Nerve Growth Factor-induced Alteration in the Response of PC12 Pheochromocytoma Cells to Epidermal Growth Factor

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ABSTRACT PC12 cells, which differentiate morphologically and biochemically into sympathetic neuronlike cells in response to nerve growth factor, also respond to epidermal growth factor. The response to epidermal growth factor is similar in certain respects to the response to nerve growth factor. Both peptides produce rapid increases in cellular adhesion and 2-deoxyglucose uptake and both induce ornithine decarboxylase. But nerve growth factor causes a decreased cell proliferation and a marked hypertrophy of the cells. In contrast, epidermal growth factor enhances cell proliferation and does not cause hypertrophy. Nerve growth factor induces the formation of neurites; epidermal growth factor does not.

When both factors are presented simultaneously, the cells form neurites. Furthermore, the biological response to epidermal growth factor, as exemplified by the induction of ornithine decarboxylase, is attenuated by prior treatment of the cells with nerve growth factor.

PC12 cells have epidermal growth factor receptors. The binding of epidermal growth factor to these receptors is rapid and specific, and exhibits an equilibrium constant of 1.9×10^{-9} M. Approximately 80,000 receptors are present per cell, and this number is independent of cell density.

Treatment of the cells with nerve growth factor reduces the amount of epidermal growth factor binding by at least 80%. The decrease in receptor binding begins after ~12–18 h of nerve growth factor treatment and is complete within 3 d. Scatchard plots indicate that the number of binding sites decreases, not the affinity of the binding sites for epidermal growth factor.

The rat pheochromocytoma clone PC12 has been used extensively as a model of neuronal differentiation. This clone, developed from an adrenal tumor by Greene and his colleagues (12, 18–20, 22, 45), is of adrenergic neural crest derivation. The cells grow readily under standard conditions and exhibit many of the properties of adrenal medullary chromaffin cells. In the presence of nerve growth factor in nanogram or even subnanogram amounts, the cells differentiate into a phenotype resembling sympathetic neurons. The changes occur within a few days and include process formation, electrical excitability, amine uptake and storage, and a cessation of cell division. The cells contain increased levels of certain transmitter-synthesizing enzymes (13, 20, 41) and will make synapses with appropriate target tissues (41). The differentiation appears to be reversible, but except for this, it resembles, as far as has been explored, the changes occurring in normal cells differentiating into sympathetic neurons.

The importance of this system for studies on nerve growth factor is hard to exaggerate. In vitro work with nerve growth factor has been limited for some time to experiments with sympathetic or sensory neurons in culture. Such experiments have been less than satisfactory because these neurons require nerve growth factor for survival under these conditions. Thus, any differences found between nerve growth factor-treated tissue and controls could be charged simply to the fact that the controls were dying. The PC12 system, because it remains quite healthy in the absence of nerve growth factor, provides a unique in vitro tool for the study of nerve growth factor-initiated changes.

The use of this system for the study of neuronal differentiation is also of immense importance. Although there are some reservations about the fidelity of this model, it does provide an early and synchronized look at the events preceding, accompanying, or even participating in the decision point leading to

the formation of a neuron. Also, it allows questions to be asked about what events are initiated and what events are terminated when the cell expresses neuronal properties. Such studies are difficult if not impossible with normal cells because of the inaccessibility of neurons or their precursor cells at such early stages of development.

The recent observation (14, 30) that the PC12 cell line responds to epidermal growth factor as well as to nerve growth factor provides a new dimension. Although epidermal growth factor is generally isolated from the same source as is nerve growth factor (2, 40), and shares some very general similarities in its mode of biosynthesis and its subunit structure (21), the proteins themselves are different both chemically and physically. More importantly, the effects of the two factors on their respective target organs are very different. Epidermal growth factor is a potent mitogen in many of its target tissues (7, 16). Nerve growth factor, in PC12 cells at least, is a terminal differentiator and stops cell division. Indeed, even the early observations, which led to the conclusion that nerve growth factor had hyperplastic effects in normal ganglia (31), have now been reinterpreted in the light of more recent studies (25).

Thus, the interaction between these two growth factors, with apparently opposing effects in a single system, appeared worthy of study. This paper describes experiments designed to explore the several actions of epidermal growth factor on PC12 cells, to contrast them with the actions of nerve growth factor, and to inspect the consequences of exposing the cell to both. The results of these studies necessitated an investigation of the epidermal growth factor receptor and the changes it undergoes when the cells differentiate in response to nerve growth factor.

MATERIALS AND METHODS

Nerve growth factor was prepared by the method of Bocchini and Angeletti (2). Nerve growth factor antiserum was raised in sheep by standard techniques and stored as a lyophilized powder at −20°C. Epidermal growth factor was prepared by the procedure of Savage and Cohen (40). Epidermal growth factor antiserum was purchased from Collaborative Research Inc., Waltham, Mass. PC12 cells were obtained from Dr. Roseanne Goodman, School of Medicine, University of California. Dulbecco's Modified Eagles Medium (Vogt modification) (DMEM) and fetal calf serum were purchased from Grand Island Biological Co. (GIBCO, Grand Island, N. Y.). DL-[1-^{14}C]Ornithine hydrochloride (45 mCi/mmol), [*methyl*-^{3}H]thymidine (56.2 Ci/mmol), 2-[1,2-^{3}H]deoxy-D-glucose (40 Ci/mmol), and L-[4,5-^{3}H]leucine (5 Ci/mmol) were obtained from New England Nuclear, Boston, Mass. Insulin and EDTA were purchased from Sigma Chemical Co., St. Louis, Mo., hyamine hydroxide and [^{125}I]NaI from New England Nuclear, dibutyryl cyclic adenosine monophosphate (dBcAMP) from Boehringer-Mannheim Biochemicals, Indianapolis, Ind., and pyridoxal phosphate and dithiothreitol from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.

Cells were grown as monolayers in DMEM supplemented with 15% fetal calf serum, 6 mM additional glutamine, penicillin (100 mU/ml), streptomycin (100 μg/ml), and 25 mM HEPES. The medium was changed twice a week and the cells were generally split once a week in a 1:4 ratio. The cells were maintained in tightly closed flasks at 37°C under an atmosphere sufficiently enriched with CO_2 to maintain a slightly acidic pH in the medium.

Adhesion assays were performed in 25-cm^2 Falcon flasks (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) essentially by the method of Schubert and Whitlock (43). PC12 cells were grown overnight in the presence of [^{3}H]leucine (2 μCi/ml). They were then rinsed and collected in a small volume of fresh serum-free medium. The labeled cells were diluted with fresh serum-free control medium or with medium containing the growth factors. This cell suspension was placed in empty Falcon flasks or in flasks containing a monolayer of unlabeled PC12 cells. The cells were left at room temperature for 60 min and then were given a standardized swirling of 10 revolutions on a metabolic shaker. The unattached cells were poured off and the attached cells were collected in fresh medium by vigorous shaking of the flasks. The cells were centrifuged and washed twice with 0.1 M Tris-HCl, pH 7.4, containing 0.9% NaCl. The cells were then dispersed in fresh Tris-buffered saline and trichloroacetic acid was added to a final concentration of 10%. The precipitated protein was collected on Whatman GFA glass fiber filters and washed with 5% trichloroacetic acid. The glass fiber discs were immersed in Liquafluor (Research Products International, Elk Grove Village, Ill.) and counted in a scintillation counter.

The uptake of 2-deoxyglucose was measured by a modification of the procedure described by Hollenberg and Cuatrecasas (27) for the study of 3-*O*-methyl-D-glucose transport. PC12 cells cultured on 35-mm tissue culture dishes were rinsed free of medium with warm phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). The cells were preincubated for 10 min at 24°C in 1 ml of PBS containing BSA. Then the test compounds were added, together with tritiated 2-deoxyglucose, to a final concentration of 0.5 μCi/ml. After 45 min at 24°C, the uptake was terminated by the addition of 1 ml of ice-cold PBS containing phloretin (30 μg/ml). The cells were harvested by scraping the dishes with a rubber policeman and a 1 ml portion of the cell suspension was layered over 1.6 ml of 4% Ficoll made up in PBS. The tubes were centrifuged at 27,000 *g* for 5 min and the pellet was washed once in cold PBS. Ice-cold 5% trichloroacetic acid was added to each pellet and the trichloroacetic acid precipitate was removed by centrifugation. A portion of the supernatant fraction was placed in a counting vial containing Aquasol (New England Nuclear) and the radioactivity was measured in a liquid scintillation spectrometer. A portion of the precipitate was used for estimation of the protein and aliquots of the original suspension were used to obtain cell counts. Values for the uptake of 2-deoxyglucose in the presence of phloretin (40 μg/ml) were subtracted from the experimental values to correct for nonspecific effects.

Ornithine decarboxylase was assayed by the method of Pegg and Williams-Ashman (37) as modified by Oka and Perry (36). 5 h after the addition of the appropriate growth factor to cells in 25-cm^2 Falcon flasks and 30 h after the last medium change, the medium was removed and the cells collected in 4.5 ml of cold 0.32 M sucrose containing 0.01 M Tris-HCl, pH 7.4. The cells were centrifuged and washed twice with 4.5-ml portions of cold sucrose-Tris. The cells were then dispersed in 200 μl of the homogenizing buffer (50 mM Tris-HCl, pH 7.4, 5 mM dithiothreitol, 0.04 mM pyridoxal phosphate) and the suspension frozen on dry ice. The frozen suspension was stored at −20°C overnight. The next day, the suspension was thawed, homogenized in a ground glass homogenizer, and centrifuged at 8,000 *g* for 15 min. The assay was performed on a portion of the homogenized, centrifuged cell supernate in the presence of 111 μM DL-[1-^{14}C]ornithine (2.5 μCi) in a total volume of 0.5 ml. The reaction was carried out in a rubber-stoppered tube with a hanging center well containing 0.2 ml of hyamine hydroxide to absorb the labeled CO_2. The reaction was terminated after 1 h at 37°C by injecting 0.6 ml of 2.5 M H_2SO_4 through the stopper, and the tubes were allowed to equilibrate at room temperature overnight. The next day, the hyamine-containing center well was placed in a scintillation vial containing Liquafluor and the radioactivity measured in a liquid scintillation spectrometer.

To measure leucine or thymidine incorporation, we grew the cells in 35-mm Costar tissue culture dishes (Costar, Data Packaging, Cambridge, Mass.). Tritiated thymidine (1 μCi/ml) or tritiated leucine (1 μCi/ml) was added and the cells kept at 37°C for 2 h. The incubation was terminated by the addition of 1 ml of ice-cold PBS. The cells were harvested by scraping the dishes with a rubber policeman. The cell suspensions were transferred to centrifuge tubes. Each dish was washed with 1 ml of fresh PBS, and the wash added to the cell suspension. The cell suspensions were centrifuged at 1,000 *g* for 5 min and washed twice with PBS. Ice-cold trichloroacetic acid was added to the pellets and the tubes centrifuged at 27,000 *g* for 15 min. A portion of the supernatant fraction was transferred to a counting vial containing scintillant to determine thymidine or leucine uptake into the soluble compartment of the cells. The pellet was partially digested by heating for 30 min at 70°C in 0.5 ml of 10% perchloric acid to solubilize DNA or in 0.5 ml of 0.5 N NaOH to solubilize protein. A portion of the digest was added to counting solution to determine the incorporation of the tritiated precursor. Another portion was taken for DNA or protein determination.

Iodinated epidermal growth factor was prepared by the chloramine T-talcum procedure of Cuatrecasas (11). The factor (4 μg) was added to 100 μl of 0.25 M sodium phosphate, pH 7.4. Then, 20 μl of chloramine T solution (5 mg/ml in water) was added, followed by 5 μl of Na ^{125}I (2.5 mCi). The mixture was kept for 40 s at room temperature and then 40 μl of sodium metabisulfite solution (10 mg/ml in water) was added. The mixture was diluted with 4 ml of 0.1 M sodium phosphate, pH 7.4, containing 0.1% BSA. Talcum (25 mg) was added, and the mixture was centrifuged at low speed. The talcum pellet was resuspended in five successive 4 ml washings of 0.1 M sodium phosphate, pH 7.4, and collected by centrifugation after each. Then the iodinated peptide was eluted with 3 ml of 0.4 M HCl containing 6% BSA. In practice, it was found necessary to filter the acid before use to remove small amounts of undissolved albumin. The eluate was centrifuged for 20 min at 8,000 *g* and the supernatant portion was removed and neutralized with 0.5 M NaOH using pH paper to indicate the end point. In a typical preparation, 5 μl contained 300,000 cpm which was at least 95% precipitable with 10% trichloroacetic acid.

The specific binding of epidermal growth factor was measured by adding labeled epidermal growth factor to monolayer cultures of PC12 cells in fresh growth medium. The cells were incubated at 37°C for 45 min, after which the radioactive medium was removed by aspiration. The monolayers were rinsed

twice gently and rapidly with cold PBS. Then, 2 ml of 1 N NaOH were added to disrupt the monolayer and the radioactivity contained in a 1-ml portion was measured in a Searle model 1185 gamma counter (G. D. Searle & Co., Des Plaines, Ill.). Specific binding was evaluated in each case by subtracting the counts bound in duplicate flasks to which 500 ng/ml of unlabeled epidermal growth factor had been added from the total binding.

Protein was determined either by the method of Lowry et al. (32) or by the method of Bradford (3). DNA was measured by the method of Burton (4). Cell numbers were estimated in a hemocytometer.

RESULTS

In the absence of growth factors, PC12 cells showed a characteristic round appearance under the phase-contrast microscope. There was some flattening of the cells as they adhered to the plastic substrate. After treatment with nerve growth factor, there was a noticeable increase in the number of phase-dark, flattened cells within 2–4 h. After the next several days of nerve growth factor treatment, thin neurites appeared which attained a length of several microns and eventually formed a thick network between the cells. Treatment with epidermal growth factor produced an initial darkening and flattening of the cells, similar to, but not as pronounced as, the flattening observed with nerve growth factor. However, even after several days of treatment with epidermal growth factor, there was little or no evidence of neurite production. In the presence of both factors, there appeared to be more initial flattening of the cells than with either alone, but the pattern of neurite formation was indistinguishable from that seen with nerve growth factor alone.

The flattening seen after treatment of the cells with either nerve growth or epidermal growth factor was reflected in an increased adhesiveness of the treated cells. When cells were labeled overnight and then treated for 1 h with nerve growth factor, they adhered more firmly to a monolayer of attached PC12 cells than did labeled cells that had not been so treated (Table I). Treatment with epidermal growth factor had a similar but less pronounced effect. The combination of the two factors was frequently more effective in this regard than either factor alone, but the increases were not additive. Dibutyryl cyclic AMP also produced an increase in the adhesion of labeled PC12 to a monolayer. Similar experiments were performed with only the plastic dish as substrate. The differences in adhesion of the labeled cells to plastic were not as marked as those seen when a monolayer was used, but they were in the same order and the same direction as the measurements presented in Table I.

Within 45 min after the addition of either nerve growth factor (Fig. 1 *A*) or epidermal growth factor (Fig. 1 *B*), the uptake of 2-deoxyglucose into the cells was increased. The stimulation in each case was concentration-dependent and appeared to be maximal at ~500 ng of nerve growth factor/ml and 50 ng of epidermal growth factor/ml.

Several hours after the addition of nerve growth factor, there was a marked increase in the activity of ornithine decarboxylase in the cells (Table II). With epidermal growth factor, there was also an induction of ornithine decarboxylase, although not so marked as that seen after nerve growth factor (Table II). These levels of induction were maximal; increases in the concentration of either growth factor did not produce increases in the levels of ornithine decarboxylase found. Thus, the ability of nerve growth factor to induce ornithine decarboxylase was severalfold greater than the ability of epidermal growth factor to induce what is presumably the same enzyme. The combination of nerve growth factor and epidermal growth factor

TABLE I

Adhesion of Labeled PC12 Cells to a Monolayer of Unlabeled PC12 Cells in the Presence of Nerve Growth Factor or Epidermal Growth Factor.

Treatment	Cell adhering
	%
None	36.8 ± 2.4
Nerve growth factor	68.6 ± 1.3
Epidermal growth factor	55.0 ± 1.4
Nerve growth factor + epidermal growth factor	73.1 ± 2.3
Dibutyryl cyclic AMP	71.6 ± 7.8

PC12 cells were labeled overnight with [^{3}H]leucine and adhesion assays were performed as described in Materials and Methods. Additions were: nerve growth factor, 100 ng/ml; epidermal growth factor, 150 ng/ml; dibutyryl cyclic AMP, 1 mM. Values reported as means (n = 3) ± SEM.

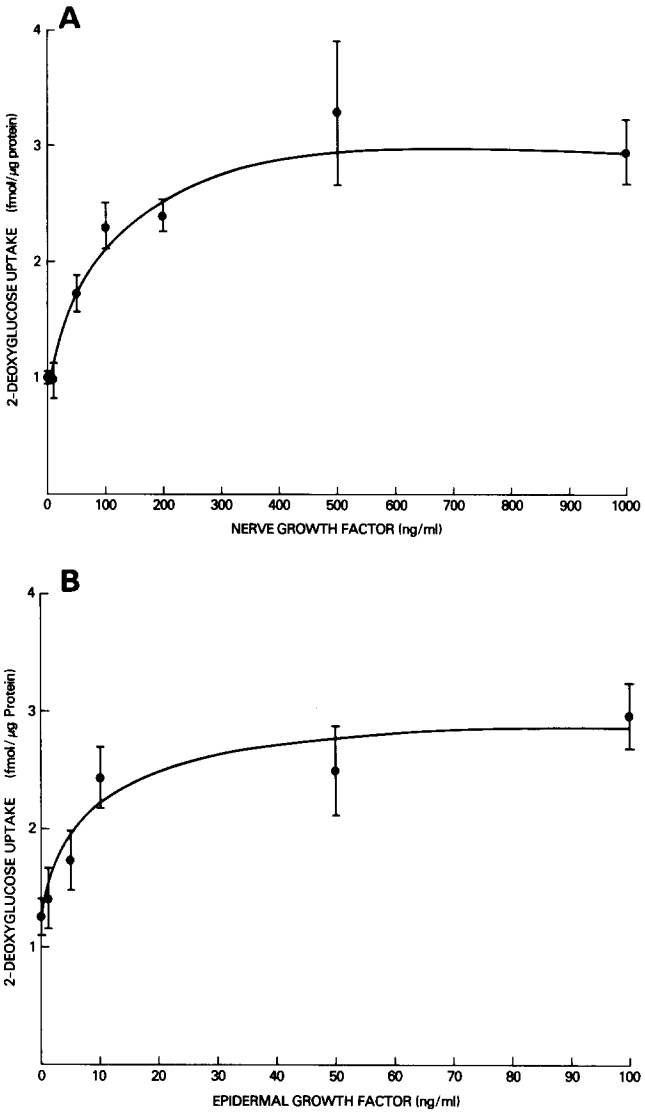

FIGURE 1 Stimulation of [^{3}H]2-deoxy-D-glucose uptake into PC12 cells by nerve growth factor (*A*) and by epidermal growth factor (*B*). PC12 were grown in 35-mm culture dishes. Nerve growth factor and [^{3}H]deoxyglucose (0.5 μCi) were added in 1.0 ml of phosphate-buffered saline containing 0.1% bovine serum albumin. After 45 minutes, the cells were harvested and treated with 5% trichloroacetic acid. The soluble radioactivity was measured. Parallel cell monolayers were treated with phloretin (30 μg/ml) to correct for [^{3}H]-deoxyglucose accumulation not mediated by the hexose carrier. Values reported as means (n = 4). Brackets indicate SEM.

TABLE II

Effect of Nerve Growth Factor, Epidermal Growth Factor, and of Specific Antisera on Ornithine Decarboxylase Activity in PC12 Cells

Treatment	Ornithine decarboxylase activity
	pmol [$^{14}CO_2$] released/μg protein
None	0.6 ± 0.1
Nerve growth factor	19.2 ± 2.2
Nerve growth factor + nerve growth factor antiserum	2.6 ± 0.6
Nerve growth factor + epidermal growth factor antiserum	21.7 ± 4.0
Epidermal growth factor	5.5 ± 0.6
Epidermal growth factor + nerve growth factor antiserum	6.4 ± 0.6
Epidermal growth factor + epidermal growth factor antiserum	2.8 ± 0.5
Nerve growth factor + epidermal growth factor	24.6 ± 4.3

Cells were treated for 6 h and ornithine decarboxylase assays were performed as described in Materials and Methods. Additions were: nerve growth factor, 30 ng/ml; epidermal growth factor, 150 ng/ml; antisera, 0.1 ml, full strength. Antisera were preincubated with the relevant growth factors for 30 min at room temperature before adding them to the medium. Values reported as means ($n = 3$) ± SEM.

seemed to produce a greater induction than either alone in several experiments, although the variability of the actual induction and the much greater magnitude of the nerve growth factor induction made it difficult to determine if these inductions were additive.

To prove that the induction by each factor was independent, and not a result of contamination of one factor by the other, antibody experiments were performed (Table II). It was shown that nerve growth factor antiserum blocked the action of nerve growth factor on ornithine decarboxylase induction by >90%, but epidermal growth factor antiserum had no effect. The reverse was true of the epidermal growth factor response. It was blocked by ~60% by epidermal growth factor antiserum, but was not influenced by nerve growth factor antiserum. Thus, the difference in the maximal activity elicited by each factor, the additional activity elicited by one in the presence of the other, and the specificity of each antiserum indicates that the cells are responding independently to each factor and not to one factor as a contaminant of the other.

As mentioned earlier, the presence of nerve growth factor and epidermal growth factor together gave the appearance, morphologically, of the presence of nerve growth factor alone. To understand this relationship fully, experiments were performed in which the biological response to one factor was measured after treatment with the other. Cells pretreated with epidermal growth factor for 3 d were then exposed to nerve growth factor. The induction of ornithine decarboxylase was similar to that seen in untreated cells when similarly exposed to nerve growth factor (Table III). The reverse, however, was not true. Cells treated for 3 d with nerve growth factor had a much lower response to epidermal growth factor than did untreated cells (Table IV). The increase in treated cells was <20% of that in controls in most experiments. That this was not because of some general inability of the nerve growth factor-treated cells to respond was shown by the fact that ornithine decarboxylase induction by dibutyryl cyclic AMP in nerve growth factor-treated cells was the same as that seen in untreated cells (Table IV).

After several days of treatment, the cultures treated with nerve growth factor showed different characteristics than those treated with epidermal growth factor. In addition to the obvious difference in morphology, there also appeared to be some difference in cell numbers. After 96 h, cell proliferation, as estimated by the number of cells (Fig. 2*A*) and the total DNA (Fig. 2*B*) in each culture dish, was moderately stimulated by epidermal growth factor and moderately inhibited by nerve growth factor. Neither treatment produced effects on cell numbers or DNA content which differed significantly from untreated controls, even after 96 h of treatment. However, the divergence in cell number and total DNA between epidermal growth factor-treated and nerve growth factor-treated cultures was statistically significant ($P < 0.05$ by analysis of variance combined with Duncan's multiple range tests). Although some initial increases in the uptake of [^{3}H]thymidine into the intracellular acid-soluble pool were noted in both nerve growth factor-treated and epidermal growth factor-treated cells, there were no significant differences in the uptake of tritiated thymidine into the cells or in the incorporation of [^{3}H]thymidine into the DNA after 72 h of treatment (Table V).

Nerve growth factor produced its characteristic hypertrophic effect on the cells, as evidenced by a marked increase in the amount of protein per cell (Fig. 2*C*). Epidermal growth factor

TABLE III

Effect of Nerve Growth Factor on Ornithine Decarboxylase Activity in Control and in Epidermal Growth Factor-treated PC12 Cells

Cells	Treatment	Ornithine decarboxylase activity
		pmol [$^{14}CO_2$] released/μg protein
Control	None	1.8 ± 0.3
	Nerve growth factor	9.9 ± 1.8
Epidermal growth factor-treated	None	3.4 ± 0.4
	Nerve growth factor	15.3 ± 1.7

Cells were grown for 3 d alone or in the presence of epidermal growth factor (150 ng/ml). The medium was changed, and fresh epidermal growth factor was added on the 2nd day. Nerve growth factor (30 ng/ml) was added and the cells harvested 6 h later. Values reported as means ($n = 3$) ± SEM.

TABLE IV

Effect of Epidermal Growth Factor and of Dibutyryl Cyclic AMP on Ornithine Decarboxylase Activity in Control and in Nerve Growth Factor-treated PC12 Cells

Cells	Treatment	Ornithine decarboxylase activity
		pmol [$^{14}CO_2$] released/μg protein
Control	None	0.9 ± 0.1
	Epidermal growth factor	5.7 ± 0.3
	Dibutyryl cyclic AMP	5.6 ± 0.9
Nerve growth factor-treated	None	1.1 ± 0.1
	Epidermal growth factor	2.4 ± 0.1
	Dibutyryl cyclic AMP	6.5 ± 0.5

Cells were grown for 3 d alone or in nerve growth factor (30 ng/ml). The medium was changed, and fresh nerve growth factor was added on the 2nd day. Epidermal growth factor (150 ng/ml) or dibutyryl cyclic AMP (1 mM) was added and the cells harvested 6 h later. Values reported as means ($n = 3$) ± SEM.

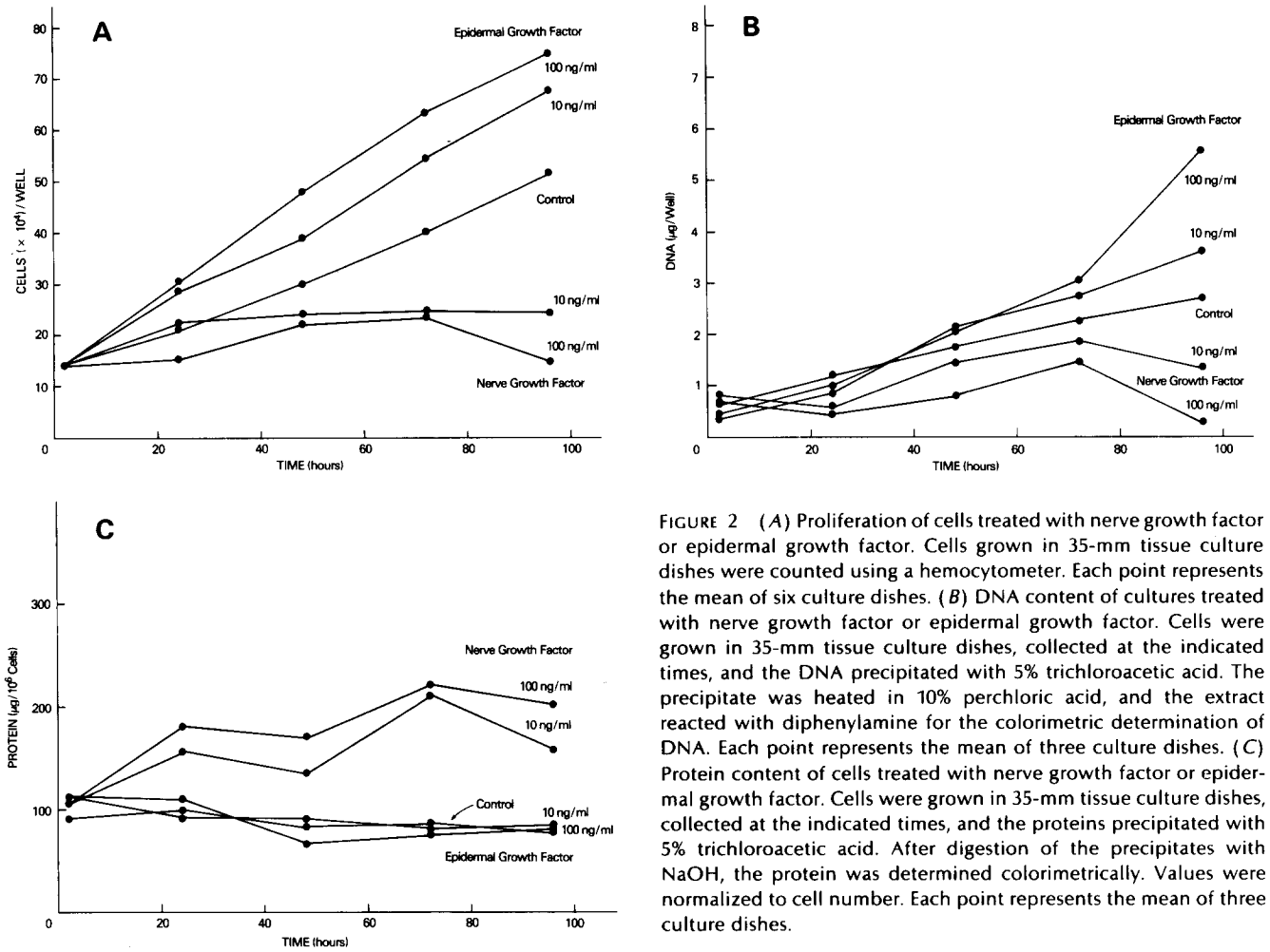

FIGURE 2 (*A*) Proliferation of cells treated with nerve growth factor or epidermal growth factor. Cells grown in 35-mm tissue culture dishes were counted using a hemocytometer. Each point represents the mean of six culture dishes. (*B*) DNA content of cultures treated with nerve growth factor or epidermal growth factor. Cells were grown in 35-mm tissue culture dishes, collected at the indicated times, and the DNA precipitated with 5% trichloroacetic acid. The precipitate was heated in 10% perchloric acid, and the extract reacted with diphenylamine for the colorimetric determination of DNA. Each point represents the mean of three culture dishes. (*C*) Protein content of cells treated with nerve growth factor or epidermal growth factor. Cells were grown in 35-mm tissue culture dishes, collected at the indicated times, and the proteins precipitated with 5% trichloroacetic acid. After digestion of the precipitates with NaOH, the protein was determined colorimetrically. Values were normalized to cell number. Each point represents the mean of three culture dishes.

TABLE V

Effect of Nerve Growth Factor or Epidermal Growth Factor on Thymidine Uptake and Thymidine Incorporation in PC12 Cells

Treatment	Thymidine	
	Uptake	Incorporation
	cpm/μg DNA	
None	1,361 ± 376	15,433 ± 4,024
Nerve growth factor, *10 ng/ml*	1,352 ± 180	18,163 ± 642
Nerve growth factor, *100 ng/ml*	1,227 ± 196	18,793 ± 741
Epidermal growth factor, *10 ng/ml*	1,421 ± 260	15,356 ± 1,016
Epidermal growth factor, *100 ng/ml*	1,050 ± 274	21,508 ± 8,089

Cells were grown for 72 h in the presence of the indicated levels of the factors. The cells were then incubated for 2 h with 1 μCi of [^{3}H]thymidine/ml. The cells were harvested, washed, and treated with 5% trichloroacetic acid. The acid-soluble and acid-precipitable radioactivity were measured as described in Materials and Methods. Values were normalized to the total DNA in the culture. Values reported are the mean (n = 3) ± SEM.

TABLE VI

Effect of Nerve Growth Factor or Epidermal Growth Factor on Leucine Uptake and Leucine Incorporation in PC12 Cells

Treatment	Leucine		
	Uptake	Incorporation	
	cpm/μg protein	*cpm/ 10⁶ cells*	*cpm/μg protein*
None	38.7 ± 6.4	3,450 ± 920	40.5 ± 2.1
Nerve growth factor, *10 ng/ml*	46.7 ± 11.5	12,800 ± 990	54.8 ± 8.0
Nerve growth factor, *100 ng/ml*	26.3 ± 3.6	9,270 ± 1,700	43.3 ± 3.3
Epidermal growth factor, *10 ng/ml*	38.4 ± 5.1	5,540 ± 1,600	57.9 ± 10.6
Epidermal growth factor, *100 ng/ml*	49.0 ± 6.6	4,330 ± 870	62.3 ± 12.9

Cells were grown for 72 h in the presence of the indicated levels of the factors. The cells were then incubated for 2 h with 1 μCi of [^{3}H]leucine/ml. The cells were harvested, washed, and treated with 5% trichloroacetic acid. The acid-soluble and acid-precipitable radioactivity were measured as described in Materials and Methods. Values reported are the means (n = 3) ± SEM.

had no comparable effect. Nerve growth factor caused a corresponding increase in the amount of leucine incorporated per cell (Table VI), but no substantial increase when the incorporation was normalized to cellular protein. Epidermal growth factor produced small, fairly consistent increases in the rate of protein synthesis per cell as evidenced by the increased specific activity of the protein isolated from epidermal growth factor-treated cells. No consistent increases in leucine uptake into the acid-soluble pool were seen after 72 h treatment with either factor, although epidermal growth factor stimulated such uptake at earlier times.

The binding of iodinated epidermal growth factor to PC12 cells could be inhibited by low concentrations of native epidermal growth factor (Fig. 3). This displacement was concentration dependent and saturable. The specific binding of radi-

oiodinated epidermal growth factor accounted for 80–95% of the total radioactivity bound to the cells. The rapid, initial binding of iodinated epidermal growth factor was complete within 40 min, although a slower gradual increase in the radioactivity associated with the cells was apparent for at least another hour (Fig. 4). The binding was quite specific for epidermal growth factor (Table VII). Neither nerve growth factor, cytochrome *c*, insulin, nor growth hormone in large amounts displaced labeled epidermal growth factor. Finally, the binding of epidermal growth factor to PC12 cells was independent of cell density (Fig. 5).

Treatment of cells with nerve growth factor markedly reduced the binding of epidermal growth factor (Fig. 6). A latency period of at least 9 h passed before the reduction in binding was observed. After the latency period, a time-dependent decrease in binding occurred, which resulted in an 80% reduction of epidermal growth factor binding within 72 h. In view of the nerve growth factor-induced hypertrophy of the cells, which resulted in a doubling of cell protein content, the results were also expressed in terms of cell number (Table VIII). In these terms, the decrease in epidermal growth factor binding was still apparent. The effects of nerve growth factor were concentration-dependent up to 20 ng/ml (Fig. 7). Kinetic analysis of the nerve growth factor-induced reduction in epidermal growth factor binding (Fig. 8) revealed that nerve growth factor reduced the number of binding sites, rather than altering their affinity for the ligand. Least squares linear regression analysis of the Scatchard plots (Fig. 9) yielded correlation coefficients of 0.840 and 0.889 for the binding data obtained from nerve growth factor-treated and untreated cells, respectively. The apparent dissociation constants were 1.97×10^{-9} M and 1.91×10^{-9} M, respectively. Treatment of cells with nerve growth factor reduced the number of epidermal growth factor binding sites in this experiment from 79,640 binding sites per cell to 42,680 binding sites per cell. It should be noted here that this experiment involved 24 h of nerve growth factor treatment rather than 72. After 72 h, the amount of epidermal

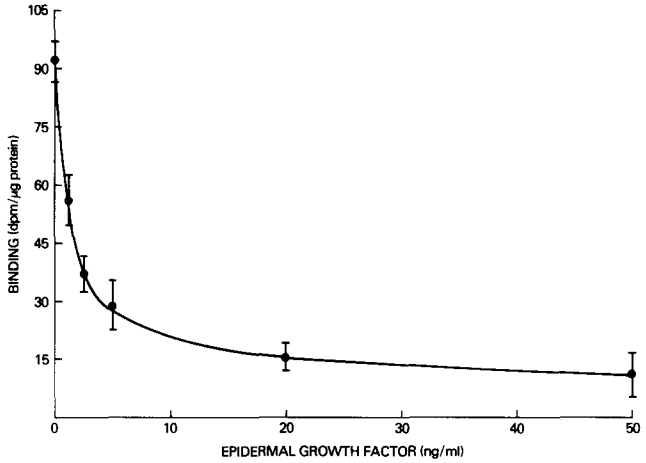

FIGURE 3 Concentration dependence of the binding of epidermal growth factor to PC12 cells. Approximately 0.5 ng of iodinated epidermal growth factor (~763,000 dpm) and the indicated amounts of native epidermal growth factor were added to PC12 cultures in 2 ml of medium. After 45 min the cells were collected and the associated radioactivity counted. Each point represents the mean of four determinations. Brackets indicate SD.

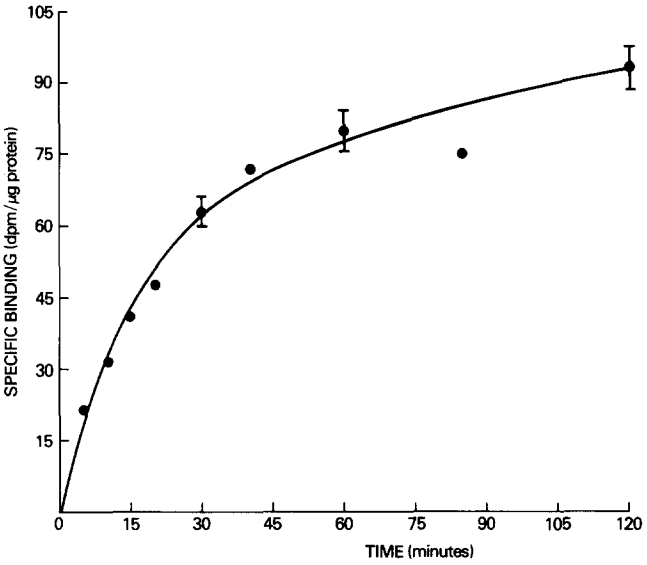

FIGURE 4 Time-course of the binding of iodinated epidermal growth factor to PC12 cells. Binding was done with 0.4 ng (135,000 dpm) of iodinated epidermal growth factor. Nonspecific binding, i.e., binding in the presence of 500 ng/ml of unlabeled epidermal growth factor, was subtracted from each point. Each point represents the mean of three determinations. Brackets indicate SD.

TABLE VII

Specificity of ^{125}I-Epidermal Growth Factor Binding to PC12 Cells

Additions	^{125}I-Epidermal growth factor bound
	dpm/μg cell protein
None	142.5 ± 4.3
Epidermal growth factor, *500 ng/ml*	8.7 ± 0.7
Nerve growth factor, *10 μg/ml*	132.5 ± 5.2
Insulin, *100 μg/ml*	152.0 ± 7.3
Cytochrome *c*, *100 μg/ml*	139.0 ± 2.4
Growth hormone, *100 μg/ml*	135.1 ± 5.8

Iodinated epidermal growth factor (0.44 ng, 420,000 dpm) was added in the presence of various unlabeled proteins. Data represent the means ($n = 4$) ± SEM.

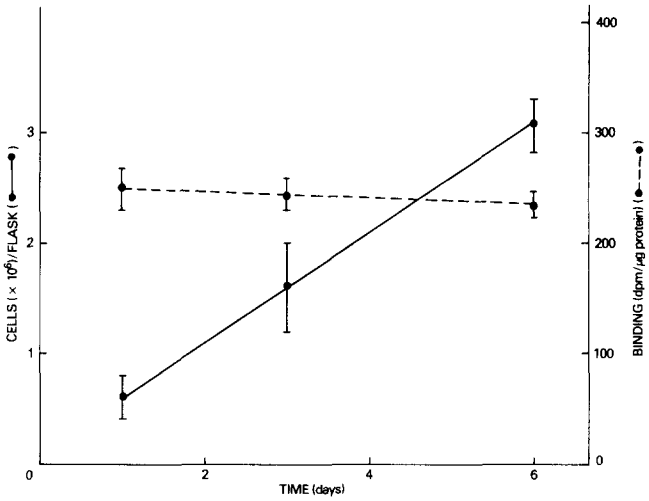

FIGURE 5 Lack of effect of cell density on the binding of iodinated epidermal growth factor to PC12 cells. PC12 cells were plated at an initial density of 5×10^5 cells per 25-cm^2 tissue culture flask and grown for 6 d under the conditions described in Materials and Methods. Binding was evaluated with 0.62 ng (521,000 dpm) of iodinated epidermal growth factor. Each point represents the mean of five determinations. Brackets indicate SD.

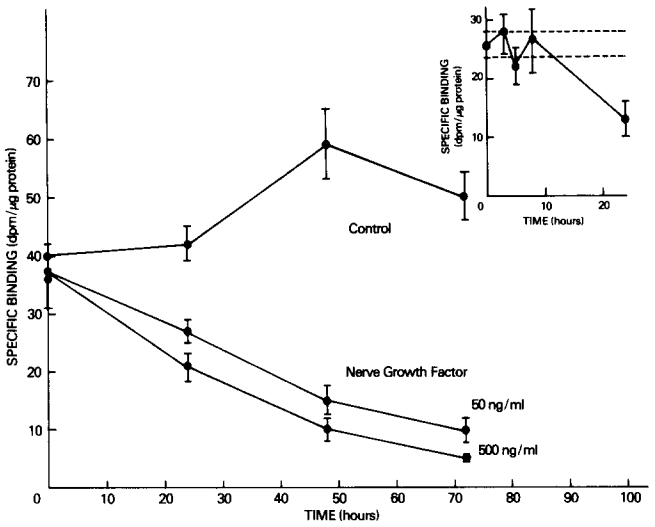

FIGURE 6 Time-course of nerve growth factor-induced loss of epidermal growth factor binding to PC12 cells. Cells were grown in control medium or in nerve growth factor-containing medium, 50 or 500 ng/ml. Binding of iodinated epidermal growth factor (0.5 ng, 415,000 dpm) was measured at the indicated times. Each point represents the mean of four determinations. Brackets indicate SD. The *inset* presents data for the loss of epidermal growth factor binding for the first 24 h after treatment with 100 ng/ml of nerve growth factor.

TABLE VIII

Reduction of Epidermal Growth Factor Binding in Nerve Growth Factor-treated Cells

Cells	Epidermal growth factor binding	
	dpm/μg protein	*dpm/10^3 cells*
Control	50.2	9,045
Nerve growth factor-treated	5.5	1,972

Cells were grown in control medium or in nerve growth factor-containing medium (500 ng/ml) for 72 h. Data from Fig. 4.

growth factor binding was so low that kinetic analysis became difficult.

DISCUSSION

Epidermal growth factor is a potent mitogen for a number of cell types (7, 16). It stimulates epithelial and endothelial cell proliferation, and interacts with receptors on cells of different derivation from a number of species. The biochemical responses it elicits include rapid increases in the transport of small molecules (1, 26, 27, 38), increases in the phosphorylation of membrane (8) and nuclear proteins (29), and increases in the synthesis of RNA and proteins (10, 26). All responses, however, are not found in all cells that interact with epidermal growth factor, and indeed, some cells have easily demonstrable receptors for epidermal growth factor but exhibit no substantial mitogenic response under the conditions studied.

Very few neural-type cells are known to respond to epidermal growth factor. It had been shown that human glial cells exhibit a density-dependent response to epidermal growth factor in serum-free medium (46). This response includes increases in multiplication rate and in thymidine incorporation. And, during this work, it has been reported (15) that epidermal growth factor induces tyrosine hydroxylase in PC-G2, another clone from rat pheochromocytoma. But the original observation that PC12 cells respond was a bit unexpected.

On the other hand, the response of PC12 to nerve growth factor has been well characterized. This response includes increases in cellular adhesiveness (43), changes in membrane structure (9) and in membrane proteins (34), alterations in cyclic nucleotide levels (43), increases in the transport of small molecules (33), induction of ornithine decarboxylase (17, 24, 30), alterations in the phosphorylation of nuclear proteins (47), and induction of transmitter-synthesizing enzymes (13, 20, 41). The cells develop excitable membranes (12), grow processes (45), and stop dividing (22). Overall, the response to nerve growth factor can be characterized as the terminal differentiation of a nonneuronal cell into a sympathetic neuron, although it differs from normal cell differentiation in the one vital respect that it is reversible.

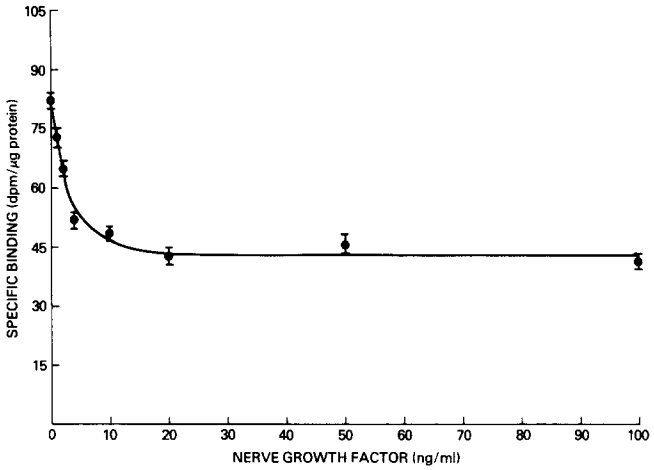

FIGURE 7 Dose response curve of nerve growth factor-induced loss of epidermal growth factor binding to PC12 cells. Cells were grown in various concentrations of nerve growth factor as described in Materials and Methods. Binding of iodinated epidermal growth factor (0.12 ng, 117,000 dpm) was measured after 24 h. Each point represents the mean of four determinations. Brackets indicate SD.

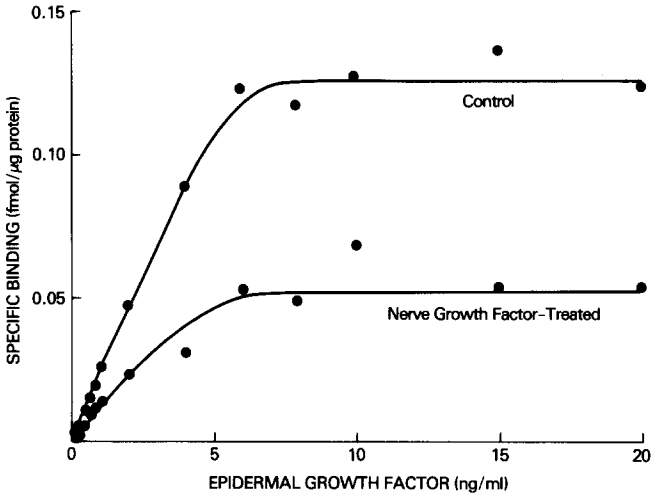

FIGURE 8 Concentration dependence of epidermal growth factor binding to control and to nerve growth factor-treated PC12 cells. Cells were treated with 100 ng/ml of nerve growth factor for 24 h. Indicated concentrations of native epidermal growth factor were mixed with iodinated epidermal growth factor (0.06 ng, 793,000 dpm). Binding was measured as described in Materials and Methods. Each point represents the mean of duplicate determinations.

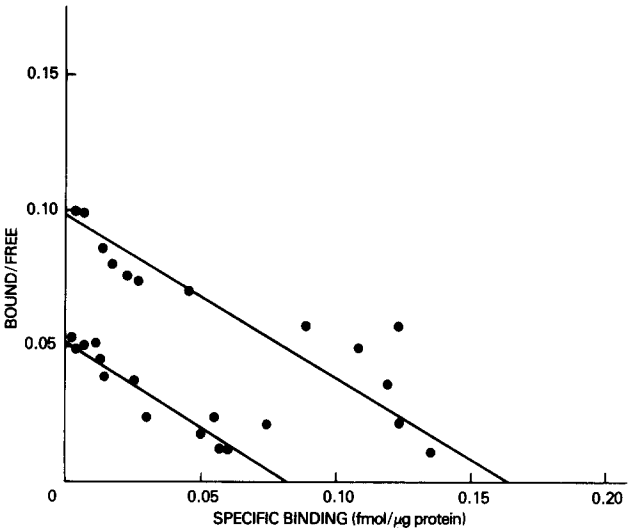

FIGURE 9 Scatchard analysis of the nerve growth factor-induced decrease in epidermal growth factor binding. Constructed from data in Fig. 8.

Thus, the effects of epidermal growth factor in other test systems are quite different than the known effects of nerve growth factor in PC12 cells. Nevertheless, these two peptides produce some apparently similar responses in these cells.

One of these responses is an increased cellular adhesion. Both peptides increase the ability of the PC12 cells to adhere to plastic or to each other. This finding does not support the concept that increased adhesion is prelude to neurite outgrowth (42) because epidermal growth factor produces little or no neurite formation. It does not, however, preclude the possibility that the two factors are increasing adhesiveness by different mechanisms. The observation that the increases produced by the two factors together were frequently greater than that produced by either alone suggests that the action of each may be different. Because so little is known about the molecular basis of the alterations in the membrane underlying adhesiveness, this question seems a difficult one to answer.

Another common response is an increase in the uptake of 2-deoxyglucose. Neither increase is unexpected because increases in the transport of small molecules, and specifically in the transport of monosaccharides, have been reported as consequences of the action of both nerve growth factor (28) and epidermal growth factor (27) on their respective target cells.

A third apparently common response is the increase in ornithine decarboxylase activity. This enzyme is rate-limiting for the synthesis of polyamines, and has been implicated in a variety of cellular changes including malignant transformations and hormonal responses (39, 44). Whatever its function or functions in the cell, it appears to be an excellent indicator of changes in nuclear activity. Both peptides produce an increase in the activity of the enzyme, presumably an induction. The increase, caused by epidermal growth factor, is less than the increase caused by nerve growth factor even with maximal amounts of each in the medium. This may suggest that they are working on the regulatory system controlling the induction in different ways. Support for this concept can be found in the frequent observation that the induction by the two factors together is greater than that by either alone. Again, because ornithine decarboxylase has been implicated in many different processes in various cells, there is no certainty that the induction by two different factors means that the factors produce the same end results in the cell. Incidentally, the observation that epidermal growth factor induces ornithine decarboxylase, but doesn't produce neurite outgrowth, supports previous studies (17) showing a dissociation between these two events, both of which are consequences of the action of nerve growth factor.

It is known that nerve growth factor inhibits or terminates cell division in PC12 cells (22). The observation here that cell proliferation in nerve growth factor-treated cultures is lower than that in controls is consistent with these previous observations. The modest stimulation of cell proliferation produced by the epidermal growth factor, although not convincing evidence that epidermal growth factor has mitogenic action in these cells, is distinct from the effect of nerve growth factor. The possible mitogenic effects of epidermal growth factor may have been masked by the culture conditions, i.e., 15% fetal calf serum. It is clear from work in other systems that the magnitude of the epidermal growth factor effect on thymidine incorporation and cell division is markedly influenced by the culture conditions, more specifically, by the serum level (7). Reducing the serum concentration in the growth medium is a manipulation that has been used to slow cell proliferation and, hence, unmask the mitogenic response to epidermal growth factor. It is reasonable to ask, however, whether epidermal growth factor actually initiates a mitogenic response or simply maintains cell viability under such suboptimal conditions.

As mentioned before, the addition of epidermal growth factor and nerve growth factor together results in cells with morphological characteristics indistinguishable from those treated with nerve growth factor alone. Indeed, the cells appear to stop growing and cell counts reveal, again, a similarity to those treated only with nerve growth factor. It appears, then, that the action of nerve growth factor predominates and it becomes of interest to inquire into the mechanism mediating this effect.

The observation that the cells treated with nerve growth factor do not respond to epidermal growth factor with as substantial an induction of ornithine decarboxylase as do untreated cells suggests that the nerve growth factor in some way limits the intracellular biological response to epidermal growth factor. This is consistent with the predominance of nerve growth factor. The fact that a cells treated with nerve growth factor respond normally to dibutyryl cyclic AMP shows that the lowered response to epidermal growth factor is a specific inhibition and does not reflect some artifactual refractoriness to ornithine decarboxylase in these treated cells. Parenthetically, it seems worth mentioning that in these experiments the cells were treated for 3 d to fully influence them with nerve growth factor and to allow ornithine decarboxylase levels to come back to baseline after induction by the nerve growth factor itself (17), that the medium was changed fully 24 h before the addition of epidermal growth factor to avoid any increases in ornithine decarboxylase caused simply by the addition of fresh medium, and that nerve growth factor was kept in the medium continually because of the reversibility of nerve growth factor effects in these cells.

Thus, nerve growth factor and epidermal growth factor act independently on PC12 cells, although some of the consequences of the actions of the two peptide factors on these cells are the same. That is, both increase cellular adhesion, 2-deoxyglucose uptake, and ornithine decarboxylase levels. But epidermal growth factor has a mild proliferative action on the cells and nerve growth factor induces hypertrophy and a

terminal differentiation. Nerve growth factor actions predominate when the two factors are added together and nerve growth factor treatment of the cells limits the biological response of the cells to epidermal growth factor.

The epidermal growth factor receptors on PC12 cells seem unremarkable. The apparent equilibrium constant of 1.9×10^{-9} is on the order of similar constants reported for epidermal growth factor receptors on other cells (5, 6, 26, 35); the number of receptors per cell is approximately the same as that found on cultured human fibroblasts (5, 26). The specificity for epidermal growth factor has not been done in detail here, but it is important to note, in view of the functional interaction between the two factors, that nerve growth factor has no apparent affinity for the epidermal growth factor receptor.

The reason that the cells treated with nerve growth factor exhibit a reduced response to epidermal growth factor appears to be that there is a diminished amount of epidermal growth factor binding to the treated cells. That is, the biological response to epidermal growth factor is diminished by a reduction of cellular receptors after nerve growth factor treatment. There are now numerous examples of cells that have lost epidermal growth factor receptors for one reason or another and exhibit a parallel loss of biological response to the factor (6, 23, 35). This is the first case, however, of a loss of receptors caused by a differentiating stimulus. The concentration dependence of the receptor loss, virtually a complete effect at ~20 ng of nerve growth factor/ml, again suggests that the interaction is not caused by a direct competition for the receptor. The time-course of the loss, a 9-h lag followed by a complete loss over a 3-d period, indicates that the loss is caused by some long-term, perhaps transcriptional alteration in the synthesis of the receptor, and not a short-term alteration in the conformation of the membrane. This seems consistent with the Scatchard data which indicate a loss of receptors rather than an alteration in their affinity.

One interpretation of the data is that the nerve growth factor acts on PC12 cells to terminate their cell division by, at least in part, limiting their receptors for, and their response to, epidermal growth factor, a known mitogen. A mild extrapolation of such a concept might lead to the suggestion that differentiating agents cause, as part of their action, a lowered response to mitogens by a similar mechanism. A bolder extrapolation would be that cells destined to become neurons respond, at some early point of decision, to nerve growth factor by limiting their response to mitogens and, thus, become neurons.

Another interpretation is that nerve growth factor usurps the trophic influences of epidermal growth factor at the level of the membrane receptor before the expression of the differentiated phenotype of the sympathetic neuron. While epidermal growth factor did enhance the proliferation of PC12 cells modestly, its major effects of increased 2-deoxyglucose uptake, increased cellular adhesion, and induction of ornithine decarboxylase are shared by nerve growth factor and resemble a generalized set of trophic responses which enhance the survival of the cells. In transferring the regulation of trophic function to the differentiating stimulus, nerve growth factor, basic cellular processes may be better synchronized with the event of differentiation. Experiments with embryonic tissue are underway to test these hypotheses.

David End was the recipient of a National Research Service award (1-F32-HD05905-01).

Received for publication 5 June 1980, and in revised form 18 August 1980.

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