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 $1.9 \times 10^{-9}$ 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 $\sim 12-18$ 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 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 physiologically. 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 antiseraum 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 antiseraum 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 Eagle Medium (Vogt modification) (DMEM) and fetal calf serum were purchased from Grand Island Biological Co. (GIBCO, Grand Island, N. Y.). dl-[3H]Orotate hydrochloride (45 Ci/mmol), [methyl-3H]thyminine (55.2 Ci/mmol), 2-[3H]deoxy-d-glucose (40 Ci/mmol), and 4,5,6,7,8-[5,6,7,8-3H]thymidine (55.2 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 [2-14C]NaI (2.5 mCi) 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 10% fetal calf serum, 6 mM additional glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml), and 25 mM HEPES. The medium was changed twice a week and stored at 37°C under an atmosphere sufficiently enriched with CO2 to maintain a slightly acidic pH in the medium.

Adhesion assays were performed in 25-cm2 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 [3H]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 then dispensed 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 0.14 Tris-HCl, pH 7.4, containing 0.9% NaCl. The cells were then dispersed in empty Falcon flasks or in flasks containing a monolayer of unlabeled PC12 cells and 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 centrifuged to remove the supernatant. Each dish was washed with 1 ml of fresh PBS, and the wash was added to the cell suspension. The cell suspensions were centrifuged at 1,000 g for 10 min and washed twice with PBS. Ice-cold trichloroacetic acid was added to the pellets and the tubes centrifuged for 20 min at 8,000 g for 15 min. A portion of the supernatant was then transferred to a counting vial containing scintillation fluid and counted 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 phlorotetrahydrobenzylamine (40 µCi/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 Okas and Perry (36). 5 h after the addition of the appropriate growth factor to cells in 25-cm2 Falcon flasks and 30 h after the last medium change, the medium was removed and the cells collected at 37°C by injecting 0.6 ml of 2.5 M HCl 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 Package, Cambridge, Mass.). Triticated 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 centrifuged to remove the supernatant. Each dish was washed with 1 ml of fresh PBS, and the wash was 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 1,000 g for 10 min. A portion of the supernatant was then 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 M 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-talunum procedure of Cuatreceas (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 NaIO4 (2.5 M). 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 8 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. The mixture was divided into five 0.5 ml portions, and 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 acid. The mixture 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 unlabelled 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. 1A) or epidermal growth factor (Fig. 1B), 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
was blocked by \(-60\%\) by epidermal growth factor antiserum, growth factor on ornithine decarboxylase induction by >90%, that nerve growth factor antiserum blocked the action of nerve factor in antibody experiments were performed (Table II). It was shown and not a result of contamination of one factor by the other, 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 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 understand the relationship fully, experiments 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.

### 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 ['CO₂] 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.

### 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 ['CO₂] released/µg protein |
|----------------------------|-------------------------------|---------------------------------------------------------------|
| Control                    | None                           | 1.8 ± 0.3                                                     |
| Epidermal growth factor- treated | Nerve growth factor          | 9.9 ± 1.8                                                     |
|                            | 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 ['CO₂] released/µg protein |
|----------------------------|-------------------------------|---------------------------------------------------------------|
| Control                    | None                           | 0.9 ± 0.1                                                     |
|                            | Epidermal growth factor        | 5.7 ± 0.3                                                     |
|                            | Dibutyryl cyclic AMP           | 5.6 ± 0.9                                                     |
|                            | 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.
TABLE V

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

| Treatment                  | Uptake cpm/µg DNA | 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 [3H]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 reported are the mean (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-
iodinated 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 PC 12 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 x 10^-9 M and 1.91 x 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.

**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 x 10^5 cells per 25-cm² 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.
DISCUSSION

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.

Epidermal 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.
Both peptides produce an increase in the activity of 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. 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 cell treated with nerve growth factor respond normally to dibutyl 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 remarkable. The apparent equilibrium constant of $1.9 \times 10^{-9}$ 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.

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