# Pit Formation and Rapid Changes in Surface Morphology of Sympathetic Neurons in Response to Nerve Growth Factor

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ABSTRACT Scanning and transmission electron microscope studies were carried out on the rapid cell surface responses of cultured newborn rat sympathetic neurons to nerve growth factor (NGF), a substance that promotes their survival and differentiation. The somas of sympathetic neurons continuously exposed to NGF or deprived of the factor for 4–5 h have a very smooth surface. After readdition of NGF to the latter type of cultures, there is rapidly initiated a transient, sequential change in the cell surface. Microvilli and small ruffles appear within 30 s and are most prominent by 1 min. By 3 min of exposure, the microvilli and ruffles decrease in prominence, and by 7 min the somal surface is again smooth. By 30 s after NGF readdition, an increase in the number of 60- to 130-nm coated pits is also detectable. This increase reaches a maximum of about threefold from 0.5 to 3 min and then gradually decreases. Alterations in the surface did not occur on the nonneuronal cell types present in the cultures and were not observed in response to another basic protein (cytochrome *c*) or to physical manipulation. Changes in cell surface architecture induced by NGF in normal sympathetic neurons and, as previously described, in PC12 pheochromocytoma cells indicate that such responses may represent or reflect primary events in the mechanism of the factor's action.

There has been increasing interest in the relationship between the surface morphology of cells and their hormonal milieu. In particular, rapidly onsetting changes in cell surface architecture may reflect events that occur in response to initial hormone binding and may themselves play a causal role in subsequent hormone-triggered responses. Recently, we have reported (1) a rapidly initiated sequence of changes in the surface architecture of PC12 pheochromocytoma cells that occurs in response to treatment with nerve growth factor (NGF). Such changes were initiated within minutes of exposure to NGF and far preceded subsequent responses such as initiation of neurite outgrowth and alterations in protein synthesis. Similar types of surface changes have also been reported (2) to occur in cultures of carcinoma A431 cells in response to epidermal growth factor (EGF).

Although tumor-derived cell lines may possess many of the physiologic characteristics of their normal counterparts, their responses may also significantly differ from those of normal cells. Therefore, we have undertaken the following study of the surface response of cultured rat sympathetic neurons to NGF.

## MATERIALS AND METHODS

Sympathetic ganglion cells were prepared from the dissected superior cervical ganglia of newborn rats as previously described (3). They were then plated on poly-L-lysine-coated (4) glass cover slips at a density of two ganglia per dish in medium and under conditions as reported in reference 1. The cells were maintained in medium containing 50 ng/ml mouse salivary NGF (5) for 4 d and were then treated in one of three ways: (*a*) washed three times with NGF-free medium and placed in NGF-free complete medium for 4–5 h before an experiment; (*b*) washed three times with NGF-containing or NGF-free medium and placed in NGF-containing medium; or (*c*) left untouched. After 4–5 h, the test substance, NGF or horse cytochrome *c* (Sigma Chemical Co., St. Louis, Mo.), was added to the NGF-deprived cultures in a concentrated form in 5 μl of complete medium to a final concentration of 50 ng/ml. Control (non-NGF-deprived) cultures received 5 μl of complete medium. The times of incubation before fixation were 0, 0.5, 1, 3, 5, 7, 60, and 120 min after readdition of NGF; other cultures were continuously treated (NGF not deleted at any time since the initial plating). Experiments were performed at least four to six times. At termination of each incubation, the cells were washed, fixed, and processed for scanning (SEM) and transmission electron microscopy (TEM) as previously reported (1).

Specific features of cells such as the presence and number of microvilli and ruffles were counted by SEM on 100 consecutive cells for each experimental condition. The counts of the number of pits per unit area on scanning images were done by placing a grid over high-magnification (× 10,000) micrographs of



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cells of similar size and counting as many areas as were readily visible. The total number of areas evaluated was between 30 and 88 on 10–18 cells. The values obtained per cell were compared using the independent samples *t* test.

The number of coated areas per unit length of membrane was evaluated by TEM in the following manner. Cell clumps embedded in Epon in the tissue culture dish were selected by phase microscopy, isolated, cut at four distinct levels in each block, and stained. One complete set of micrographs was taken at × 3,480 of the entire free surface without the aid of binoculars and evaluated with a cell perimeter program at a final magnification of 28,400 with a Bioquant II Biological image analyzer by R & M Biometrics, Nashville, Tenn. The entire free surface (excluding areas between cells or under neurites) of 10–15 cells cut at four levels per time-point (0, 0.5, 3, and 120 min) was evaluated, with a total of 475 micrographs.

## RESULTS

The cultured sympathetic ganglion neurons tended to form small clusters of cell bodies that were interconnected by numerous neurites. The somas of neurons either continuously treated with NGF or deprived of the factor for 4–5 h had very smooth surfaces. In each case, microvilli and specialized surface structures were very rare and ruffles were absent (Fig. 1).

When NGF (2 nM final concentration) was added to the medium of neurons that had been deprived of the factor for 4–5 h, there was a rapid change in the cell surface. Within 30 s, >90% of the neurons had developed small stubby microvilli and small ridges (Fig. 2). A minority of the neurons (8–10%) developed very prominent ruffles (Fig. 3). By 1 min after NGF treatment, essentially all of the neurons had prominent microvilli and 50% had small ruffles (Fig. 4). By 3 min, the neurons had fewer microvilli and ruffles. By 7 min, the somal surface had returned to the pretreated smooth state (Fig. 5).

When the neuronal somatic surfaces were examined at high magnification (× 10,000–20,000) by SEM another more subtle phenomenon was observed. Small pits 60–130 nm in diameter were present on all neurons (Fig. 6). There was a marked increase in the number of such pits after NGF treatment (Fig. 7). When viewed by TEM, these pits were of the coated variety (Fig. 8). The increase in the density of pits was seen at our earliest time-point, 0.5 min. At this time, TEM revealed many areas of nonindented, coated membrane in addition to the well-formed coated pits. Between 0.5 and 3 min of NGF exposure, the density of pits reached a level almost threefold the level observed in cultures continuously treated with NGF or deprived of the factor for 4–5 h (Figs. 6 and 7). After 3 min, the density of pits decreased but by 2 h, the maximum time observed in this study, had not returned to the level present in the untreated or continuously treated cultures (Fig. 9).

Because PC12 cells have been shown to undergo rapid surface changes in response to NGF (1), we also reexamined this model system, and preliminary data[1] show a similar threefold increase in coated pits in response to NGF.

Several types of controls were used in the experiments to

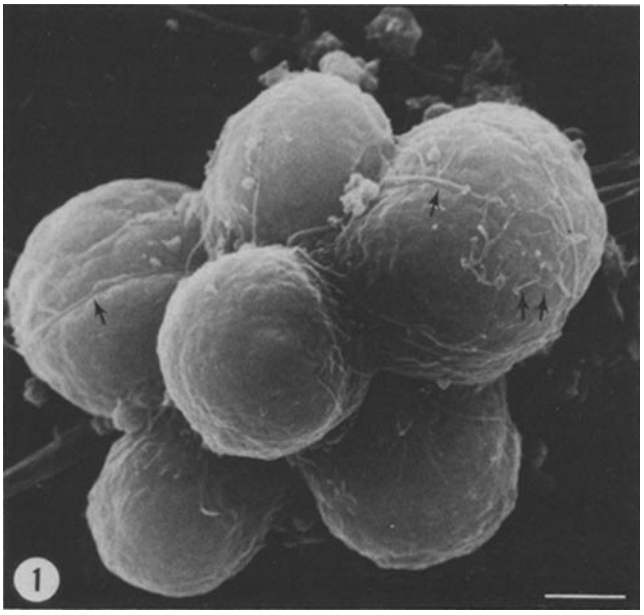


FIGURE 1 Sympathetic ganglion cells deprived of NGF for 5 h. The neuronal surfaces are quite smooth. Neurites (single arrows) and an occasional nerve ending (double arrow) can be seen. Bar, 5.0 μm. × 2,150.

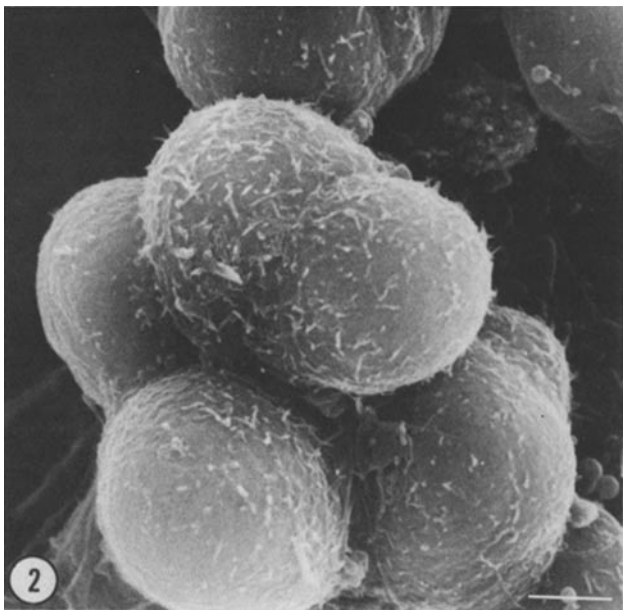


FIGURE 2 Sympathetic neurons 30 s after readdition of NGF. Small microvilli appear on >90% of the cells. Bar, 5.0 μm. × 2,150.

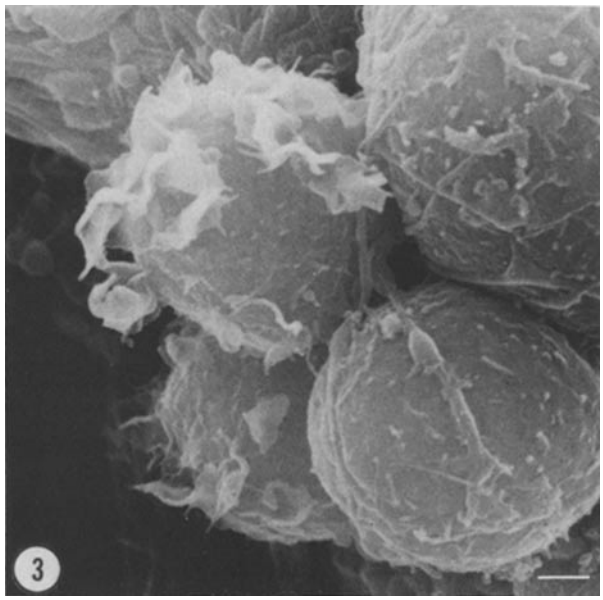


FIGURE 3 30 s after NGF treatment. 8–10% of the cells develop very prominent ruffles. Bar, 2.0 μm. × 3,200.

[1] J. L. Connolly and L. A. Greene. Unpublished observations.

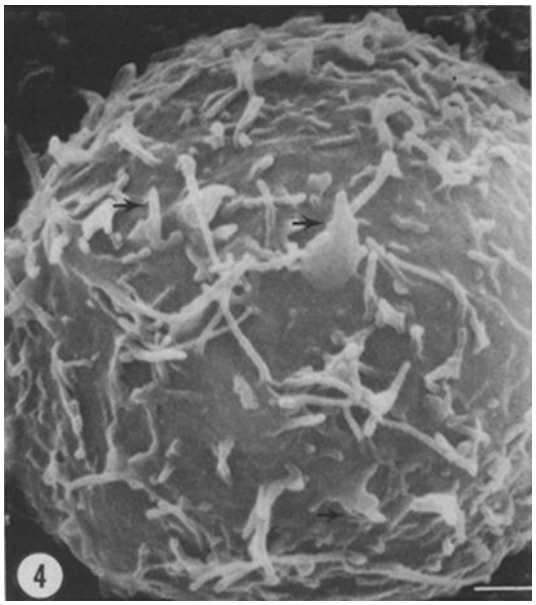

FIGURE 4 1 min after NGF treatment. Virtually all of the neurons have prominent microvilli and 50% have small ruffles. Many of the small ruffles have blunt fingerlike projections (arrows). Bar, 1.0 μm. × 7,200.

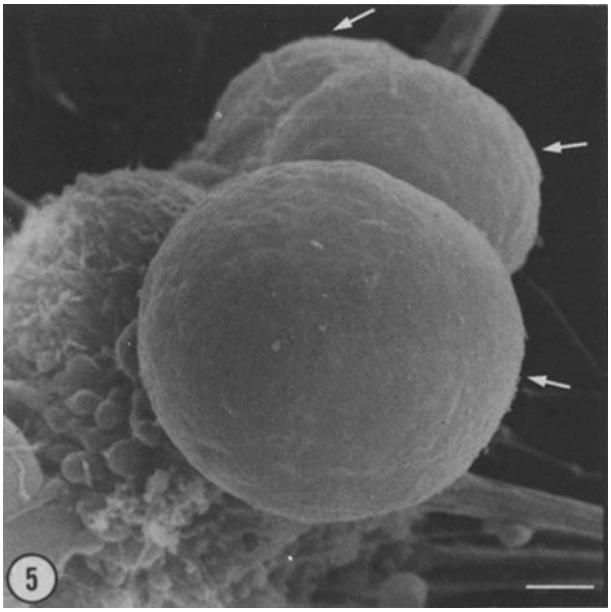

FIGURE 5 7 min after NGF treatment. This micrograph demonstrates three sympathetic cell bodies (arrows) sitting atop neurites, supporting cells and vesicles at the base of a cell clump. The cell surface is simplified; microvilli and ruffles are absent. Bar, 2.0 μm. × 4,200.

rule out artifacts. Cultures that were physically manipulated in the same fashion as NGF-treated cultures but that were not retreated with NGF or were treated with the basic protein cytochrome *c* did not show surface changes or an increase in pit formation. Also, cells in the cultures with nonneural morphologies (fibroblastlike and satellite cells) did not show changes in surface architecture in response to NGF.

## DISCUSSION

We have demonstrated here that NGF induces a rapidly onsetting set of surface architectural changes in cultured neo-

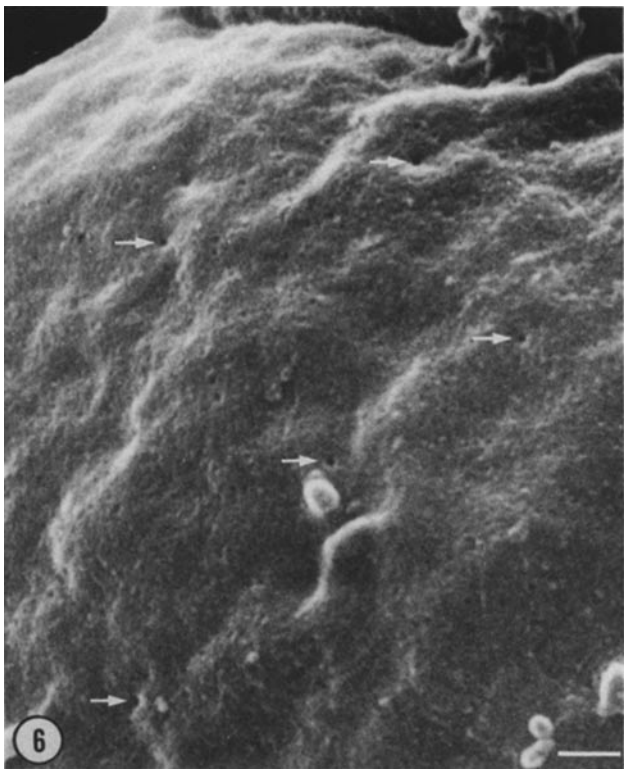

FIGURE 6 Surface of sympathetic ganglion cell deprived of NGF for 5 h, shown here at high magnification. Five 60- to 130-nm pits can be clearly defined (arrows). Bar, 0.5 μm. × 16,000.

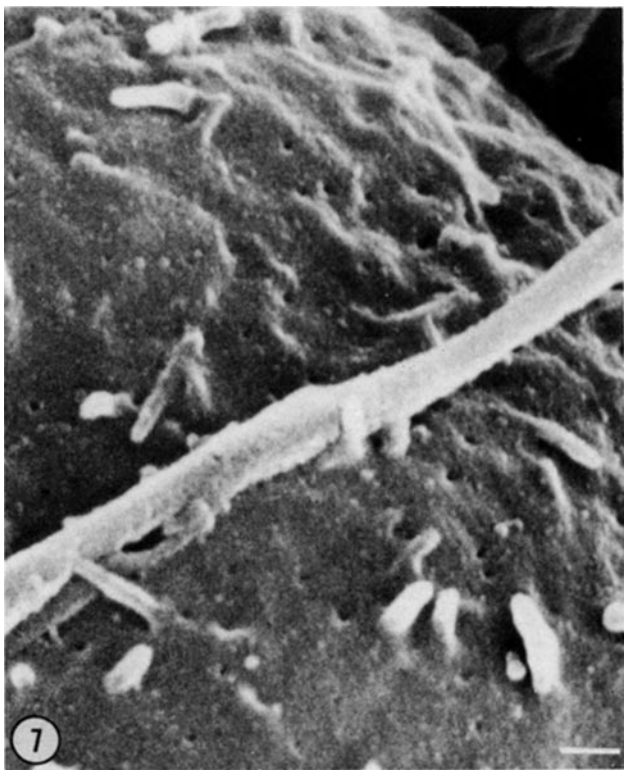

FIGURE 7 3 min after addition of NGF. There is a marked increase in the number of pits per unit area as compared with the NGF-deprived cells (Fig. 6). In concert with this change, the microvilli become more prominent. Bar, 0.5 μm. × 16,000.

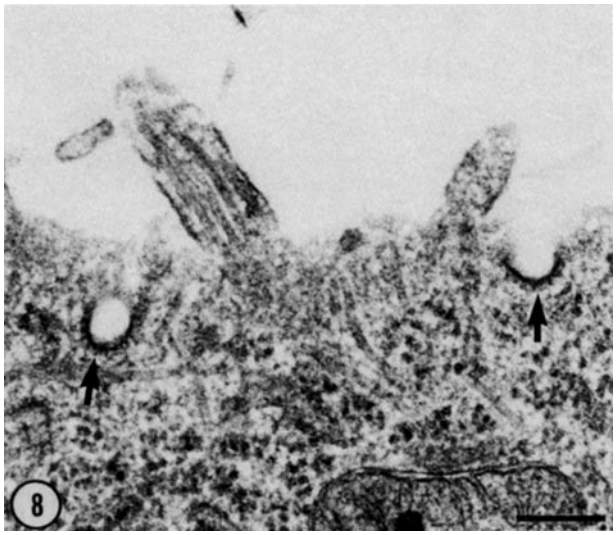

FIGURE 8 Transmission micrograph of a sympathetic ganglion cell after 30 s of NGF treatment. Note the presence of microvilli and coated pits (arrows). Bar, 0.2 μm. × 57,000.

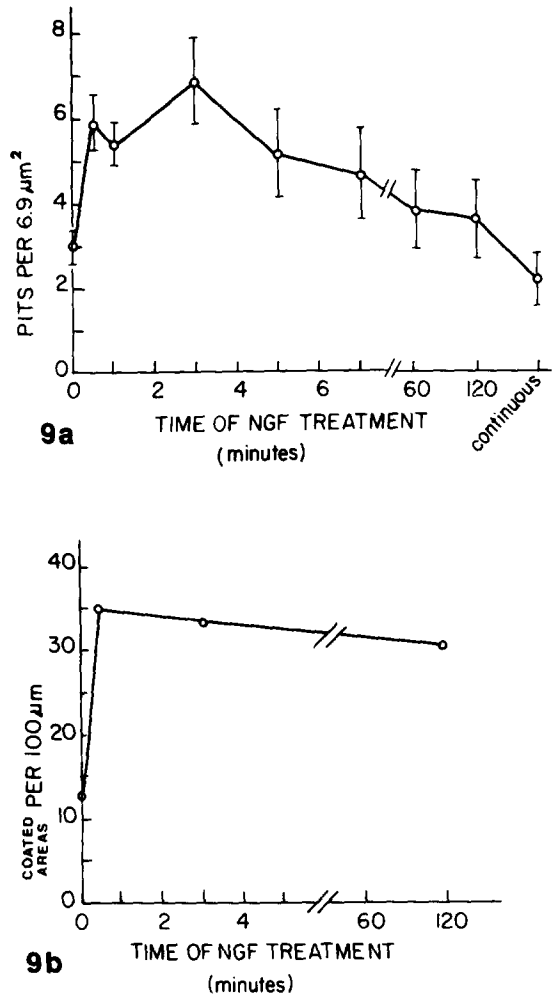


FIGURE 9 (*a*) Number of pits per unit area per cell (SEM) in NGF-deprived sympathetic ganglion cells after readdition of NGF. Error bars express standard deviation. Relative to 0 NGF, for 0.5, 1, 3, and 5 min $P < 0.001$, and for 7, 60, and 120 min $P < 0.01$. (*b*) Number of coated areas per unit length of membrane (TEM). Actual values: 0 NGF, 140 coated areas/1,099,387 nm; 30-s NGF, 214 coated areas/610,010 nm; 3-min NGF, 265 coated areas/802,604 nm; 120-min NGF, 210 coated areas/695,275 nm.

natal rat sympathetic neurons. Such cells require NGF for their survival in vitro; if they are deprived of NGF for >6 h, the neurons undergo irreversible metabolic changes and eventually die (6, 7). Thus, to carry out our experiments, NGF was withdrawn from the cultures for 4–5 h, and surface changes were observed after its replacement. The 4–5 h of incubation without NGF should result in considerable dissociation of NGF from surface membrane receptors (8) but should not result in irreversible cell damage.

The onset of the changes reported here is as rapid as any response to NGF yet detected and appears to correlate temporally with the binding of NGF to surface receptors. 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 were reported to occur within 2 min and to plateau by 12 min (8).

The surface changes found on neurons were not observed on the nonneuronal cells in our cultures. Such cells do not have other known responses to NGF and do not appear to possess high-affinity NGF receptors. The surface responses to NGF therefore appear to be restricted to otherwise responsive, receptor-bearing targets.

We have previously shown that initial exposure to NGF causes a rapidly initiated sequential change in the surfaces of PC12 rat pheochromocytoma cells. The latter is a cell line that does not require NGF for survival but that responds to the factor by ceasing mitosis and undergoing neuronal differentiation (e.g., neurite outgrowth and acquisition of electrical excitability) (9, 10). After initial addition of NGF to PC12 cultures, ruffles appear over the dorsal cell surface within 30 s, 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 cell periphery at 3 min, are prominent by 7 min, and are gone by 15 min (1). There is also a threefold increase in the density of pits at the cell surface within 3 min of initial NGF treatment.

There are both similarities and differences in the response to NGF that we observed between the sympathetic neurons and PC12 cells. The most striking similarities are in the occurrence and time-course of surface ruffling and pit formation. The presence of such changes in both differentiated neurons and in a cell line indicates that they are a basic component of the response to NGF. With respect to differences, PC12 cells initially have a complex surface that undergoes simplification and within a period of hours again becomes complex; the sympathetic neurons have a simple surface that initially becomes complex and rapidly simplifies again. The degree of NGF-induced ruffling is also more marked on PC12 cells than on sympathetic neurons. There are a number of possible reasons for such differences. For example, PC12 cells are tumor-derived, capable of survival and mitosis in the absence of NGF, and undergo neuronal differentiation in response to NGF. Thus, when NGF is added to PC12 cultures, one is observing an initial response by a previously "undifferentiated" cell. Sympathetic neurons, on the other hand, represent a postmitotic, neuronally differentiated population that requires NGF for survival. Such cells have had prior exposure to NGF and cannot be observed during their initial response to the factor.

Extensive surface architectural changes induced within seconds of peptide treatment are not limited to NGF. Human

carcinoma A-431 cells show extensive ruffling and extension of filopodia possibly beginning as early as 30 s and subsiding within 5–15 min after treatment with epidermal growth factor (EGF) (2). Also, recent studies indicate that cultured thyroid epithelial cells display pit formation within 30 min of exposure to thyroid-stimulating hormone.[2] Such changes do not appear to be nonspecific. In the present study, changes took place in the presence of a high background of serum proteins and were not caused by cytochrome *c*, a protein with a charge similar to that of NGF. In our studies with PC12 cells, the effects of NGF were not mimicked by several basic proteins or by α-bungarotoxin, a basic peptide for which PC12 cells appear to have specific receptors (11).

Neither the mechanisms nor the role of the effects we have observed are presently known. As pointed out above, temporal considerations suggest that such changes are triggered by the initial binding of NGF to surface receptors. It has been previously hypothesized that a binding-induced phosphorylation event may be responsible for the surface changes caused by EGF in A-431 cells (12). In this respect, there is conflicting evidence that NGF triggers a transient rise in intracellular cAMP levels (13), and there are several reports that NGF stimulates phosphorylation in its target cells (14, 15). Additional rapidly onsetting responses to NGF that have been reported include enhanced uptake of metabolites and extrusion of $Na^+$ (16). However, causal relationships between these events and cell surface morphology remain to be defined.

The types of surface structures we have observed have in some cases been associated with internalization of macromolecules. Membrane ruffling and macropinocytosis appear to be correlated (17, 18), and receptor-mediated uptake of several peptide hormones appears to take place via coated pits (19–21). There is abundant evidence that NGF can be internalized by its target cells (13), and recent findings (22) suggest that at least some of the uptake occurs via receptor-mediated patching and subsequent internalization at coated pits. Hence, it is quite possible that reorganization of the cell surface plays a causal role in the uptake of NGF.

In summary, we have reported a number of surface architectural changes induced by NGF on cultured sympathetic neurons. The occurrence of these surface changes in a normal differentiated cell as well as in tumor-derived cell lines indicates that such phenomena may represent a fundamental characteristic of the interaction of peptide hormones with their target cells.

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