Suppression of Nerve Growth Factor-directed Neurite Outgrowth in PC12 Cells by Sphingosine, an Inhibitor of Protein Kinase C*

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The formation of vertebrate neural circuitry is regulated in part by neurotrophic agents, such as nerve growth factor (NGF); however, the biochemical mechanisms involved in neurite outgrowth have yet to be completely resolved. Phorbol ester tumor promoters are known to influence the extension of neurites in a variety of neurodevelopmental systems, and protein kinase C, the major phorbol ester receptor, has been implicated in this process. In the present study, sphingosine, a specific pharmacological inhibitor of protein kinase C, was employed to investigate the role of this enzyme in the elaboration of neurites in PC12 pheochromocytoma cells. Normally, PC12 cells respond to NGF by morphologically differentiating into sympathetic neuron-like cells, exhibiting a marked hypertrophy and extending slender neurites piloted by well-defined growth cones. The elaboration of NGF-induced neurites was found to be reversibly inhibited by sphingosine in a dose-dependent manner (IC50 = 2.5–5 μM), while similar concentrations of several structural analogs were inactive. The suppression of neurite outgrowth by sphingosine was antagonized by the addition of 12-O-tetradecanoylphorbol 13-acetate (TPA), which binds to and directly activates protein kinase C. In the presence of NGF, TPA treatment increased the incidence of neurite outgrowth, and this increase, in turn, was antagonized by sphingosine. The binding of [3H]phorbol 12,13-dibutyrate to specific phorbol ester binding sites in PC12 cells was inhibited by sphingosine at concentrations similar to those which inhibited neurite outgrowth. The effects of sphingosine on TPA-directed protein phosphorylation were examined in situ, revealing inhibition of [32P]phosphate incorporation into cellular proteins. The specific TPA-directed phosphorylation of tyrosine hydroxylase was inhibited by sphingosine, as was the resulting increase in enzyme activity. The effects of sphingosine on the levels of α- and β-tubulin mRNAs were also examined in an effort to delimit the locus of protein kinase C action. Concentrations of sphingosine which suppressed neurite outgrowth did not inhibit the NGF-directed elevation of tubulin transcript levels. Taken together, these results reveal the presence of a sphingosine-sensitive pathway in neurite outgrowth and indicate that protein kinase C plays a role in mediating the neuritogenic effects of NGF. Furthermore, the results suggest that protein kinase C acts at a distal segment of the neurite growth pathway.

Neurotrophic agents, such as nerve growth factor (NGF), are known to influence the growth and maintenance of vertebrate neural circuitry (1, 2). Tissues that produce NGF may serve to guide the directional growth of advancing sensory and sympathetic axons. Neurites are attracted up a concentration gradient of NGF in vivo and in vitro. Although advances have been made in understanding the properties of the NGF receptor, the nature of the transmembrane events and the mechanisms by which neurotogenic polypeptides initiate neurite extension remain important but largely unresolved problems.

The formation of the axonal cytoskeleton is dependent on the synthesis and assembly of its structural elements. Although many structural proteins contribute to the assembly of neurites, the role of microtubules is among the best understood and may potentially serve as a paradigm for other studies. Microtubules, comprised of α- and β-tubulin subunits, are essential for neurite growth as well as axonal transport. NGF can promote the accumulation of microtubules (1), which, additionally, become more resistant to depolymerization (3). In PC12 pheochromocytoma cells, both the levels of tubulin and the assembly of microtubules are increased by NGF treatment (4). The recent finding that tubulin mRNA levels are also elevated by NGF in PC12 cells has led to the hypothesis that neurotogenic polypeptides may act to regulate tubulin transcript levels as a prelude to neurite extension (5).

Complementary investigations demonstrated that tumor-promoting phorbol esters, such as 12-O-tetradecanoylphorbol 13-acetate (TPA), are capable of altering the morphological differentiation, i.e. the outgrowth of neurites, in a variety of neurodevelopmental systems (6–9). It is now generally acknowledged that the Ca2+-activated phospholipid-dependent protein kinase (protein kinase C) represents the major intracellular receptor for the tumor-promoting phorbol esters, which persistently activate the enzyme and thereby circumvent several physiological controls (10, 11). Studies of the developmental expression and regional distribution of phorbol ester receptors in the mammalian brain indirectly suggest that this enzyme may play an important role in neurite outgrowth (12). Moreover, the expression of phorbol ester binding and/or protein kinase C activity is closely correlated with neurite outgrowth in cultured human neuroblastoma

* This work was supported in part by USAF-OSR Grant 86-0117 (to P. R. V.) and by National Institute of Neurological and Communicative Disorders and Stroke Grant R01-NS24787 (to D. N. I.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: NGF, nerve growth factor; TPA, 12-O-tetradecanoylphorbol 13-acetate; PDBu, phorbol 12,13-dibutyrate; RPMI, Roswell Park Memorial Institute; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KRB, Krebs-Ringer HEPES; SDS, sodium dodecyl sulfate; DOPA, 3,4-dihydroxyphenylalanine.
cells (7, 8) and rat embryonic neurons (13). While the function of phorbol ester receptors in developing neurons is not fully established, one notable possibility is that they modulate the cellular responses to neurotrophic factors (14).

PC12 pheochromocytoma cells, regarded as an important model system for the study of adrenergic neuronal differentiation (15, 16), respond to NGF by differentiating into sympathoadrenal neural-like cells, exhibiting a marked hypertrophy, and extending slender neurites piloted by well defined growth cones. In the absence of NGF, TPA treatment alone is not sufficient to induce neurite outgrowth in PC12 cells; however, TPA is capable of enhancing neurite outgrowth in PC12 cells that have concomitantly been induced to differentiate by the actions of NGF (17). Although several recent studies have indicated that protein kinase C is an important component of the NGF-sensitive phosphorylation system in PC12 cells (18–20), the associated biochemical pathways and the relationships to neurite outgrowth have yet to be elucidated. One problem that tended to hinder progress in this area was the lack of pharmacological inhibitors of protein kinase C that displayed the affinity, potency, and specificity necessary for analytical purposes. This obstacle now appears to have been overcome with the discovery that sphingosine and other long-chain (sphingoid) bases can bind to the regulatory subunit of protein kinase C, competitively inhibit the binding of phorbol esters, and selectively inhibit the activity of the enzyme in promyelocytic leukemic cells (21), normal neutrophils (22), and platelets, as well as in vitro assays (23). In the present study, sphingosine was employed to investigate the role of protein kinase C in the elaboration of neurites in PC12 cells. Specifically, this study was designed to (i) determine whether sphingosine could inhibit NGF-directed neurite outgrowth, (ii) assess the interactions between sphingosine and TPA in terms of their effects on cellular morphology, protein phosphorylation, and tyrosine hydroxylase activity, (iii) characterize the displacement of phorbol esters from specific binding sites by sphingosine in PC12 cells, and (iv) examine the effects of sphingosine on tubulin mRNA levels in an effort to delimit the locus of protein kinase C action.

**EXPERIMENTAL PROCEDURES**

Materials—TPA, PDBu, d-sphingosine, n-octylamine, ceramide, and palmitic acid were purchased from Sigma and dissolved at 20 mM in ethanol. For experimentation, the lipids were diluted into RPMI 1640 (GIBCO) containing equimolar concentrations of fatty acid-free bovine serum albumin. Control and experimental solutions both contained equivalent final concentrations of albumin and ethanol. The maximum ethanol concentration did not exceed 0.06%, previously found to have no detectable effect on neurite outgrowth (8).

Mouse submaxillary gland 2.5 S NGF was obtained from Collaborative Research (Lexington, MA) and dissolved in serum-containing medium. The [3H]phosphate, as H2PO4, was obtained from ICN Radiochemicals (Irvine, CA), and [3H]PDBu was obtained from Du Pont-New England Nuclear. The cDNA clone pKo1, obtained from human keratinocytes, containing the entire a-tubulin coding region plus 67 and 173 base pairs of the 5'- and 3'-untranslated regions, respectively (24), was the kind gift of Dr. Donald W. Cleveland. The cDNA clone pKCL1, obtained from human fetal brain, containing all but 21 bases from the 5'-end of the coding region for ß-tubulin and all 21 bases from the 3'-end of the coding region (25), was the kind gift of Dr. Nicholas Cowan.

Cell Cultures—PC12 pheochromocytoma cells were maintained in exponential growth in monolayers in 12-well Primaria flasks (Falcon) in RPMI 1640 supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin sulfate. The cells were cultured at 37°C in a water-saturated 5% CO2, 95% air atmosphere, passed routinely prior to confluence, and plated at a density of approximately 1 × 105 cells/cm2 (in 1 ml) onto protamine sulfate-coated 24-well culture plates (Falcon) for studies of neurite outgrowth or on larger 75-cm2 dishes for studies of phorbol ester binding or for the assessment of changes in tubulin mRNA. NGF was generally added to a final concentration of 50 ng/ml. Solutions were changed daily in an effort to minimize the potential effects of cellular metabolism on sphingosine concentration.

Neurite Outgrowth—The method of quantification has been previously described (7, 26). Basically, the percentage of cells bearing neurites was determined by counting more than 100 cells randomly chosen fields under low-power phase or modulation contrast microscopy. Fields were photographed to later score neurite outgrowth. Only cells with processes greater than or equal to one cell diameter were counted as positive. Cells with multiple processes were scored only once. Cell aggregates which sometimes obscured neurites were not scored. Neurites out for 15 h may be identified by the presence of well defined growth cones. The mean scores and number of replicates are indicated in the figure legends.

**Protein Phosphorylation**—PC12 cells were gently detached from the Primaria culture flasks in Ca2+- and Mg2+-free Hanks' balanced salt solution containing 1 mM EDTA and were washed twice in Krebs-Ringer (KRH) buffer (containing 125 mM NaCl, 4.8 mM KCl, 2.6 mM CaCl2, 1.2 mM MgSO4, 5.6 mM glucose, and 25 mM HEPES). Aliquots of approximately 5 × 105 cells were incubated in suspension at 37°C for 2 h in 1 ml of KRH buffer containing a 0.25 mCi of inorganic [32P]phosphate (original specific activity, 180 µCi/ml), 100 µCi/ml, in the presence or absence of 5 µM sphingosine, TPA (50 ng/ml) or a control solution was then added to certain treatment groups, and the incubations were continued for another 20 min, after which the cells were chilled, pelleted at 1000 × g, washed with KRH buffer, and lysed with a buffer (containing 0.27 M sucrose, 1 mM EDTA, 40 mM NaF, 4 µg/ml leupeptin, and 0.2% Triton X-100) for 15 min at 4°C. The homogenates were spun at 15,000 × g, and aliquots of the resulting supernatants were prepared for either protein determinations, tyrosine hydroxylase activity measurements, assessment of bulk [3H]PDBu phosphate incorporation into 25 trichloroacetic acid-precipitable proteins, or SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (27). Electrophoretic transfer of proteins from 10% polyacrylamide gels to nitrocellulose sheets (Schleicher & Schull BA85) was performed using a Bio-Rad Transblot apparatus. The efficiency of the transfer was confirmed by staining the polyacrylamide gels with 0.1% Coomassie Blue and the nitrocellulose sheets with 0.1% fast green FCF. The nitrocellulose sheets were autoradiographed on Protosol-soaked filter papers and quantified by liquid scintillation counting.

**Tyrosine Hydroxylase Activity**—PC12 cell supernatants were assayed for tyrosine hydroxylase activity using a modification of a CO2 trapping procedure involving the coupled decarboxylation of DOPA formed from L-[3,4-14C]tyrosine (28). Primary (hydroxylation) incubation conditions were: 0.1 mM L-[3,4-14C]tyrosine, 1.0 mM 6-methyl-5,6,7,8-tetrahydropteridine, 2 mg/ml catalase, 2 µg/ml bovine serum albumin (v/v) supernatant, 150 mM Tris acetate, pH 8.0, 30 min at 37°C. Secondary (decarboxylation) incubation conditions were: 0.6 mg/ml 3-iodo-L-tyrosine, 10.3 µg/ml pyridoxal 5-phosphate, 10% (v/v) partially purified DOPA decarboxylase, 150 mM Tris acetate, pH 8.0, for 30 min at 37°C, followed by acidification with 10% trichloroacetic acid and incubation for an additional 1 h at 37°C. Radiolabeled CO2 was collected onto Protosol-soaked filter papers and quantified by liquid scintillation counting.

**[3H]PDBu Binding**—PC12 cells plated at a density of 2.7 × 105 cells/cm2 on protamine sulfate-coated culture dishes were pretreated with 50 ng/ml NGF for 2 days, detached from the dishes by brief incubation in Hanks' balanced salt solution containing 1 mM EDTA, and dispersed by mild trituration. The cells were counted and resuspended in RPMI 1640 with 25 mM HEPES, pH 7.4, for the binding assay (8). Aliquots of 0.7 × 105 cells/ml were incubated in plastic tubes suspended in an RPMI 1640 incubation buffer containing 10% fetal calf serum, 25 mM HEPES, pH 7.4, 10 mCi [3H]PDBu (specific activity, 18.9 Ci/mmol), and various concentrations of sphingosine and/or control solution. Noncompetitive [3H]PDBu binding was determined in parallel incubations containing a 1000-fold excess (10 µM) of unlabeled PDBu. After incubation for 1 h at 37°C, the cell pellets were washed with 1 ml phosphate-buffered saline solution containing reduced pressure in an Amicon filtration manifold. The filters were then washed rapidly 3 times with 2 ml of ice-cold incubation buffer, air dried, and the radioactivity quantified by liquid scintillation counting.

**RNA Preparation, Electrophoresis, and Hybridization to cDNA**—The methodology used in the detection of tubulin mRNA has been previously described (5). Briefly, PC12 cells were harvested and then
RESULTS

Effects of Sphingosine on NGF-directed Neurite Outgrowth—Neurite outgrowth stimulated by NGF alone proceeds slowly in PC12 cells, and a maximum response is generally reached after about 1 week (15). In the present study, PC12 cells cultured in the presence of 50 ng/ml NGF exhibited characteristic changes in cellular morphology, extending neurites after approximately 24 h, the incidence of which increased gradually over time and included 30–35% of the cells after 72 h. Spontaneous neurites were rare in the absence of NGF (Fig. 1). This inhibition was promptly reversible upon washout of sphingosine; 93 ± 1%, 50 ng/ml NGF; 94 ± 2%, untreated; 94 ± 2%, 5 μM sphingosine; 93 ± 1%, 50 ng/ml NGF; 94 ± 1%, 50 ng/ml NGF plus 5 μM sphingosine (presented as the mean ± S.D. of four replicate cultures). At 10 μM sphingosine, viability appeared to be adversely affected in approximately 20% of the cells, which may explain why neurite outgrowth was only reversible to the extent of about 86% in Fig. 1. The data suggest that viability is affected on a dose-response curve that is different from that for inhibition of neurite outgrowth.

Antagonism of Sphingosine’s Inhibition by TPA—If sphingosine were indeed acting through the inhibition of protein kinase C, then TPA would presumably be able to antagonize the inhibition. This prediction was tested in the following series of experiments (Fig. 4). The number of neurites induced by NGF was inhibited about 63% by 5 μM sphingosine. This inhibition was completely counteracted when 50 ng/ml TPA was additionally present. In the presence of NGF, TPA treatment increased the incidence of neurite outgrowth, and this increase was, in turn, antagonized by sphingosine. The nature of this antagonism was further assessed by examining the sphingosine inhibition of NGF-directed neurites over a range of TPA concentrations (Fig. 5). Under the permissive influence of NGF, TPA synergistically potentiated neurite outgrowth. The half-maximally effective dose (ED50) was approximately 5–10 ng/ml (curve with solid circles). Increasing con-
Protein Kinase C-dependent Neurite Outgrowth

Fig. 3. Morphology of PC12 cells cultured in the presence or absence of NGF and sphingosine. The cells were plated on protamine sulfate-coated plastic dishes and incubated for 3 days in RPMI 1640 supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum and the following additions: A, none; B, 50 ng/ml NGF; C, 50 ng/ml NGF plus 5 µM sphingosine; D, 50 ng/ml NGF plus 0.5 µM sphingosine for 2 days followed by incubation in NGF only for 1 day. The cells were photographed under Hoffman modulation contrast microscopy. Bars, 50 µm.

Fig. 4. Effect of TPA and/or sphingosine on the time course of neurite outgrowth in NGF-treated PC12 cells. The proportion of neurite-bearing cells at each time is presented as the percentage of NGF-treated levels at three days of incubation. The following concentrations were employed: NGF, 50 ng/ml; TPA, 50 ng/ml; sphingosine (SPH), 5 µM. Each point represents the mean of two independent experiments; the bars represent the range.

Centrations of sphingosine caused a progressive shift in the TPA dose-response curve to the right, indicated by the progressive increase in the half-maximally effective dose. The dose-response curves appeared to approach a maximum asymptotically with increasing concentrations of TPA. While double-reciprocal plots were not constructed, due to the complex nature of the curves produced in this mixed-agonist system, the results suggest that there is a competitive mode of antagonism between TPA and sphingosine.

Effects of Sphingosine on Protein Phosphorylation and Phosphorylation-dependent Tyrosine Hydroxylase Activity—Another neuron-specific event linked to occupancy of the high affinity NGF receptor is activation of tyrosine hydroxylase (16, 19). The activity of tyrosine hydroxylase is regulated largely by protein phosphorylation mechanisms, and this activity is known to be increased in vivo by the activation of protein kinase C (31, 32). Preliminary investigations based on the assessment of tyrosine hydroxylase activity served to determine that, at a suspension concentration of 0.5 x 10⁶ PC12 cells/ml in KRH buffer, treatment with 30 ng/ml TPA for 20 min was suitable for the examination of enzyme activation. Subsequently, studies of TPA and/or sphingosine effects were undertaken in [³²P]phosphate-loaded PC12 cells that had or had not been previously exposed to NGF. As shown in Table I, treatment of [³²P]phosphate-loaded cells with TPA resulted in an overall increase in the incorporation of [³²P]phosphate into TCA-precipitable proteins, and this increase was diminished in the presence of 5 µM sphingosine. SDS-polyacrylamide gel electrophoresis of the cellular extracts followed by electrophoretic transfer to nitrocellulose and autoradiography indicated that a band migrating in the expected position of tyrosine hydroxylase (56 kDa) represents...
TABLE I

Effects of TPA and/or sphingosine on total \[^{32}P\] incorporation, tyrosine hydroxylase phosphorylation, and tyrosine hydroxylase activity

PC12 cell cultures pretreated in the presence or absence of 50 ng/ml NGF for 24 h were suspended in KRH buffer; aliquots were loaded for 2 h with \[^{32}P\]phosphate and then incubated for 20 min with one of the following treatments: control = control solutions; TPA = 30 ng/ml TPA; TPA + SPH = 30 ng/ml TPA plus 5 \(\mu M\) sphingosine; SPH = 5 \(\mu M\) sphingosine. Cellular extracts were then analyzed for 1) the incorporation of \[^{32}P\]phosphate into trichloroacetic acid-precipitable proteins, 2) the phosphorylation of the tyrosine hydroxylase (TH) band after SDS-polyacrylamide gel electrophoresis (15 \(\mu g\) of protein/lane) followed by transfer to nitrocellulose, and 3) the resulting tyrosine hydroxylase activity determined by a coupled decarboxylation procedure, as described under "Experimental Procedures." The normalized results of two independent experiments are presented; control values expressed as the means of these two experiments were 6950 cpm/\(\mu g\) protein, 401 cpm/TH band, and 0.48 nmol DOPA formed/\(\mu g\) protein/min for nonpretreated cells, and 5016 cpm/\(\mu g\) protein, 335 cpm/TH band, and 1.21 nmol DOPA formed/\(\mu g\) protein/min for NGF-pretreated cells, respectively.

| Treatment            | Protein phosphorylation | TH phosphorylation | TH activity   |
|----------------------|-------------------------|--------------------|--------------|
|                      | % of control            | % of control       | % of control |
| **No pretreatment**  |                         |                    |              |
| Control              | 100                     | 100                | 100          |
| TPA                  | 114                     | 118                | 128          |
| TPA + SPH            | 98                      | 85                 | 99           |
| SPH                  | 97                      | 87                 | 69           |
| **NGF pretreatment**|                         |                    |              |
| Control              | 100                     | 100                | 100          |
| TPA                  | 134                     | 149                | 141          |
| TPA + SPH            | 118                     | 132                | 124          |
| SPH                  | 107                     | 112                | 106          |

![Fig. 6. Effect of sphingosine on the TPA-directed phosphorylation of tyrosine hydroxylase (TH) in PC12 cells.](image)

Expected, the control levels of tyrosine hydroxylase were markedly elevated by NGF pretreatment (Table I, legend); nevertheless, similar antagonistic interactions between TPA and sphingosine were observed in both NGF-pretreated and in untreated PC12 cells.

**Displacement of Phorbol Ester Binding by Sphingosine**—The mechanism of sphingosine inhibition was further examined by assessing the effects of various sphingosine concentrations on phorbol ester binding in intact NGF-treated PC12 cells. Nonspecific \[^{3}H\]PDBu binding was determined as that radioactivity which was not competitively displaced by 1000-fold excess unlabeled PDBu. Specific phorbol ester binding, in the presence of 10 nM \[^{3}H\]PDBu, was determined by subtracting the amount of nonspecific binding from the total binding. As shown in Fig. 7, sphingosine inhibited the binding of \[^{3}H\]PDBu to PC12 cells in a concentration-dependent manner. The shape of the dose inhibition curve was remarkably similar to that for inhibition of neurite outgrowth (Fig. 2). Both curves appear to be somewhat biphasic. The displacement of one-half maximal binding at a cell density of 0.7 x 10^5 cells/ml occurred at approximately 0.3–5 \(\mu M\) sphingosine. The levels of nonspecific \[^{3}H\]PDBu binding were not affected over the range of sphingosine concentrations (0.5–10 \(\mu M\)) employed in these studies.

**Effects of Sphingosine on NGF’s Modulation of Tubulin mRNA Levels**—Previous studies have shown that the induction of neurites by NGF in PC12 cells is correlated with an increase in the levels of tubulin messenger RNA (5). Therefore, the effects of sphingosine on tubulin mRNA levels were examined on Northern blots (Fig. 8) in order to further clarify the possible locus of protein kinase C action. Both \(\alpha\)- and \(\beta\)-tubulin mRNA levels were increased by NGF. At a concentration of 5 \(\mu M\) sphingosine, NGF-directed neurite outgrowth was profoundly inhibited, yet NGF-directed increases in the amount of \(\alpha\)- and \(\beta\)-tubulin mRNAs were not affected. In other experiments, dot blot hybridization assays confirmed that sphingosine did not inhibit the NGF-directed induction of either \(\alpha\)- or \(\beta\)-tubulin mRNA over the range of sphingosine concentrations (1–10 \(\mu M\)) employed in the neurite outgrowth studies (data not shown). These results indicate that protein kinase C activity is not required for the NGF-directed modulation of tubulin transcript levels and suggest that the sphingosine-sensitive step is more distal in the biochemical pathway leading to neurite outgrowth.

![Fig. 7. Effect of sphingosine concentration on \[^{3}H\]PDBu binding in PC12 cells.](image)
binding to specific receptors on the plasma membrane. NGF directly implicated protein kinase C as an important component of the induction of numerous proteins including neuron-specific tubulin. RNA of which are known to have a direct impact upon the activity of protein kinase C and the turnover of phosphatidylinositol (44,45) of which are increased in NGF-treated PC12 cells, both of which are known to have a direct impact upon the activity of protein kinase C. Furthermore, a number of studies have directly implicated protein kinase C as an important component of the NGF-sensitive phosphorylation system in PC12 cells (18-20).

The present study focuses on the importance of protein kinase C in governing NGF-directed neurite outgrowth in PC12 cells. The results show that sphingosine inhibits NGF-directed neurite outgrowth in a reversible and dose-dependent manner. The inhibition exhibits selectivity, because similar concentrations of several structural analogs were inactive. In the presence of sphingosine at concentrations that effectively inhibited neurite outgrowth, NGF-treated PC12 cells still exhibited a characteristic flattening and an apparent morphological hypertrophy. Moreover, sphingosine did not inhibit the NGF-directed elevation of tubulin mRNA levels. These results indicate that some, but not all, of the actions of NGF were inhibited. This conclusion is further supported by the rate of neurite outgrowth observed upon the removal of sphingosine. Within 24 h of sphingosine removal, the levels of neurite outgrowth far exceeded that of newly differentiating cells and approached that of parallel cultures exposed continuously to NGF. The prompt extension of neurites observed upon the removal of sphingosine, together with the results of the dye exclusion test, also served to confirm that the inhibition of neurite outgrowth was not the result of gross cellular toxicity. These results further show that the sphingosine-sensitive step in the process of neurite outgrowth is not at the level of the NGF receptor, because several effects of NGF are not inhibited. Moreover, the neurite growth response to TPA, as well as to NGF, is inhibited by sphingosine.

The effects of sphingosine on general and specific protein phosphorylation mechanisms were also explored. Examination of overall protein phosphorylation in situ confirmed that sphingosine inhibited TPA-directed [32P]phosphate incorporation into cellular proteins. In addition, tyrosine hydroxylase, which represents a specific substrate for phorbol ester-stimulated protein phosphorylation and is activated by TPA in both PC12 cells (31) and normal adrenal chromaffin cells (32), was predictably phosphorylated and activated by TPA treatment, and these effects were consistently antagonized by sphingosine. Studies of the effects of sphingosine on phorbol ester binding in PC12 cells provided further substantiation that sphingosine inhibition occurs by interacting directly with protein kinase C. The binding of [3H]PDBo to specific phorbol ester binding sites was inhibited by sphingosine in a dose-dependent manner at concentrations very similar to those which inhibited neurite outgrowth.

Recent studies have revealed that protein kinase C activity in the cytosol of undifferentiated PC12 cells is rather low (20), which may explain why TPA alone is unable to induce neurites in these cells. NGF treatment reportedly causes a rapid and significant increase in the specific activity of this enzyme (46). Treatment of PC12 cells with NGF was found to permit the dose-dependent induction of neurites by TPA, exhibiting an EDso of 5-10 ng/ml, which is close to the EDso for TPA-dependent neurite outgrowth (7) and the occupancy of tumor promoter receptors (8) in SH-SY5Y human neuroblastoma cells. In PC12 cells, sphingosine antagonized the neuritogenic effects of TPA in a manner that was suggestive of competitive inhibition. An antagonism between sphingosine and phorbol esters was also demonstrated in PC12 cells in terms of protein phosphorylation, enzyme activation, and phorbol ester binding. Presumably, these compounds compete for the same binding site on the regulatory domain of protein kinase C (23). Taken together, the results of this study (i) reveal the presence of a sphingosine-sensitive pathway in neurite outgrowth; (ii) indicate that protein kinase C plays a role in mediating the neuritogenic effects of NGF; and (iii) suggest that protein kinase C acts at a distal segment of the neurite growth pathway.

The actual biochemical mechanism(s) by which protein kinase C activity governs neurite outgrowth remains a matter for speculation. The formation of microtubule bundles presents an attractive possibility for the assembly of microtubules is known to be promoted by both TPA (47) and NGF (1,4), and several microtubule-associated proteins have recently been identified as substrates for protein kinase C (48,49). Several microfilament-related cytoskeletal components have also been identified as substrates for protein kinase C (11).
In addition, a certain "growth-associated protein," GAP-43, a major component of neuronal growth cones and growing neurites (50), is directly correlated with neuronal growth and regeneration (51, 52) and is recognized as a putative substrate for protein kinase C (53). In view of these findings, the role of protein kinase C in neurite outgrowth should continue to merit intensive investigation.

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