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Regulation of the Differentiation of PC12 Pheochromocytoma Cells

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The PC12 clone, developed from a pheochromocytoma tumor of the rat adrenal medulla, has become a premiere model for the study of neuronal differentiation. When treated in culture with nanomolar concentrations of nerve growth factor, PC12 cells stop dividing, elaborate processes, become electrically excitable, and will make synapses with appropriate muscle cells in culture. The changes induced by nerve growth factor lead to cells that, by any number of criteria, resemble mature sympathetic neurons. These changes are accompanied by a series of biochemical alterations occurring in the membrane, the cytoplasm, and the nucleus of the cell. Some of these events are independent of changes in transcription, while others clearly involve changes in gene expression. A number of the alterations seen in the cells involve increases or decreases in the phosphorylation of key cellular proteins. The information available thus far allows the construction of a hypothesis regarding the biochemical basis of PC12 differentiation.

Introduction

Between 1948, when the first definable experiment on nerve growth factor (NGF) was published, and 1976, when the first report on PC12 cells appeared, there was relatively little progress toward an understanding of the mechanisms by which nerve growth factor acts on its target cells. The reason for this difficulty is that the classical targets of nerve growth factor, sympathetic and sensory neurons, are difficult to harvest, difficult to culture, and, above all, absolutely dependent on nerve growth factor for survival. Thus, any experiments directed toward the biochemical or molecular consequences of nerve growth factor action on these cells suffered from the criticism that the controls, those not given nerve growth factor, were dying. In short, it was difficult if not impossible to say whether a given biochemical response was a specific action of nerve growth factor or simply a result of the fact that the cells were not dying. Clearly, a tool was needed with which to study the actions of the factor in the absence of the confounding consideration of frank survival. Such a tool was provided by the development of PC12 cells. These cells are currently the premiere tool for the study of nerve growth factor, but, more than that, they have become a very important model for the study of neuronal differentiation. Indeed, the findings with PC12 cells, in some cases, have implications for differentiation of cells in general.

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Properties of PC12 Cells

PC12 cells were cloned from a solid pheochromocytoma tumor passaged subcutaneously in New England Deaconess Hospital strain white rats (*1*). The original report indicated that the cells had a chromosome number of 40 and exhibited no discernable changes in properties for the first 70 passages in culture. The cells contained dense-core, chromaffinlike vesicles and catecholamines were found by histofluorescense methodology. Upon treatment with NGF the cells appeared to stop dividing and elaborated branched, varicose processes. Removal of NGF led to a degeneration of the processes and a resumption of cell division, indicating that the effects of NGF were rather readily reversible. Subsequent studies showed that the NGF-induced cessation of cell division and elaboration of processes was accompanied by the development of electrical excitability and an increased sensitivity to iontophoretically applied acetylcholine (*2*). The neuronlike character of the NGF-differentiated cells was validated by the demonstration that the cells would form functional cholinergic synapses with myotubes from the clonal rat skeletal muscle line L6 (*3*).

PC12 cells are small (6–14 μm in diameter), round, catecholamine-containing cells that grow in standard, serum-

supplemented medium with a doubling time of between 48 and 96 hr. They attach to plastic culture surfaces, but relatively loosely, and so for normal maintenance, trypsinization is not necessary. For long-term studies with the cells, collagen or polylysine substrates are advisable. The cells are rather plastic in that the cultures become somewhat heterogeneous in morphology after 25 to 35 passages. They can be grown in serum-free medium, or in suspension cultures, although the characteristics of cells grown under such conditions have not been explored as extensively as have those of cells grown as monolayers in serum-containing medium.

Unlike the adrenal medulla, in which norepinephrine is the major catecholamine, the PC12 cells contain primarily dopamine (*1,4*), on the order of 15 nmole/mg cellular protein. The norepinephrine content is about one-third to one-tenth that of dopamine, and little or no epinephrine is found. The cells will take up catecholamines from the medium by a system similar to that found in sympathetic neurons and store them in chromaffin-type granules (*5*). The cells will release catecholamines in a Ca^{2+}-dependent fashion in response to depolarization (*6*); stimulation of the cells with nicotinic cholinergic agonists also causes catecholamine release (*7*). The cells will synthesize, store, and release acetylcholine (*8*). The storage granules that contain acetylcholine appear to be of the dense-core variety, similar to those that contain catecholamines, rather than the translucent granules usually found in cholinergic nerve terminals (*9*).

The enzymes for the synthesis of catecholamines are also present in PC12 cells (*1*). The tyrosine hydroxylase has been inspected in detail, both for the short-term influences on its activity (*10,11*) and for regulation of its transcription (*12,13*). The dopamine β-hydroxylase of these cells has been found to be a glycoprotein containing two subunits of molecular weights 73,000 and 77,000 that occur in almost equal amounts in the molecule (*14*); the biosynthesis of the soluble and the membrane-bound forms of the enzyme has also been studied (*15*). There is some suggestion in the early work that the preponderance of dopamine over norepinephrine in these cells is due, not to a deficiency of dopamine β-hydroxylase, but to a relative lack of ascorbic acid, a necessary cofactor for this enzyme (*16*). Choline acetyltransferase is found in PC12 cells (*3,8*) and its induction by NGF has been studied (*17*). Acetylcholinesterase is also, of course, present and appears to exist in three molecular forms, 4, 6.5, and 10S, in dividing cells (*18*). Treatment with NGF causes the appearance of a small amount of a 16S form.

The cells have receptors for a number of different ligands. The receptors for NGF are found in two different forms (*19,20*). These forms are distinguished on the basis of their off-time, the rate with which the ligand, NGF, is released from its binding site. About 95% of the total receptors are so-called "fast" receptors, with an off-time of some 30 sec; the remainder "slow" receptors, have an off-time on the order of 30 min. The fast receptor appears to have an affinity constant of about 1 nM, the slow receptor binds NGF about 100 times more tightly. When PC12 cells are treated with trypsin or extracted with Triton X-100, the number of fast receptors is depleted; the slow receptors are relatively resistant to these treatments (*21*). The molecular weight of the low-affinity receptor is on the order of 90,000, that of the high-affinity site somewhere between 160,000 and 200,000, although both are reported to contain the same protein at the binding site (*22*). The molecular relationship between these two forms of the receptor is not known in detail, but it is widely assumed that the high-affinity receptor is responsible for the physiological actions that nerve growth factor exerts on the cells. Indeed, there is good evidence to indicate that only the high-affinity form is internalized (*23*), and mutants that express only the low-affinity form do not respond to NGF (*24*). The general properties of the NGF receptors on PC12 cells are summarized in Table 1. The cells display receptors for the mitogen epidermal growth factor (*25,26*) and these receptors are down-regulated by NGF (*25,27*). It has also been reported that two classes of epidermal growth factor receptors can be seen (*28*). Receptors for fibroblast growth factor are also present on PC12 cells (*29*).

The acetylcholine sensitivity of the PC12 cells is due to the presence of both nicotinic and muscarinic acetylcholine receptors. Stimulation of the nicotinic receptors leads to the release of catecholamines (*7*). These receptors appear to be similar to those seen on sympathetic neurons (*30,31*), and cross-react with a monoclonal antibody prepared against chicken brain nicotinic receptors (*32*). The muscarinic receptors appear to be comparable to those found on adrenal medullary cells (*33*). Stimulation of the muscarinic receptors is reported to cause an influx of Ca^{2+} and an increase in the hydrolysis of phosphoinosi-

Table 1. Properties of the nerve growth factor receptors of PC12 cells.

Property	High affinity, slow receptors	Low affinity, fast receptors	References
Affinity	$5\text{-}20 \times 10^{-11}$M	$0.2\text{-}3.2 \times 10^{-9}$M	(*20,205-207*)
Dissociation rate	$4\text{-}5.8 \times 10^{-4}S^{-1}$	$2\text{-}2.3 \times 10^{-2}S^{-1}$	(*19,20*)
Receptor number/cell	2,500-15,000	45,000-180,000	(*20,205-207*)
Molecular weight			
Core protein	Unknown	42,478	(*198,199,208*)
Mature receptor after glycosylation and sialylation	~90,000	~70,000-83,000	(*22,198,199,208*)
After cross-linking	135,000-225,000	87,000-107,000	(*22,209-211*)
Detergent extractability	Resistant	Susceptible	(*206*)
Sensitivity to proteolysis	No	Yes	(*19,206*)
Sequestration and internalization	Yes	No	(*23,197*)
Transduction of NGF signal	Yes	No	(*212*)

tides (*34,35*). PC12 cells are rich in adenosine receptors (*36*), and at least some of these receptors are coupled to adenylate cyclase. The receptors appear to be of the A2 type (*37,38*). Enkephalin receptors have been reported (*39*) and these receptors increase in number upon treatment of the cells with NGF. There is also indirect evidence that the cells display receptors for bradykinin (*40*).

The ability of PC12 cells to conduct electrical signals is determined by the presence and characteristics of specific ion-conducting components localized within the plasma membrane. These ionic channels selectively regulate the transmembrane fluxes of Na^+, K^+, and Ca^{2+} and most are sensitive to the transmembrane voltage. Besides being involved in the propagation of electrical activity, these channels regulate neurotransmitter release, enzymatic reactions, and receptor function. A variety of ion channels have been detected in PC12 cells (*41,42*). There are voltage-dependent Na^+ channels, voltage-dependent Ca^{2+} channels, voltage-dependent K^+ channels, and Ca^{2+}-dependent K^+ channels. Ion channels are also seen on the growth cones of PC12 cells induced to form neurites with NGF. Evidence has been presented to indicate that the growth cones display Na^+ channels, two types of Ca^{2+} channels, and more than one type of K^+ channel (*43*).

Much of the present effort is directed toward an exploration of the various calcium channels. It is clear that more than one type of calcium channel can be found on the membranes of these cells. There are studies indicating that the uptake of calcium is altered by the presence of ethanol (*44–46*). A number of experiments suggest that at least one type of calcium channel is regulated by phosphorylation by protein kinase C (*47–50*). The uptake of calcium by specific channels has been correlated with transmitter release (*51*). In the absence of nerve growth factor, PC12 cells are electrically inexcitable (*42*) because of the very low density of Na^+ channels present, although a sufficient number of voltage-dependent K^+ channels are found. Upon treatment with NGF there is a gradual 10- to 20-fold increase in the density of Na^+ channels (*52*), and the cells acquire neuronal excitability. Finally, the PC12 cells appear to be one of the richest known sources of the Ca^{2+}-activated, apamin-sensitive K^+ channel (*53*).

A number of PC12 cell mutants have been described. Among the first was PC12h (*54*), a subclone that, unlike the parent PC12, exhibits an induction of tyrosine hydroxylase upon treatment with NGF. There are several variants that lack the NGF receptors (*55*), although these cells are quite different than the parent, and it is generally felt that they are more than simple receptor deletions. Clones that react slowly to NGF and those that respond faster have been studied (*56*). Adenosine kinase-deficient cells have been prepared (*57*), as have cells with an altered response to dBcAMP (*58*). A PC12 mutant that lacks a cell surface heparan sulfate proteoglycan has been reported, and this mutant has an altered distribution of one of the forms of acetylcholinesterase (*59*). One of the most interesting reports has been of the mutant, mentioned above, that lacks high-affinity or slow receptors (*24*). There are clones that are deficient in catecholamine transport (*60*), and clones that have a defect in one of the cAMP-dependent protein kinases (*61*). A subline has been reported in which cAMP mimics the actions of nerve growth factor on neurite outgrowth (*62*). Finally, there is a clone for which NGF, instead of being a differentiating agent and inhibiting proliferation as it does in the parent, appears to be a mitogen (*63*). This increasing catalog of subclones gives promise of allowing a dissection of the various properties of the PC12 cells.

Properties of the Differentiating Agent, Nerve Growth Factor

The first definable experiments on NGF showed that fragments of tumor, implanted in chick embryos, elaborated a substance that enhanced neurite outgrowth from sensory and sympathetic neurons (*64,65*). The demonstration that this phenomenon could be duplicated *in vitro* (*66*) led to the isolation and purification of a peptide of molecular weight 26,000 (*67*). The physiological relevance of this factor was demonstrated in experiments in which the peptide was used as antigen. The antiserum elicited, when injected into neonatal rodents, what is now called "immunosympathectomy," the permanent and near-total disappearance of the sympathetic nervous system (*68*). The interpretation of this experiment is that NGF is required, virtually moment to moment, for the survival of sympathetic neurons. Subsequent work has shown that this is true for sensory neurons in prenatal rodents as well (*69*).

The NGF can be isolated in two different forms. The 7S form contains three different kinds of subunits, the alphas, the beta, and the gammas, and has a molecular weight of some 140,000 (*70,71*). The exact functions of the alphas and the gammas are not known. The gamma may be involved in the processing of the active beta subunit; the complex itself may be formed for the protection, the storage, or the transport of the beta. The beta is a noncovalently linked dimer, of molecular weight 26,000, containing two atoms of zinc, and has all the biological activity. It can be isolated in a form, slightly modified by proteolysis, called the 2.5S (*72*). The monomer of the beta dimer has been sequenced. The observation that NGF has some homology to insulin (*73*) has placed it in the family of insulinlike growth factors.

The gene for NGF has been identified in both mouse and human (*74,75*). These studies show that there is 86% homology between the two species. One copy of the gene exists in each species and in humans the gene is found on chromosome 1 (*76*). The probes that have become available through this work have allowed a determination of the levels of NGF mRNA, and by inference, the rates of transcription, in various tissues. Through these studies it has been shown that the synthesis of NGF occurs in those tissues innervated by sympathetic and sensory neurons and that the levels of synthesis are proportional to the amounts of that innervation (*77,78*). The original conclusion that innervation actually increased the level of NGF synthesis has now given way to the concept that the

synthesis is controlled by the intrinsic developmental program of the cells that are targets for innervation.

NGF is found in remarkably large amounts in the submaxillary gland of the mature male mouse. The biological purpose of this richness is obscure, but these glands have provided a tractable source of NGF, and this unprecedented and ready access to milligram quantities of pure material has been responsible for the large body of biological information now available about this factor. NGF also occurs in large quantities in guinea pig prostate (*79*), and in the seminal fluid of the bull (*80*). NGFs with reasonable homology to the mouse material have been isolated from snake venom (*81*), and NGF has been isolated from conditioned media from any number of cultured cells (*82*). A material having NGF activity has been found in human placenta (*83*), but detailed characterization of this material has not been presented.

It has been thought that, in general, the cells that respond to NGF all originate in the neural crest. The original work identified sympathetic and sensory neurons as the target tissues for NGF. For these cells, NGF is absolutely required, at certain stages in their development, for their survival. More recently, it has been shown that chromaffin cells of the adrenal medulla, while not requiring NGF for survival, are profoundly affected by it. Adrenal medullary cells from young animals, whether *in vitro* (*84*) or *in vivo* (*85*), respond to NGF treatment with an elaboration of neurites and a conversion into sympathetic neurons. Clearly, a population of neurons in the central nervous system is responsive to NGF as well. The cholinergic neurons of the basal forebrain demonstrate increases in choline acetyltransferase activity, neurite outgrowth, and survival (*86–88*) when treated with the factor. Although the response of the PC12 cells is the most dramatic, a number of other tumor cell lines respond to NGF in one way or the other. Certain human neuroblastomas differentiate in culture when NGF factor is added (*89*). A few gliomas also have been shown to display increased neurite outgrowth (*90*), and there are reports of enzyme induction in some other lines of pheochromocytoma (*91*). The profound changes undergone by PC12 cells are the clearest alterations seen so far in an experimental model. Although NGF has no effect on survival, the reversible differentiation caused by the factor involves virtually every metabolic and morphological characteristic of the cells.

Rapid, Membrane-Based Actions

Perhaps the most rapid changes seen in PC12 cells exposed to NGF are alterations in the structure of the membrane (*92*). Within a few seconds after the addition of NGF, ruffles appear, the microvilli decrease in number, and the density of the coated pits increases. These changes are transient, even in the continued presence of NGF, and the membrane returns to its ground state appearance within 7 min. Short-term changes in morphology are also seen in the growth cone (*93*). Along with these changes in structure come changes in membrane properties. Among these are alterations in the ability of cells to adhere to a plastic substrate or to each other (*94*).

Changes in second messenger levels have been seen immediately after the addition of NGF. cAMP levels have been measured, and moderate, transient increases have been reported (*94*), although these increases have not been seen by others (*95*). Small changes in the flux of Ca^{2+} have also been reported (*96*), but again, these reports could not be substantiated by others (*97*). Increases in the hydrolysis of phosphoinositides are seen within seconds (*98*), perhaps corroborating data in earlier reports showing a somewhat slower increase in the incorporation of inorganic phosphate into phosphatidylinositol and phosphatidic acid (*99*). A synergistic relationship between NGF and bradykinin for phosphatidylinositol turnover in PC12 cells has been reported (*40*).

It has been shown that NGF causes an activation of the Na^+, K^+-pump of PC12 cells (*100*). This increase can be blocked by amiloride, an inhibitor of Na^+ flux, and mimicked by monensin, a Na^+ ionophore, suggesting that the pump is activated by a NGF-induced increase in Na^+ influx. Changes in the transport of a number of other materials have also been reported. The model amino acids, α-amino isobutyric acid and aminocyclopentane-1-carboxylic acid were both taken up more readily by cells treated with NGF (*101*). That these changes were specific to the uptake of amino acids was shown by studies demonstrating that there was no change in the uptake of nucleosides or of catecholamines.

Changes in Phosphorylation

Following tle first demonstrations that NGF treatment of PC12 cells changed the phosphorylation of specific cellular proteins (*102,103*), there have been a number of studies focusing on alterations in various kinases and their substrates. The phosphorylation of tyrosine hydroxylase is increased by treatment of the cells with NGF (*103*), and the activity of the enzyme increases (*104,105*). The increases in phosphorylation occur at several different sites in the molecule (*11,106*) and seem to be caused by increases in the activity of both cAMP-dependent kinases and protein kinase C. Glycogen phosphorylase activity also in increased (*107*), probably by phosphorylation. The dependence of this effect on calcium ion suggests the involvement of a calcium-dependent kinase.

A soluble protein of 100,000 molecular weight, called Nsp100, shows a decreased phosphorylation upon nerve growth factor addition to the cells (*108*). The ability to capture this effect of NGF in a cell-free preparation has allowed the design of studies on the mechanism. It has been shown that the effect of NGF is to lower the activity of the threonine-specific kinase phosphorylating Nsp100 (*109*), and that this effect of NGF is calcium-dependent (*110*). There is evidence that this lowered activity may be due to the phosphorylation of the Nsp100 kinase by protein kinase C (*111*). Recent studies indicate that Nsp100 is, in fact, elongation factor 2 (EF-2) (*112*).

The ribosomal protein S6 exhibits increased phosphory-

lation in cells treated with NGF (*103,113*). Again, the ability to capture this increase in a cell-free system prepared from treated cells has allowed some detailed information to be obtained. The kinase phosphorylating S6 has a molecular weight of some 45,000 (*114*) and is itself activated by phosphorylation, probably by a cAMP-dependent system. The changes in phosphorylation in both EF-2 and S6 indicate the significant actions of NGF on protein synthesis in these cells, although the exact functional consequences of these changes remain to be explored.

Changes in phosphorylation also take place in the nucleus (*102*) and may underlie the transcriptional alterations caused by NGF. One protein phosphorylated is part of the nonhistone group and has a molecular weight of about 30,000. A cell-free system reflecting this alteration has been obtained (*115*), but the function of the protein and the nature of the kinase phosphorylating it remain to be elucidated.

As might be expected in view of the profound morphological changes occuring in the cells, several cytoskeletal proteins show changes in phosphorylation. Vinculin is among these (*116*), as are certain of the neurofilament proteins (*117*) and a 250,000 molecular weight, cytoskeletally associated protein (*118*). The increased phosphorylation of this latter protein can be seen in a properly prepared cell-free system from NGF-treated cells. The phosphorylation of one of the microtubule-associated proteins is also increased by treatment with NGF (*119*).

An increased phosphorylation of synapsin I has been reported to occur after NGF treatment (*120*). The phosphorylation of the NGF receptor also has been seen (*121*), but does not seem to be influenced by NGF and its functional significance is not known.

Alterations in the activities of a few specific kinases have been reported. Among them are the kinase for the 250,000 dalton cytoskeletal protein (*118*), the S6 kinase (*103*), a previously unidentified NGF-activated kinase (*122*), the kinase phosphorylating Nsp100/EF-2 (*110*), and protein kinase C (*111*). The identification of specific proteins, the phosphorylation of which is altered, with at least the presumption of concomitant alterations in function, provides some indication of how at least some of the actions of NGF on the cell are expressed at the molecular level.

Transcription-Dependent Alterations

Perhaps the fastest transcriptional alterations observed thus far are the alterations in the expression of several of the protooncogenes following NGF treatment (*123*). Prominent among these is the c-*fos*, which is induced 30- to 50-fold within 30 min (*124–126*). The induction of oncogenes by NGF may play a role in the differentiative actions of the factor on the cells because the introduction of specific oncogenes, such as the v-*src* (*127*), the *ras* (*128*), the N-*ras* (*129*), or the protein product of the *ras* (*130*), causes a differentiation similar to that seen with nerve growth factor. Further, the injection into the cells of the antibody to the *ras* protein inhibits NGF-induced differentiation (*131*).

The induction of ornithine decarboxylate also appears to be an early and universal consequence of the actions of NGF on its target cells (*95,132,133*). A 50- to 100-fold increase in activity can be seen within 5 hr (*134*). The increase is clearly dependent on the synthesis of the ornithine decarboxylase mRNA (*135*). Unlike the induction of protooncogenes, where a functional link to the actions of NGF on the cells is indicated, the induction of ornithine decarboxylase has been specifically disassociated from the morphological alterations caused by the factor (*132*).

The increases in the transmitter-metabolizing enzymes are also likely to be transcriptional in nature. The increase in tyrosine hydroxylase activity in PC12h cells, about 2-fold over several days, appears to have characteristics suggestive of transcriptional regulation (*54*), although transcriptional regulation has not been demonstrated formally here. Increases in the specific activity of choline acetyltransferase have been documented repeatedly (*3,8,136*), as have increases in the activities and forms of acetylcholinesterase (*18,137–139*). It has been shown in the latter case that the increases are blocked by treatment with low concentrations of actinomycin D (*138*).

The levels of a number of other proteins in PC12 cells are increased by NGF treatment. Neuron-specific enolase increases some several fold in the first few days of treatment (*140*). Increases are also seen in the surface marker Thy-1 (*141,142*). Induction of synapsin I has been reported (*143*), and changes in actin expression have been seen (*144*), as have increases in the amount of an unidentified substrate for protein kinase C (*145*). Increases in neurotensin (*146,147*), neuropeptide Y (*148*), and MAP-2 (*119*) have been reported. Increases in the levels of sodium channels on treated cells (*52*) are almost certainly dependent on transcription as well.

Increases in the NILE protein have been extensively investigated and have been shown to be transcriptionally based (*149*) This surface constituent, the NGF-inducible large external (NILE) glycoprotein, has a molecular weight of 230,000 and is increased some 3-fold in NGF-treated cells. It is found on both peripheral and central neurons (*150*), as well as on PC12 cells. Its chemical (*151*) and biochemical (*152*) properties have been detailed. Recent work has shown that the NILE is, in fact, identical to the cell adhesion molecule L1 (*153,154*) and the neuron-glia cell adhesion molecule NgCAM (*155*).

Two interesting proteins decrease in PC12 cells after NGF treatment. One is a lactic acid dehydrogenase, found because of its nuclear localization and its ability to bind to single-stranded DNA (*156–158*). The other is the epidermal growth factor receptor (*25,27,159*). Decrease in this latter protein, the receptor for a known mitogen, has been postulated to be at least in part responsible for the ability of NGF to inhibit cell division (*25,27*), although in neither case has the formal demonstration been made that these decreases are due to decreases in transcription, this seems likely in both cases.

Although direct and detailed studies on the synthesis of various mRNAs in PC12 cells treated with NGF seem

imminent, only a few have appeared so far. An increase in a specific gene sequence induced by NGF has been reported (*160*), although no information on the identity of its protein product has been forthcoming. More recently, it has been found that NGF causes the accumulation of an RNA that has sequence homology with a known regulator of transcription (*161*).

Neurite Outgrowth

While it seems likely that the complex changes in the cell caused by nerve growth factor, such as synapse formation, hypertrophy, and neurite outgrowth, would require transcriptional change, only in the case of neurite outgrowth has the subject been explored experimentally. Early studies with neuronal explants from fetal animals indicated that the presence of actinomycin D, in amounts sufficient to block RNA synthesis, did not interfere with the ability of NGF to elicit such neurites (*162*). Thus, the conclusion, almost counter-intuitive, that transcription was not required for neurite outgrowth, became established. The PC12 model allowed a reinspection of this conclusion. It was found that, in PC12 cells, RNA synthesis inhibition did inhibit the generation of neurites (*163*). However, when cells were treated with NGF and then the neurites removed by separating the cells from the substratum, they regenerated neurites when replated, at a very rapid rate and even in the presence of inhibitors of RNA synthesis. It was suggested that these primed cells had accumulated all the RNA synthesis-dependent macromolecules necessary for neurite formation and needed only some nontranscriptional stimulus provided by NGF to be able to form neurites. It was reasoned that in the earlier studies, explants from fetal animals made neurites in an RNA-synthesis independent manner because they had been removed from an NGF-sufficient environment and were therefore primed. The present understanding is that neurite generation requires the action of NGF at two loci, one at the site of transcription and the other at the level of the membrane.

Other Factors Acting on the Cells

A number of other agents act on the PC12 cells. Among these are other neurite-promoting factors, mitogens, and agents acting to alter the phenotype of the cells. Among the neurite-promoting agents is fibroblast growth factor. Fibroblast growth factor induces clear neurite outgrowth by PC12 cells (*164*), although the amount of neurite outgrowth seen is less than that elicited by NGF. The permanence of these neurites is the subject of some moderate controversy. The original reports showed that the neurites elicited by fibroblast growth factor, like those produced by treatment with dBcAMP, were transitory, disappearing within several days even in the presence, and with the repeated addition, of fresh fibroblast growth factor (*164*). Subsequent reports from other workers have presented data showing that fibroblast growth factor produces neurites as permanent as those elicited by NGF (*29*). The reasons for this discrepancy are not known. A neurite-inducing factor, isolated from salivary glands, but different from NGF, with a molecular weight of some 20,000, has been reported (*165*). Neurites have been seen after treatment of the cells with phospholipase (*166*), and after introduction of *src* (*127*) or *ras* (*128–130*) oncogenes into the cells. A form of outgrowth, similar to that seen with NGF is produced when the cells are treated with certain cAMP derivatives (*167*). Potentiation of NGF-induced neurite outgrow occurs upon treatment of PC12 cells with adenosine (*36,168*) or with specific gangliosides (*169,170*). Neurite outgrowth, in the absence of exogenous NGF, can be seen when cells are plated on extracellular matrices generated by bovine corneal epithelial cells (*171*) or astrocytes (*172*), and NGF-independent neurite regeneration can be seen when PC12 cells are placed in contact with Schwann cells (*173*).

Epidermal growth factor has a moderate mitogenic action on these cells (*25*). This action is associated with some of the same intracellular changes seen after treatment of the cells with NGF, and some of the same surface changes take place as well (*174*).

Phenotypic changes in the cells occur upon treatment with glucocorticoids. It is generally thought that these steroids direct the PC12 cell toward a chromaffinlike phenotype. There are increases in tyrosine hydroxylase activity (*136*) caused by an increase in the transcription of the tyrosine hydroxylase gene (*12,13*). Corticosteroid-induced changes in catecholamine production have also been reported (*175–177*), as have changes in the nature of the transmitter-containing vesicles (*175,178*). The sharp antagonism between NGF and the corticosteroids seen in adrenal cell cultures (*84*) has not been seen with PC12 cells, although some inhibition of NGF-induced neurite outgrowth by dexamethasone has been reported (*178*). Recent studies have shown that treatment with dexamethasone decreases the number of NGF receptors on the PC12 cell surface (*179*). There are reports that sodium butyrate alters the phenotypic characteristics of PC12 cells and guides them into a chromaffinlike pattern (*180,181*). It seems fair to say that although factors with differentiating or neurotrophic activity for PC12 cells have been identified, none has been shown to have exactly the same combination of activities exhibited by NGF. The effects of a number of other agents acting on PC12 cells are summarized in Table 2.

Comparison with Normal Cell Counterparts

As mentioned previously, the PC12 is a clone derived from a rat adrenal medullary tumor (*1*). The classification of these tumors is based on their morphological and histochemical properties compared to their normal counterparts from different phases of embryonal development (*187*). The adrenal medullary cells are derived embryologically from the neural crest. Cells that develop in the adrenal medulla from neural crest stem cells are of three major types: sympathetic principal neurons, adrenal chro-

Table 2. Comparison of PC12 cells with normal cell counterparts

Property	PC12 cells	Chromaffin cells	Sympathetic neurons
Growth	Cell line	Primary culture	Primary culture
Morphology	Round, 20–40 μm cell body diameter; heterogeneous in size, density, and low number of granules 30–350 nm (*5*)	Round, 20 μm cell body diameter; abundant vesicles of 150–350 nm (*222*)	Long neurites, 40– 50 μm diameter, vesicle size 50 nm (*223*)
Cytoskeletal composition	β and γ actin (*144*), tubulin and microtuble-associated proteins (chartins) (*224*); epithelial-like cytokeratins, and neuronal intermediate-size filaments (*225*);	Tubulin, actin, α-fodrin, myosin (*226*)	Typical neuronal cytoskeleton (*227*)
Major catecholamine content	Dopamine, norephinephrine (*228*)	Norepinephrine, epinephrine (*229*)	Norepinephrine (*230*)
Neurotransmitters and/or neuropeptide content	Acetylcholine (*6*), γ-aminobutyric acid (*231*)	Enkephalins, substance P, vasoactive intestinal peptide, somatostatin, neurotensin (*232*)	Acetylcholine (*230*), somatostatin, substance P (*233*), enkephalins (*234*)
Responsiveness to EGF and the loss of proliferative potential	Yes (*25*); heterodown regulation of EGF receptors during NGF-induced differentiation (*25,27*)	No; absence of EGF receptors on the cell surface (unpublished)	Unknown
Neurotransmitters receptors	Acetylcholine muscarinic (*33*) and nicotinic (*2*); adenosine (*36,235*); benzodiazepines (*236*)	Acetylcholine muscarinic (*237*) and nicotinic (*237*); β-adrenergic receptors (*238*); dopaminoceptors (*239*); neuropeptide receptors (*240*)	Nicotinic (*241*); adrenergic (*242*); enkephalins, substance P (*243*)
Voltage-dependent ion channels	Sodium channels (*188,189*); voltage dependent (*41*) and calcium dependent (*41,53*) potassium channels, calcium channels (*244,245*)	Sodium (*240*), potassium (*246*), and calcium channels (*247*)	Sodium, potassium (*248*), and calcium channels (*191*)
Polysialoglycolipids	Expressed upon NGF induced differentiation (*193*)	Unknown	Present (*192,249*)

maffin cells, and small intensely fluorescent (SIF) cells. These cells all synthesize and store catecholamines, but differ in cell morphology, neurotransmitter-synthetic machinery, and type and content of neurotransmitter vesicles. In some respects SIF cells appear intermediate in phenotype between sympathetic neurons and adrenal chromaffin cells. PC12 cells, like embryonal neural crest cells, have the characteristics of immature monoaminergic neurons in that they have the ability to synthesize both adrenergic and cholinergic neurotransmitters (*178*). NGF treatment produces, reversibly, the sympathetic neuronal phenotype and glucocorticoid produces, reversibly, the chromaffinlike phenotype. Thus, it is appropriate to compare the properties of PC12 cells treated with these agents with those of their normal counterparts.

Adrenal medullary cells in primary culture, unlike PC12, have a finite lifetime. They do not require NGF for survival. The catecholamines are primarily norepinephrine and epinephrine (*182*), in contrast to the preponderance of dopamine in the PC12 cells. The adrenal cells have both nicotinic and muscarinic acetylcholine receptors (*183,184*) as do the PC12 cells. Adrenal cells contain, synthesize, and secrete enkephalins (*185*); there are no reports of comparable activities in PC12 cells. Treatment with NGF increases the tyrosine hydroxylase levels of the cells from fetal animals in culture (*186*). Under appropriate conditions, neurite outgrowth can also be produced from such cells (*84*); such outgrowth cannot be seen in cultures from mature animals. This neurite outgrowth is prevented by the simultaneous presence of corticosteroids; no comparable amount of inhibition of NGF-induced neurite outgrowth has been seen with PC12 cells. There are receptors for NGF on cultured adrenal medullary cells, receptors that seem to decline or disappear as the animal ages (*186*), but these receptors have not been described in detail.

Sympathetic neurons, of course, also have a finite lifetime in culture. They generally require NGF in the medium for survival, but, unlike with PC12 cells where removal of NGF leads simply to loss of differentiated properties, removal of NGF here leads to cell death. Sympathetic neurons, like NGF-treated PC12 cells, do not divide. The major catecholamine neurotransmitter of differentiated PC12 cells is dopamine; norepinephrine is the predominant catecholamine in sympathetic neurons; acetylcholine is present in both. The nicotinic acetylcholine receptors found on sympathetic neurons appear to be identical to those seen on PC12 cells (*30*). Although there are discrepancies in the reports from various laboratories, the broad outlines of the chemistry of the NGF receptors on PC12 cells and on sympathetic neurons, e.g., specificity, saturability, both low- and high-affinity sites, are also about the same. Differentiated PC12 cells display large numbers of voltage-dependent sodium channels

(*52,188,189*), as do sympathetic neurons, and the differentiation also produces a complement of calcium channels, mainly N and/or T type that are dihydropyrimidine-insensitive (*190*), similar to those seen on mature sympathetic neurons (*191*). Tetanus toxin binding sites, the hallmark of mature neurons in culture, found on terminally differentiated sympathetic neurons (*192*), appear on the neurites and the growth cones of PC12 cells after several days of treatment with NGF (*193*). The rapid changes in surface morphology seen when PC12 cells are exposed to NGF (*92*) are seen with sympathetic neurons in culture as well (*194*). Finally, it has been shown that antisera raised against mature sympathetic neurons grown in culture recognized antigens in differentiated PC12 cells (*195*). A number of the major characteristics of the PC12 cells and their normal counterparts are summarized in Table 3.

Mechanisms of Differentiation

Based on what is currently known, an outline of the pathway(s) by which NGF causes the differentiation of PC12 cells can be constructed. Clearly, the first step is the binding of NGF to its receptor. On the PC12 cells, as mentioned before, there are two classes of receptors, and the

Table 3. Other agents acting on PC12 cells.

Agent	Origin	Biochemical properties	Cellular response	Reference
Sodium butyrate	Synthetic		Induction of chromaffinlike phenotypic markers; increased cell adhesion; growth arrest	(*180,181*)
Corticosteroids	Synthetic		Induction of chromaffinlike phenotype; increased tyrosine hydroxylase, and decreased choline acetyltransferase activity	(*136,175*)
Neurite-inducing factor	Mouse salivary glands	Protein; Mr: 20; processed from NGF activator	Neurite outgrowth	(*165*)
Fibroblast growth factors	Brain or pituitary	Proteins; Mrs: 16–17 pI: 5–7; 9.6	Neurite outgrowth	(*29,164,171*)
Epidermal growth factor	Mouse salivary gland	Protein; Mr: 6.04 stimulates cell proliferation	Stimulates thymidine incorporation, activation of ornithine decarboxylase	(*25*)
Gangliosides	Bovine brain	Sialoglycolipids; modulate EGF receptor affinity; regulate tubulin gene expression; release of neurotropic glycoproteins	Growth arrest; neurotrophic; enhancers of NGF-induced neurite outgrowth	(*170,213–215*)
Soluble glycosamino glycans; Mg^{2+}	Animal and plant tissue	Dextran sulfate heparin, putative fibronectin cell binding tetrapeptide Arg-Gly-Asp-Ser	Inhibitors of NGF-induced neurite outgrowth	(*29,216,217*)
cAMP, forskolin	Synthetic	—	Enhancers of NGF-induced neurite outgrowth	(*36,167,168*)
Phorbol esters	Synthetic	TPA, PMA, modulate EGF response, enhancers of forskolin-induced cAMP formation	No effects on NGF activity on PC12; induction of ornithine decarboxylase activity	(*218,219*)
ras and v-*src* oncogenes; antibodies to *ras* p21	Rous sarcoma virus, Harvey murine sarcoma virus	—	Neurite outgrowth	(*127,128,131*)
Adenosine derivatives	Synthetic	Inhibitors of adenosyl-methio nine-methyl-transferases	Specific blockers of NGF action	(*220*)
K-252a	Culture broth alkaloid of *Nocardiopsis sp.*		Specific inhibitor of NGF action	(*221*)

weight of evidence is that the high-affinity, slow receptor is the physiologically relevant one. The evidence for this includes data showing that the concentration dependence for binding to the high-affinity site matches the concentration dependence of most of the actions of NGF, that it is the high-affinity receptor that is internalized, and that mutants lacking the high-affinity receptor do not respond to NGF. It should be remembered, however, that there are several normal cells that have only low-affinity receptors, and that some functions of NGF seem to be tied to that receptor, rather than to the high-affinity site (*196*).

The relationship of the high-affinity receptor to the low-affinty receptor is a subject of intense current interest, and may be central to the question of signal transduction by the receptor. Clearly, the high-affinity receptor has a higher molecular weight than the low-affinity receptor, although reports from different laboratories differ as to the amount by which it is higher. Clearly, also the binding site proteins of the two receptors are identical (*22*). There are suggestions that the high-affinity receptor has an additional protein subunit that may be a G-*ras*-like protein (*22*) for coupling the receptor to the generation of an intracellular message, or a C-*src*-like protooncogene (*197*) for introducing a tyrosine kinase into the pathway. In this latter regard, it is clear that the NGF receptor itself is not a tyrosine kinase, nor even possesses an ATP binding site on its cytoplasmic domain (*198,199*). It is also possible that the associated proteins could represent some membranal cytoskeletal structure involved in anchorage, sequestration, and/or internalization of the high-affinity receptor, thereby conferring their known resistance to detergent extraction and proteolytic attack.

It is clear that although the NGF and its receptor are internalized (*197,200*), and that there are NGF binding sites on the nucleus (*201*), the entrance of NGF into the cell is not necessary for, and probably does not play a role in, the mechanism by which NGF directs intracellular differentiative events. The evidence consists of studies in which NGF was introduced directly into the cytoplasm of PC12 cells (*202*), circumventing the surface receptor. When this was done the cells did not respond. Cognate experiments showed that when antibody to NGF was introduced into the cyoplasm, ostensibly preventing NGF from transiting to the nucleus, the cells responded normally to exogenous administration of the factor. The interpretation that has been given these studies is that the combination of NGF with its receptor is the signal initiating its action; internalization is thought to be for the purpose of signal termination, not signal generation.

Although it is assumed that some second messenger, or combination of second messengers, is involved in the actions of NGF, the identification of that second messenger has not been reported. It has been suggested that NGF differentiation requires cAMP as a second messenger. This suggestion was based mainly on the findings that NGF treatment caused a transient, modest increase in cAMP levels in PC12 cells (*94*), cAMP derivatives elicit a form of neurite outgrowth from the cells (*94*), and treatment of the cells with cAMP derivatives modulate the synthesis of the same group of proteins (*203*) and produce similar changes in phosphorylation of several proteins (*102,103*), as does NGF.

Systematic investigations, however, using established criteria by which second messenger function is validated showed that these criteria were not fulfilled for cAMP (*167,168*). It is now thought that agents that increase intracellular levels of cAMP for prolonged periods stimulate an early, transient outgrowth and may exert a maintenance effect on the neurite network established by NGF (*168*). The overall role of cAMP in the differentiating effects of NGF is poorly understood. The recent work with clonal variants of PC12 cells with defects in cAMP-dependent protein kinases (*61*) has shown that NGF induces ornithine decarboxylase normally in these cells, but the phosphorylation of certain sites on tyrosine hydroxylase and of the ribosomal protein S6 was decreased (*106*), implying a role for cAMP in the NGF-induced activation of specific anabolic enzymes. Thus, the body of evidence argues against a major, critical role of cAMP alone in NGF-induced differentiation, but a secondary role in certain of the actions of NGF seems likely.

The role of calcium ion in the actions of NGF may also be secondary. Original experiments showing that NGF induced a change in calcium fluxes (*96*) could not be substantiated (*97*). However, a recent study using Quin-2 with PC12 cells in suspension or Fura-2 in single PC12 cells have clearly shown that a rapid rise of cytosolic Ca^{2+} is induced by low concentrations of NGF (*204*). There are also recent reports that the actions of NGF on phosphoinositide turnover (*98*) and on the phosphorylations of glycogen phosphorylase (*107*) and Nsp100/EF-2 (*109*) are dependent on the presence of Ca^{2+}. Since other actions of NGF are clearly not calcium dependent, the role of Ca^{2+} may be, like the role of cAMP, secondary.

That NGF treatment stimulates the formation of inositol phosphates in the cells within 15 sec is clear (*98*). The involvement of the products of increased phosphoinositide turnover in the elevation of cellular calcium (*204*) and the activation of protein kinase C (*111*), both reported to occur after NGF addition, is also biochemically consistent. But the relationship of phosphoinositide turnover to the overall differentiating effects of NGF is intriguing but unproven.

The possibility that NGF acts by altering transmembrane fluxes of monovalent ions has been explored. Early studies uncovered a rapid effect of NGF on the Na^+,K^+ pump of PC12 cells (*100,205,206*). The stimulation of the Na^+,K^+-ATPase as measured by the ouabain-sensitive $^{22}Na^+$ efflux (*205*) or $^{86}Rb^+$ influx (*100,206*) occurs within minutes and is maximal within 30 min after NGF addition. Since the stimulation of the pump was completely blocked by amiloride, mimicked by the Na^+ ionophore monensin, and seemed due to an increase in Na^+ influx, it was suggested that the acceleration of the Na^+,K^+ pump was an event secondary to an increase in Na^+ influx mediated by a Na^+,H^+ exchanger. In support of a role for Na^+ in NGF-induced differentiation is the finding that substitution of choline for Na^+ inhibited the neurite outgrowth of PC12 cells (*207*). However, in a recent examination of the effect of NGF on the Na^+-H^+ antiport

in PC12 cells using a fluorescent probe sensitive to changes in the cytoplasmic pH, no activation was observed (*208*). It will be interesting to determine if other ion transport systems in the plasma membranes of the PC12 cells are influenced by addition of NGF.

Thus there is evidence that several second messengers play some role in the actions of NGF. The data on protein phosphorylation is consistent with such a picture. That is, there is evidence that Ca^{2+} is required for some of the phosphorylations (*107,109*), but not for others. There is evidence that cAMP is involved in some (*114*), but not in others. Indeed, there are data showing that both second messengers are needed for the complete actions of NGF on the phosphorylation of a single protein (*106*). The picture that could be drawn is that the actions of NGF are mediated by several parallel pathways, perhaps somewhat redundant in function, subsets controlled by different second messengers.

These pathways are likely to be chains of kinases. Such a picture emerges from the observation that protein kinase C is activated by NGF and phosphorylates a kinase that phosphorylates the cytoplasmic protein called Nsp100 (*111*), recently identified as elongation factor 2 (EF-2) (*112*). Another independent kinase chain appears to be involved in the phosphorylation of the ribosomal protein S6, in which the S6 kinase is itself activated by phosphorylation, probably by a cAMP-dependent kinase (*114*). These pathways lead to the phosphorylation of a number of important proteins in various compartments in the cell, enumerated in an earlier section, the phosphorylation presumably altering their functional characteristics.

In the absence of any other data, it can be suggested that these phosphorylations and altered functions, taken together, provide the fabric of the cellular alterations comprising differentiation. Since we know that at least one of these phosphorylations involves a nonhistone protein in the nucleus (*102,115*), it requires no great leap of imagination to suggest that the NGF-induced phosphorylation of nuclear proteins is responsible for the NGF-dependent alterations in the transcription of specific genes.

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