

RAPID, SEQUENTIAL CHANGES IN SURFACE MORPHOLOGY OF PC12 PHEOCHROMOCYTOMA CELLS IN RESPONSE TO NERVE GROWTH FACTOR

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ABSTRACT

The effect of nerve growth factor (NGF), a substance that promotes the differentiation and maintenance of certain neurons, was studied via scanning electron microscopy utilizing the PC12 clonal NGF-responsive pheochromocytoma cell line. After 2–4 d of exposure to NGF, these cells acquire many of the properties of normal sympathetic neurons. However, by phase microscopy, no changes are discernible within the first 12–18 h. Since the primary NGF receptor appears to be a membrane receptor, it seemed likely that some of the initial responses to the factor may be surface related.

PC12 cells maintained without NGF are round to ovoid and have numerous microvilli and small blebs. After the addition of NGF, there is a rapidly initiated sequential change in the cell surface. Ruffles appear over the dorsal surface of the cells within 1 min, become prominent by 3 min, and almost disappear by 7 min. Microvilli, conversely, disappear as the dorsal ruffles become prominent. Ruffles are seen at the periphery of the cell at 3 min, are prominent on most of the cells by 7 min and are gone by 15 min. The surface remains smooth from 15 min until 45 min when large blebs appear. The large blebs are present on most cells at 2 h and are gone by 4 h. The surface remains relatively smooth until 6–7 h of NGF treatment, when microvilli reappear as small knobs. These microvilli increase in both number and length to cover the cell surface by 10 h.

These changes were not observed with other basic proteins, with α -bungarotoxin (which binds specifically to PC12 membranes), and were not affected by an RNA synthesis inhibitor that blocks initiation of neurite outgrowth. Changes in the cell surface architecture appear to be among the earliest NGF responses yet detected and may represent or reflect primary events in the mechanism of the factor's action.

KEY WORDS nerve growth factor · PC12 · cell surface · scanning microscopy · hormone receptor

Nerve growth factor (NGF) is a polypeptide that affects the differentiation and maintenance of sympathetic and certain sensory neurons (14, 18). Among the well-known effects of NGF on its target cells are stimulation of neurite outgrowth, increase in somatic volume, and maintenance of survival (14, 18). However, since normal responsive neurons are exposed to endogenous NGF during development *in vivo* and require NGF for survival *in vitro* it has been difficult to observe the early morphological and physiological events that occur in response to initial exposure to the factor.

A clonal NGF-responsive cell line, PC12, has been established (9) from a transplantable rat adrenal medullary pheochromocytoma. When grown in the absence of NGF, this line shares many of the differentiated properties of adrenal chromaffin cells such as the presence of chromaffin granules (9) and synthesis, storage, and release of catecholamines (9, 10). In addition, within several days after the cells are exposed to physiologic levels of NGF, they begin to acquire a number of phenotypic properties of normal sympathetic neurons. These include initiation of neurite outgrowth (9, 24), cessation of cell division (9) and development of electrical excitability (6). Since PC12 cells may be maintained without NGF, but can respond to it, they provide a useful opportunity to examine the phenomena associated with initial NGF exposure.

By bright field or phase microscopy, few if any changes are discernible in PC12 cell morphology within at least the first 12 h after initial exposure to NGF. Since the first interaction of NGF with its target cells appears to be via cell membrane receptors (1, 8, 26), it is likely that at least some of the initial responses to the factor are surface-related. The purpose of the present work was to study, by means of scanning electron microscopy, the possible effects of NGF on the surface morphology of PC12 cells. We report here that among the initial responses to NGF treatment is a rapidly onset reproducible sequence of changes in cell surface architecture.

MATERIALS AND METHODS

PC12 cells, 100–150 generations after isolation, were grown in plastic tissue culture dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) in a medium consisting of 85% RPMI (Grand Island Biolog-

ical Co. [Gibco], Grand Island, N. Y.), 10% heat-inactivated horse serum (KC Serum Co., Lenexa, Kan.), 5% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.), 50 $\mu\text{g}/\text{ml}$ streptomycin and 50 mg/ml penicillin. Approximately 1×10^4 cells in 2 ml of culture medium were plated 2–3 d before each experiment into tissue culture dishes containing poly-L-lysine (Sigma)-coated (15) glass coverslips previously washed in HCl and rinsed in distilled water. All substances tested were added to the cultures in a concentrated form in 5 μl of complete culture medium. The substances tested were 2.5 S NGF prepared from mouse salivary glands (2), calf thymus histone (Sigma), horse cytochrome *c* (Sigma), and α -bungarotoxin (α -BT) (17). The final concentration of all substances (except α -BT which was 4 $\mu\text{g}/\text{ml}$) was 50 ng/ml . "Control" NGF-untreated cultures received 5 μl of complete medium containing the NGF carrier buffer alone (50 nmol of pH 5.0 sodium acetate buffer). Camptothecin (11) was added with or without NGF (50 ng/ml) to a final concentration of 0.2 $\mu\text{g}/\text{ml}$. To maintain conditions as uniform as possible in each experiment, the test substances were added at the appropriate time before fixation, and all cells were thus fixed at approximately the same time after plating. The times of observation were 0, 1, 3, 5, 7, 10, 15, 30, 45, 60, 90, and 120 min, then hourly until 10 h. Substances other than NGF were tested simultaneously with an NGF experiment. Cultures were maintained at 37°C in a water-saturated atmosphere containing 7% CO_2 .

At the termination of each experiment, the cells were rapidly washed twice with Hanks' balanced salt solution (Gibco) at 37°C and fixed in 2% cacodylate-buffered glutaraldehyde at 37°C. The cells were postfixured in 1% osmium tetroxide and dehydrated through graded ethanols, with or without final dehydration in amyl acetate. The results were identical with final dehydration in absolute ethanol or amyl acetate. The cells were then critical-point dried from liquid CO_2 , coated with 200 Å of gold palladium and observed in an Advanced Metals Research (AMR) 900 or 1000 or JEOL JSM 35 scanning electron microscope.

Cells were prepared for transmission electron microscopy in a similar manner as those prepared for scanning. After ethanol dehydration, they were embedded in Epon, cut, stained with uranyl acetate and lead citrate, and observed with a Philips 200 electron microscope.

The percentage of cells with a given feature was determined by scoring 100 consecutive cells in from one to three separate experiments.

RESULTS

PC12 cells maintained without NGF vary in shape but generally are round to ovoid (Fig. 1). After fixation and dehydration, the average diameter of the cells is between 9 and 12 μm . At the periphery, most of the cells are thinly spread in one or two areas. Long, slender filopodia 0.1–0.2 μm in diameter, but varying in length, form attachment

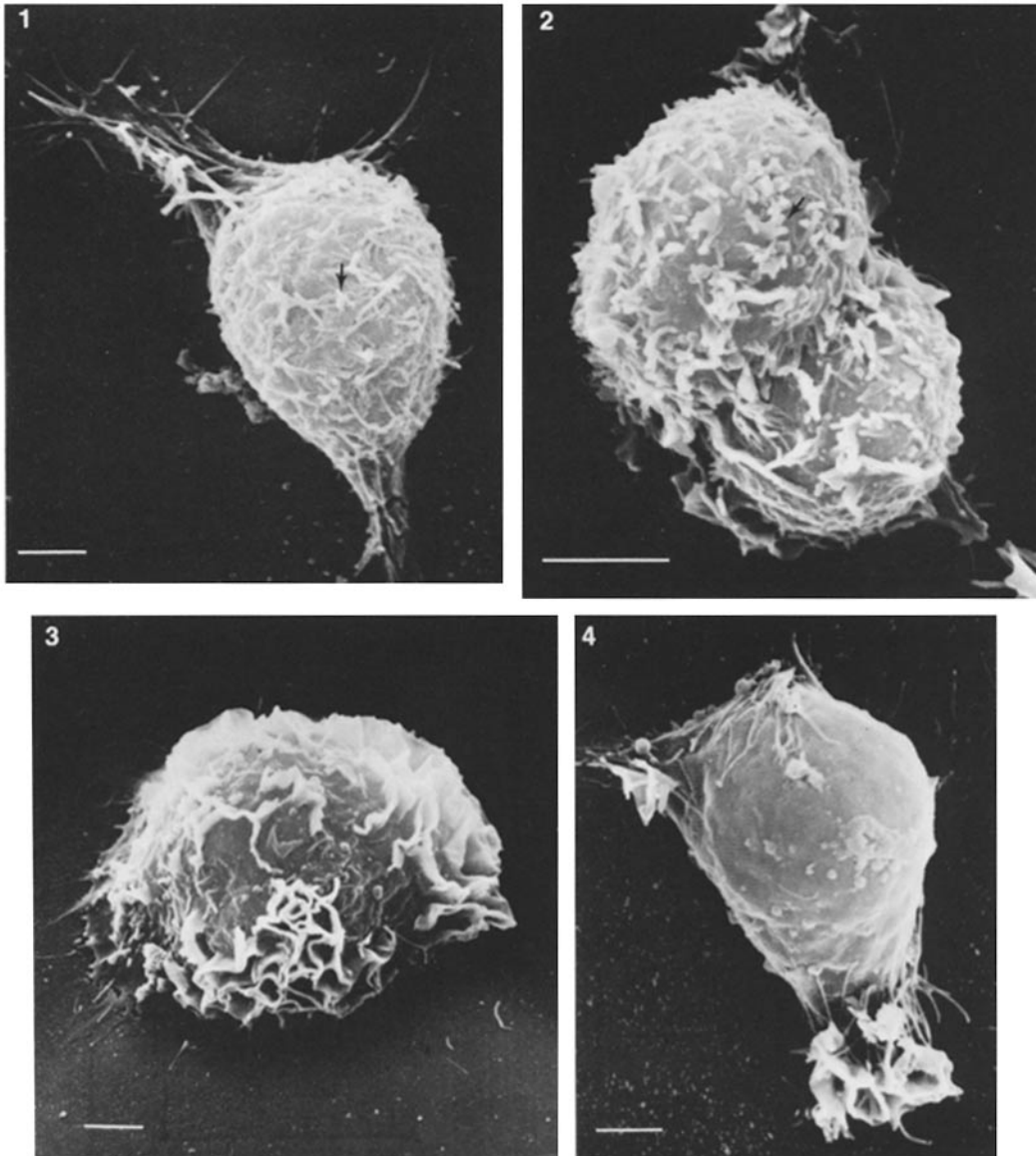


FIGURE 1 PC12 cells not treated with NGF. The cells are round to ovoid, between 9 and 12 μm . At the periphery most cells are thinly spread in one or two areas. The surface has numerous small blebs (arrow) 0.2–0.6 μm in diameter and numerous microvilli. Bar, 2.0 μm . $\times 4,700$.

FIGURE 2 1 min after NGF treatment, many small ruffles have appeared on the dorsal surface of these two cells. These ruffles often show blunt, finger-like projections (arrow). Microvilli have markedly decreased. Bar, 2.0 μm . $\times 8,500$.

FIGURE 3 3 min after NGF treatment. Large ruffles are present on the dorsal surface of this small group of cells. The ruffles at this time are smooth in contour. Microvilli are markedly decreased. Bar, 2.0 μm . $\times 4,100$.

FIGURE 4 5 min after NGF treatment. The dorsal aspect of the cells is dramatically simplified. Microvilli are almost absent, small blebs are variable, and dorsal ruffles are still present on many cells (see Figs. 3 and 5). Now, ruffles are apparent at the cell periphery. Bar, 0.2 μm . $\times 4,600$.

sites with the substratum (Fig. 1). Occasional cells have thicker, blunt processes that arise from the basal portion of the cell and that extend one to two cell diameters distal to the cell. The filopodia and short blunt processes vary from cell to cell but were not noticeably changed by NGF treatment during the time period of this study.

The free surface of PC12 cells untreated with NGF is complex. The most prominent features are, first, a variable number of randomly spaced microvilli (Fig. 1). In three separate experiments, these were present on 94, 97, and 98% of the cells. The microvilli are 0.2 μm in diameter and from 0.3–2.4 μm in length, arise directly from the cell surface or from small ridges, and are usually unbranched. A second prominent feature on virtually every cell is the presence of numerous small blebs. The blebs range in size from 0.2–0.6 μm and may be randomly dispersed or may cluster (Fig. 1). Microvilli and small blebs appear to be distinct structures. 8% of the cells had, in addition to small blebs, one or more large blebs, from 0.8 to 1.8 μm in diameter and averaging 1.2 μm , (see Fig. 6 for an example on an NGF-treated cell). None of the cells had ruffles over the dorsal surface.

After the addition of NGF to the cultures, there was a dramatic sequential change in the cell surface. This sequential change showed remarkable consistency both between different experiments and within the cell population of a given experiment. Within 1 min of exposure to NGF, small ruffles appeared on the dorsal cell surface of 97% of the cells. These ruffles sometimes had a smooth contour but commonly showed blunt finger-like projections (Fig. 2). At this time, microvilli were

present on 70% of the cells. By three minutes (Fig. 3), large ruffles were still present on the dorsal surface of almost all the cells but were generally smooth in contour, and sometimes formed elaborate branching patterns. Also, by 3 min of treatment, microvilli had markedly decreased in number and were present on only 26% of cells.

By 5 min of treatment, the dorsal aspect of the cells was significantly simplified (Fig. 4). Microvilli were seen on 11% of the cells and, when present, were few in number. Dorsal ruffles had decreased and were seen on 55% of cells. At this time peripheral ruffles became prominent and were seen on 43% of cells, whereas before this time they were seen on <5% of cells. The dorsal ruffles rapidly decreased and were seen on 3% of cells at 7 min and on 1% at 10 min of treatment. Peripheral ruffles increased as the dorsal ruffles decreased; at 7 min, they were seen on 73% of the cells. Shortly thereafter, peripheral ruffles also began to disappear so that they were seen on 29% of cells at 10 min and were almost absent by 15 min. The time course of the above described changes is summarized in graph form in Fig. 5a.

The time period between 15 and 45 min of NGF treatment was characterized by a relatively simple surface with few microvilli, ruffles or small blebs. Then, beginning at ~45 min, large blebs became apparent on an increasing percentage of cells so that, by 2 h, they were seen on 85% of the population. Such large blebs were primarily seen on the dorsal surface, but were also observed over the thinly spread peripheral area as well (Fig. 6). When studied by transmission electron microscopy (Fig. 6, *inset*), these large blebs contained

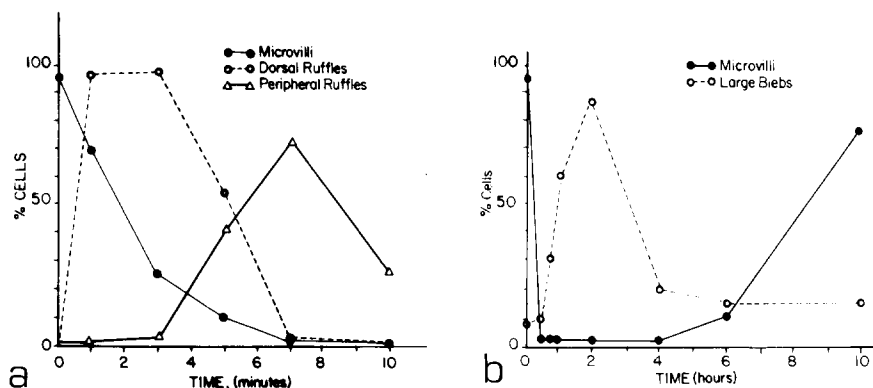


FIGURE 5 Time course after nerve growth factor treatment for the presence of specific surface features. Each point represents the results of observations on 100 consecutive cells. (a) Data for the first 10 min. (b) Data for the first 10 h.

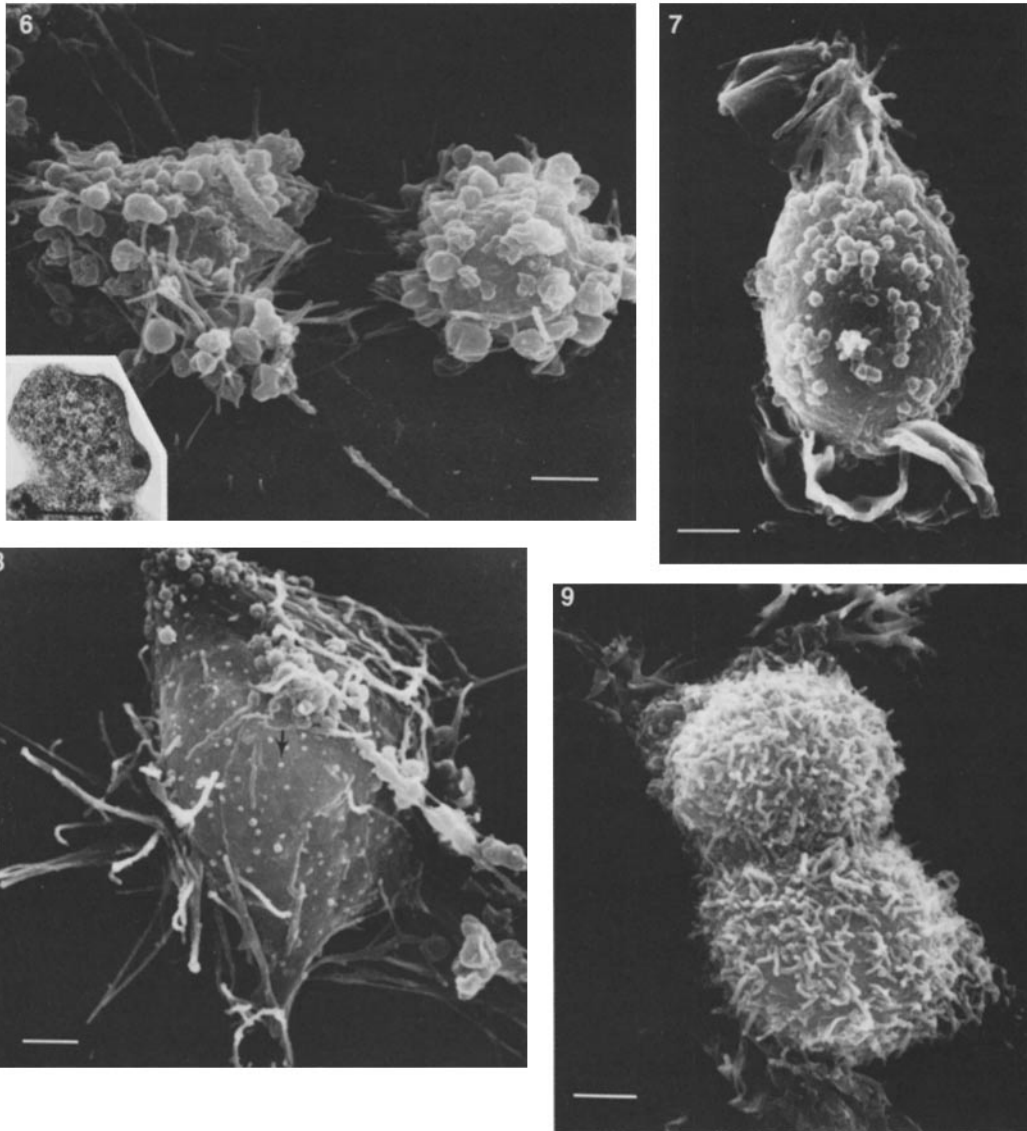


FIGURE 6 2 h after NGF treatment. Numerous large blebs, 0.8–1.8 μm in diameter, have appeared on the surface of most cells. These large blebs contain polyribosomes and an occasional dense-core secretory granule. Bar, 2.0 μm . $\times 4,400$. (inset) Bar, 0.5 μm . $\times 20,500$.

FIGURE 7 4 h after NGF treatment. The large blebs are absent from most of the cells, microvilli are rare, and small blebs are variable in number. Peripheral ruffling is seen on occasional cells. Bar 2.0 μm . $\times 4,200$.

FIGURE 8 7 h after NGF treatment. Microvilli have begun to reappear as small buds (arrow). Bar, 2.0 μm . $\times 3,700$.

FIGURE 9 10 h after NGF treatment. Two cells showing numerous microvilli covering the surface. The microvilli at this time are more numerous than on untreated cells (Fig. 1). Bar, 2.0 μm . $\times 4,300$.

aggregates of polyribosomes and dense-core chromaffin granules but larger cytoplasmic structures such as mitochondria were not present. During this period of predominance of large blebs, microvilli remain scant.

The principal changes that occurred from 10 min to 10 h of NGF treatment are summarized in Fig. 5 *b*. From 2 to 4 h after NGF treatment, there was a decrease in the number of large blebs until they were again quite scarce. Microvilli also remained scarce, and small blebs were less frequent than before treatment but were variable in number. The period between 4 and 6 h was again one marked by relatively smooth cellular surfaces; large blebs were absent from 85% of the cells, microvilli were scant, and small blebs were variable in number (Fig. 7).

The time period from seven to eight hours (Fig. 8) was marked by the reappearance of microvilli. These structures appeared as small knobs and gradually grew longer. By 10 h, the population of microvilli became quite extensive (Fig. 9). The microvilli were more numerous on cells during this period than they were on cells that were not treated with NGF. Large blebs were present on ~15% of the cells during this period.

The changes described here which took place in the presence of NGF were not blocked in the presence of camptothecin (0.2 $\mu\text{g}/\text{ml}$) (11), an inhibitor of RNA synthesis that blocks the initiation of neurite outgrowth in PC12 cultures (4). This suggests that such changes do not require transcription of new RNA.

To exclude the possibility that the observed surface changes were merely a nonspecific effect of the basic charge of NGF, two other basic proteins, histone and cytochrome *c* (50 ng/ml), were added to cultures. Also, to test for changes induced by mere binding of a substance to a membrane receptor, α -bungarotoxin (α -BT) (4 $\mu\text{g}/\text{ml}$), which is known to bind to PC12 membranes (19), was added. While cells treated with histone and α -BT showed a slight increase in blebbing after 10 h of treatment, none of the above substances caused the sequential changes associated with NGF.

DISCUSSION

We have demonstrated here that NGF treatment causes a rapidly onset set of sequential changes in the surface architecture of PC12 pheochromocytoma cells. The onset of these changes is among the most rapid response to NGF yet detected and

appears to correlate temporally with the binding of NGF to its target cells. In membrane fragments of rabbit superior cervical ganglia at 24°C and at an NGF concentration of 4 ng/ml, half-maximal levels of specific binding occurred within 2 min and plateaued by 12 min (1). Recent experiments indicate that NGF binds to receptors on PC12 cell membranes with a similar rapid time course (26).¹ In addition, there is evidence that significant amounts of NGF can be internalized by PC12 cells within several minutes after addition to cultures (reference 26, footnote 1) and that such internalized NGF is detectable in the nucleus within 20 min (26).

Several physiologic responses of PC12 cell membranes are also known to change soon after initial NGF exposure. Within 15 min of NGF treatment, PC12 cells show an increased uptake of amino acids (16). Also, starting within 10 min of NGF exposure, PC12 cells show increased cell-to-cell and cell-to-substratum adhesion as well as a change in lectin-induced cell aggregation without a change in the total number of lectin receptors (22, 23). The possible existence of causal relationships between these events and the surface architectural changes described here, however, remains to be established.

The functional significance of the surface changes caused by NGF may be interpreted with respect to a number of different possibilities. Since NGF affects PC12 cell proliferation, one possible consideration is cell cycle. The surfaces of synchronized Chinese hamster ovary (CHO) cells in confluent culture have been shown to undergo a series of sequential surface changes during the cell cycle (20), some of which resemble stages found in the present study. The cell cycle changes in CHO cell surfaces, however, do not appear to occur in unsynchronized cultures (25), nor do they appear in sparsely plated cultures (21). The PC12 cells used in the present studies have a generation time of ~48 h. These cells were at nonconfluent densities, were not synchronized, and light and transmission electron microscopy did not reveal synchrony of nuclear division at any time during the study. Moreover, PC12 cells do not cease proliferation until at least 4–7 d after addition of NGF. Thus, the remarkable uniformity in the response

¹ P. Calissano and M. L. Shelanski, Department of Neuropathology, Harvard Medical School. Manuscript in preparation.

of PC12 cultures to NGF strongly suggests that these changes are not related to cell cycle alterations.

The possibility that the presently described NGF-induced changes in PC12 membranes could represent the beginnings of neurite outgrowth can also be considered. This possibility seems unlikely since few of the surface projections that appeared, such as ruffles or large blebs, persist beyond about 10 h, while neurite outgrowth itself is not observed before 18–48 h after NGF treatment. Also, while essentially all of the cells underwent the changes shown here, the rate of initial appearance of neurites in the total PC12 population shows a rather prolonged time course requiring 4–7 d. Furthermore, initiation of neurite outgrowth is blocked by camptothecin (4) while the surface changes described here were not. On the other hand, while the surface changes may not be directly structurally related to neurite outgrowth, one cannot yet evaluate the possibility that they may represent an essential stage in the processes of preparing the cell for neurite outgrowth.

Another possibility is that the changes in cell surface caused by NGF, particularly at early times, such as ruffling or disappearance of microvilli, are related to internalization. As mentioned above, there is recent evidence for rapidly onsetting internalization of NGF by PC12 cells (26). Epidermal growth factor (EGF) has also been shown to have specific membrane receptors and to be very rapidly internalized by its target cells (5). Treatment of human glial cells with EGF has been shown to cause extensive ruffling (first studied after 6 h of EGF treatment) and there is evidence for pinocytosis at the central aspect of these ruffles (3). As for EGF, the biological significance of internalization of NGF is not yet clear.

Changes in surface morphology may represent a general response of cells to treatment with peptide hormones and growth factors. Intrinsic tryptophan fluorescence of membranes prepared from GH3 pituitary cells was shown to be quenched by thyrotropin releasing hormone (TRH), indicating that the TRH-receptor interaction in such membranes is associated with a change in membrane conformation (12). Microvilli on the surface of Balb/c 3T3 cells have been found to disappear with serum starvation and to reappear within 1 h of subsequent insulin treatment (7). Cultured rat granulosa cells undergo rapidly onsetting reversible shape changes and surface simplification during exposure to follicle-stimulating hormone (13).

In summary, the present experiments demonstrate a dynamic sequence of surface events that is triggered soon after the interaction of NGF with a target cell. These events may play an as yet undetermined role in the mechanism of action of NGF.

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