and rat 208F fibroblast cells, which show that microinjection of the transforming *ras* protein causes a rapid induction of c-*fos* (Stacey *et al.*, 1987; Riabowol *et al.*, 1988a) and that the expression of antisense-c-*fos* mRNA reverses the transforming effects of the *ras* oncogene (Ledwith *et al.*, 1990). Thus, induction of c-*fos* may constitute an essential part of the signal for differentiation by NGF and bFGF in PC12 cells. Conversely, the transfection of a mouse N-*ras* gene into a subline of PC12 cells was seen to induce neurite outgrowth without induction of the c-*fos* gene (Guerrero *et al.*, 1986, 1988), and overexpression of the c-*fos* gene in PC12 cells was reported to inhibit neurite induction by NGF (Ito *et al.*, 1989).

Recently, affinity-purified antibodies against the *fos* protein have been developed that inhibit the ability of serum to stimulate DNA synthesis in rat fibroblasts (Riabowol *et al.*, 1988b). To determine whether the *fos* protein plays a role in the neurotrophic action of NGF and bFGF, we have scored the ability of PC12 cells to respond morphologically to NGF and bFGF in the absence and presence of microinjected anti-*fos* antibodies. In addition, we have studied the effects of microinjecting a monoclonal antibody to PKC (monoclonal antibody PKC 1.9), which is reported to inhibit the activity of all PKC isozymes (Mochly-Rosen and Koshland, 1987, 1988). Our results indicate that microinjection of the PKC antibody inhibits neurite induction by NGF but that *fos* antibodies significantly potentiate the induction of neurite outgrowth in response to either NGF or bFGF.

MATERIALS AND METHODS

Materials

Monoclonal antibody PKC 1.9 was obtained from GIBCO-BRL (Grand Island, NY). The affinity-purified antibodies directed against the *fos* protein used in the present experiments were identical to those described previously (Riabowol *et al.*, 1988b). NGF was prepared as β-NGF by the method of Mobley *et al.* (1976). A bFGF analogue in which all half-cystine residues were replaced by serines (Fox *et al.*, 1988) was used in all experiments with bFGF. This analogue was kindly provided by Dr. Gary M. Fox, Amgen Inc. (Thousand Oaks, CA) and previously was found to be indistinguishable from preparations of the natural protein in stimulating neurite outgrowth and phospholipid metabolism in PC12 cells (Altin and Bradshaw, 1990).

Cell Culture and Microinjection

PC12 cells were adapted to grow in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 5% heat-inactivated horse serum, and 1% Fungi-Bact solution as described previously (Altin *et al.*, 1991b). PC12 cells were seeded in collagen-coated (Greene and Tischler, 1982) six-well plates (Linbro Flow Laboratories, McLean, VA) at a density of 4×10 cells/cm^2 in low serum media (DMEM supplemented with 2% fetal calf serum and 1% heat-inactivated horse serum) 48 h before microinjection. To buffer the medium while the cells were out of the incubator, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (20 mM, pH 7.4) was added to the medium after removal from the incubator. Immediately before microinjection, the medium was replaced with warmed Dulbecco's phosphate-buffered saline (pH 7.4), which also contained 10 mM glucose. After microinjection, the cells were kept in this solution for 1–2 h at 37°C before replacing with low serum media (as above) in the presence of either NGF (100 ng/ml) or bFGF (5 ng/ml).

Individual cells were microinjected with either a control solution or an antibody solution with the use of techniques similar to those used for microinjection of the anti-p21*ras* antibody (Altin *et al.*, 1991b). Each culture well contained both control-injected cells and antibody-injected cells; this controlled effectively for any variability in the amount of induction by the growth factor. The control solution was composed of 60 mM KCl, 10 mM NaCl, 0.1 mM EDTA, and 100 mg/ml rhodamine-conjugated dextran (70 kDa, #D-1818, Molecular Probes, Eugene, OR); the antibody solution was control solution plus either PKC 1.9 antibody (5 mg/ml) or affinity-purified antibodies to *fos* (8 mg/ml). In previous microinjection studies, it was shown that the use of this control solution gave results that were indistinguishable from those in which the control solution also contained 8 mg/ml of bovine serum albumin or an equivalent amount of antibody that was inactivated by freeze-thawing (Altin *et al.*, 1991b). Most cells received injections of ~10% of the cell volume, but due to the small volume of the PC12 cells and the consequent difficulty of their microinjection, the amount of solution expelled was variable. The total number of rhodamine-labeled cells was counted after microinjection of a group of cells, so that any cells (healthy or not) that detached immediately were not included in the count. At ~24 h postinjection, the rhodamine-labeled cells were examined and scored as described previously (Altin *et al.*, 1991b). This was the optimal time for scoring the cells because the levels of the injected antibodies declined after 24 h (see Altin *et al.*, 1991b) and because the intensity of the rhodamine fluorescence was notably decreased at 48 h compared with 24 h (Altin and Wetts, unpublished observations).

Table 1. The percentage of PC12 cells that were scored into one of four different categories 24 h after microinjection of either control solution or a solution containing the PKC 1.9 antibody

Condition	Cells (%)				Total cells injected	Number of experiments
	Neurites	Round	Abnormal	Missing		
NGF (control)	38	31	27	4	277	6
PKC-1.9 antibody[a]	27[b]	29	33[c]	11[b]	263	10

The four-by-two chi-square test for equality of two multinomials was used to compare all four categories simultaneously. There was a significant difference between the PKC-1.9 antibody-injected cells and control-injected cells treated with NGF ([a]$p < 0.05$). On finding a difference with the chi-square test, the unequal pairs were identified by the Irwin-Fisher test, using the normal approximation to the hypergeometric distribution ([b]$p < 0.01$; [c]$p < 0.10$).

Statistical Analyses

Groups of 10–50 cells were microinjected with the same micropipette, and 4–10 groups were injected for each experimental condition (Tables 1 and 2). After 24 h, each injected cell was assigned to one of four categories (as described in RESULTS). To determine whether the differences between antibody- and control-injected cells were significant, statistical analyses, recommended by Dr. Howard Tucker (Department of Mathematics, University of California, Irvine), were performed. The number of cells in each category was summed across the groups, thereby providing equal importance to each injected cell regardless of the number of cells in a group. A four-by-two chi-square test was used first to detect any difference between antibody- and control-injected cells. This test simultaneously compares all four categories and indicates if there is any difference between the antibody- and control-injected cells. If there was an overall difference, the Irwin-Fisher test was used then to independently compare each of the four categories and to determine which one(s) contributed to the difference.

Immunocytochemistry Assay for DNA Synthesis

To identify the cells undergoing DNA synthesis after stimulation with serum, the cells were incubated in DMEM containing 12% fetal calf serum, 6% horse serum, and 1% Fungi-Bact solution in the presence of 10 μM bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO) during the 24 h after microinjection. The cells were rinsed with phosphate buffer (0.1 M, pH 7.4), fixed with 70% ethanol (30 min at room temperature), air-dried (5–10 min), and treated with 2 N HCl plus 0.5% Triton X-100 to permeabilize the cells and to denature the DNA (30 min). After washing with phosphate buffer, the cells were incubated with a solution of 20% goat serum plus 0.2% Triton X-100 in phosphate buffer, followed by incubation with anti-BrdU antibody (Becton Dickinson, San Jose, CA) diluted 1:20 in the same solution, rinsed three times in the same solution, and then incubated in fluorescein-conjugated goat anti-mouse IgG antibody diluted 1:100 in the same solution (30 min per incubation at room temperature). After three washes with phosphate buffer, rhodamine and fluorescein fluorescence was visualized to detect the presence of the dextran and the BrdU, respectively.

RESULTS

Microinjection of Antibodies into PC12 Cells

Microinjections were performed on PC12 cells that had been plated in low serum medium without growth factors for 48 h. Few of these cells had prominent neurites (<1%) and about one-half were spherical and poorly attached to the substrate with the remainder slightly

Table 2. The percentage of PC12 cells that were scored into one of four different categories 24 h after microinjection of either control solution or a solution containing Fos antibodies

Condition	Cells (%)				Total cells injected	Number of experiments
	Neurites	Round	Abnormal	Missing		
NGF (control)	32	29	26	13	195	6
Fos antibody[a]	39[b]	17[c]	23	20[c]	155	5
bFGF (control)	8	27	46	19	107	5
Fos antibody[a]	21[d]	18[c]	31[c]	30[c]	118	5
No GF (control)	4	52	31	13	126	5
Fos antibody[a]	2	38[d]	40[b]	20[c]	181	7
Hi serum (control)	0	47	12	41	115	4
Fos antibody	0	44	23	33	139	5

The four-by-two chi-square test for equality of two multinomials was used to compare all four categories simultaneously. There was a significant difference between the Fos antibody-injected cells and control-injected cells for the NGF-, bFGF-, and no growth factor (No GF)-treated cells ([a]$p < 0.05$). On finding a difference with the chi-square test, the unequal pairs were identified by the Irwin-Fisher test, using the normal approximation to the hypergeometric distribution ([b]$p < 0.10$; [c]$p < 0.05$; [d]$p < 0.01$). When the medium containing BrdU and high levels of serum was present after microinjection ("Hi serum" condition), there were no significant differences between control- and antibody-injected cells.

flattened and well attached. The latter cells were microinjected with either a control solution or an antibody solution, both of which contained equal concentrations of rhodamine dextran to permit identification of the injected cells (Wetts *et al.*, 1989; Altin *et al.*, 1991b). Rhodamine-labeled cells, examined 24 h after microinjection, were scored blind regarding the injected solution (either control or antibody-containing). Each clearly labeled cell was classified into one of three groups. Cells were scored as having *neurites* if they had processes of

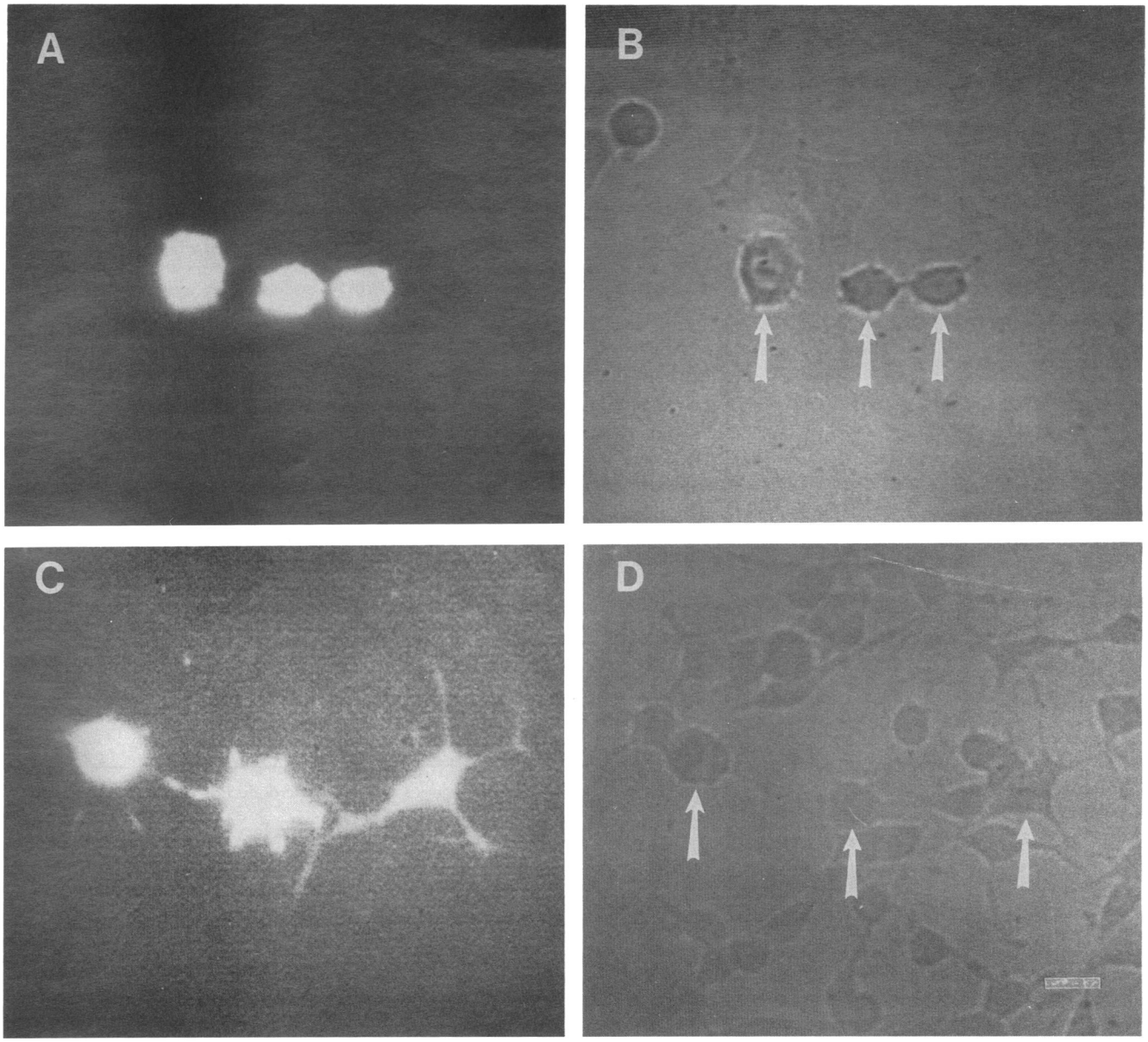


Figure 1. Effect of Fos antibodies on neurite induction by NGF. PC12 cells were plated for 48 h and then microinjected with either a control solution (A and B) or a solution containing antibodies to Fos (C and D). After exposure of the cells to NGF for 24 h, the microinjected cells were identified with rhodamine fluorescence (A and C). Bright fluorescence causes "blooming" in the image from the SIT video-camera, so the fluorescence images of these cells appear larger than their actual size. Treatment with NGF for 24 h is a relatively short period of time, so less than half of the uninjected cells had begun to extend neurites (45%). Of the cells injected with the control solution, a similar percentage responded to the NGF treatment (53%, from Table 1). This particular field (B) shows some of the cells that had not yet extended neurites (arrows); they appear indistinguishable from uninjected cells. A greater proportion of cells injected with the antibody solution had neurites (arrows) in comparison with control-injected cells (D). Similar results were seen when the cells were treated with bFGF instead of NGF (not shown). Scale bar, 20 μm.

at least one cell diameter in length or had multiple neurites. *Round* cells had small or no neurites. Round and neurite cells appeared to be normal and healthy. Other cells that may have been adversely affected by the microinjection procedure were scored as *abnormal.* These cells exhibited either very faint or punctate fluorescence. Under bright-field illumination, cells scored as abnormal were smaller, darker, and more granular than the normal cells. To determine the number of *missing* cells, the total number of labeled cells in these three groups was subtracted from the number of cells injected on the previous day. The cause(s) of this cell loss is unknown; it may reflect a decrease in cell attachment and/or cell survival.

Effect of Monoclonal Antibody PKC-1.9 on Neurite Outgrowth

To determine whether activation of PKC plays a role in the induction of neurite outgrowth by NGF, we microinjected antibodies that inhibit PKC activity (monoclonal antibody PKC 1.9) into native PC12 cells. Twenty-four hours after microinjection and continuous exposure to NGF, the percentage of neurite-bearing cells after antibody treatment (27%) was significantly lower than the percentage of cells that had neurites after microinjection of the control solution (38%) (see Table 1). However, injection of the antibody did not inhibit neurite outgrowth in all cells, because this percentage (27%) is greater than the percentage of neurite-bearing cells in the absence of NGF (2–4%) (Table 2). This incomplete inhibition could be due to the variability in the amount of antibody solution injected into each cell; that is, in some cells the amount of antibody was insufficient to block 100% of the PKC activity for the full 24 h. Alternatively, it is possible that a subpopulation of microinjected cells (perhaps those that were arrested or that were residing in a similar phase of the cell cycle) was more sensitive to PKC inhibition. Yet another possibility is that neurite outgrowth is partly mediated through PKC-independent pathways. Although it is incomplete, the observed inhibition suggests that PKC plays some role in the induction of neurite outgrowth by NGF.

Effect of PKC 1.9 Antibody on Cell Number

Microinjection of the PKC 1.9 antibody resulted in a significantly greater percentage of missing cells when scored 24 h after microinjection and exposure to NGF (Table 1). Thus, 4% of the cells that were microinjected with the control solution were missing as compared with 11% of those microinjected with the PKC 1.9 antibody ($p < 0.01$). The cause(s) of this cell loss is unknown; in the control group it could be due to the trauma of the microinjection procedure or to normal events of cell culture. However, the significant increase in the number of missing cells after antibody microinjection indicates that the antibody causes additional cell loss, perhaps by augmenting the factors involved in normal cell loss. Regardless of the mechanism, the increased cell loss is a specific effect of the PKC 1.9 antibody, because the effect was not observed after microinjection of other antibody solutions (e.g., Altin *et al.*, 1991b).

Effect of the fos Antibodies on Neurite Outgrowth

To determine whether the activity of the *fos* protein is necessary for the induction of neurite outgrowth, affin-

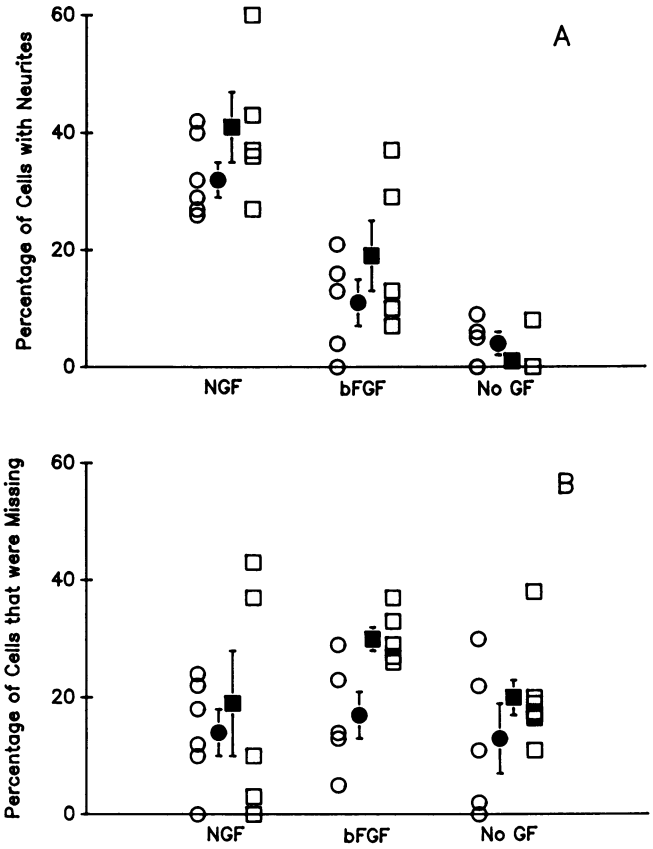
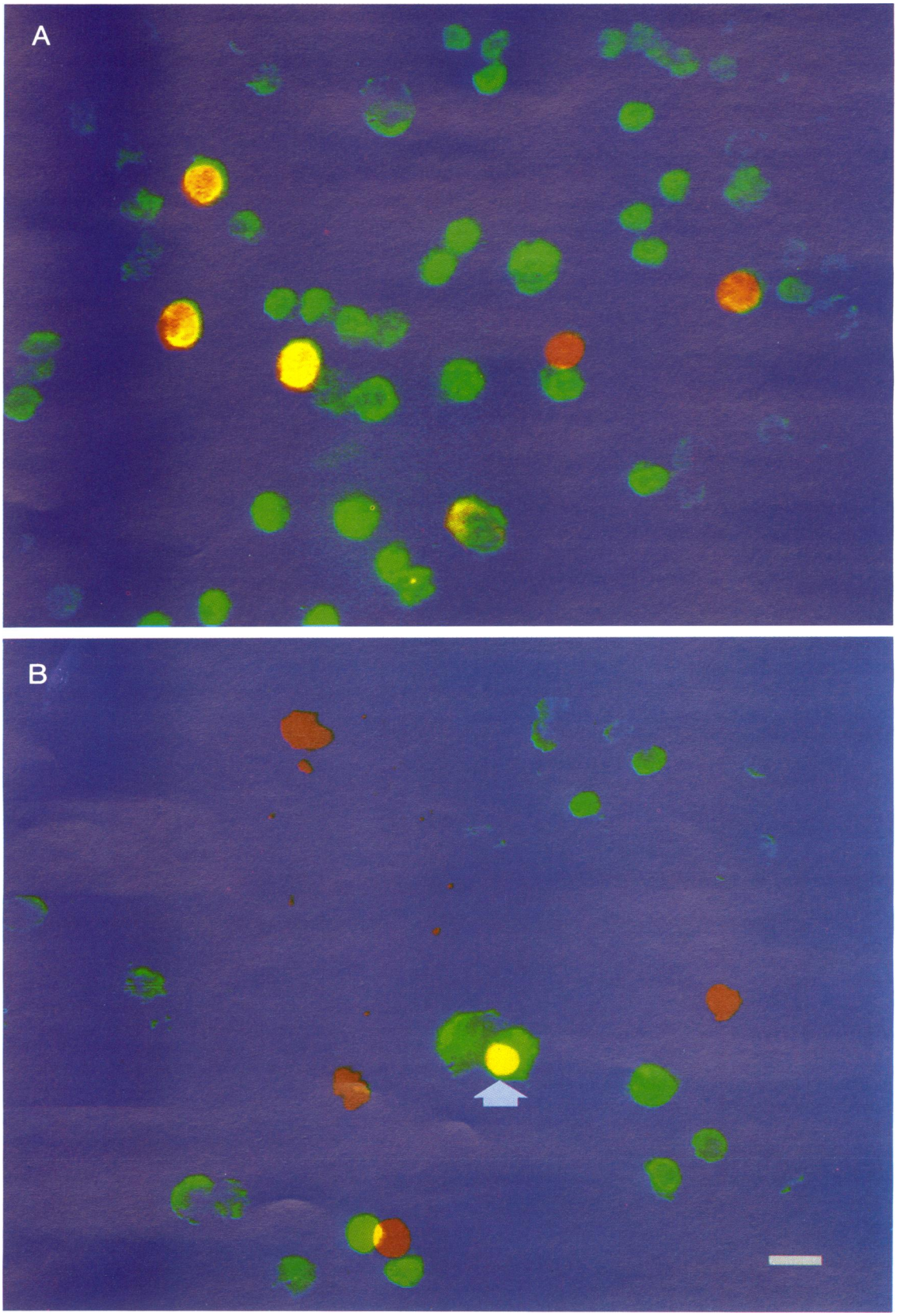


Figure 2. The percentages of microinjected PC12 cells that had neurites or that were missing 24 h after microinjection with either the control solution (circles, left) or the solution containing the Fos antibodies (squares, right). Between 10 and 50 cells were microinjected at each "site" (or experimental sitting); these microinjections were performed with the same micropipette. The mean number of cells injected per site was 26.7 ± 1.7 (SEM). Each open symbol corresponds to the percentage of microinjected cells that were scored as neurite-bearing (A) or scored as missing (B) at a particular microinjection site. The means and SEM for each set of points are shown by the open bars. The condition "No GF" corresponds to cells that had no growth factor present for the 24-h postinjection. The percentages of neurite-bearing cells (A) were different for the three conditions, indicating that NGF was more effective than bFGF (which was more effective than no GF) in inducing neurite outgrowth after 24 h. For both NGF and bFGF, microinjection of Fos antibodies resulted in an increased percentage of neurite-bearing cells in more experiments compared with control microinjections.


A
B

ity-purified antibodies directed against *fos* were microinjected into PC12 cells and the cells were scored for their ability to extend neurites in response to NGF or bFGF. In contrast to injection of the PKC-1.9 antibody, microinjection of the *fos* antibodies was associated with a significant increase in the number of cells with neurites induced by NGF (Figure 1, A and B and Figure 2A) and by bFGF (Figure 2A), relative to microinjection of the control solution (Figure 1, C and D and Figure 2A). Quantitatively, 32% of the control cells had neurites, whereas 39% of the cells microinjected with the *fos* antibodies had neurites after exposure to NGF (Table 2). Similarly, after exposure to bFGF, the percentage of cells with neurites was lower for control-injected cells (8%) than for antibody-injected cells (21%). This effect was a function of growth factor stimulation because the percentage of cells with neurites was essentially the same after control (4%) or antibody (2%) injection when growth factor was omitted during the 24 h postinjection (in addition to the 48 h preinjection) (Table 2 and Figure 2A). This suggests that the *fos* antibodies do not induce neurite outgrowth by themselves. However, in the presence of either NGF or bFGF, introduction of the antibodies appears to potentiate the action of the growth factors. These results suggest that the *fos* protein does not play a necessary role in neurite induction and in fact appears to inhibit this process.

Effect of fos Antibodies on Numbers of Round Cells and Cell Loss

In comparison with the controls, microinjection of *fos* antibodies resulted in a significant decrease in the proportion of round cells but in an increase in the percentage of missing cells 24 h after microinjection. Thus, for NGF- or bFGF-treated cells, the proportion of round cells decreased from 27 to 29% in cells microinjected with control solution to only 17–18% in cells microinjected with the antibodies (Table 2). The percentage of missing cells for NGF-treated cells increased from 13% in control-injected cells to 20% in antibody-injected cells, whereas for bFGF-treated cells the percentage increased from 19% in control-injected cells to 30% in antibody-injected cells (Table 2 and Figure 2B). In cells not treated with growth factor during the 24-h postinjection period, the percentage of round cells decreased from 52% in control-injected cells to 38% in antibody-injected cells, but the percentage of missing cells increased from 13% in control-injected cells to 20% in antibody-injected cells (Table 2 and Figure 2B). Because microinjection of the *fos* antibodies affects the proportion of round and missing cells in the absence, as well as the presence, of growth factor, the results suggest that the *fos* antibodies interfere with the basal level of *fos* activity in these cells (which were not treated with growth factor before microinjection) and that treatment with NGF or bFGF does not overcome the neutralizing effect achieved after introduction of the antibodies. This suggests that under these conditions *fos* activity (whether basal activity or induced by treatment with NGF or bFGF) plays at least some role in promoting cell attachment and/or cell survival.

Effect of fos Antibodies on DNA Synthesis

Because our studies suggest that c-*fos* expression is not required for neurite outgrowth, we tested the possibility that the *fos* protein is involved in cell growth. For these experiments, cells that had been plated for 48 h under low serum conditions (i.e., the same conditions as for the neurite outgrowth experiments) were microinjected with either the control solution or the solution containing *fos* antibodies and then were stimulated to divide with high-serum-containing medium in the presence of 10 μM BrdU for 24 h. The percentages of neurite-bearing, round, abnormal, or missing cells between control-injected and antibody-injected cells were not statistically significant (Table 2). After scoring morphology, these cells were processed for immunocytochemistry with fluorescein-labeled secondary antibodies against the BrdU-specific antibody. Fluorescein fluorescence shows the nuclei of cells that incorporated BrdU into their

Figure 3. Effect of Fos antibodies on the stimulation of DNA synthesis by serum. PC12 cells (plated for 48 h under low serum conditions) were microinjected with either a control solution (A) or a Fos antibody-containing solution (B) and then exposed to high serum medium containing the thymidine analogue BrdU. After 24 h, many of the uninjected cells were labeled by fluorescein fluorescence (green label), indicating that they had incorporated BrdU into their DNA. The microinjected cells were identified by rhodamine fluorescence (red label). Some cells microinjected with the control solution (A) had incorporated BrdU (the green and red labels together appear yellow), indicating that microinjection of cells does not inhibit passage through the cell cycle, although some cells had not begun to incorporate BrdU (red or orange label). Cells microinjected with the Fos antibodies (B) were unlabeled by fluorescein (only the red label is visible), indicating that the antibody had inhibited entry into S phase in these cells. One of the injected cells is on top of a fluorescein-labeled cell, hence it appears yellow but had not incorporated BrdU (arrow in B). A small proportion of cells injected with the antibodies were labeled by fluorescein, and this proportion is significantly smaller than the proportion of control-injected cells that were fluorescein-labeled (see Table 3). The fluorescein fluorescence of uninjected cells incubated in the presence of thymidine instead of BrdU is very low (not shown); this lack of labeling verifies the specificity of the anti-BrdU antibodies. A narrow-band emission filter was used for the fluorescein fluorescence pictures to eliminate any fluorescence from the rhodamine of the injected cells. To enhance the clarity of the video images, the original black-and-white images were pseudo-colored and combined (Vidim program courtesy of Dr. Scott Fraser, UCI). Scale bar, 20 μm.

DNA, indicating that these cells had entered into S phase during the labeling period. Most of the uninjected cells, as well as the control-injected cells (identified by rhodamine fluorescence), incorporated BrdU (Figure 3A). However, few antibody-injected cells (also rhodamine labeled) were labeled for BrdU (Figure 3B). The proportion of cells that displayed fluorescein fluorescence is significantly lower for cells microinjected with the antibody solution (30%) than for cells microinjected with the control solution (59%, Table 3), suggesting that the *fos* antibodies are effective in inhibiting the stimulation of DNA synthesis by serum in PC12 cells.

DISCUSSION

To study signal transduction pathways, we microinjected function-blocking antibodies into unfused native PC12 cells. Comparisons of antibody-injected cells with neighboring control-injected cells revealed small but significant differences in the percentage of cells bearing neurites after 24 h (Tables 1 and 2). There are several reasons why the observed differences were not larger. Some cells might not have gotten enough antibody to completely block the activity of PKC (or *fos*). The small size of native PC12 cells increased the technical difficulties of the microinjection technique, but artificially increasing the cell size by fusing together several cells might have caused changes in the normal transduction pathways. In addition, there is substantial evidence that signal transduction can involve multiple pathways, so that completely blocking one pathway may only cause a partial effect. Although we observed relatively small differences in the percentages of cells in each category, we microinjected many cells, and the statistical tests indicated that the observed differences were significant. Thus, our results provide evidence that PKC contributes to neurite outgrowth induced by NGF and that *fos* inhibits neurite outgrowth and is involved in proliferation.

The monoclonal antibody anti-PKC 1.9, raised against highly purified rat brain PKC, recognizes an epitope in the catalytic domain of the enzyme (Mochly-Rosen and Koshland, 1987). The antibody has high specificity for PKC and strongly inhibits the activity of all PKC isozymes in vitro and in vivo without affecting either the cAMP-dependent protein kinase or the calcium/calmodulin-dependent kinase (Mochly-Rosen and Koshland, 1987, 1988). Our findings that microinjection of this antibody into PC12 cells inhibits NGF-induced neurite production provides independent evidence for a role for PKC in this process. This is consistent with the observations that TPA potentiates the action of NGF in PC12 cells (Burstein *et al.*, 1982) but appears inconsistent with the reports that NGF (and bFGF) can elicit neurite outgrowth in PC12 cells in which PKC has been down-regulated by prolonged treatment with TPA (Reinhold and Neet, 1989; Damon *et al.*, 1990; Sigmund *et al.*, 1990). However, down-regulating PKC in this manner may not be a totally effective means of eliminating all PKC isozymes (Cooper *et al.*, 1989). Furthermore, this type of experiment does not exclude phosphorylation events (by activation of PKC by TPA) before the down-regulation. The partial inhibition of immediate early gene responses to NGF in such cells (Sigmund *et al.*, 1990; Altin *et al.*, 1991a) favors the view that, in PC12 cells, blocking PKC affects NGF-induced neurite outgrowth.

A major finding from the present work is that microinjection of antibodies directed against the *fos* protein into PC12 cells significantly potentiates the induction of neurite outgrowth in response to NGF and bFGF. Previous studies have shown that NGF and bFGF rapidly induce c-*fos* mRNA expression in PC12 cells and therefore suggested a role for the *fos* protein in the induction of differentiation by these growth factors (Curran and Morgan, 1985; Milbrandt, 1986). The *fos* antibodies used in the present study have been found to immunoprecipitate c-*fos* and possibly the *fos*-related antigen, *fra*-1 (Riabowol *et al.*, 1988b; Vosatka *et al.*, 1989; Riabowol, unpublished observations). Because *jun* is precipitated also under nondenaturing conditions by virtue of its tight association with *fos*, it is likely that other proteins in the AP-1 complex are inactivated by these antibodies in vivo. In vitro studies have shown that these antibodies block 90–95% of specific AP-1 gel shift activity (Riabowol, Schiff, and Gilman, unpublished data). Our results therefore suggest that in PC12 cells, *fos* and other proteins, such as *fra*-1 and *jun*, are not required for the induction of neurite outgrowth by NGF or bFGF. Instead, the results suggest that the activity of *fos* (and/or an antigenically related proteins) results in an apparent inhibition of the neurotrophic response induced by these growth factors. This result

Table 3. The percentage of dividing PC12 cells after microinjection of either control solution or Fos antibody solution.

Condition	Anti-BrdU-labeled cells (%)
Control	59
Fos antibody	30[a]
Uninjected	63

Cells were incubated with high-serum-containing medium and bromodeoxyuridine (BrdU) for 24 h before conducting immunocytochemistry to detect the incorporation of BrdU into DNA. Percentages for antibody-injected cells were significantly different from control-injected cells by chi-square test ([a]$p < 0.005$). The percentages for control-injected cells and uninjected cells were similar, indicating that the microinjection procedure does not inhibit cell division.

is consistent with recent observations that over-expression of the c-*fos* gene in PC12 cells blocks neurite outgrowth by NGF (Ito *et al.*, 1989) and that induced expression of the N-*ras* oncogene in a subline of PC12 cells can elicit neurite outgrowth in the absence of c-*fos* induction (Guerrero *et al.*, 1988).

The role of *fos* in the action of NGF and bFGF is still unclear. NGF-induced neurite induction in "unprimed" PC12 cells (unlike neurite regeneration in "primed" cells) is thought to be a transcription-dependent event (Burstein and Greene, 1978; but see Nichols *et al.*, 1989). It is not yet clear whether protein synthesis is required. There is evidence that a transcription-dependent increase in the rates of synthesis of several cytoplasmic and nuclear proteins occurs within the first few hours of NGF treatment (Tiercy and Shooter, 1986), and it has been suggested that ongoing translation is needed for the neurite response (Greene and Shooter, 1980). Increased transcription of c-*fos* mRNA and expression of *fos* protein by NGF and bFGF in PC12 cells are transient responses, with both returning to near-basal levels after 4 h (Kruijer *et al.*, 1985). c-*fos* induction may still play a role in mediating responses other than initial neurite outgrowth, as suggested by our observation that the *fos* antibodies inhibit cell attachment and/or cell survival and also by the recent report that c-*fos* is required for the induction of tyrosine hydroxylase gene expression by NGF (Gizang-Ginsberg and Ziff, 1990).

Of related significance is our observation that the microinjection of the *fos* antibodies into PC12 cells significantly inhibited the stimulation of DNA synthesis by serum (Table 3). These results are consistent with similar findings in rat fibroblasts using these same antibodies and support the proposed role of c-*fos* in traversing the G1 phase of the cell cycle and entry into DNA synthesis (Riabowol *et al.*, 1988b). This suggests that in PC12 cells *fos* is not required for induction of neurite outgrowth but instead is involved in a pathway in which the major effect culminates in cellular proliferation. Indeed, one initial effect of NGF and bFGF on PC12 cells is to stimulate a cycle of cell division (Greene and Tischler, 1982), and it is possible that the rapid induction of c-*fos* expression by these growth factors constitutes a step in that division. Recent work suggests that expression of the N-*ras* gene in a subline of PC12 cells inhibits the induction of c-*fos* by NGF and bFGF (Thomson *et al.*, 1990). Because the pathway of neurite induction by NGF and by bFGF is thought to involve *ras* (Hagag *et al.*, 1986; Altin *et al.*, 1991b), an action of these growth factors may be to block or suppress c-*fos* expression at some time after its initial induction. This action, as well as the elimination of any constitutively expressed c-*fos* by autoregulatory mechanisms, may be necessary for priming in these cells, which occurs in the early phase of the NGF and bFGF response before the stimulation of any neurite outgrowth (Greene and Tischler, 1982; Rydel and Greene, 1987).

The mechanisms by which NGF, bFGF, and other extracellular stimuli elicit increased transcription of c-*fos* or other immediate early response genes are not well understood but clearly involve both PKC-dependent and -independent pathways (Cho *et al.*, 1989; Sigmund *et al.*, 1990; Damon *et al.*, 1990; Altin *et al.*, 1991a: Graham and Gilman, 1991). Although tyrosine phosphorylation may be involved in these pathways, some early response genes can be induced by cAMP and by agents that elevate intracellular Ca^{2+} (Milbrandt, 1986; Morgan and Curran, 1986; Sheng *et al.*, 1990). Furthermore, neither the activation of PKC nor the induction of c-*fos* transcription are specific for differentiation in PC12 cells because they are induced by agents, such as epidermal growth factor, that do not lead to neurite outgrowth. The results presented here support the view that *fos* expression in PC12 cells is involved primarily in mitotic responses (that may be subsequently repressed by NGF and bFGF), whereas PKC, at least in the NGF response, plays a positive role in initial neurite extension. That PKC may not be obligatory illustrates the multifaceted and perhaps redundant responses that these factors can induce in this cell line.

ACKNOWLEDGMENTS

This work was supported by USPHS research grants AG09735 (R.A.B.) and EY08153 (R.W.) and by an American Cancer Society research grant BE-41L (R.A.B.). A portion of these experiments was performed in the laboratory of Dr. S.E. Fraser, Department of Physiology and Biophysics, University of California, Irvine, and we gratefully acknowledge both his advice and support. The assistance of Mrs. J. Jamal in maintaining stocks of PC12 cells is greatly appreciated.

REFERENCES

Alema, S., Casalbore, P., Agostini, E., and Tato, F. (1985). Differentiation of PC12 phaeochromocytoma cells induced by v-*src* oncogene. Nature *316*, 557–559.

Altin, J.G., and Bradshaw, R.A. (1990). Production of 1,2-diacylglycerol in PC12 cells by nerve growth factor and basic fibroblast growth factor. J. Neurochem. *54*, 1666–1676.

Altin, J.G., Kujubu, D.A., Raffioni, S., Eveleth, D.D., Herschman, H.R., and Bradshaw, R.A. (1991a). Differential induction of primary-response (TIS) genes in PC12 pheochromocytoma cells and the unresponsive variants PC12nnr5. J. Biol. Chem. *266*, 5401–5406.

Altin, J.G., Wetts, R., and Bradshaw, R.A. (1991b). Microinjection of a p21ras antibody into PC12 cells inhibit neurite outgrowth induced by nerve growth factor and basic fibroblast growth factor. Growth Factors *4*, 145–155.

Bar-Sagi, D., and Feramisco, J.R. (1985). Microinjection of the *ras* oncogene protein into PC12 cells induces morphological differentiation. Cell *42*, 841–848.

Burstein, D.E., Blumberg, P.M., and Greene, L.A. (1982). Nerve growth factor-induced neuronal differentiation of PC12 pheochromocytoma cells: lack of inhibition by a tumor promoter. Brain Res. *247*, 115–119.

Burstein, D.E., and Greene, L.A. (1978). Evidence for RNA synthesis-dependent and -independent pathways in stimulation of neurite outgrowth by nerve growth factor. Proc. Natl. Acad. Sci. USA *75*, 6059–6063.

Chiu, R., Boyle, W.J., Meek, J., Smeal, T., Hunter, T., and Karin, M. (1988). The c-*fos* protein interacts with c-*jun*/AP-1 to stimulate transcription of AP-1 responsive genes. Cell *54*, 541–552.

Cho, K.-O., Skarnes, W.C., Minsk, B., Palmieri, S., Jackson-Grusby, L., and Wagner, J.A. (1989). Nerve growth factor regulates gene expression by several distinct mechanisms. Mol. Cell. Biol. *9*, 135–143.

Cooper, D.R., Watson, J.E., Acevedo-Duncan, M., Pollet, R.J., Standaert, M.L., and Farese, R.V. (1989). Retention of specific PKC isozymes following chronic phorbol ester treatment in BC3H-1 myocytes. Biochem. Biophys. Res. Commun. *161*, 327–334.

Cremins, J., Wagner, J.A., and Halegoua, S. (1986). Nerve growth factor action is mediated by cyclic AMP- and Ca^{2+}/phospholipid-dependent protein kinases. J. Cell Biol. *103*, 887–893.

Curran, T., and Morgan, J.I. (1985). Superinduction of c-*fos* by nerve growth factor in the presence of peripherally active benzodiazepines. Science *229*, 1265–1268.

Damon, D.H., D'Amore, P.A., and Wagner, J.A. (1990). Nerve growth factor and fibroblast growth factor regulate neurite outgrowth and gene expression in PC12 cells via both PKC- and cAMP-independent mechanisms. J. Cell Biol. *110*, 1333–1339.

Eveleth, D.D., Hanecak, R., Fox, G.M., Fan, H., and Bradshaw, R.A. (1989). *v-src* genes stimulate neurite outgrowth in pheochromocytoma (PC12) variants unresponsive to neurotrophic factors. J. Neurosci. Res. *24*, 67–71.

Fox, G.M., Schiffer, S.G., Rohde, M.F., Tsai, L.B., Banks, A.R., and Arakawa, T. (1988). Production, biological activity, and structure of recombinant basic fibroblast growth factor and an analog with cysteine replaced by serine. J. Biol. Chem. *263*, 18452–18458.

Gizang-Ginsberg, E., and Ziff, E.B. (1990). Nerve growth factor regulates tyrosine hydroxylase gene transcription through a nucleoprotein complex that contains c-*fos*. Genes & Dev. *4*, 477–491.

Glowacka, D., and Wagner, J.A. (1990). Role of the cAMP-dependent protein kinase and protein kinase C in regulating the morphological differentiation of PC12 cells. J. Neurosci. Res. *25*, 453–462.

Graham, R., and Gilman, M.Z. (1991). Distinct protein targets or signals acting at the Fos serum response element. Science *251*, 189–192.

Greene, L.A., and Shooter, E.M. (1980). The nerve growth factor: biochemistry, synthesis, and mechanism of action. Annu. Rev. Neurosci. *3*, 353–402.

Greene, L.A., and Tischler, A.S. (1982). PC12 pheochromocytoma cultures in neurobiological research. Adv. Cell. Neurobiol. *3*, 373–414.

Guerrero, I., Pellicer, A., and Burstein, D.E. (1988). Dissociation of c-*fos* from ODC expression and neuronal differentiation in a PC12 subline stably transfected with an inducible N-ras oncogene. Biochem. Biophys. Res. Comm. *150*, 1185–1192.

Guerrero, I., Wong, H., Pellicer, A., and Burstein, D.E. (1986). Activated N-ras gene induces neuronal differentiation of PC12 rat pheochromocytoma cells. J. Cell Physiol. *129*, 71–76.

Hagag, N., Halegoua, S., and Viola, M. (1986). Inhibition of growth factor-induced differentiation of PC12 cells by microinjection of antibody to *ras* p21. Nature *319*, 680–682.

Hall, F.L., Fernyhough, P., Ishii, D.N., and Vulliet, P.R. (1988). Suppression of nerve growth factor-directed neurite outgrowth in PC12 cells by sphingosine, an inhibitor of protein kinase C. J. Biol. Chem. *263*, 4460–4466.

Hama, T., Huang, K.P., and Guroff, G. (1986). Protein kinase C as a component of a nerve growth factor-sensitive phosphorylation system in PC12 cells. Proc. Natl. Acad. Sci. USA *83*, 2353–2357.

Heasley, L.E., and Johnson, G.L. (1989). Regulation of protein kinase C by nerve growth factor, epidermal growth factor, and phorbol esters in PC12 pheochromocytoma cells. J. Biol. Chem. *264*, 8646–8652.

Huang, M., Chida, K., Kamata, N., Nose, K., Kato, M., Homma, Y., Takenawa, T. and Kuroki, T. (1988). Enhancement of inositol phospholipid metabolism and activation of protein kinase C in *ras*-transformed rat fibroblasts. J. Biol. Chem. *263*, 17975–17980.

Hsu, L., Jeng, A.Y., and Chen, K.Y. (1989). Inducers of neurite outgrowth from chick embryonic ganglia explants by activators of protein kinase C. Neurosci. Lett. *8*, 257–263.

Ito, E., Sonnenberg, J.L., and Naraynan, R. (1989). Nerve growth factor-induced differentiation in PC12 cells is blocked by *fos* oncogene. Oncogene *4*, 1193–1199.

Kruijer, W., Schubert, D., and Verma, I.M. (1985). Induction of the proto-oncogene *fos* by nerve growth factor. Proc. Natl. Acad. Sci. USA *82*, 7330–7334.

Lacal, J.C. (1990). Diacylglycerol production in *Xenopus laevis* oocytes after microinjection of $p21^{ras}$ proteins is a consequence of activation of phosphatidylcholine metabolism. Mol. Cell. Biol. *10*, 333–340.

Lacal, J.C., Moscat, J., and Aaronson, S.A. (1987). Novel source of 1,2-diacylglycerol elevated in cells transformed by Ha-*ras* oncogene. Nature *330*, 269–272.

Ledwith, B.J., Manam, S., Kraynak, A.R., Nichols, W.W., and Bradley, M.O. (1990). Antisense-*fos* RNA causes partial reversion of the transformed phenotypes induced by the c-Ha-ras oncogene. Mol. Cell. Biol. *10*, 1545–1555.

Milbrandt, J. (1986). Nerve growth factor rapidly induces c-*fos* mRNA in PC12 rat pheochromocytoma cells. Proc. Natl. Acad. Sci. USA *83*, 4789–4793.

Mobley, W.C., Schenker, A., and Shooter, E.M. (1976). Characterization and isolation of proteolytically modified nerve growth factor. Biochemistry J. *5*, 5543–5552.

Mochly-Rosen, D., and Koshland, D.E., Jr. (1987). Domain structure and phosphorylation of protein kinase C. J. Biol. Chem. *262*, 2291–2297.

Mochly-Rosen, D., and Koshland, D.E., Jr. (1988). A general procedure for screening inhibitory antibodies: application for identifying anti-protein kinase C antibodies. Anal. Biochem. *170*, 31–37.

Morgan, J.I., and Curran, T. (1986). Role of ion flux in the control of c-*fos* expression. Nature *322*, 552–555.

Nichols, R.A., Chandler, C.E., and Shooter, E.M. (1989). Enucleation of the rat pheochromocytoma clonal cell line, PC12: effect on neurite outgrowth. J. Cell Physiol. *141*, 301–309.

Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. Science *233*, 305–312.

Rausch, D.M., Dickens, G., Doll, S., Fujita, K., Koizumi, S., Rudkin, B.B., Tocco, M., Eiden, L.E., and Guroff, G. (1989). Differentiation of PC12 cells with v-src: comparison with nerve growth factor. J. Neurosci. Res. *24*, 49–58.

Reinhold, D.S., and Neet, K.E. (1989). The lack of a role for protein kinase C in neurite extension and in the induction of ornithine decarboxylase by nerve growth factor in PC12 cells. J. Biol. Chem. *264*, 3538–3544.

Riabowol, K.T., Fink, J.S., Gilman, M.Z., Walsh, D.A., Goodman, R.H., and Feramisco, J.R. (1988a). The catalytic subunit of cAMP-dependent

protein kinase induces expression of genes containing cAMP-responsive enhancer elements. Nature *336*, 83–86.

Riabowol, K.T., Vosatka, R.J., Ziff, E.B., Lamb, N.J., and Feramisco, J.R. (1988b). Microinjection of *fos*-specific antibodies blocks DNA synthesis in fibroblast cells. Mol. Cell. Biol. *8*, 1670–1676.

Rydel, R.E., and Greene, L.A. (1987). Acidic and basic fibroblast growth factors promote stable neurite outgrowth and neuronal differentiation in cultures of PC12 cells. J. Neurosci. *7*, 3639–3653.

Sassone-Corsi, P., Der, C.J., and Verma, I.M. (1989). *ras*-induced neuronal differentiation of PC12 cells: possible involvement of *fos* and *jun*. Mol. Cell. Biol. *9*, 3174–3183.

Satoh, T., Nakamura, S., and Kaziro, Y. (1987). Induction of neurite formation in PC12 cells by microinjection of proto-oncogenic Ha-*ras* protein preincubated with guanosine-5′-O-(3-thiotriphosphate). Mol. Cell. Biol. *7*, 4553–4556.

Schubert, D., Ling, N., and Baird, A. (1987). Multiple influences of a heparin-binding growth factor on neuronal development. J. Cell Biol. *104*, 635–643.

Sheng, M., Dougan, S.T., McFadden, G., and Greenberg, M.E. (1988). Calcium and growth factor pathways of c-*fos* transcriptional activation require distinct upstream regulatory sequences. Mol. Cell. Biol. *8*, 2787–2796.

Sheng, M., and Greenberg, M.E. (1990). The regulation and function of c-*fos* and other immediate early genes in the nervous system. Neuron *4*, 477–485.

Sheng, M., McFadden, G., and Greenberg, M.E. (1990). Membrane depolarization and calcium induce c-*fos* transcription via phosphorylation of transcription factor CREB. Neuron *4*, 571–582.

Sigmund, O., Naor, Z., Anderson, D.J., and Stein, R. (1990). Effect of nerve growth factor and fibroblast growth factor on SCG10 and c-*fos* expression and neurite outgrowth in protein kinase C-depleted PC12 cells. J. Biol. Chem. *265*, 2257–2261.

Stacey, D.W., DeGudicibus, S.R., and Smith, M.R. (1987). Cellular *ras* activity and tumor cell proliferation. Exp. Cell Res. *171*, 232–242.

Thomson, T.M., Green, S.H., Trotta, R.J., Burstein, D.E., and Pellicer, A. (1990). Oncogene N-ras mediates selective inhibition of c-*fos* induction by nerve growth factor and basic fibroblast growth factor in a PC12 cell line. Mol. Cell. Biol. *10*, 1556–1563.

Tiercy, J.-M., and Shooter, E.M. (1986). Early changes in the synthesis of nuclear and cytoplasmic proteins are induced by nerve growth factor in differentiating rat PC12 cells. J. Cell Biol. *103*, 2367–2378.

Togari, A., Dickens, G., Kuzuya, H., and Guroff, G. (1985). The effect of fibroblast growth factor on PC12 cells. J. Neurosci. *5*, 307–316.

Vosatka, R.J., Hermanowski-Vosatka, A., Metz, R., and Ziff, E.B. (1989). Dynamic interactions of c-*fos* protein in serum-stimulated 3T3 cells. J. Cell. Physiol. *138*, 493–502.

Wetts, R., Serbedzija, G.N., and Fraser, S.E. (1989). Cell lineage analysis reveals multipotent precursors in the ciliary margin of the frog retina. Dev. Biol. *136*, 254–263.

Wu, B-Y., Fodor, E.J.B., Edwards, R.H., and Rutter, W.J. (1989). Nerve growth factor induces the proto-oncogene c-*jun* in PC12 cells. J. Biol. Chem. *264*, 9000–9003.