A Bryostatin-Sensitive Protein Kinase C Required for Nerve Growth Factor Activity†

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ABSTRACT: Nerve growth factor (NGF) stimulates rat pheochromocytoma cells (PC12) to differentiate into a neuronal-like cell that exhibits neurite extensions. The role of protein kinase C in signal transduction has been examined in PC12 cells treated with phorbol 12-myristate 13-acetate (PMA) and bryostatin, a macrocyclic lactone that activates protein kinase C at both the nuclear and the plasma membranes [Hocevar, B. A., & Fields, A. P. (1991) *J. Biol. Chem. 266*, 28–33]. In contrast to PMA down-regulation [Reinhold, D. S., & Neet, K. E. (1989) *J. Biol. Chem. 264*, 3538–3544], chronic (24 h) treatment with bryostatin blocked the formation of neurites in response to NGF or basic fibroblast-derived growth factor stimulation, but, like PMA, bryostatin did not block the induction of c-*fos* or c-*jun* protooncogenes by NGF. Chronic bryostatin treatment down-regulated protein kinase C activity in the cytosolic, membrane, and nuclear fractions. Acute (60 min) bryostatin or NGF treatment activated cytosolic and nuclear protein kinase C activity, suggesting possible translocation to the nucleus. Bryostatin did not induce neurite outgrowth, either alone or in combination with PMA. Thus, the bryostatin-sensitive protein kinase C is distinct from PMA- or K252a-sensitive kinases previously described. The bryostatin-sensitive protein kinase C is necessary, but not sufficient, for neurite outgrowth and acts in the nucleus in a manner independent of c-*fos* and c-*jun* transcription.

Nerve growth factor (NGF)[1] plays a role in the development and maintenance of vertebrate neurons (Levi-Montalcini & Angeletti, 1968; Greene & Shooter, 1980; Yankner & Shooter, 1982; Levi-Montalcini, 1987). The rat pheochromocytoma cell line PC12 (Greene & Tischler, 1976) responds to NGF by undergoing a number of changes such as cell-surface ruffling (Connolly et al., 1979), protein phosphorylation (Halegoua & Patrick, 1980), increased transcription of some oncogenes (Greenberg et al., 1985; Wu et al., 1989), and the eventual extension of neurite processes (Greene & Tischler, 1976). This cell line is a model system for NGF action, and has the advantage that unlike neurons it does not require NGF to survive in culture.

NGF induces the phosphorylation of a number of proteins in PC12 cells (Halegoua & Patrick, 1980), including some tyrosine residues (Maher, 1988). The high-affinity NGF receptor is phosphorylated on tyrosine residues (Radeke & Feinstein, 1991; Meakin & Shooter, 1991) due to the presence of the *trk* protooncogene protein tyrosine kinase that has been identified as a component of the receptor (Kaplan et al., 1991; Klein et al., 1991). Several responsive protein kinases have now been described that may mediate the signal. Upon NGF treatment, mitogen-activated protein (MAP) kinases (or extracellular regulated kinase, ERK) are rapidly activated by phosphorylation on both tyrosine and serine/threonine residues (Landreth & Williams, 1987; Sturgill et al., 1988; Miyasaka et al., 1990, 1991; Schanen-King et al., 1991; Gomez & Cohen, 1991; Wood et al., 1992; Ohmichi et al., 1992) by MAP kinase kinase (Nakielny et al., 1992; Rossomando et al., 1992; Seger et al., 1992). MAP kinase then phosphorylates other substrates, including other kinases and microtubule-associated protein II. Ribosomal S6 protein kinase II ($p90^{rsk}$) is activated after NGF stimulation due to Ser/Thr phosphorylation by a kinase that is probably MAP kinase (Sturgill et al., 1988; Wood et al., 1992). The B-raf (Oshima et al., 1991) and raf-1 (Wood et al., 1992; Ohmichi et al., 1992) oncogene products are also rapidly stimulated by NGF and may act upstream of MAP kinase. Protein kinase N (PKN) is activated by NGF and specifically inhibited by purine analogs (Rowland et al., 1987; Rowland-Gagne & Greene, 1990). Rapid activation (Heasley & Johnson, 1989a,b) and translocation (Kondratyev et al., 1990) of protein kinase C (PKC) lead in one or more steps to phosphorylation and activation of calmodulin kinase III (Nsp 100 kinase), phosphorylation of translational elongation factor 2 (EF2) (Hama et al., 1986; Nairn et al., 1987), and, possibly, activation of MAP, raf-1, and ribosomal S6 protein kinases (Wood et al., 1992; Troppmair et al., 1992). Only two of these kinases have clearly been demonstrated to be *required* for NGF action, namely, the *trk* protein tyrosine kinase (Kaplan et al., 1991; Klein et al., 1991) and the protein *Ser*/Thr kinase PKN (Volonte et al., 1989).

Description of the role of PKC stimulation in the NGF-induced biological responses of PC12 cells has produced

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[1] Abbreviations: NGF, nerve growth factor; FGF, fibroblast-derived growth factor; PMA, phorbol 12-myristate 13-acetate; PC12 cells, rat pheochromocytoma cell line; PKC, protein kinase C; BS-PKC, bryostatin-sensitive protein kinase C; PS-PKC, phorbol-sensitive protein kinase C; MAP kinase, mitogen-activated protein kinase; DTT, dithiothreitol; DMEM, Delbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide.

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somewhat confusing results. Inhibition of NGF-induced neurite outgrowth by sphingosine and its relief by phorbol 12-myristate 13-acetate (PMA) treatment have been attributed to effects on protein kinase C (Hall et al., 1988). We, however, reported that reduction of 99% of measurable PKC activity by down-regulation with PMA did not retard neurite outgrowth or induction (Reinhold & Neet, 1989). The same phorbol ester down-regulation paradigm has subsequently been reported to have little or not effect on other NGF-induced responses in PC12 cells such as mRNA levels of the ornithine decarboxylase, c-*fos*, SCG10, d5, NGF1A (TIS8, d2), NGF1B (TIS1), or TIS12 genes (Sigmund et al., 1990; Damon et al., 1990; Altin et al., 1991). Tyrosine hydroxylase activation is also not affected by PMA down-regulation of PKC (Cahill et al., 1989), even though the hydroxylase can be activated by protein phosphorylation with protein kinase C (Cremins et al., 1986). Similarly in sympathetic neurons, Campenot et al. (1991) have shown that down-regulation by phorbol esters modulates the subsequent NGF-induced response to neurites but does not prevent it. In this same system, distal application of sphingosine caused retraction/degeneration of neurites (Campenot et al., 1991). Some effect of microinjection of antibodies to PKC into PC12 cells has also been reported (Altin et al., 1992).

Our current study was designed to delineate the involvement of the Ca^{2+}/phospholipid-dependent protein kinase C in neurite outgrowth by examining the effects of several inhibitors and down-regulation paradigms on several PC12 cell responses. We have utilized bryostatin, a macrocyclic lactone (Pettit et al., 1982) that activates PKC by binding to the regulatory domain (Kraft et al., 1986) and causes down-regulation of PKC with chronic treatment (Fields, 1988). In several cell lines (HL-60, FDC-P1, NIH/3T3, and K562), bryostatin causes translocation of a Ca^{2+}/phospholipid-dependent PKC activity to the nuclear membrane and phosphorylation of the nuclear envelope protein lamin B (Fields et al., 1988, 1989, 1990; Hocevar & Fields, 1991; Hocevar et al., 1992). In addition, we have compared these bryostatin effects to those of PMA, sphingosine, and K252a. We report here the observation of a bryostatin-sensitive protein kinase C isotype in PC12 cells that is necessary for neurite outgrowth, is distinct from PMA-sensitive kinases, and is activated in both cytosol and nuclei.

MATERIALS AND METHODS

Materials. D-Sphingosine was purchased from Sigma and dissolved at 20 mM in ethanol. PMA was purchased from Sigma. Bryostatin-1 and its inactive analog, bryostatin-13, were a kind gift of Dr. W. S. May, Johns Hopkins University. The inhibitor K252a was a gift of Y. Matsuda, Kyowa Hakko Kogyo Co., Ltd., Tokyo.

Cell Lines and Culture Conditions. PC12 cells, from a line initiated by Greene and Tischler (1976), were grown in Dulbecco's modified Eagle's medium (DMEM) enriched with 10% horse serum, 5% fetal bovine serum, 4.5 mg/mL glucose, 4.0 mM L-glutamine, 100 units/mL penicillin, 100 units/mL streptomycin, and 100 units/mL fungizone at 37 °C in a humid atmosphere of 10% CO_2. Cells were grown on Falcon tissue culture dishes (150 × 25 mm), subcultured every 3 or 4 days, and usually split at a ratio of 1:3. The β subunit of NGF was purified from the 7S NGF as previously described (Woodruff & Neet, 1986).

Neurite Assay. A modified bioassay in defined media was employed because of faster response time for neurites (Reinhold & Neet, 1989). Cells were harvested and washed 3 times in DMEM with 100 μM putresine, 4.0 mM L-glutamine, 20.0 nM progesterone, 5.0 μg/mL insulin, 5.0 μg/mL transferrin, 5.0 ng/mL sodium selenite, 100 units/mL penicillin, 100 units/mL streptomycin, and 100 units/mL fungizone. The cells were diluted to 30 000 cells/mL, and 100 μL of the cell suspension was added to collagen-coated wells of a 96-well plate. The remaining 100-μL volume of the well was made up of additional media, NGF, and the appropriate reagent. At various times after NGF addition (usually 2–3 days), the cells were counted to determine the percentage of neurite-bearing cells. At least 200 cells were counted per treatment. Only single cells were counted, and a cell was scored positive for neurite extension if it had at least one process longer than a cell body. Results are reported either as percentage of the cells that developed neurites (percent neurite response) or, so that comparisons of various additions could be made in some cases, as percentage relative to control cells that had been treated with NGF without the experimental condition (percent control); in the latter case, the percent cells responding in the control is given in the appropriate legend. Representative experiments are reported for an experimental paradigm that had been repeated 3 or more times with similar results.

The sphingosine stock solution (20 mM) in ethanol was diluted in DMEM with an equimolar concentration of fatty acid free bovine serum albumin (Sigma), and control assays contained the appropriate concentrations of ethanol and bovine serum albumin. In sphingosine bioassays, at 24 h after treatment, additional sphingosine was added to replace what was metabolized. PMA (5 mM), bryostatin (0.1 mM), and K252a (2 mM) were kept as stocks in DMSO and diluted directly into cell cultures without filtering to avoid loss of material on glass or plastic surfaces. Control assays contained appropriate concentrations of DMSO. For down-regulation, cells were incubated with 1 μM PMA or >100 nM bryostatin for 24 h prior to NGF treatment. Sphingosine and stimulatory concentrations of bryostatin or PMA were added to the cells simultaneously with NGF.

Protein Kinase C Assays. Protein kinase C was assayed either in whole cell lysates or in subcellular fractions. For determinations in whole cell lysates, washed cells were disrupted by sonication for 20 s in lysis buffer A [20 mM Tris-HCl, pH 7.5, 250 mM sucrose, 5 mM EGTA, 2.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.01% leupeptin, and 0.4 mM phenylmethanesulfonyl fluoride] containing 0.1% NP40, and assays were run on aliquots of the solubilized cell lysate without further purification. Subcellular fractions were prepared by washing PC12 cells in Hank's balanced salt solution (HBSS, Gibco), homogenization for 2 min in cold lysis buffer A, and centrifugation at 3000 rpm for 10 min in a microcentrifuge. The first pellet is the nuclear fraction. The supernatant was centrifuged at 40 000 rpm for 70 min to obtain the cytosolic and membrane (pellet) fractions. Membrane and nuclear fractions were solubilized in 1% NP40 in lysis buffer A and centrifuged at 13 000 rpm for 10 min in a microcentrifuge. Each fraction was then applied to a DEAE column, washed with 3 mL of washing buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, and 1 mM DTT), and then eluted with washing buffer containing 0.2 M NaCl to obtain protein kinase C fractions. All steps were carried out at 0–4 °C.

Ca^{2+}/phospholipid-dependent protein kinase activity (Hovis et al., 1986; Reinhold & Neet, 1989) was determined by incubating 50 μL of the enzyme fraction for 3 min at 30 °C in 50 μL of reaction mixture containing 1 μCi of [γ-^{32}P]ATP

(NEN), with final concentrations of 1 mg/mL histone III S (Sigma), 100 μM ATP, 0.125 mg/mL phosphatidylserine, 1.5 mM $CaCl_2$, 20 mM $MgCl_2$, 5–8 μg/mL diolein, 2 mM DTT, and 50 mM Tris-HCl at pH 7.5. The reaction was stopped with 20% trichloroacetic acid and filtered on a Millipore HA membrane, and the membrane was counted in a scintillation counter. Protein kinase C activity was determined by subtraction of cpm values in a reaction mixture without cofactors (phosphatidylserine, diolein, and $CaCl_2$), but containing 3 mM EGTA, from those cpm values obtained in the presence of cofactors. Note that the substrate used in all of these PKC assays was histone and may not entirely reflect the relative activities with functional endogenous protein substrates. Protein concentrations were determined by the Coomassie Blue method (BioRad). Each kinase assay was run in duplicate or triplicate and averaged; occasional outliers were discarded. With whole cell lysates, the activity was normalized to the protein concentration, and the results are reported as specific activity. With the subcellular fractions, the raw values were normalized to total activity recovered in each experiment, each subcellular fraction was averaged, and the results were multiplied by the average amount of protein found in each fraction (determined from three experiments); thus, the values represent the total cellular PKC activity in each subcellular fraction, normalized to the total cellular activity in each experiment. All conditions were run in two to five independent experiments, and data are presented as mean ± the standard deviation. Student's *t*-test was used for statistical comparison.

Slot Blot Assays for mRNA. For each experiment, about 30 million cells were harvested after the indicated treatment of the cells, total RNA was extracted in guanidine isothiocyanate, and 5 μg was blotted on nitrocellulose in a slot blot apparatus (SH1, Ann Arbor Plastics, Inc.). Specific cDNA probes were labeled by nick-translation (U.S. Biochemical kit) with α-^{32}P-labeled nucleotides, hybridized to the filters with appropriate blocking/washes, and the level of specific mRNA was assessed by autoradiography (Davis et al., 1986; Sambrook et al., 1989). Control experiments for mRNA loading were done with the Tα1 tubulin mRNA which has been shown to have a minimal response at these time intervals (Greenberg et al., 1985). Murine cDNA probes were obtained as follows: c-*jun* from the American Type Culture Collection; v-*fos* from Dr. R. W. Hanson, Case Western Reserve University; and Tα1 tubulin from Dr. M. Oblinger, Chicago Mecical School.

RESULTS

Effects of Bryostatin Down-Regulation on NGF- or FGF-Induced Neurite Outgrowth. In our defined media bioassay, NGF-induced neurite outgrowth in PC12 cells proceeds relatively quickly, reaching a maximum between 24 and 48 h (Reinhold & Neet, 1989). PC12 cells were chronically treated with bryostatin for 24 h prior to NGF treatment in order to down-regulate PKC. In contrast to PMA down-regulation, this bryostatin treatment inhibited NGF-induced neurite outgrowth (Figure 1A) with an IC_{50} of 100–200 nM and complete inhibition by 1 μM. The inactive analog of bryostatin-1, bryostatin-13, did not inhibit NGF-induced neurite outgrowth. Bryostatin-1 down-regulation caused only a slight decrease in viability over controls (data not shown). This neurogenic inhibition was both time- and concentration-dependent. When bryostatin and NGF were added simultaneously for the 24-h treatment (data not shown), the IC_{50} value was much higher at 24 h, decreased significantly by 48 h, but was still 3-fold higher than the IC_{50} value in the down-regulation study with bryostatin preincubation for 24 h prior to NGF treatment for 24 h (Figure 1A). This result suggests that the bryostatin-sensitive kinase is necessary throughout the differentiation process, not just at the initiation of the NGF response.

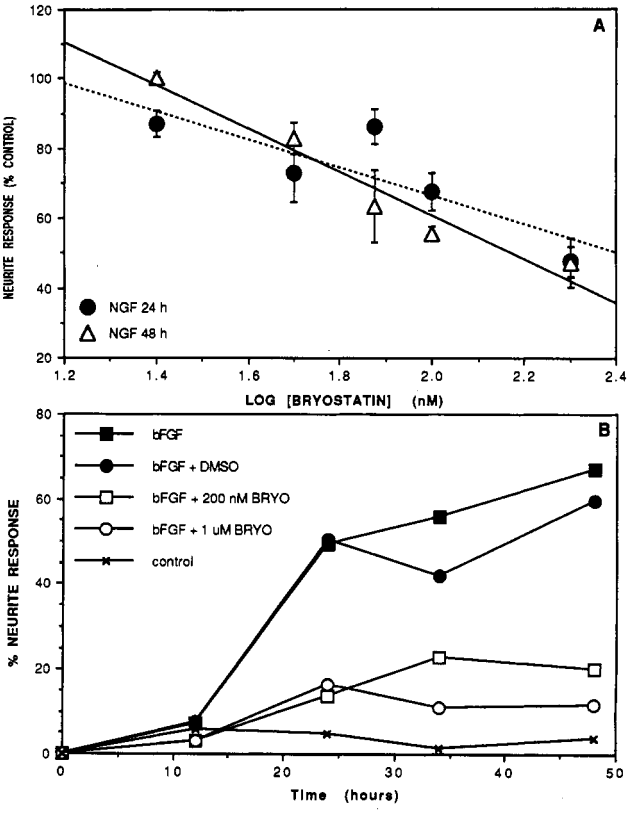

FIGURE 1: Effect of chronic bryostatin treatment on neurite outgrowth in PC12 cells. (A) NGF-treated cells. PC12 cells in defined media were treated with the indicated concentration of bryostatin-1 for 24 h prior to NGF treatment. The appropriate concentration of the inactive analog bryostatin-13 plus NGF was used as a control. Cells bearing neurites were counted at 24 h (closed symbols, dashed line) or 48 h (open symbols, solid line) after NGF addition. A higher concentration (1 μM) of bryostatin reduced the neurite extension after NGF treatment to 34% of control at 24 h and 4.5% at 48 h (data not shown). The mean of two experiments (±range) is reported as the percent of control cells that did not receive bryostatin; in the control plates, 38.8 ± 2.4% and 55.5 ± 2% (n = 14) cells extended neurites at 24 and 48 h, respectively. (B) FGF-treated cells. PC12 cells in defined medium were treated with 0, 0.2 (□), or 1.0 (○) μM bryostatin-1 in 0.02% DMSO for 24 h prior to addition of 250 ng/mL bFGF. Controls without bryostatin were run without bFGF (×), with (■), and with bFGF plus DMSO (●). Cells bearing neurites were counted at the indicated times after bFGF addition and reported as the percentage of the cell population with neurites.

Since FGF is also capable of inducing neurites in PC12 cells through its own cell-surface receptor (Togari et al., 1985; Rydel & Greene, 1987), we investigated the effects of down-regulation of PKC by either PMA or bryostatin on the signal transduction pathway of this growth factor. PMA down-regulation had no effect on FGF-induced neurite outgrowth (95% of control; data not shown), in agreement with other results (Damon et al., 1990; Sigmund et al., 1990) and consistent with the lack of effect upon NGF neuritogenesis. However, chronic treatment with bryostatin led to a block of the response to FGF (Figure 1B) with an IC_{50} of less than 200 nM. Thus, the bryostatin-sensitive step must occur subsequent to the convergence of the FGF and NGF pathways to neurite production.

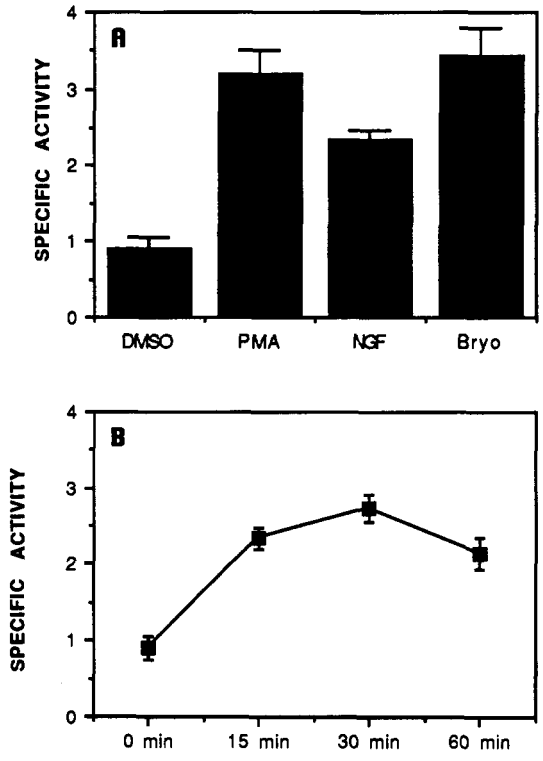

FIGURE 2: PKC activity in PC12 cell lysate. PC12 cells were incubated at 37 °C in DMEM/serum media containing 0.015% DMSO (control) or in media containing the same concentration of DMSO plus additions. The cells were then sonicated in NP40, and PKC activity was determined by histone phosphorylation. The specific activity is expressed as cpm per 3 min per milligram of protein ($\times 10^{-4}$) ● SD. All values were significantly different from the appropriate control with $p < 0.01$. (A) Acute (15 min) treatment with PMA (100 nM), NGF (2 nM), or bryostatin (10 nM). (B) Time course after treatment with NGF (2 nM).

Stimulation of Protein Kinase C Activity in Whole Cell Lysates. The role of PKC in neurite outgrowth was inititally assessed by examining PKC activity in cell lysates and subcellular fractions of PC12 cells treated with PKC activators NGF, PMA, and bryostatin. PKC specific activity in the PMA-treated cell lysate was stimulated 3.5-fold at 15 min (Figure 2A), consistent with earlier observations that PMA is a strong activator of overall PKC activity in PC12 (Hama et al., 1986; Heasley & Johnson, 1989a; Kondratyev et al., 1990). PKC specific activity in the NGF- and bryostatin-treated cell lysate was comparable to PMA treatment, 2.5–3.5-fold higher than in the control cells, consistent with observations that PKC is activated by NGF in PC12 cells (Hama et al., 1986; Reinhold & Neet, 1989; Kondratyev et al., 1990) and by bryostatin in other cell lines (Field et al., 1988, 1989, 1990; Hocevar & Fields, 1991). A time course of NGF activation of PKC was examined in the whole cell lysates of PC12 cells. After an initial 2.5-fold activation by NGF in the first 15 min, the PKC specific activity peaked modestly at 30 min and declined slightly at 1 h (Figure 2B).

NGF-Dependent Activation of Nuclear PKC Activity. Functional PKC activity normally involves an association of soluble PKC with intracellular membranes (Kraft et al., 1982). Since the effect of NGF on PKC activity in the whole cell lysate does not reveal these functional forms, we prepared subcellular fractions and examined the PKC activity in cytosol, membrane (including plasma and organelle), and nuclear fractions. Results are normalized for the amount of protein in each fraction (see Materials and Methods) and thus represent relative activities in each subcellular fraction. NGF effects on the PKC activity in the subcellular fractions revealed

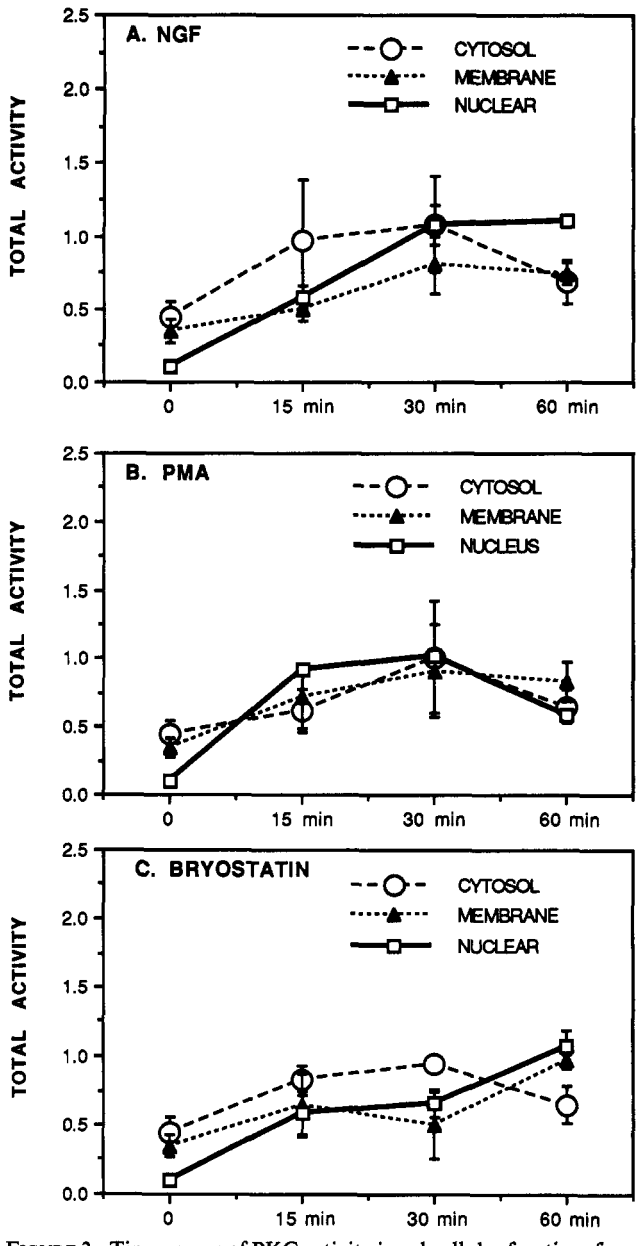

FIGURE 3: Time course of PKC activity in subcellular fractions from treated PC12 cells. Cells were incubated in DMEM/serum media containing 0.015% DMSO (control) or media containing the same concentration of DMSO and the added activator at 37 °C with increasing time. The cells were homogenized, subcellular fractions were prepared, the fractions were purified on DEAE columns, and the eluent was assayed for PKC (see Materials and Methods). Activity is expressed as the total relative activity (±SD) in a subcellular fraction (cpm/fraction $\times 10^{-5}$) after values were normalized for the average protein content of each fraction. (A) NGF (2 nM). Differences in membrane at 60 min and nuclear at 15, 30, and 60 min were significant relative to the control value with $p < 0.01$. The difference in cytosol at 30 min was significant relative to the control value with $p < 0.05$. At 60 min, nuclear values were significantly different from membrane or cytosol with $p < 0.01$. (B) PMA (100 nM). Differences in membrane at 60 min and nuclear at 15, 30, and 60 min were significant relative to the control value with $p < 0.01$. Membrane was different from control at 15 and 30 min at the $p < 0.02$ level. (C) Bryostatin (10 nM). Differences in cytosol at 15 and 30 min, membrane at 60 min, and nuclear at 15, 30, and 60 min were significant relative to the control value with $p < 0.01$. At 60 min, cytosol values were significantly different from membrane or nuclear with $p < 0.05$.

significant changes. Cytosolic PKC activity increased within 15 min after NGF treatment and declined after 30-min exposure to NGF (Figure 3A). Although PKC activity in the membrane fraction increased only slightly during the 1-h

incubation, PKC activity in the nuclear fraction increased 10-fold by 30 min and then reached a plateau by 60 min. At 1 h, cytosolic PKC activity had declined to near basal levels, whereas nuclear PKC activity was still 10-fold-activated and higher than cytosolic activity. The summation of the total activities of the individual subcellular fractions is in good agreement with the time course of the specific activity in the whole cell extracts (Figure 2B). The transient increase in cytosolic PKC activity (Hama et al., 1986) and the increased PKC activity in both cytosolic and particulate fractions at 15 min (Kondratyev et al., 1990) after incubation with NGF have previously been noted. The steady temporal increase in nuclear PKC activity and the rise and decline in cytosolic PKC activity observed here suggest a translocation of PKC from the cytosol to the nucleus, but direct activation of nuclear PKC cannot be ruled out. This nuclear activation of PKC may play a significant role in the transduction of intracellular signals to the nucleus, subsequent to NGF stimulation.

Effect of Acute PMA Treatment on the Subcellular Distribution of PKC. PC12 cells were treated with concentrations of PMA that activate PKC (Nishizuka, 1988), and the PKC activity was measured in subcellular fractions to ascertain any shift in activity (Figure 3B). In all three compartments, cytosolic, membrane, and nuclear, the PKC activity increased at 15 min, reached a maximum at 30 min, and then declined by an hour. Our data at 15 min are consistent with the data in cytosolic and particulate fractions observed at s single time point by Kondratyev et al. (1990). Although PKC activity was enhanced after PMA treatment in all three subcellular fractions, we did not observe a clear-cut "shift" of PKC activity from cytosol to particulate fractions (see Discussion). The changes with PMA were similar to those with NGF except that a decrease in the nucleus at 1 h was observed with PMA but not with NGF. The different effects of PMA and NGF on PKC activation and distribution within the cell (cf. Figure 3A,B) may be partly related to the contrasting cell morphological responses to these agents.

Effect of Acute Bryostatin Treatment on the Subcellular Distribution of PKC. Low concentrations (10 nM) of bryostatin rapidly produced a redistribution of PKC activity in PC12 cells with the highest levels occurring at 1 h in the nuclear and membrane fractions (Figure 3C). The shift in PKC distribution in the cytosolic and nuclear fractions after bryostatin stimulation is similar to that observed with NGF, but differs from PMA-treated cells. In the nuclear fractions at 1 h, PKC activity remains high in NGF- or bryostatin-treated cells, whereas in PMA-treated cells nuclear activity has declined. The similarity in nuclear activity between bryostatin and NGF responses suggests that these agents either activate the same kinase or activate kinases in the same intracellular pathway that, in the case of NGF, leads to neurite outgrowth. With bryostatin, the membrane fraction is still rising at 1 h, but with NGF or PMA, it has leveled off, which suggests that the membrane pools of PKC also respond differently to these agents. Bryostatin and NGF may activate one or more PKC isozymes which are translocated to the nucleus for phosphorylation of nuclear proteins, as has been found for bryostatin effects in other cell lines (see Discussion).

Down-Regulation of PKC by PMA or Bryostatin. A clear difference between PMA and bryostatin was evident upon prolonged exposure. Down-regulation, i.e., depletion of PKC protein and activity, occurs after chronic treatment with phorbol esters (Young et al., 1987) or bryostatin (Fields et al., 1988) due to the fact that PKC is degraded at a faster rate than it is synthesized. In order to better understand the effects

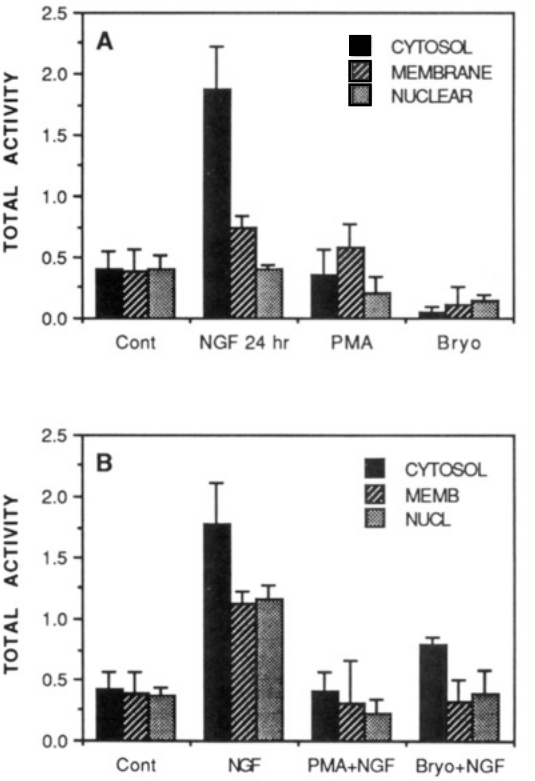

FIGURE 4: (A) PKC down-regulation by PMA and bryostatin in PC12 subcellular fractions. PC12 cells were chronically treated for 24 h in DMEM/serum media with no additions, or in media containing 0.015% DMSO (control), or in media containing the same concentration of DMSO plus NGF (2 nM), PMA (1 μM), or bryostatin (100 nM). (B) Effect of NGF on PKC activity in PC12 subcellular fractions after down-regulation by PMA or bryostatin. PC12 cells were incubated in DMEM/serum media containing 0.015% DMSO (control) or media containing DMSO plus PMA (1 μM) or bryostatin (100 nM) for 24 h. The media were discarded, and cells were incubated in fresh media containing NGF (2 nM) for 15 min. The column labeled NGF represents cells treated with DMSO for 24 h and NGF for 15 min (no down-regulation). In both panels, subcellular fractions were prepared and assayed for PKC activity as described in Figure 3, with activity expressed as the total relative activity in each fraction ($\times 10^{-5}$) $\pm$ SD. Increases in cytosol after 24-h NGF treatment (panel A) and cytosol, membrane, and nuclear fractions after 15-min NGF treatment of the 24-h DMSO control (panel B) were significant relative to the control value with $p < 0.01$. The value for the 15-min NGF treatment after chronic bryostatin down-regulation (0.80, panel B) was significantly different relative to the control (0.43, panel B, $p < 0.01$), to NGF treatment without down-regulation (1.78, panel B, $p < 0.02$), and to bryostatin down-regulation without NGF (0.06, panel A, $p < 0.01$).

of down-regulation on neurite outgrowth (Figure 1), PKC activity was measured both in cell lysates (data not shown) and in subcellular fractions subsequent to chronic treatment by either bryostatin or PMA. Chronic PMA treatment led to down-regulation in the nuclear fraction (Figure 4A), but with activity in the cytosol and membranes that was not below that of the untreated cells. Note that the residual activities in all fractions are lower than the PMA acute stimulation in Figure 3B. Chronic bryostatin treatment led to a much greater effect on all fractions relative to either control (Figure 4A). These results indicate that down-regulation with bryostatin is more effective than with PMA and may occur with different isozymes. After 24 h with NGF, cytosolic and membrane PKC activity is high.

We then assessed the ability of PKC activity to respond to NGF treatment after down-regulation of PC12 cells (Figure 4B). After chronic PMA or bryostatin treatment, NGF-stimulated PKC activity in the membrane and nucleus was

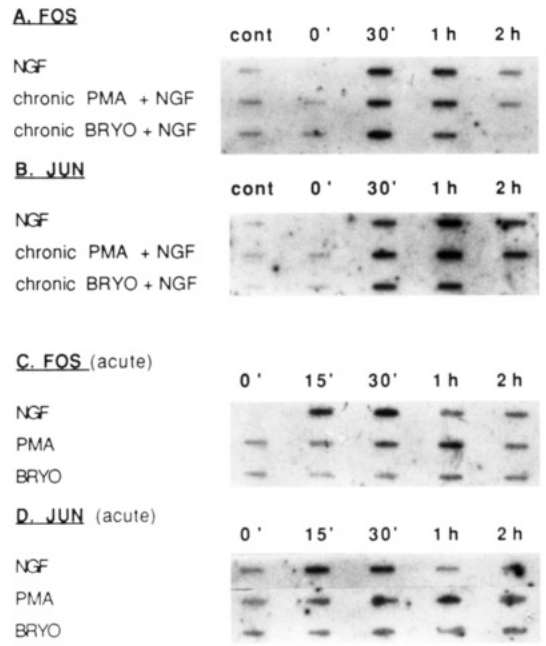

FIGURE 5: Time course of c-*fos* (A and C) and c-*jun* (B and D) expression after NGF, PMA, or bryostatin treatment. Slot blots of total RNA were probed for c-*fos* and c-*jun* at the indicated times after addition of reagent. Controls with Tα1-tubulin cDNA showed that an approximately equal amount of RNA was loaded in each lane for each experiment (data not shown). (A and B) Chronic treatment: NGF, DMSO control prior to NGF (2 nM); chronic PMA + NGF, chronic PMA (1 μM) for 24 h followed by NGF (2 nM); chronic BRYO + NGF, chronic bryostatin (100 nM) for 36 h followed by NGF (2 nM). (C and D) Acute treatment: NGF, NGF alone (2 nM); PMA, PMA alone (100 nM); BRYO, bryostatin alone (10 nM).

at the level of untreated cells but was much lower than with NGF stimulation in the absence of down-regulation. In the cytosolic fraction, NGF-stimulated PKC activity in bryostatin down-regulated cells was about 80% higher than control. PKC activity in the whole cell lysate also showed a similar response to NGF treatment after down-regulation with bryostatin (data not shown). The 100 nM concentration of bryostatin used in these chronic experiments (Figure 4B) was only at the IC_{50} for blockage of neurite extension (because of the limited amounts available) and thus would not be expected to have a complete effect on the relevant kinases. As found with the acute experiments (Figure 3C), the membrane and the nuclear site of action of bryostatin correlate with the functional effects (Figure 4B). As an aside, it may be noted that 15-min NGF stimulation after 24-h DMSO treatment (Figure 4B) appeared to give greater stimulation in all three fractions compared to just NGF/DMSO treatment for 15 min (Figure 3A).

Induction of Immediate Early Genes after Acute or Chronic Treatment by PMA or Bryostatin. We next examined the effect of PMA and bryostatin on the induction of certain genes in order to determine any correlation with the effect on neurites and PKC activity. The c-*fos* and c-*jun* protooncogenes are known to be transcriptionally regulated by NGF or phorbols in PC12 cells (Greenberg et al., 1985; Wu et al., 1989). PC12 cells that were chronically treated with either PMA or bryostatin were still capable of indicating expression of c-*fos* or c-*jun* after NGF treatment (Figure 5A,B). The time course and levels were essentially the same as those in the control in the absence of down-regulation of PKC. These results are in accord with other responses that are independent of PMA-sensitive protein kinase C isozymes (Reinhold & Neet, 1989; Sigmund et al., 1990; Damon et al., 1990; Cahill et al., 1990; Campenot et al., 1991). Furthermore, these results demonstrate that the sensitive step in the inhibition of neuritogenesis by chronic bryostatin treatment either is downstream or is on a separate pathway from the c-*fos* and c-*jun* protooncogenes. Our results are in agreement with the conclusion from other experiments that c-*fos* induction is not sufficient for generation of neurites (Volonte et al., 1989) and that c-*fos* is not required for PC12 cell differentiation (Altin et al., 1992).

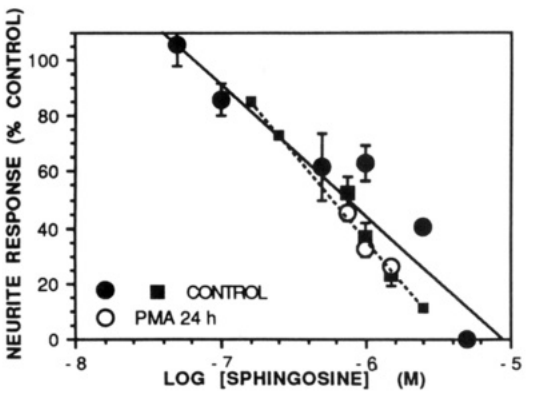

FIGURE 6: Effect of sphingosine concentration on NGF-induced neurite outgrowth. PC12 cells in defined media were chronically treated with either 1 μM PMA in DMSO (open symbols) or 0.02% DMSO (closed symbols) alone for 24 h prior to NGF addition. Sphingosine and bovine serum albumin were added with 2 nM NGF (PMA still present), and neurite response was assayed at 48 h after NGF treatment. Results are reported as the percent of response of control cells that had been treated with NGF but not sphingosine. For the control experiment with just NGF for the data shown in solid circles, 62.5 ± 3.8% (n = 17) of the cells extended neurites. For the control experiment with just NGF for the data shown in solid squares, 53.5 ± 1.4% (n = 10) of the cells had neurites; for the control experiment done on the same day and pretreated with PMA and then NGF but not sphingosine, 54.6 ± 1.6% (n = 8) of the cells had neurites. Each point represents the mean of two replicate cultures; the bars indicate the range.

Acute treatment with PMA alone produced a robust stimulation of both c-*fos* and c-*jun* mRNA levels (Figure 5C,D). This observation is consistent with previous reports with PC12 cells (Greenberg et al., 1985; Wu et al., 1989) and in other cell lines. On the other hand, the acute bryostatin stimulation of c-*fos* was weaker (Figure 5C), but significant, whereas the effect on levels of c-*jun* was negligible (Figure 5D). Although some of this diminution of effect could be due to the lack of saturation by bryostatin in these experiments, it should be noted that the 10 nM bryostatin concentration utilized in this acute experiment had significant effects on sphingosine inhibition (data not shown) and on PKC stimulation (Figures 2A and 3C). Acute treatment with NGF in combination with either PMA or bryostatin led to enhanced expression of c-*fos* and c-*jun* mRNA that appeared to be approximately additive (data not shown). This difference in the immediate early gene response indicates that kinases stimulated by PMA and kinases stimulated by bryostatin lead to distinct transcriptional responses.

Effects of Sphingosine on NGF-Induced Neurite Outgrowth after PMA Down-Regulation. Since sphingosine has been reported to inhibit NGF responses in PC12 cells through its effect on PKC (Hall et al., 1988), we did experiments to determine if the same or different steps were affected by PMA or bryostatin. In the defined medium bioassay, sphingosine inhibited NGF-induced neurite outgrowth with an IC_{50} value of approximately 750 nM (filled symbols, Figure 6). This value is 4 times lower than that found by Hall et al. (1988), or by us (data not shown) in a serum-containing bioassay; the difference in IC_{50} values is probably due to sphingosine's lipophilic properties with partitioning into serum lipids.

Table 1: Lack of Effect of PMA on NGF-Induced Neurite Outgrowth in Bryostatin Down-Regulated PC12 Cells[a]

treatment	[PMA] (nM)	neurite response (% of cells)	down-regulation (% decrease)
NGF		51.8 ± 5.3	0
bryostatin + NGF		18.2 ± 1.5[b]	65
bryostatin + NGF	50	17.1 ± 1.7	70
bryostatin + NGF	100	22.1 ± 3.3	57
bryostatin + NGF	150	18.8 ± 2.7	64

[a] PC12 cells were treated for 24 h with 200 nM bryostatin prior to NGF (2 nM) and PMA addition. Results shown indicate the percentage of cells responding at 48 h; similar results were obtained at 24 h. The values given are the mean ± range of duplicate experiments. [b] Control wells contained bryostatin, 2 nM NGF, and the appropriate concentration of DMSO; the mean ± SD of three experiments is presented.

Sphingosine also inhibits the FGF neurite response with an IC_{50} similar to that found with NGF (data not shown). In order to examine the relationship between PMA-sensitive and sphingosine-sensitive kinases, sphingosine was added to PC12 cells after down-regulation by a 24-h treatment with 1 μM PMA. Sphingosine inhibited NGF-induced neurite outgrowth with approximately the same IC_{50} value (open circles, Figure 6) and with good cell viability, despite greatly depressed PKC activity. Thus, sphingosine acts on a reaction other than a PMA-sensitive protein kinase C. Sphingosine could not be tested similarly on cells chronically treated with bryostatin because of the already depressed neurite response.

Effects of Bryostatin or PMA in Combination with Inhibitors of Neurite Outgrowth. When used alone in a defined media bioassay, bryostatin (1 or 10 nM) or PMA (10 or 50 nM) did not induce neurite outgrowth (1.7 and 2.8% of control, respectively). Simultaneous addition of low, activating concentrations of PMA plus bryostatin did not result in neurite induction. Moreover, no increase in the rate or extent of NGF-induced neurite outgrowth occurred when these concentrations of bryostatin (94.4 ± 8.8% of control) or PMA (103.5 ± 9.7% of control) were used to activate PKC in conjunction with saturating concentrations of NGF. However, low concentrations of bryostatin were able to partially relieve sphingosine inhibition of neurite outgrowth, as does PMA (Hall et al., 1988). The percentage of PC12 cells in defined media treated with 1 μM sphingosine and 2 nM NGF was 31.0% (±0.6) of the control with NGF alone; simultaneous addition of 150 nM bryostatin increased this value to 40.9% (±3.9) ($p < 0.01$) or addition of 1.5 μM PMA to 45.1% (±0.5) ($p < 0.01$). Neither PMA nor bryostatin was able to relieve inhibition by the kinase inhibitor K252a, which specifically inhibits NGF action (Koizumi et al., 1988) by acting on the NGF receptor, *trk* protooncogene product (Berg et al., 1992). Additionally, PMA was unable to reverse the inhibition of NGF-induced neurites due to down-regulation with 200 nM bryostatin (Table 1). Although cells did not tolerate severe down-regulation by a combination of PMA and bryostatin, down-regulation by low levels of PMA plus bryostatin did not produce a greater inhibition of NGF-induced neurites than bryostatin alone (data not shown). Thus, either competition occurs among pairs of activators/inhibitors, or alternate pathways of kinase stimulation exist, allowing a bypass of inhibitor-sensitive kinases (see Discussion).

DISCUSSION

We have provided evidence for a PKC in PC12 cells that is activated in the nucleus by either NGF or bryostatin. Down-regulation of the PKC by bryostatin prevents subsequent differentiation by NGF or FGF, indicating an essential role for a bryostatin-sensitive PKC in neuritogenesis. This bryostatin-sensitive PKC was not down-regulated by PMA nor required for induction of c-*fos* or c-*jun* by NGF. The activation of PKC in cellular compartments and the induction of mRNA for c-*fos* and c-*jun* were different between PMA and bryostatin, indicating that these two reagents act on distinct PKC isozymes.

Protein Kinase C Involvement in NGF Responses. NGF stimulation of PC12 cells results in the phosphorylation of phospholipase C-γ on tyrosine residues (Kim et al., 1991; Vetter et al., 1991) and leads to an early, transient increase in diacylglycerol hydrolysis from both phosphatidylinositols (Traynor et al., 1982; Contreras & Guroff, 1987; Altin & Bradshaw, 1990; Pessin et al., 1991) and glycosylphosphatidylinositols (Chan et al., 1989; Represa et al., 1991). Internal Ca^{2+} levels also increase rapidly (Pandiella-Alonso et al., 1986; Kozak et al., 1992). The diacylglycerol and Ca^{2+} signals could participate in the activation of Ca^{2+}/phospholipid-dependent PKC, and, indeed, NGF treatment has been reported to increase PKC activity within 5 min (Heasley & Johnson, 1989a,b). Nevertheless, conflicting conclusions have been made regarding the necessity of PKC in neurite outgrowth. Neuritogenesis is blocked by sphingosine treatment (Hall et al., 1988). In contrast, down-regulation of PKC by PMA (1% remaining activity on histone) does not block neurite response (Reinhold & Neet, 1989; Damon et al., 1990), ODC induction (Reinhold & Neet, 1989; Damon et al., 1990), induction of immediate early response genes (Sigmund et al., 1990; Damon et al., 1990; Altin et al., 1991), or phosphorylation of tyrosine hydroxylase (Cahill et al., 1989), S6 kinase II peptide (Heasley & Johnson, 1989a), or initiation factor IF-4E (Fredrickson et al., 1992). The interpretation of sphingosine inhibition (Hall et al., 1988) is further complicated by our present observation that this inhibition still occurs after PKC down-regulation with PMA (Figure 6) and by the lack of specificity of sphingosine (Jefferson & Schulman, 1988; Faucher et al., 1988; Zhang et al., 1990). The bryostatin experiments, however, clearly demonstrate that down-regulation is an effective means of determining the results of PKC action.

The observation that bryostatin down-regulation of PKC (>100 nM for 24 h) inhibited NGF-induced neurite outgrowth (Figure 1) shows that PKC activity is necessary for induction of neurites by NGF through a mechanism that is not sensitive to PMA (Reinhold & Neet, 1989). However, the activation of protein kinase C by bryostatin or PMA, alone or in combination, does not initiate neurite outgrowth, confirming other studies that activation of PKC is not sufficient to induce the phenotypic response. Bryostatin down-regulation of PKC activity was more effective than that with PMA in all subcellular fractions (Figure 4A). However, bryostatin down-regulation did not entirely eliminate the ability of NGF to activate PKC, particularly in the cytosolic fraction (Figure 4B), possibly because higher concentrations of bryostatin are required for complete neurite inhibition than that used in this experiment for PKC assays or because some PKC activity is not bryostatin-sensitive.

Translocation of PKC in NGF-Responsive Cells. Contrary to the generally accepted paradigm, we did not find clear-cut evidence for translocation of PKC from the cytosol to the plasma membrane. Our data showed an increase of cytosolic activity as well as nuclear activity after NGF stimulation; the latter could be due to translocation or activation of resident PKC in the nucleus. The data for whole cell extracts (Figure 2) and subcellular fractions (Figure 3) are consistent in

indicating an increase in overall PKC activity, with or without DEAE purification and whether specific or total activity was considered. Immunoblots in our laboratory support the contention that simple translocation of PKC is not the only mechanism in short-term NGF stimulation of PC12 cells (J. I. Morris et al., unpublished results). Our data are in agreement with the increase in cytosolic activity observed by Hama et al. (1986), who did not report membrane activity but hypothesized that the increase in cytosolic PKC activity toward histone was due to a shift from the membrane compartment. Our data also agree with Kondratyev et al. (1990), who reported a partial shift of PKC protein to the membranes from immunoblot experiments, but who also observed a TPA- or NGF-stimulated increase in activity toward histone in both cytosolic and membrane fractions. The data of Healy and Johnson (1989a), who measured stimulation of PKC activity toward the specific peptide substrate KRTLRR inside permeabilized PC12 cells, would be consistent with either a translocation of a direct activation of the kinase. The short-term (hours) stimulation of PKC activity presented in this paper is necessarily different from the long-term (3–6 days) effect on PKC isozyme levels reported by Wooten et al. (1992). One explanation of our results is that phosphorylation or autophosphorylation of PKC occurs and affects the activity of the kinase measured in the extracts. Autophosphorylation occurs at multiple sites in PKC (Flint et al., 1990) and has been shown to affect both activity toward histone (Mochly-Rosen & Koshland, 1987) and membrane binding (Wolf et al., 1985). Further studies will be needed to elucidate the details of these effects in PC12 cells.

Results that are not consistent with a simple translocation from cytosol to membranes have also been reported in other systems. A reverse shift of PKC activity (measured on histone) from membrane to cytosol was reported after ACTH stimulation of bovine adrenocortical cells (Vilgrain et al., 1984). A translocation-independent increase in cytosolic and membrane PKC activity toward histone upon stimulation of platelets by platelet-activating factor, thrombin, or prostacyclin was reported (Salari et al., 1990). Bryostatin stimulation of platelets produced enhanced activity toward a PKC substrate, p47, but no evidence for a shift of any of the four isozymes identifiable in the cells was noted by immunoblots of subcellular fractions (Grabarek et al., 1993). Astrocytoma cells treated with muscarinic receptor activators were still able to phosphorylate a PKC substrate, p80 (MARCKS), even though translocation to membranes was blocked with a Ca^{2+} chelator (Trilivas et al., 1991). Ca^{2+} has been suggested to play a key role in the observed distribution between subcellular fractions in neutrophils (Phillips et al., 1989), but other investigators suggest that the binding of PKC to membranes promoted by Ca^{2+} is different in affinity and character from that promoted by diacylglycerol or phorbol esters (Wolf et al., 1985; Gopalakrishna et al., 1986). These reports also demonstrate that phosphorylation of endogenous substrates may be qualitatively different from exogenous substrates such as histone or a peptide.

Role of the Bryostatin-Sensitive PKC. Several lines of reasoning suggest that the bryostatin-sensitive PKC (BS-PKC) in PC12 cells is distinct from the phorbol-sensitive PKC (PS-PKC): down-regulation by bryostatin affected the NGF neurite response while PMA did not; down-regulation by the two compounds was not additive; the effect of bryostatin down-regulation on neurites was not reversed by PMA; effects of PMA on PKC activity distribution in subcellular fractions were different from those of bryostatin; and induction of c-*jun* by the two compounds was distinct. PKC comprises a family of isozymes (Nishizuka, 1988) which are differently regulated by NGF (Clark & Lee, 1991; Wooten et al., 1992). Since the pattern of the PKC shift in NGF-treated PC12 cells more closely resembled the pattern observed in bryostatin-treated cells than in PMA treated cells, we suggest that NGF and bryostatin translocate the same PKC isozyme to the nucleus, or perhaps activate a PKC isozyme in the nucleus. The kinase may then modulate nuclear functions necessary for induction and/or stabilization of neurites.

Accumulating evidence suggests that translocation of PKC to the nucleus plays a role in the transduction of a variety of external signals (Cambier et al., 1987; Rogue et al., 1990; Fields et al., 1990; Hocevar & Fields, 1991; Hocevar et al., 1992). In two leukemic cell lines, bryostatin, but not PMA, selectively translocates PKC β_{II} to the nucleus and phosphorylates lamin B (Hocevar & Fields, 1991; Billi et al., 1991). Moreover, PC12 cell differentiation (1–14 days) is accompanied by an increased accumulation of PKC β_{II} (Wooten et al., 1992), suggesting a role for this isozyme in neuronal morphology. NGF and bryostatin translocate PKC activity to the nucleus in a time-dependent manner, and NGF induces differentiation of PC12 cells. Since bryostatin down-regulates a required kinase that is distinct from PS–PKC, an isozyme of PKC, such as β_{II} (Wooten et al., 1992; Hocevar & Fields, 1991) or ζ (Gschwendt et al., 1992; Berra et al., 1993), may function in the PC12 cell nucleus.

Signaling Pathways and the Sphingosine Effect. The concept of multiple functional signaling pathways is supported by these data. Since chronic down-regulation with PMA did not affect sphingosine inhibition, PMA and sphingosine must affect alternative pathways. However, acute PMA treatment could partly relieve sphingosine inhibition, indicating that the alternate pathway bypasses the sphingosine inhibition step. Since acute bryostatin treatment also partly relieved sphingosine inhibition of the NGF neurite response, bryostatin either could activate kinase(s) in an alternate bypass pathway *or* could compete with sphingosine for the same enzyme(s). Bryostatin and sphingosine do not compete for the same PMA-insensitive kinase (X. Z. Campbell and K. E. Neet, unpublished observations). Thus, PMA, bryostatin, and sphingosine act on three distinct alternative pathways.

Conclusions. The role of any member of the PKC family in the cascade of steps eventually leading to neurite outgrowth in PC12 cells is not yet clear. We describe here a required role for a bryostatin-sensitive, PMA-insensitive PKC that differs from other kinases that are required for NGF action. Studies are in progress to identify and characterize the kinase isozyme(s) that is (are) activated or translocated in the nucleus and its protein target(s).

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