Comparison of Rapid Changes in Surface Morphology and Coated Pit Formation of PC12 Cells in Response to Nerve Growth Factor, Epidermal Growth Factor, and Dibutyryl Cyclic AMP

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ABSTRACT Scanning and transmission electron microscopic studies were carried out on the rapid cell surface response of PC12 pheochromocytoma cells to treatment with nerve growth factor (NGF), epidermal growth factor (EGF), and dibutyryl cyclic AMP. EGF induced a rapidly initiated series of surface changes identical to those previously observed with NGF. Ruffles appear over the dorsal surface of the cells by 30 s, are prominent at 3 min, and are absent by 7 min. Microvilli disappear as dorsal ruffles become prominent. Peripheral ruffles are seen by 3 min, are prominent on most of the cells by 7 min, and are virtually absent by 15 min. Large blebs are present on 50% of the cells by 2 h and are markedly decreased by 4 h. Within 30 s after NGF or EGF addition, an increase in the density of 60–130-nm coated pits per unit membrane is detectable. This reaches a maximum of two- to threefold in from 1 to 3 min and gradually decreases. Combined treatment with NGF and EGF increases surface ruffling and, after an early peak in coated pits which at 3 min is similar in magnitude to that observed for the separately administered factors, maintains a greater number of pits per unit area than either treatment alone. 3-d pretreatment with NGF greatly reduces the response of the cells to EGF both with respect to surface ruffling and coated pit formation while 4-h NGF pretreatment has no effect on the EGF response. Dibutyryl cyclic AMP induced none of the rapidly onsetting changes caused by NGF or EGF, and therefore it seems unlikely that cyclic AMP mediates these surface changes. Changes in cell surface architecture induced by NGF and EGF on PC12 cells and by NGF in normal sympathetic neurons (as previously described) indicates that such responses may be a widespread phenomenon associated with the interaction of at least some peptide growth factors/hormones with their receptors. These responses may represent or reflect primary events in the mechanism by which these factors act.

The surface morphology of cells may vary tremendously with changes in their hormonal milieu. Rapidly onsetting changes in surface morphology may play a role in subsequent hormone-triggered responses. For instance, a rapidly initiated sequence of changes in surface architecture of PC12 pheochromocytoma cells occurs after treatment with nerve growth factor (NGF)[1] (1). These changes commence within seconds of exposure to NGF and far precede subsequent responses such as initiation of neurite outgrowth or alteration in protein synthesis. Also, rapidly initiated surface ruffling has been reported on carcinoma A431 cells after treatment with epidermal growth factor (EGF) (2). We have reported subsequent

[1] *Abbreviations used in this paper:* cAMP, cyclic AMP; DbcAMP, dibutyryl cAMP; EGF, epidermal growth factor; NGF, nerve growth factor; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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studies that have shown alterations in surface morphology of sympathetic neurons in response to NGF (3) resembling those seen on PC12 cells, thus indicating that the phenomenon is not limited to tumor-derived cells lines but is also seen in primary culture of normal tissue. In addition to the alterations in surface morphology of sympathetic neurons, a threefold increase in the number of coated pits per unit area of cell surface was seen after NGF treatment (3). In another study, PC12 cells are reported to undergo loss of short microvilli and an increase in long microvilli after 1-h treatment with NGF or EGF (4).

The PC12 cells ultimately responds to NGF both morphologically (5, 6) and electrophysiologically (7) to attain a differentiated state similar to that of sympathetic neurons. Carcinoma A431 cells show epidermoid type differentiation (2) by changing from a monolayer to multilayered colonies after 12 h of EGF treatment. The first purpose of the present study was to determine whether the observed surface changes were specific manifestations of early differentiation or reflected a more universal phenomenon associated with the early response of sensitive cells to peptide growth factor treatment. To deal with this question, we chose to take advantage of the observation that PC12 cells have receptors for, and responses to, both NGF and EGF (4, 8). These cells respond to NGF in various ways including cessation of cell division (5) and neuronal differentiation (5–7), as noted above, and respond to EGF with ornithine decarboxylase induction (8), rapid phosphorylation of several proteins (9), continued cell division, and no evident morphological differentiation (4, 8). In addition, the PC12 cells have 8×10^4 EGF receptors per cell (8), which is more in the physiologic range than the A431 cells which have $2–3 \times 10^6$ receptors per cell (10, 11).

The second purpose of this study was to determine the effects of dibutyryl 3′5′ cyclic AMP (DbcAMP) on the surface of PC12 cells and thereby to test the possible role of cyclic AMP (cAMP) in early and subsequent responses to this peptide. It has been shown that PC12 cells respond to cAMP by an increase in RNA synthesis (12) and by formation of short, blunt processes (12, 13). There are reports suggesting that cAMP is the second messenger for NGF (14, 15) while other reports do not confirm this model (12, 13, 16). Therefore we chose to study the effects of a cAMP analogue, DbcAMP, on the surface of PC12 cells to determine what, if any, alterations occurred.

MATERIALS AND METHODS

Cell Culture: PC12 cells (passages 18–29) were grown in complete medium and under conditions as previously reported (1). Cells were plated 2–3 d before each experiment at $\sim 1 \times 10^5$ per 35-mm dish. For scanning experiments, each dish contained poly-1-lysine–coated (17) glass coverslips. Experiments using DbcAMP were carried out in complete medium. In all other experiments, cells were washed with serum-free RPMI 1640 medium and maintained in this medium for 4 h prior to each experiment. This was done to avoid possible effects due to hormones or growth factors already present in the serum. For pretreatment with NGF, cells were maintained in serum-free medium containing 50 ng/ml NGF for the indicated times. Growth factors were added to the cultures in 5 μl/ml of a concentrated stock solution in serum-free medium to final concentrations of 50 ng/ml NGF, 10 ng/ml EGF, and 1 mM DbcAMP. Limited studies utilizing 0.1, 1.0, and 100 ng/ml EGF were conducted in a manner similar to that using 10 ng/ml EGF. After addition of the test substances, cells were incubated for 1, 3, 5, 15, 60, and 120 min before fixation.

NGF was isolated by the method of Mobley et al. (18), and EGF was prepared according to Savage and Cohen (19) and was the generous gift of Dr. Frederick Maxfield, Department of Pharmacology, New York University Medical Center. DbcAMP was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Scanning Electron Microscopy (SEM): Upon termination of each incubation, cells were washed twice with Hank's buffered saline solution at 37°C and fixed in 3% glutaraldehyde in 0.085 M sodium cacodylate buffer at pH 7.4 for 90 min at room temperature. Samples for SEM were washed, postfixed in 1% osmium tetroxide in cacodylate buffer, then treated with thiocarbohydrazide (Eastman Kodak Co., Rochester, NY), and OsO_4 using the OTOTO procedure (20), except that 1% OsO_4 treatments were shortened to 1 h, 1% thiocarbohydrazide treatments were 20 min, and distilled water washes were 4 × 2.5 min. Cultures were then dehydrated through graded concentrations of ethanol and critical-point dried from liquid CO_2. The coverslips were mounted on aluminum stubs, coated with 4–5 nm of gold/palladium, and viewed in a JEOL JSM-35 microscope at 25 kV.

Specific surface structures were counted by SEM on 100 consecutive cells for each experimental condition. Counts of the number of pits per unit area were done by placing a grid over high-magnification (× 12,000) scanning micrographs, and counting as many areas per micrograph as were readily visible. At least 10 cells and 50 areas were counted in each sample. The mean values per cell were compared using the independent variable *t* test.

Transmission Electron Microscopy (TEM): Two types of TEM experiments were carried out. The first type was performed to evaluate the dorsal surface of attached PC12 cells, thus duplicating conditions used in our SEM studies. For these experiments the cells were grown on poly L lysine- or collagen-coated tissue culture dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) treated and fixed in the same manner as were the SEM samples (above). The samples were then postfixed in 1% OsO_4 in cacodylate buffer, pH 7.4, dehydrated through graded ethanol concentrations, and embedded in Epon. Embedded cells were selected by phase-contrast microscopy, mounted on blocks, and sectioned. Sections were collected in order on three grids per block, stained with uranyl acetate and lead citrate, and viewed in a Philips 200 electron microscope at 60 kV.

TEM counts of coated areas (pits) per unit cell profile area on attached oriented cells were carried out as follows. One complete set of micrographs was taken of each cell surface on grid at × 3,450, and analyzed at a final magnification of × 28,700 on a Zeiss MOP-3 image analyzer. Coated areas were counted on at least 10 cells per time point.

Since the adherent cells are polarized, random sections for morphometry are very difficult to obtain. For this reason a second type of TEM experiment was carried out. PC12 cells were grown under similar conditions in spinner culture. The cells placed in suspension at 37°C were either treated with NGF for 3 min or 60 min or left untreated. All the cells were fixed at approximately the same time and underwent similar physical manipulations. They were fixed in 3% glutaraldehyde in 0.085 M sodium cacodylate buffer at pH 7.4 at 37°C and allowed to cool to room temperature for 90 min. The cells were pelleted in 2% agar (aqueous) (Beckman Microfuge B, Beckman Instruments, Inc., Palo Alto, CA), and postfixed in 1% OsO_4 in 0.085 M cacodylate buffer, pH 7.4. To improve membrane clarity, we stained the cells en bloc first in 1% tannic acid in 0.085 M cacodylate, pH 7.2, and then in 1% uranyl acetate in 50% ethanol. The pellets were dehydrated in a graded series of alcohol and embedded for electron microscopy.

Morphometric Analysis: Morphometric analysis was performed in the following manner: silver/gold sections were picked up on 300-mesh grids. All cell profiles in which the entire perimeter could be visualized were photographed at × 675 from a single section per block and analyzed at a final magnification of × 4400. Approximately 40 micrographs and 300 cell profiles were analyzed at each time point. These micrographs were used to determine cell surface and volume relationships. Coated pits were too small to be visualized at this magnification. These were quantified on micrographs of the same cell populations taken at × 3400 and enlarged to a final magnification of × 22,000. Between 34 and 39 cell profiles per time point were examined at this higher magnification. Micrographs were taken sequentially across all cell profiles in a single section per block, taking care not to overlap images. Test grids were placed over the micrographs for actual data gathering. A test grid with a higher density of test lines was used for the pit analysis. The methodologies of Weibel (21, 22), Weibel and Bolender (23), and Williams (24) were utilized. For both types of analysis the reference volume was protoplasm. Surface densities (*Sv*) and volume fractions (*Vv*) were computed by summing the individual counts for each profile and taking the rate of the sums using the following formulas:

$$Sv = \frac{Ii}{Ppr} \times \frac{2}{z}$$

where Sv = surface density in μm^{-1}, z = the test line length in μm, Ii = intersection of the test line with cell membrane, and Ppr = the number of

points of the lattice contained within the protoplasm;

$$v = \beta \left(\frac{Ppr}{N} \times K \right)^{3/2}$$

where v = volume of the cell in μm^3, β is the shape constant for spheres, 1.38 (24), N = the total number of cells, and K is the constant for the size of the point in the lattice (8.404 μm^2);

$$S = v \times Sv$$

where S = surface area in μm^2, v = cell volume, and Sv = surface density;

$$Na = \frac{\text{Total number of pits}}{Ppr}$$

where Na = the total number of pits per cell area;

$$N_v = \frac{Na}{\bar{D} + T}$$

where N_v = the number of pits per cell volume in μm^3, $\bar{D}$ = mean particle diameter (0.01 μm), and T = section thickness (0.01 μm);

$$N_v \times v = \text{pits per cell; and}$$

$\text{SD} = \sqrt{\frac{\sum(\text{points} - \text{mean})^2}{N - 1}}$. The significance was calculated using the Student's t test.

RESULTS

Cell Surface Morphology

The free surfaces of PC12 cells maintained for 4 h in serum-free medium without NGF or EGF are complex and are quite similar to those of PC12 cells continuously maintained in serum-containing medium. The most prominent features are variable numbers of randomly spaced microvilli and numerous small blebs (Fig. 1). The small blebs range in size from 0.2 to 0.6 μm and may be randomly spaced or may cluster. In addition to these small blebs, up to 25% of the cells have one or more large blebs from 0.8 to 1.9 μm diam, averaging 1.2 μm (see Fig. 4 for an example of such a bleb on a treated cell). None of the cells have ruffles over the dorsal surface. The only differences found in PC12 cells grown continuously in serum-containing medium were an increase in the proportion of cells with large blebs (25% vs. 8%) and the presence of a few cells (~10%) with peripheral ruffles. Serum removal caused no change in coated pit number per unit area.

The effects of EGF (10 ng/ml) or of NGF (50 ng/ml) on the surface morphology of PC12 cells were essentially identical and closely resembled the changes elicited by NGF in serum-containing medium (1). After the addition of either of these factors to serum-free cultures, there is a dramatic sequential change in the cell surface. Within 30 s of treatment, small ruffles appear over the dorsal surface of 97% of the cells (n = 100 for this and all quantifications of structures other than pits). When cells are observed at 1 min, small ruffles are seen on 98% of cells (Fig. 2). Ruffles at 1 min usually have jagged edges and small finger-like projections and are discontinuous. By 3 min (Fig. 3), large ruffles are prominent on the dorsal surface of >98% of the cells and sometimes form elaborate branching patterns. The ruffles at 3 min are usually smooth contoured and are long, continuous structures. These dorsal ruffles are almost gone by 7 min of treatment. Peripheral ruffles, which are present on 10% of control cells, become much more prominent after EGF and NGF treatment. Such ruffles begin to become prominent after dorsal ruffles begin to decrease and are seen on ~70% of cells by 7 min of treatment and have returned to baseline by 15 min of NGF or EGF treatment. The surface of the cells is quite smooth between 15 and 45 min of treatment. At 45 min, large blebs begin to appear on the cells, are seen on 50% of cells by 2 h (Fig. 4), and are absent by 4 h.

When 50 ng/ml NGF and 10 ng/ml EGF were added simultaneously, the same series of surface changes occurred, but the time course was shortened and the magnitude increased. The individual ruffles were larger, ruffling was more marked, and the peak of the ruffling response was seen at 1 min rather than at 3 min, as was the case with the single agents. Ruffles at 1 min of combined treatment are large, continuous structures (Fig. 5) more closely resembling those seen at 3 min of single agent treatment (Fig. 3) than those at 1 min of single agent treatment (Fig. 2). The subsequent peripheral ruffling also occurred and subsided more rapidly. Blebbing was unchanged in magnitude and time course.

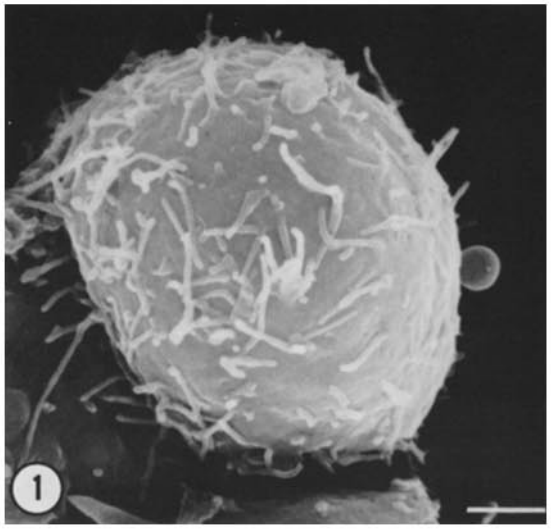
Limited experiments utilizing EGF concentrations of 0.1, 1.0, and 100 ng/ml were compared with our experiments using standard concentration of 10 ng/ml. The surface response in terms of ruffling and blebbing was not diminished at the higher concentrations. Ruffling was slightly decreased at 1.0 ng/ml and moderately decreased at 0.1 ng/ml. There was an apparent increase in ruffling at 100 ng/ml in both the rapidity of the time course and the magnitude of the individual ruffles.

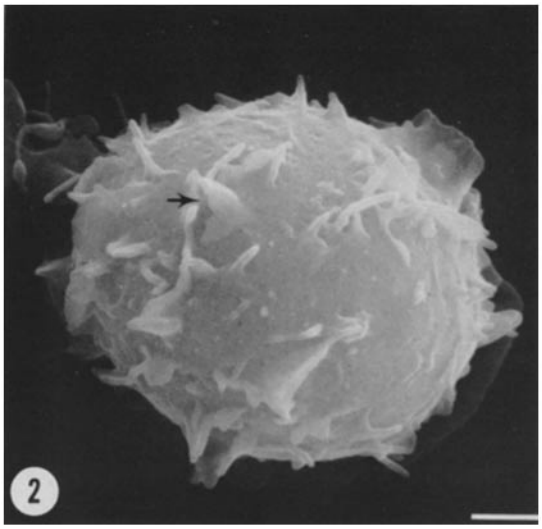
In another series of experiments, cultures were treated with NGF for 4 h and then observed after EGF addition. Despite the cells having already undergone their transitory surface changes in response to NGF, they exhibited the normal course of ruffling and blebbing with EGF. In contrast, after 3 d of NGF pretreatment, the surface changes induced by EGF were much less dramatic. Surface ruffling still occurred, but to a much more limited degree. The ruffles were smaller and fewer were seen per cell. Blebbing at 1–3 h was also less dramatic both in numbers of responding cells and in magnitude.

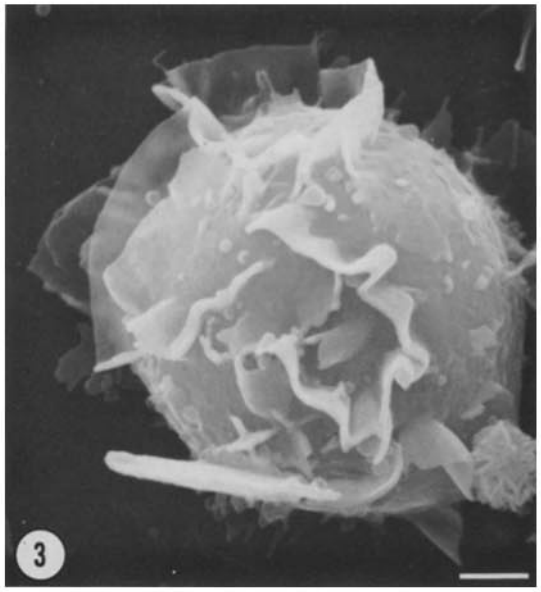
DbcAMP (1 mM) caused none of the surface changes described above. No ruffling, loss of microvilli, or blebbing was noted along the entire time course. After 5–15 min of DbcAMP treatment, occasional cells showed short, blunt processes.

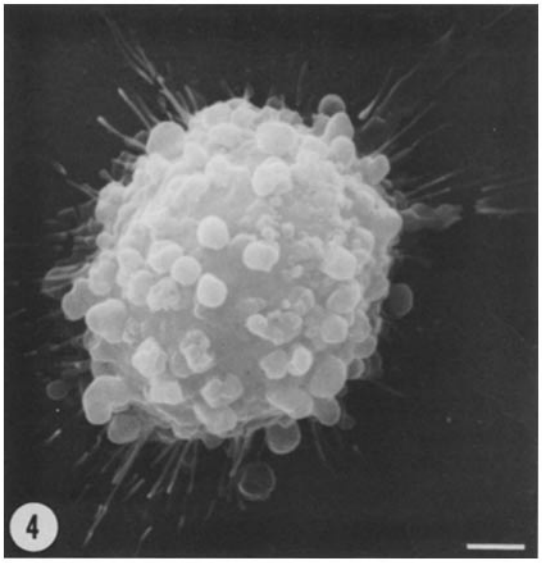
The Effects of NGF or EGF on Coated Pits

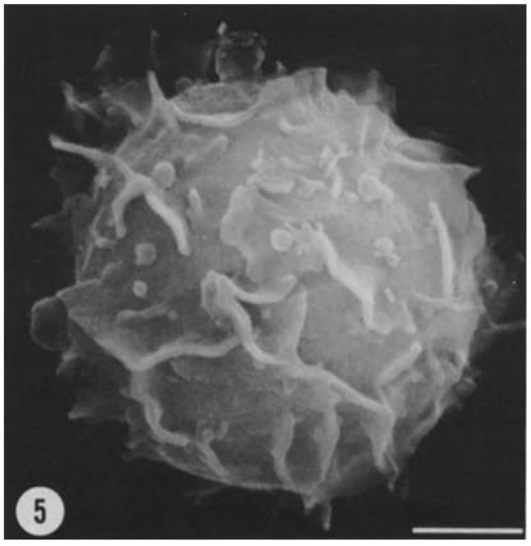
When observed at higher magnification (× 12,000), a more subtle feature of the cell surface can be observed. As was seen on sympathetic ganglion cells (3), small pits 60–130 nm diam were observed on all PC12 cells (Figs. 6 and 7). An examination of the dorsal surface of attached NGF-treated cells by TEM showed that the pits are coated (Fig. 7; see also Fig. 10). There was a marked increase in the surface density of such pits after either NGF or EGF treatment. Between 0.5 and 3 min of NGF or EGF treatment, there was an almost threefold increase in the number of these pits per unit membrane area (Fig. 9, *a* and *b*). After 3 min, the density of these pits decreased, but by 60 min, it had not returned to the level present in untreated cultures. This series of experiments demonstrates that the density of the pits on the dorsal surface of adherent PC12 cells increases with ligand stimulation. The question still remained as to whether this increase was an absolute increase in the number of coated pits per cell or represented a condensation of coated membrane from other











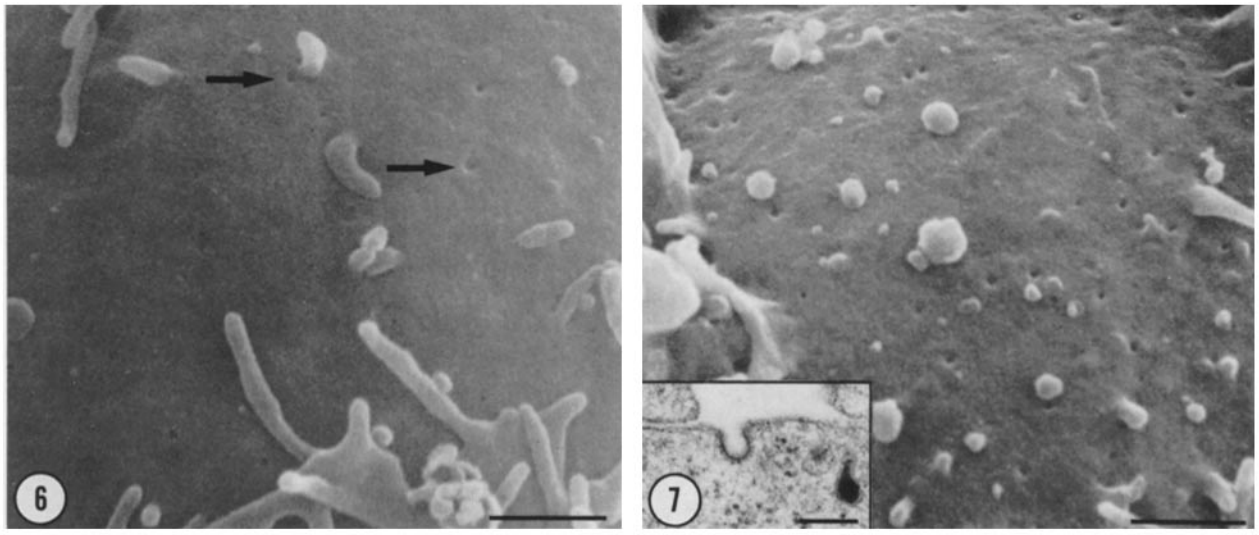
FIGURES 1–5 Fig. 1: PC12 cell maintained in serum-free medium for 4 h. In serum-free medium these cells have a variable number of microvilli and occasional small blebs. Bar, 2.0 μm. × 5,100. Fig. 2: PC12 cell 1 min after EGF treatment. Cells are beginning to develop small ruffles. Many of the ruffles at this time have jagged edges (arrow) and small finger-like projections. In contrast to later times, ruffles are discontinuous. Bar, 2.0 μm. × 4,500. Fig. 3: PC12 cell 3 min after EGF treatment. 97% of the cells develop ruffles over the dorsal surface. Ruffles at this time have smooth contours and often form continuous bands. These ruffles are greatly diminished by 5 min of treatment and are absent by 15 min. Microvilli are virtually absent by 3 min. Bar, 2.0 μm. × 4,500. Fig. 4: PC12 cell 60 min after EGF treatment. Large blebs appear on some cells by 45 min and are present on 50% of the cells at 120 min of treatment. Bar, 2.0 μm. × 3,600. Fig. 5: PC12 cell 1-min treatment with EGF plus NGF. The cells have well developed ruffles. The ruffles are larger and more numerous than cells treated for 1 min with a single agent (see Fig. 2, for example). The ruffles on these cells have smooth contours and form continuous bands. These cells bear striking resemblance to those treated for 3 min with a single agent (Fig. 3). Bar, 2.0 μm. × 7,000.

areas of the cell such as from ruffles or from the surface of the cell adherent to the substratum.

To quantify the number of pits per cell and to determine whether the increase in pits was due to a redistribution or absolute increase in number, we performed a second type of stereologic analysis. Cells were treated for 0, 3, and 60 min with NGF in suspension culture, processed, and examined as described in Materials and Methods. In addition, the cells were en bloc stained with tannic acid and uranyl acetate, a technique that produces improved membrane clarity. The use of tannic acid, as has been reported (25), improved visualization of coated membrane and allowed us to visualize flattened coated areas as well as indented ones (Fig. 8). These studies demonstrated that there was no significant change in the surface area or volume of the cells even during the peak ruffling period. Furthermore, a greater than twofold increase in coated membrane was seen between 0 and 3 min of NGF treatment (Table I). In addition, when the density of pits of the whole cells examined in suspension is compared with that seen on the dorsal surface of adherent cells obtained by either SEM or TEM, we see that the values obtained by the various methods are in close agreement (Table II). Moreover the increase in pit density induced by NGF on PC12 cells is similar to that seen in our previous experiments with sympathetic ganglion cells (3).

Pits per unit area of membrane were compared under a variety of conditions. The levels and the curves generated for the number of pits per unit area as a function of time of treatment were nearly identical for NGF and EGF each given separately as well as for EGF following the 4 h of NGF pretreatment (Fig. 11). Striking differences can be noted when one compares the effects of either substance alone (Figs. 9 and 10) with those of both combined (Fig. 12, *a* and *b*). The initial peaks are identical, but by 5 min they separate. The combined treatment maintains more pits per unit area through 60 min, and the differences between combined and noncombined treatments become more marked as time passes (Fig. 12*b*).

The cell surface response to EGF applied after 3 d of NGF pretreatment also showed a significant difference when compared with that of NGF or EGF alone or with that of EGF after 4 h of NGF. When such cells were treated with EGF, there was a much smaller increase in coated pits versus the greater increase in pits on the cells lacking previous treatment.



FIGURES 6 and 7 Fig. 6: High magnification of an untreated PC12 cell. A few 60- to 130-nm pits can be clearly seen (arrows). Fig. 7: PC12 cell 3 min after EGF treatment. Cells treated with EGF or NGF show a marked increase in the number of pits per unit area as compared with untreated cells (Fig. 6). *Inset*: Transmission electron micrograph demonstrating a coated pit on the surface of a treated cell. Note the characteristic dense-core secretory granule distinctive of pheochromocytoma and related cells. (Figs. 6 and 7) Bar, 1.0 μm. × 15,500. (*Inset*) Bar, 0.2 μm. × 38,000.

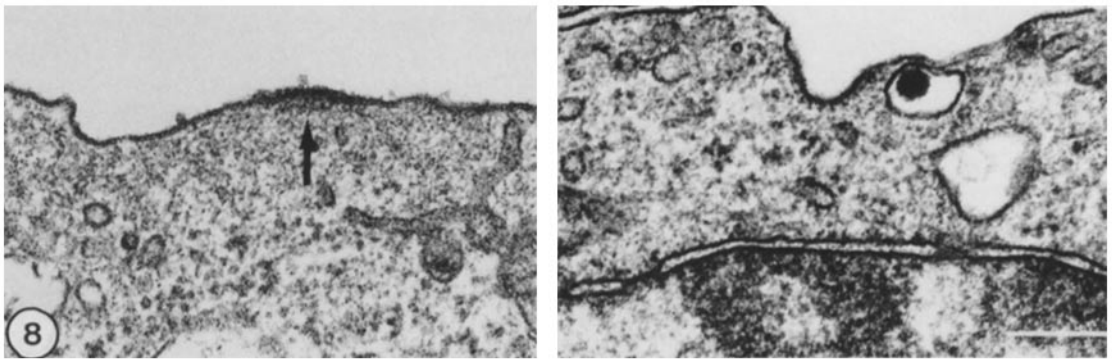


FIGURE 8 Transmission electron micrographs of PC12 cells treated with NGF in suspension, fixed, and processed with tannic acid and uranyl acetate en bloc staining. One can identify flattened coated areas (arrow) as well as the more common coated pit (adjacent to the dense-core secretory granule). Bar, 500 μm. × 82,000.

TABLE I

Comparison of Surface Area, Volume, Pits per μm^2, and Pits per Cell of PC12 Cells Treated for Various Times in Suspension with NGF

Time of NGF exposure	Cell surface area	Cell volume	Pits per μm^2	Pits per cell
min	μm^2	μm^3		
0	288 ± 45	379 ± 30	0.50 ± 0.11	144 ± 33
3	292 ± 32	418 ± 21	1.14 ± 0.15	334 ± 43
60	270 ± 44	384 ± 34	0.58 ± 0.13	159 ± 35

These data were determined as described in Materials and Methods. The difference in the number of pits per μm^2 between 0 and 3 min was significant with $P < 0.0005$. The variation is expressed as the standard error of the mean.

TABLE II

Influence of NGF on the Surface Density of Pits on PC12 Cells as Determined by Various Techniques

Means of analysis	Pits per μm^2 of PC12 cell surface after		
	0 NGF	3-min NGF	60-min NGF
SEM	0.42 ± 0.02	1.23 ± 0.06	0.60 ± 0.03
TEM of the dorsal surface of adherent cells	0.52	1.27	0.54
TEM of whole cells	0.50 ± 0.11	1.14 ± 0.15	0.58 ± 0.13

The variation is expressed as the standard error of the mean.

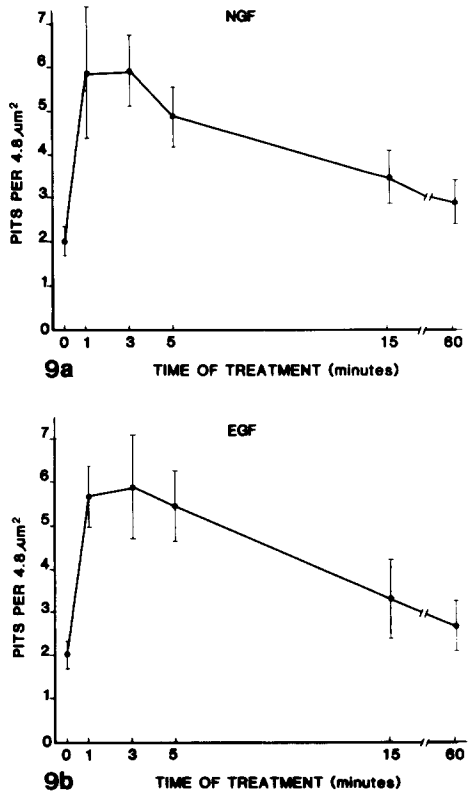


FIGURE 9 Average number of pits per unit are per PC12 cell as determined by SEM at various times after addition of NGF (*a*) or EGF (*b*) compared with 0 treatment, for 1, 3, 5, and 15 min, $P \leq 0.001$. For 60-min NGF and for 60-min EGF, the values are not significantly different than 0 treatment. Compared with 3-min treatment, for 15 and 60 min, $P \leq 0.001$. Error bars express SD.

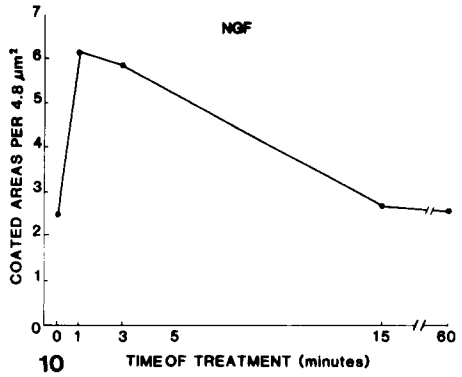


FIGURE 10 Number of coated areas per unit area of cell membrane of attached PC12 cells as determined by TEM at various times after addition of NGF.

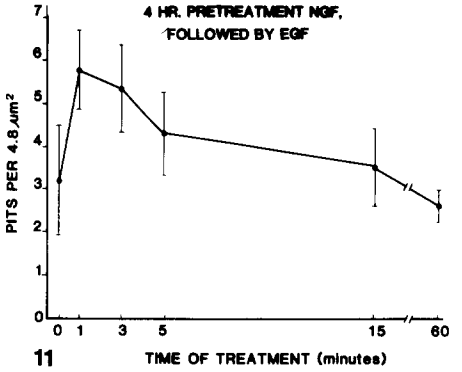


FIGURE 11 Average number of pits per unit area per cell by SEM at various times after addition of EGF to PC12 cultures pretreated with NGF for 4 h. Compared with 0 EGF for 1 and 3 min, $P < 0.001$. Compared with 3 min EGF for 15 and 60 min, $P < 0.001$. Error bars express SD.

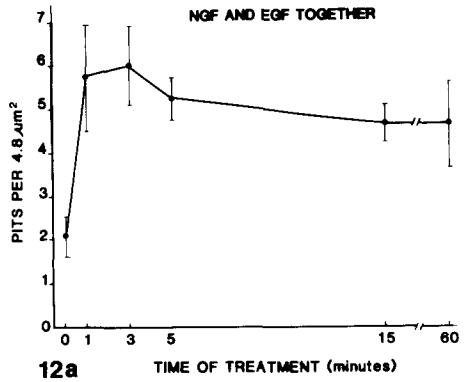
Furthermore, the level of coated pits quickly diminished and by 3 min equaled that seen in single agent treatment from 15 to 60 min (Fig. 13, *a* and *b*).

DISCUSSION

Specificity and Role of Surface Responses

Many effects of NGF and EGF on PC12 cells have been documented (for review see reference 6). Some of these, particularly those that occur with short latencies, are exhibited by both peptides, including induction of ornithine decarboxylase (8), increased cellular adhesion (4), enhanced metabolite uptake (8), and altered phosphorylation of specific cell proteins (9, 26). On the other hand, the two factors also promote rather distinct actions on the cells. In particular, NGF causes PC12 cells, by means of a slow, transcription-dependent process, to switch from a proliferating chromaffin cell-like phenotype to a sympathetic neuron-like phenotype characterized by cessation of cell division, growth of long neuronal processes, development of electrical excitability, and specific changes in molecular composition (see reference 6 for review). EGF does not induce these long-latency changes and may act, as it can for a number of cell types (27, 28), as a mitogen for PC12 cells (8).

We demonstrate here that cell surface ruffling, loss of microvilli, increased pit density, and blebbing are identically promoted by NGF and EGF treatment of PC12 cells. This



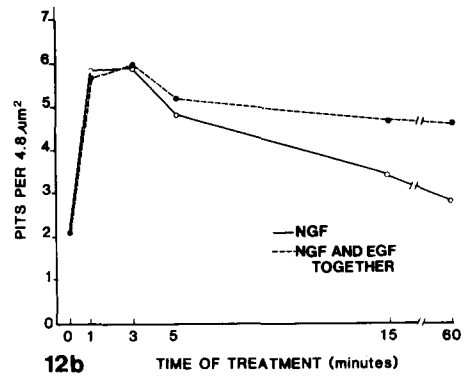
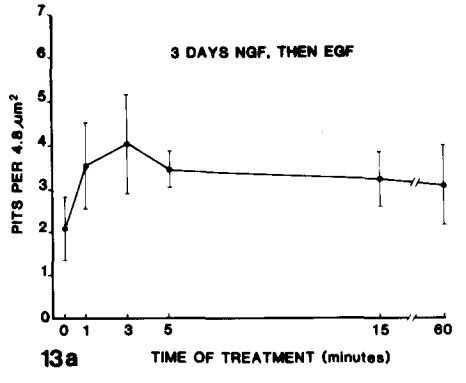


FIGURE 12 (a) Average number of pits per unit area per PC12 cell by SEM after simultaneous addition of NGF and EGF. Compared with 0 treatment, for 1, 3, 5, 15, and 60 min, $P < 0.001$. Compared with 3 min treatment, for 15–60 min, $P < 0.005$. Error bars express SD. (*b*) Comparison of the effects of NGF alone and of NGF plus EGF together on the number of pits per unit area per cell.



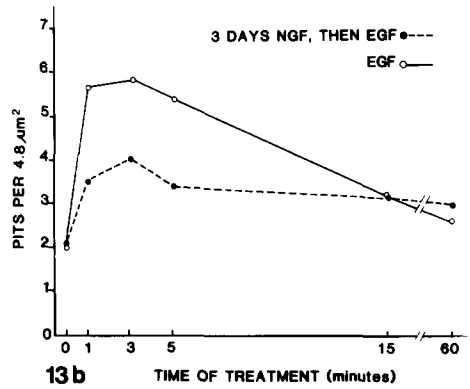


FIGURE 13 (*a*) Average number of pits per unit area per PC12 cell as determined by SEM at various times after addition of EGF to cultures pretreated with NGF for 3 d. Compared with 0 EGF, for 1 and 5 min, $P < 0.005$; for 3 min, $P < 0.001$; for 15 and 60 min, $P < 0.01$. Compared with 3 min EGF, for 15 and 60 min, $P < 0.01$. Error bars express SD. (*b*) Comparison of the effects of EGF alone and of 3-d NGF pretreatment followed by EGF addition on the number of pits per unit area per PC12 cell.

indicates that the surface changes are unlikely to be specific actions of either peptide in promoting neuronal or epithelial differentiation, respectively. On the other hand, given the many early responses that NGF and EGF trigger in common, it may be that the surface changes are manifestations of a general overall rapid response of cells to these and perhaps other growth factors or peptide hormones. In this regard, it is of interest that EGF has been reported to trigger similar alterations in the cell surfaces of several other cell types (2, 29–31).

The surface changes reported here do not appear to be nonspecific. In our previous experiments (1), such responses were not induced by calf thymus histone or cytochrome *c*, proteins with similar size and charge as NGF, or by α-bungarotoxin, a peptide for which PC12 cells appear to have specific receptors (32).

We (J. L. Connolly and L. A. Greene, unpublished observations) and Bothwell et al. (33) have noted that certain PC12 variants (34) that do not respond to NGF under normal growth conditions by undergoing neuronal differentiation nevertheless undergo surface ruffling and simplification after NGF treatment. Therefore, while the early surface changes could be necessary, they cannot themselves be sufficient for later, more specific morphologic differentiation.

Coated Pits and Internalization

Coated pits are the site of receptor-mediated internalization of many peptide hormones, growth factors, and other macromolecules (35–39). There is abundant evidence that NGF can be rapidly internalized by PC12 and other target cells (see references 40, 41 for review), and one study suggests that at least some of this uptake occurs by receptor-mediated patching and subsequent endocytosis via coated pits (42). EGF is also known to be rapidly internalized by at least certain of its target cells by a process involving receptor-mediated endocytosis at coated pits (11).

Treatment of PC12 cells with 50 ng/ml NGF or 10 ng/ml EGF leads to an identical increase in the number of coated pits per unit membrane area within 3 min. Stereologic analysis of the NGF response demonstrates that the increased number of coated pits seen per area reflects not a reduction in surface area available for formation of pits but a real increase in the number of pits present per cell. This may result from formation of coated pits at a rate that is temporarily greater than the rate of internalization of vesicles.

The onset of the changes reported here appear to correlate with the association of NGF and EGF with their respective surface receptors. In the systems thus far studied, binding of these factors reaches half-maximal levels within minutes (10, 11, 40, 41). Hence, while the ultimate role of the effects we have demonstrated here is not presently known, temporal considerations plus the increase in coated pits indicate that these rapid cell surface changes may be associated with peptide hormone/growth factor binding.

We previously documented a threefold increase in coated pits on sympathetic ganglion cells in response to NGF (3). A recent study utilizing lymphoblastoid cells with cell surface

receptor IgM molecules demonstrated a threefold increase in coated membrane after treatment with multivalent anti-IgM antibodies (43). In contrast, however, another group found no increase in coated membrane on cultured fibroblasts in response to α-2 macroglobulin after injection with anticlathrin antibodies (44). Hence, while the types of changes in coated pits reported here may also take place in other cases, their occurrence may depend on the ligand and cell in question.

In addition to coated pits, other types of surface structures observed here have been associated in some cases with internalization of macromolecules. Membrane ruffling and macropinocytosis occurrig after EGF treatment have been demonstrated in glial cells (29), carcinoma A431 cells (30), and in mouse epithelial cells (31). It is conceivable that similar effects may occur with PC12 cells.

Combined Versus Single Treatment

4 h of pretreatment with NGF had no effect on the EGF-promoted surface changes or coated pit formation. In contrast, 3 d of NGF pretreatment caused a markedly diminished series of surface changes to EGF. These results are quite interesting in view of the work of Huff and Guroff (8) who demonstrated a down-regulation of EGF binding which began after 12–18 h of NGF treatment and reached an 80% decrease by 3 d. Such findings provide further evidence that the surface changes are linked to the peptide hormone/growth factor receptor interaction.

When NGF and EGF were added simultaneously there was an increased surface response characterized by more marked and earlier peaking ruffling. This indicates that the cell surface changes induced by different ligands may be additive. This result is analogous to that of Huff and Guroff (8) who demonstrated a greater increase in ornithine decarboxylase activity with a combination of NGF and EGF than with either substance alone.

The initial increase in coated pits per unit area is essentially identical, irrespective of whether NGF and EGF were administered separately or together. In each case there was an approximately threefold increase over the unstimulated levels. The same threefold increase was seen with sympathetic ganglion cells after NGF (3) and with lymphoblastoid cells after anti-IgM antibody treatment (43). The reason why the increase in number of pits is very similar under these various conditions is unclear. Among possible explanations is that a maximum number of pits can form per unit area of membrane or that coated pit formation from the available clathrin pool is the rate-limiting step.

Possible Mechanisms of the Changes

The mechanisms of the peptide-induced surface changes are as yet unknown. There is conflicting evidence that cAMP may be a second messenger mediating the responses of PC12 cells to NGF (9, 12, 15). We found, however, that DbcAMP, at a concentration at which it is reported to mimic other of NGF's actions (9, 14, 15), initiated no surface simplification, ruffling, or blebbing. Therefore, it seems quite unlikely that cAMP mediates these surface changes.

Several other mechanisms have been raised that could play a role in the factor-induced surface changes, namely phosphorylation and methylation. NGF and EGF have been reported to promote rapid changes in the phosphorylation of specific PC12 cell proteins (9, 26). Also, EGF has been consistently linked to rapidly stimulated phosphorylation of tyrosine residues via a cAMP-independent protein kinase that either resides in, or is closely linked to, the EGF receptor (45–50). For the A431 cell system, it has been postulated that a binding-induced phosphorylation event may be responsible for the surface changes promoted by EGF (2, 45).

While we have demonstrated here that certain ligands such as NGF and EGF are capable of recruiting coated membrane to the cell surface, the mechanisms of this recruitment are at this time unclear. Among the possible events leading to increased coated pit formation are ligand-induced cross-linking of receptors (42, 51), or receptor-ligand–mediated alterations in membrane potential (52) or pH.

Methylation has been suggested to be a transducing mechanism for mediation of a variety of responses to extracellular signals (53). NGF has been shown to induce a very rapid spike of phospholipid methylation in the neurite fraction of cultured rat sympathetic neurons (54). Recent experiments show that the inhibitors of methylation block the capacity of NGF, but not EGF, to promote surface changes of the type described here (55).

Conclusions

In summary, we have reported surface architectural changes induced by EGF and NGF on PC12 pheochromocytoma cells. These changes are similar to those seen on primary cultures of sympathetic ganglion cells treated with NGF and on carcinoma A431 and mouse epithelial cells treated with EGF. The occurrence of these changes with different peptide hormones/growth factors on normal differentiated cells as well as on tumor-derived cell lines from cultures of rat, mouse, and human tumors indicates that such phenomena may represent fundamental characteristics of the interaction of at least some peptide growth factors/hormones with their target cells.

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