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Microtubules and microfilaments in fixed and permeabilized cells are selectively decorated by nerve growth factor

(indirect immunofluorescence)

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ABSTRACT **A specific antibody against nerve growth factor (NGF) and indirect immunofluorescence microscopy have been used to follow the *in vitro* binding of NGF to cells made permeable to large molecules. All cells tested, both target (sensory neurons and PC12 cells) and nontarget (3T3, BKH 2I, C6 glioma cells), revealed a decoration of cytoskeletal structures which on the basis of their form, reactivity with antibodies, and sensitivity to specific drugs may be identified as microtubules (MTs) and microfilaments (MFs). The decoration of either structure depends on the fixation and permeabilization conditions: MFs, in the form of stress fibers, are stained by NGF when the plasma membrane is permeabilized with methanol/acetone; MTs become intensely stained when the plasma membrane is solubilized with a nonionic detergent in the presence of a MT-stabilizing medium. The two procedures do not affect the staining of these structures with specific antibodies. Binding of ^{125}I-labeled NGF to PC12 cells was not competitively inhibited by a 100-fold excess of several positively charged proteins but it was markedly decreased in the presence of DNase I. ^{125}I-Labeled NGF interacted with MTs and F-actin (fixed with paraformaldehyde) in a range of concentrations similar to that used for their cellular localization with NGF–anti-NGF. Our studies show that the specificity and affinity of NGF binding to MTs and MFs is in the range of that of antibodies against tubulin and actin. The possible relevance of these findings to the mechanism of action of NGF in target cells is discussed.**

After the protein nerve growth factor (NGF) (1) binds to receptors located on the cytoplasmic membrane or at the nerve terminals of target cells (2, 3), it is internalized into the cytoplasm (4, 5). In pheochromocytoma cells (clone PC12), it is localized around the nuclear outline and within the nucleus in a paranucleolar position (6). It has been suggested that such an intracellular pool of NGF could have a role in the induction of neurite growth (7–10). An important contribution to solving this problem would be provided by a precise localization of NGF in the cell. Some studies along this line have been attempted but the weakness of the signal detected with autoradiographic or immunofluorescence techniques (6) has permitted relatively precise localization of only a fraction of the intracellular NGF.

In order to overcome the fact that only a minute amount of NGF enters living target cells, we have facilitated the intracellular diffusion of a much larger quantity of this protein into fixed and permeabilized cells and detected this ligand by indirect immunofluorescence microscopy. It has been found that NGF is not evenly distributed in the cytoplasm. It selectively binds and decorates cytoskeletal elements such as microtubules (MTs) and microfilaments (MFs) to an extent and with a specificity comparable to those obtained with specific antibodies. These findings add support to the hypothesis (11, 12) that interaction of NGF with these structures may occur in the living target cells and have an important role in the mechanism of action of this growth factor.

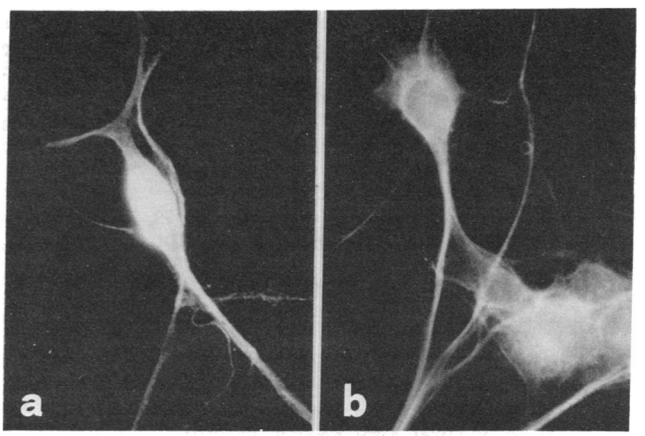

FIG. 1. PC12 cells incubated for 14 days with NGF (50 ng/ml) were treated with MSB containing 0.1% Nonidet P40, fixed with paraformaldehyde, incubated with NGF (1.0 μg/ml), and processed for indirect immunofluorescence microscopy either with purified NGF antibodies (*a*) or with tubulin antibodies (*b*). (×730.)

METHODS

Cells. Rat pheochromocytoma cells (clone PC12) (13) were cultured as described (6). Sensory ganglion cells were prepared from 9-day chicken embryos and cultured in the presence of NGF at 50 ng/ml. The other cell lines (3T3, BHK-C13 and C6 glioma) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Cells for immunofluorescence microscopy were plated on round glass coverslips in 35-mm plastic culture dishes.

Preparation of Cells for Immunofluorescence Microscopy. Cytoskeletal structures were prepared according to a published procedure (14). Briefly, cells were washed in a MT-stabilizing buffer (MSB) twice for 30 sec, incubated in MSB containing 0.5% Triton-X 100 or 0.1% Nonidet P40 for 5 min at room temperature, rinsed in MSB, and fixed in 3.7% paraformaldehyde in MSB. After extensive washing, coverslips were treated as described in the following section. An alternative procedure involved fixation in 3.7% paraformaldehyde in phosphate-buffered saline (P_i/NaCl) and sequential treatment in chilled (−10°C) methanol and acetone as described (6).

Indirect Immunofluorescence Microscopy. After fixation, cells were incubated either with NGF (1.0 μg/ml) in P_i/NaCl containing 10 mg of bovine serum albumin per ml or in the same medium without NGF for 45 min at 37°C. After extensive wash-

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Abbreviations: NGF, nerve growth factor; MT, microtubule; MSB, microtubule stabilizing buffer; MF, microfilament; Rh-NGF, rhodamine-conjugated NGF; P_i/NaCl, phosphate-buffered saline.

ing in P_i/NaCl/albumin, NGF antibodies were layered on coverslips and incubated for 45 min at 37°C. Parallel coverslips were treated with monospecific tubulin, actin, or α-actinin antibodies (50 μg/ml). After rinsing, the coverslips were incubated with fluorescein-labeled goat anti-rabbit IgG (Miles-Yeda), rinsed, and mounted on Elvanol. For each experiment, controls were performed either by omitting the NGF incubation step or by replacing the first antibody with preimmune rabbit IgG. Coverslips were examined with a Zeiss Photomicroscope III equipped with epifluorescence optic and an oil-immersion Zeiss Planapo 63 objective.

Preparation of Antibodies Specific for NGF, Rhodamine-Conjugated NGF (Rh-NGF), Tubulin, and Actin. NGF was prepared by the method described by Bocchini and Angeletti (15); purified antibodies against NGF were obtained as described (6). For the preparation of Rh-NGF, the protein was dissolved at 2.0 mg/ml in 0.5 M NaCl/2 mM Na acetate, pH 5.0. Then, 0.1 vol of 0.5 M bicarbonate buffer (pH 9.5) and 1/30th vol of tetramethylrhodamine (Cappell Laboratories, Cochranville, PA) (2.0 mg/ml in bicarbonate buffer) were added to the NGF solution. The mixture was stirred for 60 min at 0°C, dialyzed against 0.1 M phosphate buffer (pH 7.5), and chromatographed on a small DEAE-cellulose (DE-52) column equilibrated with the same buffer. The Rh-NGF recovered in the flow-through of the column had an average molar ratio of fluorochrome to protein of 1.2. Antibodies against actin and α-actinin were the generous gifts of K. Weber and B. Geiger. Purified antibodies against 6S tubulin were prepared by affinity chromatography of the IgG fraction on Sepharose 4B by the procedure described for the purification of NGF antibodies. Calf brain tubulin for binding experiments was prepared with two cycles of assembly/disassembly (16) and chicken muscle actin was purified by the procedure described by Spudich *et al.* (17). ^{125}I-labeled NGF (^{125}I-NGF) was prepared as described (6) and had a specific activity of 1.1×10^5 cpm/ng.

^{125}I-NGF Binding to PC12 Cells, MTs, and F-Actin. Confluent PC12 cells exposed for 2 days to NGF (50 ng/ml) were washed three times with P_i/NaCl and fixed with 3.7% parafor-

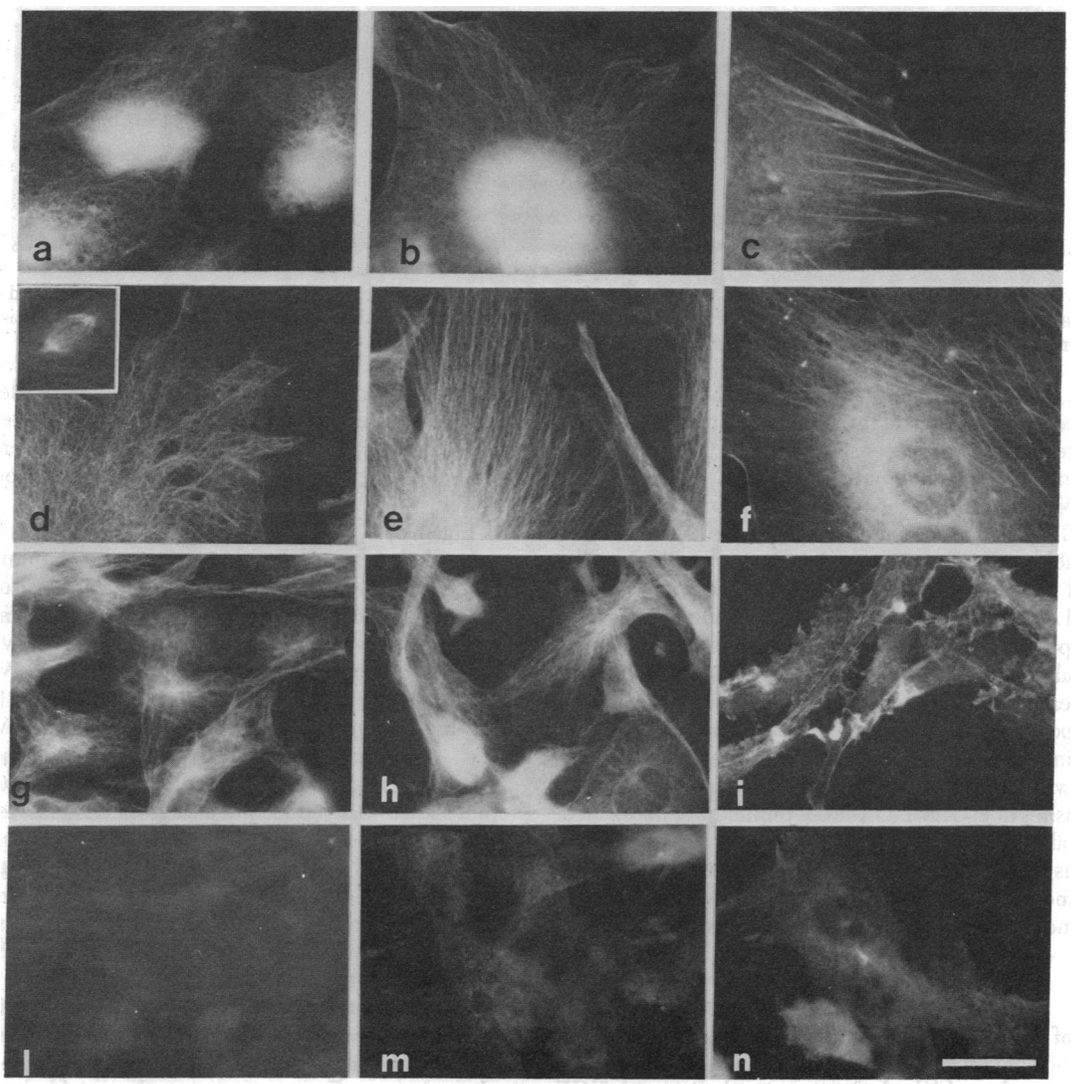

FIG. 2. 3T3 cells (*a–c*), BHK-C13 cells (*d–f* and *l–n*), and C6 glioma cells (*g–i*) were cultured. All cells were extracted with MSB containing 0.1% Nonidet P40 and fixed with paraformaldehyde. Cells were then stained for indirect immunofluorescence microscopy either after incubation with NGF at 1 μg/ml followed by purified NGF antibodies (*a, d, g, l, n*) or after incubation with tubulin (*b, e, h*), actin (*f, i*), or α-actinin (*c*) antibodies. A mitotic spindle (*d, Inset*) decorated by the NGF–anti-NGF method shows a relatively weaker staining of the interzonal fibers compared to spindle pole fibers. A control preparation in which the NGF incubation step was omitted is shown in (*l*). The cells shown in *m* were permeabilized in MSB containing 4.0 mM Ca^{2+} (5 min, 20°C); MTs are no longer detectable when stained with the NGF–anti-NGF procedure. BHK cells (*n*) were incubated in colcemid (1.0 μg/ml, 30 min, 37°C) before MSB treatment and fixation; cytoplasmic MTs were no longer detectable with antitubulin antibodies (not shown) and decoration with the NGF–anti-NGF procedure no longer stains MTs except at an elongated site which probably represents a cilium. Similar results were obtained when fixation preceded detergent extraction of the cells. (Bar = 20 μm.)

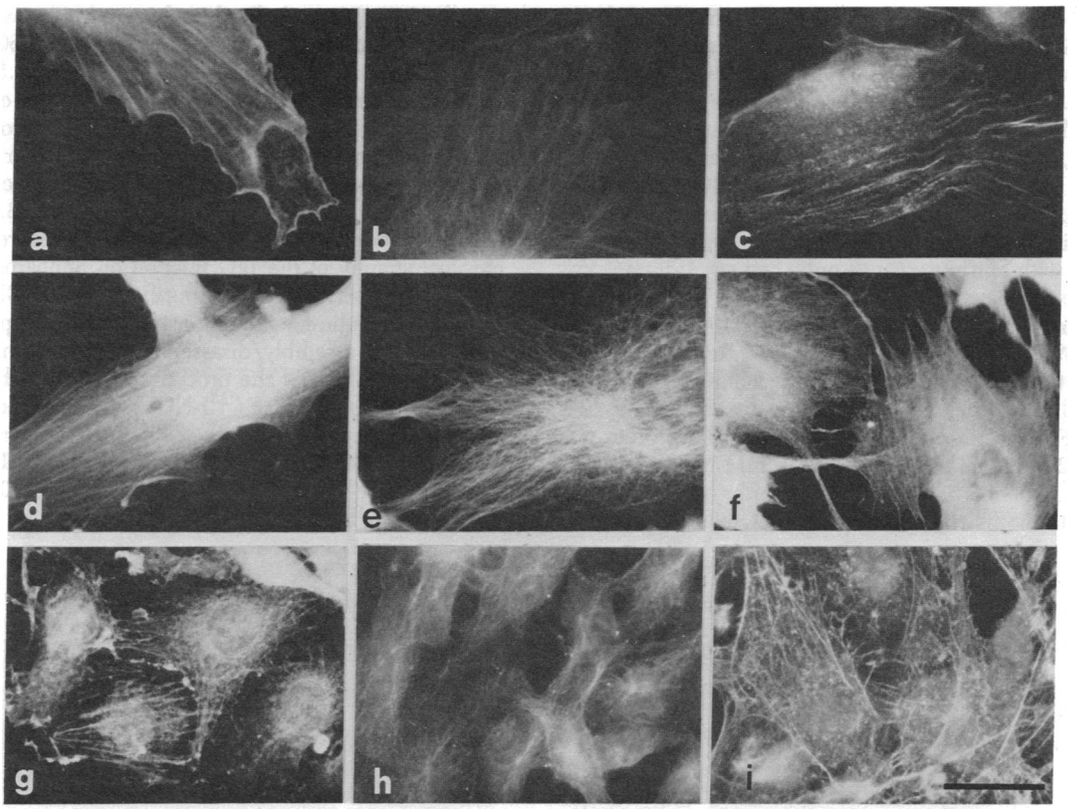

FIG. 3. 3T3 (*a–c*), BHK-C13 (*d–f*), and C6 glioma cells (*g–i*) were fixed in paraformaldehyde, extracted in chilled methanol and acetone (5 min and 5 sec, respectively), and air dried. All cells were then treated exactly as described in the legend of Fig. 2 (*a–i*) for NGF–anti-NGF antibody (*a, d, g*), tubulin antibody (*b, e, h*), α-actinin antibody (*c*), and actin antibody (*f, i*) staining. (Bar = 20 μm.)

maldehyde in P_i/NaCl. Previous studies have shown (18) that, with this procedure, 60–75% of the NGF remains bound to its receptors. After fixation and extensive rinsing, PC12 cells were soaked for 6 min in prechilled methanol (−10°C), washed, and used for ^{125}I-NGF binding in the presence of a constant amount of labeled NGF plus various concentrations of unlabeled NGF in P_i/NaCl/ablumin. After 90-min incubation at 37°C, cells were washed with P_i/NaCl; cell-bound radioactivity was removed with 1.0 M NaOH and measured. Binding to MTs and F-actin was performed as follows. Samples (200 μl) of polymerized tubulin (0.6 mg/ml) or polymerized actin (0.08 mg/ml) were layered onto tissue culture plastic dishes previously coated with polylysine and incubated for 30 min at 37°C. The excess protein was removed and the portion attached to the plastic dish was fixed with 3.7% paraformaldehyde followed by several washes in P_i/NaCl. ^{125}I-NGF binding was performed as described above for PC12 cells but with a 60-min incubation at 37°C. Nonspecific binding of ^{125}I-NGF to the plastic dishes was always measured in parallel experiments and subtracted from total binding to PC12 cells, MTs, and F-actin.

RESULTS

Staining of MTs with NGF–Anti-NGF. Fig. 1 shows the staining pattern obtained on PC12 cells after detergent extraction in MSB, paraformaldehyde fixation, incubation with NGF (1.0 μg/ml), and indirect immunofluorescence microscopy with purified NGF antibodies. The staining pattern was closely comparable to that obtained after decorating the same cells with purified tubulin antibodies. Analogous results were obtained when sensory neurons, another target cell of NGF, were treated the same way. These findings suggest that NGF binds to some filamentous structures resembling MTs and possibly other constituents of the cytoskeleton. Because PC12 cells and sensory neurons do not lend themselves to a precise identification of each component of this filamentous network, the visualization of NGF distribution in permeable cells has been extended to other cell types which are more suitable for this purpose, being flatter.

Fig. 2 *a*, *d*, and *g* shows that NGF decorates a filamentous network that is similar to that visualized with tubulin antibodies (Fig. 2 *b*, *e*, and *h*) in several types of cells (see also Fig. 2. *Inset*, showing a mitotic spindle decorated with NGF–anti-NGF). The pattern observed is markedly different from that visualized with actin or α-actinin antibodies (Fig. 2 *c*, *f*, and *i*). When the NGF incubation step was omitted before exposure to NGF antibodies, the staining pattern was abolished (Fig. 2*l*). When 4.0 mM Ca^{2+} was present in the extraction buffer, the staining by NGF–NGF antibodies (Fig. 2*m*) as well as by tubulin antibodies (not shown) was reduced to background fluorescence. This also was true when BHK-C13 or 3T3 cells first were exposed to Colcemid (1.0 μg/ml, 30 min) or colchicine (10 μm, 2 hr). Under these conditions the decoration by NGF–anti-NGF or by tubulin antibodies was abolished and the only structures still detectable were those, like the cilium shown in Fig. 2*n*, known to be resistant to these drugs (19).

F-Actin Staining with NGF–Anti-NGF. When the same set of cells first were fixed with paraformaldehyde and then extracted in chilled methanol/acetone, the NGF–anti-NGF indirect immunofluorescence procedure yielded a different staining pattern and mainly showed MFs either in the form of stress fibers or as membrane-associated MF bundles (Fig. 3 *a*, *d*, and *e*). This MF pattern was altered by pretreatment of the cells with cytochalasin B (5 μg/ml, 1 hr, 37°C) according to the typical altered distribution of MFs induced by this drug (20).

The two different staining procedures adopted for the experiments in Fig. 2 and 3 did not detectably alter the distribution and localization of MTs and MFs visualized with their

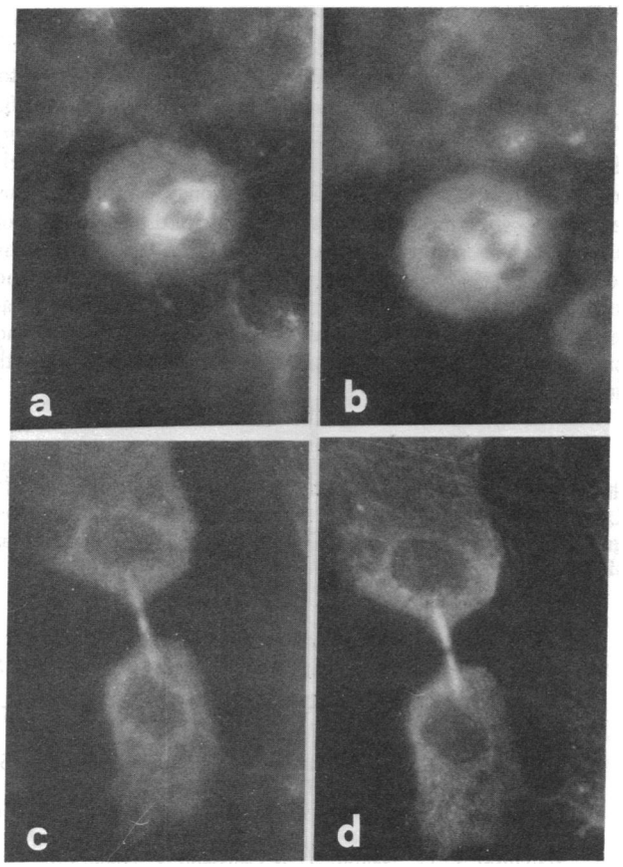

FIG. 4. 3T3 cells were grown on glass coverslips, fixed with paraformaldehyde, and extracted with MSB containing 0.1% Nonidet P40. Cells were then incubated with a mixure of Rh-NGF (20 μg/ml) and tubulin antibodies; after washing, the coverslips were incubated with fluorescein-labeled goat anti-rabbit IgG, washed, and mounted on Elvanol. The same set of cells was illuminated for rhodamine (*a*, *c*) or fluorescein (*b*, *d*) and photographed. (×650.)

respective antibodies (see Figs. 2 *b*, *e*, and *h* and 2 and 3 *c*, *f*, and *i*). The possible explanation for the preferential staining by NGF–anti-NGF of the two cytoskeletal structures according to the fixation and permeabilization procedure will be discussed later. Fig. 4 shows a double-staining experiment performed on detergent-treated 3T3 cells with Rh-NGF and tubulin antibody. Rh-NGF stained a mitotic spindle and a midbody to an extent and with a localization identical to those obtained in the same cells with tubulin antibody. Analogous experiments, performed with methanol/acetone-treated 3T3 cells, showed that Rh-NGF decorates a cytoskeletal structure (stress fibers) identical to that visualized with anti-actin antibodies. Due to the lack of amplification of the direct staining by Rh-NGF, which requires 20 μg/ml instead of the 1 μg/ml with NGF, we adopted the indirect immunofluorescence procedure for our routine experiments.

^{125}I-NGF Binding Studies. In order to assess the affinity and specificity of NGF binding to MTs and MFs under the conditions used for the immunofluorescence studies, ^{125}I-NGF binding to these structures as well as to fixed and permeabilized PC12 cells was studied. Scatchard analysis (Fig. 5A) of NGF binding to MTs previously fixed to tissue culture dishes showed that NGF binds to an apparently single set of binding sites present on MTs prepared with two cycles of assembly/disassembly as described by Shelanski *et al.* (16). Besides the basic constituent, tubulin, these MTs contain several other proteins or MAPs (21). Thus, it is not possible to ascertain whether the value of the dissociation constant (K_d=8.8 × 10^{-8} M) of NGF

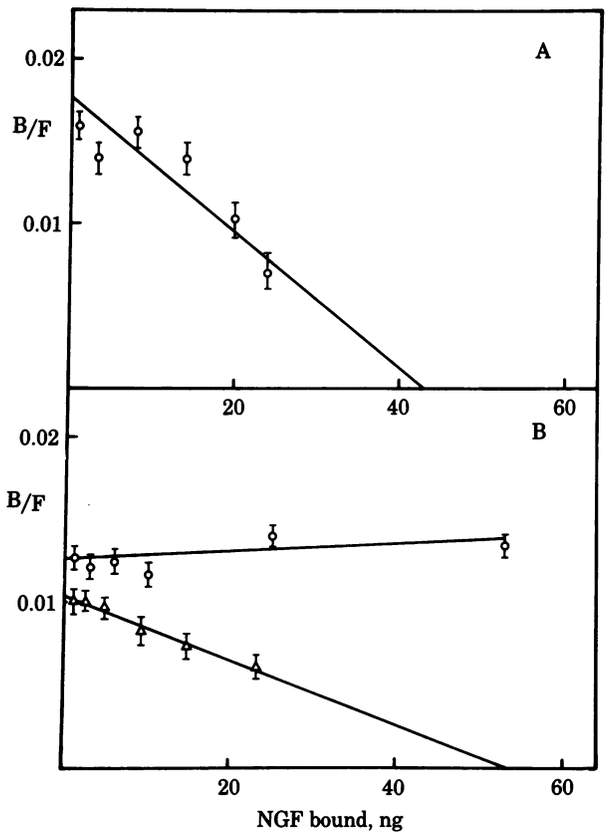

FIG. 5. Scatchard analysis of ^{125}I-NGF binding to MTs (*A*) and F-actin (*B*) fixed to tissue culture dishes. Each point represents the mean ± SD of triplicate samples; △, binding in the presence of DNase I at 20 μg/ml; ○, F-actin alone.

for these structures is due to the binding to tubulin or to some MAPs.

Fig. 5*B* shows a Scatchard plot of NGF binding to F-actin. Saturation was not attained in the same range of concentrations used for the binding to MTs. On the other hand, saturation was observable when the binding is performed in the presence of

Table 1. Inhibition of ^{125}I-NGF binding to PC12 cells

Inhibitor	^{125}I-NGF bound, cpm/well
Exp. 1	
Control	6300 ± 1200
Lysozyme	6890 ± 1500
Cytochrome *c*	6770 ± 900
Histone type II	7140 ± 1250
Polylysine	5200 ± 850
DNase I	3990 ± 1600
Exp. 2	
Control	7350 ± 600
Naia naia toxin	7590 ± 400
β-Bungarotoxin	7950 ± 750
RNase A type II	7340 ± 220
1 M NaCl	4920 ± 400
1 M KCl	5140 ± 560

Cells (0.5 × 10^6 per well) were incubated for 2 days with NGF, fixed, treated with chilled (−10°C) methanol, and used for ^{125}I-NGF binding (136,000 cpm per well); unlabeled NGF (1.0 μg/ml) and the protein (100 μg/ml) to be tested or 1.0 M NaCl or KCl were present in P_i/NaCl containing 1% bovine serum albumin. Each value represents the mean ± SD of four determinations.

a constant amount (20 μg/ml) of DNase I, an enzyme that binds tightly to actin (22) and competes for NGF binding. Notice that the higher the NGF concentration, the higher, in relative terms, the inhibition by DNase I. These findings suggest that there are two sets of NGF binding sites on actin filaments: one with low affinity which DNase I competes for, and one with a K_d of 1.9×10^{-7} M which is unique to NGF and is not blocked by the enzyme. Whether this site belongs to actin itself in a conformation not suitable for DNase binding (e.g., F-actin) remains to be elucidated.

Binding studies performed with fixed and permeabilized PC12 cells have shown intracellular binding sites in these cells with a K_d for NGF similar to that found in the *in vitro* experiments reported in Fig. 5.

These binding sites were not inhibited by a 100-fold excess of various basic proteins such as cytochrome *c*, histone, lysozyme, and RNase A and were decreased 20–30% by polylysine or 1 M NaCl or KCl (Table 1). Similar results were obtained for the immunofluorescence decoration by NGF–anti-NGF. The most effective inhibitor was DNase I, a protein that binds to actin with high affinity. These findings indicate that, if ionic interactions between negatively charged constituents of MTs and MFs and positively charged NGF play a role in the NGF binding, they are not antagonized by a group of proteins which, on the basis of their charge, should exhibit a similar tendency to interact with these filamentous structures. The slight inhibition of polylysine or high ionic strength may reflect the extent of participation of electrostatic interactions in the intracellular distribution of NGF in these cells.

DISCUSSION

When NGF is allowed to diffuse freely into cells made permeable to large molecules, indirect immunofluorescence microscopy with purified NGF antibodies reveals a network of filaments which, on the basis of their form, staining with specific antibodies, and sensitivity to drugs, are identified as MTs and MFs. Both the cytoskeleton decoration and the *in vitro* binding to these structures are not antagonized by various positively charged proteins present in a 100-fold excess over NGF. Moreover, MT and MF staining by NGF occurs at a concentration (1.0 μg/ml) similar to that used with specific antibodies (10–50 μg/ml)—i.e., with an affinity comparable to that of an antigen–antibody reaction. The only effective inhibitor of NGF binding is DNase I, an enzyme known to bind selectively and tightly to actin (22). It is interesting to note that this protein has been used to stain F-actin (23) but the concentration used was 1.0 mg/ml instead of 1.0 μg/ml as for NGF.

The finding that NGF decoration of MTs and MFs is a function of the procedure used to fix and expose the intracellular structures calls for some comment. It is not yet clear whether NGF decoration is due to binding of this ligand to the basic constituent of MTs and MFs (tubulin and actin) or to some of their associated proteins (21). Whatever the correct explanation is, our findings suggest that detergent treatment solubilizes a component of stress fibers that is responsible for NGF binding.

The present data clearly show that one major cytoplasmic target of NGF is represented by MTs and by MFs. This finding suggests that, if the pool of NGF internalized in target cells (3, 5, 9, 18), presumably via endocytosis, is subsequently released and diffuses before being degraded, as is known to occur for some internalized proteins (24, 25), the chances of an interaction with these cytoskeletal structures are high.

In vitro studies of binding of NGF to tubulin and to actin in their assembled or disassembled state have found a marked effect of this ligand on their rate of assembly, association into large bundles, and other physicochemical changes (11, 12, 26, 27). These effects have been related to the mechanism of action of NGF with special relevance to the process of neurite growth and elongation. The present studies demonstrate that such interaction occurs not only *in vitro* with purified preparations of tubulin and actin but also in a cell, provided that NGF gains access to the cytoplasmic compartment. In the living cell, the interaction of NGF with these elements may be limited to strategic points, such as those where assembly is modulated, and may occur with substoichiometric concentrations of this growth factor.

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