Comparison of the Effects of NGF, Activators of Protein Kinase C, and a Calcium Ionophore on the Expression of Thy-1 and N-CAM in PC12 Cell Cultures

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Abstract. The addition of nerve growth factor (NGF) to PC12 cells induces an approximate doubling in the cell surface expression of the Thy-1 glycoprotein and the neural cell adhesion molecule (N-CAM) after 24 h of culture. Although both responses are measured at the same time point, their sensitivity to NGF differed with half-maximal induction of Thy-1 apparent at NGF concentrations ($\sim$0.1 ng/ml NGF) that had little effect on N-CAM expression. Phorbol ester derivatives capable of activating Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C) and the calcium ionophore A23187 were found to mimic the NGF induction of Thy-1, but not N-CAM. Similar results were observed when a synthetic diacylglycerol was added to PC12 cell cultures. Increased expression of Thy-1 consequent to phorbol ester, calcium ionophore, or NGF treatment was associated with an increase in the expression of the mRNA species that encodes Thy-1. Increased expression of Thy-1 consequent to all three treatments was also reduced by treatment with the transcription inhibitor cordycepin. Treatment of PC12 cells with high concentrations of phorbol esters was found to inhibit the NGF induction of Thy-1, but not N-CAM.

Whereas the above results are consistent with activation of protein kinase C underlying the NGF induction of Thy-1, the same data are not consistent with this pathway being important in the N-CAM response.

THE polypeptide hormone nerve growth factor (NGF)[1] is required for the normal development of the sympathetic nervous system (for review see Thoenen and Barde, 1980). Accumulating evidence suggests that NGF may also act as a target-derived retrogradely acting messenger in the central nervous system (Shelton and Reichardt, 1986; Levi-Montalcini and Calissano, 1986). NGF has been shown in vitro to elicit a wide variety of cellular responses including promoting the survival and morphological differentiation of neurons dissociated from embryonic sensory and sympathetic ganglia (Thoenen and Barde, 1980; Barde et al., 1980). However, despite extensive studies on the morphological and biochemical changes that accompany NGF-induced differentiation, the question of its precise mechanism of action remains unanswered (Levi-Montalcini and Calissano, 1986).

Biochemical changes underlying morphological differentiation have been extensively studied in a cell line (PC12) derived from a pheochromocytoma tumor. In the presence of NGF, PC12 cells undergo mitotic arrest and differentiate both morphologically and biochemically into a nerve cell

capable of forming functional synapses with skeletal muscle (Greene and Tischler, 1976; Greene, 1984; Schubert et al., 1977). A diverse set of biochemical changes accompanying NGF-induced differentiation have been documented and NGF has been shown to influence protein expression at transcriptional, posttranscriptional, and translational levels (Burstein and Greene, 1978; Gunning et al., 1981; Greene, 1984; Dickson et al., 1986a). Morphological differentiation of PC12 cells in response to NGF is dependent on synthesis of new mRNA species (Burstein and Greene, 1978; Greene and Shooter, 1980; Greene et al., 1982). We have recently fully characterized two early transcription-dependent responses in PC12 cells. NGF treatment of PC12 cells results directly in a fourfold increase in the rate of Thy-1 gene transcription with accumulation of the encoding mRNA species readily detectable within 24 h of growth factor addition (Dickson et al., 1986a). Similarly, NGF activates the neural cell adhesion molecule (N-CAM) gene with an increased expression of the 140-kD N-CAM isoform detected within 24 h (Prentice et al., 1987). As both of these responses can be readily indexed by measuring the increased expression of immunoreactive protein by ELISA, they can be conveniently used to test the underlying mechanism whereby NGF activates transcription of genes encoding cell surface glycoproteins.

In the present study, we have determined the effects of acti-

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1. *Abbreviations used in this paper:* MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; N-CAM, neural cell adhesion molecule; NGF, nerve growth factor; PdBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate, 13-acetate.

vators of protein kinase C and the calcium ionophore A23187 on the expression of Thy-1 and N-CAM for cultures grown both in the presence and absence of NGF. Our results are consistent with NGF initiating gene transcription via at least two pathways, one of which may involve the activation of protein kinase C.

Materials and Methods

Cell Culture

PC12 cells were grown in SATO media (Bottenstein, 1985) consisting of DME supplemented with progesterone (0.062 mg/liter), putrescine (16.6 mg/liter), thyroxine (0.4 mg/liter), selenium (0.039 mg/liter), human transferrin (100 mg/liter), bovine pancreas insulin (10 mg/liter), and triiodothyronine (0.377 mg/liter). (All reagents were from Sigma Chemical Co., St. Louis, MO.) The media was further supplemented at 1% (vol/vol) with Path-o-cyte 4 (BSA solution from Miles Scientific Div., Naperville, IL). For continuous culture, PC12 cells were grown on 100-mm collagen-coated tissue culture plates at a starting density of ∼2 × 10^6 cells/plate. For subculture, the cells were washed once with versene solution (Gibco, Grand Island, NY) followed by incubation at 37°C for 5–7 min with 0.05% (wt/vol) trypsin in the same buffer. Detached cells were centrifuged and resuspended by trituration with a pipette followed by a 19-gauge syringe needle. This procedure yielded a single cell suspension. For experimental studies, single-cell suspensions were seeded at 10,000 cells in 50 μl SATO media into individual wells of a collagen-coated 96-well microtiter plate. In all experiments, the cells were allowed a 12–16-h period to attach to the microwell and recover from trituration before the addition of test reagents. At this time >98% of the PC12 cells were viable as judged by their ability to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to an insoluble blue crystalline reaction product. Under the given culture conditions, PC12 cells do not enter into the logarithmic phase of growth until after day 3, and we are unable to detect any increase in cell number in control or NGF-treated cultures over the initial 24–48-h period of culture (Doherty and Walsh, 1987; Doherty et al., 1987a). Consistent with this observation, we find no change in the basal expression of Thy-1 over the longest period of study (see Fig. 3).

Reagents

The following phorbol esters were obtained from Sigma Chemical Co.: phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PdBu), and phorbol 13-monoacetate. The former two have been established as highly specific and potent activators of protein kinase C, whereas the latter phorbol has no activity (Castagna et al., 1982). All phorbols were stored as 1-mM stock solutions in ethanol at −20°C. Cordycepin (3-deoxyadenosine), a potent and specific inhibitor of polyadenylation (Penman et al., 1970), and 1,2,-dioctanolyl-sn-glycerol were obtained from Sigma Chemical Co. The 2.5S subunit of NGF was a generous gift from Dr. A. Leon (Fidia Research Laboratories, Abano Terme, Italy). The calcium ionophore A23187 was from Sigma Chemical Co.

Immunochemical Analysis of Cell Surface N-CAM and Thy-1 Levels

Cultures were carefully fixed by a 50% medium exchange with 4% paraformaldehyde in DME for 60 min at 20°C, followed by a total medium exchange for a further 60 min. Cultures were washed twice with PBS and the relative level of Thy-1 antigen and N-CAM surface immunoreactivity was determined essentially as previously described (Doherty and Walsh, 1987; Prentice et al., 1987). Briefly, after incubation with PBS containing 0.5% gelatin to block nonspecific protein-binding sites, cultures were incubated for 60 min at 20°C with a 1:2,000 dilution of rabbit anti-N-CAM Ig or, alternatively, a 1:50 dilution of the anti-Thy-1 mAb OX7 (Mason and Williams, 1980). The latter reagent was obtained as a tissue culture supernatant and was a generous gift from Dr. Neil Barclay (Medical Research Council Cellular Immunology Unit, Oxford). After incubation, cultures were washed three times with PBS containing 1% BSA and incubated for a further 60 min with a 1:1,000 dilution of horseradish peroxidase–conjugated anti-mouse Ig (Thy-1 assay) or anti-rabbit Ig (N-CAM assay; both Igs from Sigma Chemical Co.). Cultures were then washed four times with PBS, twice with distilled H_2O, and finally incubated with 50 μl of 0.2% (wt/vol)

o-phenylenediamine and 0.02% (vol/vol) H_2O_2 in citrate buffer. After 10–30 min, the conversion of o-phenylenediamine to its oxidized product was stopped by the addition of 50 μl H_2SO_4 and optical density determined at 490 nm using a microplate reader (No. MR700; Dynatech Laboratories, Inc., Alexandria, VA).

Colorimetric MTT (Tetrazolium) Assay

The colorimetric MTT assay was as described by Mosmann (1983) and included the modifications of Manthorpe et al. (1986). The assay determines the activity of various dehydrogenase enzymes, and is based on the cleavage of the tetrazolium ring of MTT that occurs only in active mitochondria. MTT was obtained from Sigma Chemical Co., and was dissolved in DME at 2.5 mg/ml. Stock solutions were held at −20°C. After a given period of culture, 10 μl of sterile MTT solution was added to each culture well containing 50 μl of growth media. After 2 h, the conversion of MTT to its formazin product was stopped by adding 50 μl of 0.08 N HCl in isopropanol. The microtiter plates were vigorously shaken and culture media in individual wells were mixed to dissolve the dark blue reaction product (MTT formazin). Plates were immediately read on a microplate reader (No. MR700; Dynatech Laboratories, Inc.) set at a test wavelength of 570 nm with a reference wavelength of 630 nm. Under the assay conditions there was a linear relationship between cell number and final reaction product over the range of 1 × 10^3–50 × 10^3 PC12 cells.

Northern Blot Analysis

Isolation of total RNA, glyoxal–agarose gel electrophoresis, and capillary blotting to Genescreen transfer membrane (New England Nuclear, Boston, MA) was performed as previously described (Dickson et al., 1986b). The cDNA probes to rat Thy-1 (Moriuchi et al., 1983) and neurofilament protein (Julien et al., 1985) were labeled by a replacement synthesis method and used for filter hybridization as described in Dickson et al. (1986a).

Protein Determination

Total protein levels were determined for 1 × 10^6 PC12 cells grown on 35-mm tissue culture plates by use of the Bradford method (Bradford, 1976).

Indirect Immunofluorescence

Indirect staining of live PC12 cultures was carried out as previously described (Doherty et al., 1987b). A 1:50 dilution of mAb OX7 was used, and as detecting antibody a 1:100 dilution of rhodamine-labeled anti-mouse Ig was used.

Results

NGF-induced Increases in Thy-1 Expression

PC12 cells were grown in the presence of NGF (0–25 ng/ml) over a 24-h period. Cultures were fixed and the relative expression of the Thy-1 antigen at the cell surface determined as described in Materials and Methods. Fig. 1 a shows a typical example of the relationship between NGF concentration and Thy-1 induction. From a total of six full dose–response curves, significant increases in Thy-1 were always induced by the lowest NGF concentration tested (0.008–0.014 ng/ml) with half-maximal responses consistently found at <0.1 ng/ml. Maximal responses were apparent at 1–5 ng/ml NGF and values ranged from a 31 to 124% increase in mAb OX7 binding over control (mean 64.9, 13%, ± SEM, n = 8). Over the above experimental period there was no increase in cell number in control as well as NGF-treated cultures (Doherty and Walsh, 1987). Protein levels were determined for 1 × 10^6 PC12 cells grown over 24 h in the presence and absence of NGF (10 ng/ml). There was an insignificant increase of ∼10% in treated as compared to control cultures (measured values of 110.6 ± 11.9 and 99.4 ± 2.2 μg protein, respectively, both values mean ± SD, n = 3). Thus the in-
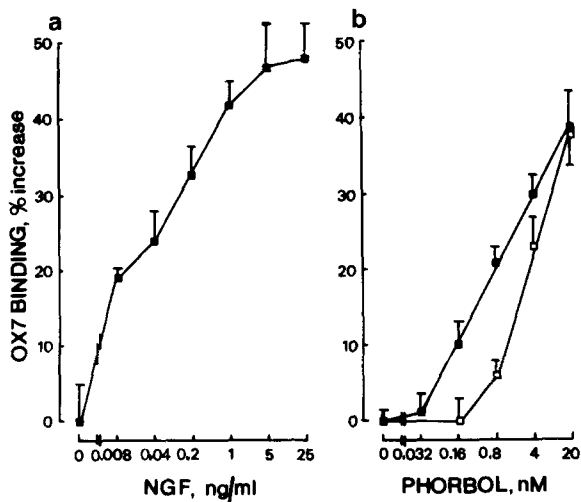
Figure 1. Cell surface-associated Thy-1 antigen levels were determined for PC12 cultures grown over 24 h in the presence of 0–25 ng/ml NGF (*a*) or 0–20 nM PMA and PdBu (*b*). The results show the percentage increase in mAb OX7 binding over cultures grown in control media, and each value is the mean + 1 SEM of six independent determinations. 100% control binding was measured as 0.383 ± 0.008 OD units (mean ± SEM, *n* = 12). ■, PMA; □, PdBu.

creased accumulation of Thy-1 in NGF-treated cultures can be considered to reflect a specific increase in expression at the cellular level.

Effects of Phorbol Esters on Thy-1 Expression

Phorbol esters were added to cultures of PC12 cells and Thy-1 levels were determined 24 h later. Fig. 1 *b* shows that in sister cultures to those illustrated in Fig. 1 *a*, both PMA and PdBu can induce dose-dependent increases in the expression of Thy-1. In a further series of experiments, PMA was consistently found to be more active than PdBu; however, the maximal response elicited by both phorbols was similar (Fig. 1, *b*). Maximal responses to both PMA and PdBu were observed at ∿20 nM, and at higher concentrations (>200 nM) both showed reduced responses. Similar biphasic responses have been commented upon in other systems (Blumberg et al., 1985) and most probably reflect phorbol ester–induced desensitization of protein kinase C (Rodriguez-Pena and Rozengurt, 1984). From a series of experiments in which PMA and NGF were added to sister cultures, the percentage increase in Thy-1 expression induced by 20 nM PMA was 74.4 ± 6.2 (mean ± SEM, *n* = 5) of that induced by 5 ng/ml NGF. A third phorbol ester, phorbol 13-monoacetate, which does not activate protein kinase C, had no effect on Thy-1 expression (data not shown).
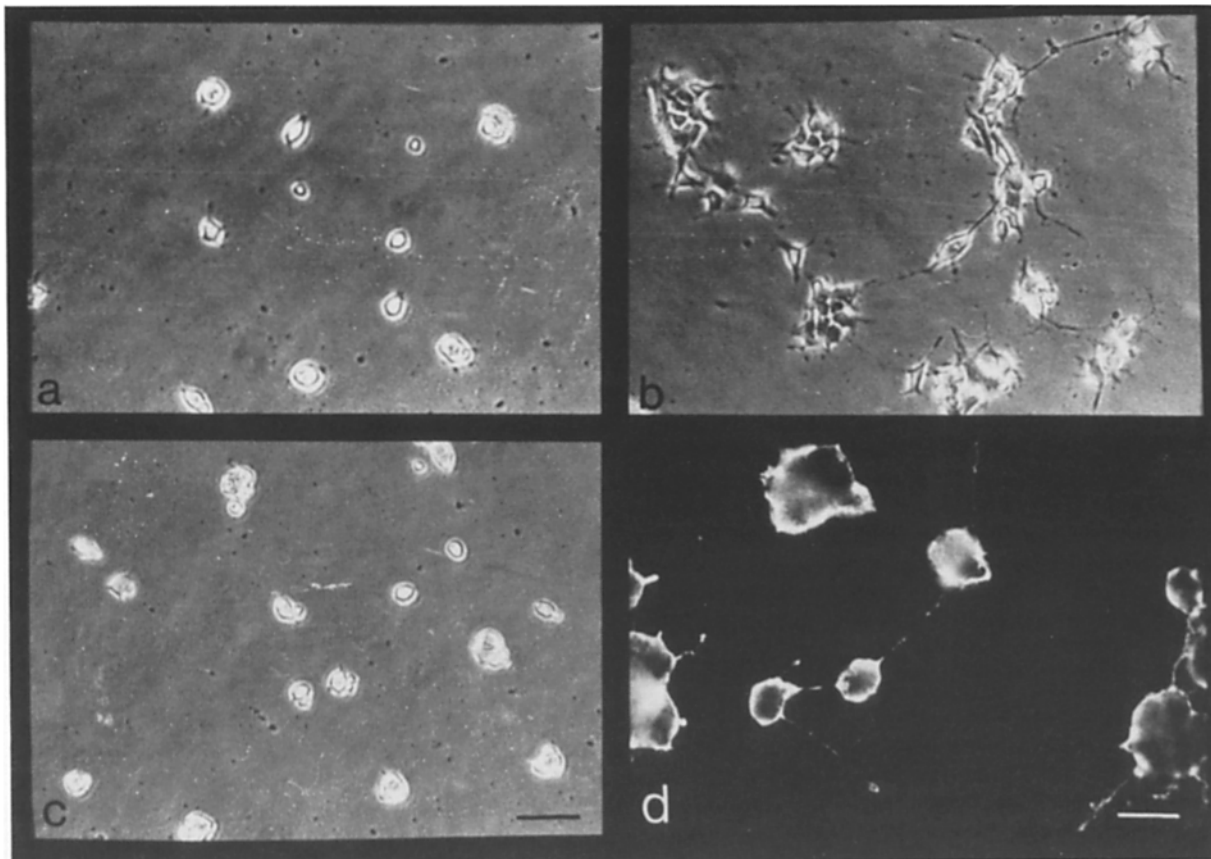
Figure 2. PC12 cells were grown over 24 h in control media (*a*), media supplemented with 10 ng/ml NGF (*b*), or 20 nM PMA (*c*). *a–c* show representative fields after assay for OX7 binding. *d* shows an NGF-treated culture stained with mAb OX7. Immunoreactivity is present over the entire cell surface and is also found in association with growth cones. Bars: (*a–c*) 50 μm; (*d*) 200 μm.
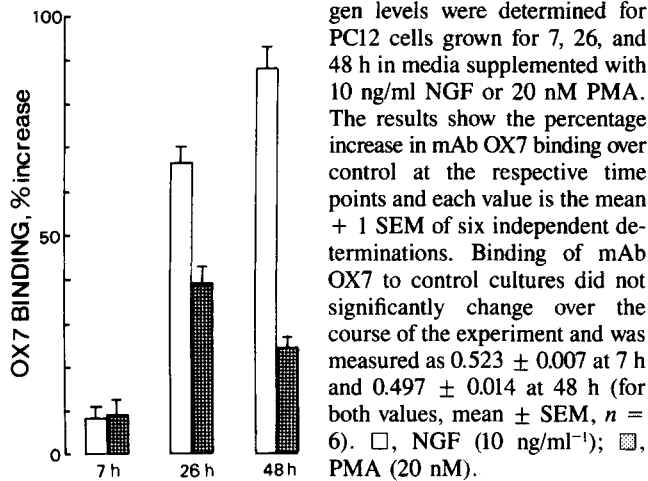
Figure 3. Cell surface Thy-1 antigen levels were determined for PC12 cells grown for 7, 26, and 48 h in media supplemented with 10 ng/ml NGF or 20 nM PMA. The results show the percentage increase in mAb OX7 binding over control at the respective time points and each value is the mean + 1 SEM of six independent determinations. Binding of mAb OX7 to control cultures did not significantly change over the course of the experiment and was measured as 0.523 ± 0.007 at 7 h and 0.497 ± 0.014 at 48 h (for both values, mean ± SEM, n = 6). □, NGF (10 ng/ml⁻¹); ▨, PMA (20 nM).

Figure 4. Immunoreactive N-CAM levels were determined for PC12 cells grown over 24 h in the presence of 0–25 ng/ml NGF (a) or, alternatively, in media supplemented with 20 nM PMA, 200 nM PMA, or 50 ng/ml NGF (b). The results show the percentage increase in Ig binding over cultures grown in control media, and each value is the mean of seven independent determinations. Bars show 1 SEM and 100% control value was 0.184 ± 0.006 OD units (mean ± SEM, n = 12). □, PMA (20 nM); ■, PMA (200 nM); ▨, NGF (50 ng/ml⁻¹).
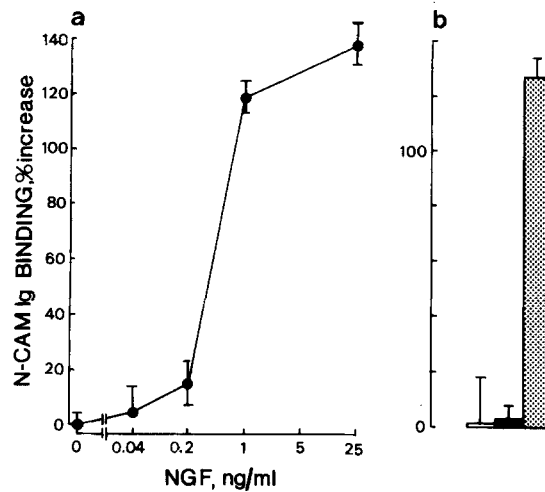
Under the present culture conditions, the vast majority of PC12 cells displayed neuritic processes after 24 h in response to 10 ng/ml NGF. In contrast, PMA and PdBu, at concentrations ranging from 1 to 1,000 nM, had no comparable effect on the morphology of PC12 cells (Fig. 2). Thy-1 immunocytochemistry highlights the morphological differentiation of NGF-treated cells.

Initial Time Course of Thy-1 Responses to NGF and Phorbol Esters Are Similar

PC12 cells were grown in media supplemented with NGF at 10 ng/ml or PMA at 20 nM. Cultures were fixed at various time points and assayed for the relative expression of Thy-1 at the cell surface. The basal level of Thy-1 expression did not change over this experimental period. However, small but significant ($P < 5\%$) increases in cell-surface expression of Thy-1 were apparent after only 7 h for both the PMA- and NGF-treated cultures (Fig. 3). Thy-1 induction by NGF continued to increase over the full 48-h period of culture. In contrast, the increased expression in response to PMA treatment was maximal at the 26-h time point.

### Effects of NGF and Phorbol Esters on N-CAM Expression

We have previously shown that NGF can induce a rapid increase in the expression of the 140-kD molecular species of N-CAM in PC12 cells, and that this response can be readily quantified by an ELISA (Prentice et al., 1987). In the present study, PC12 cells were grown in the presence of 0–50 ng/ml NGF and up to 200 nM PMA for 24 h. Fig. 4 shows NGF to be without effect on N-CAM immunoreactivity at up to 0.2 ng/ml. However at 1–25 ng/ml, NGF induced a greater than twofold increase in N-CAM immunoreactivity at the cell surface. In contrast to the results obtained with Thy-1, PMA had no effect on N-CAM expression when tested at 20 nM and 200 nM (Fig. 4 b). We can therefore conclude that the phorbol ester effects on Thy-1 expression do not simply reflect generalized changes in cell number in the synthesis of cell surface glycoproteins.

High Concentrations of Phorbol Esters Antagonize NGF Induction of Thy-1, but Not N-CAM

PC12 cells were grown as normal over a wide range of NGF concentrations in the presence and absence of a submaximally active concentration of PMA (0.33 nM). Fig. 5 shows the effects of the phorbol ester and NGF on Thy-1 expression to be additive rather than synergistic. In a separate series of experiments, PC12 cells were grown over a wide concentration range of phorbol ester in the presence and absence of a maximally active concentration of NGF (5 ng/ml). Fig. 6 again shows low concentrations of PdBu to have additive effects with NGF; however, increasing the concentration of PdBu was found to progressively abolish the NGF induction
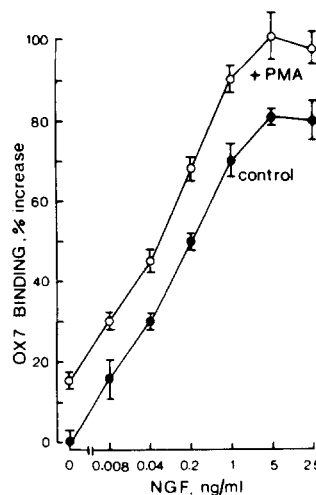
Figure 5. Immunoreactive Thy-1 levels were determined for PC12 cells grown for 24 h over a wide range of NGF concentration in the presence (O) and absence (●) of 0.33 nM PMA. The results show the percentage increase in the binding of mAb OX7 over cultures grown in control media and each value is the mean of six independent determinations. Bars show 1 SEM and 100% control value was 0.432 ± 0.011 OD units (mean ± SEM, n = 12).
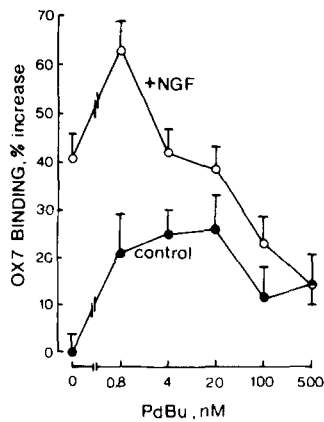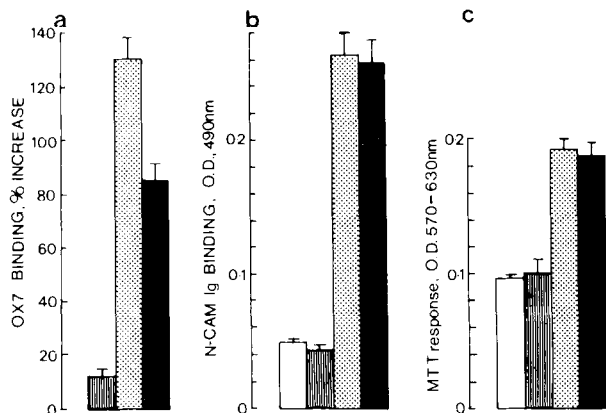
Figure 6. PC12 cells were grown over a wide range of PdBu concentrations (0–500 nM) in control media (●) or, alternatively, media supplemented with 5 ng/ml NGF (O). After 24 h the cultures were analyzed for Thy-1 binding, and the results show the percentage increase in mAb OX7 binding over cultures maintained only in SATO media. Each value is the mean of six independent determinations. Bars show 1 SEM and 100% control was 0.355 ± 0.012 OD units (mean ± SEM, n = 6).

Figure 8. Cell surface–associated Thy-1 and N-CAM levels were determined in sister cultures grown over 24 h in the presence of (□) A23187 (a–c: 62, 125, and 250 ng/ml, respectively) or, alternatively, (▨) NGF at 50 ng/ml. The results show the mean percentage increase in mAb OX7 binding or alternatively N-CAM Ig binding over cultures grown in control media. 100% control binding was measured as 0.423 ± 0.027 and 0.126 ± 0.008 (both values mean ± SEM, n = 7), for OX7 and N-CAM Ig, respectively. Each value is the mean of seven independent determinations, and bars show 1 SEM.
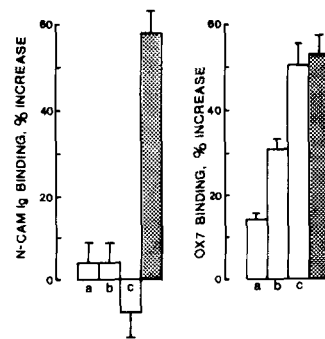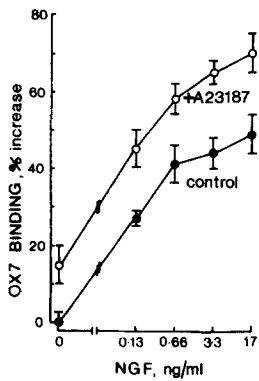
of Thy-1. A similar result was obtained in PC12 cultures treated with PMA (data not shown).

To determine if the inhibitory effects of phorbol esters reflected cytotoxicity or a generalized inhibition of the synthesis of membrane glycoproteins, PC12 cells were maintained over a 40-h period in the presence of NGF (50 ng/ml), PMA (500 nM), or both agents together. The induction of Thy-1 (Fig. 7 *a*), N-CAM (Fig. 7 *b*), and the general metabolic activation state of the cell population, as measured by the conversion of MTT to its formazan product (Fig. 7 *c*), were determined in sister cultures.

On its own, PMA induced a small increase in Thy-1 expression in the absence of any effect on N-CAM immunoreactivity or cell activity and viability. NGF increased Thy-1 expression by ∼130% and N-CAM immunoreactivity by more than fivefold. NGF activation of individual PC12 cells

was reflected in a twofold increase in the conversion of MTT to its formazan product. In the presence of NGF, PMA had no effect on N-CAM immunoreactivity, nor did it affect the general metabolic activation state of the cell population. However, treatment with PMA together with NGF limited the increased expression of Thy-1 to <60% of the sum of the individual responses. In a second independent experiment, NGF (5 ng/ml) induced a 98 ± 3.3% increase in Thy-1 expression, and PdBu (500 nM) a 29 ± 2.1% increase; when added together the increase was limited to 57 ± 3.8% (or ∼45% of the sum of the individual responses). The addition of phorbol 13-monoacetate (500 nM) had no effect on the NGF response (all values mean ± SEM, n = 9).

### A23187 and Dioctanoylglycerol Mimic Phorbol Ester Responses

An NGF-stimulated influx of extracellular calcium has been postulated to mediate the NGF stimulation of phospholipase C–mediated hydrolysis of inositol-containing phospholipids in PC12 cells (Contreras and Guroff, 1987). In the present studies, PC12 cells were grown in the presence of the calcium ionophore A23187 over a 24-h period. Fig. 8 shows the ionophore to mimic the NGF induction of Thy-1, but not N-CAM. However, as with phorbol esters and in contrast to NGF, the ionophore on its own had no obvious effect on the morphology of PC12 cells (data not shown). In several experiments A23187 was added together with NGF. Over the full range of active concentrations of A23187, additive effects were consistently observed for Thy-1 induction (Fig. 9). However, in the same experiments the ionophore was consistently without effect on basal or NGF induction of N-CAM (data not shown). A synthetic diacylglycerol, dioctanoylglycerol, that has previously been shown to be a potent and highly specific activator of protein kinase C (Lapetina et al., 1985), was also tested for its ability to modulate Thy-1 and N-CAM expression. In three independent experiments, treatment with dioctanoyl-glycerol (10 µg/ml) for 24 h resulted, in each instance, in a significant (<0.1%) increase in Thy-1 without affecting N-CAM immunoreactivity. The mean increase over the three experiments was 30.7 ± 3.2%, with the corresponding N-CAM immunoreactivity measured as 99.7 ± 4.9% control value (both values mean ± SD, n = 3).

Figure 7. PC12 cells were grown over 40 h in control media (□), media supplemented with 500 nM PMA (▦), 50 ng/ml NGF (▥), or 500 nM PMA and 50 ng/ml NGF (■). *a* shows the percentage increase in mAb OX7 binding over control with 100% control measured as 0.373 ± 0.028 (mean ± SEM, n = 7). *b* shows the absolute binding of N-CAM Ig to the four sets of cultures, and *c* shows the relative conversion of MTT to MTT-formazin by the respective cultures at 40 h. In each case, results show the mean + SEM of seven independent determinations.

Figure 9. Immunoreactive Thy-1 levels were determined for PC12 cells grown for 24 h over a wide range of NGF concentration in the presence (O) and absence (●) of 125 ng/ml A23187. The results show the percentage increase in the binding of mAb OX7 over cultures grown in control media and each value is the mean ± SEM of four independent determinations. 100% control value was 0.61 ± 0.017 OD units (mean ± SEM, $n = 4$).

Thy-1 Responses Are Dependent on the Synthesis of mRNA

Control experiments established that in the presence of NGF, up to 1 μm cordycepin could be added to PC12 cells for 24 h without any detrimental effect on cell metabolic activity (conversion of MTT in treated cultures measured as 103 ± 5% of respective control, mean ± SEM, $n = 6$). Under these conditions cordycepin was found to fully block the NGF induction of Thy-1 with a half-maximal inhibition apparent at ∼0.1–0.2 μm (Fig. 10 a). In contrast, cordycepin (1 μm) had no effect on the NGF induction of Thy-1 from primed PC12 cells, confirming the latter response to be independent of transcription (Doherty and Walsh, 1987; Mann, D. A., unpublished observation). In the presence of phorbol ester or calcium ionophore, or in control media, cordycepin could only be added at up to 0.1 μm without detrimental effect on metabolic activity. At this concentration, cor-
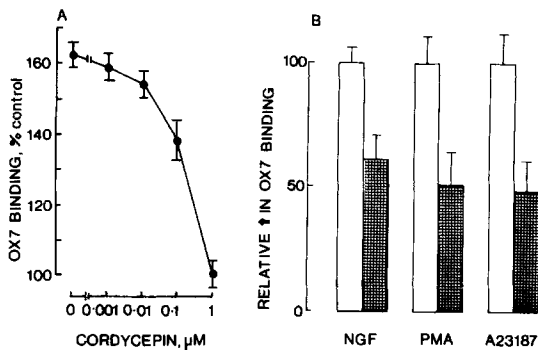
Figure 10. (A) Cell surface Thy-1 antigen levels were determined for PC12 cells grown in the presence of cordycepin (0–1 μM) in media supplemented with 10 ng/ml NGF. mAb OX7 binding was determined at 24 h and each value is the mean ± 1 SEM of six independent determinations. Results are expressed as percentage of mAb OX7 binding to cultures grown only in SATO media, with this value measured as 0.316 ± 0.004 OD units (mean ± SEM, $n = 12$). (B) PC12 cells were grown in media supplemented with 10 ng/ml NGF, 125 ng/ml A23187, or 20 nM PMA. Cultures were grown in both the presence (▨) and absence (□) of 0.1 μM cordycepin. In each instance the increase in mAb OX7 binding induced by test agent in the absence of cordycepin has been normalized to 100 arbitrary units. Each value is the mean + 1 SEM of six independent determinations.
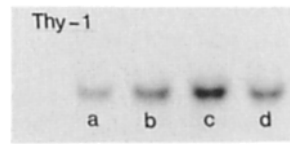
Figure 11. Level of Thy-1 mRNA in control (a), NGF- (b), phorbol ester- (c), and calcium ionophore- (d) treated PC12 cells. Cultures were grown over 24 h and total RNA samples collected. RNA samples (10 μg) were processed for Northern blot analysis, hybridized to Thy-1 cDNA probe, and the filter was autoradiographed at −80°C in the presence of intensifying screens.

dycepin had no effect on the basal expression of Thy-1 in control cultures and inhibited the induction of Thy-1 by NGF, PMA, and A23187 to a similar extent (Fig. 10 b).

To determine if changes in Thy-1 expression were accompanied by changes in the level of the mRNA species that encodes this protein, Northern blot analysis was carried out as previously described (Dickson et al., 1986a, b). PC12 cells were grown for 24 h in control media or alternatively in the presence of NGF (10 ng/ml), PMA (20 nM), and A23187 (125 ng/ml). Fig. 11 shows treatment with all three agents to induce a substantial increase in the level of the mRNA-encoding Thy-1. In contrast, and as a control, after 24 h of treatment, PMA and A23187 were without effect on the relative level of expression of the mRNA species that encodes the 68-kD subunit of the neurofilament protein (Dickson et al., 1986a; Mann, D. A., and H. Prentice, unpublished observation).

Discussion

Morphological differentiation of PC12 cells consequent to NGF treatment is a highly complex response that requires transcriptional changes in protein expression (Greene, 1984). Although the mechanism of action of NGF remains unknown, accumulating evidence suggests that activation of protein kinase C may be involved in at least the early NGF responses. For example, NGF treatment of PC12 cells and sympathetic neurons has been shown to stimulate hydrolysis of inositol phospholipids (Lakshmannan, 1978; Traynor et al., 1982; Contreras and Guroff, 1987) with the resultant accumulation of diacylglycerol providing for a physiological activation of protein kinase C (for review see Nishizuka, 1984). Direct evidence for such an activation has recently been obtained (Hama et al., 1986). What remains to be determined is to what extent activation of protein kinase C can mimic NGF responses.

We have previously shown that NGF can rapidly activate the genes that encode the N-CAM and Thy-1 cell surface glycoproteins. As considerable evidence exists to support the notion that these glycoproteins may function as molecules involved in cell recognition and/or transduction of biological signals across cell membranes (Rutishauser et al., 1983; Edelman, 1984; Morris, 1985; Kroczek et al., 1986), elucidation of the cellular mechanisms controlling their expression is of particular interest.

In the present study, we have shown that NGF can induce an approximate doubling of immunoreactive Thy-1 and N-CAM at the cell surface within the first 24 h of culture. An unexpected observation suggesting that the expression of these glycoproteins may be controlled along different pathways was that half-maximal induction of Thy-1 was consis-

tently apparent at NGF concentrations that had little or no effect on N-CAM expression. In support, induction of Thy-1, but not N-CAM, could be mimicked by low concentrations of the protein kinase C activators PMA and PdBu as well as by the synthetic diacylglycerol, dioctanoylglycerol. A third phorbol ester that has no effect on protein kinase C activity was without effect on Thy-1 expression. Induction of Thy-1 by both NGF and PMA was accompanied by a relative increase in the expression of the mRNA that encodes this protein, and both responses could be inhibited by cordycepin.

It has been suggested from observations in several cellular systems that full physiological responses are dependent on both protein kinase C activation and internal calcium mobilization (Nishizuka, 1984; Isakov and Altman, 1983; Omann et al., 1987). It has recently been shown that in PC12 cells the calcium ionophore A23187 can fully mimic the NGF-induced hydrolysis of inositol phospholipids and that the latter response is dependent on extracellular calcium (Contreras and Guroff, 1987). In the present study, we now report that as with direct activators of protein kinase C, the calcium ionophore can induce a rapid dose-dependent increase in Thy-1 expression. As with NGF and activators of protein kinase C, this response could be inhibited by cordycepin and was accompanied with an increased level of the mRNA species that encodes Thy-1. However the calcium ionophore failed to mimic the NGF induction of N-CAM. Thus, whereas these data are entirely consistent with NGF-induced hydrolysis of inositol phospholipids underlying the induction of Thy-1, they are not consistent with this pathway mediating the N-CAM response.

Low concentrations of both PMA and PdBu showed additive effects with maximally active concentrations of NGF in promoting increased expression of Thy-1. Similarly, A23187, over its full range of active concentration, also showed an additive effect with NGF. However, high concentrations of both PMA and PdBu were found to substantially inhibit the NGF induction of Thy-1. In addition to serving as an internal control for nonspecific effects on glycoprotein synthesis, the clear demonstration that high concentrations of PdBu do not inhibit the NGF-induced increases in N-CAM expression provides further evidence that NGF activates at least two independent pathways in the control of cell surface glycoprotein expression.

The mechanism whereby high concentrations of phorbol esters inhibit the Thy-1 response remains to be determined. However, the higher concentrations of both PMA and PdBu used in the present study have previously been shown to cause a highly specific and rapid loss in total protein kinase C activity in many cells, including PC12 (Rodriguez-Pena and Rozengurt, 1984; Rozengurt et al., 1985; Matthies et al., 1987). Thus one possibility is that NGF-induced activation of protein kinase C is required for the induction of Thy-1, but not N-CAM, and that inhibition of the former is a direct consequence of phorbol ester-induced inactivation of protein kinase C. A second possibility is that phorbol ester-induced activation of protein kinase C results in the inactivation of an alternative pathway involved in the NGF induction of Thy-1, but not N-CAM. However, this seems less likely in view of the observation that A23187 does not inhibit the NGF response.

Activators of protein kinase C and A23187 did not mimic the NGF-induced morphological differentiation of PC12

cells. Thus increased expression of Thy-1 is not consequential to neurite outgrowth, nor does it trigger the latter. However, increased expression of Thy-1 and other as yet unidentified molecules may constitute an important component of the neuritogenic response. Receptor-mediated increases in activation of adenylate cyclase can inhibit receptor-linked degradation of inositol phospholipids in membranes and thereby block cellular functions by counteracting activation of protein kinase C (Billah et al., 1979; Nishizuka, 1984; Isakov and Altman, 1985; Omann et al., 1987). Over a 3-d period, cholera toxin can inhibit NGF-induced increases in Thy-1 expression in cultures of naive PC12 cells by up to 50% (Doherty and Walsh, 1987). Over longer periods of culture (>6 d), increased intracellular cAMP can also inhibit neurite outgrowth from naive PC12 cells (Richter-Landsberg and Jastorff, 1986). In cultures of primed PC12 cells, a full inhibition of Thy-1 induction by NGF can be obtained over a 48-h culture period with an associated inhibition of neurite regeneration (Doherty and Walsh, 1987).

The data in the present paper suggest that a second distinct pathway is likely to act in concert with or independently of hydrolysis of inositol phospholipids in promoting a full cellular response to NGF. One possibility is that activation of protein kinase A together with protein kinase C underlies the NGF response (Cremins et al., 1986). However, we have failed to detect any change in N-CAM levels by addition of cholera toxin to PC12 cells (Prentice et al., 1987) and, as discussed above, increased cAMP can antagonize NGF responses.

In conclusion, the results of the present study demonstrate that increased expression of Thy-1 can be induced independently of changes in N-CAM expression. Differential inhibition of the NGF-induced increases in the expression of these glycoproteins supports the notion that the NGF signal can bifurcate to reveal two independently acting pathways of gene activation. Whereas the presented data is entirely consistent with hydrolysis of phospholipids and consequent activation of protein kinase C that underlies the NGF induction of Thy-1, the same data are not consistent with this pathway being involved in the N-CAM response. On the basis of these observations, we would postulate that activation of the phosphatidylinositol pathway is not the primary event in transduction of the NGF signal in PC12 cells.

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