Effect of Tumor Promoters on the Response of Cultured Embryonic Chick Ganglia to Nerve Growth Factor

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ABSTRACT

The tumor-promoting plant diterpene 12-O-tetradecanoyl-phorbol-13-acetate (TPA) inhibits nerve growth factor (NGF)-provoked neurite outgrowth in cultured embryonic chick sensory and sympathetic ganglia. Other plant diterpenes that are tumor promoters on the mouse skin carcinogenesis system also inhibit ganglia response to NGF, but structurally related non-promoting compounds are inactive. There is no evidence that the inhibitory effect of TPA is due to cytotoxicity. In fact, it appears that TPA can enhance the survival of neuronal and nonneuronal cells in culture. Although neurite outgrowth is prevented, established neurites do not retract in the presence of TPA. After 24 hr, ganglia can slowly overcome the block, even in the presence of fresh TPA; thus, inhibition is transient. The concentration of NGF that does induce a half-maximal neurite outgrowth response in sensory ganglia is approximately $0.6 \times 10^{-11}$ M. The antagonism of NGF by TPA is dose dependent and apparently noncompetitive. TPA concentrations that extensively inhibit neurite outgrowth do not affect the amount of $^{125}$I-labeled NGF bound to specific sites on dissociated dorsal root ganglion cells, which supports the contention that TPA acts at a stage beyond the initial interaction of the factor with its receptor site.

INTRODUCTION

The process of neurite outgrowth and arborization is an important determinant of the function of nerve cells, yet little is known of how neuronal topography is regulated. The structural features of NGF have been studied in detail (1, 44, 46); this polypeptide possesses the remarkable property of being able to promote neurite extension from embryonic vertebrate sensory (13) and sympathetic (29) ganglia and can accelerate maturation of sensory and sympathetic neuroblasts (28, 29). NGF can also accelerate the sprouting of transected axons of central catecholaminergic neurons (9). The detailed mechanism by which NGF promotes neurite outgrowth is not known. Production of NGF or NGF-like activity by a number of cell types in vitro (34, 51), growth of neurites toward explants of target tissues (16), confinement of neurite extension to local regions in culture containing adequate amounts of NGF (15), prevention of sympathetic ganglion cell degeneration normally following axotomy in young rats by the administration of NGF (21), and retrograde transport studies (22) support the assertion that the target cells could maintain and perhaps attract contact with appropriate neurons through NGF release.

This report concerns the effects of TPA on NGF-induced neurite outgrowth from cultured chick embryonic ganglia. As neurite outgrowth can be regulated by local concentrations of NGF (15), this process may also be considered an aspect of morphological differentiation. TPA and other plant diterpenes are among a class of compounds that are potent tumor promoters in the 2-stage mouse skin carcinogenesis system (6, 20, 42). Some of the metabolic effects of promoters have been reviewed (10, 35), but the mechanism of promotion remains obscure. Of particular interest are the recent observations that TPA can inhibit fusion and myogenesis of cultured chick myoblasts (17), spontaneous and induced differentiation of Friend erythroleukemia cells (37, 49), and conversion of 3T3 fibroblasts to adipose-like cells in culture (18). If tumor promotion involves inhibition of cellular differentiation, neurite outgrowth might also be affected. This possibility was tested, and my results show that TPA and other tumor-promoting plant diterpenes can specifically and reversibly inhibit neurite outgrowth provoked by NGF in cultured embryonic chick ganglia, in a manner unrelated to cytotoxicity.

MATERIALS AND METHODS

Materials. Carrier-free Na$^{125}$I was obtained from New England Nuclear, Boston, Mass., and BSA, Fraction 5, was obtained from Consolidated Midland Corp., Brewster, N. Y. Ingenol dibenzate and mezerein were obtained from Dr. I. B. Weinstein of the Cancer Research Institute, Columbia University, N. Y. The phorbol esters were dissolved at 1 mg/ml in ethanol and stored at $-20^\circ$.

Preparation and Iodination of NGF. The $\beta$ subunit, in which all of the NGF activity resides, was prepared from the 7S NGF complex of mouse submaxillary glands by procedures described by others (38, 43, 44). Purity was confirmed by the presence of a single band on isoelectric focusing (pH 3.5 to 10) in 7.5% polyacrylamide gels.

Purified $\beta$ NGF was iodinated to 20 to 30 cpm/pg (0.5 iodines/mol NGF), as described by Herrup and Shooter (23). The protein-bound iodine was separated from free iodine by elution through a BioGel P-10 column (1 × 10 cm) with the BSA buffer [0.5 mM NaCl, 50 mM sodium acetate (pH 4.0), sodium azide (0.2 mg/ml), and BSA (1 mg/ml) included.

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as a precaution against nonspecific adsorption of NGF. The stock solution of ¹²⁵I-labeled NGF was stored in the BSA buffer at 4° and used within 20 days of preparation. Prior to incubation with cells, this stock was diluted more than 1000-fold; hence, the azide would have no deleterious effect.

**NGF Bioassay.** A modification of the original bioassay procedure used by Levi-Montalcini et al. (30) was used. Dorsal root ganglia from 8- to 10-day-old or lumbar sympathetic ganglia from 13-day-old white leghorn chick embryos were cultured in Roswell Park Memorial Institute Tissue Culture Medium 1640 containing BSA (1 mg/ml) on a rat-tail collagen-coated microwell plate (Model FB 16-24C Disposo trays; Linbro Chemical Co., New Haven, Conn.). Test samples were incubated at 37° with ganglia (5 or 6) for 18 to 24 hr in humidified air. The extent of fiber outgrowth was scored on an arbitrary scale of 0 to 5, in which the maximum score was given for a symmetrical halo of nerve fibers when the fiber length exceeded the diameter of the ganglia.

**Ganglia Cell Cultures.** Excised dorsal root ganglia were washed in Hanks' salt solution and were then incubated for 20 min at 37° in Hanks' salt solution containing 1 mM EDTA and 0.05% trypsin. The ganglia were dissociated by trituration through a Pasteur pipet. Ganglionic debris was removed by an initial centrifugation at 300 g for 1 min, and then the cells were collected by centrifugation at 600 × g for 5 min. The pellet was resuspended in Roswell Park Memorial Institute Tissue Culture Medium 1640 containing 10% fetal calf serum. The cells (10⁶ cells/ml) were seeded onto collagen-coated microwells and incubated under various conditions, as explained in the individual figure legends. The cultures were examined under low-power phase-contrast with a Leitz Diavert microscope equipped with an Orthomat-W automatic 35-mm camera.

**Binding of ¹²⁵I-labeled NGF.** The cells obtained from the dissociated ganglia were resuspended in Gey's balanced salt solution, modified so that the phosphate salts were increased to 25 mM (pH 7.4), with the NaCl reduced to 110 mM to maintain the initial ionic strength; BSA (1 mg/ml) was also included. The modified Gey's solution was filtered through a Nalgene filter of 0.20 µm (Sybron Corp., Rochester, N. Y.), and all other solutions were centrifuged at 15,000 × g for 30 min to remove all suspended particulate matter prior to use. In other respects the assay for binding of ¹²⁵I-labeled NGF followed closely that described by Herrup and Shooter (23) and modified by Sutler et al. (39). In essence the cells (10⁶) were incubated for 30 min at 37° with ¹²⁵I-labeled NGF (1 ng/ml) and other additions as explained in the legends to the experiments. The incubations were discontinued by immersion in an ice bath, and the samples were immediately layered over a discontinuous gradient of 150 µl of the modified Gey's solution containing 0.3 M sucrose overlaid with 100 µl of the modified Gey's solution containing phenol red (20 µg/ml) and 0.15 M sucrose prepared in a 400-µl Microfuge tube (Beckman Instruments, Inc., Palo Alto, Calif.). The tubes were then centrifuged for 5 min in a Beckman Model 152 Microfuge. The tubes were immersed in an acetone-dry ice bath and were then quickly severed at approximately one-fourth of the distance from the bottom. The cell pellet fraction in the upper portion of the tube were assayed for radioactivity content by a Nuclear-Chicago well-type γ-scintillation detector. The pellet fraction obtained from control incubations conducted in the absence of cells did not significantly increase in radioactivity content over background. All assays were performed in quadruplicate.

**RESULTS**

**Time Course and Concentration Dependence of TPA Effect.** Dorsal root ganglia from embryonic chicks were explanted onto collagen-coated tissue culture wells and incubated at 37°. In the absence of NGF, there was no detectable neurite outgrowth, but in the presence of NGF (1 ng/ml) a maximum response was attained in about 24 hr and sustained for at least 48 hr (Chart 1), which is consistent with the observations of most workers who do not include serum in their incubation medium (serum can cause neurite outgrowth and has components that can bind to NGF). When the potent tumor-promoting compound TPA (100 ng/ml) (1.7 × 10⁻⁷ M) was introduced to NGF-treated cultures after 3.7 hr incubation, further neurite outgrowth was inhibited for up to 24 hr. TPA did not cause a detectable retraction of established neurite outgrowth in this experiment nor in other experiments when added to sensory ganglia cultures previously exposed to NGF for 24 hr (data not shown). Thereafter, a gradual increase in neurite outgrowth was observed. There may have been inactivation of the TPA due to degradation or metabolism, but there are other plausible possibilities that shall subsequently be discussed. Neurite outgrowth provoked by NGF from 13-day-old embryonic chick sympathetic ganglia was also inhibited by TPA; thus, the effect of TPA is not restricted to sensory ganglia. The remaining studies in this report were conducted on dorsal root ganglia, which are more accessible to dissection.

A log dose-response curve for inhibition by TPA of dorsal

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**Chart 1. Time course of TPA action.** Two groups of embryonic 8-day-old dorsal root ganglia were incubated in the presence of NGF (1 ng/ml). After 3.7 hr (arrow), TPA (100 ng/ml) was added to 1 group, and the rate of neurite outgrowth was observed for 2 days. △, TPA-treated cultures; ○, controls. The symbols show the mean ± S.E. of the ganglia response (TPA-treated, n = 5; controls, n = 10).
root ganglia response to an optimum concentration of NGF is presented in Chart 2. The ganglia were incubated in the presence of NGF (1 ng/ml) and various concentrations of TPA and were then scored for neurite outgrowth after 1 day. Half-maximum inhibition occurred at about 10 ng/ml (1.7 × 10⁻⁸ M) TPA, and near maximum inhibition was obtained in the presence of 300 ng/ml (5.1 × 10⁻⁷ M) TPA.

**Morphology of Ganglia Exposed to TPA and NGF.** Ganglia incubated in the absence of NGF extended no neurites and were poorly anchored to the collagen substrate, and migratory cells were only occasionally observed at the periphery of explants (Fig. 1A). In the presence of NGF (10 ng/ml), there was profuse outgrowth of long fibers, which formed bundles or plexuses (Fig. 1B). Cells do migrate away from the ganglia but can be obscured by the extensive neurite network. When ganglia were incubated in the presence of TPA (200 ng/ml), very few processes were evident and, in contrast to control cultures, a small but substantial number of cells migrated out from the ganglia (Fig. 1C). Neurites formed in the presence of both NGF (10 ng/ml) and TPA (200 ng/ml) were shorter, thicker, and sparser than those of NGF-treated cultures (Fig. 1D). Also, it again seemed that more cells had migrated away from the ganglia. A greater number of cells around the periphery of the ganglia may be a consequence of the increased viability of cells in the presence of TPA, which shall be examined.

**Reversibility of TPA Inhibition.** The data in Chart 1 showed that after 24 hr there was a slow escape from inhibition by TPA. For examination of the basis for this escape, the following experiment was performed (data not shown). Ganglia were preincubated for 20 hr in the presence of both NGF (1 ng/ml) and TPA (100 ng/ml), and then the ganglia were incubated for an additional 50 hr under a variety of conditions. When the incubations were simply continued with no change in the conditions, there was escape from inhibition at a slow and constant rate so that by 50 hr the bioassay response had increased from 1.9 to approximately 3.9. Interestingly, the rate of neurite outgrowth was not affected when the ganglia were washed free of TPA or when fresh TPA was added to the incubations. Thus, the escape is not due to degradative or metabolic inactivation of TPA. The failure to obtain a more rapid rate of outgrowth upon washout of TPA may simply reflect the persistence of the compound trapped within the confines of the ganglia capsule or a relatively slow rate of its dissociation from lipophilic binding sites. One gains the overall impression that the cells can undergo a metabolic change to overcome eventually the inhibitory effect of TPA, but any attempt at explanation remains unsatisfactorily speculative at this stage of understanding. The neuronal cells, then, may overcome the inhibition of neurite outgrowth by TPA, and inhibition of neurite outgrowth is transient.

**Effect of TPA on Cell Survival in Culture.** Experiments were conducted to examine whether TPA was cytotoxic to ganglion cells. Ganglia were dissociated, and cultures consisting primarily of single cells were incubated for 20 hr (Table 1). The total number of cells and the number of phase-bright cells were determined and compared to the numbers present after only 3 hr in culture. Assuming that the phase-bright cells represent the neuronal cell population, there was a loss of 68% of the neuronal cells and 32% of the nonneuronal cells in the absence of NGF. When NGF (1 ng/ml) was present, loss of neuronal cells did not occur, but loss of nonneuronal cells remained the same. Interestingly, in the presence of TPA (100 ng/ml) alone there was substantially enhanced survival of ganglion cells. There was a better than 2-fold increase in the number of surviving neuronal cells and only a 7% loss of nonneuronal cells. The combination of TPA and NGF appeared to increase the number of surviving cells over that of either acting alone, although this mixture should only improve survival of nonneuronal cells over that of NGF-treated cultures and may have increased the total number of cells in culture over that present in the 3-hr control cultures. These experiments do not reveal whether TPA is acting as a maintenance factor or as a mitogen, and it has not yet been established whether the ability of TPA to support neuronal cells in culture is related to the tumor-promoting property of this diterpene. At any rate TPA is certainly not cytotoxic to ganglion cells at concentrations that antagonize NGF. On the contrary, it seems to enhance their survival in culture.

**Effect of Tumor-promoting and Inactive Plant Diterpenes on Neurite Outgrowth.** The ability of plant diterpenes to inhibit ganglia response to NGF correlates with their tumor-promoting property in the mouse skin 2-stage carcinogenesis system (6, 20, 42). Ganglia explants were incubated for 24 hr with NGF alone or together with 1 of the plant diterpenes indicated in Table 2. PDD shares the phorbol structural nucleus with TPA, is a potent promoting agent, and also inhibits neurite outgrowth. But, the stereoisomer 4-a-phorbol-12,13-didecanoate, as well as phorbol itself, are not promoters in the mouse skin system (10, 20, 42) and were not antagonists of neurite outgrowth. The stereospecificity of inhibition suggests that the tumor-promoting plant diterpenes might inhibit neurite outgrowth by interaction with a receptive macromolecule. Phorbol in tissues other than mouse skin is a promoter. For example, it can promote leukemia and also tumors in liver and lung.
Table 1

**Tumor Promoters and Ganglia Response to NGF**

A single-cell suspension was prepared from 8-day-old embryonic chick dorsal root sensory ganglia. The cells (10^6) were seeded onto collagen-coated wells containing 1 ml of Roswell Park Memorial Institute Tissue Culture Medium 1640 with 10% fetal calf serum, BSA (1 mg/ml), and the additions indicated in the table. After 3 and 20 hr, 2 fields were selected at random, and the total number of cells and the number of phase-bright cells in culture were determined by microscopic examination.

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Time (hr)</th>
<th>Total</th>
<th>Phase-bright</th>
<th>Difference^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>298 ± 26^b (100)^c</td>
<td>85 ± 14 (100)</td>
<td>213 ± 15 (100)</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>171 ± 13 (57)</td>
<td>27 ± 2 (32)</td>
<td>144 ± 13 (68)</td>
</tr>
<tr>
<td>NGF (1 ng/ml)</td>
<td>20</td>
<td>235 ± 9 (79)</td>
<td>83 ± 4 (98)</td>
<td>151 ± 5 (71)</td>
</tr>
<tr>
<td>TPA (100 ng/ml)</td>
<td>20</td>
<td>260 ± 30 (87)</td>
<td>62 ± 10 (73)</td>
<td>199 ± 21 (93)</td>
</tr>
<tr>
<td>NGF (1 ng/ml) plus</td>
<td>20</td>
<td>347 ± 32 (116)</td>
<td>97 ± 14 (114)</td>
<td>251 ± 21 (118)</td>
</tr>
<tr>
<td>TPA (100 ng/ml)</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

^a The difference between total and phase-bright values is the number of non-phase-bright cells.
^b Mean ± S.E. for 4 replicate cultures.
^c Numbers in parentheses, percentages calculated relative to the 3-hr control values.

Table 2

**Effect of tumor-promoting and inactive plant diterpenes on the response of embryonic chick sensory ganglia to NGF**

Embryonic chick (8-day) dorsal root ganglia were incubated for 24 hr on collagen-coated tissue culture wells in Roswell Park Memorial Institute Tissue Culture Medium 1640 containing BSA (1 mg/ml) and NGF (1 ng/ml) in the presence or absence of the compounds indicated (200 ng/ml).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Ganglia response^a</th>
<th>Tumor-promoting activity^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.8 ± 0.1^c</td>
<td>-</td>
</tr>
<tr>
<td>TPA</td>
<td>1.6 ± 0.2</td>
<td>+</td>
</tr>
<tr>
<td>Phorbol</td>
<td>4.8 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>PDD</td>
<td>2.1 ± 0.4</td>
<td>+</td>
</tr>
<tr>
<td>4-α-phorbol-12,13-didecanoate</td>
<td>4.7 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>Ingenol dibenzoate</td>
<td>2.1 ± 0.3</td>
<td>+</td>
</tr>
<tr>
<td>Mezerein</td>
<td>1.9 ± 0.1</td>
<td>+</td>
</tr>
</tbody>
</table>

^a Ganglia were scored on an arbitrary scale ranging from 0 to 5.
^b Tumor-promoting activity was determined in the mouse skin assay system by others (3, 10, 21, 42).
^c Mean ± S.E. (control, n = 10; others, n = 5 or 6).

in mice when administered systemically (2, 8). But metabolism of phorbol may be required to produce an active principle. Ingenol dibenzoate and mezerein are nonphorbol macrocyclic plant diterpenes. Both of these compounds inhibited neurite outgrowth and are promoting agents, although mezerein is considered less potent than TPA in the latter respect (20). Mezerein does share with TPA the ability to inhibit differentiation of erythroblastic cells (49) and murine neuroblastoma cells (24) in culture.

Although it would have been desirable to correlate inhibition of neurite outgrowth with a quantitative rather than a qualitative assessment of tumor-promoting activity, the mouse skin test system does not readily lend itself to this preference. Test compounds are repeatedly applied in a volatile solvent to mouse skin over a prolonged period of time, and a quantitative interpretation of dosage must also consider possible differences in permeability, stability, metabolism, and excretion between test compounds.

**Effect of TPA on Bioassay Dose-Response Curve.** When ganglia are incubated in the presence of various concentrations of NGF, a bell-shaped dose-response curve is obtained. The maximum response is near 1 ng NGF per ml. It is often reported that 10 ng NGF per ml are required, but the presence of BSA (1 mg/ml) shifts the dose-response curve to the left (data not shown) by simply blocking nonspecific adsorption to incubation vessel walls. Complications may arise when serum is present, as was previously mentioned. Concentrations of NGF larger than 1 ng/ml result in an apparently diminished response due to entanglement of exuberant neurite outgrowth within the confines of the ganglia capsule (27). In fact, a normal plateau in the dose-response curve is obtained at high concentrations of NGF in single-cell cultures, and the maximum response occurs between 0.3 and 1.0 ng NGF per ml (19, 33). In this laboratory, when the bioassay is scored, an attempt is made to adjust the score in a manner proportionate to the fractional response. Thus, the left-hand rising portion of the ganglia dose-response curve may be analyzed in the normal way. There was a reduction in the maximum response when the dose-response curve was determined in the presence of 3 different concentrations of TPA (not shown). TPA did not alter the affinity of NGF binding, as the position of the maximum response was the same. The results indicate that TPA is not a competitive inhibitor of NGF, a situation that would tend to shift the curve to the right with no change in the maximum response. Instead, the diminished maximum response and the reduced slope of the rising portion of the curve are consistent with the behavior of a noncompetitive antagonist. For confirmation of this supposition, the data from the rising portion of the curves were subjected to the Lineweaver-Burk type of double-reciprocal plot (Chart 3). The maximum attainable response to NGF steadily declined in the presence of increasing TPA concentrations, but all of the response curves gave the same ED_{50} (0.6 × 10^{-11} M). The ED_{50} may be an underestimate of the equilibrium dissociation constant, K_{d}, as will be discussed later. This pattern of response is characteristic for noncompetitive inhibition, but the appearance of noncompetitive behavior could arise through mechanisms not directly involving the NGF receptor. However, although the data are apparently linear on a double-reciprocal plot, scoring is not on a
The abscissa are to be multiplied by 10. The calculations are based on the TPA, as indicated in the chart. After 1 day the ganglia response was scored continuous scale and is only semiquantitative. The mean ganglia response is expressed as a fraction relative to the theoretical maximum bioassay presented as a plot of the reciprocal of the mean ganglia response (A) against the reciprocal of the NGF concentration. The data from the rising portion of the dose-response curves are

suggestion that TPA inhibits a later step in the process leading to neurite outgrowth.

DISCUSSION

TPA and other tumor-promoting plant diterpenes can inhibit neurite outgrowth provoked by NGF from cultured embryonic chick sensory ganglia (Chart 1; Table 2). Structurally related but nonpromoting congeners are inactive. Ganglia eventually escape from the TPA inhibition (Chart 1); hence, the antagonism is transient and, in this sense, reversible and unrelated to a cytotoxic effect (Table 1). TPA gives the appearance of noncompetitive antagonism (Chart 3), and neither alters the ED₅₀ for NGF nor inhibits the occupancy of the NGF-binding sites on ganglion cells (Table 3). The ED₅₀ was 0.6 × 10⁻¹¹ M. Sutter et al. (39) reported a high-affinity class of binding sites (their Site I) with an equilibrium dissociation constant (Kₘ) of 2 × 10⁻¹¹ M which, when 10% occupied, caused a half-maximal response from cultures of single ganglion cells. The observed ED₅₀ does underestimate the dissociation constant, as expected when spare receptors are present.

TPA can inhibit neurite outgrowth provoked by serum deprivation, prostaglandin E₁, 5-bromo-2'-deoxyuridine, and papaverine in cultured mouse neuroblastoma (24). Inhibition of neurite outgrowth is, therefore, not secondary to TPA effects on ganglionic nonneuronal elements. Moreover, TPA can inhibit neurite outgrowth from a variety of neurons responding to a variety of stimuli. Mouse neuroblastoma cells are not known to be responsive to NGF and are not known to have NGF-binding sites which satisfies expectations for a specific receptor. There are reports of NGF binding-to these cells (36) but only at concentrations several orders of magnitude above the physiologically relevant range. These observations limit the likelihood that TPA inactivates the receptor while binding of NGF remains unaffected and indicate that the NGF receptor is not the site of action.

**Table 3**

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Bound ¹²⁵I-labeled NGF</th>
<th>%a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1860 ± 208b</td>
<td>100</td>
</tr>
<tr>
<td>NGF (10 µg/ml)</td>
<td>174 ± 59</td>
<td>9</td>
</tr>
<tr>
<td>TPA (200 ng/ml)</td>
<td>1996 ± 199</td>
<td>107</td>
</tr>
<tr>
<td>TPA (200 ng/ml) plus NGF (10 µg/ml)</td>
<td>172 ± 71</td>
<td>9</td>
</tr>
</tbody>
</table>

a The percentages were calculated relative to the control value. b Mean ± S.E. for replicate incubations (n = 4).
The role of microtubules in neurite outgrowth under the direction of NGF has been examined, and there is no change in the colchicine-binding capacity in the neuroplasm during neurite formation (32). It is possible that NGF might increase microtubule assembly from a previously existing tubulin subunit pool. Colchicine can, of course, prevent neurite outgrowth through binding to tubulin and disruption of microtubules resulting in neurite retraction (47). TPA appears unlike colchicine, as its presence does not promote fiber retraction (Chart 1), but the possibility that TPA can alter polymerization of tubulin or the assembly of other important structural proteins is worth consideration.

The most attractive present hypothesis is that TPA acts at or near the growth cone. Neurite extension and the shape of arborization is regulated by the activity of the growth cone (11, 12). The only organelles visible in the advancing portion of the growth cone are the plasma membrane and a microfilamentous lattice (14, 31, 40); neurite extension appears to occur only at or just behind the growth cone (11). Removal of NGF from the local vicinity of extending neurites will lead to retraction, even though the soma remains exposed to the factor (15). Since TPA does not cause fiber retraction, phorbolesters probably do not interfere with the early cellular response to NGF. Indeed, the simplest of several explanations for escape from TPA inhibition (Chart 1) is that accumulation of a metabolite occurs, which, upon exceeding a certain threshold, will overcome the blockade. TPA may act in a manner similar to that of cytochalasin B, which causes retraction of filopodia and cessation but not retraction of neurite outgrowth (48), or it could alter the adhesivity of growth cones to the substratum, which seems an important determinant of cell process extension (26). The possibility that TPA acts at or near the growth cone is presently being examined.

The survival of ganglion cells in culture is enhanced by TPA (Table 1). The means by which this is accomplished are not clear. One possibility is that TPA might stimulate cell division, as in mouse skin epidermis (3, 35) and in rat thymic lymphocytes in vitro (45). Neurons at the stage of development used in this study are essentially nondividing (50) and do not incorporate thymidine in culture (41). But if neurons are arrested in a particular phase of the cell cycle by chalone-like substances, as seems to be true in epidermal tissue (25), TPA might be able to release the cells. Alternatively, TPA may mimic NGF in being able to maintain the survival of cultured neurons (5). There are no convincing data that NGF is a mitogen (33).

The tumor-promoting phorbol esters have similar effects in other cell systems. These compounds inhibit differentiation of cultured muscle cells (17), 3T3 fibroblast cells (18), and erythroleukemia cells (37, 49). Inhibition in these systems is reversible and occurs at nontoxic concentrations of the compound. The addition of TPA to committed cultures of erythroleukemia cells does not suppress the eventual expression of differentiation (49). The 3T3 fibroblast cells could escape from the inhibitory effect of the promoters (18), as could the ganglion cells in this study. If we view neurite outgrowth from embryonic ganglia and from neuroblastoma as an aspect of morphological differentiation, this study and the studies cited in this paragraph support the speculation made by Berenblum (7) many years ago that tumor promoters act on initiated mouse skin stem cells by inhibition of the process of terminal differentiation.

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REFERENCES

D. N. Ishii


Fig. 1. Morphology of chick sensory ganglia cultured in the presence of NGF and TPA. Dorsal root ganglia from 8-day-old embryonic chicks were incubated for 20 hr on collagen-coated coverslips in Roswell Park Memorial Institute Tissue Culture Medium 1640 with BSA (1 mg/ml) and the following additions: A, none; B, NGF (10 ng/ml); C, TPA (200 ng/ml); D, NGF (10 ng/ml) plus TPA (200 ng/ml). The ganglia were photographed under phase-contrast microscopy. A and B, \( \times 125 \); C and D, \( \times 250 \).
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