Molecular Biology of the Cell
Vol. 6, 449–458, April 1995

Selective Translocation of Protein Kinase C-δ in PC12 Cells during Nerve Growth Factor-induced Neuritogenesis

Kevin R. O'Driscoll,*†‡ Kenneth K. Teng,§‖ Doriano Fabbro,¶ Lloyd A. Greene,*§ and I. Bernard Weinstein*#

*Columbia-Presbyterian Cancer Center, †Department of Neurology, Division of Pediatric Neurology, The Colleen Giblin Laboratories, §Department of Pathology, Center for Neurobiology and Behavior, #Departments of Medicine and of Genetics and Development, and the School of Public Health, College of Physicians and Surgeons of Columbia University, New York, New York 10032; and ¶Department of Pharmaceutical Research, Oncology K125, Ciba Geigy, Ltd, CH-4002 Basel, Switzerland

Submitted April 19, 1994; Accepted March 28, 1995
Monitoring Editor: Martin Raff

The specific intracellular signals initiated by nerve growth factor (NGF) that lead to neurite formation in PC12 rat pheochromocytoma cells are as of yet unclear. Protein kinase C-δ (PKCδ) is translocated from the soluble to the particulate subcellular fraction during NGF-induced–neuritogenesis; however, this does not occur after treatment with the epidermal growth factor, which is mitogenic but does not induce neurite formation. PC12 cells also contain both Ca^{2+}-sensitive and Ca^{2+}-independent PKC enzymatic activities, and express mRNA and immunoreactive proteins corresponding to the PKC isoforms α, β, δ, ε, and ζ. There are transient decreases in the levels of immunoreactive PKCs α, β, and ε after 1–3 days of NGF treatment, and after 7 days there is a 2.5-fold increase in the level of PKCα, and a 1.8-fold increase in total cellular PKC activity. NGF-induced PC12 cell neuritogenesis is enhanced by 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) in a TPA dose- and time-dependent manner, and this differentiation coincides with abrogation of the down-regulation of PKCδ and other PKC isoforms, when the cells are treated with TPA. Thus a selective activation of PKCδ may play a role in neuritogenic signals in PC12 cells.

INTRODUCTION

Protein kinase C (PKC)[1] mediates intracellular signaling initiated by transmembrane-receptor–linked hydrolysis of phospholipids, and by phorbol esters or related compounds, thus modulating diverse biological processes, which include differentiation and malignant transformation (reviewed by Weinstein *et al.*, 1991; Hug and Sarre, 1993). In addition, PKC has been implicated in a variety of cellular events in the central nervous system (reviewed by Tanaka and Saito, 1992). Molecular cloning studies have revealed that at least 10 members of the PKC family exist, which are termed PKC α, βI, βII, γ, δ, ε, ζ, η, θ, and λ (reviewed by Nishizuka, 1992; Hug and Sarre, 1993). Based on sequence homology and biochemical properties, PKC isoforms can be divided into three classes: the Ca^{2+}-sensitive conventional PKCs (PKCs α, βI, βII, and γ), the Ca^{2+}-independent novel PKCs (PKCs δ, ε, η, and θ), and the Ca^{2+}– and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA)–insensitive atypical PKCs (PKCs ζ

‡ Corresponding author: The Neurological Institute of New York, Columbia-Presbyterian Medical Center, 710 West 168th St., Box 52, New York, NY 10032.
‖ Present address: Division of Hematology and Oncology, Cornell University Medical College, New York, NY 10021.

[1] Abbreviations used: EGF, epidermal growth factor; kDa, kilodaltons; NGF, nerve growth factor; PKC, protein kinase C; PS, 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine]; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

and λ). Although this heterogeneity of structure suggests that individual isoforms perform distinct functions, there is a limited knowledge of how each isoform of PKC is coupled to specific biological responses.

The rat pheochromocytoma PC12 cell line (Greene and Tischler, 1976) has been a valuable model system for studying the molecular events that underlie the biological actions of nerve growth factor (NGF) (reviewed by Levi-Montalcini and Angeletti, 1968; Levi and Alema, 1991). PC12 cells are proliferative neoplastic counterparts of immature neural crest-derived adrenal chromaffin cells. When exposed to NGF, PC12 cells cease division and gradually express many of the properties characteristic of mature sympathetic neurons (reviewed by Greene and Tischler, 1982). The most striking phenotypic alteration induced by NGF is the extension of neurite-like processes from these cells, a differentiation process termed neuritogenesis. Neuritogenesis in PC12 cells is also induced by treatment of the cells with basic fibroblast growth factor (Togari *et al.*, 1983; Rydel and Greene, 1987). Previous studies suggest that the Trk NGF-receptor tyrosine kinase as well as a variety of subsequently activated serine/threonine protein kinases are involved in NGF-promoted neuritogenesis and neuronal differentiation (reviewed by Halegoua *et al.*, 1991; Levi and Alema, 1991; Chao, 1992; Raffioni *et al.*, 1993); however, to date, most of the intracellular signaling events in PC12 cells known to occur after NGF treatment are also produced by epidermal growth factor (EGF). Because EGF-treatment of these cells causes cell proliferation and does not induce neuritogenesis (unlike NGF and basic fibroblast growth factor treatment of PC12 cells), the identification of signaling events specific to the neuronal differentiation factors remains a current challenge (Chao, 1992).

Several previous studies suggest an involvement of one or more isoforms of PKC in differentiation of PC12 cells. For instance, it was shown that treatment of PC12 cells with the tumor promoter TPA enhances neuritogenesis in response to low concentrations of NGF (Burstein *et al.*, 1982). Neuritogenesis in PC12 cells induced by expression of the N-*ras* oncogene is accompanied by interference in the phorbol 12, 13-dibutyrate–induced down-regulation of phorbol ester receptors, in vitro PKC enzyme activity, and immunoreactive PKC (Lacal *et al.*, 1990). In addition, microinjection of a PKC-neutralizing monoclonal antibody (Altin *et al.*, 1992), and down-regulation of PKC by treatment of PC12 cells with the phorbol ester agonist bryostatin (Singh *et al.*, 1994), have been reported to inhibit neuritogenesis in NGF-treated PC12 cells. The present study examines the effects of long-term treatment with NGF and with the tumor-promoting phorbol ester TPA, on the expression of various PKC isoforms in PC12 cells. Taken together, our data suggest that a selective activation of PKCδ may play an important role in NGF-evoked neuritogenesis in PC12 cells.

MATERIALS AND METHODS

Materials and Reagents

NGF was purified from adult mouse submaxillary glands as previously described (Mobley *et al.*, 1976). TPA and EGF were obtained from Sigma Chemical (St. Louis, MO). 1,2-Dioleoyl-*sn*-glycero-3-[phospho-L-serine] (PS) was obtained from Avanti Polar Lipids (Alabaster, AL). Monoclonal antibody preparations were obtained from the following sources: PKCα (M5) was obtained from Amersham (Arlington Heights, IL); PKCα (M6) was obtained from Dr. S. Jaken (Alton Jones Cell Science Center, Lake Placid, NY) (Leach *et al.*, 1988); PKCβ (MC-2a) and PKCγ (MC-Ia) (Hidaka *et al.*, 1988) were obtained from Seikagaku America (Rockville, MD). The generation and characterization of the polyclonal antisera for PKCs ε, δ, and ζ were described previously (Borner *et al.*, 1992). Radiolabeling reagents, molecular mass standards, and the Enhanced Chemi-Luminescence detection system were obtained from Amersham. Protein determinations (Bradford, 1976) were performed with reagents purchased from Bio-Rad Laboratories (Richmond, CA), using bovine serum albumin as the standard.

Cell Cultures and Measurement of Neurite Outgrowth

PC12 cells were cultured on collagen-coated dishes in RPMI 1640 medium supplemented with 10% horse serum and 5% fetal bovine serum (Greene and Tischler, 1976). For NGF treatment, the cells were plated at low densities (5×10^5 cells per 35-mm dish, 2×10^6 cells per 100-mm dish, or 5×10^6 cells per 150-mm dish) in RPMI 1640 medium containing 1% horse serum and NGF (50 ng/ml). For TPA treatment, PC12 cells were plated in RPMI 1640 medium containing 1% horse serum, and the appropriate concentration of TPA was added to the medium in dimethyl sulfoxide solvent at a final concentration of 0.1% (vol/vol). Control cultures were plated in RPMI 1640 medium with 1% horse serum, and in experiments involving treatment with TPA, received an equivalent amount of dimethylsulfoxide alone, which produced no observable effects on the cellular morphology of PC12 cells. The percent of neurite-bearing PC12 cells was determined as previously described (Burstein *et al.*, 1982) and only individual/discernible cells, but not cell clumps, were scored. Only processes greater than 2 cell body diameters in length (i.e., about 20 μm) were considered neurites. For each culture condition, at least 100 cells from randomly chosen fields were assessed.

PKC Enzyme Assays

PKC was partially purified from 0.5% (vol/vol) Triton X-100 detergent cell extracts by DEAE-Sephacel column chromatography, as previously described (Solomon *et al.*, 1991). Three different PKC enzyme assay conditions were used in this study. A nonrestrictive PKC assay condition (O'Brien *et al.*, 1985) utilized the EGF receptor-derived synthetic peptide RKRTLRRL as a substrate in the presence of 0.8 mM Ca^{2+}, and 85 μg/ml PS to detect PKC activity. Under these conditions the RKRTLRRL peptide is phosphorylated in vitro by both conventional as well as novel PKC isoforms (Khaled *et al.*, 1995). Therefore, we assume that this assay system simultaneously detects both calcium-sensitive and calcium-independent classes of PKC activities. Partially restrictive assay conditions to distinguish calcium-independent from Ca^{2+}-sensitive PKC activities utilized either the synthetic peptide RVARRGSLRQKNVHEVKN, whose sequence is based on the pseudosubstrate domain of PKCε (Sossin and Schwartz, 1992), as substrate in the absence of Ca^{2+}, or alter-

natively utilized Histone-IIIs (Sigma Chemical) as a substrate in the presence of Ca^{2+}, as described previously (Schaap *et al.*, 1989). These latter assays utilized 85 μg/ml PS and 100 ng/ml TPA for stimulation of PKC activities. Specific PKC activities were expressed in units of nmol [^{32}P] transferred min^{-1} per mg of protein in the respective DEAE fractions tested, and represent the means of triplicate determinations that differed by less than 15%.

Northern Blotting Analysis

Total cellular RNA was extracted from PC12 cells using 5 M guanidine isothiocyanate and was purified on a 5.7 M CsCl density gradient (Sambrook *et al.*, 1989). Polyadenylated RNA was further purified by oligo dT cellulose chromatography, and was resolved by electrophoresis on gels containing 1% (wt/vol) agarose and 6% (vol/vol) formaldehyde. Blot transfers on Hybond-N membrane (Amersham) were probed with the PKC isoform-specific cDNA probes described in the legend to Figure 1, as described previously (Feinberg and Vogelstein, 1983; Church and Gilbert, 1984). Autoradiographic images were obtained by exposing the blots to Kodak XAR-5 film at −80°C with intensifying screens.

Immunoblotting Analysis

Cultures of PC12 cells were placed on ice, and were rinsed and resuspended in phosphate-buffered saline containing protease inhibitors (10 μg/ml aprotinin and 10 μg/ml leupeptin). After centrifugation at 500 × *g* for 10 min at 4°C, the cell pellets were sonicated briefly in lysis buffer (20 mM Tris [pH 7.5], 2 mM [ethylenebis(oxyethylene-nitrilo)]tetraacetic acid, 2 mM EDTA, 50 mM KF, 50 mM NaH_2PO_4, 100 μM Na_3VO_4, 6 mM 2-mercaptoethanol, 1.25 mM phenylmethane-sulfonate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Subcellular fractions were isolated by sedimentation at 100,000 × *g* for 40 min at 4°C as described previously (Borner *et al.*, 1992). The soluble fraction was taken from the supernatant, and the pellet was rinsed and resuspended by sonication in lysis buffer containing 1% Triton X-100 to yield the particulate fraction. Equal masses of each protein sample (50–100 μg) were then separated by 8% polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS-PAGE) (Sambrook *et al.*, 1989) and were then electroblotted (400 mA for 1–4 h) onto either Immobilon PVDF membranes (Millipore, Bedford, MA), or alternatively, onto nitrocellulose membranes (Amersham ECL grade). Protein molecular mass markers (Amersham) were used as follows: 200 kilodaltons (kDa), myosin; 92 kDa, phosphorylase b; 69 kDa, bovine serum albumin; 46 kDa, ovalbumin; 30 kDa, carbonic anhydrase. Blots were then blocked in TN-Tween buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.2% [vol/vol] Tween 20) containing 5% (wt/vol) nonfat dry milk powder for 1 to 2 h, washed in five to seven changes of TN-Tween buffer, and incubated with primary antibodies as described in the figure legends. After further washing, the blots were processed for visualization using the Enhanced Chemi-Luminescence system according to the manufacturer's recommendations (Amersham). The blots were then exposed to Kodak XAR-5 film for between 1 s and several minutes to obtain the fluorographic images. Densitometric scanning of fluorograms was performed on a Molecular Dynamics computing densitometer, model 300A (Sunnyvale, CA).

RESULTS

Characterization of PKC Activities in PC12 Cells

To characterize their content of PKC activities, PC12 cell extracts were subjected to DEAE-Sephacel chromatography, and column fractions were assayed using selective PKC substrates and under conditions designed to optimize detection of either the Ca^{2+}-sensitive PKC isoforms (using histone type III-S as the substrate) or the Ca^{2+}-independent PKC isoforms (using the PKCε-derived RVARRGSLRQKNVHEVKN peptide as the substrate) (Schapp *et al.*, 1989) (Table 1). Evidence for the presence of both classes of PKC isoforms was obtained, with the relative level of calcium-independent PKC activity being about twice that of conventional calcium-sensitive PKC activity. To determine if cellular PKC activity was affected by long-term treatment of the cells with NGF, total PKC was purified from PC12 cells treated for 0, 7, or 31 days with NGF. These time points were chosen because they correspond to times before neurite formation (0 day), during maximal neurite outgrowth and differentiation (7 days), and after full differentiation has occurred (31 days). PKC was partially purified by chromatography on DEAE Sephacel columns and the 0.1 M NaCl eluate was assayed using the EGF-receptor–derived RKRTLRRL peptide for substrate, in the presence of 0.8 mM Ca^{2+} and PS vesicles (85 μg/ml). These conditions allowed the detection of all classes of PKC isoforms (for additional details see MATERIALS AND METHODS). The mean level of PKC activity in DEAE-Sephacel–purified fractions from untreated PC12 cells was 21.7 (± 1.1) U/mg protein, the value in fractions from NGF-treated PC12 cells after 7 days was 39.3 (± 2.9) U/mg, and the level in fractions from NGF-treated cells after 31 days was 22.5 (± 0.4) U/mg. Thus at the time of maximal NGF-induced neurite formation (7

Table 1. Assays for total PKC activity in untreated PC12 cells utilizing two different assay conditions

	PKC activity[a] nmol[^{32}P]/min/mg	
DEAE-Sephacel eluate (NaCl concentration)	Histone III-S[b]	RVARRGSLRQKNVHEVKN[c]
0.1 M	3.4	6.2
0.2 M	1.4	2.2
0.3 M	0.1	0.2
0.4 M	0	0.1

[a]Total cell extracts of untreated PC12 cells were applied to a DEAE-Sephacel column and eluted with a step gradient with the indicated concentrations of NaCl, and the respective fractions were assayed for PKC activity. The data are expressed as specific activities after subtraction of the blank values, i.e., activity obtained in the absence of PS and the additional cofactors described below. The values represent the mean specific activities determined in duplicate PKC assays, which differed by less than 10%. For additional details see MATERIALS AND METHODS.

[b]Assays were performed with Histone III-S (1.25 mg/ml) as the substrate, and in the presence of 0.8 mM Ca^{2+} and PS vesicles (85 μg/ml), to optimize the detection of Ca^{2+}-sensitive PKC isoforms.

[c]Assays were performed with the PKCε-derived RVARRGSLRQKNVHEVKN peptide (0.1 mM) as the substrate, and in the presence of 2 mM [ethylenebis(oxyethylene-nitrilo)]tetraacetic acid, PS vesicles (85 μg/ml), and TPA (100 ng/ml), to optimize the detection of Ca^{2+}-independent PKC isoforms.

days) there was a 1.8-fold increase in total PKC activity compared with the levels in naive and fully differentiated PC12 cells.

PC12 Cells Express Multiple PKC Isoforms

We next used Northern blot analyses to determine the expression of various PKC isoform-encoding mRNAs in untreated PC12 cells. Hybridization of polyadenylated RNA with PKC isoform-specific cDNA probes revealed that untreated PC12 cells express mRNA species of the characteristic sizes encoding PKCs α, β, γ, δ, ϵ, and ζ (Figure 1). These results suggest that mRNAs encoding PKCs δ and ζ are expressed abundantly, whereas those encoding PKCs α and β are expressed to moderate levels, and those encoding PKCs ϵ and γ are barely detectable. Our finding that two transcripts of different sizes were detected for both PKCs α and ζ is consistent with previous results obtained in studies with rat fibroblasts (Borner *et al.*, 1992). Additional isoforms of PKC (PKCs η, θ, and λ) were not examined in the present study.

PKC Isoforms Are Differentially Modulated during NGF-induced Neuritogenesis

In view of our finding that PC12 cells treated with NGF for 7 days display about a twofold increase in total PKC enzyme activity, and that multiple PKC isoforms are expressed in PC12 cells, we examined the effects of NGF treatment on the levels and subcellular distribution of individual PKC isoforms. PC12 cells were treated with NGF for 0, 1, 3, or 7 days, soluble and particulate cell fractions were prepared, and these were analyzed separately by immunoblotting (see MATERIALS AND METHODS). Figure 2 shows representative immunoblots obtained using isoform-specific antibodies for PKCs α, β, δ, and ϵ.

The level of the 80-kDa PKCα protein in the untreated PC12 cells decreased transiently in both the soluble and particulate fractions after NGF treatment. This was followed by an increase of PKCα in the soluble fraction so that by day 7 its level, as determined by densitometer scanning of fluorograms, was about 2.5-fold higher than that in control PC12 cells. The abundance of PKCα in the particulate fraction was increased on day 3, and returned to the control level by day 7 of NGF exposure. In contrast, the 80-kDa PKCβ protein was detected exclusively in the soluble fraction of control and NGF-treated cells. The level of PKCβ was transiently reduced at days 1 and 3 after NGF treatment, but by day 7 expression returned to near the control level.

PKCδ was present mainly in the soluble fraction of untreated PC12 cells as a 74- to 76-kDa diffuse band; however, after treatment with NGF for 3 or 7 days, there was a decline in the level of PKCδ in the soluble fraction, and a concomitant increase of PKCδ in the particulate fraction (Figure 2). The detection of PKCδ in the particulate fraction required longer fluorographic exposures, and as a consequence several nonspecific bands were also detected under these conditions. Control experiments using as a competitor a peptide corresponding to the carboxyl-terminus of PKCδ, which was the original immunogen used to produce the PKCδ antibody (Borner *et al.*, 1992), eliminated the 74- and 76-kDa proteins but not the 90-, 55-, and 45-kDa proteins. It appears therefore that the latter proteins are not related to PKCδ. The identities of these proteins remain to be determined.

In both control and NGF-treated cultures, the 90-kDa PKCϵ protein was detected in both the soluble and particulate fractions. The abundance of PKCϵ in the soluble fraction decreased after 1 day of NGF treatment but then gradually returned to the control level. The level of PKCϵ in the particulate fraction was somewhat reduced throughout the 7-day time course of NGF treatment, relative to the untreated control cells.

NGF-specific Translocation of PKCδ

To determine if the observed translocation of PKCδ correlates with neuritogenic-specific signals, PC12 cells were maintained in the absence of growth factors,

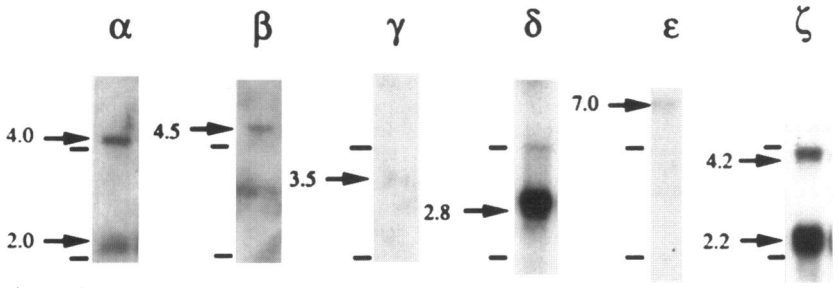

Figure 1. Expression of PKC isoform-specific mRNA species in PC12 cells. Northern blots of polyadenylated RNA (2 μg/lane) isolated from untreated PC12 cells were probed with the following ^{32}P-labeled DNA restriction fragments, as described in MATERIALS AND METHODS: PKCα, the 1.3-kb *Eco*RI fragment encoding a human cDNA clone (Coussens *et al.*, 1986); PKCβ, a 2.4-kb *Spe*I-*Hin*dIII fragment encoding a full length cDNA from rat brain (Housey *et al.*, 1988); PKCγ, a 2.6-kb *Eco*RI fragment encoding a full length cDNA from rat brain (Knopf *et al.*, 1987); PKCϵ, a 3.3-kb *Xho*I fragment encoding a full length cDNA from rat brain; PKCδ, a 0.2-kb *Eco*RI fragment derived from the V3 domain of rat brain PKCδ; PKCζ, a 0.2-kb *Eco*RI fragment derived from the V3 domain of rat brain PKCζ (Genetics Institute, Cambridge, MA). The predicted sizes of the PKC isoform-specific transcripts are indicated by the arrows, and the positions of the 28s and 18s ribosomal RNA markers are also indicated.

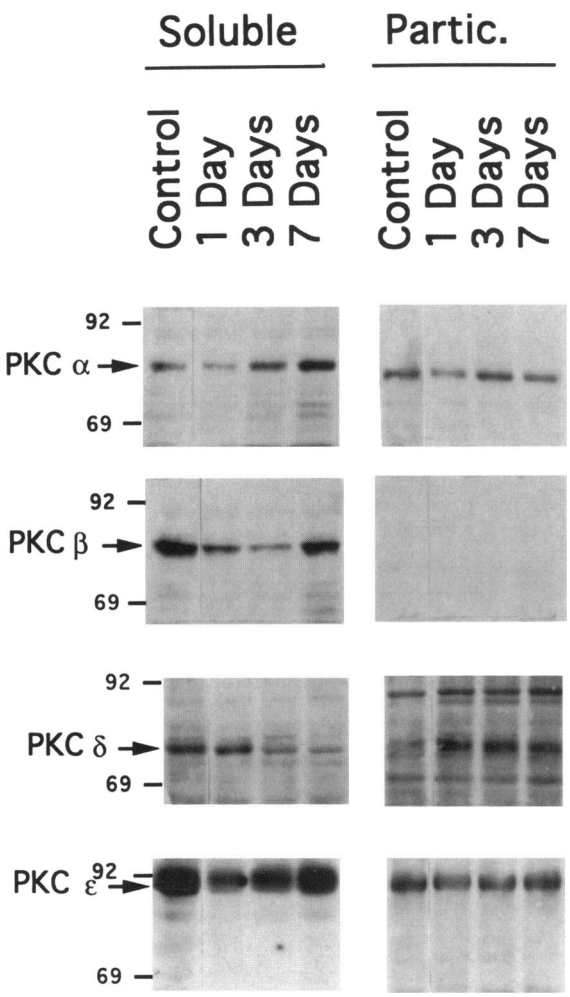

Figure 2. Expression of PKC isoforms at various times after treatment of PC12 cells with NGF. Cultures of PC12 cells were incubated in the absence or presence of NGF (50 ng/ml) for either 1, 3, or 7 days. Soluble and particulate subcellular fractions were prepared as described in MATERIALS AND METHODS, resolved by SDS-PAGE, and resulting replicate immunoblots were probed with PKC isoform-specific antibodies at the following dilutions: PKCα, 1:1000; PKCβ, 1:500; PKCδ, 1:500; PKCε, 1:500. Subsequently the blots were washed and probed with horseradish peroxide-conjugated secondary antibodies diluted to 1:10,000. The latter antibodies were raised in either sheep anti-mouse IgG in the case of the PKCα, β, and γ blots, or alternatively in donkey anti-rabbit IgG in the case of the PKCδ, ε, and ζ blots. The positions of migration of the proteins phosphorylase b (92 kDa) and bovine serum albumin (69 kDa) are indicated as molecular mass standards. Similar results were obtained in three independent experiments. For additional details see MATERIALS AND METHODS.

or were treated either with NGF or the mitogenic growth factor EGF. Subcellular fractions were prepared and subjected to immunoblotting with the PKCδ-specific antibody, as described above. Translocation of 74- to 76-kDa PKCδ-related proteins from the soluble to the particulate fraction was again seen with NGF but not EGF treatment of the cells (Figure 3).

Effects of NGF on TPA-induced Down-Regulation of PKC Isoforms

It was also of interest to examine the effects of NGF treatment on expression of these isoforms in the soluble and particulate fractions of cells treated with various concentrations of TPA (Figure 4). The extent of down-regulation of PKCs α and β from the soluble fractions of cells treated with 100 nM TPA was greater in the cells not exposed to NGF than in NGF-differentiated cells. The data also revealed a complete loss of immunologically reactive calcium-sensitive PKCs α and β in the soluble fractions of PC12 cells treated with 1000 nM TPA regardless of the presence of NGF. Exposure of PC12 cells to TPA also reduced the levels of PKCα in the particulate fraction, but some immunoreactive PKCα in this fraction persisted even after 7 days in the presence of 1000 nM TPA. This was observed both in the presence and absence of NGF treatment.

The calcium-independent PKCs δ and ε were also more resistant to down-regulation by a given concentration of TPA in cells that were treated with NGF. This phenomenon was especially apparent in both the soluble and particulate fractions when the cells were exposed to intermediate concentrations of TPA (i.e., 10–100 nM). An additional 73-kDa protein recognized in soluble extracts of the NGF-treated cells by the PKCδ-specific antibody was not down-regulated after 7 days of exposure to 1000 nM TPA (Figure 4); however, this 73-kDa protein was not recognized on replicate immunoblots with two independent PKCδ-specific antibodies, and furthermore, antibody recognition was not reduced in the presence of the PKCδ peptide used as the original immunogen (our unpublished observations). The identity of this NGF-induced 73-kDa protein is not known. The 72-kDa PKCζ protein was resistant to TPA-induced down-regulation, in both the absence or

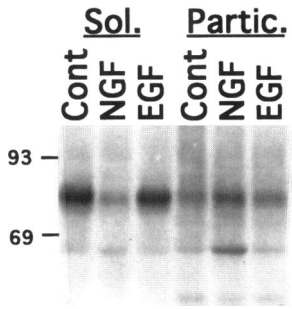

Figure 3. Effects of treatment of PC12 cells with various agents on expression of PKCδ. PC12 cells were treated for 7 days with NGF (50 ng/ml), EGF (10 ng/ml), or maintained in RPMI 1640 medium containing 1% horse serum (Cont). Subcellular fractions were prepared, and after protein determination, 100 μg of each cell extract was resolved by SDS-PAGE, and PKCδ was detected immunologically as described in MATERIALS AND METHODS and in the legend to Figure 2. Similar results were obtained in an independent experiment.

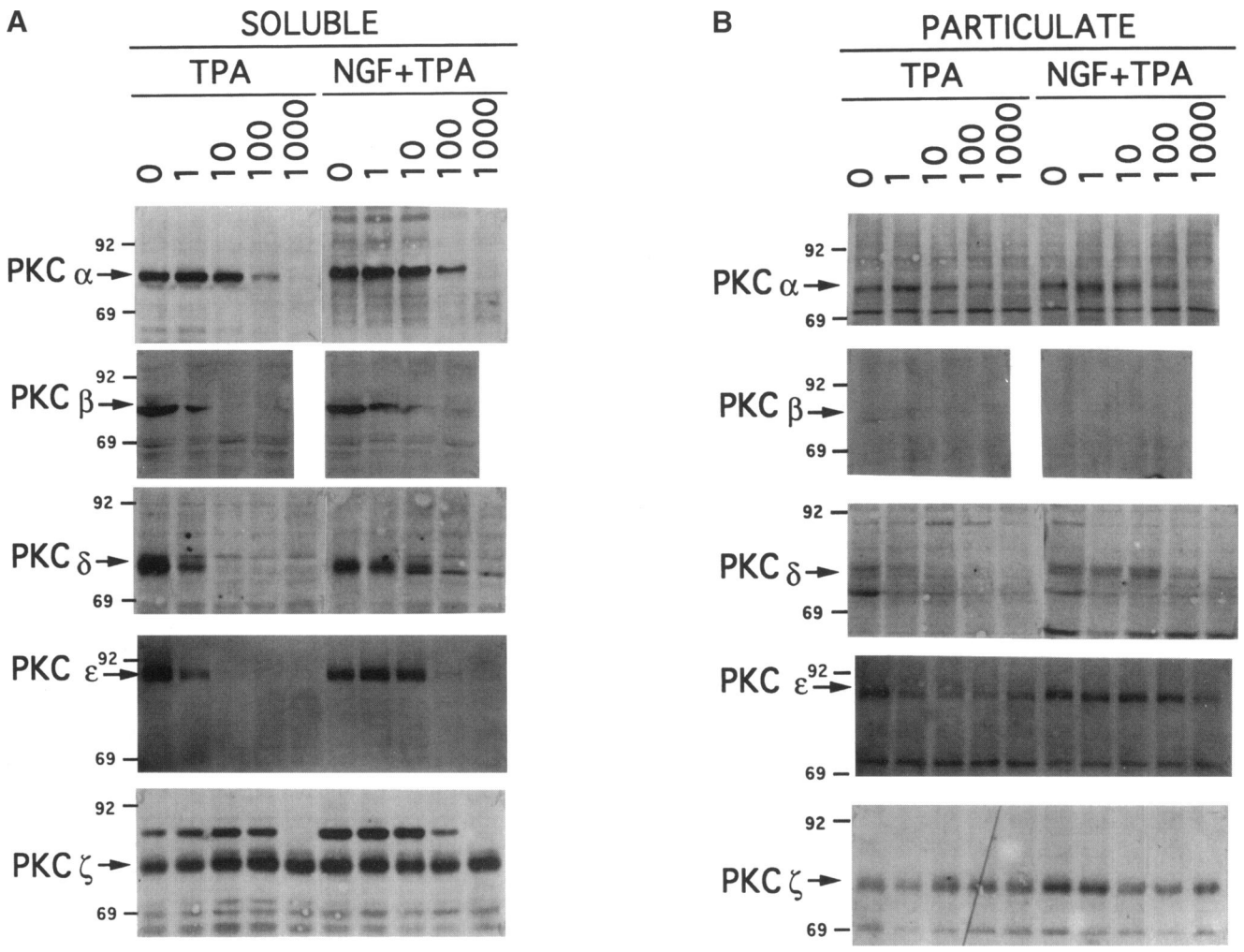

Figure 4. Effects of treatment of PC12 cells with TPA or TPA plus NGF. PC12 cells were treated with the solvent control dimethyl sulfoxide (0.1%) or alternatively with TPA at 1, 10, 100, or 1000 nM, for 7 days, either in the presence or absence of NGF, and SDS-PAGE immunoblotting was performed on both the soluble and particulate subcellular fractions, as described in MATERIALS AND METHODS. The migration of the molecular mass markers phosphorylase b (92 kDa) and bovine serum albumin (69 kDa) are indicated. (A) Soluble subcellular fractions and (B) particulate subcellular fractions. Similar results were obtained in three independent experiments.

presence of NGF (Figure 4). An 80-kDa protein seen with the PKCζ antibody is PKCα, because as demonstrated previously (Borner *et al.*, 1992), this antibody reacts with PKCα.

TPA Potentiates NGF-induced Neurite Outgrowth in PC12 cells

A previous study suggested that although TPA alone does not stimulate neurite outgrowth, it reduces the concentration of NGF required for neuritogenesis by PC12 cells (Burstein *et al.*, 1982). Because of this finding and the results shown in Figure 4, PC12 cells were exposed to NGF in the absence or presence of different concentrations of TPA (1–1000 nM), and the percentage of neurite-bearing cells in each set of cultures was compared at 2, 5, and 7 days of treatment. The results indicated both time- and dose-dependent effects of TPA in potentiating NGF-induced neuritogenesis (Figure 5). Thus, at 2 days of treatment, neurite formation was negligible in cells treated with only NGF, whereas cultures treated with NGF in the presence of 1 or 10 nM TPA contained >20% neurite-bearing cells. Neurite formation at 5 and 7 days of NGF exposure was also potentiated by 1 or 10 nM TPA. The maximal number of neurite-bearing PC12 cells (>75%) was observed after 7 days of NGF treatment in the continued presence of either 1 or 10 nM TPA. Interestingly, higher concentrations of TPA (100 or 1000 nM) were less effective in enhancing NGF-

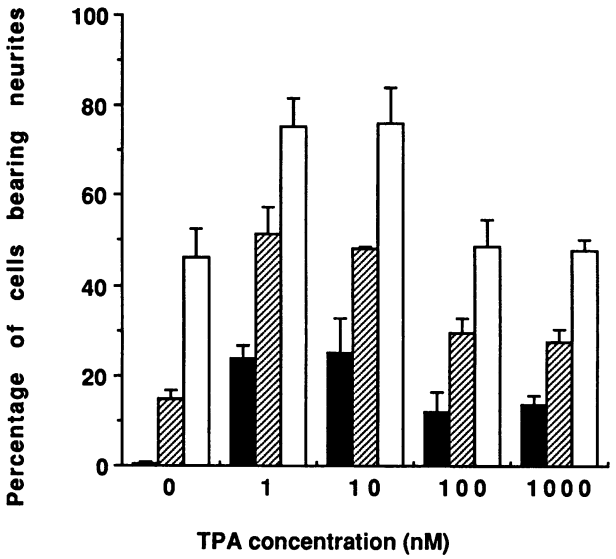

Figure 5. Enhancement of NGF-induced neurite outgrowth by TPA in PC12 cells. Cells were grown in triplicate 60-mm tissue culture plates, and were treated with NGF in the presence of TPA (1, 10, 100, or 1000 nM), or alternatively with NGF in the presence of the dimethyl sulfoxide solvent alone (0.1%) as a control (0 nM TPA). Neurite outgrowth was scored after 2 days (solid bars), 5 days (cross-hatched bars), and 7 days (open bars) as described in MATERIALS AND METHODS, and is expressed as the mean of triplicate determinations of the percentage of neurite-bearing cells. The error bars represent the SD. Qualitatively similar results were obtained in an additional experiment.

induced neurite formation and their effects were only transient (i.e., between 2 and 5 days).

DISCUSSION

NGF Differentially Regulates Individual PKC Isoforms in PC12 Cells

A previous investigation reported a 1.3- to 2-fold increase in PKC activity in permeabilized PC12 cells after a brief (1–5 min) incubation with NGF (Heasley and Johnson, 1989). Additional previous studies of short-term (less than 1 h) NGF-treated PC12 cells have demonstrated the translocation to the particulate subcellular fraction of PKCα (Kondratyev *et al.*, 1990) and PKCζ (Wooten *et al.*, 1994), and a selective activation of PKCϵ kinase activity (Ohmichi *et al.*, 1993). These rapid effects may be related to the early signal transduction events initiated by NGF binding to the Trk proto-oncogene receptor expressed in PC12 cells (Kaplan *et al.*, 1991); however, because NGF-induced neuritogenesis by PC12 cells occurs over a prolonged course (1–7 days) (Greene and Tischler, 1976), in the present study we examined the expression of several PKC isoforms at later time points. Our results suggest the possible involvement of one or more isoforms of PKC in the sustained events that produce functional neurites in these cells. Our finding that PC12 cells treated with NGF for 1 wk, the time at which neuritogenesis is maximal, exhibited a 1.8-fold increase in total PKC activity when compared with untreated control cells is generally consistent with a previous report (Wooten *et al.*, 1992). In addition, the present study suggests that this increase in total PKC activity reflects mainly an increased abundance of PKCα. Furthermore, we have found that changes in the levels of PKC α and β proteins in NGF-treated PC12 cells occur in parallel with changes in the abundance of the corresponding species of mRNA (our unpublished observations). Our findings on the profile of PKC isoforms expressed in untreated PC12 cells are in general agreement with one set of prior studies (Messing *et al.*, 1991; Roivainen *et al.*, 1993; Roivainen and Messing, 1993), yet differ significantly from findings in other studies in which PKCγ-related protein was detected in PC12 cells, with a polyclonal antiserum (Wooten, 1992; Wooten *et al.*, 1992). Our failure to detect the PKCγ protein may be a matter of sensitivity because in our studies the PKCγ mRNA species was barely detected on Northern blots (Figure 1). It is not clear why we did not detect the increased expression of PKCs βII and γ in NGF-treated PC12 cells described by Wooten and collaborators. This discrepancy is, however, not due to a failure of the antibodies used in the present study to recognize rat PKCs βI, βII, and γ, because positive results were obtained in control experiments using extracts of rat brain, and of mammalian, insect, or bacterial cells engineered to express these PKC isoforms (our unpublished observations).

The persistent expression of PKCs α, δ, ϵ, and ζ in cells treated with both NGF and TPA is consistent with a previous study (Lacal *et al.*, 1990), which found that neuronal differentiation of PC12 cells induced by the N-*ras* oncogene blunted the down-regulation of PKC activity and phorbol ester binding obtained when the cells were treated with phorbol-12, 13-dibutyrate. These effects may be related to the finding that differentiation of PC12 cells is accompanied by decreased calpain protease activity, which itself is due to increased expression of calpstatin, a cellular inhibitor of calpain activity (Oshima *et al.*, 1989). Perhaps NGF treatment blunts TPA-induced down-regulation of certain isoforms of PKC by reducing their rate of post-translational degradation (Young *et al.*, 1987) by calpain (Kishimoto *et al.*, 1989). Alternate explanations, such as increased de novo synthesis of these isoforms of PKC in NGF-treated PC12 cells, are also possible. Regardless of the mechanism, the blunting of TPA-induced down-regulation of PKC isoforms in NGF-treated PC12 cells is indicative of a potential functional involvement of one or more of these isoforms in neuritogenesis. Previous studies (Burstein *et al.*, 1982; Hall *et al.*, 1988; Glowacka and Wagner, 1990; Sigmund *et al.*, 1990; Glowacka *et al.*, 1992; Roivainen

et al., 1993), as well as the results in the present study, indicate that treatment with TPA increases the responsiveness of PC12 cells to NGF with respect to neurite outgrowth. The mechanism of this effect has, however, been unclear because TPA can both activate as well as down-regulate PKC isoforms. The observations that treatment of PC12 cells with the PKC inhibitor sphingosine (Hall *et al.*, 1988) or microinjection of a PKC-specific neutralizing antibody (Altin *et al.*, 1992) causes inhibition of NGF-induced neurite formation, are also consistent with the hypothesis that one or more isoforms of PKC play a critical role in NGF-induced neuritogenesis. Despite a report that NGF-treatment produces a rapid activation of PKCζ in PC12 cells (Wooten *et al.*, 1994), it is unlikely that the TPA-enhancement of NGF-evoked neuritogenesis in PC12 cells is mediated by PKCζ because this isoform does not bind to, or become activated by or down-regulated by, phorbol esters (Ono *et al.*, 1989; McGlynn *et al.*, 1992; Ways *et al.*, 1992).

PKCδ Is Selectively Activated during Neuritogenesis and Also Mediates Cellular Differentiation in Additional Cell Types

Intracellular translocation of PKC isoforms is commonly associated with allosteric activation by second messengers and tumor promoters (reviewed by Hug and Sarre, 1993). Our present findings are therefore consistent with the interpretation that the PKCδ isoform is activated in a highly specific fashion, and concurrently with NGF-evoked neuritogenesis. A recent study (Singh *et al.*, 1994) has shown that treatment of PC12 cells with bryostatin, a non-phorbol ester compound that down-regulates PKC activity in these cells, markedly reduces NGF-induced neuritogenesis. Also consistent with our present findings is the fact that exposure of PC12 cells to ethanol leads to an increased expression of PKCδ and also enhances NGF-induced neuritogenesis (Messing *et al.*, 1991; Roivainen *et al.*, 1993). The phosphorylation of PKCδ on tyrosine residues was also recently described (Denning *et al.*, 1993; Li *et al.*, 1994a,b). In this regard, it may be relevant that treatment of PC12 cells with orthovanadate, a specific inhibitor of tyrosine phosphatases, inhibits NGF- and basic fibroblast growth factor-evoked neuritogenesis (Wu and Bradshaw, 1993). The present study suggests that a selective activation of PKCδ may play a critical role in NGF-induced neuritogenesis.

PKCδ has also been linked to other differentiation pathways because transfection and stable overexpression of this isoform in a mouse myeloid cell line enhances TPA-induced monocytic differentiation (Mischak *et al.*, 1993b). PKCδ expression is also modified during the transformation of murine keratinocytes (Denning *et al.*, 1993), during the treatment of PKCδ-overexpressing mouse fibroblasts with TPA (Li *et al.*, 1994a), or in response to the treatment of a mouse myeloid cell line with platelet-derived growth factor (Li *et al.*, 1994b). In addition both PKCs δ and ε are translocated in murine erythroleukemia cells during differentiation induced by exposure of the cells to hexamethylene bisacetamide (Leng *et al.*, 1993). Furthermore, overexpression and activation of PKCδ in CHO and NIH3T3 cell cultures inhibits cell proliferation (Watanabe *et al.*, 1992; Mischak *et al.*, 1993a), which often correlates with differentiation. It is notable in this regard that in a rat neuroblastoma cell line, malignant transformation induced by the N-*myc* oncogene leads to a reduced level of PKCδ expression (Bernards, 1991). The compound bryostatin also has specific effects on PKCδ in murine keratinocytes that are consistent with a putative role for this PKC isoform in cornification in the squamous epithelium (Szallasi *et al.*, 1994). Taken together these findings suggest the possibility of a role for PKCδ in terminal cellular differentiation in a variety of cell types.

ACKNOWLEDGMENTS

This work was funded in part by a National Cancer Institute grant, CA-02656 (to I.B.W.); by an award from the Lucille Markey Charitable Trust (to I.B.W.); and by a grant from the National Institute of Neurologic Disease and Stroke NS16036 (to L.A.G.). K.R. O'D. was the recipient of a National Research Service Award CA09529-05 postdoctoral fellowship and of continuing support by the Colleen Giblin Charitable Foundation. We thank Drs. S. Jaken, W. Jiang, J. Knopf, W. Sossin, M. Ueffing, A. Ullrich, and G. Yancopoulous for valuable reagents and other assistance as well as H. Kwok and B. Feuer for voluntary technical assistance.

REFERENCES

Altin, J.G., Wetts, R., Riabowol, K.T., and Bradshaw, R.A. (1992). Testing the in vivo role of protein kinase C and c-*fos* in neurite outgrowth by microinjection of antibodies into PC12 cells. Mol. Biol. Cell *3*, 323–333.

Bernards, R. (1991). N-*myc* disrupts protein kinase C-mediated signal transduction in neuroblastoma. EMBO J. *10*, 1119–1125.

Borner, C., Guadagno, S.N., Fabbro, D., and Weinstein, I.B. (1992). Expression of four protein kinase C isoforms in rat fibroblasts. J. Biol. Chem. *267*, 12892–12899.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal. Biochem. *76*, 248–254.

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

Chao, M.V. (1992). Growth factor signaling: where is the specificity? Cell *68*, 995–997.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA *81*, 1991–1995.

Coussens, L., Parker, P.J., Rhee, L., Yang-Feng, T.L., Chen, E., Waterfield, M.D., Francke, U., and Ullrich, A. (1986). Multiple and distinct forms of bovine and human protein kinase C suggest diversity in cellular signalling pathways. Science *233*, 859–864.

Denning, M.F., Dlugosz, A.A., Howett, M.K., and Yuspa, S.H. (1993). Expression of an ondogenic rasHa gene in murine keratinocytes induces tyrosine phosphorylation and reduced activity of protein kinase C delta. J. Biol. Chem. *268*, 26079–26081.

Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabeling DNA. Anal. Biochem. *132*, 6–13.

Glowacka, D., Ginty, D.D., and Wagner, J.A. (1992). Synergistic effects of nerve growth factor and phorbol 12-myristate 13-acetate on rapid motility and process formation in PC12 cells: the role of laminin. J. Neurosci. Res. *31*, 263–272.

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.

Greene, L.A., and Tischler, A.S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA *73*, 2424–2428.

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

Halegoua, S., Armstrong, R.C., and Kremer, N.E. (1991). Dissecting the mode of action of nerve growth factor. Curr. Top. Microbiol. Immunol. *165*, 119–170.

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.

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.

Hidaka, H., Tanaka, T., Onoda, K., Hahiwara, M., Watanabe, M., Ohta, H., Ito, Y., Tsurudome, M., and Yoshida, T. (1988). Cell type-specific expression of protein kinase C isozymes in the rabbit cerebellum. J. Biol. Chem. *263*, 4523–4526.

Housey, G.M., Johnson, M.D., Hsiao, W.L.-W., O'Brian, C.A., Murphy, J.P., Kirschmeier, P., and Weinstein, I.B. (1988). Overproduction of protein kinase C causes disordered growth control in rat fibroblasts. Cell *52*, 343–354.

Hug, H., and Sarre, T.F. (1993). Protein kinase C isoenzymes: divergence in signal transduction? Biochem. J. *291*, 329–343.

Kaplan, D.R., Martin-Zanca, D., and Parada, L.F. (1991). Tyrosine phosphorylation and tyrosine kinase activity of the *trk* proto-oncogene product induced by NGF. Nature *350*, 158–160.

Khaled, Z., Rideout, D., O'Driscoll, K.R., Petrylak, D., Cacace, A., Patel, R., Chiang, L.C., Rotenberg, S., and Stein, C.A. (1995). Effects of suramin-related and other clinically therapeutic polyanions on protein kinase C activity. Clin. Cancer Res. *1*, 113–122.

Kishimoto, A., Mikawa, K., Hashimoto, K., Yasuda, I., Tanaka, S., Tominaga, M., Kuroda, T., and Nishizuka, N. (1989). Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain). J. Biol. Chem. *264*, 4088–4092.

Knopf, J.L., Lee, M.-H., Swultzman, L.A., Kriz, R.W., Loomis, C.R., Hewick, R.M., and Bell, R.M. (1987). Cloning and expression of multiple protein kinase C cDNAs. Cell *46*, 491–502.

Kondratyev, A.D., Popova, O.N., Severin, S.E., Choladze, M.A., Shmyrev, I.I., Tubasheva, I.A., Zotova, E.E., Posypanova, G.A., and Severin, E.S. (1990). Nerve growth factor stimulates protein kinase C translocation in PC12 cells. FEBS Lett. *264*, 75–77.

Lacal, L.C., Cuadrado, A., Jones, J.E., Trotta, R., Burstein, D.E., Thomson, T., and Pellicer, A. (1990). Regulation of protein kinase C activity in neuronal differentiation induced by the N-*ras* oncogene in PC-12 cells. Mol. Cell. Biol. *10*, 2983–2990.

Leach, K.L., Powers, E.A., McGuire, J.C., Dong, L., Kiley, S.C., and Jaken, S. (1988). Monoclonal antibodies specific for type 3 protein kinase C recognize distinct domains of protein kinase C and inhibit in vitro functional activity. J. Biol. Chem. *263*, 13223–13230.

Leng, L., Yu, F., Dong, L., Busquets, X., Osada, S., Richon, R., Marks, P.A., and Rifkind, R.A. (1993). Differential modulation of protein kinase C isoforms in erythroleukemia during induced differentiation. Cancer Res. *53*, 5554–5558.

Levi, A., and Alema, S. (1991). The mechanism of action of nerve growth factor. Annu. Rev. Pharmacol. Toxicol. *31*, 205–228.

Levi-Montalcini, R., and Angeletti, P.U. (1968). Nerve growth factor. Physiol. Rev. *48*, 1359–1362.

Li, W., Mischak, H., Yu, J.-C., Wang, L.-M., Mushinski, J.F., Heidaran, M.A., and Pierce, J. (1994a). Tyrosine phosphorylation of PKC-δ in response to its activation. J. Biol. Chem. *269*, 2349–2352.

Li, W., Yu, J.-C., Michieli, P., Beeler, J.F., Ellmore, N., Heidaran, M.A., and Pierce, J. (1994b). Stimulation of the platelet-derived growth factor β receptor signaling pathway stimulates protein kinase C-δ. Mol. Cell. Biol. *14*, 6727–6735.

McGlynn, E., Liebetanz, J., Reutener, S., Wood, J., Lydon, N.B., Hofstetter, H., Vanek, M., Meyer, T., and Fabbro, D. (1992). Expression and partial characterization of rat protein kinase C-δ and protein kinase C-ζ in insect cells using recombinant baculovirus. J. Cell. Biochem. *49*, 239–250.

Messing, R.O., Petersen, P.J., and Henrich, C.J. (1991). Chronic ethanol exposure increases levels of protein kinase C δ and ε and protein kinase C-mediated phosphorylation in cultured neural cells. J. Biol. Chem. *266*, 23428–23432.

Mischak, H., Goodnight, J.A., Kolch, W., Martiny-Baron, G., Schaechtle, C., Kazanietz, M.G., Blumberg, P.M., Pierce, J.H., and Mushinski, J.F. (1993a). Overexpression of protein kinase C-δ and -ε in NIH3T3 cells induces opposite effects on growth, morphology, anchorage dependence, and tumorigenicity. J. Biol. Chem. *268*, 6090–6096.

Mischak, H., Pierce, J.H., Goodnight, J.A., Kazanietz, M.G., Blumberg, P.M., and Mushinski, J.F. (1993b). Phorbol ester-induced myeloid differentiation is mediated by protein kinase C-α and -δ and not by protein kinase C-βII, -ε, -ζ and -η. J. Biol. Chem. *268*, 20110–20115.

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

Nishizuka, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science *258*, 607–614.

O'Brien, C.A., Liskamp, R.M., Solomon, D.H., and Weinstein, I.B. (1985). Inhibition of protein kinase C by tamoxifen. Cancer Res. *45*, 2462–2465.

Ono, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U., and Nishizuka, Y. (1989). Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger–like sequence. Proc. Natl. Acad. Sci. USA. *86*, 4868–4871.

Ohmichi, M., Zhu, G., and Saltiel, A.R. (1993). Nerve growth factor activates calcium-insensitive protein kinase C, -ε in PC12 rat pheochromocytoma cells. Biochem. J. *295*, 767–772.

Oshima, M., Koizumi, S., Fujita, K., and Guroff, G. (1989). Nerve growth factor-induced decrease in the calpain activity of PC12 cells. J. Biol. Chem. *264*, 20811–20816.

Raffioni, S., Bradshaw, R.A., and Buxser, S.E. (1993). The receptors for nerve growth factor and other neurotrophins. Annu. Rev. Biochem. *62*, 823–850.

Roivainen, R., McMahon, T., and Messing, R.O. (1993). Protein kinase C isoforms that mediate enhancement of neurite outgrowth by ethanol and phorbol esters in PC12 cells. Brain Res. *624*, 85–93.

Roivainen, R., and Messing, R.O. (1993). The phorbol derivatives thymeleatoxin and 12-deoxyphorbol-13-*O*-phenylacetate-10-acetate cause translocation and down-regulation of multiple protein kinase C isozymes. FEBS Lett. *319*, 31–34.

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.

Sambrook, J., Fritsch, E., and Maniatis, T. (ed.) (1989). Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schaap, D., Parker, P.J., Bristol, A., Kriz, R., and Knopf, J. (1989). Unique substrate specificity and regulatory properties of PKC-epsilon: a rationale for diversity. FEBS Lett. *243*, 351–357.

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

Singh, K.R., Taylor, L.K., Campbell, X.Z., Fields, A.P., and Neet, K.E. (1994). A bryostatin-sensitive protein kinase C required for nerve growth factor activity. Biochemistry *33*, 542–551.

Solomon, D.H., O'Driscoll, K.R., Sosne, G., Weinstein, I.B., and Cayre, Y. (1991). 1α,25-Dihydroxyvitamin D3-induced regulation of protein kinase C gene expression during HL-60 cell differentiation. Cell Growth Differ. *2*, 187–194.

Sossin, W.S., and Schwartz, J.H. (1992). Selective activation of Ca^{2+}-activated PKCs in *Aplysia* neurons by 5-HT. J. Neurosci. *12*, 1160–1168.

Szallasi, Z., Smith, C.B., Pettit, G.R., and Blumberg, P.M. (1994). Differential regulation of protein kinase C isozymes by bryostatin 1 and phorbol 12-myristate 13-acetate in HIN3T3 fibroblasts. J. Biol. Chem. *269*, 2118–2124.

Tanaka, C., and Saito, N. (1992). Localization of subspecies of protein kinase C in the mammalian central nervous system. Neurochem. Int. *21*, 499–512.

Togari, A., Baker, D., Dickens, G., and Guroff, G. (1983). The neurite-promoting effect of fibroblast growth factor on PC12 cells. Biochem. Biophys. Res. Comm. *114*, 1189–1193.

Watanabe, T., Ono, Y., Taniyama, Y., Hazama, K., Igarashi, K., Ogita, K., Kikkawa, U., and Nishizuka, Y. (1992). Cell division arrest induced by phorbol ester in CHO cells overexpressing protein kinase C-delta subspecies. Proc. Natl. Acad. Sci. USA *89*, 10159–10163.

Ways, D.K., Cook, P.P., Webster, C., and Parker, P.J. (1992). Effect of phorbol esters on protein kinase C-zeta. J. Biol. Chem. *267*, 4799–4805.

Weinstein, I.B., Borner, C.M., Krauss, R.S., O'Driscoll, K., Choi, P.M., Morotomi, M., Hoshina, S., Hsieh, L.L., Tchou-Wong, K.M., Guadagno, S.N., Ueffing, M., and Guillem, J.G. (1991). The concept of multistage carcinogenesis as a progressive disorder in signal transduction. In: Origins of Human Cancer: A Comprehensive Review, ed. J. Brugge, T. Curran, E. Harlow, and F. McCormick, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 113–124.

Wooten, M.W. (1992). Differential expression of PKC isoforms and PC12 cell differentiation. Exp. Cell Res. *199*, 111–119.

Wooten, M.W., Seibenhener, M.L., Soh, Y., Ewald, S.J., White, K.R., Lloyd, E.D., Olivier, A., and Parker, P.J. (1992). Characterization and differential expression of protein kinase C isoforms in PC12 cells, differentiation parallels an increase in PKC betaII. FEBS Lett. *298*, 74–78.

Wooten, M.W., Zhou, G., Seibenhener, M.L., and Coleman, E.S. (1994). A role for ζ protein kinase C in nerve growth factor-induced differentiation of PC12 cells. Cell Growth Differ. *5*, 395–403.

Wu, Y.Y., and Bradshaw, R.A. (1993). Effect of nerve growth factor and fibroblast growth factor on PC12 cells: inhibition by orthovanadate. J. Cell. Biol. *121*, 409–422.

Young, S., Parker, P.J., Ullrich, A., and Stabel, S. (1987). Down-regulation of protein kinase C is due to an increased rate of degradation. Biochem. J. *244*, 775–779.